728. *Cyto-active Amino-acids and Peptides. Part VIII.*[1] N^{α}-*Acyl, Amide, Ester and Peptide Derivatives of Melphalan.*

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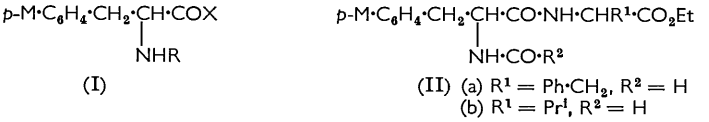
Various simple derivatives of melphalan have been prepared in which the α-amino-group is formylated or the carboxyl group converted into an ester or amide group, or in which both changes have been made. Some di- and tri-peptides and a tetrapeptide are described in which, with one exception, melphalan is the *C*-terminal amino-acid. The deacylation of benzyloxy-carbonyl-dipeptide esters with ethanolic hydrogen chloride has been studied. Preliminary biological results are briefly discussed.

Melphalan * [*p*-di-(2-chloroethyl)amino-L-phenylalanine] [2] [I; X = OH, R = H, M = $(Cl{\cdot}CH_2{\cdot}CH_2)_2N$ here and in all succeeding formulæ] has a very pronounced anti-tumour activity but also powerfully affects the blood-forming organs and circulating blood elements and has a high general toxicity.[3] Such effects are especially undesirable if the drug is to

* Melphalan is the " open name " adopted by the British Pharmacopœia Nomenclature Commission. It is composed from Mel and the standard symbols for phenylalanine.
M (= " mustard ") *el* (= L-configuration) and *ph*enyl*alan*ine

[1] Part VII, Bergel and Wade, *J.*, 1959, 941.
[2] (*a*) Bergel and Stock, *J.*, 1954, 2409; (*b*) Bergel, Burnop, and Stock, *J.*, 1955, 1223.
[3] Stock, in Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity, London, Churchill, 1958, p. 89.

be used against solid tumours in man, and we therefore sought derivatives possessing the high carcinolytic activity of the parent compound but showing a diminished hæmo- and general toxicity. The derivatives of melphalan we have prepared fall into two main groups: (i) simple derivatives, including amides and esters; (ii) di-, tri-, and tetra-peptides.



(I) (II) (a) $R^1 = Ph\cdot CH_2$, $R^2 = H$ (b) $R^1 = Pr^i$, $R^2 = H$

Larionov and Sof'ina [4] have reported that two members of a series of N^{α}-formyl-dipeptides (II) of sarcolysin (merphalan, the racemic form of melphalan) are effective experimental tumour inhibitors with relatively low toxicity and little effect on the bone marrow. These compounds are the phenylalanine and valine derivatives (IIa and b respectively), prepared in their " all-DL " forms by Knunyants and his co-workers,[5] and they resemble the *N*-benzoyl analogues (II; R′ = Ph) which we described in Part IV [6] of this series. It seemed of interest to determine whether the biological properties of these formyl-dipeptides could be simulated by simpler compounds. We therefore prepared a number of derivatives of melphalan in which the carboxyl group was blocked by ester or amide formation, or the α-amino-group by acylation. Relevant data are given in Table 1,

TABLE 1. *Simple melphalan derivatives* (I).

No.	X	R	C.B. No.*	Cryst. from	M. p.	$[\alpha]_D$	Yield (%)
1	OH	HCO	3208	A [a]	139—140°	+72°	85
2	OEt	HCO	3239	A [a]	70—71	+37 [d]	71
3	NH_2	HCO	3249	B [b]	140—145	+25 [d]	76
4	NHEt	HCO	3209	C [a]	141—144	+20 [d]	65
5	OEt	$HO_2C\cdot[CH_2]_2\cdot CO$	3236	A [b]	112—114	+26 [d]	79
6	OEt	H,HCl	3177	D [a]	165—167	+12 [e]	87
7	NH_2	H,HCl	3215	E [b]	241—243	+12 [f]	30

		Found (%)				Required (%)			
No.	Formula	C	H	Cl	N	C	H	Cl	N
1	$C_{14}H_{18}Cl_2N_2O_3$	50·5	5·4	21·3	8·4	50·5	5·4	21·3	8·4
2	$C_{16}H_{22}Cl_2N_2O_3$	52·8	5·9	20·0	7·7	53·2	6·1	19·6	7·75
3	$C_{14}H_{19}Cl_2N_3O_2$	50·7	5·4	21·5	—	50·6	5·75	21·3	—
4	$C_{16}H_{23}Cl_2N_3O_2$	53·2	6·5	19·9	11·55	53·3	6·4	19·7	11·65
5	$C_{19}H_{26}Cl_2N_2O_5$	52·8	6·1	16·8	6·4	52·7	6·05	16·4	6·5
6	$C_{15}H_{22}Cl_2N_2O_2$,HCl	48·7	6·4	28·5	7·5	48·7	6·3	28·8	7·6
7	$C_{13}H_{19}Cl_2N_3O$,HCl	45·65	6·0	30·7	12·1	45·8	5·9	31·2	12·3

A, Aq. MeOH. B, Aq. EtOH. C, Aq. Pr^nOH. D, $CHCl_3$–EtOH. E, EtOH. [a] Colourless rods or needles. [b] Amorphous. [c] *c* 2·00 in EtOH at 24°. [d] *c* 1·00 in MeOH at 25°. [e] *c* 5·00 in EtOH at 24°. [f] *c* 1·00 in EtOH at 24°. * Chester Beatty Research Institute reference no.

and it can be seen that in four cases (nos. 2—5), both carboxyl and amino-functions are blocked. Compounds nos. 1 and 2 were prepared by formylation of melphalan and melphalan ester respectively; the ester (no. 6) was made by the action of ethanolic hydrogen chloride on the free amino-acid. The amide (no. 3) and ethylamide (no. 4) were prepared from the formyl derivative (no. 1) by the mixed anhydride procedure using isobutyl chloroformate.[7] Deacylation of the N^{α}-formyl derivative of the amide by brief warming with 3 equivalents of hydrogen chloride in ethanol gave the crystalline hydrochloride of the amide of melphalan (no. 7); the use of 3N-ethanolic hydrogen chloride (24 hr. at room temperature) led to substantial hydrolysis of the amide group. An attempt to prepare the ethylamide in a similar way from the corresponding formyl compound (no. 4) gave a very

[4] Larionov and Sof'ina, *Doklady Akad. Nauk S.S.S.R.*, 1957, **114**, 1070.
[5] Knunyants, Kil'disheva, and Golubeva, *Izvest. Akad. Nauk S.S.S.R., Otdel. khim. Nauk*, 1956, 1418.
[6] Bergel and Stock, *J.*, 1957, 4563.
[7] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547.

hygroscopic hydrochloride, and the corresponding picrate could not be obtained solid. Reaction of the ester (no. 6) with succinic anhydride gave the ethyl N^{α}-β-carboxypropionyl-ester (no. 5). A similar reaction with free melphalan gave a gum, as did the sodium salt.

