[Chem. Pharm. Bull. 20(8)1612--1616(1972)]
UDC 547.879.09 : 615.31.015.4.076.9

Studies on Cyclophosphamide Metabolites and Their Related Compounds. I

Akira Takamizawa, Yoshihiro Tochino, Yoshio Hamashima, and Tsuyoshi Iwata

Shionogi Research Laboratory, Shionogi & Co., Ltd.[1]

(Received October 5, 1971)

Two crystalline metabolites (**2**, **3**) of cyclophosphamide (**1**) were isolated from rabbit urine. The structures of **2** and **3** were determined from their physicochemical data, and confirmed by synthesis. The positions of **2** and **3** in the metabolic pathway of **1** in living animals were discussed briefly.

The activation mechanism of cyclophosphamide (**1**), an agent widely used in the treatment of various types of tumor, has been a matter of significant interest for over a decade.[2] In *in vitro* experiments, cyclophosphamide does not show any significant activity against tumors, while it has considerable activity in living animals. We also have been interested in studying its metabolic pathway and the chemical applications of its metabolites. A recent publication by Hill, Kirk, and Struck[3] prompts us to report some of our results in this field. This report deals with the isolation and chemical identification of cyclophosphamide metabolites from rabbit urine, and includes a brief discussion on the situation of these metabolites in the metabolic pathway of cyclophosphamide in the living animal.

Isolation of Two Metabolites

We have isolated two crystalline metabolites from rabbit urine. Urine from twenty rabbits each injected intraperitonealy with 0.75 g of cyclophosphamide was collected for 24 hours (total amount, 2.7 liter). From a chloroform extract of the urine were isolated colorless crystals which showed a sharp melting point at 148° after recrystallization. Mass spectral analysis gave a molecular ion at *m/e* 274 ($C_7H_{13}O_3N_2Cl_2P$) and a base peak at *m/e* 225. Infrared (IR) spectrum exhibited bands at 1695 (C=O). 1236 (P=O), and 1029 (P-O-C), and nuclear magnetic resonance (NMR) spectrum showed signals due to C-5 methylene shifted down field at 7.32 τ as shown in Figure 1. These data indicated that cyclophosphamide was oxidized at a position α to the imino group resulting in the lactam (**2**). Next, extraction of the residue with ethanol gave colorless crystals, mp 117—119°, whose structure was determined to be N,N-bis-(2-chloroethyl)-O-2-carboxyethylphosphorodiamide from the following data. Elemental analysis of this compound corresponded to $C_7H_{15}O_4N_2P$, and the IR spectrum showed bands at 2475

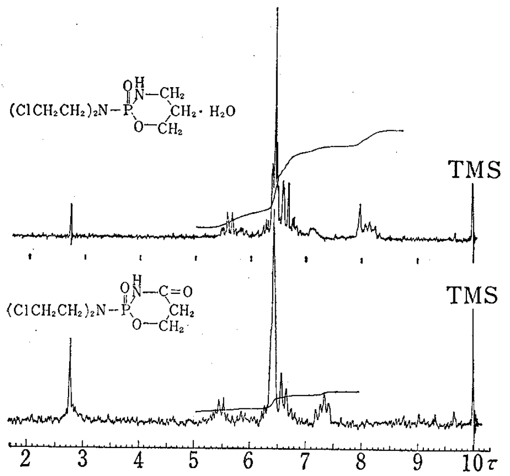


Fig. 1. NMR Spectra of Cyclophosphamide (1) and Lactam (2u) in $CDCl_3$

---

[1] Location: *Fukushima-ku, Osaka.*

[2] See, for example: H. Arnold, F. Bourseaux, and N. Brock, *Naturwissenschaften*, **45**, 64 (1958); G.E. Foley, O.M. Friedman, and B.P. Drolet, *Cancer Res.*, **21**, 57 (1961); N. Brock and H. Hohorst, Jr., *Arzneim. Forsch.*, **13**, 1021 (1963); H.M. Rauen and K. Norpoth, *Naturwissenschaften*, **52**, 477 (1965).

