O-(2-Ethylphenyl) O, O-Diphenyl Phosphate から 4-Alkylidene-1,3,2-benzodioxaphosphorinane 2-Oxide 類の代謝生成(農芸化学科)

Metabolic Formation of 4-Alkylidene-1,3,2-benzodioxaphosphorinane 2-Oxides from *O*-(2-Ethylphenyl) *O*,*O*-Diphenyl Phosphate**

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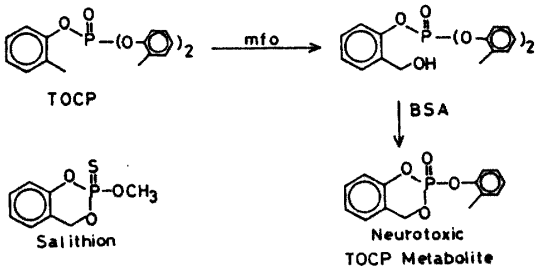
Summary

O-(2-Ethylphenyl) *O*,*O*-diphenyl phosphate and *O*,*O*-di(2-ethylphenyl) phenylphosphonate are metabolized in rats and mice to give its 2-acetophenyl derivatives, 2-(α-hydroxyethyl)phenol, and small amounts of 4-alkylidene-1,3,2-benzodioxaphosphorin 2-oxides. The alkylidene derivatives are both cyclic and enol phosphates, thereby combining some structural features and biological properties of 2-substituted-benzodioxaphosphorin 2-oxides and 2-chloro-1-(substituted-phenol)ethenyl dialkyl phosphate (e.g., the insecticide tetrachlorvinphos). The toxicity and esterase inhibition are attributable to the reactivity of the P-O-aryl bond of salioxon, but the high biological activity of 4-alkylidene derivative is probably due to their action in phosphorylating esterases or proteases by the P-O-ethenyl bond cleavage, because the alkylidene cyclic phosphonates yielded *O*-(2-alkylketophenyl) *O*-methyl phenyl phosphonates on alkaline methanolysis.

Introduction

The saligenin cyclic phosphates were first reported by Casida and coworkers in 1961[1]. Tri-*o*-cresyl phosphate(TOCP) is first oxidized by the mixed-function oxidase system to give a hydroxymethyl derivative. In the presence of albumin, the hydroxymethyl compound cyclizes to form the neurotoxic TOCP metabolite, a saligenin cyclic phosphate as shown in Graph 1. This compound served as a model



Graph 1

** The study is related to the previous paper in this issue.

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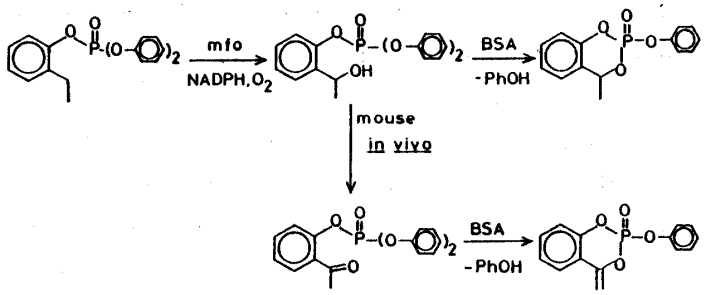
Sci. Bull. Coll. Agr. Univ. Ryukyus, **30** : 217～226 (1983)

for development of an insecticide without delayed neurotoxic properties[5]. Salithion was reported by Eto *et al.* in 1963[5,6]. The studies on TOCP, just described, have analogy in possible metabolic activation reactions of tri-*o*-ethylphenyl phosphate. This compound probably undergoes metabolic hydroxylation at the benzylic methylene position. The intermediate α-hydroxyethylphenyl compounds might cyclize, as shown in Graph 2, based on speculation by Eto in 1962[4]. It might also undergo oxida-



Graph 2

tion to the acetophenyl compound according to the finding of Eto and coworkers in 1971, who found formation of *p*-acetyl-phenyl-phosphorus esters from the analogous *p*-ethylphenyl phosphorus compound[2,3]. It appears possible that an alternative method for formation of a potent phosphorylating agent might be as shown in Graph 2, that is, an enolization with loss of *o*-ethylphenol. The author has examined these metabolic reactions using *o*-ethylphenyl diphenylphosphonate in a series of *in vitro* and *in vivo* reactions to establish the possibility of metabolic activation of *o*-ethylphenyl phosphate.