We have also prepared a number of compounds in which, in general, the phenylalanine " mustard " is linked to other amino-acids or peptides through its α-amino-group. These comprise some di- and tri-peptide esters, a tetrapeptide ester, and a free dipeptide where melphalan, with one exception (VI), is the *C*-terminal amino-acid. The intermediate benzyloxycarbonyl and formyl compounds and four acetyl-peptides are set down in Table 2. The acyl-dipeptides (III; R = Ph·CH_2·O·CO or Ac) and (II; R = R′ = H) were prepared by the chloroformate mixed anhydride procedure [7] from various acylamino-acids and the ethyl ester (I; X = OEt, R = H) of melphalan. The acetyl-dipeptide esters (Table 2, nos. 7—10) were of biological interest in their own right and no attempt was made to hydrolyse them to the free dipeptides. Although racemisation is usually insignificant [8] in the chloroformate synthesis of dipeptides, the use of the same method in the condensation of acyl-dipeptides with amino-acid or peptide esters may lead to some racemisation.[9] It is generally accepted, however, that Curtius's azide method of peptide synthesis [10] is completely safe in this respect.[11] The benzyloxycarbonyl-tripeptides (IV; R^1 = Ph·CH_2·O·CO; Table 2, nos. 14 and 15) were therefore prepared by the azide procedure from the appropriate acyl-dipeptide hydrazide.

R^1·NH·CHR^2·CO·NH·CH(·CO_2Et)·CH_2·C_6H_4M-*p* (III)

R^1·NH·CHR^2·CO·NH·CHR^3·CO·NH·CH(·CO_2Et)·CH_2·C_6H_4M-*p* (IV)

This stereochemical consideration does not apply in the case of the benzyloxycarbonyl-tripeptide ester no. 12 of Table 2, since the intermediate benzyloxycarbonyl-dipeptide has glycine as the *C*-terminal amino-acid, and the chloroformate method of synthesis would doubtless be satisfactory. However, unless a different intermediate were used, this method would require the initial alkaline hydrolysis of benzyloxycarbonylvalylglycine ester to the free acid, and dipeptides with a *C*-terminal glycine residue are not always hydrolysed in a straightforward way.[12] This ester no. 12 was therefore synthesised by the azide procedure also. The intermediate benzyloxycarbonyl-dipeptide esters required for the synthesis of the melphalan tripeptides were prepared by the chloroformate procedure.

$[$R·NH·CH(·CH_2·S·)·CO·NH·CH(CO_2Et)·CH_2·C_6H_4M-*p*$)]_2$ (V)

R·NH·$CHPr^I$·CO·NH·CH(·CH_2·C_6H_4M-*p*)·CO·NH·CH_2·CO_2Et (VI)

R·NH·$CHPr^I$·CO·$[NH·CH_2·CO]_2$·NH·CH(CO_2Et)·CH_2·C_6H_4M-*p* (VII)

The ester (V; R = Ph·CH_2·O·CO) (Table 2, no. 16), though formally a tripeptide, is of the dipeptide type, and was prepared by the chloroformate method from dibenzyloxycarbonyl-L-cystine and two molar proportions of the ethyl ester of melphalan. The acyl-tripeptide ester (VI; R = Ph·CH_2·O·CO; Table 2, no. 13) contains melphalan as the central amino-acid. In the preparation of this compound, the amorphous, hygroscopic " melphalanyl "-glycine ester hydrochloride [prepared by deformylation of the corresponding formyl derivative (Table 2, no. 11)] was condensed with benzyloxycarbonyl-L-valine by the chloroformate procedure. The yield of purified acyl-tripeptide ester was low, possibly because of the questionable purity of the dipeptide ester intermediate. We have so far prepared only one tetrapeptide containing melphalan. The acyl intermediate (VII; R = Ph·CH_2·O·CO; Table 2, no. 17) was synthesised by the azide method from the ethyl ester of melphalan and benzyloxycarbonyl-L-valylglycylglycine hydrazide. Although it

[8] Vaughan and Osato, *J. Amer. Chem. Soc.*, 1952, **74**, 676; but see North and Young, ref. 11*a*.
[9] Vaughan, *J. Amer. Chem. Soc.*, 1952, **74**, 6137.
[10] Fruton, *Adv. Protein Chem.*, 1949, **5**, 1.
[11] (*a*) North and Young, *Chem. and Ind.*, 1955, 1597; (*b*) Goodman and Kenner, *Adv. Protein Chem.*, 1957, **12**, 465.
[12] McLaren, *Austral. J. Chem.*, 1958, 360.

TABLE 2.

No.	Compound [a]	Formula	C.B. No.	Cryst. from	M. p.	$[\alpha]_D$	Yield (%)
1	Z-L-Ala.Mel.OEt [b]	III; R^1 = Z, R^2 = Me	—	A [d]	95—96°	+35° [e]	71
2	Z-D-Ala.Mel.OEt [b]	III; R^1 = Z, R^2 = Me	—	A [f]	100—101	+19 [g]	60
3	Z-L-Val.Mel.OEt [b]	III; R^1 = Z, R^2 = Pr^i	—	B [d]	159—160	+35 [e]	72
4	Z-D-Val.Mel.OEt [b]	III; R^1 = Z, R^2 = Pr^i	—	C [f]	160—161	+26 [g]	55
5	Z-L-Leu.Mel.OEt [b]	III; R^1 = Z, R^2 = Bu^i	—	*D*	90—92	+32 [g]	84
6	Z-L-Phe.Mel.OEt [b]	III; R^1 = Z, R^2 = Ph·CH_2	—	E [d]	164—165	+21 [g]	61
7	Ac-L-Phe.Mel.OEt [b]	III; R^1 = Ac, R^2 = Ph·CH_2	3224	B [d]	156—158	+31 [g]	71
8	Ac-*p*-NO_2-L-Phe.Mel.OEt [b]	III; R^1 = Ac, R^2 = *p*-NO_2·C_6H_4·CH_2	3205	C [h]	167—170 [i]	+39 [g]	76
9	Ac-*p*-NO_2-D-Phe.Mel.OEt [bj]	III; R^1 = Ac, R^2 = *p*-NO_2·C_6H_4·CH_2	3242	F [f]	125—127	+42 [g]	62
10	Ac-*p*-NH_2-L-Phe.Mel.OEt(HCl)	III; R^1 = Ac, R^2 = *p*-NH_2·C_6H_4·CH_2	3231	G [k]	211—214	−7 [l]	78
11	Fo.Mel.Gly.OEt [b]	II; R^1 = R^2 = H	3243	B [f]	120—122	+15 [g]	53
12	Z-L-Val.Gly.Mel.OEt [c]	IV; R^1 = Z, R^2 = Pr^i, R^3 = H	—	A [f]	106—109	+25 [e]	82
13	Z-L-Val.Mel.Gly.OEt [b]	VI; R = Z	—	I [f]	178—180	−22 [r]	16
14	Z-L-Ala-L-Leu.Mel.OEt [c]	IV; R^1 = Z, R^2 = Me, R^3 = Bu^i	—	B [d]	150—152	−21 [g]	44
15	Z-L-Val-L-Leu.Mel.OEt [g]	IV; R^1 = Z, R^2 = Pr^i, R^3 = Bu^i	—	B [m]	156—159	−14 [g]	54
16	Z-L-Cys.Mel.OEt [b] S S Z-L-Cys.Mel.OEt	V; R = Z	—	H [f]	160—162	−8 [e]	60
17	Z-L-Val.Gly.Gly.Mel.OEt [e]	VII; R = Z	—	B [f]	133—135 [p]	+36 [q]	82

