Dipeptide Nitrogen Mustards of Glycine and γ-Aminobutyric Acid[1]

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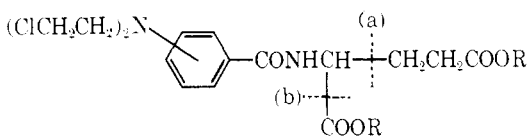
The position isomers of N-[bis(2-chloroethyl)aminobenzoyl]glycine and of N-{bis(2-chloroethyl)aminobenzoyl}-γ-aminobutyric acid were synthesized. The biological activities of these dipeptide nitrogen mustards and their ethyl esters were studied in eight microbial systems, in Sarcoma 180 and KB cell cultures, and in the Ehrlich ascites tumor. The *ortho* isomers of both the glycine and γ-aminobutyric acid series showed the highest antineoplastic activity, analogous to that of the *ortho* isomer in dipeptide nitrogen mustards of glutamic acids.

In our investigation of dipeptide nitrogen mustards, we have synthesized the optical and position isomers of N-[bis(2-chloroethyl)aminobenzoyl]glutamic acid.[2] The biological activities of these dipeptide nitrogen mustards and their benzyl[2a] and ethyl[2] esters were studied in microbial systems and in KB line cell culture in which moderate activities were demonstrated. In the Ehrlich ascites mouse tumor, the antineoplastic activities are largely restricted to the L compounds.[2] Among the position isomers in both the L and D series, the *ortho* isomers exceed the corresponding *para* and *meta* isomers in activity. N-{*o*-[Bis(2-chloroethyl)-amino]benzoyl}-L-glutamic acid and its ethyl ester consequently showed significant antineoplastic activity against Ehrlich ascites mouse carcinoma. This is comparable to methyl nitrogen mustard (HN2) and the phenylalanine nitrogen mustard (sarcolysin) but at higher effective and toxic dose levels.[2c]

Further investigation of the relation of chemical structure to biological activity seems of importance for elucidation of the mode of action and for future design of biologically active peptide nitrogen mustards as carcinolytic agents. Replacement of parts of the peptide chain in the N-[bis(2-chloroethyl)amino-benzoyl]glutamic acid by hydrogen at (a) to eliminate the CH_2CH_2COOR, or at (b) to eliminate the



COOR functional groups would result, respectively, in position isomers of N-[bis(2-chloroethyl)amino-benzoyl]glycine and N-[bis(2-chloroethyl)aminoben-zoyl]-γ-aminobutyric acid. This article presents the synthesis of these dipeptide nitrogen mustards and the evaluation of their biological activities.

Experimental Section[3]

Ethyl γ-Aminobutyrate Hydrochloride.—The preparation of the ester hydrochloride by esterification of γ-aminobutyric acid was previously reported.[4] The modified esterification procedure below was found practical. It was found that the variation in melting point reported, 65–72°[4a] and 72°,[4b,5] was attributable to moisture in the compound and the solvent used for recrystallization.

γ-Aminobutyric acid (5 g) was suspended in 60 ml of absolute ethanol which was then saturated with dry HCl in approximately 0.5 hr. The reaction mixture was heated under reflux for 20 hr, at the end of which time a clear solution had resulted. After evaporation of the excess ethanol, a semisolid was obtained. This was dried over P_2O_5 and soda lime under reduced pressure, and a crystalline product was formed. After two recrystallizations from absolute ethanol–ether (1:5), the pure hygroscopic ester hydrochloride (75%) was obtained; mp 70–72° (sealed tube). If absolute ethanol was used for recrystallization a melting point of 65–72° often resulted. The elemental analyses are correct in both instances.

Ethyl N-[Bis(2-chloroethyl)aminobenzoyl]glycinates.—The isomeric ethyl bis(2-chloroethyl)aminobenzoates were prepared according to the procedures previously reported.[2] The bis(chloroethyl)aminobenzoic acid esters were hydrolyzed in concentrated HCl to their respective acids which were converted to the acid chlorides. Condensation of the bis(2-chloroethyl)aminobenzoyl chlorides with ethyl glycinate, in $NaHCO_3$ solution, was carried out by the procedure previously described.[2]

N-[Bis(2-chloroethyl)aminobenzoyl]glycines.—The esters obtained above were hydrolyzed in 0.5 *N* NaOH in 50% methanol as described in the previous procedure.[2] The dipeptide nitrogen mustards were purified by recrystallization from various solvent systems (see Table I). However, N-{*o*-[bis(2-chloroethyl)amino]-benzoyl}glycine has not been obtained in a pure form by repeated recrystallization.

