347. *Cyto-active Amino-acids and Peptides. Part XI.*[1] *Nitrogen Mustard Derivatives of Glutamic Acid and Related Compounds*

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The synthesis of a series of derivatives in which glutamic acid was linked by peptide bonds to various bases containing aromatic nitrogen mustard groups is described. Both L and D isomers of glutamic acid were joined in α and γ peptide linkage. Basic nitrogen mustard derivatives used were melphalan * ethyl ester, *NN*-dichloroethylphenylenediamine, and *NN*-dichloroethylaminophenylethylamine. In addition α- and β-aspartyl- and seryl-melphalan esters were prepared and a polyglutamylmelphalan ester. Comparative results of anti-tumour tests are briefly discussed.

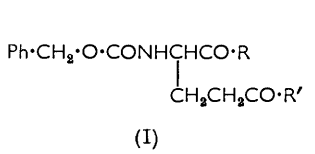
The preparation of a series of peptide esters in which melphalan * ester was acylated with various amino-acids and peptides containing (with the exception of cystine) non-functional side chains, was described in Part VIII.[2] Part X[1] included the synthesis of L-arginyl-melphalan ester, representing basic side-chain derivatives, a compound which exhibited anti-tumour activity to a high degree. Considering amino-acids with other than basic functional side chains, we recall the work of Roberts and his co-workers [3] among others; they have shown that glutamine plays a key role in the metabolism of tumour cells, being absorbed rapidly. This encouraged the supposition that some analogues of glutamine might share this property and possess high permeability into tumour cells. Accordingly a number of derivatives of glutamine and isoglutamine have been prepared in which variations have been made both in the configuration of the glutamic acid used and in the nature of the basic molecule carrying the nitrogen mustard group.

Preliminary experiments carried out by J. M. Johnson [4] in this Institute had indicated that α-benzyl *N*-benzyloxycarbonylglutamate prepared by the method of Bergmann, Zervas, and Salzmann [5] was not a suitable intermediate for the preparation of pure γ-glutamyl derivatives in this particular series. Coupling of the ester-acid with *NN*-dichloroethylphenylenediamine gave a mixture of α and γ isomers which could not be separated by crystallisation (cf. preparation of glutamine [5]). Consequently the analogues of glutamine were prepared using α-benzyl *N*-benzyloxycarbonyl-L-glutamate made as described by

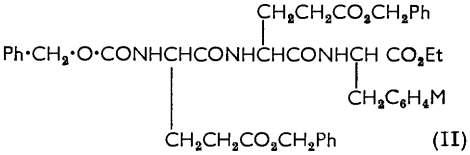
* Melphalan is the Pharmacopoeia name for L-*p*-di-(2-chloroethyl)aminophenylalanine. Mel is used as an abbreviation for the residue.

[1] Part X, J. M. Johnson and J. A. Stock, *J.*, 1962, 3806.
[2] F. Bergel and J. A. Stock, *J.*, 1960, 3658.
[3] E. Roberts, K. K. Tanaka, and D. G. Simonsen, *Cancer Res.*, 1956, **16**, 970.
[4] J. M. Johnson, Ph.D. Thesis, 1963, London University.
[5] M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, 1933, **66**, 1288.

Klieger and Gibian.[6] The isoglutamine analogues were synthesised from γ-benzyl *N*-benzyloxycarbonyl-L-glutamate available from γ-benzyl glutamate which was itself readily obtained free from the α half-ester by the procedure of Hayakawa and his collaborators.[7] Optical enantiomorphs of the same two protected glutamic acid half-esters were also coupled with melphalan ethyl ester either by the carbodi-imide or mixed anhydride procedures [I; R = Ph·CH_2·O, R′ = *p*-M·C_6H_4·CH_2·CH(CO_2Et)·NH and R = *p*-M·C_6H_4·CH_2·CH(CO_2Et)·NH, R′ = Ph·CH_2·O].* Protected glutamine analogues containing *p*-di-(2-chloroethyl)aminophenylethylamine residues were prepared in a similar