No.	Formula	Found (%) C	H	Cl	N	Required (%) C	H	Cl	N
1	$C_{26}H_{33}Cl_2N_3O_5$	58·0	5·8	13·1	8·0	58·0	6·15	13·2	7·8
2	,,	58·0	6·2	13·6	7·9	,,	,,	,,	,,
3	$C_{28}H_{37}Cl_2N_3O_5$	59·8	6·7	12·1	7·4	59·4	6·6	12·5	7·4
4	,,	59·6	6·7	—	7·5	,,	,,	,,	,,
5	$C_{29}H_{39}Cl_2N_3O_5$	59·9	6·6	12·2	6·9	60·0	6·8	12·2	7·2
6	$C_{32}H_{37}Cl_2N_3O_5$	62·5	6·3	11·4	7·1	62·5	6·1	11·5	6·8
7	$C_{26}H_{33}Cl_2N_3O_4$	60·2	6·4	13·9	8·3	60·0	6·4	13·6	8·0
8	$C_{26}H_{32}Cl_2N_4O_6$	55·3	5·8	12·5	10·0	55·0	5·7	12·5	9·9
9	,,	55·3	5·7	12·2	9·7	,,	,,	,,	,,
10	$C_{26}H_{34}Cl_2N_4O_4$·HCl	54·6	6·3	18·4	9·9	54·4	6·15	18·5	9·8
11	$C_{18}H_{25}Cl_2N_3O_4$	51·4	6·0	17·3	9·7	51·7	6·0	17·0	10·0
12	$C_{30}H_{40}Cl_2N_4O_6$	57·8	6·4	—	9·2	57·8	6·5	—	9·0
13	,,	57·4	6·4	11·3	9·3	,,	,,	11·4	,,
14	$C_{32}H_{44}Cl_2N_4O_6$	58·7	6·7	10·6	8·9	59·0	6·8	10·9	8·6
15	$C_{34}H_{48}Cl_2N_4O_6$	60·0	7·0	10·1	8·3	60·1	7·1	10·4	8·2
16	$C_{52}H_{64}Cl_4N_6O_{10}S_2$	54·4	5·6	12·5	7·4	54·8	5·7	12·5	7·4
17	$C_{32}H_{43}Cl_2N_5O_7$	56·4	6·4	10·9	10·5	56·5	6·4	10·4	10·3

A, Aq.EtOH. B, Aq.MeOH. C, MeOH. D, Pr^nOH, then aq.EtOH. E, EtOH. F, aq.Pr^nOH. G, MeOH–EtOAc. H, C_5H_{11}·OH. I, Pr^nOH. [a] The nomenclature follows that proposed by Brand and Edsall.[11b] Z = benzyloxycarbonyl; Fo = formyl; OEt indicates ethyl ester; Mel = "melphalanyl" [*p*-di-(2-chloroethyl)amino-L-phenylalanyl]. [b] "Chloroformate" synthesis. [c] "Azide" synthesis. [d] Needles. [e] 1·00 in AcOEt at 23°. [f] Amorphous. [g] *c* 1·00 in EtOH at 23—27°. [h] Yellow irregular prisms. [i] 1st m. p. 125—127° (glass), 130° (fluid), then resolidification. [j] Prepared from the appropriate isomer of acetyl-*p*-nitrophenylalanine.[2b] [k] Plates. [l] 1·00 in MeOH at 23°. [m] Prisms softening to glass at 142—144°, then resolidification. [p] Softening to glass at 125—127°. [q] *c* 2·00 in $CHCl_3$ at 20°. [r] *c* 1·00 in $CHCl_3$ at 20°.

seemed likely that catalytic hydrogenolysis of the benzyloxycarbonyl-peptide esters would be a satisfactory route to the free dipeptide esters, we decided to investigate the practicability of hydrolytic deacylation. In a trial experiment, a saturated solution of hydrogen chloride in formic acid did not deacylate the benzyloxycarbonyl-ester no. 6 (Table 2) to any significant extent in 16 hr. at room temperature. This result was unexpected, for the

reagent had been successfully used in an earlier series of compounds for a similar purpose.[13] We then tried ethanolic hydrogen chloride which had been used occasionally for removing benzyloxycarbonyl groups from dipeptides [14] though there seems to have been no general study of its potentialities. Details of a limited investigation are given in the Experimental section. Table 4 indicates that dilution of saturated ethanolic hydrogen chloride causes a very marked reduction in the speed of deacylation of benzyloxycarbonylglycylglycine at room temperature. In solutions saturated with hydrogen chloride (*ca.* 10N) at room temperature hydrolysis is 50—100 times faster than in solutions of half this acid concentration. 10N-Ethanolic hydrogen chloride completely deacylates benzyloxycarbonylphenylalanine in 2—3 days at room temperature; and peptide hydrolysis was negligible during 72 hr. for benzyloxycarbonylphenylalanylglycine ester in 10N-ethanolic hydrogen chloride at room temperature, though it occurred to a very slight extent under the same conditions with benzyloxycarbonylglycylphenylalanine ester. It appeared that peptide-bond hydrolysis did not occur in the case of benzyloxycarbonylglycylglycine, used in the time and concentration studies mentioned above, but the R_F values of the esters of glycine and of glycylglycine in the solvents were too similar for certainty in this limited study.

This preliminary work suggested that the hydrolytic method would be applicable to the benzyloxycarbonylaminoacyl-esters of melphalan, and a number of the corresponding dipeptide esters (Table 3, nos. 1—4, 6 and 7) were prepared in this way. The purity of some of the products was checked chromatographically; no contamination by melphalan ester or by other amino-acids was detected. The glycyl- and L-valyl-dipeptide esters (Table 3, nos. 1 and 4) were also prepared by catalytic hydrogenolysis of the corresponding benzyloxycarbonyl compounds. Some of these dipeptide ester hydrochlorides were unstable and decomposed slowly in the dark at room temperature. The presumed " D-valylmelphalan " ester hydrochloride decomposed substantially to a brown gum within a few days (the compound was not analysed). The ester hydrochlorides of the L-valyl, L-leucyl, and L-phenylalanyl derivatives of melphalan became brown within a few months. The well-known [15] resistance of valine peptides to acid-hydrolysis encouraged us to attempt a simultaneous deacylation and ester hydrolysis of the benzyloxycarbonyl-ester no. 3 of Table 2 by heating the compound under reflux with concentrated aqueous hydrochloric acid. The free dipeptide (VIII) (Table 3, no. 5) was thus obtained chromatographically pure and in reasonable yield.

$$NH_2{\cdot}CHPr^i{\cdot}CO{\cdot}NH{\cdot}CH(CO_2H){\cdot}CH_2{\cdot}C_6H_4M\text{-}p \quad \text{(VIII)}$$

With the exception of the cystine derivative (Table 2, no. 16), where ethanolic hydrogen chloride was used, the benzyloxycarbonyl-tripeptide esters were deacylated by hydrogenation over palladium–charcoal, the free tripeptide esters being isolated as their hydrochlorides (Table 3, nos. 8—10 and 12). The product of deacylation of the benzyloxycarbonyl-L-valyl-L-leucyl derivative (Table 2, no. 15) became gummy and discoloured on storage for a day or two, and was not analysed. The tetrapeptide, no. 13 of Table 3, was obtained in the same way by hydrogenolysis of the benzyloxycarbonyl derivative (Table 2, no. 17). The amino-acids used throughout this peptide work were either of the L- or the D-configuration: DL-forms were not used since it seemed advisable to avoid the preparation of products of uncertain stereochemical composition.

