Synthesis of ϵ -Aspartyl-lysines and of *iso*Hexylamides of Aspartic Acid

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Lockhart & Abraham (1956) found that two compounds which appeared to be aspartyl-lysine and aspartyl(isoleucyl)-lysine respectively were formed when bacitracin A was hydrolysed with 11*N*-hydrochloric acid at 80°. The aspartic acid residue in these compounds had the *L*-configuration and was linked to the ϵ -amino group of *L*-lysine. However, both compounds carried a net positive charge at pH 7 and this raised the question whether they were simple peptides. The synthesis of peptides in which aspartic acid is joined through its α - and β -carboxyl group respectively to the ϵ -amino group of lysine has therefore been undertaken. For purposes of comparison the α - and β -*iso*hexylamides of aspartic acid have also been synthesized. *L*-Aspartic acid and *L*-lysine have been used in this work but no attempt has been made to discover whether significant racemization occurs in the course of any of the reactions.

It seemed possible that the reaction of *N*-benzyloxycarbonyl-*L*-aspartic anhydride (I) with the copper complex of *L*-lysine or with α -toluene-*p*-sulphonyl-*L*-lysine ester (α -tosyl-*L*-lysine ester) would provide a simple route to both ϵ -*N*-(α -*L*-aspartyl)-*L*-lysine (II) and ϵ -*N*-(β -*L*-aspartyl)-*L*-lysine (III). The anhydride (I) was used by Bergmann & Zervas (1932) in peptide synthesis and was later shown to yield a mixture of α - and β -aspartyl derivatives on reaction with several amino acid esters (LeQuesne & Young, 1952; John & Young, 1954).

Interaction of the anhydride with the copper complex of *L*-lysine in aqueous dioxan, followed by removal of copper and of the benzyloxycarbonyl group, led to a product from which only one com-

pound, subsequently shown to be ϵ -(α -*L*-aspartyl)-*L*-lysine, was obtained by crystallization from water. The non-crystalline material from the mother liquors contained some of the β -aspartyl isomer.

Reaction of the anhydride with α -tosyl-*L*-lysine ethyl ester in a mixture of ethyl acetate and aqueous potassium bicarbonate appeared to occur in the expected fashion, but difficulty was experienced in the removal of the ethyl ester group. This difficulty was avoided by use of the corresponding benzyl ester and subsequent removal of the benzyl and benzyloxycarbonyl group together by catalytic hydrogenation. The final product was shown by electrophoresis on paper to have no significant net charge at pH 7.0 and 5.0 and to yield equivalent amounts of aspartic acid and lysine on hydrolysis. When subjected to electrophoresis on paper at pH 2.3, however, it was resolved into two components which migrated towards the cathode at different rates. The faster-moving component was assumed to be the α -aspartyl peptide (II) and the slower moving the β -aspartyl peptide (III), since the free β -carboxyl group of the former should be a weaker acid than the corresponding α -carboxyl group of the latter. The faster-moving component migrated at the same rate as the peptide obtained from the copper complex of lysine.

This assumption was shown to be correct when the two isomers were synthesized by unambiguous routes. The syntheses involved the coupling, by the mixed-anhydride method (Vaughan, 1951), of an α -tosyl-*L*-lysine benzyl ester with the β - and α -benzyl esters respectively of *N*-benzyloxycarbonyl-*L*-aspartic acid. The benzyl and benzyloxycarbonyl groups were removed from the products by catalytic hydrogenation and the tosyl group by sodium in liquid ammonia. ϵ -(α -*L*-Aspartyl)-*L*-lysine, obtained from the β -benzyl aspartate, migrated as a single compound, and at the same rate as the faster-moving of the two isomers prepared earlier, when subjected to electrophoresis on paper at pH 2.3. ϵ -(β -*L*-Aspartyl)-*L*-lysine, obtained from the α -benzyl aspartate, proved to have the same mobility at pH 2.3 as the slower-moving isomer. It was contaminated, however, with a trace of the α -aspartyl peptide. This indicated that the method used for the preparation of α -benzyl

N-benzyloxycarbonylaspartate (Bergmann, Zervas & Salzmann, 1933) had given a product containing a small proportion of the β -benzyl ester.

