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An Examination of the Use of Carbobenzyloxy-γ-L-glutamyl Hydrazide in the Synthesis of γ-Peptides[1]

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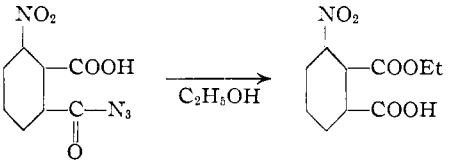
The reported synthesis of γ-glutamyl peptides *via* N-carbobenzyloxy-γ-L-glutamyl azide is shown to yield mixtures of α- and γ-peptides. These mixtures were fractionated and the products identified. A possible mechanism is proposed.

In the past, the α-ester γ-chloride of carbobenzyloxyglutamic acid[3-7] [symbol: Z·Glu·OEt][8] or
└Cl
phthalylglutamic anhydride[9] have been used in the synthesis of γ-peptides. Both of these methods are open to objection since at some stage they involve the opening of an N-acylglutamic anhydride ring which may yield mixtures of α- and γ-derivatives.[10] In order to circumvent this difficulty, the γ-hydrazide of carbobenzyloxy-L-glutamic acid, [Z·Glu·OH (L)], has been prepared[11,12] and its use
└NHNH$_2$
claimed to provide an unequivocal pathway for the synthesis of γ-peptides.

We have found that coupling the pure N-carbobenzyloxy-γ-L-glutamyl azide with an amino acid ester leads to a mixture of α- and γ-peptide derivatives.[13] The nature of the isolable product will therefore depend upon the reaction conditions, the purification procedures, and the solubilities of both the isomeric intermediates and free peptides.

The above finding resembles the observations of Curtius and Semper[14] who found that treatment of 2-carboxy-3-nitrobenzazide with ethanol yielded 2-carboethoxy-3-nitrobenzoic acid.

Somewhat analogous rearrangements have also been reported in the glutaric acid series.[15,16]



When N-carbobenzyloxy-γ-L-glutamyl azide (I), [Z·Glu·OH (L)] was treated with H·Ala·OBz (L),
└N$_3$
(IV), in ethyl acetate at 0° and the product worked up as described in the Experimental part, the pure α-dipeptide derivative, Z·Glu-Ala·OBz (L-L) (Table I, compound 2A) was obtained. In addition, a second fraction (*cf.* Table I, compound 2B) consisting of a mixture of γ- and α-isomers was isolated. The assignment of the structure Z·Glu-Ala·OBz (L-L) for compound 2A is based upon the following evidence: (1) compound 2A is identical with the α-derivative obtained by coupling N-carbobenzyloxy-L-glutamic anhydride (III) with H·Ala·OBz (L)[17]; (2) treatment of compound 2A with phenyldiazomethane gives a derivative identical with authentic Z·Glu-Ala·OBz
└OBz
(L-L)[18] (VIII) (ref. 18, Table I, compound 4); (3) catalytic reduction of compound 2A yields a peptide which in all respects (*e.g.*, paper chromatography, optical rotation, carboxyl and amino N values) is identical with authentic H·Glu-Ala·OH (L-L) (V) (ref. 18, Table II, compound 15). This series of reactions starting with the N-carbobenzyloxy γ-azide is described in the accompanying scheme.

Similarly, when the N-carbobenzyloxy-γ-azide was coupled with H·Ala·OBz (D) and H·Glu-OBz
└OBz
(L) and (D), mixtures of α- and γ-dipeptides were obtained, separable either by fractional crystallization (Table II, compounds 8A, 8B) or paper chromatography.

The most obvious pathway leading to the formation of α-peptides from the N-carbobenzyloxy-γ-azide would appear to be *via* carbobenzyloxyglutamic anhydride or a pseudo anhydride intermediate (II).

