64. Michimasa Izumi***: N-Bis(β-chloroethyl)amino Acids and their Effect upon the Yoshida Sarcoma*.

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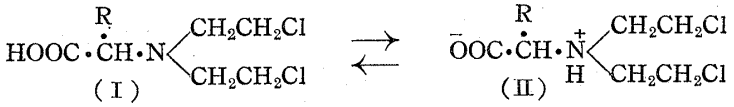
Nitrogen mustard N-oxides[1], which were found in the course of our study to have more favorable efficacy against the Yoshida sarcoma or the ascites hepatoma of rats when compared with the original nitrogen mustards like HN_2 had been assumed from the results of several model experiments *in vitro* to be reduced to the tertiary nitrogen mustards *in vivo* before entering into the reaction with the cell constituents of the tumor[2]. This added process of the prior reduction was supposed to give the compounds more or less selectiveness in action, because the redox-potentials of the tissues, normal and neoplastic, may happen to differ from each other under certain circumstances.

These results were hopeful and the continued search for a compound having particular and proper physical properties, viz. solubility in fat and water, basicity and polarity of a molecule, under consideration of the specific character of the tumor tissues, might lead to a promising substance having such special activities.

In this paper, the preparation, toxicity, and inhibitory action against the Yoshida sarcoma of N-bis(β-chloroethyl)-alanine, -glycine, -taurine, and their derivatives are reported.

Since nitrogen mustard behaves as an alkylating agent when it exists in the state of free base in a solution, its reactivity depends both upon the basicity of the amine and pH of the reacting medium. In a physiological liquid, all the hydrochlorides of nitrogen mustards or their N-oxides, so far obtained, existed as a hydrolyzed free base, but N-bis(β-chloroethyl)amino acid (I) was supposed to exist, practically under the same conditions, as an amphoteric ion (II), in which the reactivity of the chlorine atoms of (II) was controlled by the formation of an ammonium ion.



(I) (II)

Such property of the amino acid derivatives of nitrogen mustard, together with its hydrophilic character of the base, was expected to contribute to pacify its action *in vivo* and it was also expected that the selectivity of action may arise from this cause if there is any difference in pH between the normal and neoplastic tissues.

The compounds prepared herein and tested as to their anticancer effect are summarized in Table I.

N-Bis(β-hydroxyethyl)glycine was prepared by heating diethanolamine with chloroacetic acid in anhyd. alcohol and it was found to be identical with the product obtained by Kiprianow[3] from glycine ethyl ester and ethylene oxide. Its chlorination using thionyl chloride led to the mixture of N-β-hydroxyethylmorpholone and N-β-chloroethylmorpholone. These morpholone derivatives could in part be changed to N-bis(β-chloroethyl)-

* M. Ishidate, Y. Sakurai: Studies on Cancerocidal Substances. X.
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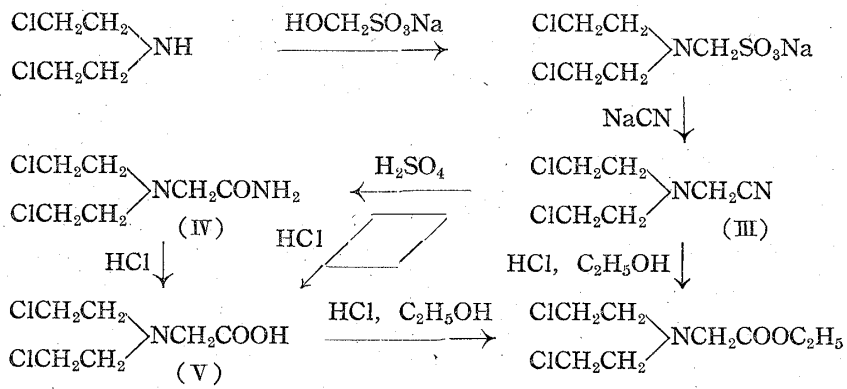
1) M. Ishidate, T. Yoshida, Y. Sakurai, *et al.*: Proc. Japan Acad., **27**, 493 (1951); S. Owari, I. Aiko, M. Torigoe: J. Pharm. Soc. Japan, **72**, 1297 (1952); Y. Sakurai, M. Izumi: This Bulletin, **1**, 297 (1953).
2) M. Torigoe: This Bulletin, **1**, 349 (1953).
3) A. Kiprianow: C. (1927) 2654.

