The authors wish to express their gratitude to Prof. T. Yoshida, Dr. H. Satoh, and Dr. H. Imamura for their advices and collaboration especially in the biological screening experiments. A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education and from the Ministry of Health and Welfare, to which the authors' thanks are due.

Summary

Several derivatives of N-(2-substituted ethoxy)-2,2'-dichlorodiethylamine were prepared and tested for their latent antitumor activity against Yoshida sarcoma. Mode of activation of these compounds was also discussed.

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109. Morizo Ishidate,*2 Yoshio Sakurai,*2 and Ken-ichi Sawatari*3 : Studies on Carcinostatic Substances. XXXVII.*1 Preparation of Derivatives of Nitrogen Mustard having Metal Chelate-forming Activity.

(*Iatrochemical Institute of Pharmacological Research Foundation**2 *and Yoshitomi Pharmaceutical Co., Ltd.**3)

The most important problem is to improve specificity of action of derivatives of simple nitrogen mustard on tumor by modification of the molecular structure. One probable method for this purpose may be the control or restriction of reactivity of the two functional groups of this compound by some means and, from this point of view, copper chelate formation of amino acid derivatives having two 2-chloroethyl groups was investigated.

Needless to say, the reactivity of this functional group depends on the property of lone-pair electrons of nitrogen, which should change when the electrons participate in formation of a chelate. Therefore, it was expected that such a compound in a form of chelate might behave as an antitumor agent having an improved chemotherapeutic index or a long-lasting effect.

The compounds prepared and discussed in this work are shown *en bloc* in Table I and their synthetic procedures are shown in Chart 1.

The chelate-forming activity of 2,2'-dichlorodiethylamine derivative (No. 602) was found to be nearly of the same order as that of the derivative of 2-(2-chloroethylamino)-acetic acid (No. 652) by determination of stability constants of their copper chelates, data of which are listed in Table II.

As will be mentioned below, the derivatives of ethylenediamine have greater tendency to form a chelate with copper ion than those of propylenediamine. The reaction of No. 601 in an aqueous solution is supposed to proceed as shown in Chart 2.

Alkylating velocity depends on the concentration of the aziridinium intermediate (III), which is necessarily dependent on the stability of the chelate in the reaction solution and,

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*1 Part XXXVI : This Bulletin, **9**, 676 (1961).

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Table I.

Compd. No.	Structure		m.p.	LD_{50}[a] mg./kg.	MTD[b] mg./kg.	MED[c] mg./kg.	MEC[d] mM
638	$(NCCH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2CN)(CH_2CH_2Cl)$	Hydrochloride	120~121°	75	50	5	1×10^{-2}
659	$(C_2H_5OOCCH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2CH_2COOC_2H_5)(CH_2CH_2Cl)$	Dipicryl-sulfonate	124~125°	175	100	1	2.5×10^{-3}
660	$(H_2NOCCH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2CONH_2)(CH_2CH_2Cl)$	Picrate	166~167°	0.75	0.5	0.01	(−)
652	$(HOOCCH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2COOH)(CH_2CH_2Cl)$	Dihydrochloride	187~188°	30	10	1	2.5×10^{-1}
651	$(ClCH_2CH_2)(CH_2)NCH_2CH_2N(CH_2CH_2Cl)(CH_2)\cdot H_2O$, $OC-O-Cu-O-CO$		169~170°	30	10	5	(−)
602	$(ClCH_2CH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2COOH)(CH_2COOH)$	Dihydrochloride	114~115°	75	50	10	2.5×10^{-1}
601	$(ClCH_2CH_2)(ClCH_2CH_2)NCH_2CH_2N(CH_2COO)(CH_2COO)Cu\cdot H_2O$		118~120°	30	10	10	(−)
662	$(ClCH_2CH_2)(ClCH_2CH_2)NCH_2CH_2CH_2N(CH_2CH_2COO)(CH_2CH_2COO)Cu$	Hydro-chloride	120~121°	30	10	1	(−)
723	$(HOOCCH_2)(ClCH_2CH_2)NCH_2CH_2CH_2N(CH_2CH_2Cl)(CH_2COOH)$	Dipicrate	87~90°	75	50	5	2.5×10^{-2}
	$(HOOCCH_2)(HOOCCH_2)NCH_2CH_2N(CH_2COOH)(CH_2COOH)$			500	200	(−)	(−)
	$(NaOOCCH_2)(H_2C)NCH_2CH_2N(CH_2COONa)(CH_2)$, $O=C-O-Cu-O-C=O$			75	50	(−)	(−)