From hydrolysis experiments (*cf.* Experimental part) we may conclude that racemization probably does not occur during the synthesis of γ-peptides by either the γ-azide method or the procedure devised by the present authors[18] which starts with the pure α-benzyl ester of N-carbobenzyloxy-L-glutamic acid, [Z·Glu·OBz (L)]. In view of this finding and the results with the γ-azide, the reported discrepancies in optical rotation of a number of γ-peptides (*cf.* ref. 18, Table II, footnotes *j* and *k*, prepared

(1) From a dissertation to be submitted by Howard Sachs in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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(8) The following abbreviations and symbols are used (*cf.* E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946); ref. 18, footnote 3): Z: carbobenzyloxy, $C_6H_5CH_2OCO$; Bz: $C_6H_5CH_2$; Ala: $NH{\cdot}CH(CH_3){\cdot}CO$, C_3H_5ON; Glu: $NH{\cdot}CH(CH_2{\cdot}CH_2{\cdot}COOH){\cdot}CO$, $C_5H_7O_3N$; peptide linkage indicated by dash: -; configuration follows compound in parentheses. *E.g.*, L-glutamic acid γ-ethyl ester: H·Glu·OH (L); N-carbo-
└OEt
benzyloxy-γ-L-glutamyl-D-alanine benzyl ester: Z·Glu·OH (L-D);
└Ala·OBz
N-carbobenzyloxy-α-L-glutamyl-L-glutamyl dibenzyl ester: Z·Glu-Glu·OBz (L-L); γ-L-glutamyl-D-glutamic acid: H·Glu·OH (L-D).
└OBz └Glu·OH

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(10) For a more complete discussion, *cf.* (a) F. Sorm and J. Rudinger, *Czech. Chem. Comm.*, **XV**, 8-9, 491 (1950); (b) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, 1954 (1950).

(11) (a) W. J. LeQuesne and G. T. Young, *ibid.*, 1959 (1950); (b) D. A. Rowlands and G. T. Young, *ibid.*, 3937 (1952).

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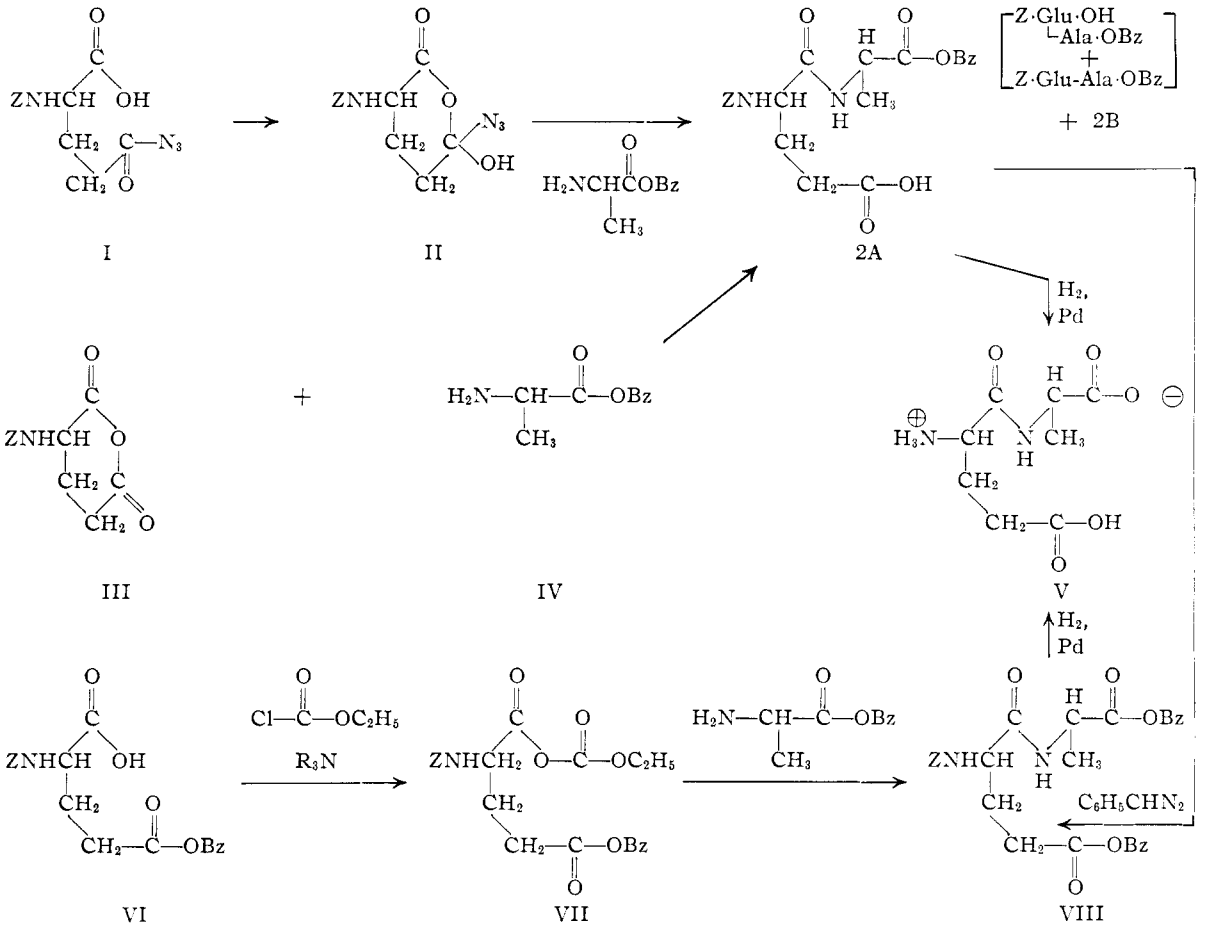