While the biological effects of the melphalan derivatives described in this paper have not yet been fully assessed, some points are already clear. The general toxicity, hæmotoxicity, and anti-tumour activity (with special reference to the Walker rat carcinoma 256)

[13] Bergel and Stock, *J.*, 1959, 97.
[14] Barkdoll and Ross, *J. Amer. Chem. Soc.*, 1944, **66**, 951; Goldschmidt and Jutz, *Chem. Ber.*, 1953, **86**, 1116; Gawron and Draus, *J. Org. Chem.*, 1958, **23**, 1040.
[15] Syngè, *Biochem. J.*, 1945, **39**, 351; Hirohata, Kanda, Nakamura, Izumiya, Nagamatsu, Ono, Fujii, and Kimitsuki, *Z. physiol. Chem.*, 1953, **295**, 368.

TABLE 3.

No.	Compound [a]	Formula	C.B. No.	Cryst. from	M. p.	$[\alpha]_D$	Yield (%)
1	Gly.Mel.OEt(P) [bj]	III; $R^1 = R^2 = H$	—	A [f]	146—147°	+20° [g]	53 [h]
2	L-Ala.Mel.OEt(H) [e]	III; $R^1 = H$, $R^2 = Me$	3225	B [i]	193—195	+21 [g]	61
3	D-Ala.Mel.OEt(P) [ej]	III; $R^1 = H$, $R^2 = Me$	—	C [k]	137—139	+18 [l]	50
4	L-Val.Mel.OEt(H) [bly]	III; $R^1 = H$, $R^2 = Pr^i$	3206	B [m]	187—189	+30 [g]	93 [v]
5	L-Val.Mel.OH [t]	VIII	3261	D [i]	240—243 [z]	+54 ‡	75
6	L-Leu.Mel.OEt(H) [elv]	III; $R^1 = H$, $R^2 = Bu^i$	3262	B [m]	170—172	+12 [g]	88
7	L-Phe.Mel.OEt(H) [cy]	III; $R^1 = H$, $R^2 = Ph{\cdot}CH_2$	3207	B [m]	204—206 [n]	+20 [g]	96
8	L-Val.Gly.Mel.OEt(Di-H) [d]	IV; $R^1 = H$, $R^2 = Pr^i$, $R^3 = H$	3232	E [o]	153—155	−54 [g]	80
9	L-Val.Mel.Gly.OEt(Di-H) [dt]	VI; R = H	3263	B [m]	153—156 *	−30 [g]	53
10	L-Ala-L-Leu.Mel.OEt(Di-H) [d]	IV; $R^1 = H$, $R^2 = Me$, $R^3 = Bu^i$	3248	B [m]	155—158 [q]	0 [l]	75
11	,, ,, (P) [e]			F [m]	158—163 [r]	+100 [s]	—
12	L-Cys.Mel.OEt | S : S | L-Cys.Mel.OEt (Di-H) [et]	V; R = H	3247	B [m]	140—142	−34 [g]	92
13	L-Val.Gly.Gly.Mel.OEt(Di-H) [dt]	VII; R = H	3252	B [m]	124—127 †	+34 [p]	82

No.	Formula	Found (%) C	H	Cl	N	Required (%) C	H	Cl	N
1	$C_{23}H_{28}Cl_2N_6O_{10}$	44·9	4·3	11·0	13·8	44·6	4·5	11·4	13·6
2	$C_{18}H_{27}Cl_2N_3O_3,HCl$	49·1	6·3	24·6	9·4	49·1	6·4	24·1	9·5
3	$C_{24}H_{30}Cl_2N_6O_{10}$	45·3	5·0	11·2	13·0	45·5	4·8	11·2	13·3
4	$C_{20}H_{31}Cl_2N_3O_3,HCl,H_2O$	49·2	7·0	21·5	8·4	49·3	7·0	21·8	8·6
5	$C_{18}H_{27}Cl_2N_3O_3,H_2O$	51·2	6·8	16·5	9·9	51·2	6·9	16·8	9·9
,,	$C_{18}H_{27}Cl_2N_3O_3$ [x]	53·7	6·6	17·8	10·6	53·5	6·7	17·5	10·4
6	$C_{21}H_{33}Cl_2N_3O_3,HCl,H_2O$	49·8	6·8	21·5	8·1	50·4	7·2	21·3	8·4
7	$C_{24}H_{31}Cl_2N_3O_3,HCl$	55·4	6·5	20·7	8·4	55·8	6·2	20·6	8·1
8	$C_{22}H_{34}Cl_2N_4O_4,2HCl$ [w]	47·3	6·5	24·9	10·3	47·0	6·5	25·2	10·0
9	$C_{22}H_{34}Cl_2N_4O_4,2HCl,2H_2O$	44·3	6·4	—	9·6	44·2	6·7	—	9·4
10	$C_{24}H_{38}Cl_2N_4O_4,2HCl$ [x]	48·7	6·8	—	9·3	48·8	6·8	—	9·5
11	$C_{30}H_{41}Cl_2N_7O_{11}$	48·1	5·5	—	—	48·3	5·5	—	—
12	$C_{34}H_{52}Cl_4N_6O_6S_2,2HCl,2H_2O$	42·7	6·1	22·25	8·8	42·3	5·9	21·8	8·7
13	$C_{24}H_{37}Cl_2N_5O_5,2HCl,H_2O$	44·8	6·4	—	11·4	45·2	6·5	—	11·0

