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Studies on the Urinary Metabolites of Isophosphamide and Its Activated Species in Rabbits[1,2]

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Investigations on the urinary metabolites of isophosphamide, 4-hydroxyisophosphamide and 4-hydroperoxyisophosphamide including stereoisomers of the C_4-oxidized derivatives in rabbits revealed that their metabolic behaviors were different from each other and also form those of cyclophosphamide. Administration of isophosphamide to rabbits resulted in the urinary excretion of carboxyisophosphamide and two N-dechloroethylated metabolites besides considerable amount of unchanged isophosphamide, while 4-hydroxyisophosphamide was metabolized principally into carboxyisophosphamide. In the case of 4-hydroperoxyisophosphamide, carboxyisophosphamide was excreted as a major metabolite, but considerable amount of a new metabolite which might be produced from 4-ketoisophosphamide *via* a hitherto unknown pathway was also excreted besides small amount of 4-ketoisophosphamide. Mechanism of the formation of this new metabolite was proposed based on the chemical conversion of a suggested intermediate into the metabolite. Phosphorus configuration of the C_4-oxidized isophosphamides was found to have no significant effect upon their metabolism. It was suggested that the results of the present studies could account for the great differences in *in vivo* antitumor activities between isophosphamide and its pre-activated derivatives and also between isophosphamide and cyclophosphamide.

Keywords——cyclophosphamide; antitumor agent; *in vivo* oxidation; 1,3,2-oxazaphosphorinane; 1,3,2-oxazaphospholidine; nucleophilic substitution at phosphorus; ring transformation

The antitumor agent cyclophosphamide (NSC 26271) (**1**), a representative of the nitrogen mustard alkylating agents having 1,3,2-oxazaphosphorinane ring, is activated *in vivo* to a cytotoxic species after enzymatic C_4-hydroxylation of the ring.[4] Isophosphamide (NSC 109724) (**2**) is a cyclophosphamide analogue differing only in the position of alkylating functionalities and its activation mechanisms have been expected to resemble those for cyclophosphamide.[5-7] Recently, we have synthesized the C_4-oxidized cyclophosphamide and isophosphamide derivatives which exhibited pronounced antitumor activities in both *in vivo* and *in vitro* experiments, confirming that the C_4-hydroxylation is an essential step for the activation of these drugs.[2,8] Although isophosphamide shows considerable activity against experimental

1) This paper forms Part VI of Studies on Cyclophosphamide Metabolites and Their Related Compounds. Part V: A. Takamizawa, S. Matsumoto, T. Iwata, and I. Makino, *Chem. Pharm. Bull.* (Tokyo), **25**, 1877 (1977).
2) Preliminary account of this work was reported partly in A. Takamizawa, S. Matsumoto, T. Iwata, Y. Tochino, K. Katagiri, K. Yamaguchi, and O. Shiratori, *J. Med. Chem.*, **17**, 1237 (1974). A part of this work was also presented at the Symposium on Metabolism and Mechanism of Action of Cyclophosphamide (London, July 1975) of which proceedings appeared in *Cancer Treatment Reports*, **60**, 361 (1976).
3) Location: *Fukushima-ku, Osaka 553, Japan.*
4) A.R. Torkelson, J.A. LaBudde, and J.H. Weikel, Jr., *Drug. Metabolism Reviews*, **3**, 131 (1974), and references cited therein.
5) L.M. Allen and P.J. Creaven, *Cancer Chemother. Rept. Part I*, **56**, 603 (1972).
6) R.A. Alarcon, J. Meienhofer, and E. Atherton, *Cancer Res.*, 32, 2519 (1972).
7) D.L. Hill, W.R. Laster, Jr., M.C. Kirk, S. ElDareer, and R.F. Struck, *Cancer Res.*, 33, 1016 (1973).
8) *a)* A. Takamizawa, S. Matsumoto, T. Iwata, K. Katagiri, Y. Tochino, and K. Yamaguchi, *J. Am. Chem. Soc.*, **95**, 985 (1973); *b)* A. Takamizawa, S. Matsumoto, T. Iwata, Y. Tochino, K. Katagiri, K. Yamaguchi, and O. Shiratori, *J. Med. Chem.*, **18**, 376 (1975).

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animal tumors, it has been reported to show no increased antitumor effect in clinical trials.[9] Our recent preclinical experiments, however, have revealed that enhancement of activity by the C_4-oxidation was much greater for isophosphamide than for cyclophosphamide.[2,8] Other workers have indicated by *in vitro* experiments that isophosphamide differed from cyclophosphamide in the activation kinetics[5] and also in the pattern of formation of acrolein[6] which is a possible metabolic fragment produced after C_4-hydroxylation.[10] Considering these results, the observed lower antitumor effect of isophosphamide might be attributed to the less efficient *in vivo* C_4-oxidation due to steric hindrance by 2-chloroethyl substituent at the ring nitrogen atom. It is therefore of interest to investigate the *in vivo* metabolism of isophosphamide. This paper is concerned with the comparative studies on the urinary metabolites of isophosphamide and its C_4-oxidized derivatives in rabbits, which reveal that the isophosphamide metabolism is greatly different from that of cyclophosphamide producing considerable amount of N-dechloroethylated metabolites *via* pathways which should be ineffective for its activation and that 4-hydroperoxyisophosphamide is partly metabolized *via* a hitherto unknown pathway producing a novel five-membered metabolite.

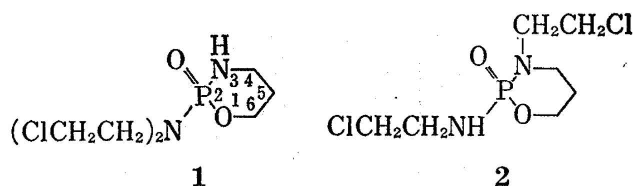

Chart 1

Urinary Metabolites of Isophosphamide

Isophosphamide (2) (6.2 g) in saline was subcutaneously administered with 200 mg/kg to ten rabbits (♂, 2—3.5 kg), and their urine was collected after 24 hr. Thin-layer chromatography (TLC) of the urine on a silica gel plate (precoated with Kieselgel 60 F-254, Merck) in acetone revealed the presence of four components ($Rf<0.1$, Rf 0.15, Rf 0.19 and Rf 0.37) which gave positive Epstein test.[11] The collected urine (2.2 liters) was divided into two equal volumes (1.1 liters) and the each half of the urine were treated separately according to the procedures A and B as described in Fig. 1. The most polar metabolite ($Rf<0.1$) which could be isolated according to the procedure A was carboxylisophosphamide (**3**) and was identified by converting into a crystalline 4-phenylphenacyl ester (**6**) (mp 106—109°) with an authentic synthetic specimen. Separation of other metabolites which are less polar than **3** could be performed by the procedure B, giving unchanged isophosphamide (**2**) (Rf 0.37) and two N-dechloroethylated metabolites **4** (mp 99—100°, Rf 0.19) and **5** (mp 109—110°, Rf 0.15). The metabolite **4** was convertible into a readily crystallizable acetate **4'** (mp 116—118°) by treating with acetyl chloride and pyridine. The structures of these metabolites **4** and **5** including the acetate **4'** were confirmed by synthesis (see Experimental).