[3] D.L. Hill, M.C. Kirk, and R.F. Struck, *J. Amer. Chem. Soc.*, **92**, 3207 (1970).

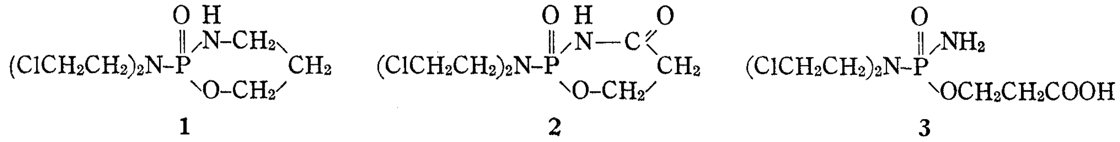


Chart 1

and 1702 cm⁻¹ (COOH). Structural identification of **2** and **3** were confirmed by synthesis.

Syntheses of the Metabolites

We synthesized **2** and **3** as follows. Two ways were considered for obtaining the key intermediate (**8**) from phosphorous oxychloride. Compound (**6**) was not very reactive to benzyl 2-hydroxypropionate (**4**). The reaction proceeded at a high temperature, resulting in large amounts of side products. Compound (**8**) was therefore synthesized by treating **7** with bis(2-chloroethyl)amine hydrochloride in the presence of triethylamine. Addition of ammonia to an ethereal solution of **8** gave **9**, which was hydrogenated with palladized charcoal to afford **3**[4] as colorless crystalline powder. The structure of **3** was determined by IR, NMR spectral data and elemental analysis. The identification of this compound with the metabolite (**3**) isolated from urine was made by comparison of the spectroscopic data. Further, the IR, and NMR spectra of the esters obtained from each acid were entirely identical. Treatment of acid (**3**) with dicyclohexylcarbodiimide or thionyl chloride afforded colorless crystals melting at 148—149° after recrystallization. The structure of this compound was assigned as 2-bis(2-chloroethyl)amido-1,3,2-oxazaphosphoridin-4-one-P-oxide (**2**) from the IR, NMR spectral data and from elemental analysis. The structure of the metabolite (**2**) was confirmed by comparison with this synthetically prepared sample, to which it was identical in every respect.