The results of electrometric titrations of peptides (II) and (III) are shown in Fig. 1. ϵ -(α -L-Aspartyl)-L-lysine contained ionizable groups with pK_a values of <2.7, 3.4, 8.0 and 9.9. These values could be assigned to the α -carboxyl group of the lysine residue, the β -carboxyl group of the aspartic acid residue, the amino group of the aspartic acid residue, and the α -amino group of the lysine residue respectively. In ϵ -(β -L-aspartyl)-L-lysine the corresponding pK_a values were <2.5, 2.8, 9.1 and 10.1. As expected, the second carboxyl of the β -aspartyl isomer was a stronger acid than that of the α -isomer, whereas the first amino group of the α -isomer (being in the α position to a peptide bond) was a weaker base than the first amino group of the β -isomer.

The titration curves of the two ϵ -aspartyl-lysines accounted for the results obtained when the compounds were subjected to electrophoresis on paper in different buffers. At pH 7.0 and pH 5.0 both peptides behaved as neutral substances. At pH 2.3 ϵ -(α -L-aspartyl)-L-lysine migrated 11 cm. towards the cathode under the conditions used (14 v/cm. for 2.5 hr.) and ϵ -(β -L-aspartyl)-L-lysine migrated 8 cm. towards the cathode. At pH 9.1 the α -isomer migrated 5.6 cm. towards the anode and the β -isomer 2.5 cm. towards the anode.

Fig. 1. Electrometric titrations in water at 20° with 0.1N-HCl and 0.1N-NaOH. ●, ϵ -*N*-(α -L-Aspartyl)-L-lysine (1.5 mg./ml.); ×, ϵ -*N*-(β -L-Aspartyl)-L-lysine (1.8 mg./ml.).

The two ϵ -aspartyl-lysines were not separated by paper chromatography in butanol-acetic acid-water, both compounds showing an R_f close to that of lysine. In a phenol-water-ammonia system both compounds showed an R_f of 0.56. Attempts to separate the compounds by chromatography in other solvent systems (see Experimental) were also unsuccessful.

ϵ -(α -Aspartyl)-lysine gave a biuret colour, whereas the β -isomer did not. Liwshitz & Zilkha (1954) have previously reported that the biuret reaction can be used to distinguish between α - and β -aspartyl peptides. When ϵ -(α -aspartyl)-lysine was allowed to react with ninhydrin under the conditions used by Van Slyke, Dillon, MacFadyen & Hamilton (1941), 1.49 molecular proportions of carbon dioxide were evolved after 6 min., but this value rose to 1.83 when heating was continued for 25 min. This is consistent with the finding of John & Young (1954) that α -aspartylvaline evolved a significant amount of carbon dioxide after 7 min. and about one molecular proportion after 20 min.

The α - and β -isohexylamides of aspartic acid were synthesized by three methods, two of which were similar to methods used for the preparation of ϵ -aspartyl-lysines. The reaction of benzyloxycarbonyl-L-aspartic anhydride with isohexylamine led to a mixture of α - and β -isohexylamides, the α -isomer predominating. The β -isohexylamide was obtained from α -benzyl *N*-benzyloxycarbonyl-L-aspartate and isohexylamine by the mixed-anhydride method; the presence of some α -isomer in the mother liquors from the crystalline product again indicated that the α -benzyl *N*-benzyloxycarbonyl aspartate used was contaminated with a small amount of the corresponding β -benzyl ester. The third procedure was based on the method of Liwshitz & Zilkha (1955) for synthesizing β -aspartyl peptides. *N*-isohexylmaleamic acid (IV) was prepared

from maleic anhydride and isohexylamine under conditions similar to those used by King, Clark-Lewis, Wade & Swindin (1957) for obtaining maleamic acids. Addition of benzylamine to the double bond of (IV) gave a mixture of the α - and β -isohexylamides of *N*-benzyl-DL-aspartic acid, but the two isomers had widely different solubilities in hot dioxan and were readily separated. Removal of the benzyl group from the two compounds by catalytic hydrogenation gave the corresponding isohexylamides of aspartic acid.

The α - and β -isohexylamides of aspartic acid were readily separated by electrophoresis on paper

(14 v/cm. for 2.5 hr.) at pH 2.3, the former migrating 8.7 and the latter 4.2 cm. towards the cathode. The two compounds could also be distinguished on paper chromatograms run with butanol-acetic acid-water, the R_f of the α -isomer being 0.80 and that of the β -isomer 0.75.