Table I.

Compound	Free base m.p. °C	Picrate m.p. °C	Picryl-sulfonate m.p. °C	LD_{50}[a] mg./kg.	M.E.D.[b] mg./kg.	Response of tumor cells[c]	Life prolongation of tumor rats[d]
$X\cdot CH_2\cdot N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}$							
X : $\cdot CN\cdot HCl$	94	65	130	75	5	+	/
: $\cdot COOC_2H_5\cdot HCl$	115	90	180	3	0.1	+	−
: $\cdot CONH_2$	70	137	178	/	/	/	/
: $\cdot CONH_2\cdot HCl$	145	〃	〃	0.8	/	+	+
: $\cdot CONH_2\cdot H_2SO_4$	192 (decomp.)	〃	〃	/	/	/	/
: $\cdot COOH\cdot HCl$	76	—	220	15	0.5	+	+
$Y\cdot CH(CH_3)\cdot N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}$							
Y : $\cdot CN\cdot HCl$	108	97	99~100	/	/	/	/
: $\cdot CONH_2$	88~89	157	170 (decomp.)	/	/	/	/
: $\cdot CONH_2\cdot H_2SO_4$	170	〃	〃	/	/	/	/
: $\cdot CONH_2\cdot HCl$	190	〃	〃	3	0.01	+	/
: $\cdot COOC_2H_5\cdot HCl$	—	—	158	/	/	/	/
: $\cdot COOH\cdot HCl$	97	—	142 (decomp.)	30	0.05	+	+
$HO_3S\cdot CH_2CH_2\cdot N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}$	177~179	—	—	55	0.5	+	−
$CH_3\cdot N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}\cdot HCl$ [e]				1.6	0.05	+	+
$CH_3\cdot N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}\cdot HCl$ [f]				80	1	+	+

a) Intraperitoneal dose in rats.
b) Minimum dose which caused the characteristic response in the tumor cell.
c) + : An appearance of the definite and characteristic aberration in the nuclei of tumor cells.
d) + : Half number of the treated tumor rats were alive more than 20 days after transplantation. Life-length of the untreated tumor rat is 10~11 days.
e, *f*) The compounds given for comparison.

glycine ethyl ester, when they were first heated in anhyd. alcohol containing hydrochloric acid and then chlorinated with thionyl chloride. The yield from this process was very poor and the following scheme of synthesis gave far better yield in preparation of both glycine and alanine derivatives.



N-bis(β-chloroethyl)aminoacetonitrile (III) was prepared easily after Knoevennagel's procedure[4] on dialkylaminoacetonitrile. This nitrile (III) gave either the corresponding acid (V) by hydrolysis with conc. hydrochloric acid for two hours at 60° or the amide (IV) with conc. sulfuric acid for twelve hours at room temperature. The latter (IV) was readily hydrolysed to the former (V) with conc. hydrochloric acid in a preferable yield.

4) E. Knoevennagel : Ber., **37**, 4073 (1904).

In case of N-bis(β-chloroethyl)alanine, the intermediate nitrile should be saponified first with sulfuric acid into the amide and then treated with conc. hydrochloric acid, because such nitriles, except compound (III), seemed to decompose into their components, viz. N-bis(β-chloroethyl)amine, aldehyde, and hydrocyanic acid in hot conc. hydrochloric acid solution. The similar decomposition was also experienced in the case of N-bis(β-chloroethyl)aminobenzyl cyanide.

N-Bis(β-chloroethyl)taurine was obtained by alkylation of taurine with ethylene oxide and subsequent chlorination with thionyl chloride.

