7. *Amino-acids and Peptides. Part VI.* Synthesis of* L-*Aspartyl Peptides from Carbobenzyloxy-*L-*aspartic Anhydride.*

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The reaction of carbobenzyloxy-L-aspartic anhydride (I) with amino-acid esters has been found to yield both α- and β-aspartyl derivatives (II and III) which may be separated by fractional extraction with alkali. In this way α- and β-L-aspartyl-glycine, -L-tyrosine, and -L-glutamic acids have been prepared. From the products of the reaction of the anhydride with ethanol, crystalline α-ethyl carbobenzyloxy-L-aspartate has been isolated. The β-aspartyl peptides are distinguishable from their α-isomers in that they give a blue colour with ninhydrin.

In Part I (Le Quesne and Young, *J.*, 1950, 1954) it was shown that the reaction of carbobenzyloxy-L-glutamic anhydride with amino-compounds and with alcohols gives both α- and γ-glutamyl derivatives, the former normally preponderating. Carbobenzyloxy-L-aspartic anhydride (I) presents an analogous problem. There has long been evidence

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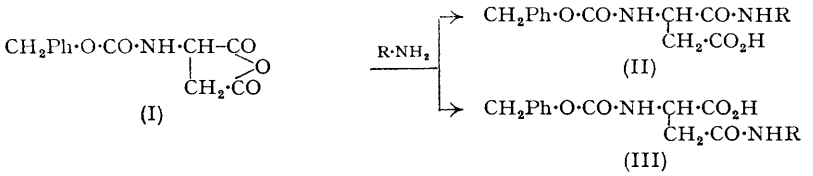
that this anhydride ring may open to yield both α- and β-aspartyl compounds (II and III). Although Bergmann and Zervas (*Ber.*, 1932, **65**, 1192) reported that with ammonia carbobenzyloxy-L-*iso*asparagine (II; R = H) was formed, with L-tyrosine ethyl ester



hydrochloride in pyridine solution they obtained a substance subsequently shown (Bergmann, Zervas, Salzmann, and Schleich, *Z. physiol. Chem.*, 1934, **224**, 17) to be β-L-aspartyl-L-tyrosine ethyl ester, together with a low-melting material which was not investigated further. From the reaction with glycine ethyl ester, Grassmann and Schneider (*Biochem. Z.*, 1934, **273**, 452) obtained carbobenzyloxy-α-L-aspartylglycine ethyl ester of melting point 113°; Bergmann, Zervas, and Fruton later (*J. Biol. Chem.*, 1935, **111**, 225) raised the melting point to 128° by repeated crystallisation, and it seemed likely that the contaminant was the β-isomer.

We have therefore reinvestigated the reaction of carbobenzyloxy-L-aspartic anhydride with amino-acid esters. The preparation of the anhydride itself calls for some comment; Bergmann and Zervas (*loc. cit.*) originally gave its melting point as 84°, but a year later (Bergmann, Zervas, and Salzmann, *Ber.*, 1933, **66**, 1288) this figure was corrected to 124°. Subsequently, Miller, Behrens, and du Vigneaud (*J. Biol. Chem.*, 1941, **140**, 411) used cold acetic anhydride to effect the ring closure but recorded no constants for their product. Using the latter method we obtained an anhydride melting, after recrystallisation, at 109—111°; more recently, in this laboratory, Mr. J. B. Capindale has obtained, by a slightly modified procedure (to be published), material melting sharply at 111°.

The products of the reaction of the anhydride with glycine ethyl ester in ethyl acetate solution were separated by fractional extraction with aqueous sodium carbonate (compare Part I, *loc. cit.*); β-aspartyl peptides, having the α-carboxyl group free, would be expected to be slightly stronger acids than their α-isomers. Both carbobenzyloxy-α- and -β-L-aspartylglycine ethyl ester were isolated in this manner from the reaction products, in nearly equal yield and having very similar melting points; the mixed melting point was depressed. Hydrolysis gave carbobenzyloxy-α- and -β-L-aspartylglycine (again similar in melting point) and hydrogenation yielded α- and β-L-aspartylglycine.

