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17. Morizo Ishidate, Yoshio Sakurai, and Eiichi Matsui : Studies on Carcinostatic Substances. XXIV.*1 Preparation of Diphenylphosphoric Acid Esters of N-Methyl- and N-Phenyl-bis(2-hydroxyethyl)amine and their Alkylating Activity.

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It is well known that nitrogen mustards react readily at room temperature with hydroxyl, amino, carboxyl, mercapto, or phosphoric acid group of other compounds in neutral and dilute aqueous solutions.

Recently it was reported[1] that the process of mitosis of fertilized sea-urchin egg was inhibited or remarkably retarded by nitrogen mustard (HN_2). However, inhibition was removed after washing and subsequent addition of a quantity of L-cysteine into the medium.

Two explanations are possible for this phenomenon. One is that L-cysteine inactivated the remaining portion of HN_2 absorbed on the cells, which could cause further damage upon the mitotic nuclei if it were not eliminated. The second is that L-cysteine could deprive the cell constituents of already chemically combined nitrogen mustard residue toward its mercapto group by transalkylation and free the once affected cells.

If the biological action of HN_2 should be due, at least in part, to its esterification in the phosphoric acid portion of nucleic acids of the cells, as suggested by Alexander, *et al.*,[2] it might be worth testing whether or not nitrogen mustard residue, once reacted with the phosphoric acid portion of a certain model compound, could be transferred to the mercapto group of L-cysteine in *in vitro* condition. This does not seem to be completely improbable, as it is known that the ester of bis(2-hydroxyethyl)amine with a strong acid, such as sulfonic acid, exhibits strong alkylating activity, among whichbis-toluenesulfonic ester of N, N-bis(2-hydroxyethyl)aniline or *p*-chloroaniline has already been reported[3] as anti-tumor agents.

In view of the above, diphenylphosphoric esters of N-methyl- and, N-phenyl-bis-(2-hydroxyethyl)amine were prepared and their alkylating activity *in vitro* on mercapto group of L-cysteine or thiosulfate was examined.

The preparation of these compounds was undertaken by referring to the report of Ross[4] concerning the synthesis of diphenylphosphoric esters of N,N-bis(2-hydroxyethyl)-naphthylamine.

The ester of N-phenyl-bis(2-hydroxyethyl)amine (I) was isolated as a stable crystalline substance, while that of N-methyl-bis(2-hydroxyethyl)amine (II) was obtained only as a syrupy mass which was freely soluble in ether. It was however found to be so labile that it changed instantly to a different crystalline compound, which was not soluble in ether and was identified by analysis to be its dimer (IV). Although the monomer could be kept unchanged longer when it was dissolved in ether, the crystalline dimer began to separate within 20 minutes even at a low temperature (0~5°).

On the contrary, (I) or its ether solution showed no tendency of dimerization. The reaction process is shown in Chart 1.

In order to determine the alkylating activity of the two compounds, each of the

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*1 Part XXIII : This Bulletin, **8**, 89(1960).