(I)



(II)

manner (I; R = *p*-M·C_6H_4·CH_2·CH_2·NH, R′ = Ph·CH_2·O). Furthermore benzyloxycarbonylglutamic acid was coupled on both acid groups simultaneously to melphalan ester and in addition a protected tripeptide (II) was synthesised by joining γ-benzyl benzyloxycarbonyl-L-glutamic acid to γ-benzyl α-L-glutamylmelphalan ethyl ester. Benzyloxycarbonyl-L-serine and the two benzyl half esters of benzyloxycarbonyl-L-aspartic acid were also combined with melphalan ethyl ester. The physical characteristics of these protected intermediates are recorded in Table 1.

Protecting groups were removed from these intermediates by hydrogenolysis with palladium–charcoal. The final products (Table 2) were usually isolated as the hygroscopic hydrochlorides; exceptions were the four derivatives of *NN*-dichloroethyl-*p*-phenylenediamine which were obtained as the free amino-acids.

In addition to these low molecular weight compounds, the use of larger molecules containing cytotoxic groups has been investigated in a preliminary fashion in this series of glutamic acid derivatives. This was foreshadowed by our experiments of several years ago.[8] Melphalan ester was used to initiate the polymerisation of γ-benzyl *N*-carboxy-L-glutamate anhydride to give poly(γ-benzyl α-L-glutamyl)melphalan ethyl ester. Benzyl ester groups were removed by treatment with hydrogen bromide in dioxan and the acid polymer isolated by precipitation with ether. Paucity of material prevented full characterisation of the product.

Most of the compounds described have been tested for anti-tumour activity on the Walker rat carcinoma 256 and a detailed account of these tests will be published elsewhere. In general, compounds derived from melphalan ester were no more active on a molar basis than the parent compound but some had a more favourable chemotherapeutic index (notably α-L-glutamylmelphalan ester). In cases where melphalan ester was acylated by an L-amino-acid the derivative was more active and usually had a better chemotherapeutic index than the corresponding D-diastereoisomer. In fact α-D-glutamylmelphalan ester failed to give complete tumour inhibition even at toxic doses. α- and β-L-Aspartylmelphalan esters showed an unexpectedly large (10-fold) difference in activity although the chemotherapeutic index was the same in each case. The polyamino-acid derivatives were of considerable interest. Preliminary tests showed that these gave complete inhibition of the Walker 256 tumour and no toxic symptoms at doses of the order of 1000 mg./kg. Poly-α-L-glutamic acid of a similar degree of polymerisation gave no inhibition indicating that this part of the molecule was acting as a carrier.

* Throughout this Paper, M = *NN*-dichloroethylamino, $(Cl·CH_2·CH_2)_2N$–.

[6] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.

[7] T. Hayakawa, H. Nishi, J. Noguchi, S. Ikeda, T. Yamashita, and T. Isemura, *J. Chem. Soc. Japan*, 1961, **82**, 601.

[8] F. Bergel, J. A. Stock, and R. Wade, " Peptides and Macromolecules as Carriers of Cytotoxic Groups " in Biological Approaches to Cancer Chemotherapy, Academic Press, London, 1961, p. 125.

