780. *Amino-acids and Peptides. Part XIV.* Further Studies on the Synthesis of Aspartyl-peptides.*

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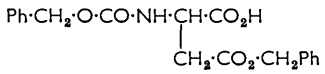
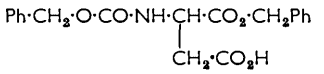
α-L-Aspartyl-L-leucine, and α- and β-L-aspartyl-L-aspartic and -L-glutamic acid, have been synthesised through the appropriate monobenzyl ester (I) or (II) of benzyloxycarbonyl-L-aspartic acid. A new route to α-benzyl L-aspartate has been developed. The esters (I) and (II) have been coupled also with ε-benzyloxycarbonyl-L-lysine benzyl ester.

The classical method of synthesis of aspartyl-peptides, by the action of an amino-ester on benzyloxycarbonylaspartic anhydride,[1] yields a mixture of α- and β-aspartyl derivatives,[2] and although in some cases these can be separated,[2,3] in others (*e.g.*, the preparation of the aspartyl-leucines) [4] the separation is unsatisfactory. An alternative route to α-aspartyl-peptides [5] is through β-benzyl benzyloxycarbonyl-L-aspartate (I) prepared by the partial hydrolysis of the dibenzyl ester.[6] We have coupled this intermediate with the appropriate amino-acid benzyl esters for the synthesis of α-L-aspartyl-L-leucine, -L-aspartic acid, and



(I)



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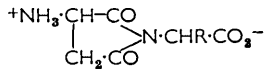

(III)

-L-glutamic acid. In the first case, we used the carbonic mixed anhydride [7] and the ethylene phosphorochloridite ("amide procedure" [8]) methods of coupling, but in the later work we found the use of dicyclohexylcarbodi-imide [9] most satisfactory. The subsequent hydrogenation to remove the benzyloxycarbonyl and the benzyl ester protecting groups was effected without added acid, as evaporation of solutions of the peptides in the presence of mineral acid gave traces of an impurity which is probably the corresponding aspartimide (III) (cf. ref. 10).

The analogous intermediate for the synthesis of β-aspartyl-peptides, α-benzyl benzyloxycarbonyl-L-aspartate (II), is well known,[11] but its preparation from benzyloxycarbonyl-L-aspartic anhydride involves careful purification from its β-isomer. We have developed an alternative preparation, in which α-benzyl L-aspartate is obtained by the controlled cleavage of dibenzyl L-aspartate with hydriodic acid in acetic acid, a reaction similar to that by which α-benzyl L-glutamate can be prepared.[12] Benzyloxycarbonylation gave the required intermediate, from which β-L-aspartyl-L-aspartic acid and -L-glutamic acid were prepared. In each case, dicyclohexylcarbodi-imide was used for the coupling.

By controlled hydrolysis of 3,6-biscarboxymethyl-2,5-dioxopiperazine, Fischer and Koenigs [13] obtained a product which they provisionally formulated as α-aspartylaspartic acid; Greenstein [14] titrated electrometrically a solution of the dipeptide prepared in this

* Part XIII, *J.*, 1959, 2626.

[1] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.
[2] Le Quesne and G. T. Young, *J.*, 1952, 24.
[3] John and G. T. Young, *J.*, 1954, 2870.
[4] *Idem*, unpublished work.
[5] Miller, Neidle, and Waelsch, *Arch. Biochem. Biophys.*, 1955, **56**, 11.
[6] Berger and Katchalski, *J. Amer. Chem. Soc.*, 1951, **73**, 4084.
[7] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547; Wieland and Bernard, *Annalen*, 1951, **572**, 190.
[8] R. W. Young, Wood, Joyce, and Anderson, *J. Amer. Chem. Soc.*, 1956, **78**, 2126.
[9] Sheehan and Hess, *ibid.*, 1955, **77**, 1067.
[10] Swallow, Lockhart, and Abraham, *Biochem. J.*, 1958, **70**, 359.
[11] Bergmann, Zervas, and Salzmann, *Ber.*, 1933, **66**, 1288; Miller, Behrens, and du Vigneaud, *J. Biol. Chem.*, 1941, **140**, 411.
[12] Sachs and Brand, *J. Amer. Chem. Soc.*, 1953, **75**, 4610.
[13] Fischer and Koenigs, *Ber.*, 1907, **40**, 2048.
[14] Greenstein, *J. Biol. Chem.*, 1931, **93**, 479.