EXPERIMENTAL

L-Aspartic acid and L-lysine were obtained from British Drug Houses Ltd., and isohexylamine from L. Light and Co. Ltd., Colnbrook, Bucks. Analyses were by Weiler and Strauss, Oxford.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1953).

Paper chromatography and electrophoresis of peptides. Paper chromatograms were run on Whatman no. 1 paper. The solvent systems used were butanol-acetic acid-water (Woiwod, 1949), phenol-water-NH₃ (Underwood & Rockland, 1954), triethylamine-acetone-water (Wright & Stadtman, 1956), *tert.*-butanol-methanol-water (Boissonnas, 1950) and butan-2-one-propionic acid-water (Clayton & Strong, 1954).

Electrophoresis on Whatman no. 1 paper was carried out as described by Newton & Abraham (1954). The buffers used were 0.05 M-collidine acetate (0.05 M to acetate, pH 7), pyridine acetate (0.05 M to acetate, pH 5), 10% (v/v) acetic acid (pH 2.3), and 0.02 M-sodium borate, pH 9.1. The potential gradient was 14 v/cm. and electrophoresis was continued for 2.5 hr. Spots were located by spraying with ninhydrin.

2,4-Dinitrophenyl (DNP) derivatives. These were prepared by the method of Sanger & Thompson (1953) under the conditions used by Lockhart & Abraham (1956). DNP-aspartic acid was identified by chromatography on paper in the system of Blackburn & Lowther (1951). α -DNP-lysine and ϵ -DNP-lysine were separated by electrophoresis on paper at pH 9.1 as described by Lockhart & Abraham (1956).

N-Benzylloxycarbonyl-L-aspartic acid. This was prepared by a modification of the method of Bergmann & Zervas (1932), for which we are indebted to Dr W. D. John and Dr G. T. Young. L-Aspartic acid (2.0 g.) was dissolved in a solution of NaOH (1.85 g.) in water (6.1 ml.). The solution was covered with a layer of ether, stirred vigorously and cooled to 0° in ice. Benzyl chloroformate (2.5 ml., containing 1.14 g. of chloroformate/ml.) was added in small portions over 30 min. The mixture was stirred for a further 30 min. at 0° and for 2 hr. at room temperature. The ethereal layer was removed and the aqueous layer washed twice with 10 ml. portions of ether. The solution was filtered and made acid to Congo red with 11 N-HCl, when an oil separated. The oil and supernatant solution were shaken and traces of ether removed *in vacuo* (water pump). The oil then solidified and the solid product was collected and dried. Yield 66%, m.p. 117°.

N-Benzylloxycarbonyl-L-aspartic anhydride. This was prepared by the method of John & Young (1954), but the product was not washed with cold ether before crystallization. Needles, m.p. 113°, were obtained (John & Young give m.p. 111°) in 85% yield (Found: C, 57.6; H, 4.3; N, 5.4. Calc. for C₁₅H₁₁O₅N: C, 57.8; H, 4.4; N, 5.6%).

Dibenzyl N-benzylloxycarbonyl-L-aspartate. The method of

Berger & Katchalski (1951) was used. Distillation under high vacuum (0.05 mm.) was required to remove excess of benzyl alcohol from the reaction mixture. Otherwise the product would not crystallize on trituration with light petroleum (b.p. 40–60°).

β -Benzyl N-benzylloxycarbonyl-L-aspartate. The method of Berger & Katchalski (1951) was followed, but very small yields of the product were obtained and 75% of the starting material was recovered. Somewhat higher, but still small, yields were obtained when the reaction was carried out at 37° instead of at room temperature. The product crystallized from a small volume of benzene, giving needles, m.p. 108–109°.

α -Benzyl N-benzylloxycarbonyl-L-aspartate. The method of Bergmann *et al.* (1933) was used.

ϵ -N-Benzylloxycarbonyl-L-lysine. This was prepared by the method of Neuberger & Sanger (1943). The lysine copper complex was isolated by precipitation with acetone as a pale-blue amorphous powder. It was kept as such for use in future experiments.

