Metabolic Formation of 4-Methyl-4*H*-1,3,2-benzodioxaphosphorins from Bis(*o*-ethylphenyl) Phenylphosphonates in House Flies

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Bis(4-chloro-2-ethylphenyl) phenylphosphonate was metabolically transformed into the corresponding cyclic ester, *i.e.*, 6-chloro-4-methyl-2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide, in houseflies *in vivo*. In a *p*-unsubstituted analog, hydroxylation at the para-position of an ester linkage occurred preferably to alpha-hydroxylation with subsequent cyclization. The cyclization was diastereomerically selective, giving predominantly the *cis* ester. The biological activities of synthesized and related cyclic esters were similar to but weaker than saligenin cyclic phosphorus esters lacking a methyl group at the 4-position.

The metabolic activation of tri-*o*-tolyl phosphate (TOCP) by the hydroxylation of a methyl group and subsequent cyclization to form a saligenin cyclic phosphate, *i.e.*, 2-*o*-tolyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide, has already been established.¹⁾ The insecticide salithion, 2-methoxy-4*H*-1,3,2-benzodioxaphosphorin 2-sulfide, has been developed by modifying the structure of the active metabolite.²⁾ Triaryl phosphates, which show biological activity similar to TOCP, always carry at least one primary alkyl group, if not a methyl, at the *ortho*-position.³⁾ It is reasonable to presume that they are activated in organisms by a similar metabolic transformation into the corresponding α -alkylsaligenin cyclic phosphate.

This paper confirms this hypothesis, including the stereochemistry in the cyclization process. For simplicity, the phenylphosphonate esters of *o*-ethylphenols were used as the substrates.

MATERIALS AND METHODS

Synthesis

Bis(o-ethylphenyl) phenylphosphonate (BEPP). To a mixture of *o*-ethylphenol (5 g) and pyridine (3.2 g), phenyl-

phosphonic dichloride (4 g) dissolved in benzene (10 ml) was added dropwise at 0°C. The mixture was stirred for 22 hr at room temperature and then at 50°C for 4 hr with the addition of pyridine in a small excess. The mixture was successively washed with 5% hydrochloric acid, saturated sodium bicarbonate and water, and was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was submitted to a silica gel column and eluted with a hexane-ether mixture (95:5) to get 3.2 g of an oil. ¹H-NMR δ : 1.1 (6H, t, $J=8$ Hz, CH₃), 2.6 (4H, q, $J=8$ Hz, CH₂), 6.9~8.1 (13H, m, aromatic). MS m/z : 366 (M⁺). ³¹P-NMR δ : -0.04 ppm.

Bis(4-chloro-2-ethylphenyl) phenylphosphonate was prepared from 4-chloro-2-ethylphenol in the same manner. ¹H-NMR δ : 1.1 (6H, t, $J=8$ Hz, CH₂CH₃), 2.6 (4H, q, $J=8$ Hz, CH₂Me), 7.1~8.2 (11H, m, aromatic).

1-(*o*-Hydroxyphenyl)ethanol. To a suspension of lithium aluminum hydride (3g) in dry ether (20 ml) was added dropwise *o*-hydroxyacetophenone (14.6 g) dissolved in the same solvent (10 ml). After stirring for 30 min, 3% hydrochloric acid (20 ml) was slowly added to decompose the formed chelate compounds. The ether layer that separated was worked up to give colorless crystals after recrystallization from benzene, mp 53~54°C; yield, 68%. ¹H-NMR δ : 1.55 (3H, d, $J=6$ Hz, CH₃), 2.90 (1H, alcoholic OH), 5.00 (1H, q, $J=6$ Hz, CH), 6.60~7.20 (4H, m, aromatic), 8.00 (1H, phenolic OH).

3-Chloro- and 3-methyl-6-hydroxyphenylethanols were prepared in a similar manner from the corresponding ring-substituted hydroxyacetophenone, mp 101~102 and 99~100°C, respectively.

Cyclic phosphoryl esters of 1-(*o*-hydroxyphenyl)ethanols.

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These were synthesized from the corresponding *o*-hydroxyphenylethanol and an appropriate phosphoryl dichloride by modifying a procedure for the synthesis of saligenin cyclic phosphoryl esters.⁴⁾ The following is a typical example.

