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34. Morizo Ishidate, Yoshio Sakurai, and Sakahiko Owari : Studies on Cancerocidal Substances. XV.* Preparation of Some New Azo Derivatives of Bis(β-chloroethyl)amine.

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In the preceding paper of this series, preparation of some anti-cancer azo dyes, derived from bis(β-chloroethyl)-β-hydroxyethylamine, was reported and it was found that these compounds were retained far longer in the ascites of rat after an intraperitoneal injection than the ordinary nitrogen mustards.[1]

It has been believed that the relative length of time the drug concentration is maintained in the ascites had a considerable influence upon its therapeutic efficacy against ascites tumors such as the Yoshida sarcoma. Furthermore, it was reported by Odashima[2] that an inducement of hepatoma of rat fed with butter yellow diet was markedly retarded if this azo derivative of nitrogen mustard was injected repeatedly at a proper interval.

On the contrary, methyl-bis(β-chloroethyl)amine or its N-oxide, a well-known potent anti-cancer agent, could give entirely no influence upon the inducement of hepatoma even if it was given every successive day.

Azo derivative of nitrogen mustard seemed therefore to be very characteristic in its biological action at least from our experiences and further attempt was made to prepare some new azo compounds and to analyze their detailed biological action on the Yoshida sarcoma and other tumors.

The compounds derived from N,N-bis(β-chloroethyl)aniline, viz. aromatic nitrogen mustard, had already been reported by Ross[3] and some new derivatives are here added in this paper. They are summarized in Table I and were prepared in general by coupling N,N-bis(β-chloroethyl)aniline in a weak acid solution with equivalent amount of the corresponding diazotized amino or amino-sulfonic compound of aromatic character.

Among the compounds listed in Table I, some were unquestionably active on the Yoshida sarcoma, although their effectiveness was not found to be promising enough for practical purposes.

It has been our experience, however, that aliphatic bis(β-chloroethyl)amines are much different from aromatic ones either in their biological effect or in their chemical behavior. The dyes described in the preceding paper[1] are derivatives of aliphatic nitrogen mustard but had an unstable ester linkage in their molecule. It was supposed that it might be easily hydrolyzed *in vivo* yielding bis(β-chloroethyl)-β-hydroxyethylamine, which might exhibit secondary anti-cancer activity. In fact, these esters were easily hydrolysed *in vitro* even in a very dilute acid solution.

Two compounds (XII, XIII), listed in Table II, were therefore prepared as examples of stable azo dyes having aliphatic mustard group. Moreover, it must be mentioned that these compounds were derived from benzyl-bis(β-chloroethyl)amine, which itself or N-oxide thereof was reported by Gotō[4] to be the most active among the compounds he tested as a virucide upon rabies *in vitro*.

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1) M. Ishidate, T. Yoshida, Y. Sakurai, H. Satoh : Gann, **46**, 469(1955).
2) S. Odashima : Gann, **47**, No. 3~4, 586(1956).
3) W. C. J. Ross : J. Chem. Soc., **1949**, 185.
4) M. Gotō : Private communication to the authors (1955).

TABLE I. R= -N=N-⟨⟩-N(C$_2$H$_4$Cl)(C$_2$H$_4$Cl) (Aromatic Azo-Nitrogen Mustards)

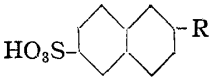
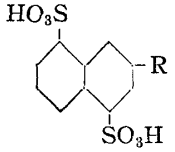
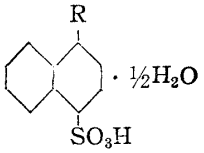
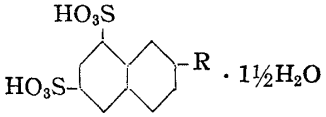
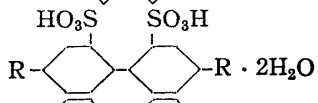
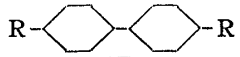
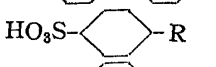
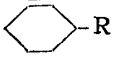
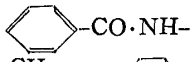
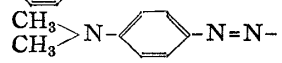
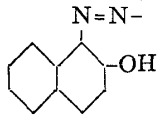
No.	Compound	Properties	LD_{50} on rat, mg./kg. (intraperitoneal)	MED
(I)		Bluish green plates	350	200
(II)		Greenish purple prisms	350	100
(III)		Dark purple prisms	—	—
(IV)		Brown needles	—	—
(V)		Dark purple prisms	≫ 1000	150
(VI)		Brownish red needles, m.p. 204~205°	≫ 1000	—
(VII)		Orange red prisms, m.p. 185~186°	150	100
(VIII)		Orange plates, m.p. 73~75°	350	200