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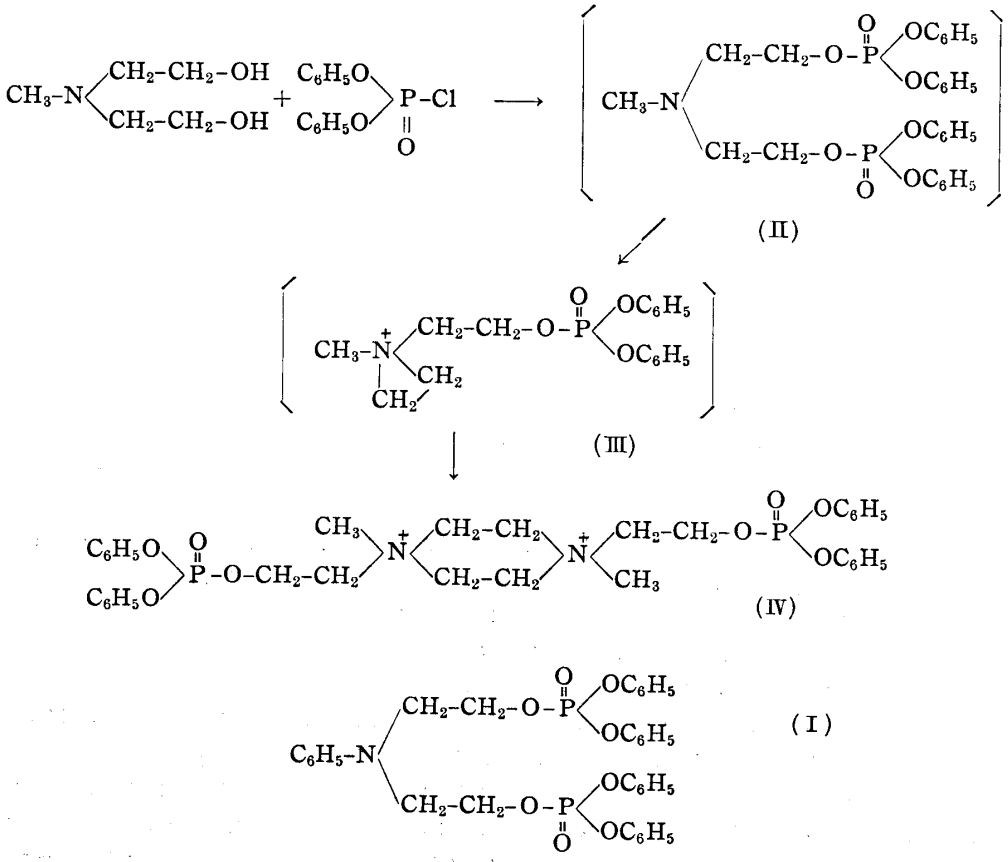
1) Y. Iwata : Nagoya Igaku, **76**, 85(1958).
2) P. Alexander : Nature, **169**, 226, 572(1952).
3) M. Ishidate, Y. Sakurai, S. Owari : This Bulletin, **5**, 203(1957).
4) W. C. J. Ross, W. Davis : J. Chem. Soc., **1952**, 4296.



compounds as acetone or ether solution was added into or laid over a hydrogencarbonate-buffered solution of L-cysteine or thiosulfate and incubated for 24 hours at 25°, shaking if necessary. At the end of incubation, consumption of L-cysteine[5] or thiosulfate[3] was determined by known procedures. The result is shown in Table I.

Table I.

Compd. No. \ Mol. equiv.	L-Cysteine uptake	Thiosulfate uptake
Control	0.3	0.0
(II)	0.2	0.0
(I)	0.3	0.0
HN_2	2.0	0.9 (10 min.) 1.9 (2 hr.)

Incubation : 24 hr., 25°.

As seen in Table I, both esters showed no alkylating activity under this condition, under which the anti-tumor alkylating agent had never failed to react with L-cysteine or thiosulfate.

It was also surprising that the compound (II) did not react with mercapto compounds under this condition in spite of its rapid trend to dimerization as stated above. It has been suggested by Bergmann[6] and Ross[7] that hydrolysis, dimerization, and alkylation of these compounds in an aqueous solution all proceeded through the formation of a three-membered ring intermediate in the initial step of the reaction, viz. ethyleneimmonium compound.

It is considered that compound (II) also changed to the intermediate in the solution,

5) A. Schöberl, *et al.* : Biochem. Z., **295**, 377(1938).
6) M. Bergmann, M. A. Stahmann : J. Org. Chem., **11**, 518(1946).
7) W. C. J. Ross, W. Davis : J. Chem. Soc., **1950**, 3056.

but the polymerization reaction was dominant and the alkylation on mercapto group could never compete with it even when concentration of the mercapto group was sufficient in the same solution.

It was further observed that dimerization was strongly retarded by the addition of mercapto compounds into the reaction mixture but accelerated by the addition of oxidizing agents. Another example of such an exceptional compound had also been reported by Owari[3] who found that N,N-bis(2-thiocyanoethyl)methylamine dimerized easily but had no alkylating activity on thiosulfate. It is interesting to know that the orientation of the reaction of the intermediate towards dimerization or alkylation was determined by its characteristic property or chemical structure.