way and found the expected ionisable groups, but the material was not isolated. α- and β-L-Aspartyl-L-glutamic acid had previously been prepared, with some difficulty, from benzyloxycarbonyl-L-aspartic anhydride, by Le Quesne and Young.[2] Shiba, Yamakita, and Kaneko [15] later reported the synthesis of these dipeptides by a similar route. No specific rotation is quoted for the second-named compound, but for the first they found $[\alpha]_D^{19}$ −3·6°, whereas Le Quesne and Young reported $[\alpha]_D^{25}$ +5·6°, in water. We were therefore glad of the opportunity which the present synthesis, by an entirely different route, gave to redetermine this constant, and we find $[\alpha]_D^{18}$ +6·3°. Since the β-isomer has $[\alpha]_D^{18}$ −10·5° in water, it seems likely that Shiba, Yamakita, and Kaneko's separation of isomers was incomplete. Our dipeptide melted at 145—150°, in comparison with 150—155° [2] and 134—135°,[15] but in our experience melting points are unreliable for the characterisation of peptides.

We record also in this paper the coupling of the esters (I) and (II) with ε-benzyloxycarbonyl-L-lysine benzyl ester. These reactions could not be effected under normal conditions. The use of ethylene phosphorochloridite ("amide" or "standard" procedure [8]) gave little product; the ester (I) was converted into the phenyl thiolester, through the carbonic mixed anhydride,[16] but again condensation with the lysine component was unsuccessful. The *p*-nitrophenyl thiolesters were prepared similarly from (I) and (II), and gave satisfactory yields of coupling products, but it proved difficult to remove all traces of di-*p*-nitrophenyl disulphide, and catalytic hydrogenation failed to remove the benzyloxycarbonyl and the benzyl ester groups completely. Removal of these protecting groups by the action of hydrogen bromide in acetic acid at 60° [17] was accompanied by the formation of a substantial amount of a by-product which moved as a cation during electrophoresis at pH 5·1 and gave a yellow colour with ninhydrin; this may have been the aspartimide {III; R = $[CH_2]_4{\cdot}NH_3^+Br^-$}, analogous to that formed by the action of acid on ε-aspartyl-lysines.[10] Removal of the protecting groups by sodium in liquid ammonia [18] also gave a by-product which behaved similarly on electrophoresis. Finally, successful couplings were effected in moderate yield by using the mixed anhydride with isobutyl hydrogen carbonate under forcing conditions. Hydrogenation of the products gave materials which are clearly α-(α- and β-L-aspartyl)-L-lysine respectively, but we have been unable to recrystallise the small amounts available satisfactorily, and we record here only the protected derivatives.

An unusual side reaction was encountered during the acylation of α-benzyl L-aspartate by benzyl chloroformate, in the presence of aqueous sodium hydrogen carbonate. On two occasions, early in the reaction a considerable amount of a white solid separated, and there was isolated from the product a compound we believe to be α-*O*-benzyl-*N*-benzyloxycarbonyl-β-L-aspartic anhydride. The same material was prepared by the action of cold acetic anhydride on α-benzyl benzyloxycarbonyl-L-aspartate; the infrared absorption differed notably from that of α-benzyl benzyloxycarbonyl-L-aspartate in having a strong peak at 1805 $cm.^{-1}$ (in Nujol paste).*

In earlier papers of this series [2,3] it was noted that the β-aspartyl peptides described there were distinguishable from the α-isomers by the characteristic blue colour given when paper chromatograms were developed with ninhydrin, and this distinction has been confirmed by other workers.[19] The β-aspartyl-peptides reported here give the typical

* From an attempted coupling, by the mixed carbonic anhydride method, of benzyloxycarbonyl-asparagine, Leach and Lindley (*Austral. J. Chem.*, 1954, **7**, 173), isolated a substance which was considered to be the symmetrical anhydride.