This paper considers the reactivity and possible metabolic formation of these cyclic enol phosphates.

Experimental Section

1. Chromatography and spectroscopy

Thin-layer chromatography (TLC) utilized silica gel 60 F-254 (20 × 20 cm) chromatoplates with 0.25 mm layer thickness and the following solvent systems: dichloromethane, chloroform-dichloromethane (1:1) and hexane-acetone (7:2). Products on TLC plates were detected by UV and by 2,6-dichloroquinone-4-chloroimide or vaniline-sulfuric acid spray reagent. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined with a Perkin-Elmer Model R 32-B spectrometer at 90 MHz or a Nicolet NT-180 spectrometer at 180 MHz by using $CDCl_3$ for nonpolar compounds with tetramethylsilane as the internal standard (δ =0). ^{31}P NMR spectra were obtained at ambient temperature with a UCB-180 spectrometer. Chemical

ionization-mass spectra(CI-MS) were obtained on the Finnigan 1015D mass spectrometer by using a direct introduction probe and isobutane as the reagent gas at a source pressure of 0.5 torr.

2. Synthesis of *O*-(2-alkylphenyl) *O,O*-diphenyl phosphates (1,2,4)

The compounds **3,5,14,15,16** and **17** were prepared as previously reported[9]. *O*-(2-Alkylphenyl) *O,O*-diphenyl phosphates were made by adding diphenyl chlorophosphate to equivalent 2-substituted-phenol in 20% NaOH and product isolations were by distillation (**1**) or TLC (**2**) (dichloromethane-acetone 9:1). *O,O*-Di(2-ethylphenyl) phenylphosphonate (**4**) was prepared in an analogous manner from phenylphosphonyl dichloride and two equivalents of 2-ethylphenol with TLC purification.

3. 2-Phenoxy- or 2-pheny-1,3,2-benzodioxaphosphorin 2-oxide (11,12)

Phenylphosphonic dichloride or phenyl dichlorophosphate (0.02 mol) was slowly added to 2-hydroxybenzyl alcohol (0.02 mol) in pyridine (50 ml) at 0°C. After stirring 2 hr at 0°C, filtration and evaporation of excess pyridine, the product was dissolved in chloroform and washed twice each with 0.01 N HCl and water. Drying (Na_2SO_4), solvent evaporation and recrystallization from methanol gave **11** in 50% yield and **12** in 80% yield. **11**: mp 75-76°C; ^{1}H NMR ($CDCl_3$) δ5.33-5.36 (dd,2H,J_{H_a-P}= 13 Hz, J_{H_b-P}=17 Hz); ^{13}C NMR ($CDCl_3$) δ69.3 (d, J_{C-P}=7.4 Hz); ^{31}P NMR ($CDCl_3$) δ-18.3; CI-MS, m/z (relative intensity) 291 $(M+29)^+$(9), 263 $(M+1)^+$(100). **12**:mp 149-150°C; ^{1}H NMR ($CDCl_3$) δ5.60-4.92 (dq,2H,$J_{H_a-H_b}$=14 Hz, J_{H_a-P}=10 Hz, J_{H_b-P}=19 Hz); ^{13}C NMR ($CDCl_3$) δ66.3 (d, J_{C-P}=6.8 Hz); ^{31}P NMR ($CDCl_3$) δ10.5; CI-MS, m/z (relative intensity) 275 $(M+29)^+$(13), 274 $(M+1)^+$(100).