Z = $C_6H_5CH_2OC(=O)-$; Bz = $C_6H_5CH_2-$; configuration, (L)

via the γ-azide[11a,b]) and these compounds synthesized by our method[18] may be attributed to the presence of some α-isomer in the former case. A previous paper[18] has dealt with the synthesis of pure α- and γ-peptides of glutamic acid and alanine.

Experimental[19]

Starting Materials.—The synthesis and properties of some of the starting materials have been previously described: L- and D-alanine,[20] H·Ala·OBz (L) and (D) (ref. 20, compounds 5, 6), L- and D-glutamic acid,[21] and H·Glu·OBz (L) and (D) (ref. 21, compounds 1, 2). Other starting materials used were: Z·Glu·OH (L),[11,12] Z·Glu·OH (L)[22] and H·Glu·OH·HCl (L).[23]

Anal. Calcd. for $C_7H_{13}O_4N\cdot HCl$ (211.6): carboxyl N, 6.6. Found: carboxyl N[24] (ninhydrin, 100°, 7 min., *p*H 2.5), 6.8.

H·Glu·OH·HCl (L).—The preparation of this compound by the catalytic reduction of N-carbobenzyloxy-γ-L-glutamyl hydrazide served as an additional check[25] on the homogeneity of the latter. Z·Glu·OH (L), 2.0 g. (0.007 mole) was hydrogenated in the usual manner[18] in 50 ml. of 50% acetic acid containing 0.007 mole of HCl. The product was recrystallized from H_2O–ethanol yielding 1.2 g. (90%) of pure material, m.p. 178–179°, $[\alpha]^{25}D$ +26.2° (1.9% in 0.1 *N* HCl).

Anal. Calcd. for $C_5H_{11}O_3N_3$ (197.6): N, 21.3; carboxyl N, 7.1. Found: N, 21.3; carboxyl N,[24] 6.9.

Carbobenzyloxy Dipeptide Benzyl Esters (Compounds 2A–5).—N-Carbobenzyloxy-γ-L-glutamyl azide was prepared in the usual manner,[11] extracted into cold (0°) ethyl acetate, washed 3–5 times with ice-water until neutral to congo red, and then dried briefly over Na_2SO_4. This was filtered and added in one portion to a cold (0°) ethyl acetate solution containing either two moles of amino acid benzyl ester or a slight excess of ester and one mole of tri-*n*-butylamine (the results were identical in either case). It also made little difference whether the reaction was allowed to proceed by standing overnight at room temperature or in the ice-box (0–10°). On the next day, the reaction mixture, which smelled strongly of hydrazoic acid, was washed with 0.5 *N* HCl and then H_2O. It was then dried over Na_2SO_4 and the ethyl acetate removed *in vacuo*. The resulting sirups were easily crystallized from ethyl acetate–petroleum ether or ethanol–H_2O. In general, the final yield (10–54%) was low and the product melted over a range of several degrees.

The analytical data and specific rotations of the azide coupling products and of the free peptides are presented in Tables I and II, respectively. The coupling products (Table I) are designated by number and with respect to the amino acid ester (columns 2 and 1, respectively) reacted with N-carbobenzyloxy-γ-L-glutamyl azide. The free peptides (Table II) refer to the hydrogenated coupling products.