a) On rat (i. p.)
b) Maximum tolerance dose on rat (i. p.)
c) Minimum effective dose on Yoshida sarcoma rats
d) Minimum effective concentration on the *in vitro*-cultured Yoshida sarcoma cells (cf. This Bulletin, **7**, 873 (1959))

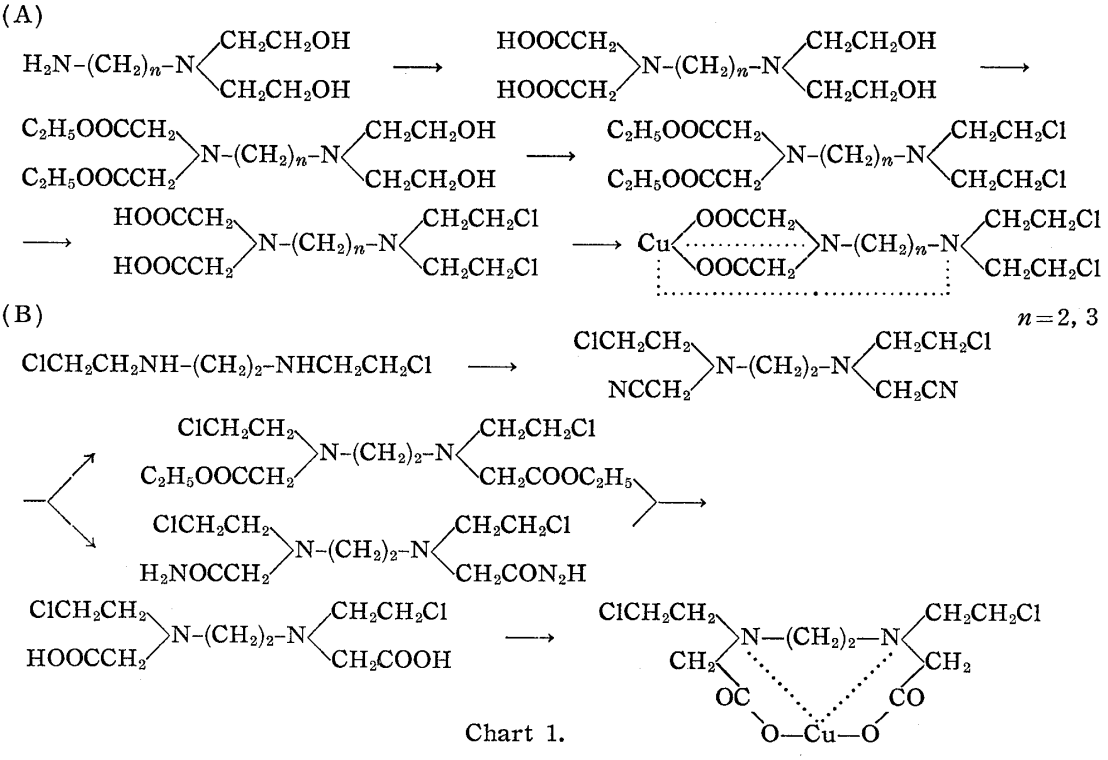


Chart 1.