A, C_6H_6–EtOAc. B, EtOH–Et_2O. C, EtOAc–light petroleum. D, MeOH–EtOH. E, EtOH–Me_2CO. F, EtOH–light petroleum. H, Hydrochloride. P, Picrate. [a] See footnote *a*, Table 2. [b] Prepared both by catalytic hydrogenolysis and by hydrolysis of the corresponding benzyloxycarbonyl derivative. [c] Prepared by the hydrolytic method. [d] Prepared by catalytic hydrogenolysis. [e] Prepared from no. 10. [f] Orange prisms. [g] *c* 1·00 in EtOH at 22—25°. [h] Calc. on the benzyloxycarbonylglycine originally used; hydrolytic deacylation. [i] Needles. [j] *Via* the deliquescent hydrochloride. [k] Yellow needles. [l] *c* 1·00 in MeOH at 24°. [m] Amorphous. [n] With decomp. [o] Prisms. [p] *c* 2·00 in EtOH at 20°. [q] Glass; softening from 140°; fluid at *ca.* 163°. [r] Not sharpened by reprecipitation. [s] *c* 0·20 in EtOH at 25°. [t] Hydrated. [u] Glass at 123—125°. [v] Yield in hydrolytic experiment; catalytic hydrogenation gave a slightly lower yield. [w] Dried at 80°/0·1 mm. [x] Dried at 100°/0·1 mm. [y] Slowly decomposes on storage in the dark at room temp.; decomposition substantial within a few months. [z] Melts to glass at 124—127° which crystallises on cooling and remelts at the temperature indicated. * Softening to glass at 136—139°. † Glass at 117—120°. ‡ *c* 1·9 in MeOH at 21°.

are greatest in compounds carrying a free primary amino-group, be they simple derivatives of melphalan or peptides of it—the activity is approximately equivalent to that of the melphalan they contain. This applies even to the tripeptides, such as no. 8 of Table 3, and the tetrapeptide (Table 3, no. 13) where the free primary amino-group is two or three amino-acids "removed" from the melphalan moiety. Acylation of the primary amino-group in the simple derivatives or peptides of melphalan substantially reduces the biological activity, although, in most cases, the ratio between the toxic and the effective anti-Walker tumour dose (the therapeutic index) is about the same. Thus, the *N*-formyl derivative of melphalan (Table 1, no. 1) has LD_{50} (median lethal dose), in rats, of 125 mg./kg. and

completely inhibits the Walker tumour at 15 mg./kg.; the corresponding figures for melphalan are about 8 mg. and 1 mg. respectively. However, the acetyl-L-phenylalanyl derivative (Table 2, no. 7) of the melphalan ester has a substantially more favourable therapeutic index. Conversion of the carboxyl group in the simple derivatives or peptides of melphalan into an ester or amide group does not significantly alter the biological activity.

TABLE 4. *Deacylation of benzyloxycarbonylglycylglycine by ethanolic hydrogen chloride at room temperature.*

Reaction time (hr.)	Normality (H^+) 1·25	2·5	5·0	10 *	Reaction time (hr.)	Normality (H^+) 1·25	2·5	5·0	10 *
0·5	—	—	—	+	24	±	±	±	++
1	—	—	—	+	48	Not done	Not done	+	++
2	—	—	±	++	96	,,	,,	Not done	++
4	—	—	±	++	120	,,	,,	,,	++
7	—	—	±	++					

Ninhydrin colour intensity of glycylglycine ester spots: — not detectable or extremely faint; ± very faint; + positive; ++ approaching or equivalent to intensity produced by 100% deacylation (≡13·5 μg. of ester). * Trace of glycylglycine present in all "10N"-samples.

For example, the ethyl ester hydrochloride of melphalan is as toxic and as active as melphalan. These findings recall those of Larionov and his colleagues concerning (*a*) the relatively non-toxic dipeptides (II*a* and *b*) mentioned above, and (*b*) the ethyl and isopropyl esters of sarcolysin (I; X = OEt or OPr, R = H), and formyl- and acetyl-sarcolysin (I; X = OH, R = HCO or Ac).[16] It is of interest that the introduction of an *aromatic* primary amino-group into "acetylphenylalanylmelphalan ester" (Table 2, no. 10) does not, however, increase the biological activity. The chemical activity of some of the compounds described here was measured by determining the extent of hydrolysis in boiling aqueous acetone in the presence of calcium carbonate under specified conditions. These results, together with the detailed biological findings, will be presented elsewhere,[17] but they indicate that the variations in biological activity in this series do not reflect variations (which are relatively slight) in the chemical reactivity of the "mustard" group. For example, *N*-formylation of melphalan ester does not significantly affect the chemical reactivity of the "mustard" group, though it very markedly reduces biological activity. It is apparent, therefore, that the nature of the carrier of the "nitrogen mustard" group in this series has a profund effect on biological activity. The extension of the kind of work described above to the preparation of peptides carrying a cytotoxic group may lead to a series of "artificial antibiotics" having useful antitumour effects. This possibility is perhaps of special interest at a time when certain naturally occurring substances of a peptide nature, such as actinomycin D,[18] are being studied as inhibitors of malignant growth.

EXPERIMENTAL

Simple Derivatives of Melphalan.—Formylation by Sheehan and Yang's procedure [19] was used, except that, after dilution of the formic acid–acetic anhydride mixture with water, the product was extracted with ether, washed with water, dried ($MgSO_4$), and recovered. The *formyl compound* (Table 1, no. 1) was obtained crystalline by the careful dropwise addition of water to its solution in methanol. The DL-isomer (*N*-formylsarcolysin) has been prepared by a similar method.[5]

Isobutyl chloroformate (2·0 ml., 15 mmoles) was added with shaking to an ice-cold solution of the formyl derivative (5·0 g., 15 mmoles) and dry triethylamine (2·1 ml., 15 mmoles) in dry

[16] Vodolazskaya, Novikova, Shkodinskaya, Vasina, Berlin and Larionov, *Bull. Biol. Med. exp. U.R.S.S.*, 1957, **44**, No. 11, 76.
[17] Elson, Bergel, and Stock, in preparation for *Biochem. Pharmacol.*
[18] Farber, in Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity, London, Churchill, 1958, p. 138.
[19] Sheehan and Yang, *J. Amer. Chem. Soc.*, 1958, **80**, 1154.

tetrahydrofuran (23 ml.). A white precipitate was rapidly formed. The mixture was set aside for 15 min. at 0°, and aqueous ammonia (*d* 0·88; 1·5 ml.; *ca.* 24 mmoles) was then added with shaking. After 2 hr. at room temperature, the mixture was evaporated under reduced pressure, and the residue extracted with ethyl acetate, washed with 0·1N-hydrochloric acid (the use of more concentrated acid may take the product into the aqueous phase as the hydrochloride of the weakly basic dichloroethylamine), sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and recovered by evaporation under reduced pressure. The residual gum was converted into the crystalline N^{α}-*formyl-amide* (Table 1, no. 3) by addition of water to its solution in ethanol.

Preparation of the N^{α}-*formyl-ethylamide* (no. 4 of Table 1) was similar to that of the amide but with ethylamine (33% w/v).