In contrast to the cyclophosphamide metabolism in rabbits[8] which excreted 4-ketocyclophosphamide and carboxycyclophosphamide in urine, it is notable that isophosphamide was metabolized into carboxyisophosphamide and N-dechloroethylated metabolites with excretion of considerable amount of unchanged isophosphamide. This suggests that the *in vivo* C_4-oxidation of isophosphamide in rabbits might be less rapid than that of cyclophosphamide, therefore oxidation of the exocyclic side-chain carbons might concurrently occur to give N-dechloroethylated metabolites. The metabolic pathways producing N-dechloroethylated metabolites are considered to be ineffective for the activation of isophosphamide because such metabolites are cytostatically inactive,[12] which might be responsible for the lower antitumor effect of iso-

9) *a*) D.L. Ahmann, H.F. Bisel, and R.G. Hahn, *Cancer Chemother. Rept. Part I*, **58**, 861 (1974); *b*) D.N. Bremner, J. St. C. McCormick, and J.W.W. Thomson, *ibid.*, **58**, 889 (1974).
10) R.A. Alarcon and J. Meienhofer, *Nature New Biol.*, **233**, 250 (1971).
11) J. Epstein, R.W. Rosenthal, and R.J. Ess, *Anal. Chem.*, **27**, 1435 (1955).
12) K. Norpoth, G. Müller, and H. Raidt, *Arzneim. Forsch.*, **26**, 1376 (1976).

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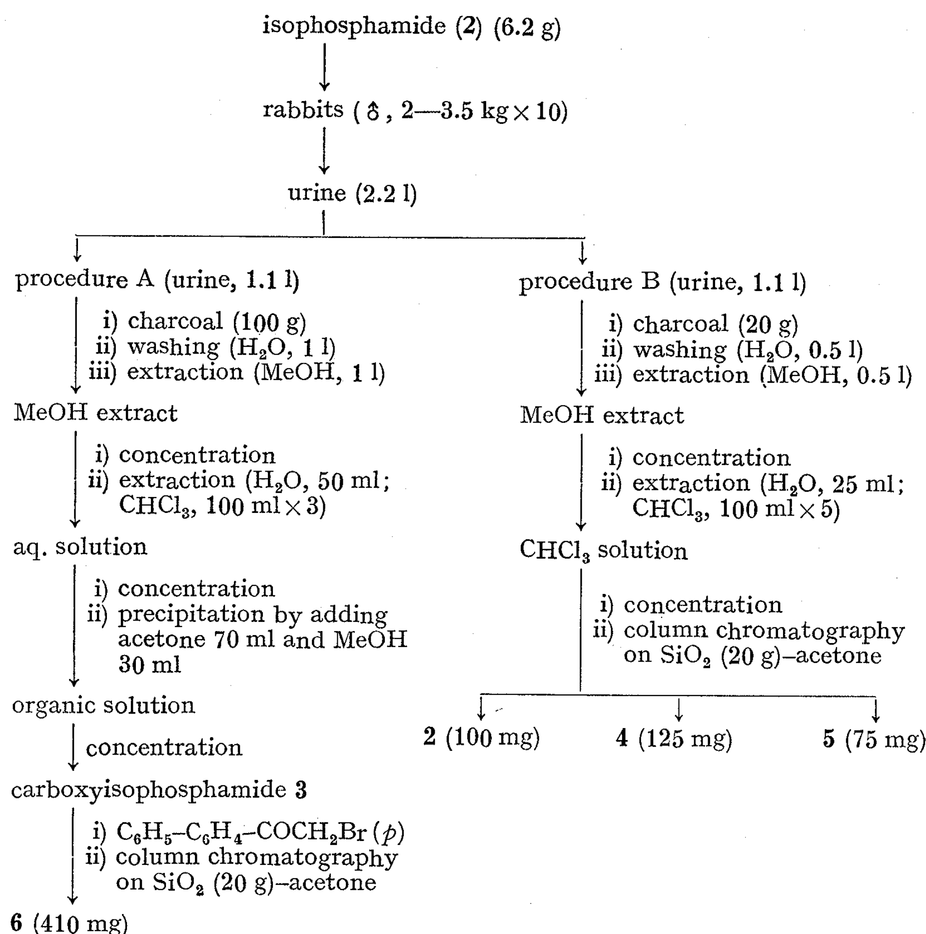

Fig. 1. Isolation Procedures for the Isophosphamide Metabolites

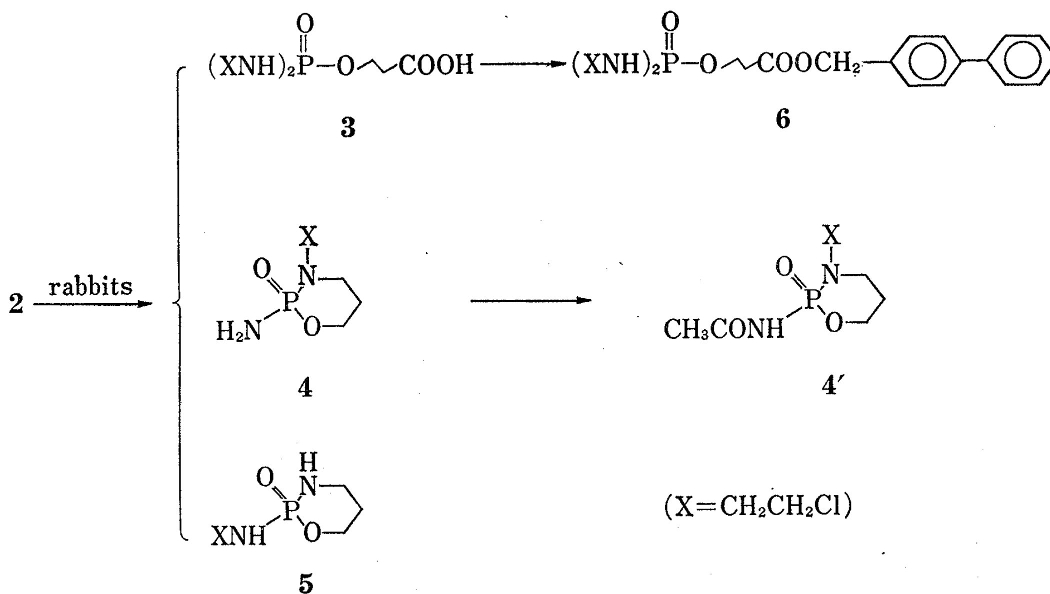

Chart 2

phosphamide. Recently, urinary excretion of N-dechloroethylated isophosphamide metabolites was observed in man,[12] and formation of such metabolites was also reported in the microsomal oxidation of isophosphamide *in vitro*.[13] 4-Methylcyclophosphamide was also known to

13) T.A. Conners, P.J. Cox, P.B. Farmer, A.B. Foster, and M. Jarman, *Biochem. Pharmacol.*, 23, 115 (1974).