ϵ -N-Benzylloxycarbonyl- α -tosyl-L-lysine. ϵ -Benzylloxycarbonyl-L-lysine (2.0 g.) was dissolved in 2 N-NaOH (46 ml.) and shaken vigorously for 4 hr. with finely powdered toluene-*p*-sulphonyl (tosyl) chloride (8 g.). The solution was filtered, the residual solid well washed with 2 N-NaOH, and the combined filtrate and washings were acidified with 2 N-H₂SO₄. The liberated oil was extracted from the mixture with ether (3 $\times$ 1 vol.), the ethereal extracts were dried over anhydrous Na₂SO₄, and the ether was removed *in vacuo*. The residue (2.5 g.) was crystallized from benzene, giving fine needles. ϵ -N-Benzylloxycarbonyl- α -tosyl-L-lysine, which appeared to contain benzene of crystallization, had m.p. 126° (Found: C, 62.8; H, 6.2; N, 5.1; S, 5.7. C₂₁H₂₆O₆N₂S requires C, 58.1; H, 6.0; N, 6.4; S, 7.4. C₂₁H₂₆O₆N₂S $\cdot$ C₆H₆ requires C, 63.3; H, 6.3; N, 5.4; S, 6.3%).

α -N-Tosyl-L-lysine. ϵ -N-Benzylloxycarbonyl- α -tosyl-L-lysine (14.2 g.) was dissolved in 80% (v/v) methanol (200 ml.) to which acetic acid (1.0 ml.) was added. Pd-charcoal (1.0 g., 10% of Pd, L. Light and Co. Ltd.) was added and the mixture hydrogenated until no more CO₂ was evolved. The product, which had precipitated from solution, was extracted from the charcoal with boiling water and the solution filtered hot. The mother liquors and washings were evaporated to dryness in the rotary evaporator (Craig, Gregory & Hausmann, 1950) at 30° and the product (7.5 g., 77%) obtained as a mass of minute hair-like crystals. It was insoluble in hot ethanol or benzene, but was appreciably soluble in cold water. α -Tosyl-L-lysine did not melt at temperatures up to 220°, behaved as a neutral substance on paper electrophoresis at pH 7.0, and ran with R_f 0.81 on chromatography in butanol-acetic acid-water.

α -Tosyl-L-lysine ethyl ester hydrochloride. α -Tosyl-L-lysine (480 mg.) was refluxed for 3 hr. with anhydrous ethanol (6.5 ml.) saturated with dry HCl. The solvent and HCl were removed *in vacuo*, leaving a colourless oil which slowly crystallized at room temperature. The product (445 mg., yield 76%) was recrystallized from ethanol-ether as fine needles. α -Tosyl-L-lysine ethyl ester hydrochloride had m.p. 180.5° (Found: C, 48.9; H, 7.0; N, 7.4; S, 8.5; Cl, 9.3. C₁₅H₂₀O₄N₂SCl requires C, 49.4; H, 6.9; N, 7.7; S, 8.9; Cl, 9.7%).

α -Tosyl-L-lysine benzyl ester hydrochloride. Freshly distilled benzyl alcohol (b.p. 86°/7 mm. Hg) was saturated

with anhydrous HCl, and the solution (6.0 ml.) added to α -tosyl-L-lysine (0.4 g.). The mixture was heated on a boiling-water bath under a reflux condenser closed by a tube of anhydrous CaCl_2 . After 3 hr. the mixture was distilled under vacuum to remove excess of benzyl alcohol. The residual oil slowly crystallized after standing for 2 hr. at room temperature. The solid was recrystallized from ethanol-ether, giving small colourless needles, m.p. 164–165° (0.45 g., yield 79%). This material was dissolved in a small volume of water and conc. HCl added. The ester hydrochloride was precipitated as fine colourless needles and was collected, washed with 2N-HCl, then with acetone, and finally dried in a vacuum desiccator in the presence of NaOH. α -Tosyl-L-lysine benzyl ester hydrochloride had m.p. 170° (Found: C, 55.9; H, 6.5; N, 6.3; S, 7.5; Cl, 8.6. $\text{C}_{20}\text{H}_{27}\text{O}_5\text{N}_2\text{S}\text{Cl}$ requires C, 56.3; H, 6.3; N, 6.5; S, 7.5; Cl, 8.3%).

An attempt to prepare this substance by the method of Erlanger & Hall (1954), with polyphosphoric acid as condensing agent, gave a very poor yield.