4-Methyl-2-phenyl-4H-1,3,2-benzodioxaphosphorin 2-oxide (MPBO). A chloroform solution (5 ml) of phenylphosphonic dichloride (0.63 g) was added dropwise to a mixture of 1-(2-hydroxyphenyl)ethanol (0.44 g) and pyridine (0.51 g) at 0°C. The mixture was stirred for 24 hr at room temperature, worked up in the usual manner and chromatographed on a silica gel column by eluting with a hexane-ethyl acetate mixture (3:1). Yield, 0.36 g. ¹H-NMR δ : *cis* isomer, 1.82 (3H, d, $J=6$ Hz, CH₃), 5.50 (1H, q, $J=6$ Hz, CH), 6.90~7.90 (9H, m, aromatic); *trans* isomer, 1.78 (3H, d, $J=6$ Hz, CH₃), 5.78 (1H, q, $J=6$ Hz, CH), 6.90~7.90 (9H, m, aromatic). ³¹P-NMR (in CDCl₃) δ : *cis* isomer, -0.55; *trans* isomer, 1.86. MS m/z : 260 (M^+).

***o*-Ethylphenyl 2-ethyl-4-hydroxyphenyl phenylphosphonate (BEPP-OH).** To the mixture of ethylhydroquinone (0.184 g), which was obtained by the Wolff-Kishner reduction of 2,5-dihydroxyacetophenone, and *t*-butyldimethylchlorosilane (0.239 g) in dry dimethylformamide (5 ml) was added imidazole (0.220 g). After stirring for 4 hr, the reaction mixture was poured into a 5% sodium bicarbonate solution (50 ml) and extracted with hexane (30 ml $\times$ 3). The combined hexane layers were washed with water, dried over sodium sulfate and chromatographed on a silica gel column to get 2-ethyl-4-*t*-butyldimethylsiloxyphenol (0.32 g). ¹H-NMR δ : 0.16 (6H, s, Si(CH₃)₂), 0.96 (9H, s, SiC(CH₃)₃), 1.20 (3H, t, $J=7$ Hz, CH₂CH₃), 2.56 (2H, q, $J=7$ Hz, CH₂Me), 6.40~6.65 (3H, aromatic). MS m/z : 252 (M^+).

To a solution of the siloxyphenol (0.14 g) in benzene (2 ml) containing pyridine in excess was added *o*-ethylphenyl phenylphosphonochloridate (0.31 g), which had been prepared from *o*-ethylphenol and phenylphosphonic dichloride and purified by distillation *in vacuo*. After being kept subsequently at 25, 65 and 110°C for 0.5, 12 and 12 hr, respectively, the reaction mixture was worked up in the usual manner and the product was purified by preparative thin-layer chromatography (hexane:ethyl acetate=5:2) to get a colorless oil of *o*-ethylphenyl 4-(*t*-butyldimethylsiloxy)-2-ethylphenyl phenylphosphonate (0.21 g). ¹H-NMR δ : 0.16 (6H, s, Si(CH₃)₂), 0.96 (9H, s, SiC(CH₃)₃), 1.08 (3H, t, $J=8$ Hz, CH₂CH₃), 1.10 (3H, t, $J=8$ Hz, CH₂CH₃), 2.52 (2H, q, $J=8$ Hz, CH₂Me), 2.56 (2H, q, $J=8$ Hz, CH₂Me), 6.40~8.08 (12H, m, aromatic). MS m/z : 496 (M^+).

The butyldimethylsilyl group was removed by treating the siloxyphenyl phenylphosphonate (210 mg) with a tetrahydrofuran solution of tetrabutylammonium fluoride (1 M, 1 ml) at room temperature for 20 min. The product was purified by thin-layer chromatography (hexane:ethyl acetate=2:1). Yield, 28 mg. ¹H-NMR δ : 0.98 (3H, t, $J=8$ Hz, CH₂CH₃), 1.12 (3H, t, $J=8$ Hz, CH₂CH₃), 2.40

(2H, q, $J=8$ Hz, CH₂Me), 2.62 (2H, q, $J=8$ Hz, CH₂Me), 6.16~8.12 (12H, m, aromatic). MS m/z : 382 (M^+ 100%), 262 (25%).

Reaction of ethylhydroquinone with *o*-ethylphenyl phenylphosphonochloridate. A mixture of ethylhydroquinone (0.14 g), *o*-ethylphenyl phenylphosphonochloridate (0.28 g) and pyridine (in excess) in benzene (5 ml) was stirred at 40°C for 12 hr, and then at 110°C for 2 hr. After being worked up and purified by TLC (hexane:ethyl acetate=10:7), *o*-ethylphenyl 3-ethyl-4-hydroxyphenyl phenylphosphonate (84 mg) was obtained. ¹H-NMR δ : 1.06 (3H, t, $J=8$ Hz, CH₂CH₃), 1.12 (3H, t, $J=8$ Hz, CH₂CH₃), 2.46 (2H, q, $J=8$ Hz, CH₂Me), 2.60 (2H, q, $J=8$ Hz, CH₂Me), 6.20~8.08 (12H, m, aromatic). MS m/z : 382 (M^+ 29%), 137 (100%).