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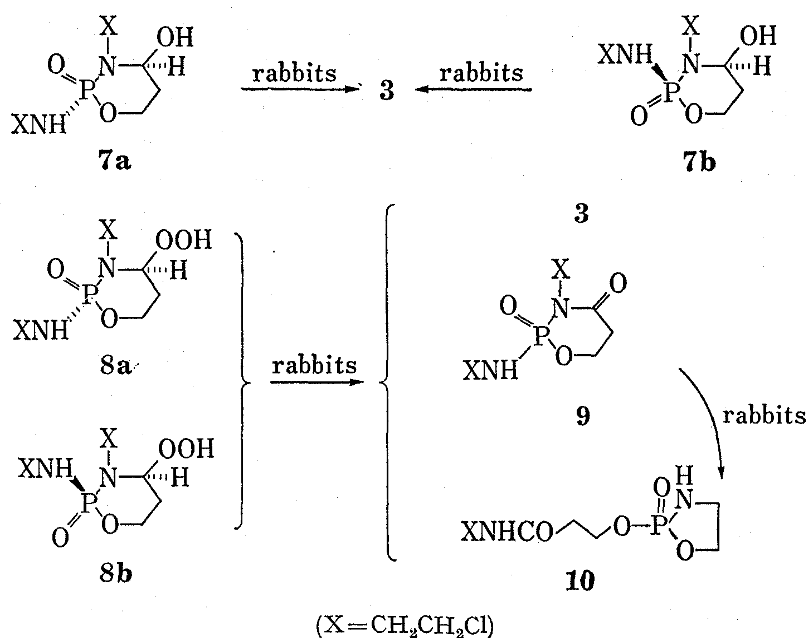
produce N-dechloroethylated metabolite, while 6-methyl analogue produced principally C_4-oxidized metabolite *in vitro*.[14] These observations including ours suggest that N-dechloroethylation might occur when C_4-position is sterically hindered, although cyclophosphamide has also been reported to be N-dechloroethylated in sheep.[15]

Urinary Metabolites of the C_4-Oxidized Isophosphamides

Chemically synthesized 4-hydroxyisophosphamide (**7a**)[2] and its stereoisomer 2-*epi*-4-hydroxyisophosphamide (**7b**)[16] were administered to rabbits and their urine was similarly treated according to the procedures described in Fig. 1. The TLC patterns of the urine were found to be equal for both isomers **7a** and **7b**, but were different from those of isophosphamide, showing only the presence of a single metabolite which was slightly mobile on silica gel in acetone ($Rf<0.1$) and gave alkylating activity on the Epstein test. By the procedure A, as expected, carboxyisophosphamide (3) was isolated as the 4-phenylphenacyl ester **6**, while no metabolite could be isolated by the procedure B. In the cases of 4-hydroperoxyisophosphamide (**8a**)[2] and 2-*epi*-4-hydroperoxyisophosphamide (**8b**),[16] both of which are the stabilized derivatives of **7a** and **7b** and give high antileukemic activities almost comparable to those of **7a** and **7b**, carboxyisophosphamide (3) was isolated as a major metabolite by the procedure A, and a small amount of 4-ketoisophosphamide (9) and a considerable amount of a new metabolite **10** (mp 159—161°; Rf 0.19, SiO_2-acetone) were isolated by the procedure B. Approximate ratios of the amounts of the isolated metabolites were **3**: **9**: **10**=40: 1: 8 for both isomers **8a** and **8b**. The metabolite 4-ketoisophosphamide (9) was identified with a synthetic specimen,[17] while the metabolite **10** was found to be a hitherto unknown compound whose structure was elucidated to be [N-(2-chloroethyl)-3-(2-oxo-1,3,2-oxazaphospholidin-2-yl)oxy]propionamide based on chemical and spectroscopic evidences (*vide infra*). Interestingly, administration of 4-ketoisophosphamide (9) to rabbits resulted in the excretion of **10** as a sole isolable metabolite, indicating that **9** might be a precursor for **10**.



($X=CH_2CH_2Cl$)

Chart 3

14) P.J. Cox, P.B. Farmer, and M. Jarman, *Biochem. Pharmacol.*, 24, 599 (1975).
15) J.E. Bakke, V.J. Feil, C.E. Fjelstul, and E.J. Thacker, *J. Agr. Food Chem.*, 20, 384 (1972).
16) A. Takamizawa, S. Matsumoto, T. Iwata, and I. Makino, *Heterocycles*, 3, 787 (1975).
17) See the reference cited in footnote 1).

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Elemental analysis of the metabolite **10** agreed with a formula $C_7H_{14}ClN_2O_4P$, which corresponds to the structure that one chlorine atom of 4-ketoisophosphamide ($C_7H_{13}Cl_2N_2O_3P$) is replaced by an OH group. However, its infrared (IR) spectrum in nujol mull showed strong bands at 1659 and 1563 cm^{-1} which are indicative of the presence of a secondary amide group (–CONH–), while 4-ketoisophosphamide showed a carbonyl band at 1690 cm^{-1}. The nuclear magnetic resonance (NMR) spectrum of **10** in DMSO-d_6 solution showed two H–D exchangeable broad peaks at δ 5.17 and δ 7.63 corresponding to PO–NH and CO–NH protons respectively, besides multiplets of twelve protons between δ 2.0—4.6. These spectral data are agreeable with the assigned structure which was unequivocally confirmed by synthesis. The synthesis of this metabolite was performed as follows. N-(2-Chloroethyl)-3-hydroxypropionamide **13** was prepared from 3-benzyloxypionyl chloride **11** *via* the the synthetic pathway described in Chart **4**. Triethylamine-mediated reaction of phosphoryl chloride with