The latter dipeptide was prepared earlier by another route by Grassmann and Schneider (*loc. cit.*) who recorded a melting point of 153° for the anhydrous compound and $[\alpha]_D^{22}$ +7·2° for the monohydrate (in water containing one mol. of hydrochloric acid). Our product melted initially at 153—156°, but analysis showed it to be the monohydrate; drying gave the anhydrous material melting at 190—200° (with decomposition). Moreover, our hydrate had $[\alpha]_D^{21}$ +13·9° ± 0·8°, in a similar solvent. Our constants for α-L-aspartylglycine agree with those of Grassmann and Schneider.

From the products of the reaction of the anhydride with L-tyrosine ethyl ester in ethyl acetate solution we have isolated the new compound, carbobenzyloxy-α-L-aspartyl-L-tyrosine ethyl ester, together with its β-isomer. Hydrolysis gave carbobenzyloxy-α-L-aspartyl-L-tyrosine, which on hydrogenation gave α-L-aspartyl-L-tyrosine as the monohydrate. Under similar conditions diethyl L-glutamate gave the previously unreported diethyl carbobenzyloxy-α- and β-L-aspartyl-L-glutamates, and thence the isomeric carbobenzyloxy-L-aspartyl-L-glutamic acids and the corresponding dipeptides.

It would appear therefore that this method of synthesis leads normally to a mixture of α- and β-aspartyl derivatives, which may be separated by fractional extraction with alkali.

Alcohols and alkoxides would similarly be expected to yield both α- and β-esters. Pauly and Weir (*Ber.*, 1910, **43**, 661) obtained α-methyl benzoyl-L-aspartate from benzoyl-L-aspartic anhydride and methanol; and Bergmann, Zervas, and Salzmann (*loc. cit.*)

prepared crystalline α-benzyl carbobenzyloxy-L-aspartate in an analogous manner. By the action of sodium methoxide on toluene-*p*-sulphonyl-L-aspartic anhydride, Harington and Moggridge (*J.*, 1940, 707) isolated a substance provisionally assigned the structure of the α-ester, by analogy.

The product obtained by heating carbobenzyloxy-L-aspartic anhydride with ethanol was fractionally extracted with aqueous sodium carbonate. The early fractions failed to crystallise, but later extracts yielded crystalline α-ethyl carbobenzyloxy-L-aspartate, which on hydrogenation gave α-ethyl L-aspartate. With aqueous ammonia the former compound gave carbobenzyloxy-L-*iso*asparagine of melting point 167—169° (uncorrected); Bergmann and Zervas's original product, obtained by direct ring-opening of the anhydride with ammonia, had melting point 164° (corrected).

It is of interest that although the α-aspartyl peptides so far prepared by us give a normal purple colour with ninhydrin, that from the β-isomers is blue.

Experimental

M. p.s are uncorrected. Combustion analyses are by Drs. Weiler and Strauss and by Mr. F. C. Hall.

Carbobenzyloxy-L-aspartic Anhydride.—The method of Miller, Behrens, and du Vigneaud (*loc. cit.*) was used. The crude product was dried in a vacuum-desiccator over calcium chloride and sodium hydroxide and purified by extraction with ether in a Soxhlet apparatus. Recrystallisation from acetone–ether–light petroleum gave m. p. 109—111°.