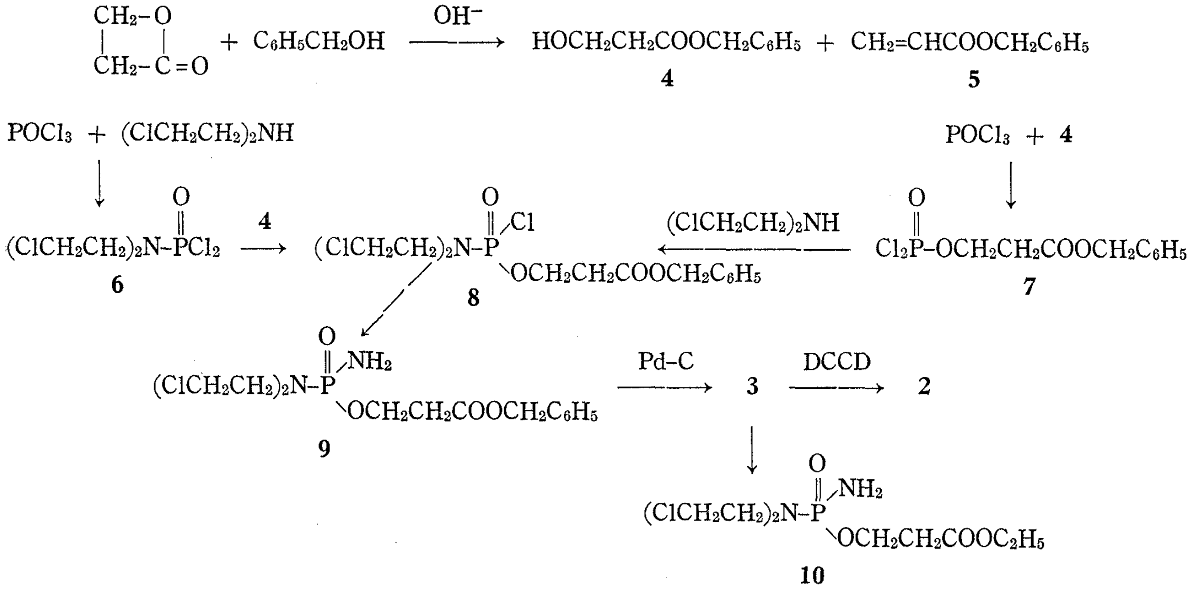


Chart 2

Discussion

Compound (**2**) is very stable in the living animal and is recovered unchanged when administered to rabbit. On the contrary, **3** is fairly labile in the body and only about forty percent

---

[4] A. Takamizawa, Y. Hamashima, T. Minesita, and Y. Tochino, Japan. Patent 7016215 (1970).

Table I. Minimal Effective Dose of Cytotoxicity of Cyclophosphamide (1), Normustard, Lactam (2), and Acid (3) against Yoshida Sarcoma Cell *in Vitro* and *in Vivo*

Compound	*in vitro* (μg/ml)	*in vivo* (mg)/kg)
1	>1000	2.5
Normustard	<5— 10	100
2	>1000	>200
3	>50—100	>100

is recovered unchanged when administered to rabbit. In this case, no lactam is detected in the metabolites. These facts indicate that **2** and **3** are not the precursors of each other. The cytotoxicity of **2** and **3** was tested *in vitro* and *in vivo* against Yoshida Sarcoma cell (Table I). Compound (**3**) inhibited cell growth at 50—100 μg/ml *in vitro*, an activity much less than normustard, but did not show any significant activity *in vivo*. Compound (**2**) did not show any significant activity either *in vivo* or *in vitro*. These data indicate that neither of these is a true active metabolite. In rat, however, **2** and **3** constitute about seventy percent of the metabolites produced, based on the amounts isolated and on calculation of the recoveries after administration of **2** and **3**. Thus, it may be considered that the pathways leading to **2** and **3** should be included in the main metabolic map. The appearance of the maximum cytotoxic activity in rat blood occurs shortly after the administration of cyclophosphamide (**1**), and the increase in **2** and **3** was shown by thin layer chromatography (TLC) to be concurrent with the decrease of the activity.[5] This suggests that the active metabolite is produced during the change of **1** into **2** or **3**. Thus, the pathway cited in Chart 3 might be considered as the main route. Further investigation of the metabolic pathway of the cyclophosphamide is now in progress.