TABLE 1

No.	Compound	Method of prepn.	M. p.[a]	$[\alpha]_D$ [b]	Yield (%)	Formula	Found (%) C	H	Cl	N	Required (%) C	H	Cl	N
1	Z-L-Glu.$OCH_2C_6H_5$ └Mel.OEt	A or B	173—174°	+36·7°	60—65	$C_{35}H_{41}Cl_2N_3O_7$	61·4	6·2	9·9	6·3	61·2	6·0	10·3	6·1
2	Z-D-Glu.$OCH_2C_6H_5$ └Mel.OEt	A	134—135	+24·3	68	,,	60·9	6·3	10·3	6·0	,,	,,	,,	,,
3	Z-L-Glu.Mel.OEt └$OCH_2C_6H_5$	B	117—118	+27·9	61	,,	60·8	5·9	10·0	6·3	,,	,,	,,	,,
4	Z-D-Glu.Mel.OEt └$OCH_2C_6H_5$	B	111—112	+22·5	63	,,	61·2	5·9	10·2	6·0	,,	,,	,,	,,
5	Z-L-Glu.$OCH_2C_6H_5$ └$NHCH_2CH_2C_6H_4M$	B	132—133	−3·5 [c]	52	$C_{32}H_{37}Cl_2N_3O_5$	62·1	6·2	11·0	7·1	62·4	6·1	11·5	6·8
6	Z-D-Glu.$OCH_2C_6H_5$ └$NHCH_2CH_2C_6H_4M$	B	132—133	+3·2 [c]	56	,,	62·2	6·3	11·1	7·0	,,	,,	,,	,,
7	Z-L-Glu.$OCH_2C_6H_5$ └NHC_6H_4M	A	124—125	−13·7 [d]	71	$C_{30}H_{33}Cl_2N_3O_5$	61·2	5·6	12·1	7·2	61·4	5·6	12·1	7·2
8	Z-D-Glu.$OCH_2C_6H_5$ └NHC_6H_4M	A	124—125	+13·6 [d]	61	,,	61·1	5·8	12·0	7·0	,,	,,	,,	,,
9	Z-L-Glu.NHC_6H_4M └$OCH_2C_6H_5$	A	106—107	−9·0 [e]	59	,,	61·2	5·5	12·1	7·0	,,	,,	,,	,,
10	Z-D-Glu.NHC_6H_4M └$OCH_2C_6H_5$	A	105—106	+8·4 [e]	63	,,	61·1	5·7	11·6	6·9	,,	,,	,,	,,
11	Z-L-Glu.Mel.OEt └Mel.OEt	A	112—113	+20·8	30	$C_{43}H_{55}Cl_2N_5O_8$	56·8	6·2	15·6	7·8	56·6	6·1	15·6	7·7
12	Z-L-Glu.-L-Glu.Mel.OEt └$OCH_2C_6H_5$ └$OCH_2C_6H_5$	B	118—119	+11·8	33 [a]	$C_{47}H_{54}Cl_2N_4O_{10}$	61·8	6·2	8·0	6·3	62·3	6·0	7·8	6·2
13	Z-L-Ser-Mel.OEt	B	119—120	+26·8	70	$C_{26}H_{33}Cl_2N_3O_6$	56·4	6·0	12·9	7·6	56·3	6·0	12·8	7·6
14	Z-L-Asp.$OCH_2C_6H_5$ └Mel.OEt	B	95—96	+58·9	50	$C_{34}H_{39}Cl_2N_3O_7$	60·7	5·9	10·8	6·5	60·7	5·8	10·5	6·3
15	Z-L-Asp.Mel.OEt └$OCH_2C_6H_5$	B	57—58	+41·6	63	,,	60·4	5·9	11·0	6·2	,,	,,	,,	,,

A = Mixed anhydride. B = Carbodi-imide. [a] Over 2 stages. [b] *c* 1 in $CHCl_3$ unless indicated otherwise. [c] *c* 4 in $CHCl_3$. [d] *c* 1 in Me_2CO. [e] *c* 2 in $CHCl_3$.