ϵ -L-Aspartyl-L-lysine. α -Tosyl-L-lysine benzyl ester hydrochloride (0.1 g.) was dissolved in water (2.5 ml.) together with KHCO_3 (0.2 g.), and the solution covered with a layer of ethyl acetate (4.0 ml.). A solution of benzyloxycarbonyl-L-aspartic anhydride (0.12 g.) in ethyl acetate (2 ml.) was added and the mixture stirred vigorously for 5.5 hr. The aqueous layer was separated, washed with ethyl acetate and acidified with 2N-HCl. The liberated oil was extracted with three portions of ethyl acetate (each 1 ml.), the solvent evaporated and the residue dried in a vacuum desiccator. This product (0.14 g.) was dissolved in 65% (v/v) ethanol (9.0 ml.) to which acetic acid (0.2 ml.) was added. The solution was hydrogenated in the presence of Pd-charcoal (50 mg.) until no more CO_2 was evolved. The charcoal was filtered off and washed with water and the filtrate and washings were evaporated to dryness in a rotary evaporator. A small sample of the residue was subjected to paper electrophoresis at pH 7 and appeared to contain mainly aspartyl- α -tosyl-lysine, together with some aspartic acid and traces of lysine and α -tosyl-lysine. The main part of the product was dissolved in liquid NH_3 (about 5 ml.) and Na added until a permanent blue colour resulted. The colour was discharged with NH_4Cl and the NH_3 allowed to evaporate. The residue was dissolved in water, the pH adjusted to 6.0 with N-HCl, and the solution extracted with ether. This removed toluene-*p*-sulphonamide. The remaining aqueous phase was submitted to ionophoresis in a four-compartment cell (Synge, 1951) until the current fell to 25 mA at 500 v. The solution from the specimen compartment was then evaporated to dryness in a rotary evaporator. Paper electrophoresis showed that the amorphous residue (13.7 mg.) was mainly neutral at pH 7, but contained a trace of lysine.

The product was shown to consist of ϵ -aspartyl-lysine by hydrolysis with 6N-HCl at 105° for 24 hr., after which only aspartic acid and lysine (in approximately equal amounts) were revealed by electrophoresis on paper, and by the preparation of its DNP derivative, which gave DNP-aspartic acid and α -DNP-lysine on hydrolysis. However, electrophoresis on paper in 10% (v/v) acetic acid showed that the product was a mixture of ϵ -(α -aspartyl)-lysine and ϵ -(β -aspartyl)-lysine.

ϵ -(α -L-Aspartyl)-L-lysine. (a) Prepared from lysine copper complex (0.67 g., 1.0 mol. prop.) and benzyloxy-

carbonylaspartic anhydride (1.12 g., 1.5 mol. prop.). The copper complex was dissolved in a mixture of water (7 ml.) and dioxan (8 ml.) and the solution cooled to 0° in ice. The anhydride was dissolved in dioxan (4 ml.) and added in portions, with stirring, over 0.5 hr., together with N-NaOH (3.0 ml.). The homogeneous mixture was kept at 0° for a further 1.5 hr. The solution was adjusted to pH 7 with N-NaOH and allowed to stand overnight. After acidification with acetic acid, the solution was extracted 6 times with 30 ml. of ether to remove dioxan, and the aqueous phase was then saturated with H_2S . The precipitate of CuS was centrifuged off and the solution boiled and aspirated until no more H_2S could be detected. The clear solution was hydrogenated in the presence of Pd-charcoal until no more CO_2 was evolved, filtered and submitted to ionophoresis in the 4-compartment cell. When the current had fallen to a minimum (25 mA at 500 v) the contents of the specimen compartment were evaporated to dryness, and the residue (0.39 g.) crystallized from water (1.0 ml.). Fine white needles (98 mg.) were obtained, which appeared to be pure ϵ -(α -aspartyl)-lysine. No β -isomer was detected by electrophoresis in 10% (v/v) acetic acid. ϵ -(α -L-Aspartyl)-L-lysine did not melt below 300° and, after drying *in vacuo* at 100°, rapidly took up water from the atmosphere to the extent of 2 H_2O /molecule: for the dihydrate $[\alpha]_{\text{D}}^{20} + 13.2^\circ$ in 0.5N-HCl (c, 1.36) (Found: C, 40.5; H, 7.6; N, 14.2. $\text{C}_{10}\text{H}_{18}\text{O}_5\text{N}_2 \cdot 2\text{H}_2\text{O}$ requires C, 40.4; H, 7.7; N, 14.1%).