(19) We are indebted for analytical work to T. Zelmenis (total and amino N).

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(25) *Cf.* ref. 11a; LeQuesne and Young established homogeneity of the N-carbobenzyloxy γ-hydrazide by Curtius degradation.

TABLE I

CARBOBENZYLOXY DIPEPTIDE BENZYL ESTERS; ANALYTICAL DATA AND SPECIFIC ROTATIONS IN GLACIAL ACETIC ACID

Amino acid ester reacted with the γ-azide[a]	Coupling product no.	Yield, %	M.p., °C. (cor.)	Nitrogen, % Calcd.	Nitrogen, % Found	Neut. equiv. Calcd.	Neut. equiv. Found	$[\alpha]^{24}D$ (c, 2)
H·Ala·OBz (L)	2A	10–17	153–154	6.3	6.3	442	449	−23.5°
	2B[b]	12	74–95	6.3	6.3	442	430	
H·Ala·OBz (D)	3	54	118–122	6.3	6.3	442	449	
H·Glu·OBz (L) └OBz	4	30	118–121	4.7	4.7	591	593	−2.8
H·Glu·OBz (D) └OBz	5	35	86–110	4.7	4.5	591	600	+4.6

[a] *Cf.* Experimental part for reaction conditions, etc. [b] Compounds 2B, 3–5 consist of mixtures of the α- and γ-derivatives.

TABLE II

DIPEPTIDES OF GLUTAMIC ACID AND ALANINE; ANALYTICAL DATA[a] AND SPECIFIC ROTATIONS IN 0.5 *N* HCl

Coupling product hydrogenated[b] no.	Peptide isolated[c] no.	Molecular formula	Mol. wt.	Nitrogen, % Calcd.	Nitrogen, % Found	Amino N, %[d] Calcd.	Amino N, %[d] Found	Carboxy N, %[e] Calcd.	Carboxy N, %[e] Found	$[\alpha]^{24}D$ (c, 2)
2A	6	$C_8H_{14}O_5N_2$	218.2	12.8	13.0	6.4	6.3	6.4	0.0	+7.6°
2B	7[f]	$C_8H_{14}O_5N_2$	218.2	12.8	13.1	6.4	11.8	6.4	5.9	−8.7
3	8A	$C_8H_{14}O_5N_2$	218.2	12.8	12.6	6.4	12.6	6.4	6.4	+63.1
	8B	$C_8H_{14}O_5N_2$	218.2	12.8	12.6	6.4	6.6	6.4	0.7	+81.0
4	9[g]	$C_{10}H_{16}O_7N_2$	276.2	10.1	10.1	5.1	9.0	5.1	4.0	+ 6.1
5	10	$C_{10}H_{16}O_7N_2$	276.2	10.1	10.1	5.1	7.8	5.1	3.9	+38.7

[a] Compounds 6, 7, 8A, 9, 10 dried at 78° *in vacuo*, compound 8B at 56°. [b] *Cf.* Table I. [c] *Cf.* Experimental part, "Dipeptides." [d] Reaction time with nitrous acid was 3 minutes, the high values for compounds 7, 8A–10 are attributed to the contribution of the γ-peptide N (*cf.* ref. 18; H. Sachs and E. Brand, unpublished work). [e] Reaction time with ninhydrin was 7 minutes at *p*H 2.5; *cf.* ref. 24. [f] Previously prepared (*cf.* ref. 11b) with $[\alpha]^{18}D$ −22.1° (5.0% in H_2O). [g] Previously prepared (*cf.* ref. 11a) with $[\alpha]^{17}D$ +6.0° (1.1% in H_2O + 1 equiv. HCl).

As described above, Z·Glu·OH (L) derived from 3.2 g. └N_3 (0.011 mole) of Z·Glu·OH (L) in 200 ml. of ethyl acetate └$NHNH_2$ (0°) was added to 150 ml. of ethyl acetate (0°) containing 2.6 ml. (0.011 mole) of tri-*n*-butylamine and H·Ala·OBz (L) (prepared from 3.5 g. (0.016 mole) of H·Ala·OBz·HCl) and the mixture allowed to stand in the ice-box overnight. The final sirup was crystallized from acetone–H_2O and gave 1.7 g. (35%) of crude material, which sintered at 85° and had m.p. 127–140°. This was recrystallized from ethyl acetate–petroleum ether and then ethanol–H_2O, yielding 0.5 g. of compound 2A, m.p. 153–154°.