TABLE 2

No.	Compound	R_F [a]	M. p.	$[\alpha]_D$	Yield (%)	Formula	Found (%) C	H	Cl	N	Required (%) C	H	Cl	N
1	H-L-Glu.OH └Mel.OEt	0·77	[b]	+23·8° [c]	78°	$C_{20}H_{30}Cl_3N_3O_5$	48·1	6·4	20·8	8·6	48·2	6·1	21·3	8·4
2	H-D-Glu.OH └Mel.OEt	0·77	[b]	+10·8 [e]	72	,,	48·3	6·2	20·9	8·3	,,	,,	,,	,,
3	H-L-Glu.Mel.OEt └OH	0·87		+20 [c]	78	,,	48·0	6·3	20·9	8·5	,,	,,	,,	,,
4	H-D-Glu.Mel.OEt └OH	0·87		+7·4 [c]	85	,,	47·9	6·4	21·2	8·1	,,	,,	,,	,,
5	H-L-Glu.OH └$NHCH_2CH_2C_6H_4M$	0·73	[b]	+12·5 [e]	75	$C_{17}H_{26}Cl_3N_3O_3$	47·6	6·8	25·0	9·4	47·8	6·1	24·9	9·9
6	H-D-Glu.OH └$NHCH_2CH_2C_6H_4M$	0·73		−12 [c]	73	,,	47·4	6·4	25·0	9·5	,,	,,	,,	,,
7	H-L-Glu.OH └NHC_6H_4M	0·75	175—176°	+19·1 [d]	75	$C_{15}H_{21}Cl_2N_3O_3$	49·2	6·0	19·3	11·2	49·7	5·8	19·6	11·6
8	H-D-Glu.OH └NHC_6H_4M	0·75	175—176	−18·6 [d]	68	,,	49·3	6·0	19·7	11·4	,,	,,	,,	,,
9	H-L-Glu.NHC_6H_4M └OH	0·88	152—154	+4·8 [e]	71	,,	49·9	6·1	19·3	11·2	,,	,,	,,	,,
10	H-D-Glu.NHC_6H_4M └OH	0·88	151—153	−5·0 [e]	76	,,	50·0	5·7	19·5	11·3	,,	,,	,,	,,
11	H-L-Glu.Mel.OEt └Mel.OEt	0·97	97—98	+18·9 [c]	70	$C_{35}H_{50}Cl_5N_5O_6$	51·9	6·2	22·2	8·6	51·6	6·2	21·8	8·6
12	H-L-Glu-L-Glu.Mel.OEt └OH └OH	0·81	108—109	+12·4 [c]	79	$C_{25}H_{37}Cl_3N_4O_8$	48·3	6·2	17·2	9·3	47·8	5·9	16·9	8·9
13	H-L-Ser-Mel.OEt	0·78	[b]	+21·9 [c]	85	$C_{18}H_{28}Cl_3N_3O_4$	47·5	6·6	22·8	9·2	47·3	6·2	23·3	9·2
14	H-L-Asp.OH └Mel.OEt	0·65	[b]	+26·1 [c]	60	$C_{19}H_{28}Cl_3N_3O_5$	47·2	5·7	22·2	8·6	47·1	5·8	21·9	8·7
15	H-L-Asp.Mel.OEt └OH	0·77		+20·6 [e]	65	,,	47·1	5·8	21·9	8·7	,,	,,	,,	,,

[a] Determined by upward development on Whatman No. 1 paper using $Bu^nOH : EtOH : EtCO_2H : H_2O = 200 : 100 : 40 : 100$ solvent. [b] Hygroscopic. [c] *c* 1 in MeOH [d] *c* 2 in 5N-hydrochloric acid. [e] *c* 2 in MeOH.

Experimental

All m. p.s were determined on the Kofler block.

Starting Materials.—The optical isomers of α-benzyl benzyloxycarbonylglutamate were prepared *via* the dicyclohexylammonium salts using Klieger and Gibian's [6] adaptation of the general procedure of Weygand and Hunger.[9] The D-*isomer* crystallised from ethyl acetate–light petroleum as needles, m. p. 97—98°, $[\alpha]_D = +24{\cdot}7°$ (*c* 1 in MeOH) (Found: C, 64·5; H, 5·6; N, 4·1. $C_{20}H_{21}NO_6$ requires C, 64·7; H, 5·7; N, 3·8%).