[15] Shiba, Yamakita, and Kaneko, *J. Inst. Polytechnics, Osaka City Univ., Ser. C*, 1956, **5**, 144.
[16] Wieland, Schäfer, and Bokelman, *Annalen*, 1951, **573**, 99.
[17] Ben-Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[18] Sifferd and du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.
[19] Liwschitz and Zilkha, *J. Amer. Chem. Soc.*, 1954, **76**, 3698; 1955, **77**, 1265; Liwschitz, Edlitz-Pfeffermann, and Lapidoth, *ibid.*, 1956, **78**, 3069; Liwschitz and Zilkha, *J.*, 1957, 4394; Zilkha and Liwschitz, *J.*, 1957, 4397.

blue colour; it is, however, important in this test that after spraying with ninhydrin the paper should be dried rapidly at 100—120°; drying at lower temperatures gives various colours, but never, with the β-peptides we have examined, the purple which is obtained from their α-isomers.

Experimental

M. p.s were determined on the Kofler block. Infrared spectra were recorded on a Perkin-Elmer model 21 spectrophotometer. The solvents used for paper chromatography (descending flow) were (*a*) butan-1-ol–water–acetic acid (62 : 26 : 12) and (*b*) phenol–water (80 : 20) in the presence of 0·1% aqueous ammonia. In these solvents, aspartic acid had R_F 0·15 and 0·06, respectively. Electrophoresis (at 7 v/cm.) was carried out in a Durrum type of apparatus, (*a*) in 10% acetic acid, pH 2·2, and (*b*) in pyridine–2N-acetic acid–water (1 : 5 : 80), pH 5·1. In each case Whatman no. 1 paper was used, and development was with 0·2% ninhydrin solution in butan-1-ol saturated with water, except where otherwise stated.

β-*Benzyl Benzyloxycarbonyl-*L*-aspartate.*—We have found it convenient to modify Berger and Katchalski's procedure [6] by using lithium hydroxide in aqueous acetone in place of sodium hydroxide in aqueous dioxan, a modification kindly communicated to us by Dr. D. F. Elliott. Dibenzyl benzyloxycarbonyl-L-aspartate (4·5 g.) was dissolved in aqueous acetone (1 : 4; 250 ml.), and a solution of lithium hydroxide (0·255 g.) in water (10 ml.) was added during ½ hr., with stirring at room temperature. The acetone was removed below 40° and unchanged diester was recovered by extraction into ether. The aqueous layer was acidified with 6N-hydrochloric acid, giving the β-ester as an oil, which soon crystallised. The product (2·5 g., 70%), after recrystallisation from benzene, had m. p. 107—109°, $[\alpha]_D^{17}$ +13·1° (*c* 10·0 in glacial acetic acid) (lit.,[6] m. p. 108°, $[\alpha]_D^{25}$ +12·1°).

*Benzyloxycarbonyl-α-*L*-aspartyl-*L*-leucine Dibenzyl Ester.*—(a) *Carbonic mixed anhydride method.*[7] β-Benzyl benzyloxycarbonyl-L-aspartate (5·34 g.) and dried triethylamine (2·1 ml.) were dissolved in toluene (30 ml.) and chloroform (10 ml.) at −5°; s-butyl chloroformate (2·0 ml.) was added dropwise with stirring, the temperature being kept at −5°. After 5 min., a similarly cooled solution of L-leucine benzyl ester toluene-*p*-sulphonate (7·07 g.) and triethylamine (2·4 ml.) in toluene (5 ml.) and chloroform (15 ml.) was added. The temperature was allowed to rise and the mixture was left overnight. The solution was washed with 2N-hydrochloric acid, N-potassium hydrogen carbonate, and water, dried ($MgSO_4$), and evaporated at reduced pressure. The residual oil slowly crystallised in the refrigerator; the solid was washed with light petroleum (b. p. 60—80°) and collected (3·65 g., 43·5%; m. p. 63—66°); recrystallisation from ether–light petroleum (b. p. 60—80°) and from ethanol–water gave *product* of m. p. 69·5—70·0°, $[\alpha]_D^{19}$ −22·5° (*c* 1·38 in acetone), $[\alpha]_D^{19}$ −29·8° (*c* 1·41 in ethanol) (Found: C, 68·5; H, 6·6; N, 5·35. $C_{32}H_{36}O_7N_2$ requires C, 68·5; H, 6·5; N, 5·0%).