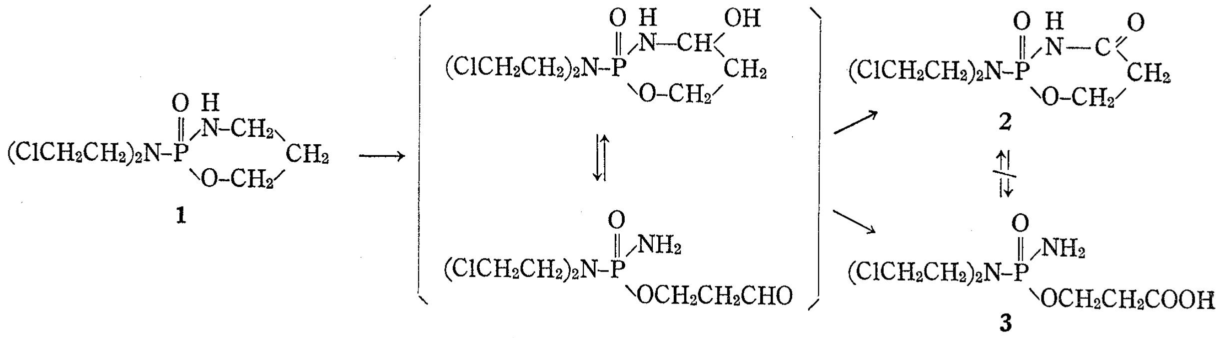


Chart 3

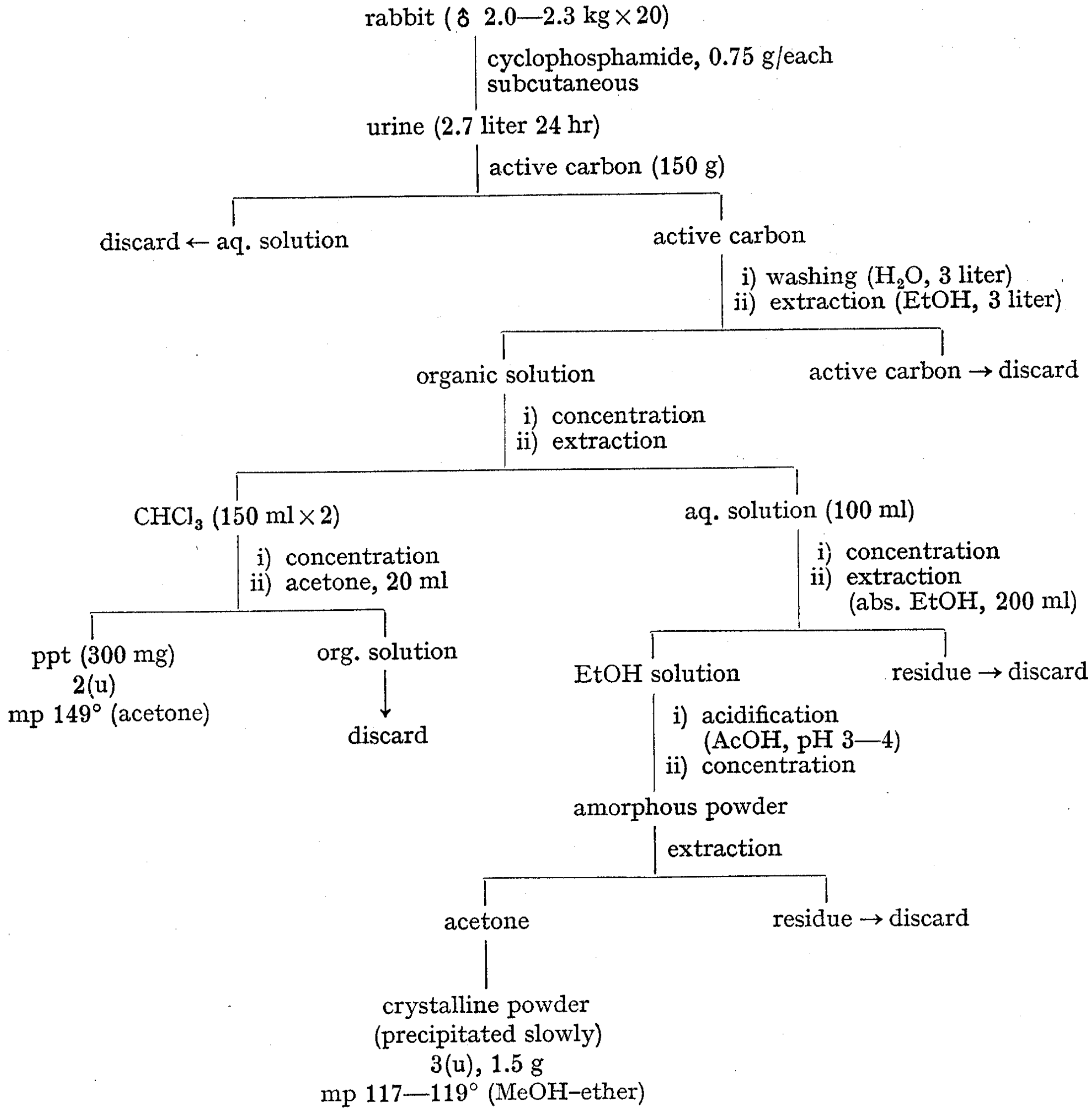
Experimental

General——All melting points were obtained using a stirred Yamato Kagaku silicon oil bath. IR spectra were measured using a JASCO DS-201B recording spectrometer, and mass spectra were measured using a Hitachi RMU-6 mass spectrometer. Proton magnetic resonance spectra were obtained using Varian A-60 Mc apparatus with tetramethylsilane as internal standard.