Spectroscopy. A JEOL JNM-FX 100 spectrometer was used to record NMR spectra for protons, ¹³carbon and ³¹phosphorus at 100, 25 and 40 MHz, respectively. Deuteriochloroform was used as a solvent, and tetramethylsilane (for protons and ¹³C) and 85% phosphoric acid (for ³¹P) were used as references. Tris-(2,2,6,6-tetramethylheptane-3,5-dionato)europium (Eu(dpm)₃), a shift reagent, was purchased from Wako Pure Chemical Industries Ltd. Mass spectra were measured with an ESCO EMD-05A mass spectrometer at 70 eV.

Chromatography. For column chromatography, a column packed with Merck silica gel (0.063~0.200 mm, 70~230 mesh) was used. Merck pre-coated silica gel 60 F254 TLC plates (2, 1 and 0.5 mm) were utilized for thin-layer chromatography.

High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-5A liquid chromatograph equipped with a Shimadzu SPD-1 spectrophotometric detector unit and a Shimadzu C-R1A CHROMATOPAC recorder. The conditions utilized for HPLC were as follows: column, Shim-pack CLC-ODS (0.15 m $\times$ 6 mm ϕ , reversed phase); mobile phase, methanol:water=7:3; pressure, 100 kg/cm²; flow rate, 1 ml/min; temperature, 40°C; detector, 273 nm.

A Shimadzu C-7A equipped with an FPD-7 flame photometric detector was used for gas chromatography. A glass column (3 mm $\times$ 250 cm) packed with 5% SE-30 on chromosorb (80/100 mesh) carried nitrogen gas at 250°C and 50 ml/min.

Treatment of houseflies and extraction of metabolites. Twenty thousand 3- to 5-day old male and female houseflies (287 g in total), *Musca domestica* WHO's standard susceptible strain, were anesthetized with ether and treated with an acetone solution (35 ml) containing 460 mg of BEPP by gentle stirring in a beaker. After being kept in housefly cages at 25°C for 2 days, the flies were homogenized in cold acetone to extract the metabolites. The acetone extract was dried over anhydrous sodium sulfate and evaporated. The residue was submitted to a silica gel column and eluted with a mixture of hexane and ether

(3:2, 250 ml) and ether (200 ml). The hexane-ether fraction was purified by TLC and developed with a mixture of hexane and ether (1:1.3). From the UV-absorptive zone, the metabolite M-1 (13 mg) was obtained. The ether fraction was submitted twice to TLC. The first TLC was developed with a mixture of hexane and ethyl acetate (1:1) to give 17 mg of a metabolite, which was then purified by TLC and developed with an ether-chloroform mixture (1:3) to obtain 2 mg of the metabolite M-2.

Bis(4-chloro-2-ethylphenyl) phenylphosphonate was also applied to houseflies and the metabolites were extracted in a similar manner.

Assays for biological activity. Anticholinesterase activity was assayed by applying Hestrin's colorimetric method for the determination of acetylcholine as described previously,⁵⁾ human plasma being used as a cholinesterase source.

Insecticidal activity was determined by topically treating the 3- to 4-day old female houseflies in the usual manner.⁶⁾

RESULTS

Isolation of metabolites of bis(*o*-ethylphenyl) phenylphosphonate (BEPP)

Two UV-absorptive metabolites, M-1 (13 mg) and M-2 (2 mg), were obtained from the acetone extract of 20,000 houseflies, which had been treated with BEPP (460 mg in total), by fractionating with silica gel column chromatography and subsequent preparative TLC. The major metabolite M-1 gave almost a single peak on GC and HPLC (see Fig. 1). Figure 1 also shows that M-2 was accompanied by some minor peaks.

Identification of M-1

The retention times of M-1 were apparently different from both those of the substrate BEPP and of the expected cyclic metabolite MPBO (see Table I). MS suggested that M-1 (m/z 382(M^+ , 100%), 262(30%)) was a mono-oxygenated metabolite of BEPP (m/z 366). The $^1\text{H-NMR}$ spectrum of M-1, in comparison with those of BEPP and sesamol, clearly indicates that it had two non-equivalent ethyl groups and one 1,2,4-trisubstituted benzene ring as shown in Fig. 2. Thus, M-1 was presumed to be *o*-ethylphenyl 2-ethyl-4-hydroxyphenyl phenylphosphonate (BEPP-OH).