(b) Prepared by the mixed-anhydride method. β -Benzyl N-benzyloxycarbonyl-L-aspartate (0.21 g.) was dissolved in dry CHCl_3 (10 ml.) and one equivalent (0.084 ml.) of triethylamine added. After being shaken for a few minutes the solution was cooled to 0° and one equivalent of freshly distilled ethyl chloroformate (0.057 ml.) was added. The solution was kept at 0° for 45 min. to allow the formation of the mixed anhydride to go to completion.

α -Tosyl-L-lysine benzyl ester hydrochloride (0.25 g.) was converted into the free ester by the method of Hillmann (1946). The CHCl_3 solution (0.75 ml.) of the ester was added to that of the mixed anhydride, and the mixture shaken well and left to stand overnight at room temperature. The solution was washed once with water, twice with NaHCO_3 solution, and finally twice with water. The CHCl_3 layer was dried with anhydrous MgSO_4 , filtered and diluted with a large volume of light petroleum (b.p. 60–80°). The precipitate of fully protected peptide (0.25 g.) had m.p. 120–122°.

The benzyl and benzyloxycarbonyl groups were removed from this material by catalytic hydrogenation (Pd-charcoal) in 75% (v/v) methanol containing 1.5% (v/v) acetic acid and the tosyl group was then removed with Na in liquid NH_3 in the usual manner. The product was subjected to ionophoresis in the 4-compartment cell until the current fell to 25 mA at 500 v. Evaporation of the solution from the specimen compartment yielded 54 mg. of ϵ -(α -aspartyl)-lysine. This showed only a single spot when subjected to paper electrophoresis in 10% (v/v) acetic acid, or in borate buffer (pH 9.1). It was indistinguishable, under these conditions, from the product obtained by method (a).

ϵ -(β -L-Aspartyl)-L-lysine. This was prepared in a similar way to the α -isomer by the mixed-anhydride method, α -benzyl N-benzyloxycarbonyl-L-aspartate being used. The product was shown by paper electrophoresis in 10% (v/v) acetic acid to be mainly the β -isomer but to be contaminated with a trace of α -isomer.

β -isoHexylamide of L-aspartic acid. This was prepared by the mixed-anhydride method as above from α -benzyl *N*-benzyloxycarbonyl-L-aspartate and isoheptylamine. Catalytic hydrogenation (Pd-charcoal) of the protected amide in 80% (v/v) ethanol gave the free amide (71 mg., yield 47%). This product was crystallized from water and gave fine rosettes of needles. On paper electrophoresis in 10% (v/v) acetic acid the crystals behaved as though they were pure β -amide (single brown spot with ninhydrin). A second crop of crystals, obtained on partial evaporation of the mother liquors, was also pure β -isomer, but third and fourth crops contained about 20 and 40% respectively of the α -amide (purple spot with ninhydrin).

The β -isoheptylamide of L-aspartic acid had m.p. 208° (decomp.) (Found: C, 55.3; H, 9.1; N, 13.2. $C_{10}H_{20}O_3N_2$ requires C, 55.5; H, 9.3; N, 13.0%).

***N*-isoHexylmaleamide.** A solution of maleic anhydride (1.0 g.) and isoheptylamine (1.0 g., 1.32 ml.) in acetic acid (20 ml.) was allowed to stand at room temperature overnight. The acetic acid was removed by distillation *in vacuo* and the brownish yellow oil partly solidified on cooling. It was dissolved in methanol and precipitated as an oil by the addition of a large volume of water. The oil solidified on cooling in ice and the solid was collected and dried (yield 1.46 g., 72.5%), m.p. 83–84°. The solid was crystallized with difficulty from ether-light petroleum (b.p. 60–80°). Four recrystallizations, in which only the first crop of colourless needles from each process were taken, gave a final product of constant m.p. *N*-isoHexylmaleamide had m.p. 110° (Found: C, 60.6; H, 8.6; N, 7.0. $C_{10}H_{17}O_3N$ requires C, 60.3; H, 8.6; N, 7.0%).

The method of Liwshitz & Zilkha (1955) for the preparation of maleamic acids, in which ether was used as a solvent, gave a product which was slightly easier to purify, but only in 32% yield.