TABLE II. R-⟨⟩-CH$_2$-N(C$_2$H$_4$Cl)(C$_2$H$_4$Cl) (Benzylamino Compounds)

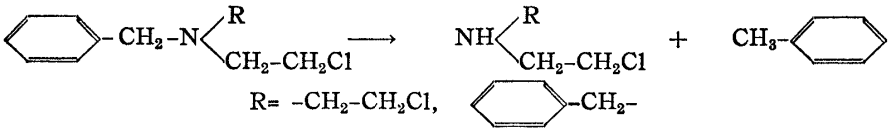
No.	R	Properties	LD_{50} on rat mg./kg.(i.p.)	MED
(IX)	NO_2-	Hydrochloride : Pale yellow prisms, m.p. 138~148°	100	1
(X)	NH_2-	Dihydrochloride : Colorless prisms, m.p. 300°	30	1
(XI)		Colorless prisms, m.p. 134~135°	150	—
(XII)		Orange prisms, m.p. 99.5~100.5°	70	—
(XIII)		Orange red prisms, m.p. 184°(decomp.)	1000	—

4-Aminobenzyl-bis(β-chloroethyl)amine dihydrochloride was not obtained in a pure state and a hydrogenated acid solution of exactly weighed 4-nitrobenzyl-bis(β-chloroethyl)-amine was immediately diazotized and coupled with β-naphthol or N,N-dimethylaniline in a weak acid solution.

It was observed that by catalytic reduction of (IX) at room temperature in a dilute hydrochloric acid over ordinary palladium-carbon catalyst, 4 moles of hydrogen was absorbed at a stretch and nothing but *p*-toluidine and bis(β-chloroethyl)amine were isolated. Partial hydrogenation to obtain (X) succeeded only in a fairly concentrated (15~20%) hydrochloric acid containing a small amount of aged or inactivated palladium-carbon catalyst. Reduction in organic solvents containing no mineral acid, e.g. ethanol or acetic anhydride, invariably effected a perfect cleavage of N-C bond.

Under the same condition, benzyl-bis(β-chloroethyl)amine was reduced to toluene

and the aliphatic amine component, while hydrogenation of dibenzyl-β-chloroethylamine stopped with one mole of hydrogen absorption and yielded toluene and benzyl-β-chloroethylamine alone as the end products. It was observed that the secondary benzylamine thus formed could not be easily cleaved by hydrogenation.



As shown in Table II, the azo compounds of this type were less toxic and highly effective. Maximum tolerable dose (MTD) of (XIII) was over 1000 mg./kg., while its minimum antimitotic dose on the Yoshida ascites sarcoma of rat was less than 10 mg./kg. The colorless derivatives of benzyl-bis(β-chloroethyl)amine (IX, X and XI) proved themselves to be highly active agents and their therapeutic efficacy in treatment of the Yoshida sarcoma and ascites hepatomas of rat seemed to be noteworthy.

A survey of biological activity of the compounds described has already been reported[5] and detailed experiments in this field of research are now continuing.

The authors acknowledge with thanks Prof. T. Yoshida and Dr. H. Satoh for their kind advices and collaboration in animal experiment and they are also indebted to Mr. D. Ohata for microanalysis in this work.

Experimental

6-[4-Bis(β-chloroethyl)aminophenylazo]-2-naphthalenesulfonic Acid (I)—Five grams of finely powdered diazonium chloride obtained from 6-amino-2-naphthalenesulfonic acid was suspended in 150 cc. of water, poured into 150 cc. of EtOH solution of N,N-bis(β-chloroethyl)aniline (4 g.), and stirred at 40° for 1 hr. After 24 hrs., the precipitated crystalline powder was filtered and washed with warm MeOH. It was dissolved in a small amount of water and precipitated by gradual addition of acetone. Yield, 8 g. *Anal.* Calcd. for $C_{20}H_{19}O_3N_3Cl_2S$: C, 53.10; H, 4.23; N, 9.29. Found: C, 53.14; H, 4.23; N, 9.22.

Sodium salt: Orange yellow prisms (from water or EtOH).

3-[4-Bis(β-chloroethyl)aminophenylazo]-1,5-naphthalenedisulfonic Acid (II)—3-Aminonaphthalene-1,5-disulfonic acid (3 g.) was dissolved in a mixture of 10 cc. each of 10% NaOH and 10% $NaNO_2$, and the solution was then poured into a mixture of cracked ice (50 g.) and conc. HCl (20 cc.). After 1 hr., a small amount of urea was added to the reaction mixture to decompose excess HNO_2. This was poured into 50 cc. of EtOH solution of N,N-bis(β-chloroethyl)aniline (1.8 g.), the mixture was maintained at 40° for 30 mins., and then at room temperature for 24 hrs. The precipitated crystalline dye was filtered and washed with MeOH. Yield, 4 g. It was purified by the same procedure as in case of (I). *Anal.* Calcd. for $C_{20}H_{19}O_6N_3Cl_2S_2$: C, 45.11; H, 3.57; N, 7.89. Found: C, 45.05; H, 3.62; N, 7.64.