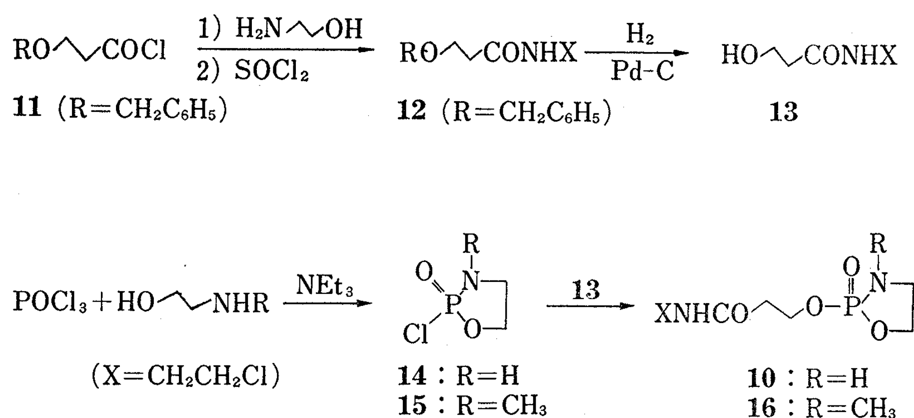

Chart 4

ethanolamine, followed by treatment of the resulting 2-chloro-1,3,2-oxazaphospholidine-2-oxide (**14**) with the compound **13**, afforded a five-membered product **10** which was identical with the metabolite in all respects (see Experimental). As a plausible mechanism of the formation of this metabolite **10** one can suppose that 4-ketoisophosphamide (9) might be first converted into an intermediate **17**, which undergoes intramolecular nucleophilic displacement reaction at phosphorus atom as illustrated in Chart 5. Such intramolecular displacement reaction at phosphorus atom, however, seems to be somewhat anomalous when one assumes a pentacoordinate bipyramidal intermediate,[18] while direct substitution mechanism as shown in Chart 5 appears more plausible because the carbonyl group at C_4-position will greatly assist the fission of the ring P–N bond as found for the ring opening reaction of 4-keto-1, 3,2-oxazaphospholidine-2-oxides.[19]

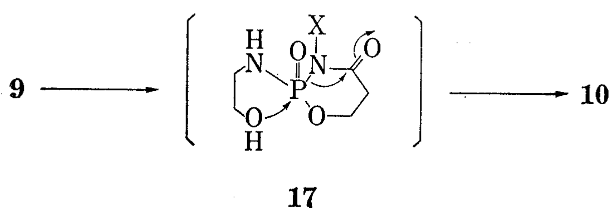

Chart 5

The *in vivo* conversion of the proposed intermediate **17** into **10** could also be demonstrated chemically as follows. Ozonolysis of O-(3-butenyl)-N-(2-chloroethyl)-N'-(2-benzyloxyethyl)phosphorodiamidate (**18a**), followed by treatment with ferrous sulfate, afforded the lactam **19a**. Catalytic hydrogenation of **19a** over palladium-charcoal gave the alcohol

18) F.H. Westheimer, *Accounts Chem. Res.*, **1**, 70 (1968).
19) M. Mulliez, *Tetrahedron Lett.*, **1974**, 2351.

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17 which was treated with aqueous NaOH at 0° to give readily the metabolite 10. N-Methyl analogue 20, which was similarly prepared from 18b *via* 19b, also produced readily a recyclized product 16 on alkali treatment, and the structure of the product was confirmed by synthesis from 15 and 13 as described in Chart 4 (see Experimental).

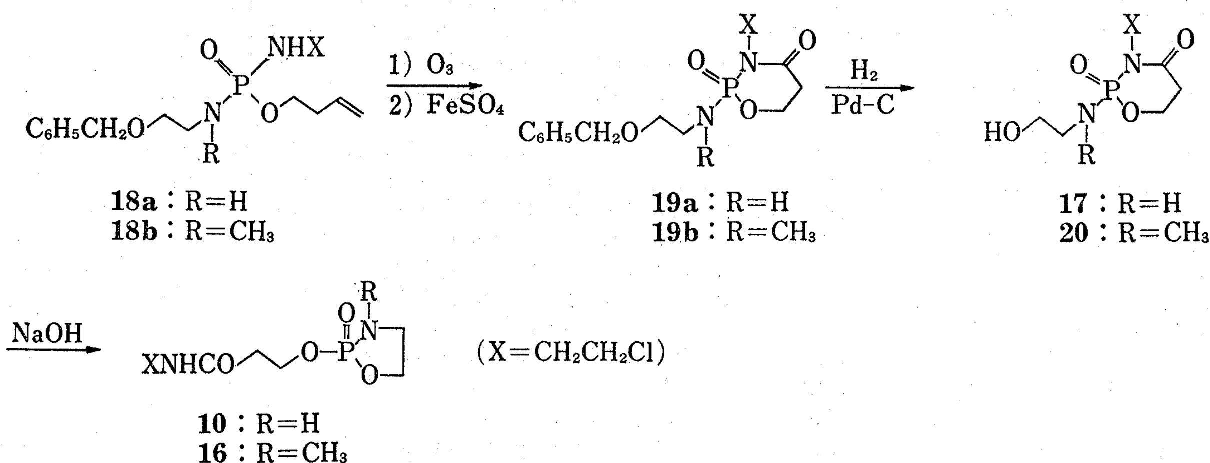

Chart 6

The experimental results described so far led us to the following conclusions: a) rabbits oxidize isophosphamide at C_4-position, but oxidation at side-chain carbons concurrently occurs to give N-dechloroethylated metabolites; b) isophosphamide is less rapidly metabolized than cyclophosphamide in rabbits, excreting considerable amount of unchanged isophosphamide in urine; c) the metabolism of 4-hydroxyisophosphamide differs from that of isophosphamide in respect that the former gives carboxyisophosphamide as a only metabolite; d) 4-hydroperoxyisophosphamide is metabolized into carboxyisophosphamide, but it is partly metabolized into 4-ketoisophosphamide which is further metabolized into a new five-membered compound 10; e) stereochemistry of the alkylating group at phosphorus atom in both 4-hydroxy and 4-hydroperoxyisophosphamide has no significant effect upon their metabolic behaviors. Among these findings, the fact that no N-dechloroethylated compouhd was isolated as the metabolite of C_4-oxidized isophosphamides is of particular interest, because this seems to account for the greater *in vivo* antitumor effect of the pre-oxidized isophosphamides.

Experimental

Melting points were determined in open glass capillary tubes using a Yamato MP-1 apparatus and were uncorrected. IR data were determined with a JASCO IRA-1 spectrometer in nujol mull or in film. NMR data were determined with a Varian Model A-60 spectrometer using tetramethylsilane as an internal standard. Column chromatography was carried out on silica gel (Kieselgel 60, Merck). TLC chromatography was carried out on precoated silica gel plate (Kieselgel 60 F-254, 0.25 mm, Merck).