Table II. Ionization Constant and Stability Constant[a]
$t=25\pm0.5°C$ $\mu^{b)}=0.10$ $\gamma H^{+c)}=0.80$

No.	Compound	Ionization constant		Stability constant of copper chelate	
		pK_1	pK_2	$\log K_1$	$\log K_2$
602	N,N-Bis(2-chloroethyl)-N′,N′-bis-(2-carboxymethyl)ethyleediamine	3.74	9.24	11.9	4.2
652	N,N′-Bis(2-chloroethyl)-N,N′-bis-(2-carboxymethyl)ethyleediamine	3.90	7.81	12.1	

a) Calculated by Bjerrum's method *b*) Ionic strength
c) Activity coeffecient determined by Dr. A. Hanaki

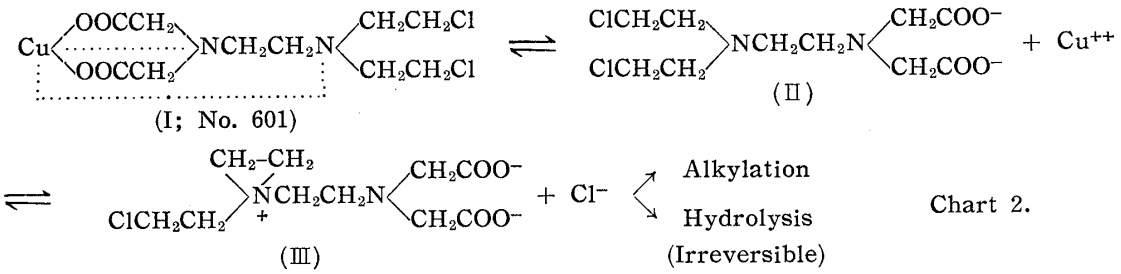
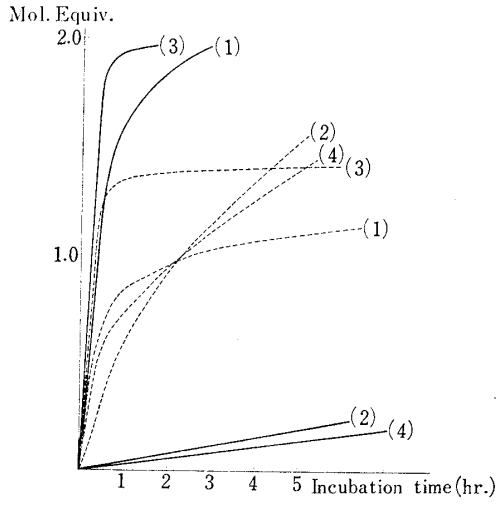


Chart 2.

therefore, the action of the functional group is retarded by chelate formation. Moreover, if there are some different factors that chang the stability of the chelate in each different tissue or organ, organ selectivity of the action might be expected to some extent.

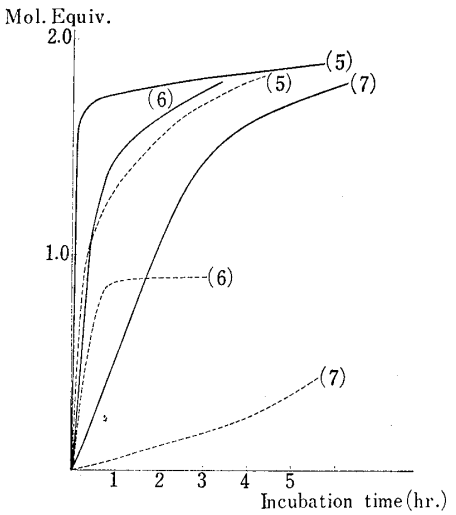
The parent compounds corresponding to the chelates, viz. Nos. 652, 602, and 723, and their related compounds in Table I were examined as derivatives of nitrogen mustard of amino acid structure. Rates of hydrolysis and alkylation on thiosulfate of Nos. 652, 651, 602, and 601 in a neutral aqueous solution are shown in Fig. 1 (A) and the data of other compounds are also given in Fig. 1 (B).