4-[4-Bis(β-chloroethyl)aminophenylazo]-1-naphthalenesulfonic Acid (III), 7-[4-Bis(β-chloroethyl)aminophenylazo]-1,3-naphthalenedisulfonic Acid (IV), and 4,4′-Bis[4-bis(β-chloroethyl)aminophenylazo]-2,2′-biphenyldisulfonic Acid (V)—All these compounds were prepared by a similar process as for (I) and (II). Two grams of (III) was obtained from 2.3 g. diazotized naphthionic acid and 2.2 g. of N,N-bis(β-chloroethyl)aniline. Purification by recrystallization did not succeed but a precipitate from the reaction mixture was fairly pure after being washed. *Anal.* Calcd for $C_{20}H_{19}O_3N_3Cl_2S\cdot 1\frac{1}{2}H_2O$: C, 50.15; H, 4.63; N, 8.77. Found: C, 50.40; H, 4.58; N, 8.90.

Yield of (IV) was 2.7 g. starting from 3 g. of amino-G-acid and 2.2 g. of N,N-bis(β-chloroethyl)-aniline. *Anal.* Calcd. for $C_{20}H_{19}O_6N_3Cl_2S_2\cdot 1\frac{1}{2}H_2O$: C, 42.97; H, 3.97; N, 7.52. Found: C, 43.14; H, 3.97; N, 7.59.

The pure crystals of the hydrochloride of (V) was obtained when acetone-water solution of the crude product was poured into a large volume of acetone followed by an addition of a small quantity of conc. HCl. Yield, 3 g. of (V) from 1.7 g. of 2,2′-benzidinedisulfonic acid and 2.2 g. of N,N-bis-(β-chloroethyl)aniline. *Anal.* Calcd. for $C_{32}H_{32}O_6N_6Cl_4S_2\cdot 2H_2O$: C, 45.85; H, 4.30; N, 10.02. Found: C, 46.08; H, 4.28; N, 10.03.

4,4′-Bis[4-bis(β-chloroethyl)aminophenylazo]biphenyl (VI)—The coupling mixture of diazotized benzidine hydrochloride (1.3 g.) and N,N-bis(β-chloroethy)aniline (2.2 g.) was poured into a large volume of water, the precipitate was collected, and recrystallized from acetone or benzene. Yield, 1.5 g. *Anal.* Calcd. for $C_{32}H_{32}N_6Cl_4$: C, 59.81; H, 4.98; N, 13.09. Found: C, 59.78; H, 4.98; N, 13.05.

5) M. Ishidate, T. Yoshida, Y. Sakurai, *et al.*: Gann, **47**, 375(1956).

4-[4-Bis(β-chloroethyl)aminophenylazo]benzenesulfonic Acid (VII)— *p*-Diazobenzenesulfonic acid (2 g.) and N,N-bis(β-chloroethyl)aniline (2.8 g.). Yield of (VII), 4.6 g. It was purified by the same process as (I). *Anal.* Calcd. for $C_{32}H_{32}N_6Cl_4$: C, 59.81; H, 4.98; N, 13.08. Found : C, 59.76; H, 4.95; N, 13.28.

Bis(β-hydroxyethyl)-4-nitrobenzylamine—One hundred grams of 4-nitrobenzyl bromide was dissolved in EtOH and 100 g. of diethanolamine was added to it drop by drop under stirring. After the addition was complete, the mixture was heated on a boiling water bath for 0.5 hr., the solvent was distilled off, and the residue was washed with water and recrystallized from ether. Yield, 80 g. of pale yellow prisms, m.p. 77~77.5°. *Anal.* Calcd. for $C_{11}H_{16}O_4N_2$: C, 54.99; H, 6.71; N, 11.66. Found: C, 55.28; H, 6.66; N, 11.74.

Bis(β-chloroethyl)-4-nitrobenzylamine (IX)—The afore-mentioned hydroxy derivative (50 g.) was chlorinated by refluxing with a mixture of $CHCl_3$(100 cc.) and $SOCl_2$(100 cc.) on a water bath for 1 hr. After the reaction ended, an excess of $SOCl_2$ and $CHCl_3$ were distilled off *in vacuo*. The residue solidified to a crystalline mass and was recrystallized from acetone. Yield, 40 g. *Anal.* Calcd. for $C_{11}H_{14}O_2N_2Cl_2 \cdot HCl$: C, 42.11; H, 4.78; N, 8.93. Found : C, 42.22; H, 4.80; N, 9.01.