It can generally be said that these amino acid derivatives having free acid residue possess more hydrophilic property and no vesicant action upon human skin. They were 10 to 20 times less toxic on rats than the ordinary aliphatic nitrogen mustards like HN_2, but they maintained predominant effectiveness against the Yoshida sarcoma as shown in Table I. The fact that the toxicity of their derivatives, such as their esters or amides, was the same or higher than that of HN_2, seemed to indicate the presumption that the amphoteric ion formation *in vivo* might play a chief role in the biological action of these compounds.

Results of the screening tests[5] are also shown in Table I in which N-bis(β-chloroethyl)alanine is shown to have the most surpassing chemotherapeutic index, viz. the ratio of LD_{50} to M.E.D. (minimum effective dose).

Further study on chemical and biological properties of the compounds are now being continued and N-bis(β-chloroethyl)alanine hydrochloride has been submitted to clinical tests for leucemia in Japan. The detail of animal experiments referred to in this report had been reported by T. Yoshida, *et al.* at the Annual Meeting of the Japanese Cancer Association held at Nagoya on April 4, 1954.

The author expresses his appreciation to Prof. T. Yoshida for his advice in this study and is also indebted to Messrs. H. Satoh and H. Imamura for the animal experiments.

Experimental

N-Bis(β-chloroethyl)aminoacetonitrile (III)—Bis(β-chloroethylamine) (100 g.) was gradually added at 5~10° into a mixture of 236 g. of 30% $NaHSO_3$ and 54 g. of 38% formaldehyde solution, then it was stirred for 2~3 hrs. Then conc. solution of 50 g. NaCN was added in portions into the reaction mixture and stirred again for 3 hrs. at 25~30°. It was kept at room temperature overnight and extracted with ether. The ether extract, dried with anhyd. K_2CO_3, was treated with dry HCl. A colorless amine hydrochloride separated, melted at 92~94° after recrystallization from acetone. *Anal.* Calcd. for $C_6H_{11}N_2Cl_3$: C, 33.10; H, 5.07; N, 12.85. Found: C, 33.12; H, 5.37; N, 12.94. It was soluble in acetone and MeOH, and insoluble in benzene and ether. When dissolved in water it was converted to free base which distilled at 114~117°. Its picrate easily recrystallized from EtOH to yellow needles, m.p. 55°.

N-Bis(β-chloroethyl)glycine Ethyl Ester—Hydrochloride of (III) (20 g.) was refluxed for 2 hrs. with 120 cc. abs. EtOH containing 36 g. HCl and 1.6 g. water. After cooling, NH_4Cl that separated was removed and the filtrate was evaporated to dryness. The hydrochloride of N-bis(β-chloroethyl)-glycine ethyl ester was obtained as a crystalline residue which was recrystallized from AcOEt to white needles, m.p. 115°, yield, 19 g. It was easily soluble in acetone, MeOH, and EtOH, less soluble in AcOEt, and insoluble in ether and benzene. It was converted to an oily base in water, which dissolved again in a large quantity of water. *Anal.* Calcd. for $C_8H_{16}O_2NCl_3$: C, 36.30; H, 6.05; N, 5.29. Found: C, 35.60; H, 5.43; N, 5.55. Picrate, m.p. 89~90° (from acetone). This ethyl ester hydrochloride was also obtained by heating 2.0 g. of (V) hydrochloride with 20 cc. of 30% alcoholic HCl for 6 hrs. Yield, 1.5 g.

N-Bis(β-chloroethyl)aminoacetamide (IV)—(III) hydrochloride (10 g.) was dissolved in cold conc. H_2SO_4 containing 0.8 g. (1 mole) of water. After standing overnight at room temperature, it was poured on 20 g. of ice and the crystals that separated were filtered. Yield, 11 g. This crude bisulfate was recrystallized from MeOH to colorless scales, m.p. 192° (decomp.). It was soluble in EtOH and

5) Experimental procedure of screening test will be published by T. Yoshida, M. Ishidate, Y. Sakurai, and H. Satoh in Gann, Nos. 2 and 3, 1954.