As will be seen in Fig. 1 (A), hydrolysis of chlorine in the two chelates is strongly suppressed when compared with that of parent compounds, while the rate of alkylation,



—— Cl^- Liberation
------ Thiosulfate uptake
(1) No. 602 (H_2O)
(2) No. 601 (H_2O)
(3) No. 652 (H_2O)
(4) No. 651 (H_2O)

Fig. 1. (A) Cl^- Liberation and Thiosulfate Uptake of the Compounds at 37° in Hydrogencarbonate Buffer



—— Cl^- Liberation
------ Thiosulfate uptake
(5) No. 662 (50% MeOH)
(6) No. 660 (30% Me_2CO)
(7) No. 638 (50% MeOH)

Fig. 1. (B) Cl^- Liberation and Thiosulfate Uptake of the Compounds at 37° in Hydrogencarbonat Buffer

viz. thiosulfate-uptake during 5 hours, of the two compounds is not so different from each other, although the initial velocity of alkylation of the chelates is of course more or less retarded. In other words, disappearance of the compound in the reaction medium by hydrolysis is fairly prevented by chelate formation without decrease of its alkylating activity.

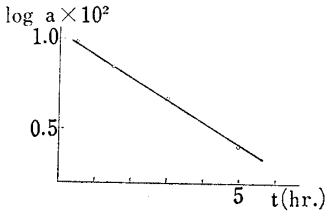


Fig. 2. Relation between Thiosulfate Concentration in Reaction Solution and Incubation Time

a : Concentration of thiosulfate in case of Fig. 1(B)
t : Incubation time

It is also shown in Fig. 2 that alkylating action of No. 601 on thiosulfate satisfies the condition of first-order reaction concerning the concentration of the latter reagent and this means that the concentration of the aziridinium intermediate remains nearly constant through the progress of the reaction. From these facts, it may be realized that the stability constant of the chelate chiefly regulates the alkylating velocity of these compounds.

As described above, No. 662, a derivative of propylenediamine, has only a weak chelating activity and its Cl^- liberation seems not to be retarded by chelate formation (Fig. 2), and it easily separates copper ion in a hydrogencarbonate-buffered solution.

Antitumor activity of these compounds, including their copper chelates, on Yoshida sarcoma is shown in Table I. Contrary to the anticipation from the mode of reaction of the chelates *in vitro*, minimum effective concentration of the chelate compound on the tumor does not differ so much from that of their parent compounds, while the toxicity of the former is the same or rather increased by chelate formation.

As it is known that the toxicity of EDTA is enhanced by chelation with copper (Table I), the higher toxicity of these chelates might be due to toxic nature of copper. Among the derivatives of amino acid, Nos. 652, 602, and 723, which have chelate-forming activity, have rather less antitumor effect than the compounds having no chelate-forming activity (Nos. 659 and 660). No. 659 was proved to have a very promising property from the view of chemotherapeutic index (LD_{50}/MED).

Experimental

Measurement of Liberation of Cl^- and Thiosulfate Uptake——Titration was carried out at 37° by the method already reported.[1]

N,N-Bis(2-hydroxyethyl)-N′-acetylethylenediamine——Two moles of ethylene oxide (88 g.) was absorbed into a solution of 1 mole of monoacetylethylenediamine (102 g.) in H_2O (100 cc.) at 35~40° with stirring. After standing overnight, water was evaporated *in vacuo* and the oily residue, which distilled out at $b.p_{0.5}$ 185~200° with partial decomposition, was used for the next process.
Picrate : m.p. 65~70°.

N,N-Bis(2-hydroxyethyl)ethylenediamine——A mixture of the crude N,N-bis(2-hydroxyethyl)-N′-acetylethylenediamine (50 g.) and 20% NaOH solution (250 cc.) was warmed at 70~75° for 3 hr. After cool, the reaction mixture was extracted with $CHCl_3$ and the $CHCl_3$ layer was separated as soon as possible. $CHCl_3$ was distilled off and the oily residue was fractionated, $b.p_{0.5}$ 130~135°.
Picrate : m.p. 181~182°. *Anal.* Calcd. for $C_{18}H_{22}O_{16}N_4$: C, 35.64; H, 3.66; N, 18.47. Found : C, 36.00; H, 3.77; N, 18.74.