Esterification was carried out by setting aside a solution of melphalan in 10N-ethanolic hydrogen chloride overnight, or by heating the amino-acid in 6N-ethanolic hydrogen chloride for 2 hr. under reflux. In both cases the mixture was evaporated almost to dryness under reduced pressure, the residual gum taken up in ethanol, and the product precipitated by addition of ether. This material was taken up in a minimum volume of ethanol, excess of chloroform added, and the solution evaporated on a hot plate with occasional addition of chloroform, until crystallisation of the *ethyl ester hydrochloride* (Table 1, no. 6) began. Recrystallisation gave an analytically pure product. The DL-isomer has been described.[16]

The preceding ester hydrochloride (3·7 g., 10 mmoles) and sodium formate (0·7 g., 10 mmoles) were dissolved in 98% formic acid (40 ml.) and acetic anhydride (10 ml.). The solution was set aside for 2 days, treated with water (5 ml.), and evaporated under reduced pressure. Extraction of the oily residue into ethyl acetate, followed by washing with 0·1N-hydrochloric acid, sodium hydrogen carbonate solution, and water, drying ($MgSO_4$), and evaporation, gave the N-*formyl ethyl ester* (Table 1, no. 2), obtained crystalline by addition of water to its solution in methanol.

The ethyl ester hydrochloride (3·7 g., 10 mmoles), dry triethylamine (1·4 ml., 10 mmoles), and chloroform was shaken until the solid had dissolved. Powdered succinic anhydride (1·0 g., 10 mmoles) were added, and the mixture shaken until dissolution was complete and then set aside overnight. Evaporation under reduced pressure yielded a gum which was converted into the solid *ester* (Table 1, no. 5) by addition of water to a methanolic solution of the product.

A solution of the N^{α}-formylamide (no. 3 of Table 1) (0·5 g., 1·5 mmoles) in 0·5N-ethanolic hydrogen chloride (10 ml.) was warmed at 40° for 5 min., cooled, and treated with an excess of ether. The precipitate was taken up in warm ethanol and set aside for 1 hr., during which the *amide hydrochloride* (no. 7, Table 1) (0·15 g.) separated [m. p. 225—228° (decomp.)]. Dissolution in ethanol and addition of ether yielded the pure product. When the formyl compound in an excess of 3N-ethanolic hydrogen chloride was set aside for 24 hr. at room temperature, the product was contaminated with ammonium chloride.

Benzyloxycarbonyl- and Acetyl-dipeptide Ethyl Esters of Melphalan (III; R^1 = Ph·CH_2·O·CO or Ac) *and Dibenzyloxycarbonyl-L-cystinylbismelphalan Ethyl Ester* (V; R = Ph·CH_2·O·CO).—The mixed carbonic–carboxylic acid anhydride method [7] was used. In general, isobutyl chloroformate (5 mmoles) was added to an ice-cooled solution of the acylamino-acid (5 mmoles) and dry triethylamine (5 mmoles) in dry tetrahydrofuran. The mixture was kept in ice for 20 min., then to it was added a freshly prepared solution of the ethyl ester hydrochloride (5 mmoles) of melphalan and triethylamine (5 mmoles) in chloroform. Next day, the mixture was taken to dryness in a vacuum, and the ethyl acetate extract of the residue was washed with 0·1N-hydrochloric acid, sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated under reduced pressure. The residual gummy or solid *acyl-peptide ester* was purified by crystallisation (Table 2). In the preparation of the cystine derivative (Table 2, no. 16), two moles of melphalan ester and of the other reagents were used per mole of the diacylcystine. Benzyloxycarbonylglycylmelphalan ester was exceptional; it could not be obtained solid, but deacylation (see below) led to a crystalline picrate (Table 3, no. 1).

N-*Formylmelphalanyl-glycine Ethyl Ester* (II; R^1 = R^2 = H).—*N*-Formylmelphalan (10 g., 30 mmoles) and glycine ethyl ester [freshly prepared from the ester hydrochloride (4·2 g., 30 mmoles)] were condensed by the chloroformate procedure described above, the *product* being worked up in the usual way (Table 2, no. 11).

Deacylation of N-*Benzyloxycarbonyl-dipeptide Esters.*—(a) *Catalytic hydrogenolysis.* Two melphalan dipeptide esters (Table 3, nos. 1 and 4) were prepared in this way. A 5% palladium–charcoal catalyst was used and the reaction was complete in about 4 hr. at room temperature

and pressure in ethanol or ethanol–ethyl acetate solution containing about 3 equivs. of ethanolic hydrogen chloride. The products were isolated as hydrochlorides; the deliquescent glycine derivative was converted into the picrate.

(b) *Attempted hydrolysis with formic acid–hydrogen chloride.* The method followed that first used in earlier work.[13] Benzyloxycarbonyl-L-phenylalanylmelphalan ethyl ester (Table 2, no. 6; 80 mg.) was dissolved in formic acid saturated with hydrogen chloride and set aside for 16 hr. The water-insoluble product crystallised from ethanol in needles, m. p. 164—165°, unchanged on admixture with the starting material.

(c) *Hydrolysis with ethanolic hydrogen chloride.* (c i) Time and concentration study with benzyloxycarbonylglycylglycine. Portions (18 mg. each) of benzyloxycarbonylglycylglycine (Z.Gly.Gly) were severally dissolved in equal volumes (10 ml. each) of 1·25N-, 2·5N-, 5N-, and 10N-ethanolic hydrogen chloride, and the containers were stoppered. Dissolution in the three weakest acids was slow, but was assisted by shaking for a few minutes. 0·01 ml. (18 μg. of Z.Gly.Gly) was removed at intervals up to 5 days and spotted on Whatman No. 1 chromatography paper, together with glycine, glycine ester (as hydrochloride), glycylglycine, and glycylglycine ester (as hydrochloride). The chromatograms (ascending) were developed in 10 : 5 : 2 : 5 butan-1-ol–ethanol–propionic acid–water, then dried and passed through a 0·25% solution of ninhydrin in acetone. The results are summarised in Table 4. Glycylglycine ester appeared to be the only product (but see footnote * below Table); however, it is possible that traces of glycine ester, formed by peptide fission, would not easily be detected since the R_F values are rather close (*e.g.*, 0·57 for the dipeptide and 0·63 for glycine ester in one experiment) for the short runs (*ca.* 16 cm.) employed; moreover, a mixture of glycine ester and glycylglycine ester had an intermediate R_F of 0·60.

(c ii) Quantitative time study with benzyloxycarbonyl-DL-phenylalanine. A 10% w/v solution of benzyloxycarbonyl-DL-phenylalanine in ethanol previously saturated with hydrogen chloride (*ca.* 10N) was prepared and protected by a calcium chloride guard-tube (a stopper tends to blow out, if used in the early stages of the experiment). Samples (2 ml. each) were withdrawn at intervals. Each sample was evaporated in a vacuum at room temperature and the residue shaken with ether (2 × 5 ml.); this rendered the residue solid. The ether extracts were decanted and discarded, and the residual ether was removed from the residue on a steam-bath. The weight of the residual phenylalanine ester hydrochloride was recorded. In a control experiment with the ester hydrochloride, 100% recovery was obtained. The results tabulated below refer to 2 ml. samples (200 mg. of Z-Phe). Removal of the acyl group is seen to be complete within 3 days.