Carbobenzyloxy-α- and -β-L-aspartylglycine Ethyl Esters.—Carbobenzyloxy-L-aspartic anhydride (4·0 g.) in ethyl acetate (20 ml.) was added to glycine ethyl ester (from 8·5 g. of hydrochloride) in ethyl acetate (50 ml.) slowly, with shaking. After this had been kept overnight, aqueous hydrochloric acid was added; after shaking, the ethyl acetate layer was separated, washed with water, and then extracted with successive portions of aqueous sodium carbonate (each containing 0·12 g.). On acidification, the products obtained rapidly solidified in all cases. The fractionation was repeated on the first three fractions, giving *carbobenzyloxy-β-L-aspartylglycine ethyl ester* (1·2 g., 21%), m. p. 122—124°, unchanged by recrystallisation from chloroform–ether (Found: C, 54·6; H, 5·9; N, 7·7. $C_{16}H_{20}O_7N_2$ requires C, 54·5; H, 5·7; N, 8·0%). The mixed m. p. with the α-isomer was 104—108°.

From the later fractions by a further fractionation was obtained carbobenzyloxy-α-L-aspartylglycine ethyl ester (1·1 g., 19%), m. p. 120—122° (Found: C, 54·7; H, 5·8; N, 8·0%).

Carbobenzyloxy-β-L-aspartylglycine.—Carbobenzyloxy-β-L-aspartylglycine ethyl ester (1 g.) in N-sodium hydroxide (7 ml.) was kept at 14° for 1½ hours and acidified with 5N-hydrochloric acid. The precipitated carbobenzyloxy-β-L-aspartylglycine (0·7 g., 75%) was filtered off after 1 hour at 0°; it had m. p. 158—161°, raised to 160—162° by recrystallisation from ethyl acetate.

β-L-*Aspartylglycine.*—Carbobenzyloxy-β-L-aspartylglycine (0·5 g.) in aqueous methanol was hydrogenated at atmospheric pressure in the presence of palladium black, giving β-L-aspartylglycine monohydrate (0·30 g., 93%), m. p. 153—156°, $[\alpha]_D^{20}$ +13·9° (*c*, 2·59 in water containing 1 mol. of hydrochloric acid) (Found: C, 34·7; H, 5·6; N, 14·0. Calc. for $C_6H_{10}O_5N_2,H_2O$: C, 34·6; H, 5·8; N, 13·5%). After drying for 4 hours at 110° and 18 mm. it had m. p. 190—200° (decomp.).

α-L-*Aspartylglycine.*—Carbobenzyloxy-α-L-aspartylglycine ethyl ester was hydrolysed to carbobenzyloxy-α-L-aspartylglycine, m. p. 162—165°, which was hydrogenated as above, giving α-L-aspartylglycine, m. p. 184—185°, $[\alpha]_D^{20}$ +35·0° (*c*, 2·14 in water containing 1 mol. of hydrochloric acid).

Carbobenzyloxy-α- and -β-L-aspartyl-L-tyrosine Ethyl Esters.—Carbobenzoyloxy-L-aspartic anhydride (2 g.) in ethyl acetate (10 ml.) was added portionwise, with shaking, to tyrosine ethyl ester (3·8 g.) in ethyl acetate (40 ml.). After being kept overnight, the solution was washed with hydrochloric acid and water and then extracted with successive portions of aqueous sodium carbonate (each containing 0·08 g.). The oils obtained on acidification of the earlier fractions crystallised when washed with ether, giving carbobenzyloxy-β-L-aspartyl-L-tyrosine ethyl ester (0·55 g., 15%), m. p. 191—196°, raised to 200—201° by recrystallisation from ethanol.

The later fractions, and the material extracted from the earlier ones with ether, were combined and recrystallised from ethyl acetate–light petroleum, giving *carbobenzyloxy-α-L-aspartyl-L-tyrosine ethyl ester* (1·5 g., 40%), m. p. 142—144° (Found: C, 59·9; H, 5·9; N, 6·4. $C_{23}H_{26}O_8N_2$ requires C, 60·2; H, 5·7; N, 6·1%).