The authentic BEPP-OH was synthesized

FIG. 1. High-performance Liquid Chromatograms of BEPP Metabolites (M-1 and M-2) and Authentic MPBO.

TABLE I. RETENTION TIMES OF METABOLITES, BEPP AND AUTHENTIC MPBO

Compound	t_R (min)	
	HPLC	GC
M-1	17.0	19.7
M-2a*	4.1	3.4
M-2b*	4.6	nd**
cis-MPBO	4.1	3.4
trans-MPBO	4.6	3.4
BEPP	>17.4	9.8

* M-2a and b are the major and minor components of metabolite M-2, respectively (see Fig. 1).

** nd, not detected.

FIG. 2. ^1H -NMR Spectra of BEPP, M-1 and Sesamol at 100 MHz.

by reacting 2-ethyl-4-*t*-butyldimethylsiloxyphenol with *o*-ethylphenyl phenylphosphonochloridate and subsequent desilylation. The direct reaction of ethylhydroquinone without protecting the 4-hydroxyl group gave only an isomeric BEPP-OH, *o*-ethylphenyl 3-ethyl-4-hydroxyphenyl phenylphosphonate. All the spectra from MS, ^1H -NMR and ^{13}C -NMR (Fig. 3) show that the isolated M-1 and the synthesized BEPP-OH were identical except for a few peaks from impurities.

Identification of M-2

M-2 appears to have been the cyclic metabolite MPBO on the basis of HPLC and GC (see Fig. 1 and Table I). This was also

supported by MS; both the isolated M-1 and MPBO gave the same molecular ion peak at m/z 260 and a fragment ion peak at 245 ($\text{M}^+ - \text{CH}_3$), with the same intensity ratio of 1:1.4.

Cis and *trans* isomerism relative to 4 methyl and $\text{P}=\text{O}$ substituents is possible in MPBO. The synthesized MPBO could not be separated into each isomer by GC under any conditions tried, but was by HPLC in the ratio of 3:2 (Fig. 1). Two 4-methine proton signals at δ : 5.44 and 5.68 ppm in the ^1H -NMR spectrum (Fig. 4) also indicate the presence of two isomers in the MPBO preparation. Since protons in a 1,3-*cis* relationship to a phosphoryl oxygen in cyclic phosphorus compounds are

FIG. 3. ^{13}C -NMR Spectra of Isolated M-1 and Synthesized BEPP-OH at 25 MHz. FIG. 4. ^1H -NMR Spectra of *cis*- and *trans*-MPBO in the Presence and Absence of $\text{Eu}(\text{dpm})_3$ in CDCl_3 .

deshielded,^{7,8)} the isomer whose 4-H resonates at the lower field was tentatively assigned as *trans*-MPBO.

This was further supported by applying a shift reagent, $\text{Eu}(\text{dpm})_3$. The effect of the reagent to move the signals of 4-H and 4- CH_3 downfield was apparently different between the isomers, as shown in Fig. 4. Figure 5 shows that the shift of the 4-H signal per increment of the reagent was greater in the minor isomer

than in the major one, whereas the reverse was true for that of 4- CH_3 . This means that the 4-H of the minor isomer and the 4- CH_3 of the major isomer were closer to the phosphoryl oxygen. It is consequently concluded that the major isomer ($t_R=4.1$ min in Fig. 1) was *cis* and the minor one ($t_R=4.6$ min) was *trans*.

Thus, the metabolite M-2 consisted mainly of *cis* MPBO (M-2a). This was accompanied by a small peak (M-2b), which was probably due to

FIG. 5. Effect of Eu(dpm)_3 on the Chemical Shift of 4-H and 4-CH₃ of MPBO Isomers (in CDCl_3).

TABLE II. R_f VALUES OF A METABOLITE OF Cl-MPBO AND RELATED AUTHENTIC COMPOUNDS ON TLC

Compound	Solvent system*	
	I	II
M-3	0.46	0.52
Cl-MPBO	0.47	0.52
Cl-BEPP	0.71	0.81
4-Chloro-2-ethylphenol	0.71	0.70

* Solvent system I, benzene: ether = 3:2; II, chloroform: ether = 4:1.

the *trans* isomer, at t_R 4.6 min in HPLC (Fig. 1). Their area intensity ratio is about 10:1.