α-Benzyl *N*-benzyloxycarbonyl-L-aspartate was prepared by a modification [10] of Bergmann and Zervas' [5] method.

γ-Benzyl L- and D-glutamates and β-benzyl L-aspartate [7] were converted into their *N*-benzyloxycarbonyl derivatives in aqueous sodium hydrogen carbonate solution [11,12] and if necessary were purified *via* dicyclohexylammonium salts.

2-[*p*-Di-(2-chloroethyl)aminophenyl]ethylamine [13] and melphalan ethyl ester hydrochloride [2] were prepared by methods previously described.

NN-*Di*-(2-*chloroethyl*)*phenylenediamine.*—*p*-Aminoacetanilide (5 g.) was dissolved in methanol (50 ml.) containing toluene-*p*-sulphonic acid (50 mg.), the solution cooled in ice and ethylene oxide (20 ml.) added with stirring. The mixture was stored overnight at room temperature in a stoppered flask. Saturated aqueous sodium hydrogen carbonate solution (5 ml.) was added and the solution taken to dryness, the residue dried azeotropically (ethanol–benzene), and the *dihydroxyethyl* derivative recrystallised from ethanol (charcoal) as needles (72% yield), m. p. 141—142° (Found: C, 60·1; H, 7·3; N, 11·4. $C_{12}H_{18}N_2O_3$ requires C, 60·5; H, 7·6; N, 11·8%).

Chlorination was effected by treating a suspension of the dihydroxy-derivative (4·8 g.) in pyridine (20 ml.) with methanesulphonyl chloride (4·2 ml.). After the first vigorous reaction, initiated by gentle warming if necessary, had subsided the solution was boiled under reflux for 20 min. then poured on ice (250 ml.). The oily precipitate solidified on scratching, the solid was filtered off, sucked dry, and recrystallised from benzene to give plates (60%) of the chloro-compound, m. p. 126—127° (lit.,[14] m. p. 124—126°).

The chlorinated compound (9 g.) was de-acetylated by boiling in concentrated hydrochloric acid (150 ml.) for 2 hr. After evaporation to dryness, water (30 ml.) was added and the pale pink crystalline product, the mono-hydrochloride of *NN*-dichloroethylphenylenediamine, filtered off, m. p. 240° (decomp.) (lit.,[14] m. p. 250—260° decomp.).

Benzyloxycarbonylaminoacyl-derivatives of Melphalan Ester and Other Bases.—(a) *Mixed carbonic–carboxylic anhydride method.* The procedure described in Part VIII [2] was used for the coupling reaction.

(b) *Carbodi-imide method.* This was preferred when the protected acid derivative to be coupled was obtained as the dicyclohexylammonium salt (Table 1, Nos. 1, 3, 4 etc.). The salt (5 mmoles) was suspended in ethyl acetate and decomposed with 5N-hydrogen chloride in ethyl acetate. After stirring for 2—3 hr. at room temperature the mixture was washed with water, filtered, separated, the organic layer dried ($MgSO_4$), and the solvent removed *in vacuo*. The residue was dissolved in tetrahydrofuran and coupled as described in Part IX.[15]

In both (a) and (b) the crude product was purified by passage down a column of activated alumina in ethyl acetate or ethyl acetate–chloroform (1 : 1). The products were recrystallised from ethyl acetate–light petroleum and possessed the characteristics recorded in Table 1.