The anti-tumor effect of these compounds against Yoshida sarcoma was tested by the routine procedure described by Ishidate, Yoshida, *et al.*[8] The minimum effective dose (MED) of both (I) and (II) was $^1/_{2000}$ of that of HN_2 and the minimum effective concentration (MEC) of the former on the tissue-cultured Yoshida sarcoma[9] was $^1/_{700}$ of that of HN_2. Ethyl diphenylphosphate[10] (V) exhibited no cytological effect on Yoshida sarcoma cells *in vivo* or *in vitro*.

Considering that there is no activity against L-cysteine *in vitro*, such slight cytological effect of the compound may not be regarded as the result of mere alkylation. The results obtained by biological experiments are shown in Table II.

Table II.

Compound No.	CE[a]	MTD[b] (mg./kg.)	LD_{50} (mg./kg.)	MED (mg./kg.)	MEC (m*M*)
(II)	+	>600		100	
(I)	+	500	750	100	5×10^{-2}
(V)	−	250	375		——
HN_2	+	1.0	1.7	0.05	2.5×10^{-4}

a) CE : Cytological effect on the Yoshida sarcoma.
b) MTD : Maximum tolerable dose.
CE : Cytological effect, MTD : Min. tolerable dose, MED : Min. effective dose, MEC : Min. effective concentration.

Experimental

Diphenyl chlorophosphate—Prepared according to the report of Brigl and Müller.[11] $b.p_6$ 177～179°.

Diphenyl Hydrogenphosphate—Diphenyl chlorophosphate was hydrolyzed with 5% NaOH. Colorless plates (from H_2O), m.p. 51°. It contained 2 molecules of water of crystallization. Titration with alkali : 0.1*N* NaOH (indicator : phenolphthalein). Calcd. for $C_{12}H_{11}O_4P\cdot2H_2O$: 3.45 cc. Found : 3.57 cc.

Toluidine salt : m.p. 147～148°. *Anal.* Calcd. for $C_{19}H_{20}O_4NP$: C, 63.85; H, 5.64; N, 3.92. Found: C, 63.66; H, 5.51; N, 4.20, 4.27.

N-Methyl-bis(2-hydroxyethyl)amine Bis(diphenylphosphate) (II)—To a solution of N-methyl-bis-(2-hydroxyethyl)amine (25 m. moles) in 2,6-lutidine (10 cc.), previously chilled to −10°, diphenyl chlorophosphate (60 m. moles) was added in small portions over a period of 15 min. The temperature rose to 0° during the reaction. After keeping for 4 hr. in an ice-bath, the reaction mixture was left to stand overnight at room temperature and water was added. An oily substance separated, which was extracted with Et_2O, and the ether extract was consecutively washed with H_2O, 0.2*N* HCl until the water layer became acid to Congo Red, H_2O, 1% $NaHCO_3$, and finally with H_2O. The ether solution was dried over anhyd. Na_2SO_4, when a small quantity of white crystals already separated out, and evaporated to dryness, the residue changed rapidly to a crystalline mass. This compound was very stable and was identified as a dimer, viz. 1,4-dimethyl-1,4-bis(2-diphenylphosphorylethyl)piperazin-

8) M. Ishidate, Y. Sakurai, T. Yoshida, *et al.* : Gann, **44**, 342(1953).
9) M. Ishidate, Y. Sakurai, H. Imamura : This Bulletin, **7**, 873(1959).
10) M. A. Morel : Bull. soc. chim. France. [3], **21**, 491(1899).
11) P. Brigl, H. Müller : Ber., **72**, 2124(1939).

ium bis(diphenylphosphate) (IV). Colorless needles (from EtOH or H_2O), m.p. 201~203°. *Anal.* Calcd. for $C_{58}H_{62}O_{16}N_2P_4$: C, 59.56; H, 5.34; N, 2.40. Found: C, 59.41; H, 5.16; N, 2.65.

Picrate: Yellow needles (from dil. EtOH), m.p. 222~224°. *Anal.* Calcd. for $C_{46}H_{46}O_{22}N_8P_2$: C, 49.11; H, 4.12; N, 9.96. Found: C, 48.95, 49.17; H, 4.13, 4.31; N, 10.09.

The ether-soluble and syrupy substance was believed to be the monomer, viz. N-methyl-bis(2-hydroxyethyl)amine bis(diphenylphosphate) from the analytical data of its dimer, but it could not be identfied, because of its extremely labile character.