Isolation of Cyclophosphamide Metabolites——Isolation procedure of cyclophosphamide metabolites is as follows: Analytical data of 2(u) and 3(u): 2(u): *Anal.* Calcd. for $C_7H_{13}O_3N_2Cl_2P$: C, 30.57; H, 4.76; N, 10.18; Cl, 25.78; P, 11.26. Found: C, 30.51; H, 4.84; N, 10.16; Cl, 26.05; P, 11.01. 3(u): *Anal.* Calcd. for $C_7H_{15}O_4N_2Cl_2P$: C, 28.69; H, 5.16; N, 9.56; Cl, 24.26; P, 10.57. Found: C, 29.03; H, 5.52; N, 9.32; Cl, 23.76; P, 10.68.

---

5) T. Minesita, "Abstracts of Scientific Lectures," 17th General Assembly of Japan Medical Congress, Nagoya, April 1967, Part II, p. 1059.



Benzyl 2-Hydroxypropionate (4)——Benzyl alcohol (324 g) containing sodium (0.46 g) was cooled at -5——$-18°$, in which was added β-propiolactone (36 g) with stirring. After that the reaction mixture was stirred at room temperature for 3 hr. Neutralization with conc. HCl and distillation gave 60 g of the mixture of benzyl acrylate and benzyl 2-hydroxypropionate. Fractional distillation gave pure **4** as colorless liquid, bp_2 135—136°, which was gas chromatographically pure. *Anal.* Calcd. for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71; O, 26.64. Found: C, 66.32; H, 6.57; O, 26.10.

Benzyloxycarbonylethylphosphorodichloridate (7)——To phosphorylchloride (13 g) was added **4** (2.2 g) with ice-salt cooling, then the mixture was stirred at room temperature for 2 hr. The mixture was concentrated in vacuum below 40° to leave colorless oil (3.6 g, undistillable), which was used without further purification.

2-Benzyloxycarbonylethyl-N,N-bis(2-chloroethyl)phosphoramide Chloride (8)——To a mixture of **7** (3.6 g) and normustard hydrochloride (1.92 g) in CH_2Cl_2 (20 ml) was added dropwise triethylamine (2.4 g) with ice-salt cooling, then the mixture was stirred at room temperature for 1.5 hr to complete the reaction (checked by TLC). The suspension was filtered, and the filtrate was submitted to SiO_2 column chromatography and eluted with ether to give crude **8** (2.6 g) as an oil, which was used without further purification. IR $\nu_{\max}^{\text{film}}$ cm^{-1}: 1744, 1180 (CO), 1265 (P=O), 1031, 1007 (P-O-C).

N,N-Bis(2-chloroethyl)-O-2-benzyloxycarbonylethylphosphorodiamide (9)——To a solution of **8** (5.5 g) in CH_2Cl_2 (30 ml) was added NH_3 (5.0 g in CH_2Cl_2 20 ml), and the mixture was allowed to stand at room temperature for 1 hr. Filtration and concentration left colorless oil, which was submitted to SiO_2 column chromatography affording **9** (3.5 g) as colorless oil. IR $\nu_{\max}^{\text{film}}$ cm^{-1}: 3360, 1565 (NH), 1735, 1180 (CO), 1215 (P=O), 1040, 1006 (P-O-C).

N,N-Bis(2-chloroethyl)-O-2-carboxyethylphosphorodiamide (3)——Catalytic hydrogenation of **9** (1.5 g) in MeOH (80 ml) with 10% Pd-C (2 g) consumed 114 ml of H_2 within 1 hr (theoretical amount 95 ml). Filtration and concentration of the reaction mixture left crystalline residue, which was recrystallized from MeOH–ether to give **3** (0.75 g) as colorless prisms, mp 119°; IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1}: 3340, 3242, 1559 (NH_2), 2460, 1702 (COOH), 1160 (P=O), 1030, 997 (P-O-C). *Anal.* Calcd. for $C_7H_{15}O_4N_2Cl_2P$: C, 28.69; H, 5.16; N, 9.56; Cl, 24.26. Found: C, 28.77; H, 5.29; N, 9.61; Cl, 24.40.