Isolation of the Urinary Metabolites of Isophosphamide (2)——a) Carboxylisophosphamide (3): The rabbits urine (1.1 l) was treated according to the procedure A described in Fig. 1, and the organic solution containing carboxyisophosphamide was allowed to react with 4-phenylphenacyl bromide (1.5 g) and triethylamine (3.0 g) for 4 hr at room temperature. The mixture was concentrated *in vacuo* after standing overnight at room temperature, then the resulting residue was extracted with $CHCl_3$ (30 ml × 3). The $CHCl_3$ extracts were combined, washed with water, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (2.5 × 8 cm) eluting with acetone, giving the 4-phenylphenacyl ester 6 as a crystalline solid (410 mg). Recrystallization of the solid with acetone–ether gave colorless prisms, mp 106—109°; IR ν_{max}^{Nujol} cm^{-1}: 3230, 3195 (NH), 1765 (CO), 1693 (CO), 1210 (PO), 1178 (COO); NMR ($CDCl_3$) δ: 2.88 (2H, triplet, J=6.0 Hz, $-CH_2COO-$), 3.17—3.80 (10H, multiplet, $ClCH_2CH_2NH \times 2$), 4.38 (2H, quartet, J=6.5 Hz, $PO-O-CH_2-$), 5.43 (2H, singlet, $-CO-CH_2-$), 7.28—8.08 (9H,

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multiplet, aromatic protons). *Anal.* Calcd. for $C_{21}H_{25}Cl_2N_2O_5P$: C, 51.75; H, 5.13; Cl, 14.58; N, 5.75; P, 6.37. Found: C, 51.77; H, 5.28; Cl, 14.70; N, 5.64; P, 6.23.

b) N-Dechloroethylated Metabolites[2-Amino-3-(2-chloroethyl)-1,3,2-oxazaphosphorinane-2-oxide (**4**) and 2-(2-Chloroethyl)amino-1,3,2-oxazaphosphorinane-2-oxide (**5**)]: After treatment of the rabbit urine (1.1 l) according to the procedure B, the $CHCl_3$ solution containing the metabolites **4**, **5** and isophosphamide (**2**) was concentrated *in vacuo*, and the resulting residue was chromatographed on a column (2.5×8 cm) eluting with acetone. From the first eluate, isophosphamide (**2**) (100 mg) was obtained and identified with an authentic specimen by IR comparison. From the next eluate the metabolite **4** was obtained as a solid (125 mg) which was recrystallized from acetone–ether to give colorless prisms, mp 98—100°; IR ν_{max}^{Nujol} cm^{-1}: 3230, 3150 (NH_2), 1240 (PO); NMR ($CDCl_3$) δ: 1.73—2.00 (2H, multiplet, C_5–H), 3.05—3.78 (8H, multiplet, $CH_2\times3$, NH_2), 4.10—4.52 (2H, multiplet, C_6–H). *Anal.* Calcd. for $C_5H_{12}ClN_2O_2P$: C, 30.25; H, 6.10; Cl, 17.86; N, 14.12; P, 15.62. Found: C, 30.40; H, 6.20; Cl, 18.02; N, 14.32; P, 15.41. Acetylation of this metabolite with CH_3COCl and pyridine according to the usual manner gave an acetate **4'**, mp 116—118°; IR ν_{max}^{Nujol} cm^{-1}: 3200, 3160 (NH), 1690 (CO), 1245 (PO); NMR ($CDCl_3$) δ: 1.80—2.40 (2H, multiplet, C_5–H), 2.10 (3H, doublet, J=1.9 Hz, $COCH_3$), 2.80—3.90 (6H, multiplet, $3\times CH_2$), 4.57 (2H, multiplet, C_6–H), 9.17 (1H, broad doublet, J=8 Hz, PO–NH). *Anal.* Calcd. for $C_7H_{14}ClN_2O_3P$: C, 35.50; H, 5.87; Cl, 14.74; N, 11.65; P, 12.92. Found: C, 35.66; H, 5.89; Cl, 15.10; N, 11.60; P, 12.67. From the third eluate **5** was obtained as a crystalline solid (75 mg) of which recrystallization from ether–hexane gave colorless prisms, mp 109—110°; IR ν_{max}^{Nujol} cm^{-1}: 3195 (NH), 1220 (PO); NMR ($CDCl_3$) δ: 1.83 (2H, multiplet, C_5–H), 2.83—3.80 (8H, multiplet, $3\times CH_2$, $2\times NH$), 4.35 (2H, multiplet, C_6–H). *Anal.* Calcd. for $C_5H_{12}ClN_2O_2P$: C, 30.25; H, 6.10; Cl, 17.86; N, 14.12; P, 15.62. Found: C, 30.13; H, 6.16; Cl, 18.03; N, 14.11; P, 15.22.

Isolation of the Urinary Metabolites of the C_4-Oxidized Isophosphamides——a) Metabolite of 4-Hydroxy-isophosphamide (**7a**) and 2-*Epi*-4-hydroxyisophosphamide (**7b**): To twelve rabbits (♂, 2—2.5 kg) was administered **7a** (or **7b**) subcutaneously with 200 mg/kg. Total amount of the administered drug was 2.3 g for each isomer. The urine was collected (1.4 l) 24 hr after administration of the drug, and each half of the collected urine were treated according to the procedures A and B, respectively. Crude carboxyisophosphamide obtained by the procedure A was treated with 4-phenylphenacyl bromide (1.5 g) and triethylamine (3.0 g) in the similar way as described above, giving the ester **6** (95 mg) which was identified with an authentic specimen by IR comparison. By the procedure B, no metabolite giving positive Epstein test could be isolated for both isomers **7a** and **7b**.

b) Metabolite of 4-Hydroperoxyisophosphamide (**8a**) and 2-*Epi*-4-hydroperoxyisophosphamide (**8b**): To sixteen rabbits (♂, 2.5—2.8 kg) was administered **8a** (or **8b**) subcutaneously with 100 mg/kg. Total amount of the administered drug was 4.0 g for each isomer. The collected urine (2 l) was similarly treated according to the procedures A and B. By the procedure A, carboxyisophosphamide was isolated after esterification by 4-phenylphenacyl bromide (1.5 g) and triethylamine (3.0 g) to the ester **6** (240 mg) which was identified with an authentic specimen by IR comparison. The $CHCl_3$ solution fractionated by the procedure B was concentrated *in vacuo*, and the residue was chromatographed on a column (2.5×7 cm) eluting with acetone. From the first eluate 4-ketoisophosphamide (**9**) (mp 118—119°) (4 mg) was isolated and identified with a synthetic specimen[17] by IR comparison. From the second eluate, the metabolite **10** (21 mg) was isolated as a crystalline solid of which recrystallization from EtOH–ether gave colorless prisms, mp 159—160°; IR ν_{max}^{Nujol} cm^{-1}: 3260, 3200, 3080 (NH), 1659, 1563 (CONH), 1225 (PO); NMR (DMSO-d_6) δ: 2.0—4.6 (12H, multiplet, $6\times CH_2$), 5.17 (1H, broad, PO–NH), 7.63 (1H, broad, CO–NH). *Anal.* Calcd. for $C_7H_{14}ClN_2O_4P$: C, 32.78; H, 5.50; Cl, 12.15; N, 10.92; P, 13.83. Found: C, 32.89; H, 5.52; Cl, 11.90; N, 10.85; P, 14.05.