4. 2-Phenyl-4-methyl-1,3,2-benzodioxaphosphorin 2-oxide (13)

2-Hydroxyacetophenone (0.1 mol) in ethanol (50 ml) was added dropwise to sodium borohydride (0.1 mol) in ethanol (200 ml). The mixture was refluxed 5 hr and the solvent evaporated. The solid residue was dissolved in 0.1 N HCl (100 ml) and chloroform (100 ml) added for extraction of 2-(α-hydroxyethyl) phenol, after drying (Na_2SO_4) and solvent evaporation: ^{1}H NMR ($CDCl_3$) δ5.02 (q,1H), 1.53 (d,3H, J=7 Hz); CI-MS, m/z (relative intensity) 139 $(M+1)^+$(6), 138 (17), 121 $(M-H_2O)^+$(83). This phenol (without further purification) in excess pyridine was slowly treated with phenylphosphonic dichloride in an analogous manner to **12** above with final isolation by column chromatography (silica gel, dichloromethane) giving an oil in 45% yield. **13** has *cis* and *trans* isomers relative to the C-Me and P=O substituents. The *cis* isomer is tentatively assigned the lower field NMR signals. ^{1}H NMR ($CDCl_3$) δ, *cis* 5.92-5.32 (m,1H), 1.84 (d,3H, J=7 Hz), *trans* 5.92-5.32 (m,1H), 1.82 (d,3H, J=7 Hz); ^{13}C NMR ($CDCl_3$) δ, *cis* 76.9 (d, J_{C-P}=6.8 Hz), 23.0(d, J_{CH_3-P}= 4.4 Hz), *trans* 72.6 (d, J_{C-P}=7.2 Hz), 19.1 (d, J_{CH_3-P}=10.5 Hz); ^{13}P NMR ($CDCl_3$)

δ, *cis* 10.8, *trans* 8.6; CI-MS, m/z (relative intensisy) 289 $(M+29)^+$ (34), 261 $(M+1)^+$ (50), 121 (100).

5. *O*-(2-Alkylketophenyl) or *O*-(2-hydroxybenzyl) *O*-methyl phenylphosphonates

Several cyclic phosphonates (**12-16**) (100 mg) were individually subjected to methanolysis in a mixture of 2 ml each of Et_3N, methanol and chloroform. After 2 hr at 25°C, TLC (dichloromethane) revealed starting material, the initial cleavage product (**6-10**) and the corresponding free phenol. Phenylphosphonate derivatives **6-10** were isolated by preparative TLC (hexane-acetone 7:2).

6. Reaction of cyclic phosphorus esters with chymotrypsin (Xtr)

Xtr (3X crystallized, Sigma) (1 mg, 40 nmol) in 0.01M pH 7.6 sodium phosphate buffer (0.5 ml) was mixed individually with **12-16** in ethanol (10 μl). Following incubation for 15 min at 25°C and addition of 3 ml of the same phosphate buffer, the esteratic activity was assayed with 4-nitrophenyl acetate to determine the level of organophosphorus compound for 50% inhibition.

Total phenolic derivatives liberated durring 15 min of reaction with Xtr (40 nmol) in a buffer (0.5 ml) with 14 (600 nmol) or 12 (50 nmol) (90% inhibition) were analyzed by addition of 3 ml buffer, 0.2 ml aqueous 4-aminoantipyrine (0.2% w/v) and then 0.2 ml aqueous $K_3Fe(CN)_6$ (0.6% w/v) with mixing. After 5 min at 25°C, the absorbance was determined at 510 nm. For comparison, the incubated reaction mixture plus 3 ml buffer were extracted with chloroform (2.5 ml x 2) prior to addition of aminoantipyrine and color development to determine bound phenolics (recovery in aqueous phase after chloroform extraction) and free phenolics (the difference for compounds removed on chloroform extraction). This procedure provides adequate sensitivity for free and bound phenolics released on reaction of Xtr with equimolar **12** or **14**, since the dye from 2-hydroxyacetophenone has a similar specific absorbance to that from 2-hydroxybenzyl alcohol. As a control for spontaneous chemical reactions not dependent on the esteratically-active site, the Xtr was treated with diisopropyl fluorophosphate (DFP, 100 nmol) for 15 min before addition of the cyclic phosphorus compound.