Bis(β-chloroethyl)-4-aminobenzylamine (X)—Three grams of (IX) was dissolved in 50 cc. of 15~20 % HCl and shaken at room temperature with H_2 over Pd-C catalyst (from 5 cc. of 1% $PdCl_2$ and 0.5 g. of activated carbon), which was aged or exposed in air for a few days. Reduction stopped after absorption of about 640 cc. of H_2 (3 moles). The solution was evaporated *in vacuo* and the residue solidified to a crystalline powder. Yield was fairly quantitative, but perfect purification did not succeed because of mixing a small quantity of by-products of reduction.

Bis(β-chloroethyl)-4-benzamidobenzylamine (XI)—Crude (X) (from 3 g. of the nitro compound) was heated with BzCl(20 g.) and $CHCl_3$(20 cc.) for 4 hrs. on a water bath, excess of BzCl and $CHCl_3$ were distilled off *in vacuo*, and the residue was recrystallized from EtOH. Yield, 4.5 g. *Anal.* Calcd. for $C_{18}H_{20}ON_2Cl_2$: C, 61.54; H, 5.90; N, 7.98. Found : C, 61.54; H, 5.70; N, 7.98.

Hydrochloride : Colorless prisms, m.p. 138~148°. *Anal.* Calcd, for $C_{18}H_{20}ON_2Cl_2 \cdot HCl$: C, 55,89; H, 5.43; N, 7.24. Found : C, 55.88; H, 5.42; N, 7.42.

4-(4-Dimethylaminophenylazo)benzyl-bis(β-chloroethyl)amine (XII)—A solution of (X), prepared by the catalytic reduction of (IX) (3 g.), in 15% HCl(50 cc.), was immediately diazotized with $NaNO_2$ (0.8g.). After addition of urea, the mixture was poured into an ice-cooled solution of N,N-dimethylaniline (1.2 g.) in water(50 cc.) under stirring. After the acidity was adjusted with AcONa, the reaction mixture was extracted with ether. The ethereal solution was washed with $NaHCO_3$ solution and ether was distilled off. The residue became crystalline and was recrystallized from EtOH. Yield, 2 g. *Anal.* Calcd. for $C_{19}H_{24}N_3Cl_2$: C, 60.16; H, 6.38; N, 14.81. Found : C, 60.03; H, 6.44; N, 14.99.

1-[4-Bis(β-chloroethyl)aminomethylphenylazo]-2-naphthol (XIII)—Three grams of (IX) was reduced and immediately diazotized without isolating the amine. EtOH solution of β-naphthol (1.2 g.) was added to this diazonium solution, and neutralized with a mixture of Na_2CO_3 and AcONa. An oily substance separated from the reaction mixture which was extracted with ether, the ethereal solution was dried, and dry HCl gas was passed through it. A red oily layer separated and solidified to a crystalline mass, which was recrystallized from EtOH-ether mixture. Yield, 2 g. *Anal.* Calcd. for $C_{21}H_{21}ON_3Cl_2$: C, 57.46; H, 5.02; N, 9.58. Found : C, 57.18; H, 5.32; N, 9,77.

Reductive Cleavage of Benzylated Amines—A solution of (IX) (9 g.) in 5% HCl(50 cc.) was shaken with H_2 over Pd-C catalyst (10 cc. of 1% $PdCl_2$ and 1 g. of activated carbon). Absorption of H_2 stopped nearly at 2720 cc.(4 moles). The solution was evaporated *in vacuo* and the residue was acetylated with AcCl by the usual process and crystals melting at 153° were obtained which was identified as *p*-acetotoluidide. Yield, 4 g.

Bis(β-chloroethyl)benzylamine (2.3 g.) was dissolved in EtOH(50 cc.) and shaken with H_2 over Pd-C catalyst. Absorption of H_2 stopped nearly at 250 cc.(1 mole). The solution was distilled off *in vacuo* and the residue was treated with picric acid solution. The picrate was recrystallized from EtOH and melted at 112~113°. It was identified with bis(β-chloroethyl)amine picrate by mixed melting point with the authentic sample.

EtOH solution of (β-chloroethyl)dibenzylamine (2.6 g.) was reduced by H_2 over Pd-C catalyst. Absorption of H_2 stopped nearly at 250 cc.(1 mole) and did not go further. The solvent was evaporated *in vacuo* and the residue was recrystallized from EtOH or acetone to colorless prisms, m.p. 195°. Its analytical data agreed well with those of the hydrochloride of (β-chloroethyl)benzylamine. *Anal.* Calcd. for $C_9H_{12}NCl \cdot HCl$: C, 52.43; H, 6.31; N, 6.80. Found : C, 52.46; H, 6.41; N, 6.97.

Summary

A number of new azo derivatives of N,N-bis(β-chloroethyl)aniline and bis(β-chloroethyl)benzylamine were prepared for the purpose of screening their anti-cancer activity against Yoshida sarcoma.

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