Syntheses of the Metabolites——a) 4-Phenylphenacyl Ester of Carboxyisophosphamide (**3**): O-(2-Benzyloxycarbonyl)ethyl-N,N'-bis(2-chloroethyl)phosphorodiamidate (210 mg, 0.54 mmol), which was prepared by reaction of O-(2-benzyloxycarbonyl)ethylphosphorodichloridate with 2-chloroethylamine according to the procedure described in an earlier report,[20] was dissolved in MeOH (10 ml) and the solution was hydrogenated over 10% Pd-C (100 mg) at room temperature. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* to give an oily residue. The residue was dissolved in acetone (7 ml), and to the solution was added 4-phenylphenacyl bromide 275 mg (1 mmol) and triethylamine (0.2 ml). The mixture, after stirring for 1.5 hr at room temperature, was concentrated *in vacuo* and the residue was extracted with $CHCl_3$ (20 ml) and H_2O (20 ml). The $CHCl_3$ extract was washed with water, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (2×8 cm) eluting with AcOEt. From the AcOEt eluate, the ester **6** was isolated as a crystalline solid (170 mg, 65%) which was recrystallized from acetone–ether to give colorless prisms, mp 109—112°. IR spectrum of this synthetic ester in nujol mull was superimposable with that of the product **6** produced from the metabolite **3**.

b) 2-Amino-3-(2-chloroethyl)-1,3,2-oxazaphosphorinane-2-oxide (**4**): 1,3-Propanediol (152 g, 2 mol) and benzyl chloride (57 g, 0.5 mol) were allowed to react in abs. xylene (50 ml) in the presence of KOH (56 g, 1 mol) for 1.5 hr under stirring at 100°. The reaction mixture was extracted with benzene (200 ml)–water

20) A. Takamizawa, Y. Tochino, Y. Hamashima, and T. Iwata, *Chem. Pharm. Bull.* (Tokyo), **20**, 1612 (1972).

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(200 ml). The benzene layer was dried over Na_2SO_4 and concentrated to give an oliy residue which was distilled *in vacuo* to give 1,3-propanediol monobenzyl ether (bp_{30} 165—169°) (48 g, 63%). The benzyl ether (57 g, 0.37 mol) was chlorinated with $SOCl_2$ (67 g, 0.55 mol) in $CHCl_3$ (50 ml) in the presence of N,N-dimethylaniline (54 g, 0.44 mol) with stirring in an ice-water bath. After heating the reaction mixture for 30 min at 50—60°, $CHCl_3$ (100 ml) was added to the mixture and the $CHCl_3$ solution was washed with 1 N-HCl (150 ml×3) and with water (150 ml×2), dried over Na_2SO_4 and distilled *in vacuo* after evaporation of $CHCl_3$ to give 3-chloro-1-benzyloxypropane (bp_{14} 125—130°) (46 g, 70%). The chloride (5 g) and β-ethanolamine (10 g) were allowed to react at 80—90° with stirring for 1 hr, and the reaction mixture was extracted with $CHCl_3$ (100 ml)–10% NaOH (50 ml). The $CHCl_3$ extract was washed with water (50 ml×2), dried over Na_2SO_4. To the dried $CHCl_3$ solution was added dropwise $SOCl_2$ (5.0 ml) at room temperature, and the mixture was stirred for 1 hr at 40—50° and concentrated *in vacuo* to give an residue which was dissolved in $CHCl_3$ (50 ml) and washed with saturated aq. NaCl solution (30 ml). The $CHCl_3$ layer was dried over Na_2SO_4 and concentrated *in vacuo* to give a crystalline residue which was recrystallized from acetone–ether, giving N-(2-chloroethyl)-3-benzyloxypropylamine hydrochloride (3.0 g, 41%) as colorless needless, mp 95—98°. The resulting hydrochloride (1 g, 3.8 mmol) was hydrogenated over 10% Pd-C (100 mg) in MeOH (20 ml) and the reaction mixture was filtered and concentrated *in vacuo* to give crude N-(2-chloroethyl)-3-hydroxypropylamine hydrochloride as a syrup (600 mg) which was used in the next run without purification. The hydrogenated product was suspended in CH_2Cl_2 (20 ml), then was allowed to react with $POCl_3$ (580 mg, 3.8 mmol) in the presence of triethylamine (1.5 g, 15 mmol) according to the procedure described in a literature[21] and the resulting chlorophosphoramidate was aminated with anhydrous ammonia in ether (50 ml) after purification by column chromatography with SiO_2–ether. The aminated product was obtained as a crystalline solid (400 mg, 53% from $POCl_3$) which was recrystallized from acetone–ether to give colorless prisms, mp 99—100°, of which IR spectrum in nujol mull was identical with that of the metabolite **4**. *Anal.* Calcd. for $C_5H_{12}ClN_2O_2P$: C, 30.25; H, 6.10; Cl, 17.86; N, 14.12; P, 15.62. Found: C, 30.41; H, 6.22; Cl, 18.10; N, 14.41; P, 15.89. The aminated product **4** (100 mg) was acetylated with CH_3COCl (0.2 ml) in CH_2Cl_2 (4 ml) in the presence of pyridine (0.1 ml) under stirring at −50°, and the mixture was stirred for 2 hr at room temperature. The mixture was washed with 10% aq. $NaHCO_3$ solution (5 ml), dried over Na_2SO_4 and concentrated *in vacuo* to give an acetate **4′** (40 mg, 33%) as a crystalline solid which was recrystallized from acetone–ether to give colorless prisms, mp 118—119°, of which IR spectrum in nujol mull was identical with that of the acetate derived from the metabolite **4**. *Anal.* Calcd. for $C_7H_{14}ClN_2O_3P$: C, 35.50; H, 5.87; Cl, 14.74; N, 11.65; P, 12.92. Found: C, 35.24; H, 6.15; Cl, 14.97; N, 11.67; P, 12.56.

c) 2-(2-Chloroethyl)amino-1,3,2-oxazaphosphorinane-2-oxide (**5**): $POCl_3$ (1.53 g, 10 mmol), 3-aminopropan-1-ol (0.75 g, 10 mmol) and 2-chloroethylamine hydrochloride (1.16 g, 10 mmol) were allowed to react in CH_2Cl_2 in the presence of triethylamine (4.04 g, 40 mmol) by the similar procedures reported by Feil *et al.*[21] The product was purified by column chromatography on a column (3.5×14 cm) eluting with acetone and was obtained as a crystalline solid (0.79 g, 4.0%) which was recrystallized from ether–hexane to give colorless prisms, mp 110—111°. The IR spectrum in nujol mull of this purified product was identical with that of the metabolite **5**.