(b) *Using ethylene phosphorochloridite* (*amide procedure* [8]). L-Leucine benzyl ester hydrochloride (2·36 g.) and triethylamine (1·6 ml.) were dissolved in diethyl phosphite (20 ml.). Ethylene phosphorochloridite [20] (0·54 ml.) was added, and the mixture was heated on a boiling-water bath for 5 min. β-Benzyl benzyloxycarbonyl-L-aspartate (2·14 g.) in diethyl phosphite (5 ml.) was added; after 1 hr. at 100°, the mixture was diluted with water, and the product which crystallised was collected and washed with water. Purification gave 1·66 g. (49%) of *product*, m. p. 68—68·5°.

α-L-*Aspartyl-*L*-leucine.*—Hydrogenation with palladium black of the product from coupling (*b*), in aqueous ethanol containing acetic acid, gave the *dipeptide*, which was recrystallised from aqueous acetone and dried at 100° for 12 hr. at 12 mm. (Found: C, 48·6; H, 7·4; N, 11·3. $C_{10}H_{18}O_5N_2$ requires C, 48·8; H, 7·3; N, 11·4%); it had $[\alpha]_D^{17}$ −10·6° (*c* 3·3 in water), $[\alpha]_D^{17}$ −9·7° (*c* 3·42 in 0·1N-hydrochloric acid), R_F in butanol–water–acetic acid, 0·60.

*Tribenzyl Benzyloxycarbonyl-α-*L*-aspartyl-*L*-aspartate.*—β-Benzyl benzyloxycarbonyl-L-aspartate (0·89 g.) was dissolved in dry methylene chloride (5 ml.), and a solution of dibenzyl L-aspartate toluene-*p*-sulphonate (m. p. 159—160°, 1·21 g.) and triethylamine (0·33 ml.) in methylene chloride (5 ml.), was added to it. Dicyclohexylcarbodi-imide (0·57 g.), in methylene chloride (20 ml.), was added to the mixture of esters. The solution was stirred for 6 hr., a few drops of glacial acetic acid were added (to decompose unchanged di-imide), and stirring was continued for a further ½ hr. Dicyclohexylurea, which had separated, was filtered off and the

[20] Lucas, Mitchell, and Scully, *J. Amer. Chem. Soc.*, 1950, **72**, 5491.

solvent was removed under reduced pressure, leaving a white solid. This was extracted into ethyl acetate (100 ml.) and washed successively with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and water. After drying ($MgSO_4$), the solvent was removed under reduced pressure and the product (1·36 g., 81%) was washed with light petroleum (b. p. 60—80°) and filtered off. After recrystallisation from absolute ethanol, the *product* had m. p. 108·5—109·5°, $[\alpha]_D^{16}$ +23·1° (*c* 5·0 in dry chloroform), $[\alpha]_D^{16}$ −6·1° (*c* 5·06 in acetone) (Found: C, 68·2; H, 5·6; N, 4·4. $C_{37}H_{36}O_9N_2$ requires C, 68·1; H, 5·6; N, 4·3%).