MeOH, sparingly soluble in acetone, and insoluble in benzene and AcOEt. *Anal.* Calcd. for $C_{16}H_{14}O_5N_2Cl_2S$: C, 24.24; H, 4.78; N, 9.42. Found: C, 24.07; H, 4.56; N, 9.16.

This amide was also obtained as the hydrochloride when 2 g. of (III) was dissolved in 10 cc. conc. HCl, saturated with dry HCl gas, allowed to stand overnight, and the solution evaporated *in vacuo* under 40°. The residue was recrystallized from MeOH, to white scales, m.p. 145°. Yield, 1.4 g. It was soluble in water, EtOH, and hot MeOH, but insoluble in benzene and AcOEt. When a solution of the bisulfate or hydrochloride was neutralized with $NaHCO_3$, the free base of (IV) separated, which was purified by recrystallization from a mixture of $CHCl_3$ and petroleum ether to colorless needles, m.p. 70°. *Anal.* Calcd. for $C_6H_{12}ON_2Cl_2$: C, 36.20; H, 6.06; N, 14.07. Found: C, 36.20; H, 5.76; N, 14.29. Picrate of (IV): m.p. 136~137°.

N-Bis(β-chloroethyl)glycine (V)—Ten grams of the hydrochloride of (III), (IV), or N-bis(β-chloroethyl)glycine ethyl ester was heated with 50 cc. of 35% HCl at 60° for 2 hrs. The reaction mixture was distilled *in vacuo* under 40° and the hydrochloride of (V), obtained as a residue, was recrystallized from AcOEt, m.p. 76°. Yield, 6.4 g. (starting from (IV)), 5 g. (starting from the ethyl ester), and 3.5 g. (starting from (III)). It was easily soluble in water, acetone, MeOH, and EtOH, comparatively less soluble in AcOEt, and insoluble in benzene, $CHCl_3$, and ether. *Anal.* Calcd. for $C_6H_{12}O_2NCl_3$: C, 30.40; H, 5.08; N, 5.90. Found: C, 30.64; H, 5.28; N, 5.88. Picrylsulfonate, m.p. 220° (decomp.) (from MeOH). *Anal.* Calcd. for $C_{12}H_{14}O_{11}N_4Cl_2S$: C, 29.20; H, 2.89; N, 11.37. Found: C, 29.20; H, 2.94; N, 11.33.

N-Bis(β-hydroxyethyl)glycine—A mixture of 120 g. of diethanolamine and 55 g. of monochloroacetic acid dissolved in 200 cc. of abs. EtOH was refluxed for 5 hrs. After being cooled, the free base of N-bis(β-hydroxyethyl)glycine crystallized and recrystallized from 60% EtOH, m.p. 190~191° (decomp.). Yield, 70%. It was freely soluble in water, less soluble in EtOH and MeOH and insoluble in acetone and benzene. *Anal.* Calcd. for $C_6H_{13}O_4N$: C, 44.16; H, 8.03. Found: C, 44.11; H, 7.94. Hydrochloride: m.p. 108~110° (from 94% EtOH). *Anal.* Calcd. for $C_6H_{14}O_4NCl$: C, 36.10; H, 7.07; Cl, 17.77. Found: C, 36.10; H, 7.02; Cl, 17.80.

N-β-Hydroxyethylmorpholone—Its hydrochloride was obtained either by heating N-bis(β-hydroxyethyl)glycine in 30% alcoholic HCl for 4 hrs. or by heating it alone at 190° and subsequent neutralization with HCl, m.p. 138~139° (from abs. EtOH). *Anal.* Calcd. for $C_6H_{13}O_3NCl$: Cl, 19.65. Found: Cl, 19.70. By refluxing it in dil. EtOH, it was again converted to the hydrochloride of N-bis(β-hydroxyethyl)glycine.