N,N-Bis(2-hydroxyethyl)-N′,N′-bis(carboxymethyl)ethylenediamine——30% solution of NaCN (17 g.) was added to a solution of N,N-bis(2-hydroxyethyl)ethylenediamine (14.8 g.) in H_2O (50 cc.). The mixture was then diluted to 150 cc., adjusted to pH 10 by addition of *N* NaOH, and added with 3% HCHO solution (100 g.). After warming for 8 hr. at 70°, the mixture was evaporated to dryness *in*

1) M. Ishidate, Y. Sakurai, S. Owari : This Bulletin, **5**, 203 (1957).

vacuo. The residue was dissolved in H_2O and the same amount of NaCN and HCHO solution as the first addition were added. The process was repeated three times in all. The residue after the final evaporation was dissolved in 7 volumes of H_2O, Amberlite IRC-50 (100 cc.) was then added, and the mixture was stirred for 1 hr. The filtrate was evaporated to dryness *in vacuo*. After standing in an ice box for five days, the residue was recrystallized from H_2O-MeOH, m.p. 190~195°. Yield, 40%. *Anal.* Calcd. for $C_8H_{20}O_6N_2$: C, 45.43; H, 7.66; N, 10.60. Found : C, 45.66; H, 7.66; N, 10.55.

N,N-Bis(2-chloroethyl)-N′,N′-bis(carboxymethyl)ethylenediamine (No. 602)——N,N-Bis(2-hydroxyethyl)-N′,N′-bis(carboxymethyl)ethylenediamine (20 g.) was added to 60 cc. of dehyd. EtOH saturated with dry HCl at 0°. After standing overnight, the solution was heated to 90° and maintained at the same temperature for 10 hr. with stirring. The solvent was evaporated, the residue was treated again with dehyd. EtOH saturated with dry HCl, and heated for 5 hr. After EtOH was completely evaporated, the residue was dissolved in $CHCl_3$, and $SOCl_2$ was added in small portions with stirring. The reaction mixture was mildly refluxed for 6 hr. The whole liquid was removed by distillation *in vacuo*. Crude N,N-bis(2-chloroethyl)-N′,N′-bis(ethoxycarbonylmethyl)ethylenediamine hydrochloride was dissolved in conc. HCl and warmed at 80° for 2 hr. After evaporation of the solvent, the residue was recrystallized from EtOH and N,N-bis(2-chloroethyl)-N′,N′-bis(carboxymethyl)ethylenediamine dihydrochloride (No. 602) was obtained as white crystals, m.p. 115~117°.
Picrate : m.p. 120°. *Anal.* Calcd. for $C_{22}H_{24}O_{18}N_6Cl_2$: C, 34.79; H, 3.18; N, 14.76. Found : C, 35.40; H, 3.49; N, 15.02.

Copper Chelate of N,N-Bis(2-chloroethyl)-N′,N′-bis(carboxymethyl)ethylenediamine (No. 601)——The hydrochloride of No. 602 (1 g.) was dissolved in H_2O (5 cc.) and a solution of $(AcO)_2Cu$ (0.42 g.) in 10 cc. of H_2O was added. The mixture was adjusted to pH 5.5 by addition of dil. NaOH and Cu chelate of No. 602, which had 1 mole of crystal water, precipitated quantitatively as fine blue crystals, m.p. 218~220° (decomp.). *Anal.* Calcd. for $C_{10}H_{16}O_4N_2Cl_2Cu \cdot H_2O$: C, 31.50; H, 4.74; N, 7.32. Found: C, 31.22; H, 4.69; N, 7.41.

N,N′-Bis(2-chloroethyl)ethylenediamine——Into a mixture of N,N′-bis(2-hydroxyethyl)ethylenediamine (10 g.) and benzene (50 cc.), $SOCl_2$ (20.3 g.) was added in small portions. After refluxing for 20 hr., crystals that appeared were collected and recrystallized from MeOH.
Dihydrochloride : m.p. 219~220°. *Anal.* Calcd. for $C_6H_{14}N_2Cl_4$: C, 27.82; H, 6.23; N, 10.82. Found: C, 28.29; H, 6.37; N, 10.56.