α-L-*Aspartyl*-L-*aspartic Acid*.—Tribenzyl benzyloxycarbonyl-α-L-aspartyl-L-aspartate in aqueous ethanol was hydrogenated in the presence of palladium black. The solution was filtered and the solvents were removed under a high vacuum. The sticky residue was washed with dimethylformamide, and the solid product (70%) was collected and dried. The crude material dissolved in the minimum of water, the solution filtered under gentle suction, and the solvent was removed under a high vacuum. The peptide was collected under absolute ethanol, filtered off, and dried at room temperature and 0·01 mm. for 6 hr. Further purification was effected by taking the peptide into water and adjusting the pH to 2; water was removed under a high vacuum and the product was collected under absolute ethanol as before, then having $[\alpha]_D^{20}$ +16·8° (*c* 5·0 in water), $[\alpha]_D^{20}$ +8·4° (*c* 5·0 in 0·5N-hydrochloric acid) (Found: C, 38·3; H, 5·4; N, 11·8. Calc. for $C_8H_{12}O_7N_2$: C, 38·7; H, 4·9; N, 11·3%), R_F in butanol–water–acetic acid 0·11, in phenol–water 0·05. After electrophoresis on paper, the movement relative to aspartic acid was 1·4 at pH 2·2, and 1·0 at pH 5·1.

Tribenzyl Benzyloxycarbonyl-α-L-*aspartyl*-L-*glutamate*.—The coupling procedure was identical with that described above, and the *product* was obtained as a white solid (78%). After recrystallisation from absolute ethanol it had m. p. 99—100°, $[\alpha]_D^{16}$ +12·5° (*c* 5·72 in dry chloroform), $[\alpha]_D^{15}$ −10·5° (*c* 5·25 in acetone) (Found: C, 68·8; H, 5·9; N, 4·4. $C_{38}H_{38}O_9N_2$ requires C, 68·5; H, 5·8; N, 4·2%).

α-L-*Aspartyl*-L-*glutamic Acid*.—Hydrogenation in aqueous methanol gave a product (85%) which was collected under absolute ethanol. After purification (as described for α-aspartylaspartic acid) it had m. p. 145—150° (lit., 150—155°,[2] 134—135° [15]), $[\alpha]_D^{18}$ +6·3° (*c* 5·0 in water), (lit., $[\alpha]_D^{25}$ +5·6° [2]; $[\alpha]_D^{19}$ −3·6° [15]), $[\alpha]_D^{18}$ +4·6° (*c* 5·0 in 0·5N-hydrochloric acid) (Found: C, 41·5; H, 5·4; N, 10·5. Calc. for $C_9H_{14}O_7N_2$: C, 41·2; H, 5·4; N, 10·7%), R_F in butanol–water–acetic acid 0·17, in phenol–water 0·04. After electrophoresis on paper, the movement relative to aspartic acid was 1·4 at pH 2·2, and 1·0 at pH 5·1.

α-*Benzyl* L-*Aspartate*.—55% Hydriodic acid was decolorised by dropping hypophosphorous acid into the boiling solution, and was then distilled. The constant-boiling hydriodic acid (8 ml.) so obtained was added to dibenzyl L-aspartate toluene-*p*-sulphonate (9·9 g.) dissolved in "AnalaR" glacial acetic acid (60 ml.), and the solution was kept at 45° for 29 hr. The solvent was then removed by distillation under reduced pressure. To remove traces of benzyl alcohol, small quantities of benzene were added to the residue in the flask and distilled off under reduced pressure. The dark, viscous residue was cooled to −10° and covered with absolute ethanol. Triethylamine (2·5 ml.) was added and the syrup triturated; slow dissolution of the dark material occurred with simultaneous deposition of yellow crystals. More triethylamine was added until the suspension had pH 7; it was then left in the refrigerator overnight. The solid (2·25 g., 50%) was collected, washed with alcohol and ether, and recrystallised from water, pH being adjusted to 5·5 with aqueous sodium hydroxide before filtration. The *product* had m. p. 174—175°, $[\alpha]_D^{18}$ −15·4° (*c* 5·0 in N-hydrochloric acid) (Found: C, 59·2; H, 5·9; N, 6·4. $C_{11}H_{13}O_4N$ requires C, 59·2; H, 5·9; N, 6·3%).