d) [N-(2-Chloroethyl)-3-(2-oxo-1,3,2-oxazaphospholidin-2-yl)oxy]propionamide (**10**): To a stirred solution of 3-benzyloxypropionyl chloride (**11**)[22] (13.8 g, 70 mmol) in CH_2Cl_2 (60 ml) was added dropwise a solution of β-ethanolamine (4.2 g, 70 mmol) and triethylamine (9 g, 90 mmol) in CH_2Cl_2 (20 ml) at −30—−20°, then the mixture was stirred for 30 min at −20—0°. After additional stirring for 10 min at room temperature, CH_2Cl_2 (150 ml) and saturated aq. NaCl solution (50 ml) were added to the mixture and the CH_2Cl_2 layer was washed with water (10 ml×2), dried over Na_2SO_4 and concentrated *in vacuo* to give a pale yellow residue (16 g). The residue was dissolved in CH_2Cl_2 (100 ml), then $SOCl_2$ (20 ml) was added dropwise to the stirred solution at room temperature. After stirring for 1 hr at 30°, the mixture was concentrated *in vacuo* and the residue was extracted with $CHCl_3$ (200 ml)–water (150 ml). The $CHCl_3$ layer was washed with water (50 ml×3), dried over Na_2SO_4 and concentrated *in vacuo* to give N-(2-chloroethyl)-3-benzyloxypropionamide **12** (11 g, 65%) as a crystalline solid of which recrystallization from acetone–ether gave colorless leaflets, mp 66—68°; IR ν_{max}^{Nujol} cm^{-1}: 3260 (NH), 1637, 1563 (CONH); NMR (DMSO-d_6) δ: 2.43 (2H, triplet, $COCH_2$), 3.30—3.65 (5H, multiplet, $NHCH_2CH_2Cl$), 3.64 (2H, triplet, $-OC\underline{H}_2CH_2-$), 4.47 (2H, singlet, $-C\underline{H}_2-C_6H_5$), 7.31 (5H, singlet, C_6H_5). *Anal.* Calcd. for $C_{12}H_{16}ClNO_2$: C, 59.67; H, 6.67; Cl, 14.68; N, 5.80. Found: C, 59.61; H, 6.70; Cl, 14.92; N, 5.92. Catalytic hydrogenation of **12** (2.4 g, 10 mmol) over 10% Pd-C (200 mg) in MeOH (50 ml) gave N-(2-chloroethyl)-3-hydroxypropionamide (**13**) as an crude oil (1.43 g, 95%) which was used for the next run without purification. To a stirred solution of $POCl_3$ (1.45 g, 9.5 mmol) in CH_2Cl_2 (20 ml) was added dropwise a solution of β-ethanolamine (0.58 g, 9.5 mmol) and triethylamine (1.91 g, 19 mmol) in CH_2Cl_2 (5 ml) with stirring at −30—−25°. After the addition has been completed (3 min), the reaction mixture was refluxed for 1 hr at 50—60°. Then a solution of a mixture of the crude product **13** (1.43 g, 9.5 mmol) and triethylamine (0.95 g, 9.5 mmol) in CH_2Cl_2 (5 ml) was added dropwise to the stirred

21) V.J. Feil and C.H. Lamoureux, *Cancer Res.*, **34**, 2596 (1974).
22) J.H.S. Weiland, *Chem. Abstr.*, **60**, 9227*h* (1964).

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reaction mixture of $POCl_3$ and β-ethanolamine at 50—60°. After refluxing for 3 hr, the mixture was allowed to stand overnight at room temperature, and the precipitated triethylamine hydrochloride was removed by filtration. The filtrate was concentrated *in vacuo* and the resulting residue was chromatographed on a column (2.5×6 cm) eluting with acetone to give **10** (1.02 g, 40%) as a crystalline solid of which recrystallization from EtOH–ether afforded colorless prisms, mp 160—161°. IR spectrum in nujol mull of this product was identical with that of the metabolic product.

e) Synthesis of [N-(2-Chloroethyl)-3-(2-oxo-3-methyl-1,3,2-oxazaphospholidin-2-yl)oxy]propionamide (**16**): $POCl_3$ (4.6 g, 30 mmol) and N-methyl-β-ethanolamine (2.1 g, 30 mmol) were allowed to react in CH_2Cl_2 (50 ml) in the presence of triethylamine (7.0 g, 70 mmol) at -50 ± 5°. After stirring for 3 hr at 0°, the reaction mixture was filtered, and the filtrate was washed with water, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (5×25 cm) eluting with ether. The ether eluate was concentrated *in vacuo* to give crude 2-chloro-3-methyl-1,3,2-oxazaphospholidine-2-oxide (**15**) as a colorless oil (1.6 g, 33%). The oily product (1.5 g, 10 mmol) was dissolved in CH_2Cl_2 (20 ml), and to the solution was added dropwise a solution of N-(2-chloroethyl)-3-hydroxypropionamide (**13**) (1.35 g, 10 mmol) and triethylamine (1.0 g, 10 mmol) in CH_2Cl_2 (10 ml) with stirring at room temperature, then the mixture was stirred overnight at room temperature. After filtration, the reaction mixture was washed with water, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (3×15 cm) eluting with acetone giving **16** as an oily residue (1.08 g, 40%). The IR spectrum in film of this product was identical with that of the product obtained by the alkali treatment of **20** (*vide infra*).

Experiments for the Ring Transformation Reaction of 4-Keto-1,3,2-oxazaphosphorinane-2-oxides——a) O-(3-Butenyl)-N-(2-chloroethyl)-N′-(2-benzyloxyethyl)phosphorodiamidate (**18a**): $POCl_3$ (4.6 g, 30 mmol), 3-buten-1-ol (2.1 g, 30 mmol), 2-benzyloxyethylamine hydrochloride[23] and 2-chloroethylamine hydrochloride were allowed to react in CH_2Cl_2 in the presence of triethylamine by the similar procedure described in an earlier report.[8b] After column chromatographic purification on a column (5×25 cm) eluting with acetone, **18a** was obtained as an oil (6.0 g, 56%); NMR ($CDCl_3$) δ: 2.35 (2H, quartet, $-\underline{CH_2}-CH=CH_2$), 3.00—3.75 (8H, multiplet, $3\times CH_2$, $2\times NH$), 3.95 (2H, quartet, $PO-CH_2$), 4.53 (2H, singlet, $-\underline{CH_2}C_6H_5$), 4.90—6.15 (3H, multiplet, $-CH=CH_2$), 7.32 (5H, singlet, C_6H_5).

b) O-(3-Butenyl)-N-(2-chloroethyl)-N′-(2-benzyloxyethyl)-N′-methylphosphorodiamidate (**18b**): $POCl_3$ (4.6 g, 30 mmol), 3-buten-1-ol (2.1 g, 30 mmol), N-methyl-2-benzyloxyethylamine hydrochloride (This compound, mp 107—108°, was prepared by reaction of methylamine with 2-benzyloxy-1-chloroethane[24]) and 2-chloroethylamine hydrochloride were allowed to react quite similarly, and the product was purified by column chromatography on a column (5×20 cm) eluting with acetone to give **18b** as an oil (6.2 g, 55%); NMR ($CDCl_3$) δ: 2.40 (2H, quartet, $-\underline{CH_2}-CH=CH_2$), 2.72 (3H, doublet, $J=9.0$ Hz, NCH_3), 3.00—4.15 (11H, multiplet, $5\times CH_2$, NH), 4.53 (2H, singlet, $-\underline{CH_2}C_6H_5$), 4.90—6.15 (3H, multiplet, $-CH=CH_2$), 7.33 (5H, singlet, C_6H_5).