From the mother liquors was isolated 0.65 g. of compound 2B, m.p. 74–95°.

In another experiment (in which 2 moles of ester was used and no tri-*n*-butylamine) the reaction product (sirup) was taken up in 50 ml. of ethyl acetate–ether (1:1), and extracted with 5 portions of Na_2CO_3, according to the procedure of LeQuesne and Young[10b] for separating α- from γ-isomers. The pure α-derivative (m.p. 153–154°) was obtained in approximately 15% yield from the last two Na_2CO_3 extracts. The first three extracts yielded mixtures.

Reaction of Phenyldiazomethane[26] with Compound 2A.—0.0004 mole of phenyldiazomethane in 6 ml. of ether was added to 0.25 g. (0.0006 mole) of compound 2A in 25 ml. of ethyl acetate and the mixture allowed to stand at room temperature until decolorization took place (3 hours). This was washed with 5% $NaHCO_3$, and H_2O; dried over Na_2SO_4 and the solvent removed *in vacuo* leaving an oil which crystallized on trituration with petroleum ether. It was recrystallized from methanol–H_2O and *n*-propyl alcohol–H_2O; yield 75%, m.p. 102–104°; mixed m.p. with authentic Z·Glu-Ala·OBz (L),[18] 102–104°; $[\alpha]^{25}D$ −20.8° └OBz (0.8% in glacial acetic acid). The authentic α-derivative had $[\alpha]^{25}D$ −21.2° (1.2% in glacial acetic acid).

Anal. Calcd. for $C_{30}H_{32}O_7N_2$ (532.6): N, 5.3. Found: N, 5.2.

Dipeptides (Compounds 6–10).—The carbobenzyloxy dipeptide benzyl esters were hydrogenated in the usual way,[18] using 80–90% acetic acid as solvent.

Hydrogenolysis of compound 2A gave in near quantitative yield a peptide (compound 6) having zero carboxyl N and differing in no way from the authentic α-isomer.

Compound 2B yielded material which reacted with ninhydrin[24] to give 0.9 mole of CO_2 and also showed two spots on paper chromatograms (*cf.* below) corresponding to the authentic α- and γ-peptides.

From the reduction of compound 3 the pure γ-peptide, H·Glu·OH (L-D) (compound 8A), was obtained which └Ala·OH crystallized from H_2O (yield 45%). The water-soluble fraction was isolated and shown to consist of a mixture of the α- and γ-peptides (compound 8B).

Compounds 9 and 10 also contained varying proportions (*cf.* Table II, carboxyl N values) of the α- and γ-components which were separable on Whatman no. 1 paper.

Chromatography of Peptides.—In order to ensure adequate separation of the α- and γ-isomers, the peptides were chromatographed as previously described[18] (butanol–acetic acid–H_2O). Only compound 6 and 8A ran as single spots with R_{Glu}, 1.7 and 1.1, respectively. Compound 7, 8B, 9 and 10 each gave two spots corresponding to the authentic α- and γ-peptides.

Hydrolysis of Peptides.—Compound 9, 0.0766 g. (0.28 mmole), was dissolved in 5 cc. of 6 *N* HCl and the solution was refluxed for 20 hours. It was then brought to a volume of 10 cc. with 6 *N* HCl and the specific rotation of the L-glutamic acid determined. L-Glutamic acid control, 0.1429 g., and pure H·Glu·OH (L-L) (ref. 18, compound 21), └Glu·OH 0.1122 g. (0.4 mmole), was treated as described above. L-Glutamic acid control: $[\alpha]^{24}D$ +29.7° (1.4% in 6 *N* HCl); compound 9 hydrolysate, $[\alpha]^{24}D$ +30.1° (0.82% in 6 *N* HCl); H·Glu·OH (L-L) hydrolysate, $[\alpha]^{24}D$ +29.3° (1.2% in 6 *N* └Glu·OH HCl).

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