Carbobenzyloxy-α-L-aspartyl-L-tyrosine.—Carbobenzyloxy-α-L-aspartyl-L-tyrosine ethyl ester (1 g.) in N-sodium hydroxide (7 ml.) was left for 2 hours at 17°. After acidification with 5N-hydrochloric acid and storage for 1 hour at 0°, the *carbobenzyloxy-α-L-aspartyl-L-tyrosine* (0·9 g., 96%) was filtered off. It was dried in a vacuum-desiccator over phosphoric oxide and recrystallised from ethyl acetate–light petroleum; it then had m. p. 149—151° (Found: C, 58·7; H, 4·8; N, 6·3. $C_{21}H_{22}O_8N_2$ requires C, 58·6; H, 5·1; N, 6·5%).

α-L-Aspartyl-L-tyrosine.—Carbobenzyloxy-α-L-aspartyl-L-tyrosine (0·7 g.) was hydrogenated in aqueous methanol in the usual fashion. Evaporation of the solution (to approx. 1 ml.) and addition of acetone (25 ml.) gave *α-L-aspartyl-L-tyrosine monohydrate* (0·35 g., 68%), m. p. 177—178°, which retained its water after an hour at 100°/18 mm. (Found: C, 49·3; H, 5·9; N, 8·8. $C_{13}H_{16}O_6N_2,H_2O$ requires C, 49·7; H, 5·8; N, 8·9%); it had $[\alpha]_D^{21}$ +17·8° (*c*, 2·59 in water containing 1 mol. of hydrochloric acid).

Diethyl Carbobenzyloxy-α- and -β-L-aspartyl-L-glutamates.—Carbobenzyloxy-L-aspartic anhydride (3 g.) in ethyl acetate (15 ml.) was added slowly, with shaking, to a solution of diethyl L-glutamate (from 9 g. of hydrochloride) in ethyl acetate (50 ml.). Next morning the solution was washed with dilute hydrochloric acid and water, then extracted with successive portions of aqueous sodium carbonate (each containing 0·12 g.). All the fractions obtained by acidification slowly crystallised; the middle ones were refractionated. Recrystallisation from ethyl acetate–light petroleum gave *diethyl carbobenzyloxy-α-L-aspartyl-L-glutamate monohydrate* (1·2 g., 21%), m. p. 82—84° (Found: C, 53·8; H, 6·4; N, 6·0. $C_{21}H_{28}O_9N_2,H_2O$ requires C, 53·6; H, 6·4; N, 6·0%), and the *β-isomer* (*monohydrate*) (1·1 g., 19%), m. p. 71—73° (Found: C, 53·7; H, 6·3; N, 6·1%); the mixed m. p. of the two isomers was 62—68°.

Carbobenzyloxy-α-L-aspartyl-L-glutamic Acid.—Diethyl carbobenzyloxy-α-L-aspartyl-L-glutamate (0·5 g.) in N-sodium hydroxide (5 ml.) was set aside for 1 hour and acidified with 5N-hydrochloric acid. After being kept at 0° the *carbobenzyloxy-α-L-aspartyl-L-glutamic acid* (0·3 g., 71%) was filtered off; it had m. p. 151—152° (Found: C, 51·9; H, 5·2; N, 7·2. $C_{17}H_{20}O_9N_2$ requires C, 51·5; H, 5·1; N, 7·1%).

α-L-Aspartyl-L-glutamic Acid.—Carbobenzyloxy-α-L-aspartyl-L-glutamic acid (0·4 g.) in aqueous methanol was hydrogenated in the normal manner. Evaporation of the filtrate under reduced pressure (to 1 ml.) and addition of ethanol (25 ml.) precipitated *α-L-aspartyl-L-glutamic acid* (0·2 g., 75%); it was recrystallised from aqueous ethanol and dried at 100° and 18 mm., then having m. p. 150—155°, $[\alpha]_D^{25}$ +5·6° (*c*, 3·39 in water) (Found: C, 40·7; H, 5·7; N, 10·7. $C_9H_{14}O_7N_2$ requires C, 41·2; H, 5·4; N, 10·7%).