α-*Benzyl Benzyloxycarbonyl*-L-*aspartate*.—α-Benzyl L-aspartate (0·77 g.) and sodium hydrogen carbonate (0·8 g.) were dissolved in water (20 ml.) and stirred. Benzyl chloroformate (0·6 g.) was added dropwise, and stirring was continued for 5 hr. at room temperature. The aqueous solution was washed with ether, and concentrated hydrochloric acid was added to pH 2. An oil separated and was extracted into ethyl acetate. Solvent was removed under reduced pressure from the dried ($MgSO_4$) solution, and the sticky residue was left to crystallise under light petroleum (b. p. 60—80°). The product (70%) was recrystallised from toluene; it then had m. p. 84—85° (lit.,[11] 84—85°, 85°), $[\alpha]_D^{18}$ −9·7° (*c* 5·59 in acetic acid), $[\alpha]_D^{17}$ +8·8° (*c* 5·0 in dry chloroform), $[\alpha]_D^{17}$ −14·8° (*c* 5·0 in acetone) (Found: C, 63·8; H, 5·1; N, 4·0. Calc. for $C_{19}H_{19}O_6N$: C, 63·9; H, 5·3; N, 3·9%).

On two occasions, a considerable amount of a white solid separated during the reaction, and the isolated product had m. p. 109—111°. After several recrystallisations from toluene the

m. p. of this material was raised to 116—116·5°, depressed on admixture with α-benzyl benzyloxycarbonyl-L-aspartate or with β-benzyl benzyloxycarbonyl-L-aspartate. The material absorbed strongly at 1801, 1719, 1672 cm.$^{-1}$ (in Nujol), in contrast with the authentic α-ester which had peaks at 1718 and 1658 cm.$^{-1}$ (Found: C, 66·0; H, 5·0; N, 4·1. Calc. for $C_{19}H_{19}O_6N$: C, 63·9; H, 5·3; N, 3·9. $C_{38}H_{36}O_{11}N_2$ requires C, 65·5; H, 5·2; N, 4·0%). After trituration of the crude material under sodium carbonate solution, the insoluble portion was filtered off and recrystallised once from toluene, then having m. p. 119—120°. This product was shown by infrared spectra (in Nujol and in chloroform), and by mixed melting point, to be identical with the material described below as α-O-*benzyl*-N-*benzyloxycarbonyl*-β-L-*aspartic anhydride.*

α-O-*Benzyl*-N-*benzyloxycarbonyl*-β-L-*aspartic Anhydride.*—α-Benzyl benzyloxycarbonyl-L-aspartate (m. p. 84—85°) was dissolved in acetic anhydride and left at room temperature overnight. The *product* was precipitated with light petroleum (b. p. 60—80°) and after recrystallisation from toluene had m. p. 120—121°, $[\alpha]_D^{17}$ +20·9° (*c* 5·0 in dry chloroform) (Found: C, 65·4; H, 5·2; N, 4·1%). It absorbed strongly at 1805, 1724, and 1678 cm.$^{-1}$ (in Nujol). A solution in glacial acetic acid containing a trace of water had $[\alpha]_D^{17}$ −9·8° (*c* 5·0), and a solution in moist acetone had $[\alpha]_D^{17}$ −14·9° (*c* 5·0). Material recovered from these solutions had m. p. 83—85°, confirming that hydrolysis had occurred in the moist solvents.

Tribenzyl Benzyloxycarbonyl-β-L-*aspartyl*-L-*aspartate.*—The coupling procedure was analogous to that described above for the α-isomer. The *product* (83%) was recrystallised from aqueous ethanol; it then had m. p. 121·5—123°, $[\alpha]_D^{16}$ +25·2° (*c* 5·0 in dry chloroform), $[\alpha]_D^{16}$ −5·7° (*c* 5·0 in acetone) (Found: C, 68·1; H, 5·7; N, 4·2. $C_{37}H_{36}O_9N_2$ requires C, 68·1; H, 5·6; N, 4·3%).