Ethyl N-[Bis(2-chloroethyl)aminobenzoyl]-γ-aminobutyrates.—The formation, in $NaHCO_3$, and isolation of the isomeric ethyl N-[bis(2-chloroethyl)aminobenzoyl]-γ-aminobutyrates were carried out as described for the preparation of the corresponding glutamate analogs.[2]

N-[Bis(2-chloroethyl)aminobenzoyl]-γ-aminobutyric Acids.—The hydrolysis of the ethyl N-[bis(2-chloroethyl)aminobenzoyl]-γ-aminobutyrates in 0.5 *N* NaOH in 50% methanol was carried out by the procedure previously described.[2] The *para* and *meta* isomers were purified by recrystallization from various solvent systems.

N-{*o*-[Bis(2-chloroethyl)amino]benzoyl}-γ-aminobutyric acid was first obtained as a thick oil. On standing at 4° for 7 days, large, colorless crystals formed in the oil. The crystals, which were isolated manually and washed with a small amount of cold methanol and then a small amount of cold ether, had a distinct melting point and gave the correct elemental analysis for the dipeptide ester hydrochloride (see Table II). The oil remained unchanged on prolonged standing at 4° and gave varying and inconsistent elemental analyses.

(1) Supported in part by Public Health Service Research Grants CY-3335 and C-6516 from the National Cancer Institute, National Institutes of Health.

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(3) All melting points are corrected and, unless specified, the compounds always become a clear liquid at the melting range. The yield is given for the purified compound. The elemental analyses were performed by Dr. C. K. Fitz, Needham Heights, Mass.

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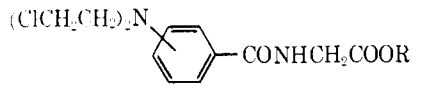
TABLE I

ISOMERS OF N-[BIS(2-CHLOROETHYL)AMINOBENZOYL]GLYCINE[a]

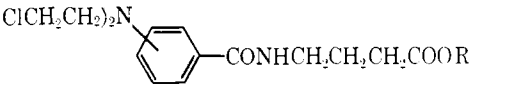


Compd[b]	Isomer	R	Recrystn solvent[c]	Yield, %	Mp, °C (cor)	Found, %[d] C	H	Cl	N
I	*p*	C_2H_5	C	74	74–75 (108 clear)	51.8	5.9	20.5	8.1
II	*m*	C_2H_5	C	88	93–94 (103 clear)	52.0	5.6	20.4	8.1
III	*o*	C_2H_5	A	75	56–60	51.8	5.6	20.4	8.1
IV	*p*	H	A	78	144–146	48.9	4.9	22.4	8.7
V	*m*	H	B	80	Oil	49.5	4.9	22.6	8.9

[a] Formulas for I–III and IV–VI are $C_{15}H_{20}Cl_2N_2O_3$ and $C_{13}H_{16}Cl_2N_2O_3$, respectively. [b] N-{*o*-[Bis(2-chloroethyl)amino]benzoyl}-glycine (VI) has not been obtained in a pure form. [c] A = acetone, B = ethyl acetate, C = ethyl acetate–hexane (1:5). [d] *Anal.* Calcd for I–III: C, 51.9; H, 5.8; Cl, 20.4; N, 8.1. Calcd for IV–VI: C, 48.9; H, 5.1; Cl, 22.2; N, 8.8.

TABLE II

ISOMERS OF N-[BIS(2-CHLOROETHYL)AMINOBENZOYL]-γ-AMINOBUTYRIC ACID[a]



Compd	Isomer	R	Recrystn solvent[b]	Yield, %	Mp, °C (cor)	Found, %[c] C	H	Cl	N
VII	*p*	C_2H_5	D	70	Semisolid	54.2	6.2	19.3	7.5
VIII	*m*	C_2H_5	C	73	71–72.5 (125 clear)	54.3	6.3	19.1	7.4
IX	*o*	C_2H_5	B	73	Oil	54.3	6.1	18.5	7.7
X	*p*	H	C	65	124–126 (129 clear)	51.6	5.9	20.5	8.0
XI	*m*	H	A	60	Semisolid	52.4	5.8	20.5	8.0
XII[d]	*o*	H	A	8	149–157 (164 clear)	47.0	5.5	27.6	7.2

[a] Formulas for VII–IX and X-XI are $C_{17}H_{24}Cl_2N_2O_3$ and $C_{15}H_{20}Cl_2N_2O_3$, respectively. [b] A = acetone, B = ethyl acetate, C = ethyl acetate–hexane (1:5), D = benzene. [c] *Anal.* Calcd for VII–IX: C, 54.4; H, 6.4; Cl, 18.9; N, 7.5. Calcd for X–XI: C, 51.9; H, 5.8; Cl, 20.4; N, 8.1. [d] Isolated as a hydrochloride. *Anal.* Calcd for $C_{15}H_{20}N_2O_3 \cdot HCl$: C, 47.0; H, 5.5; Cl, 27.7; N, 7.3.