N,N′-Bis(2-chloroethyl)-N,N′-bis(2-cyanoethyl)ethylenediamine (No. 638)——A solution of N,N′-bis(2-chloroethyl)ethylenediamine hydrochloride (40 g.) in H_2O (150 cc.) was cautiously added with stirring into a solution of NaCN (14 g.) in H_2O (50 cc.) and the temperature was kept below 0°. After stirring for 30 min. at 0°, 35% HCHO solution (36 g.) was added in small portions and the mixture was stirred for an additional 1 hr. It was extracted with Et_2O (200 cc.), the extract was dried over anhyd. Na_2SO_4, treated with dry HCl gas, and evaporated to dryness. The residue (crude No. 638) was recrystallized from EtOH, m.p. 120~121°. *Anal.* Calcd. for $C_{10}H_{17}N_4Cl_3$: C, 40.08; H, 5.72; N, 18.70; ionic Cl, 11.86. Found : C, 40.18; H, 5.70; N, 18.52; ionic Cl, 11.82.

N,N′-Bis(2-chloroethyl)-N,N′-bis(carboxymethyl)ethylenediamine Diethyl Ester (No. 659)——A mixture of 10 g. of No. 638 and EtOH (400 cc.) saturated with HCl gas was refluxed for 5 hr. After cool, NH_4Cl was removed and the filtrate was evaporated to dryness. The residue was dissolved in Me_2CO, filtered, and evaporated.

The residue was converted into the picrylsulfonate, which was recrystallized from MeOH, m.p. 124~125°. *Anal.* Calcd. for $C_{26}H_{32}O_{22}N_8Cl_2S_2$: C, 33.09; H, 3.42; N, 11.82. Found : C, 33.15; H, 3.68; N, 12.18.

N,N′-Bis(2-chloroethyl)-N,N′-bis(carbamoylmethyl)ethylenediamine (No. 660)——No. 638 hydrochloride (5 g.) was dissolved in a cold 96% H_2SO_4 solution (8 cc.) and kept standing overnight at room temperature. The mixture was poured on cracked ice and neutralized with $NaHCO_3$. The crude hydrolysate was recrystallized from MeOH, m.p. 102~103°. *Anal.* Calcd. for $C_{10}H_{20}O_2N_4Cl_2$: C, 40.14; H, 6.74; N, 18.73. Found : C, 40.17; H, 6.61; N, 18.24.
Dipicrate : m.p. 166~167°. *Anal.* Calcd. for $C_{22}H_{26}O_{16}N_{10}Cl_2$: C, 34.88; H, 3.46; N, 18.93. Found: C, 35.13; H, 3.56; N, 18.65.

N,N′-Bis(2-chloroethyl)-N,N′-bis(carboxymethyl)ethylenediamine (No. 652)——The crude No. 659 hydrochloride (5 g.) was dissolved in conc. HCl (50 cc.) and heated at 80° for 4 hr. After evaporation, the residue was recrystallized from AcOEt, m.p. 187°. *Anal.* Calcd. for $C_{10}H_{18}O_4N_2Cl_4$: C, 32.11; H, 5.29; N, 7.49. Found : C, 32.40; H, 5.44; N, 7.45.

Copper Chelate of N,N′-Bis(2-chloroethyl)-N,N′-bis(carboxymethyl)ethylenediamine (No. 651)——No. 652 hydrochloride (1 g.) was dissolved in a minimum amount of H_2O and a saturated solution of 0.42 g. of $(AcO)_2Cu$ was added. After standing for several hr., crystalline Cu chelate of No. 652 separated and was recrystallized from 90% MeOH to crystals with 1 mole of crystal water, m.p. 189~190° (decomp.). *Anal.* Calcd. for $C_{10}H_{16}O_4N_2Cl_2Cu \cdot H_2O$: C, 31.54; H, 4.79; N, 7.35. Found : C, 31.31; H, 4.72; N, 7.28.