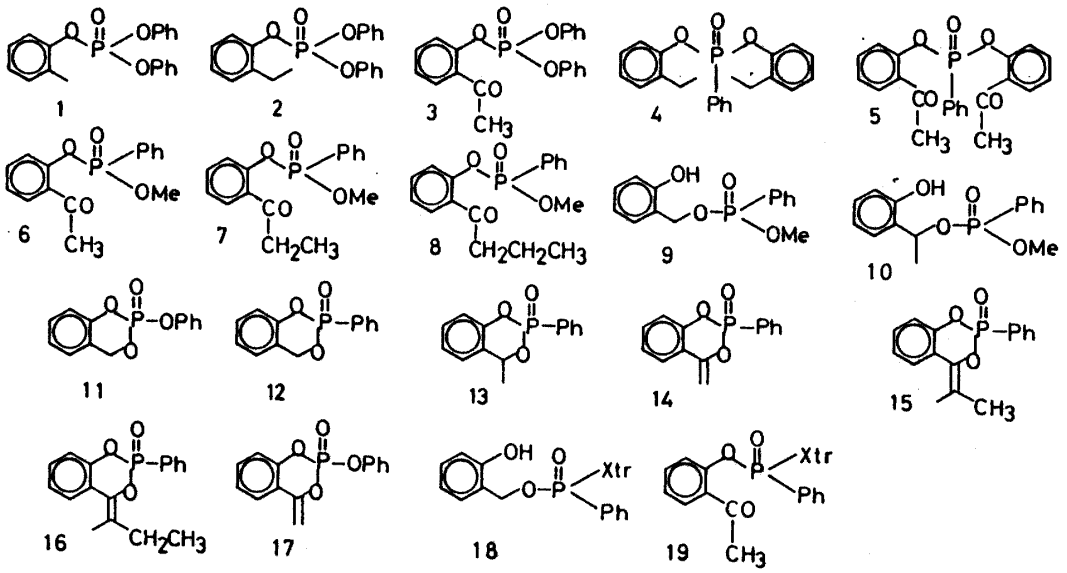
7. Metabolites of triaryl phosphonates and phosphates

Male rats treated orally by stomach tube with **1,2,3** and **4** at 100 mg/Kg were killed after 3 hr, the small intestine and liver were removed, the acetone extract was prepared and dried (Na_2SO_4), and the products were examined by ^{31}P NMR and TLC. Female mice were administered with **2** and **17** intraperitoneally at 1 g/Kg and killed after 30 min for liver analysis in the same manner. Mouse liver microsomes (equivalent to 40 mg fresh weight) in 2 ml 0.1 M phosphate buffer (pH 7.4) were incubated with **2** (0.5 mol) for 30 min at 37°C in the presence and absence of

reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2 μmol). A chloroform extract (dried over Na_2SO_4) was used for TLC examination of the products.

Results and Discussion

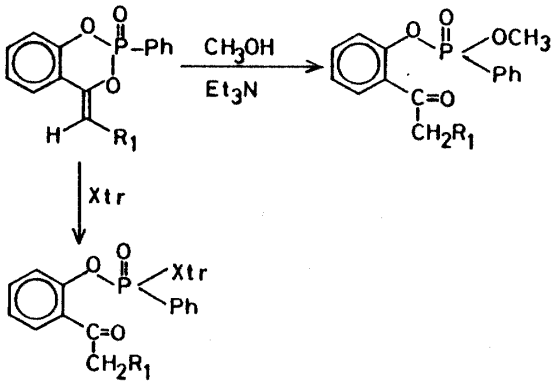
O-(2-Methylphenyl) and *O*-(2-ethylphenyl) *O,O*-diphenyl phosphates, the possible metabolic intermediates and alkylidene cyclic phosphorus compounds were synthesized to elucidate the metabolic formation of 4-alkylidene compounds. The chemical structures of prepared compounds are shown in Graph 3.