β-L-*Aspartyl*-L-*aspartic Acid.*—Hydrogenation in aqueous methanol gave a product (97%) which was collected under absolute ethanol. After purification (as described for α-aspartyl-aspartic acid) the *dipeptide* had $[\alpha]_D^{18}$ +4·8° (*c* 5·0 in water), $[\alpha]_D^{18}$ +18·2° (*c* 5·0 in 0·5N-hydrochloric acid) (Found: C, 39·0; H, 5·1; N, 11·4. $C_8H_{12}O_7N_2$ requires C, 38·7; H, 4·9; N, 11·3%), R_F in butanol–water–acetic acid 0·08, in phenol–water 0·06. After electrophoresis on paper, the movement relative to aspartic acid was 0·8 at pH 2·2, and 1·0 at pH 5·1.

Tribenzyl Benzyloxycarbonyl-β-L-*aspartyl*-L-*glutamate.*—The coupling procedure was analogous to that described above for the α-isomer. The *product* (87%), recrystallised from absolute ethanol, had m. p. 111·5—112·5°, $[\alpha]_D^{15}$ +10·7° (*c* 5·0 in dry chloroform), $[\alpha]_D^{15}$ −10·8° (*c* 5·25 in acetone) (Found: C, 68·7; H, 5·5; N, 4·3. $C_{38}H_{38}O_9N_2$ requires C, 68·5; H, 5·8; N, 4·2%).

β-L-*Aspartyl*-L-*glutamic Acid.*—Hydrogenation in aqueous methanol gave a product (79%) which was collected under absolute ethanol. After purification it had $[\alpha]_D^{18}$ −10·5° (*c* 5·25 in water), $[\alpha]_D^{18}$ +5·5° (*c* 5·05 in 0·5N-hydrochloric acid) (Found: C, 41·5; H, 5·4; N, 10·7. Calc. for $C_9H_{14}O_7N_2$: C, 41·2; H, 5·4; N, 10·7%), R_F in butanol–water–acetic acid 0·12, in phenol–water 0·07. After electrophoresis on paper, the movement relative to aspartic acid was 0·7 at pH 2·2, and 1·0 at pH 5·1.

α-(*Benzyloxycarbonyl*-α-L-*aspartyl*)-ε-*benzyloxycarbonyl*-L-*lysine Dibenzyl Ester.*—(*a*) p-*Nitrophenylthiolester procedure.*[16] β-Benzyl benzyloxycarbonyl-L-aspartate (0·89 g.) and 1-ethylpiperidine (0·34 ml.) were dissolved in anhydrous tetrahydrofuran. Ethyl chloroformate (0·24 ml.) was added dropwise to the vigorously stirred solution at 0°. After 15 min., *p*-nitrothiophenol (0·45 g.) in tetrahydrofuran was added, and the mixture was stirred for 4 hr. at room temperature. The solvent was removed under reduced pressure, and the product solidified under water at 5°. It was recrystallised from butan-1-ol (yield 1·0 g., 81%) and used directly. A small amount of 4,4′-dinitrodiphenyl disulphide (m. p. 177—178°) remained insoluble in the butanol.

The α-*p*-nitrophenyl β-benzyl benzyloxycarbonyl-α-thiol-L-aspartate (0·84 g.) thus prepared was dissolved in anhydrous tetrahydrofuran (10 ml.), and added to a mixture of ε-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (0·70 g.) and 1-ethylpiperidine (0·24 ml.), and the whole was heated under reflux for 2 hr. The solvent was removed under reduced pressure, and the residue was dissolved in chloroform and washed successively with water, 2N-hydrochloric acid, N-sodium hydrogen carbonate, and water. The chloroform solution was dried ($MgSO_4$) and filtered, and the solvent removed. Yellow crystals remained (1·2 g., 100%) which recrystallised from acetone–ether, yielding cream-coloured crystals (0·7 g., 60%), m. p. 116—117·5°.

Attempts to free the product from traces of 4,4′-dinitrodiphenyl disulphide by means of

bromine, potassium permanganate, hydrogen peroxide, or deactivated alumina, were not sufficiently effective to allow successful and reproducible catalytic hydrogenations to α-(α-L-aspartyl)-L-lysine.