N,N-Bis(2-Hydroxyethyl)trimethylenediamine——This and the following trimethylenediamine derivatives were obtained by a method similar to that for the corresponding ethylenediamine derivatives.

A solution of 3-bis(2-hydroxyethyl)aminopropionitrile (100 g.) in EtOH (100 cc.) containing NH_3 (5 g.) was placed in an autoclave with Raney Ni catalyst (10 g.) and the mixture was shaken with H_2 at 50 atm. and 60° for 1 hr. The catalyst was removed by filtration and EtOH was distilled off. The oily residue was fractionated *in vacuo*. N,N-Bis(2-hydroxyethyl)trimethylenediamine, b.$p_{0.5}$ 150～165°. (yield, 70%).

N,N-Bis(2-hydroxyethyl)-N′,N′-bis(carboxymethyl)trimethylenediamine——This was prepared by a method similar to that for the corresponding ethylenediamine derivative except that Amberlite IRC-120 was used instead of IRC-50. The free acid was not obtained as crystals. The corresponding Cu chelate with 1 mole of crystal water was obtained as fine blue crystals, m.p. 95～100°. The analytical data did not agree acurately with the theoretical values because of the difficulty in its complete combustion. *Anal.* Calcd. for $C_{11}H_{20}O_6N_2Cu \cdot H_2O$: N, 7.84. Found : N, 7.94.

N,N-Bis(2-chloroethyl)-N′,N′-bis(carboxymethyl)trimethylenediamine (No. 723)——This was obtained by the usual chlorination procedure.

Picrate : m.p. 87～90°. *Anal.* Calcd. for $C_{23}H_{26}O_{18}N_8Cl_2$: N, 14.49. Found : N, 15.05.

Copper Salt of N,N-Bis(2-chloroethyl)-N′,N′-bis(2-carboxymethyl)trimethylenediamine (No. 662)——The monohydrochloride of this compound was obtained by the similar method as in the case of No. 601; m.p. 155～160° (decomp.). *Anal.* Calcd. for $C_{11}H_{18}O_4N_2Cl_2Cu \cdot HCl$: Ionic Cl, 8.6; total Cl, 24.16. Found : Ionic Cl, 8.8; totat Cl, 24.57.

The authors wish to thank Prof. T. Yoshida for his kind advices through this investigation and Dr. H. Satoh and Dr. H. Imamura for their collaboration in animal experiments. The determination of constants of the chelates was carried out by Dr. A. Hanaki, to whom they are indebted. Expenses for this work were defrayed by the Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

Summary

Derivatives of nitrogen mustard having metal chelate-forming activity and their copper chelates were prepared and their chemical properties and their antitumor effect on Yoshida sarcoma were discussed.

(Received December 20, 1960)

UDC 547.92 : 582.572.2

110. Kanzo Sasaki : Studies on the Steroidal Components of Domestic Plants. XXXII.[1] Constituents of *Reineckia carnea* KUNTH. (4). Structure of Kitigenin. (1).

(*Research Laboratory, Shionogi & Co., Ltd.*[*1])

Kitigenin is a sapogenin isolated from *Reineckia carnea* KUNTH, of which the following facts have been clarified previously[1,2] : 1) The empirical formula of the sapogenin (I) is $C_{27}H_{44}O_6$ and it belongs to the 25D-series. 2) Acetylation of the sapogenin yields a triacetate, $C_{33}H_{50}O_9$, which has still one free hydroxyl group. 3) By periodic acid oxidation followed by silver oxide oxidation, the sapogenin affords des-A-spirostan-5-one (V). These facts show that kitigenin is a tetrahydroxy-sapogenin and all the hydroxyl groups in kitigenin are located in ring A.

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1) Part XXXI : K. Takeda, T. Okanishi, K. Sasaki, A. Shimaoka : This Bulletin, **9**, 631 (1961).

2) K. Takeda, T. Okanishi, A. Shimaoka : Yakugaku Zasshi, **75**, 560 (1955).