Graph 3

1. Phosphorylation reaction of cyclic phosphorus esters with methanol and Xtr

4-Alkylidene-1,3,2-benzodioxaphosphorin 2-oxides are phosphorylating agents which might react by cleavage, either of the P-O-aryl bond as with the salioxon or of the P-O-ethenyl bond. A suitable model reaction involves alkaline methanolysis. The high biological activity[10] of many analogs of 4-alkylidene derivatives is probably due to their action in phosphorylating esterases or proteases for which Xtr is often



Graph 4

an experimentally-convenient example. Cyclic alkylidene phosphonates **14-16** yielded *O*-(2-alkylketophenyl) *O*-methyl phenylphosphonates **6-8** on alkaline methanolysis establishing that phosphorylation involves P-O-ethenyl bond cleavage (Table 1). On the other hand, the P-O-phenyl bond is cleaved on alkaline methanolysis of **12, 13** since *O*-(2-hydroxybenzyl) and *O*-(2-hydroxy-*O*-methylbenzyl) *O*-methyl phenylphosphonates (**9,10**) were obtained (Table 1).

Table 1. *O*-(2-Alkylketophenyl) or *O*-(2-hydroxybenzyl) *O*-methyl phenylphosphonates

Cpds.	^{1}H NMR ($CDCl_3$) δ[a]	^{31}P NMR ($CDCl_3$) δ	CI-MS, $(M+1)^+$, m/z (rel. int.)	
			parent[b]	cyclic[c]
6.	3.92(d,3H,J=13), 2.59(s,3H)	14.8	291 (100)	259 (25)
7.	3.87(d,3H,J=13), 2.90(q,2H), 1.18(t,3H)	14.7	305 (100)	273 (45)
8.	3.89(d,3H,J=13), 2.88(t,2H), 1.73(m,2H), 0.90(t,3H)	14.7	319 (100)	287 (43)
9.	4.46-4.25(m,2H), 3.45(d,3H,J=12)	11.6		
10.	6.00-5.10(m,1H), 3.56(d,3H,J=12), 1.60(q,3H)	11.8		

* a Aliphatic substituents only.
b Prominent $(M+29)^+$ of **6-8**.
c Cyclic product from loss of methanol. $(M+29)^+$ of cyclic product also evident with **7**.

Cyclic phosphorus esters **11,12** are more potent inhibitors of Xtr than their analogs with 4-alkylidene substituents (**14-16**) or a 4-methyl group (**13**) (Table 2). It appeared possible that not only the potency but also the phosphorylation mechanism might change on introducing the 4-alkylidene substituent. Accordingly, **12** and **14** reacted with Xtr to obtain 90% inhibition of esteratic activity and the liberated phenolic compounds were analyzed as their aminoantipyrine derivatives. Phenyl phosphate cleavage on reaction of **12** with Xtr yields *O*-(2-hydroxy benzyl) phenylphosphonyl enzyme (**18**) in the protein-bound phenolic fraction and 2-hydroxybenzyl alcohol both protein-bound and free (differentiated by chloroform extraction). In

Table 2. Inhibitory potency of cyclic phenylphosphonates for Xtr.

Cpds.	[M] for 50% inhibition
11.	9×10^{-7}
12.	1×10^{-6}
13.	7×10^{-6}
14.	1×10^{-5}
15.	5×10^{-4}
16.	1×10^{-6}

contrast, **14** reacts with Xtr to yield chloroform-extractable 2-hydroxyacetophenone but no protein-bound phenolic compounds, i.e., on *O*-[1-(2-hydroxyphenyl)ethenyl] phenylphosphonyl enzyme or protein-bound 2-hydroxyacetophenone. These reactions of both **12** and **14** are blocked by DFP, indicating the involvement of the esteratically-active site. It appears likely that **14** phosphorylates Xtr by P-O-enol cleavage to form **19**, thereby paralleling its methanolysis reaction.

2. Metabolic formation of cyclic phosphorus esters

Cyclic phosphate **11** is formed metabolically in rats from TOCP by methyl hydroxylation and cyclization with release of 2-methylphenol (Table 3). The cyclization

Table 3. ^{31}P NMR Analysis of triaryl phosphonates and phosphates and their metabolites in rat small intestine and liver

	^{31}P NMR ($CDCl_3$)				
		metabolites[a]			
cpds. admin.	parent cpds.	cyclic	other		
1.	−19.8[c]	−18.3 (**11**)[b]	−20.3,	−20.1,	−19.7, −17.9
2.	−20.7[c]	−23.0 (**17**)[b]	−20.4,	−20.1,	−14.0
3.	none	−22.9 (**17**)[b]	−21.9,	−18.9,	−9.2
4.	8.7[a]	10.8 (**13**,*cis*)[bd]	−11.3		