(*b*) *Carbonic mixed anhydride method.*[7] β-Benzyl benzyloxycarbonyl-L-aspartate (2·55 g.) and triethylamine (1·0 ml.) were dissolved in anhydrous tetrahydrofuran and cooled to −5°. Isobutyl chloroformate (1·0 g.) was added dropwise with shaking, with protection against moisture. After 7 min., ε-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (2·90 g.) and triethylamine (1·20 ml.) in tetrahydrofuran were added. The mixture was allowed to reach room temperature slowly, and after 22 hr. was boiled under reflux for 1½ hr. The solvent was evaporated and replaced by ethyl acetate, and the solution was washed thoroughly with water, dilute hydrochloric acid, aqueous hydrogen carbonate, water, and brine. The ethyl acetate layer was dried ($MgSO_4$), filtered, and evaporated, leaving white crystals (3·1 g., 61%). The *product* recrystallised from acetone–di-isopropyl ether and had m. p. 116—117°. A further recrystallisation from methanol raised the m. p. to 118°, unchanged by further recrystallisation; $[\alpha]_D^{17}$ was −4·6° (*c* 3·5 in dioxan) (Found: C, 68·0; H, 6·2; N, 5·6. $C_{40}H_{43}O_9N_3$ requires C, 67·7; H, 6·1; N, 5·9%).

Couplings to form the above compound were attempted also by the ethylene phosphorochloridite " amide " and " standard " procedures, and through the phenyl thiolester. In all cases very low yields were obtained together with much unchanged starting materials. This was shown by hydrogenation of the crude products, followed by paper electrophoresis at pH 5·1, which gave three ninhydrin-positive spots, corresponding in position to aspartic acid, lysine, and the dipeptide respectively.

α-(*Benzyloxycarbonyl*-β-L-*aspartyl*)-ε-*benzyloxycarbonyl*-L-*lysine Dibenzyl Ester.*—(*a*) This compound was prepared by the procedure described for the α-isomer under (*a*) above. The crude product (75% yield) was considerably contaminated with 4,4′-dinitrodiphenyl disulphide, but after six recrystallisations from acetone–di-isopropyl ether, the colourless crystals had constant m. p. 153·5—154°, $[\alpha]_D^{17}$ −4·1° (*c* 2·93 in dioxan) (Found: C, 67·5; H, 6·3; N, 6·0%).

(*b*) This compound was also prepared by procedure (*b*) described for the α-isomer above. The product was obtained in 60% yield and after one recrystallisation had m. p. 153—154°, not depressed on admixture with the product obtained by method (*a*).

Hydrogenation of these α- and β-aspartyl derivatives, obtained by the carbonic mixed anhydride procedure, failed to give analytically pure dipeptides, although the products moved as single spots during paper electrophoresis at pH 5·1. Alternative methods of removing the benzyloxycarbonyl and the benzyl ester groups were tried. The use of sodium in liquid ammonia or hydrogen bromide in glacial acetic acid at 60° gave a by-product, which on paper electrophoresis at pH 5·1 moved as a cation and gave a bright yellow colour with ninhydrin; the dipeptides moved very slightly towards the cathode. The α-derivative gave a purple colour, and the β-derivative a blue colour, with ninhydrin.

Colour Reactions of α- *and* β-*Aspartyl-peptides.*—The following tests were applied to α- and β-aspartyl-L-valine, -L-tyrosine, -L-aspartic acid, and -L-glutamic acid, and to α-L-aspartyl-L-leucine. Aqueous solutions of the dipeptides (1%) were spotted on filter paper and treated with a 1% solution of ninhydrin in butan-1-ol or in ethanol, and the paper was *rapidly* dried at 100—120°. The α-aspartyl-peptides gave the normal purple colour, and the β-peptides a blue colour. Drying at lower temperatures gave various colours, usually shades of pink, instead of blue. With dilute aqueous copper sulphate and sodium hydroxide, solutions of the α-peptides gave a deep blue, and the β-isomers a pale blue colour (cf. ref. 19). Aqueous solutions of all the β-peptides dissolved copper carbonate to give a blue solution, as did α-aspartyl-aspartic and -glutamic acid; solutions of the remaining α-aspartyl-peptides failed to dissolve copper carbonate (cf. ref. 19).

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