N-Phenyl-bis(2-hydroxyethyl)amine Bis(diphenylphosphate) (I)—It was obtained by the reaction of N-phenyl-bis(2-hydroxyethyl)amine (22 m.moles), 2,6-lutidine(10 cc.), and diphenyl chlorophosphate (50 m.moles) by a similar process as in the case of above compound. From the dried ether extract of the reaction mixture, a light brown oily substance was obtained by removing the solvent by distillation, which changed to a crystalline mass when it was kept for a long time in a desiccator (NaOH). Colorless prisms, m.p. 40~41° (dry Et_2O-petr. ether). *Anal.* Calcd. for $C_{34}H_{33}O_8NP_2$: C, 63.25; H, 5.15; N, 2.17. Found: C, 63.04; H, 4.94; N, 2.33.

This substance had no trend to dimerize in solution, even when it was brought in contact with H_2O_2.

Ethyl Diphenylphosphate (V)—Prepared after the method of Morel.[10] Colorless oil, $b.p_6$ 183~184°. Beilstein reaction, negative. *Anal.* Calcd. for $C_{14}H_{15}O_4P$: C, 60.42; H, 5.43. Found: C, 60.30; H, 5.07.

Colorimetric Determination of L-Cysteine Uptake—1) L-Cysteine (4 m.moles) and $NaHCO_3$ (15 m. moles) were dissolved in H_2O (50 cc.), an ether solution of (II) (1 m.mole) was added to this mixture, and the two-layer solution was shaken for 24 hr. at 25°. The ether layer was removed by distillation *in vacuo* and the residual aqueous solution was diluted exactly to 100 cc. with distilled water. An oily substance separated which adhered to the glass wall, from which the aqueous solution was removed by decantation. Two cc. of the aqueous solution was first reduced with Na-Hg according to the method of Torigoe[12] and its L-cysteine content, measured colorimetrically.[5] The oily substance should be the unreacted (II), because, without any special treatment, it changed gradually into a crystalline mass which was proved identical with (IV) by the melting point of its picrate. It was presumed that (IV) did not exist at all in the aqueous layer, since its solubility in water was very slight (less than 0.075% at 28°) and, furthermore, the Dragendorff reaction of the solution was barely positive.

2) L-Cysteine (4 m.moles), (I) (1 m. mole), and $NaHCO_3$ (15 m. moles) were dissolved in 80% acetone and the volume of the mixture was made exactly to 100 cc. in a closely stoppered glass container. After 24-hr. incubation at 25°, 50 cc. of the reaction mixture was removed. Acetone was removed by distillation *in vacuo* below 30° and the aqueous residue was adjusted exactly to 50 cc. with distilled water. The separated oily substance was removed by extraction with Et_2O (50 cc.). Two cc. of the solution was pipetted out and treated as in the case of (II).

3) An aqueous mixture of L-cysteine (4 m. moles) and $NaHCO_3$ (12 m.moles) was incubated under the same conditions as above and results of colorimetric determination thus obtained were used as the blank value for (I) or (II).

Determination of Thiosulfate Uptake—1) An ether solution of (II) (1 m.mole) was added to 10 cc. of 0.05*N* $Na_2S_2O_3$ containing $NaHCO_3$ (2 m.moles). The two-layered solution was shaken for 24 hr. at 25°, Et_2O was evaporated *in vacuo* at room temperature, and the aqueous residue was titrated with 0.05*N* iodine solution under ice-cooling.

2) (I) (1 m. mole) was dissolved in 35 cc. of Me_2CO, and 0.1*N* $Na_2S_2O_3$ (10 cc.) and $NaHCO_3$ (2 m. moles) were mixed. Its volume was made exactly to 50 cc. by addition of distilled water and the solution was kept at 25° for 24 hr. An aliquot (10 cc.) was taken out and titrated with 0.02*N* iodine solution under ice-cooling after the usual procedure.

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Summary

Bis(diphenylphosphate) of N-methyl- and N-phenyl-bis(2-hyproxyethyl)amine was proved to have alkylating activity against mercapto group of L-cysteine and thiosulfate in a neutral aqueous solution. The compounds exhibited only a slight anti-tumor cytological effect on Yoshida sarcoma *in vitro* and *in vivo*.

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12) M. Torigoe: This Bulletin, **1**, 349(1953).