*a Detected in small intestine but not liver.
b Trace levels but sufficient for clear signal.
c Detected both liver and small intestine.
d trans isomer, if present, would overlap and be obscured by parent compound.

reaction to yield **11** is catalyzed by albumin[7], e.g., bovine serum albumin (BSA). It appears that 2-ethylphenyl derivatives **2** or **4** might undergo metabolic conversion, *via* α-hydroxyethylphenyl and acetophenyl derivatives, to either **13**,**14** or **17**, respectively, depending on the intermediate cyclization. The conversion in rats of **1** to **11**, anticipated from earlier studies on TOCP[1,4], was established in the present investigation by ^{31}P NMR (Table 3). Thus, acetone extracts of both the small intestine and liver 3 hr after an oral dose of **1** revealed the parent compound (^{31}P NMR δ-19.8 ppm) but only with the small intestine extract was **11** also evident (^{31}P NMR δ-18.3 ppm). This finding establishes the utility of ^{31}P NMR monitoring in studies on metabolic formation of cyclic phosphorus esters.

An analogous experiment with **4** administered orally to rats revealed the parent

compound and possibly the cyclic phosphate metabolite **13** in the intestine but not in the liver (^{31}P NMR δ10.8 ppm appropriate for *cis*-**13** but the position for the *trans*-**13** signal was obscured). Appropriate TLC properties in three solvent systems are shown in Table 4.

More extensive investigations were made with **2** seeking **17** and possible intermediates in its formation. *In vivo* studies in rats revealed the parent compound in the liver and intestine and **17** in trace amounts in the intestine (^{31}P NMR δ-23.0 ppm (Table 3); appropriate TLC values in one solvent system but not detected with two other solvent systems (Table 4). Alkylidene cyclic phosphate **17** was also estabilised by ^{31}P NMR and TLC in the small intestine of rats treated orally with **3** (Table 4). The analogous 4-methyl compound (the PhO analog of **13**) was not

Table 4. TLC Analysis of triaryl phosphonates and phosphates and their metabol- metabolites in rat small intestine and liver

Cpds.	TLC Rf[a] CH_2Cl_2	CH_2Cl_2 - $CHCl_3$ (1:1)	hexane-acetone(7:2)
Triaryl phosphonates and phosphates			
1.	0.29[c]	0.36[c]	0.23[c]
2.	0.32[c]	0.39[c]	0.26[c]
3.	0.17[b]	0.19[b]	0.13[b]
4.	0.22[c]	0.27[c]	0.25[c]
5.	0.03	0.04	0.05
Cyclic phosphonates and phosphates			
12.	0.13[e]	0.16[e]	0.08[e]
13. *cis*	0.07[f]	0.09[f]	0.11[f]
13. *trans*		0.06[f]	0.06[f]
14.	0.13	0.18	0.09
17.	0.27[d]	0.31	0.13
Phenols			
unsubst.	0.18	0.17	0.17
2-Me	0.22	0.26	0.26
2-Et	0.32	0.33	0.27
$2-HOCH_2$	0.04	0.03	0.06
2-MeCHOH	0.08[b]	0.07[b]	0.11[b]
2-MeC(O)	0.45	0.50	0.37

*a Silica gel 60 F-254 chromatoplates, 0.25 mm gel thickness.

b Detected as metabolite of **2**.

c Unmetabolized compound detected in small intestine and liver.

d Rf of metabolite of **3** but insufficient TLC evidence for identification. Unknown metabolites of **3** appear at 0.15 in CH_2Cl_2-$CHCl_3$ (1:1) and 0.17 in hexane-acetone (7:2).

e Detected as metabolite of **1**. Unknown metabolites appear at Rf 0.10, 0.07 and 0.06 and 0.03 in hexane-acetone (7:2).

f Detected as metabolite of **2**.

available as a synthetic standard for comparison with ^{31}P NMR signals associated with unidentified metabolites. TLC studies establised the following metabolites of **2**: in *in vivo*; **3**, 2-hydroxyacetophenone and 2-(α-hydroxyethyl) phenol were observed in the liver of mice 30 min after intraperitoneal administration. In *in vitro*; **3**, 2-(α-hydroxyethyl) phenol and phenol were observed in the mouse liver microsome system in the presence, but not in the absence of NADPH. Mice injected with **17** contained no detectable (TLC) parent compound in the liver 30 min later.