TABLE III

INHIBITORY EFFECT ON EIGHT MICROBIOLOGICAL SYSTEMS (ID_{50} in μg/ml)[a]

	1		2		3		4		5		6			
Compd[b]	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	7	8
I	45	45	I	I	280	65	I	I	28	33	100	250	30	160
II	48	51	I	I	33	60	I	I	29	33	160	100	28	30
III	80	80	I	I	I	I	I	I	43	45	I	I	I	35
IV	45	70	I	I	I	I	I	I	35	33	60	100	30	35
V	51	50	I	I	300	85	I	I	31	33	33	60	25	37
VII	55	60	I	I	I	I	80	100	30	27	I	I	30	50
VIII	60	60	I	I	I	I	100	I	34	35	I	I	30	50
IX	60	65	I	I	I	I	I	I	43	41	I	I	I	33
X	45	70	I	I	I	100	I	I	50	33	700	650	30	35
XI	61	60	I	I	I	60	I	I	35	30	I	I	41	52
XII	41	70	I	I	I	100	I	I	42	33	I	35	27	40

[a] I (inactive) = $ID_{50} \geqq 1000$ μg/ml. Systems are: 1, *Streptococcus faecalis* in PGA, (a) 0.01 μg/ml and (b) 0.001 μg/ml; 2, *Lactobacillus arabinosus* in nicotinic acid, (a) 0.1 μg/ml and (b) 0.01 μg/ml; 3, *Lactobacillus arabinosus* in pantothenate, (a) 0.1 μg/ml and (b) 0.01 μg/ml; 4, *Pediococcus cervisiae* in citrovorum factor, (a) 0.01 μg/ml and (b) 0.001 μg/ml; 5, *Lactobacillus fermenti* in thiamine, (a) 0.1 μg/ml and (b) 0.01 μg/ml; 6, *Lactobacillus casei* in riboflavin, (a) 0.1 μg/ml and (b) 0.01 μg/ml; 7, *Escherichia coli* in synthetic medium; 8, *Canadida albicans* in semisynthetic medium. [b] For compound VI, see footnote *b* in Table I.

Thin Layer Chromatography.—The compounds synthesized were chromatographed on silica gel thin layer on poly(ethylene terephthalate) (Eastman Chromatogram Sheet, type K301R). Each of the dipeptide nitrogen mustards and their ethyl esters gave a single yellow spot on the chromatogram when sprayed with 0.5% iodine in $CHCl_3$. The R_f values are 0.81–0.84 in 2-butanol–98% formic acid–H_2O (75:13:12, v/v)[6a] and 0.70–0.75 in 2,6-lutidine–ethanol–H_2O–diethylamine (55:25:20:2, v/v).[6b] The *o*-dipeptide nitrogen mustards and their esters also appeared as blue fluorescent spots under long-wavelength ultraviolet light.

Biological Activities. A. Antimicrobial Assay.—The dipeptide nitrogen mustards and their ethyl esters were tested in eight microbial systems currently in use for screening in the Laboratories of Microbiology of this institution. The assay methods have been reported in detail elsewhere.[7] The results are summarized in Table III.

B. Inhibitory Activities in Sarcoma 180[8a,b] and KB Cell Cultures.[8c]—The dipeptide nitrogen mustards and their ethyl esters were assayed for inhibitory activities in Sarcoma 180 cell culture. Four of the compounds were also tested in KB cell culture. The procedures used were previously reported.[8d]

C. Mouse Tumor Assay against Ehrlich Ascites Carcinoma.—The tetraploid Ehrlich ascites (4N) carcinoma was maintained in CAF/JAX mice by transplanting 0.1 ml of 1:7 dilution ascites

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fluid in sterile Locke's solution at 10-day intervals. For treated and control groups, 5 mice and 10 mice were used, respectively. A solution of 12% ethanol–10% Tween 80 (1:9) was used as the carrying vehicle. Both normal saline and the carrying vehicle were utilized as controls. The suspensions of the nitrogen mustards in 12% ethanol–Tween 80 solution were given intraperitoneally daily, starting 24 hr after inoculation of the tumor cells. The treatment was continued until death of the last animal. The suspensions of the nitrogen mustards remained stable at 4° for 1 week, and suitable for injection. The results are given in Tables IV. Animals were autopsied at death and gross observations, including weight of the ascites fluid were made. Tissue samples were taken for microscopic examination.[9]