Carbobenzyloxy-β-L-aspartyl-L-glutamic Acid.—Diethyl carbobenzyloxy-β-L-aspartyl-L-glutamate (0·5 g.) in N-sodium hydroxide (5 ml.) was left for 1½ hours acidified with 5N-hydrochloric acid and cooled to 0° for 15 minutes. The *carbobenzyloxy-β-L-aspartyl-L-glutamic acid* (0·3 g., 71%) was filtered off: it had m. p. 153—157° unaltered by recrystallisation from hot water (Found: C, 51·8; H, 5·2; N, 7·5. $C_{17}H_{20}O_9N_2$ requires C, 51·5; H, 5·1; N, 7·1%).

β-L-Aspartyl-L-glutamic Acid.—Carbobenzyloxy-β-L-aspartyl-L-glutamic acid (0·1 g.) in aqueous methanol was hydrogenated in the normal manner. The filtrate was evaporated to dryness and absolute ethanol added; *β-L-aspartyl-L-glutamic acid* separated as a white solid (0·02 g.). Analysis was made difficult by the extreme hygroscopicity of the product which was dried at 100°/18 mm. (Found: C, 37·8; H, 5·5; N, 9·5. $C_9H_{14}O_7N_2,H_2O$ requires C, 38·6; H, 5·7; N, 10·0%).

Paper-partition Chromatography of some Aspartyl Peptides.—By use of phenol saturated with water in an atmosphere containing ammonia, the following R_F values were obtained; each peptide gave a single spot with ninhydrin, purple in the case of the α-peptides and blue from the β-isomers: α-L-aspartyl-glycine 0·13, -L-tyrosine 0·26, and -L-glutamic acid 0·05; β-L-aspartyl-glycine 0·16, -L-tyrosine 0·29, and -L-glutamic acid, 0·05.

α-Ethyl Carbobenzyloxy-L-aspartate.—Carbobenzyloxy-L-aspartic anhydride (4·0 g.) and ethanol (25 ml.) were heated in a sealed tube at 120—130° for 4 hours. The resulting solution was evaporated under reduced pressure, dissolved in ether, and extracted with successive portions of aqueous sodium carbonate; the later fractions crystallised on acidification, to give *α-L-ethyl carbobenzyloxy-L-aspartate* which was recrystallised from ether–light petroleum, then having m. p. 80—82° (1·55 g., 33%) (Found: C, 57·0; H, 5·9; N, 4·7. $C_{14}H_{17}O_6N$ requires C, 57·0; H, 5·8; N, 4·7%). The earlier fractions gave oils which did not crystallise.

α-Ethyl L-Aspartate.—α-Ethyl carbobenzyloxy-L-aspartate (0·5 g.) in aqueous ethanol was hydrogenated in the usual manner. The filtrate was evaporated under reduced pressure (to 1 ml.) and ethanol (25 ml.) added. After ½ hour the *α-ethyl L-aspartate* (0·2 g., 74%) was filtered off; it had m. p. 180—181°, raised to 181—183° by recrystallisation from 80% aqueous

ethanol (Found : C, 44·7; H, 7·0; N, 8·4. $C_6H_{11}O_4N$ requires C, 44·7; H, 6·9; N, 8·7%); the mixed m. p. with β-ethyl L-aspartate was 151—157°.

Carbobenzyloxy-L-iso*asparagine*.—A solution of α-ethyl carbobenzyloxy-L-aspartate (0·5 g.) in aqueous ammonia (*d* 0·880; 5 ml.) was set aside for 4 days and then acidified, with cooling, by dropwise addition of concentrated hydrochloric acid. After 1 hour, the solution was filtered and the carbobenzyloxy-L-*iso*asparagine recrystallised from hot water, giving needles of m. p. 165—167° (0·3 g., 67%), raised to 167—169° by another crystallisation. The mixed m. p. with carbobenzyloxy-L-asparagine (m. p. 157—159°) was 153—157°.

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