2-Bis(2-chloroethyl)amido-1,3,2-oxazaphosphoridin-4-one-P-oxide (2)——To a solution of **3** (0.5 g) in dioxane (20 ml) was added dicyclohexylcarbodiimide (0.7 g), and the mixture was stirred at 80° for 3 hr. The reaction mixture was filtered and the filtrate was concentrated to leave crystalline residue, which was washed with *n*-hexane and was column chromatographed over SiO_2. Elution with acetone gave colorless crystals as second fraction, which were recrystallized from $CHCl_3$ or acetone affording **2** as colorless needles, mp 149°. Yield, 0.17 g. *Anal.* Calcd. for $C_7H_{13}O_3N_2Cl_2P$: C, 30.57; H, 4.76; N, 10.18; Cl, 25.78; P, 11.26. Found: C, 30.82; H, 4.63; N, 10.26; Cl, 25.27; P, 11.21.

N,N-Bis(2-chloroethyl)-O-2-ethoxycarbonylethylphosphorodiamide (10)——Thionyl chloride (2 ml) was added to EtOH (20 ml) precooled at −50°. To the mixture was then added **3** (1 g) in EtOH (10 ml) dropwise. After stirring for 1 hr at the temperature, the mixture was allowed to become to room temperature. Concentration of the reaction mixture left colorless oil, which was extracted with $CHCl_3$, and the extract was washed with dil. $NaHCO_3$ solution, and dried. Column chromatography of the oily residue over SiO_2 afforded **10** as colorless oil (0.8 g undistillable). IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1}: 3350, 3245, 1720, 1566, 1254, 1212, 1086, 1035. NMR ($CDCl_3$) τ: 5.54—6.0 (m, 2H, OCH_2), 5.83 (q, 2H, OCH_2, $J=7.0$), 6.20—6.83 (m, 8H, $ClCH_2$-$CH_2 \times 2$), 6.90 (b, 2H, NH_2), 7.35 (t, 2H, CH_2, $J=6.1$), 8.73 (t, 3H, CH_3, $J=7.0$). *Anal.* Calcd. for $C_9H_{19}O_4N_2Cl_2P$: C, 33.67; H, 5.97; N, 8.73; Cl, 22.09; P, 9.65. Found: C, 33.90; H, 6.56; N, 8.27; Cl, 21.65; P, 9.66.

Assay of Cytotoxic Activity of Compounds *in Vitro* and *in Vivo*——Yoshida Sarcoma cells which were transplanted intraperitonealy to a male wister rat weighing about 100 g at one week before experiments were employed for cytotoxic activity *in vitro* and *in vivo*.

In Vitro Assay: A half ml of Yoshida Sarcoma ascites (2×10^5/mm^3 cells) was mixed with saline solution containing each test compound at various concentrations and allowed to stand at 4° for 20—24 hr. The treated ascites tumor cells were transplanted subcutaneously to new wister rats without washing with physiological saline. When the appearance of subcutaneous tumors was not recognized until 2 weeks after the transplantation the tested compounds had significant cytotoxic activity *in vitro*.

In Vivo Assay: Yoshida Sarcoma cells (2×10^7) were transplanted subcutaneously (at interscapulal area) to male wister rats weighing 100 g. At 24 hr after the transplantation, various amounts of a physiological saline containing test compounds were injected intraperitoneally to the tumor bearing rats. The rats were sacrificed at 14th day after the injection and growth of subcutaneous tumor was inspected. When subcutaneous tumor was recognized, it is decided that the tested compound did not show a significant cytotoxic activity against Yoshida Sarcoma cells *in vivo*.

The minimal effective dose of each compound *in vitro* and *in vivo* is the lowest concentration of amount at which the test compounds show any significant cytotoxic activities against tumor cells.