A metabolite of bis(4-chloro-2-ethylphenyl) phenylphosphonate (Cl-BEPP)

Eight hundred houseflies were treated with Cl-BEPP (50 mg in total) and extracted in the same manner as that already mentioned. The extract was fractionated and examined for metabolites on TLC by comparing with authentic compounds. A metabolite, M-3, had R_f values identical with the authentic sam-

ple of 6-chloro-4-methyl-2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (Cl-MPBO) in two kinds of solvent systems (Table II), and gave positive color tests for phenols and phosphorus after hydrolysis on a TLC plate as has been briefly reported.⁹⁾ The metabolite obtained in a trace amount from TLC gave the molecular ion peak at m/z 294, corresponding to the expected cyclic phosphonate.

Biological activities of 4-methyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxides

Several α -methylsaligenin cyclic phosphonates and phosphates relating to the cyclic metabolite were prepared and examined for their anticholinesterase and insecticidal activities. The isolated metabolite M-2 itself was not used. The activities were assayed without separating the diastereomers and are compared with salioxon in Table III. They show certain biological activities but were weaker than the saligenin cyclic phosphate, salioxon, which lacks the methyl group at the 4-position. The aryl phosphates appear to be somewhat more active than the arylphosphonates.

TABLE III. ANALYTICAL DATA AND BIOLOGICAL ACTIVITIES OF 4-METHYL-1,3,2-BENZODIOXAPHOSPHORIN 2-OXIDES (I)

(I)

R	X	Y	Anal. P(%)		I ₅₀ ^a ChE (M)	LD ₅₀ μg/fly
			Calcd.	Found		
MeO	Me	Me	13.6	13.1		3.5
EtO	Me	Me	12.8	12.5	1.6×10^{-5}	3.5
<i>n</i> -PrO	Me	Me	12.1	12.1	5.5×10^{-6}	> 10
<i>o</i> -Me-PhO	Me	Me	10.5	10.1	1.4×10^{-6}	> 10
Ph	Me	Me	11.8	12.0	1.3×10^{-5}	
Ph	Me	H	12.4	12.3	2.1×10^{-5}	
MeO	H	H	(salioxon)		1.1×10^{-7}	0.035

^a Plasma cholinesterase. FIG. 6. Metabolic Hydroxylation and Cyclization of Bis(*o*-ethylphenyl) Phenylphosphonate.

DISCUSSION

By choosing bis(*o*-ethylphenyl) phenylphosphonates as simple representatives, it was confirmed that the *o*-alkylphenyl phosphates were activated by alpha-hydroxylation and subsequent cyclization in the same way as TOCP (Fig. 6). The cyclization proceeded stereoselectively, producing predominantly the *cis* form in contrast with the chemical synthesis, which gave the *cis* and *trans* isomers in the ratio of 3:2. Tawata has found a similar result by ³¹P-NMR without isolating the metabolite from

the tissue extract of rats administered with BEPP.¹⁰⁾ This stereoselectivity suggests that the *in vivo* cyclization proceeded by the catalytic action of a certain biopolymer, as was reported previously for the cyclization of diaryl *o*-hydroxymethylphenyl phosphates.¹⁵⁾

The hydroxylation occurred preferably at the *p*-position of the phenyl ester linkage rather than at the alpha carbon, unless the *p*-position has been occupied. A similar hydroxylation at the *p*-position occurred *in vivo* in houseflies in the metabolism of triphenyl phosphate (TPP).¹¹⁾ The hydroxylated meta-

bolite had only the same level of activity for esterase inhibition as TPP, although the related quinol phosphates showed some inhibitory activity against alcohol dehydrogenase.¹²⁾ Thus, the metabolite BEPP-OH may not be the active metabolite in the sense of TOCP-type bioactivity.

On the other hand, tris(*p*-ethylphenyl) phosphate (TPEP), an exceptional triaryl phosphate having TOCP-like biological activity without the *o*-alkyl group, was activated by alpha-hydroxylation and subsequent dehydrogenation into the alpha-oxo metabolites.¹³⁾ Based on this observation and on the chemical cyclization of enolyzed *o*-alkylketophenyl phosphates,¹⁴⁾ the metabolic transformation of *o*-ethylphenyl diphenyl phosphate into 4-alkylidene-2-phenoxy-1,3,2-benzodioxaphosphorinane 2-oxide has been suggested as an activation mechanism.¹⁰⁾ This may not be extensive, if at all, from the aspect that *o*-hydroxymethylphenyl phosphates cyclize so readily that the hydroxylated metabolite of TOCP could not be isolated in contrast with the stable hydroxylated metabolite of TPEP.^{1,13,15)}

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