Reaction time (hr.)	3	6	16	48	64
Ester,HCl recovd. (mg.)	61	85	126	142	154
Deacylation (%)	40	55	82	92	100

(c iii) Check on stability of peptide link in 10N-ethanolic hydrogen chloride. (1) Benzyloxycarbonyl-DL-phenylalanylglycine ethyl ester [20] (10 mg.) was dissolved in saturated ethanolic hydrogen chloride (2 ml.), set aside for 72 hr. at room temperature, and evaporated at room temperature under reduced pressure, and the residue was evaporated twice with a little ethanol and chromatographed as in (c i) above. Only one ninhydrin-positive spot (R_F 0·81) was revealed, corresponding to phenylalanylglycine ester. No phenylalanine ester or glycine ester was detected.

(2) The procedure for benzyloxycarbonylglycyl-DL-phenylalanine ethyl ester [8] was as outlined in (1) above. Glycylphenylalanine ester (R_F 0·75) was essentially the only product, but a trace of glycine ester was detected, indicating slight fission of the peptide bond (the corresponding phenylalanine ester spot was evidently obscured by the main dipeptide ester spot).

(c iv) Preparation of aminoacyl-melphalan ethyl ester hydrochlorides (III; R^1 = H,HCl) and of L-cystinylbismelphalan ethyl ester (V; R = H,HCl). In general, the esters (III; R^1 = $Ph{\cdot}CH_2{\cdot}O{\cdot}CO$) were dissolved in saturated ethanolic hydrogen chloride (*ca.* 10 ml. per g.) ($CaCl_2$ guard-tube), and set aside for 3—4 days; filtration if necessary, and evaporation in a vacuum at <35°, gave a residue which was taken up in a little ethanol and reprecipitated by ether. The *dipeptide hydrochlorides* were purified by redissolution and reprecipitation as before, except in two cases where the hydrochlorides were deliquescent and were converted into picrates (Table 3, nos. 1 and 3). The *cystine compound* (Table 3, no. 12) was prepared similarly.

[20] Schlögl, Wessely, and Wawersich, *Monatsh.*, 1954, **85**, 957.

The purity of some of the peptide ester hydrochlorides was checked by ascending chromatography on Whatman No. 1 paper; R_F values in three solvent systems are tabulated below, together with the values for reference compounds. Each dipeptide gave one ninhydrin-positive spot only.

	Developing solvent				Developing solvent		
Compound	A *	B	C	Compound	A *	B	C
Gly.Mel.OEt	0·80	—	0·52	Mel.OEt	0·84 (0·94)	0·69	0·63
L-Ala-Mel.OEt	0·89 (0·95)	0·69	—	Gly.OEt	0·57	—	0·95
L-Val.Mel.OEt	0·84	—	0·51	DL-Phe.OEt	0·87	—	—
L-Phe.Mel.OEt	0·86	—	0·01	DL-Ala.OEt	(0·66)	0·97	—
[(L-Cys.Mel.OEt)]$_2$	—	0·01	—				

A, Bu^nOH–EtOH–Et·CO_2H–H_2O (10 : 5 : 2 : 5). B, 1% aq. NH_4Cl. C, 2% aq. NH_4Cl. * R_F values vary somewhat with age of solvent mixture A. Figures in parentheses are for freshly-prepared A.

L-*Valylmelphalan* (VIII).—Benzyloxycarbonyl-L-valylmelphalan ethyl ester (Table 2, no. 3) (0·32 g., 0·65 mmole) was heated for 25 min. under reflux with concentrated hydrochloric acid (4 ml.). The solution was cooled, concentrated in a vacuum, and extracted with ether. Concentrated sodium acetate solution was then added to the cooled aqueous solution. The colourless, granular precipitate was washed with water, dried, and crystallised (Table 3, no. 5). The *peptide* gave a single ninhydrin-positive spot of R_F 0·88 on paper (Whatman No. 1) when run in solvent A (preceding paragraph). The reference compounds, melphalan and valine, had R_F 0·69 and 0·38 respectively.

Benzyloxycarbonyl-L-valylglycine Ethyl Ester.—Benzyloxycarbonyl-L-valine was condensed on a 22 mmolar scale with glycine ester by the chloroformate method used for the melphalan dipeptide esters. The acyl-dipeptide ester crystallised from ethanol in needles (64%), m. p. 169—170°, $[\alpha]_D^{24}$ −6° (*c* 1·00 in EtOAc) (Found: C, 61·1; H, 7·1; N, 8·3. Calc. for $C_{17}H_{24}N_2O_5$: C, 60·7; H, 7·2; N, 8·3%). The compound has been prepared by Grassman and Wünsch [21] by the phosphorazo-method; they record m. p. 166°, but give no optical rotation.

Benzyloxycarbonyl-L-valylglycine Hydrazide.—Benzyloxycarbonyl-L-valylglycine ethyl ester (2·0 g., 6 mmoles) and hydrazine hydrate (1·20 g., 24 mmoles) were heated for 1 hr. under reflux in methanol (30 ml.). The solution was set aside overnight, and evaporated to dryness in a vacuum. The residual *hydrazide* crystallised from methanol (yield, 1·63 g., 85%), and had m. p. 175—176°, $[\alpha]_D^{24}$ −4° (*c* 2·25 in EtOH) (Found: C, 55·8; H, 6·9; N, 17·45. $C_{15}H_{22}N_4O_4$ requires C, 55·9; H, 6·9; N, 17·4%).

Benzyloxycarbonyl-L-valylglycylmelphalan Ethyl Ester (IV; R^1 = Ph·CH_2·O·CO, R^2 = Pr^i, R^3 = H).—The preparation was carried out in a refrigerated room (*ca.* 3°). Benzyloxycarbonyl-L-valylglycine hydrazide (1·13 g., 3·5 mmoles) was dissolved in acetic acid (10 ml.), and water (25 ml.) was added. The solution was cooled in ice and to it was added concentrated hydrochloric acid (1 ml.; *d* 1·16), then a solution of sodium nitrite (0·27 g., 3·9 mmoles) in water (2 ml.). A bulky solid was immediately formed. This was extracted into ice-cold ethyl acetate, washed with cold saturated sodium hydrogen carbonate solution, then with water, dried ($MgSO_4$), and filtered. Meanwhile triethylamine (0·66 ml., 4·7 mmoles) was added to melphalan ester hydrochloride (1·74 g., 4·7 mmoles) in cold ethyl acetate (12 ml.), and the mixture stirred to assist dissolution of the free melphalan ester. This solution was filtered into the previously prepared azide solution, and the stoppered mixture set aside at 0° for 48 hr. The solution was then washed at room temperature with 0·1N-hydrochloric acid, sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated to dryness in a vacuum. Relevant data for the *tripeptide ester* are recorded in Table 2 (no. 12).

Benzyloxycarbonyl-L-alanyl-L-leucine Ethyl Ester.—This compound was prepared on a 20 mmolar scale from benzyloxycarbonyl-L-alanine and L-leucine ethyl ester hydrochloride by the method used for the synthesis of benzyloxycarbonylvalylglycine ester described above. The pale yellow oily ester (69% yield) resisted attempts to crystallise it, and was not analysed.