c) 2-[2-(Benzyloxyethyl)]amino-3-(2-chloroethyl)-1,3,2-oxazaphosphorinan-4-one-2-oxide (**19a**): **18a** (6.0 g, 17 mmol) was dissolved in a mixture of acetone (18 ml) and water (12 ml). To the solution excess amount of O_3 (*ca.* 2 eq. mol) was bubbled with stirring in an ice-water bath, then 30% H_2O_2 (5 ml) was added to the ozonized solution. After standing overnight at room temperature, acetone was removed by evaporation *in vacuo* and the resulting aqueous residue was extracted with $CHCl_3$ (50 ml×4). To the $CHCl_3$ extract was added an aqueous solution of $FeSO_4\cdot 7H_2O$ (4.73 g, 17 mmol), and the mixture was vigorously stirred for 2 hr at room temperature. The $CHCl_3$ layer was washed with water, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (6×25 cm) eluting with AcOEt giving **19a** as a colorless oil (2.2 g, 39%); IR ν_{max}^{film} cm^{-1}: 3200 (NH), 1690 (CO), 1255 (PO), 1040 (POC); NMR ($CDCl_3$) δ: 2.73 (2H, multiplet, $COCH_2$), 2.98—4.45 (11H, multiplet, $5\times CH_2$, NH), 4.52 (2H, singlet, $-\underline{CH_2}-C_6H_5$), 7.33 (5H, singlet, C_6H_5).

d) 2-[N-(2-Benzyloxyethyl)-N-methyl]amino-3-(2-chloroethyl)-1,3,2-oxazaphosphorinan-4-one-2-oxide (**19b**): **18b** (2.4 g, 6.4 mmol) was similarly ozonolyzed in aqueous acetone (H_2O 4 ml, acetone 8 ml). After treating with 30% H_2O_2 (3 ml), followed by $FeSO_4\cdot 7H_2O$ (1.78 g, 6.4 mmol) solution, the ozonolyzed product was purified on a column (4×15 cm) eluting with AcOEt to give **19b** as a colorless oil (0.9 g, 41%); IR ν_{max}^{film} cm^{-1}: 1690 (CO), 1252 (PO), 1030 (POC); NMR ($CDCl_3$) δ: 2.58—2.88 (2H, multiplet, $COCH_2$), 2.72 (3H, doublet, $J=10.4$ Hz, NCH_3), 3.10—4.62 (10H, multiplet, $5\times CH_2$), 4.54 (2H, singlet, $-\underline{CH_2}C_6H_5$), 7.33 (5H, singlet, C_6H_5).

e) Catalytic Hydrogenation of **19a**: **19a** (2.2 g, 6.6 mmol) was dissolved in MeOH (20 ml) and was hydrogenated over 10% Pd-C (200 ml) according to the usual procedure. The hydrogenated solution was filtered and the filtrate was concentrated *in vacuo* to give **17** as an oily residue which spontaneously but slowly turned out to **10** at room temperature. Thus the product could not be isolated in a pure state, therefore the freshly prepared product was dissolved in $CHCl_3$ and used for the alkali promoted ring transformation reaction without purification.

23) U. Harder, E. Pfeil, and K.-F. Zenner, *Chem. Ber.*, **97**, 510 (1964).
24) M. Kulka and F.G. Van Stryk, *Can. J. Chem.*, **33**, 1130 (1955).

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f) Catalytic Hydrogenation of **19b**: **19b** (0.8 g, 2.3 mmol) was hydrogenated over 10% Pd-C (200 mg) in MeOH (20 ml), and after filtration the hydrogenated solution was concentrated *in vacuo* to give **20** as a crystalline residue (240 mg, 41%) which was considerably stable at room temperature and was recrystallized from acetone–MeOH giving colorless prisms, mp 110—111°; IR ν_{max}^{Nujol} cm^{-1}: 3380 (NH), 1680 (CO), 1225 (PO), 1030 (POC); NMR ($CDCl_3$) δ: 2.49 (1H, broad, OH), 2.68—2.95 (2H, multiplet, $COCH_2$), 2.73 (3H, doublet, J=10.0 Hz, NCH_3), 3.10—4.72 (10H, multiplet, 5×CH_2). *Anal.* Calcd. for $C_8H_{16}ClN_2O_4P$: C, 35.54; H, 5.96; Cl, 13.10; N, 10.36; P, 11.47. Found: C, 35.73; H, 6.02; Cl, 13.07; N, 10.09; P, 11.20.

g) Alkali Treatment of **17**: The freshly prepared **17** generated by the hydrogenation of **19a** (2.2 g, 6.6 mmol) over 10% Pd-C (200 mg) in MeOH (20 ml) was dissolved in $CHCl_3$ (50 ml) and 0.5 N–NaOH (2 ml) was added to the solution. After vigorous stirring for 2 min at room temperature, the reaction mixture was dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was purified on a column (2×10 cm) eluting with acetone giving a crystalline solid (250 mg). Recrystallization of this product from EtOH–ether afforded colorless prisms, mp 159—161°; whose IR spectrum in nujol mull was identical with that of the metabolite **10**.

h) Alkali Treatment of **20**: **20** (170 mg, 0.63 mmol) was dissolved in $CHCl_3$ (10 ml), then 0.5 N–NaOH (0.3 ml) was added to the solution. After vigorous stirring for 2 min at room temperature, the reaction mixture was dried over Na_2SO_4 and concentrated *in vacuo* to give an oily residue which was chromatographed on a column (1.5×8 cm) eluting with acetone giving [N-(2-chloroethyl)-3-(2-oxo-3-methyl-1,3,2-oxazaphospholidin-2-yl)oxy]propionamide (**16**) as a colorless oil (50 mg, 29%); IR ν_{max}^{film} cm^{-1}: 3270 (NH), 1655, 1550 (CONH), 1278, 1245 (PO), 1024 (POC); NMR ($CDCl_3$) δ: 2.71 (3H, doublet J=10.1 Hz, NCH_3), 2.73 (2H, triplet, J=8.8 Hz, $-COCH_2-$), 3.18—3.63 (6H, multiplet, CH_2CH_2Cl, NCH_2), 4.13—4.52 (4H, multiplet, 2×OCH_2), 7.37 (1H, broad, CONH). *Anal.* Calcd. for $C_8H_{16}ClN_2O_4P$: C, 35.50; H, 5.96; Cl, 13.10; N, 10.35; P, 11.44. Found: C, 35.41; H, 5.70; Cl, 13.21; N, 10.26; P, 11.59.

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