N-β-Chloroethylmorpholone—Into a mixture of 200 cc. of toluene and 25 g. of pyridine, was added first 35 g. of hydroxyethylmorpholone and then 35 g. $SOCl_2$ in small portions at 15°. After heating at 100° for 2 hrs,, the toluene layer was separated and evaporated. The residue of the toluene solution was submitted to vacuum distillation, $b.p_5$ 148~150°. Picrate: m.p. 158~159°. *Anal.* Calcd. for $C_{12}H_{13}O_9N_4Cl$: C, 36.68; H, 3.24; N, 14.27. Found: C, 36.72; H, 3.53; N, 14.50.

Direct Chlorination of N-Bis(β-hydroxyethyl)glycine with Thionyl Chloride—A mixture of 10 g. N-bis(β-hydroxyethyl)glycine and 50 cc. $SOCl_2$ was refluxed at 80°. After 8 hrs., an excess of the reagent was removed *in vacuo* and, from the residue, a small amount of N-β-chloroethylmorpholone was isolated as its picrate of m.p. 158~159°. The total amount of this syrupy residue was dissolved in abs. EtOH and the solution, first saturated with dry HCl, was heated at 100° for 1 hr. After that, it was evaporated to a syrup *in vacuo*. The procedure was repeated twice more and the final residue was refluxed again with 30 cc. $SOCl_2$ at 80°. When the evolution of gas ceased, the excess $SOCl_2$ was removed and the residue was dissolved in acetone. An amorphous precipitate separated from the acetone solution by the addition of ether was dissolved in AcOEt and kept in a cool place. Prismatic crystals separated, which melted at 114~115° after purification and showed no depression on admixture with the authentic sample of the hydrochloride of N-bis(β-chloroethyl)glycine ethyl ester.

α-[N-Bis(β-chloroethyl)amino]-propionitrile (VI)—It was obtained by the same procedure as (III), starting from 15.5 g. of acetaldehyde, 50 g. of bis(β-chloroethyl)amine, 17.7 g. of sodium cyanide, and 122 g. of 30% $NaHSO_3$ solution. The ether extract of the reaction mixture was dried and dry HCl gas was bubbled through the ether solution. The hydrochloride of (VI) that separated was recrystallized from acetone, m.p. 107~108°. It was soluble in acetone and MeOH, less soluble in AcOEt, and insoluble in ether and benzene. It separated an oily base in water. *Anal.* Calcd. for $C_7H_{13}N_2Cl_3$: C, 36.31; H, 5.65; N, 12.09. Found: C, 36.12; H, 5.48; N, 12.09. Picrate, m.p. 96~97°.

α-[N-Bis(β-chloroethyl)amino]-propionamide (VII)—The bisulfate of (VII) was obtained by the hydrolysis of 10 g. of (VI) hydrochloride with 20 g. of conc. H_2SO_4 after the preparation procedure of (IV) bisulfate. Yield, 10 g. of m.p. 169~170° (from MeOH). It was easily soluble in water, hot MeOH and EtOH, and insoluble in benzene and acetone. *Anal.* Calcd. for $C_7H_{14}ON_2Cl_2 \cdot H_2SO_4$: C, 27.01; H, 5.21; N, 9.00. Found: C, 27.08; H, 5.46; N, 8.85. The free base of (VII) was prepared by the neutralization of the bisulfate with $NaHCO_3$. m.p. 79~80° (from MeOH). It was sparingly soluble in water, soluble in acetone, benzene, and MeOH. The hydrochloride of (VII) was also obtained.

from a benzene solution of the base by the addition of dry HCl, m.p. 189~190° (from methanol). It was soluble in water, hot MeOH and EtOH, and insoluble in benzene and ether. *Anal.* Calcd. for $C_7H_{15}ON_2Cl_3$: C, 33.69; H, 6.05; N, 11.12. Found: C, 33.60; H, 6.40; N, 11.22. Picrate: m.p. 155~157° (from acetone). *Anal.* Calcd. for $C_{13}H_{17}O_8N_5Cl_2$: C, 35.30; H, 3.88; N, 15.83. Found: C, 35.30; H, 4.20; N, 15.52.