Benzyloxycarbonyl-L-alanyl-L-leucine Hydrazide.—The oily benzyloxycarbonylalanyl-leucine ester was converted into the hydrazide as above. Two crystallisation from aqueous ethanol gave waxy needles (70%) of indefinite m. p. (152—162°, softening from 115°). The product was

[21] Grassman and Wünch, *Chem. Ber.*, 1958, **91**, 449.

not pure but was suitable for conversion into the tripeptide derivative described below. Recrystallisation from ethyl acetate–light petroleum sharpened the m. p. to 158—162°, but involved substantial loss and the product was again not analytically pure; it had $[\alpha]_D^{22}$ −55° (*c* 1·00 in EtOH).

Benzyloxycarbonyl-L-alanyl-L-leucylmelphalan Ethyl Ester (IV; R^1 = Ph·CH_2·O·CO, R^2 = Me, R^3 = Bu^i).—The azide method was used (2 mmolar scale), and the procedure followed that described above for benzyloxycarbonylvalylglycylmelphalan ester. Data for the *tripeptide ester* are given in Table 2 (no. 14).

Benzyloxycarbonyl-L-valyl-L-leucine Ethyl Ester.—Benzyloxycarbonyl-L-valine was condensed with L-leucine ester on a 7 mmolar scale by the method used for benzyloxycarbonylvalylglycine ester. The product was isolated as a straw-coloured oil which gradually crystallised. Two successive crystallisations from aqueous ethanol gave needles, m. p. 103—105°, of the *dipeptide ester* (1·7 g., 60%), $[\alpha]_D^{24}$ −42° (*c* 1·00 in EtOH) (Found: C, 64·2; H, 8·2; N, 7·0. $C_{21}H_{32}N_2O_5$ requires C, 64·3; H, 8·2; N, 7·1%).

Benzyloxycarbonyl-L-valyl-L-leucine Hydrazide.—Benzyloxycarbonyl-L-valyl-L-leucine ester (1·0 g., 2·8 mmoles) was converted into the hydrazide by the method used for benzyloxycarbonyl-L-valylglycine hydrazide. Two crystallisations of the product from aqueous methanol gave the granular hydrazide, m. p. 135—140° (glass), 172—173° (fluid). The compound was not pure, but was suitable for conversion into the tripeptide described below.

Benzyloxycarbonyl-L-valyl-L-leucylmelphalan Ethyl Ester (IV; R^1 = Ph·CH_2·O·CO, R^2 = Pr^i, R^3 = Bu^i).—The preparation (1·5 mmolar) followed the azide procedure used for benzyloxycarbonylvalylglycylmelphalan ethyl ester. Data for the *tripeptide ester* are presented in Table 2 (no. 15).

Benzyloxycarbonyl-L-valyl-" melphalanyl "-glycine Ethyl Ester (V; R = Ph·CH_2·O·CO).—A solution of formyl-" melphalanyl "-glycine ester (1·5 g., 3·6 mmoles) in N-ethanolic hydrogen chloride (20 ml.) was warmed for 5 min. at *ca.* 45°. Evaporation gave a deliquescent gum; this material was evaporated twice with a little ethanol and then taken up in chloroform (10 ml.), and triethylamine (0·49 ml., 3·6 mmoles) was added. The resulting solution was added to a cold solution prepared 15 minutes earlier by adding dry triethylamine (0·49 ml., 3·6 mmoles), then isobutyl chloroformate (0·47 ml., 3·6 mmoles), to an ice-cold solution of benzyloxycarbonyl-L-valine (0·825 g., 3·6 mmoles). The mixture was set aside overnight and worked up in the usual way. The gummy product solidified on being rubbed with ether and was purified by crystallisation (Table 2, no. 13).

Tripeptide Esters of Melphalan (VI; R = H,HCl).—Three of the benzyloxycarbonyl-tripeptide esters (Table 2, nos. 12—14) were each (1—2 mmoles) deacylated by catalytic hydrogenation over 5% palladium–charcoal in ethanol to which a little (4—8 mequivs.) 10N-ethanolic hydrogen chloride had been added. The product obtained by evaporation of each filtered reaction mixture was recrystallised or reprecipitated, to give the pure *tripeptide ester dihydrochloride* (Table 3, nos. 8—10). Hydrogenolysis of benzyloxycarbonyl-L-valyl-L-leucylmelphalan ester (Table 2, no. 15) (0·28 g., 0·4 mmole) under the same conditions yielded a colourless granular product (0·18 g.), m. p. 182—189°. This material was converted by two successive crystallisations from ethanol–ether into colourless needles of a lower but sharp m. p. [155—157° (decomp.), somewhat dependent on the rate of heating]. The product was presumably the hydrochloride of L-valyl-L-leucylmelphalan ethyl ester (IV; R^1 = H, R^2 = Pr^i, R^3 = Bu^i). Heating part of this material at 100° under reduced pressure converted it into a gum; this was not analysed. The remainder of the product decomposed to a dark gum within a few days in the dark at room temperature.

Benzyloxycarbonyl-L-valylglycylglycine Ethyl Ester.—Benzyloxycarbonyl-L-valine was condensed with glycylglycine ethyl ester on a 27 mmolar scale by the method used for benzyloxycarbonyl-L-valylglycine ester, the isolation procedure being similar. The product was crystallised twice from aqueous ethanol, then propan-1-ol. The tiny crystals of *acyl-tripeptide ester* (73%) had m. p. 155—157° and $[\alpha]_D^{21}$ +1° (*c* 1·0 in EtOH) (Found: C, 58·1; H, 6·7; N, 10·6. $C_{19}H_{27}N_3O_6$ requires C, 58·0; H, 6·9; N, 10·7%).

Benzyloxycarbonyl-L-valylglycylglycine Hydrazide.—The above ester (3·93 g., 10 mmoles) was heated for 1·5 hr. under reflux with hydrazine hydrate (2·0 g., 40 mmoles) in methanol (40 ml.). The colourless granular solid obtained by evaporation to dryness under reduced pressure crystallised from ethanol as needles of the *hydrazide* (3·1 g., 82%), m. p. 178—180° (Found: C, 53·4; H, 6·7. $C_{17}H_{25}N_5O_5$ requires C, 53·8; H, 6·6%).

Benzyloxycarbonyl-L-valylglycylglycylmelphalan ester (VII; R = $Ph{\cdot}CH_2{\cdot}O{\cdot}CO$).—This acyltetrapeptide ester (Table 2, no. 17) was prepared from benzyloxycarbonyl-L-valylglycylglycine hydrazide and melphalan ester by the azide method (8 mmolar scale). The procedure was that used for the benzyloxycarbonyl-tripeptides (IV; R^1 = $Ph{\cdot}CH_2{\cdot}O{\cdot}CO$) described above.

L-*Valylglycylglycylmelphalan Ethyl Ester* (VII; R = H).—Benzyloxycarbonyl-L-valylglycylglycylmelphalan ester (1·0 g.) was deacylated by catalytic hydrogenation by the procedure used for the analogous tripeptide esters. The tetrapeptide ester was isolated as the *dihydrochloride monohydrate* (Table 3, no. 13).

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