Benzyloxycarbonyl-α-L-(γ-benzyl glutamyl)-α-L-(γ-benzyl glutamyl)melphalan Ethyl Ester (II).—γ-Benzyl *N*-benzyloxycarbonyl-α-L-glutamylmelphalan ethyl ester (Table 1, No. 3) (5 mmoles) was treated at room temperature with hydrogen bromide in glacial acetic acid (50%) for 20 min. Dilution with dry ether (5 vol.) precipitated the pale yellow hydrobromide. This was quickly filtered off, washed thoroughly with ether, and dried overnight in a desiccator over phosphoric oxide and potassium hydroxide. γ-Benzyl *N*-benzyloxycarbonyl-L-glutamate dicyclohexylammonium salt was decomposed as described above and the free acid coupled with γ-benzyl α-L-glutamylmelphalan ethyl ester hydrobromide in chloroform in the presence of triethylamine

[9] F. Weygand and K. Hunger, *Z. Naturforsch.*, 1958, **13***b*, 50.
[10] G. L. Miller, O. K. Behrens, and V. du Vigneaud, *J. Biol. Chem.*, 1941, **140**, 141.
[11] W. E. Hanby, S. W. Waley, and F. Watson, *J.*, 1950, 3239.
[12] L. Benoiton, *Canad. J. Chem.*, 1962, **40**, 570.
[13] F. Bergel, J. L. Everett, J. J. Roberts, and W. C. J. Ross, *J.*, 1955, 3835.
[14] J. L. Everett and W. C. J. Ross, *J.*, 1949, 1972.
[15] F. Bergel, J. M. Johnson, and R. Wade, *J.*, 1962, 3802.

(5 mmoles) by the carbodi-imide method in the usual way. The *product* was recrystallised from ethyl acetate–light petroleum; physical data are in Table 1, No. 12.

Catalytic Hydrogenolysis of Protected Derivatives.—The various intermediates were dissolved in ethanol–ethyl acetate–dioxan (2 : 1 : 1) and hydrogenolysed in a stream of hydrogen at room temperature in the presence of palladium–charcoal (5%). Usually the hydrogenolysis was carried out in the presence of ethanolic hydrogen chloride (1 equiv.) and the *product* isolated as the hydrochloride, but in the case of the glutamyl derivatives of *NN*-di-2-chloroethylphenylenediamine (Nos. 7—10) it was found to be preferable to isolate the free amino-acids. Pertinent data are in Table 2.

Poly-α-L-glutamylmelphalan Ethyl Ester.—Melphalan ester was liberated from its hydrochloride by treating the salt in chloroform with triethylamine (1 equiv.). After the suspension had been stirred vigorously for 15 min., anhydrous ether (8 vol.) was added and the precipitated triethylammonium chloride filtered off. The filtrate was concentrated under reduced pressure and the residue dissolved in a little anhydrous chloroform. This solution was used to initiate the polymerisation of γ-benzyl *N*-carboxy-L-glutamate anhydride in dry dioxan at room temperature. A/I was 26 and 13. In each case the polymerisation was allowed to proceed for 4 days at room temperature and the solution was then diluted with an excess of ethanol. The nearly white fibrous material was filtered off and thoroughly washed with ethanol and ether (A/I 26. Found: Cl, 1·06; N, 8·04%. A/I 13. Found: Cl, 1·63; N, 7·3%).

Fission of benzyl ester groups was achieved with hydrogen bromide in dioxan (40%) solution. The material dissolved rapidly and solid began to separate from the solution after 15 min. After 90 min. the solution was diluted with ether, the *polymer* filtered off and washed thoroughly with ether. After drying in a desiccator (P_2O_5) the material was pale yellow and insoluble in water (A/I 26. Found: C, 46·3; H, 6·4; N, 9·45% $[\alpha]_D$ −73·4° (*c* 1 in N-NaOH). A/I 13. Found: C, 44·4; H, 5·6; N, 10·4% $[\alpha]_D$ −67·7° (*c* 1 in N-NaOH). $H(C_5H_7NO_3)_{20}$, $C_{15}H_{21}Cl_2N_2O_2$ requires C, 47·2; H, 5·5; N, 10·5.

Molecular-weight determination by sedimentation gave values of 16,000 and 10,400 respectively. We are indebted to Mr. P. A. Edwards for these.

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