N-Bis(β-chloroethyl)alanine (VIII)—A mixture of 50 g. of the hydrochloride of (VII) and 300 cc. conc. HCl was heated at 80° for 1 hr. The reaction product was treated as in the case of (V). m.p. 96~97° (from acetone). It was soluble in water, acetone, and MeOH, sparingly soluble in AcOEt, and insoluble in benzene and ether. *Anal.* Calcd. for $C_6H_{12}O_2NCl_3$: C, 33.56; H, 5.63; N, 5.59. Found: C, 33.55; H, 5.97; N, 5.84. Picrylsulfonate: m.p. 142° (decomp.)(from MeOH). *Anal.* Calcd. for $C_{13}H_{16}O_{11}N_4Cl_2S$: C, 30.78; H, 3.16; N, 11.03. Found: C, 30.78; H, 3.18; N, 10.89.

N-Bis(β-chloroethyl)alanine Ethyl Ester—The hydrochloride of (VIII) was esterified by refluxing with 30% alcoholic HCl, but the ester of this hydrochloride did not crystallize. Picrylsulfonate: m.p. 157~158° (from acetone). *Anal.* Calcd. for $C_{15}H_{20}O_{11}N_4Cl_2S$: C, 33.65; H, 3.76; N, 10.46. Found: C, 33.79; H, 3.72; N, 10.47.

N-Bis(β-hydroxyethyl)taurine (IX)—Into a mixture of 10 g. of taurine, 11 g. of $Ba(OH)_2 \cdot 8H_2O$, and 50 cc. water, 8 g. of ethylene oxide was added, stoppered with a cork, and held at a room temperature for 48 hrs. After removing Ba^{++} with an approximately equivalent amount of H_2SO_4, the filtrate was condensed *in vacuo* until (IX) began to crystallize out: m.p. 150~151° (from 70% EtOH). *Anal.* Calcd. for $C_6H_{15}O_5NS$: C, 33.77; H, 7.09. Found: C, 33.24; H, 6.73. Yield, 10 g. It was soluble in dil. EtOH and sparingly soluble in abs. EtOH.

N-Bis(β-chloroethyl)taurine—A mixture of 10 g. of (IX) and 60 cc. of $SOCl_2$ was heated at 90~100° on a water bath. After refluxing for 30 mins., the upper layer was removed from the excess $SOCl_2$ by decantation and kept warm for a while under a reduced pressure to remove the accompanying reagent completely. The residue was dissolved in a small quantity of water and added gradually with water. (IX) crystallized from the solution as colorless prisms, m.p. 177~179° (decomp.)(from EtOH). Yield, 8 g. *Anal.* Calcd. for $C_6H_{13}O_3NCl_2S$: C, 28.79; H, 5.24. Found: C, 28.76; H, 5.24.

Summary

N-Bis(β-chloroethyl)-alanine, -glycine, -taurine, and their derivatives were prepared and their properties, toxicity, and effects upon the Yoshida sarcoma of rats were examined. Among these compounds, N-bis(β-chloroethyl)alanine showed the most predominant efficacy by the screening tests and it was presumed that its superiority was at least due in part to the formation of an amphoteric ion in the animal body.

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65. Michimasa Izumi***: N-Bis(β-chloroethyl)amino Acid N-Oxides and their Effect upon the Yoshida Sarcoma*.

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In the preceeding paper of this series[1], the amino acid derivatives of nitrogen mustard were shown to have predominant anticancer efficacy. According to previous experiences[2] that the N-oxide had a more favorable inhibitory effect upon neoplastic growth when they were compared with the original tertiary nitrogen mustards, the N-oxides of N-bis-(β-chloroethyl)amino acids were prepared in the hope of getting a still more effective and less toxic chemotherapeutics.

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1) M. Izumi: This Bulletin, **2**, 275 (1954).