The direct observation of toxic metabolites by ^{31}P NMR on extract of tissues may be the first report. Thus, the metabolic formation of the 4-alkylidene derivatives from the 2-ethylphenyl phosphorus compounds is now more completely elucidated using the new ^{31}P NMR technique in this report.

References

1. Casida, J.E., Eto, M. and Baron, R.L. 1961 Biological activity of a tri-*o*-cresyl phosphate metabolite, Nature, **191**: 1396-1397
2. Eto, M. and Abe, M. 1971 Metabolic activation of alkylphenyl phosphates, Biochem. Pharmacol., **20**: 976-969
3. ————————. and Takehara, H. 1971 Metabolism of tri-*p*-ethylphenyl phosphate and neurotoxicity of the metabolites, Agr. Biol. Che., **35**: 929-940
4. ————., Casida, J.E. and Eto, T. 1962 Hydroxylation and cyclization involved in the metabolism of tri-*o*-cresyl phosphate, Biochem. Pharmacol., **11**: 337-352
5. ————., Kinoshita, Y., Kato, T. and Oshima, Y. 1963 Saligenin cyclic methyl phosphate and its thiono analogue: new insecticides related to the active metabolite of tri-*o*-cresyl phosphate, Nature, **200**: 171
6. ————————————————. 1963 Saligenin cyclic alkyl phosphates and phosphorothionates with insecticidal activity, Agr. Biol. Chem., **27**: 789-794
7. ————., Oshima, Y. and Casida, J.E. 1967 Plasma albumin as a catalyst in cyclization of diaryl *O*-(α-hydroxy) tolyl phosphates, Biochem. Pharmacol., **16**: 295-308
8. ————., Sakata, M. and Sasayama, T. 1972 Biological activities of *p*-ethylphenyl and *p*-acetylphenyl phosphates and their thiono analogs, Agr. Biol. Chem., **36**: 645-650
9. Tawata, S., Eto, M.-Y. and Casida, J.E. 1982 [Z]-4-Alkylidene-1,3,2-benzodioxaphosphorinane 2-oxides from stereospecific cyclization of 2-alkylketo-phenyl phosphonates and phosphates, Bioorg. Chem., **11**: 457-462
10. ————————————————. 1982 Insecticidal 4-alkylidene-1,3,2-benzodioxaphosphorinane derivatives, J. Agric. Food Chem., **30**: 198-199

琉球大学農学部学術執告　第30号（1983）

O-(2-Ethylphenyl) *O*,*O*-Diphenyl Phosphate から 4-Alkylidene-1,3,2-benzodioxaphosphorinane 2-Oxide 類の代謝生成

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摘　要

O-(2-Ethylphenyl) *O*,*O*-diphenyl phosphate のラット及びマウス体内での代謝により，2-acetophenyl類，2-(α-hydroxyethyl)phenol，及び少量の4-alkylidene-1,3,2-benzodioxaphosphorin 2-oxide が代謝生成される事を^{31}PNMR 及びTLC法で確認した。最終産物の4-alkylidene 類は環状であると同時にエノールリン酸類の構造も有しており，2- 置換-benzo-dioxaphosphorin 2-oxide(salioxon) 及び2-chloro-1-(substituted-phenol) ethenyl dialkyl phosphate（例えば殺虫剤 tetrachlorvinphos）の構造的特徴及び生物活性的性質をかね備えている。毒性発現及びエステラーゼ阻害はsalioxon の場合，P-O-aryl 結合の反応性によるが，4-alkylidene 類の高い活性発現は，アルカリ加水分解によりP-O-ethenyl 結合が切れる事から，おそらくこの部位で酵素をリン酸化するためと思われた。

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