Table IV

Results on Mammalian Cell Culture and Ehrlich Ascites Tumor (4N) Bearing Mice

	Mammalian cell culture[b] ID_{50}, μg/ml		Ehrlich ascites tumor bearers	
Compd[a]	S-180	KB	(T/C − 1) × 100[c]	Dose, mg/kg[d]
I	300	70	−10	100
II	150			
III	250	62	+66	100
IV	275	400	−2	6.25
V	300			
VII	275		+15	100
VIII	225			
IX	150	100	+83	50
X	275		+34	400
XI	300			
XII	150			

[a] For compound VI, see footnote *b* in Table I. [b] See ref 8. [c] T/C = treated mice/control mice, based on survival time. [d] Daily effective dose, given intraperitoneally. The figures given are those of the most effective dose in 4–6 dosage levels.

Results and Discussion

The physical data of the compounds synthesized are shown in Tables I and II. Most of the dipeptides and their esters possess wide-range melting points similar to those in the glutamic acid series.[2] Thin layer chromatograms revealed only one component for each compound. N-{*o*-[Bis(2-chloroethyl)amino]benzoyl}-glycine has not been obtained in a pure form by repeated recrystallization from various solvent systems, and N-{*o*-[bis(2-chloroethyl)amino]benzoyl}-γ-aminobutyric acid was obtained in too low yield to permit antineoplastic tests in mice.

Data on the inhibitory activity observed in the eight microbial systems are given in Table III. These indices indicate that most compounds are moderately active, ID_{50} = 50–150 μg/ml. They are most effective in *Streptococcus faecalis, Lactobacillus fermenti, Escherichia coli,* and *Candida albicans.* The magnitude and pattern of inhibition are similar to those of N-[bis(2-chloroethyl)aminobenzoyl]glutamic acids.[2c] The replacement of the glutamic acid in the peptide chain with a glycine or γ-aminobutyric acid apparently has little or no effect on the activity. The microbiological activity of several L dipeptide nitrogen mustards of glutamic acid in *S. faecalis* and *L. fermenti* assay systems was studied recently in detail in the Laboratories of Microbiology of this institution.[10] The data so obtained do not seem to support our early speculation[2b] that the nitrogen mustards related to the folic acid side chain, *p*-aminobenzoyl-L-glutamic acid, might possess specific antifolic acid activity. The observed inhibitory activity seems to be solely attributable to the nitrogen mustard moiety.

The inhibitory activity in Sarcoma 180 cell culture is in the range of ID_{50} = 150–300 μg/ml. For comparison with our previous results, four of the dipeptide nitrogen mustards synthesized were also tested in KB cell culture. The inhibition indices are in the same range for the dipeptide nitrogen mustards of glutamic acid.[2c] The data are shown in Table IV.

The *meta* and *para* isomers of N-[bis(2-chloroethyl)-aminobenzoyl]glutamic acid showed nearly identical biological activities. The *meta* and *para* isomers of N-[bis(2-chloroethyl)aminobenzoyl]glycine and N-[bis(2-chloroethyl)aminobenzoyl]-γ-aminobutyric acid showed similar inhibitory activities in the microbial and cell culture systems. Therefore, only the *para* and *ortho* isomers of the dipeptide nitrogen mustards of glycine and γ-aminobutyric acid were studied against tetraploid Ehrlich ascites tumor in CAF/JAX mice. The data are shown in Table IV. The *ortho* dipeptide nitrogen mustards in both the glycine and the γ-aminobutyric acid series showed the highest activity. The mice treated with the two active compounds, III and IX, survived 66 and 83% longer, respectively, than the control mice. The prolongation of survival, however, was only slightly more than half the survival time for the corresponding glutamic acid analog. The alteration of the terminal amino acid reduced the antineoplastic activity. The fact that the *ortho* isomers of both the glycine and γ-aminobutyric acid series exhibited highest antineoplastic activity is in agreement with our earlier observation on the activity of dipeptide nitrogen mustard glutamic acids.[2c] The same phenomenon has been observed in the isomers of phenylalanine nitrogen mustard by other investigators.[11] The most effective dose levels for compounds III and IX are at 50 and 100 μg/kg, respectively, which are in the same range for the active dipeptide nitrogen mustard glutamic acids studied.[2c]

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(9) The pathological findings will be reported elsewhere.

(10) G. E. Foley, unpublished results.

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