α - and β -isoHexylamides of *N*-benzyl-DL-aspartic acid. *N*-isoHexylmaleamide (1.05 g.) was dissolved in redistilled dry dioxan (10 ml.) and freshly distilled benzylamine (0.8 g.) was added. The solution was refluxed on an oil bath at 120°. After about 25 min. a white suspension formed. The mixture was refluxed for a further 30 min., cooled, and the solid collected. It was washed with dioxan and dried, yielding 0.57 g., m.p. 198° (decomp.). The slightly turbid filtrate was refluxed for a further hour, but no more precipitate formed. The dioxan was distilled off, leaving an oily residue. Acetone (10 ml.) was added and, on shaking, the oil solidified. The white amorphous powder was collected, washed with acetone and dried, yielding 0.63 g., m.p. 150–152°.

Both products were crystallized from 30% (v/v) methanol. The β -isoheptylamide of *N*-benzyl-DL-aspartic acid, which was insoluble in boiling dioxan, gave rosettes of needles, m.p. 199° (decomp.) (Found: C, 66.8; H, 8.3; N, 8.8. $C_{17}H_{26}O_3N_2$ requires C, 66.7; H, 8.5; N, 9.1%). The α -isoheptylamide of *N*-benzyl-DL-aspartic acid, which was readily soluble in boiling dioxan, crystallized in small leaflets, m.p. 157.5° (Found: C, 67.0; H, 8.4; N, 8.8. $C_{17}H_{26}O_3N_2$ requires C, 66.7; H, 8.5; N, 9.1%).

The oily residues from the preparation of *N*-isoheptyl-maleamide gave a good yield of the β -isoheptylamide of *N*-benzyl-DL-aspartic acid when treated with benzylamine.

α - and β -isoHexylamides of DL-aspartic acid. The benzyl group was removed from the α - and β -isoheptylamides of *N*-benzyl-DL-aspartic acid by catalytic hydrogenation at

75° in 66% (v/v) ethanol in the presence of Pd-charcoal (10% Pd) (cf. Velluz, Amiard & Heymès, 1955). The solution was filtered hot, the Pd-charcoal washed once with hot 66% (v/v) ethanol and the filtrate and washings evaporated to dryness in a rotary evaporator. The residue was crystallized from slightly aqueous ethanol. The identity of the products was confirmed by examination on paper electrophoresis in 10% (v/v) acetic acid and by paper chromatography in butanol-acetic acid-water. The β -isoheptylamide of *N*-benzyllaspartic acid gave the β -isoheptylamide of DL-aspartic acid, m.p. 208° (decomp.) (brown spot with ninhydrin). The α -isoheptylamide of *N*-benzyllaspartic acid gave the α -isoheptylamide of DL-aspartic acid, m.p. 197° (purple spot with ninhydrin) (Found: C, 55.4; H, 9.0; N, 11.7; 14.8. $C_{10}H_{20}O_3N_2$ requires C, 55.5; H, 9.3; N, 13.0%).

SUMMARY

1. ϵ -*N*-(α -L-Aspartyl)-L-lysine has been obtained by interaction of *N*-benzyloxycarbonyl-L-aspartic anhydride with the copper complex of L-lysine.

2. ϵ -*N*-(α -Aspartyl)-L-lysine and the corresponding β -aspartyl-lysine have been synthesized by the mixed-anhydride method from α -toluene-*p*-sulphonyl-L-lysine benzyl ester and the β - and α -benzyl esters respectively of *N*-benzyloxycarbonyl-L-aspartic acid.

3. The β -isoheptylamide of L-aspartic acid has been synthesized by the mixed-anhydride method from α -benzyl *N*-benzyloxycarbonyl-L-aspartate and isoheptylamine. The α - and β -isoheptylamides of DL-aspartic acid have been synthesized from *N*-isoheptylmaleamic acid.

4. The two ϵ -aspartyl-lysines are readily separated by electrophoresis on paper at pH 2.3 or 9.1, but not by paper chromatography in a number of solvent systems. The two isoheptylamides of aspartic acid can be separated in paper chromatograms run in butanol-acetic acid-water.

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Formation of ϵ -(Aminosuccinyl)-lysine from ϵ -Aspartyl-lysine from Bacitracin A, and from the Cell Walls of Lactobacilli

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Work in two Laboratories has led to the conclusion that the polypeptide antibiotic bacitracin A contains the sequence $L\text{-Asp} \xrightarrow{\epsilon} L\text{-Lys} \xleftarrow{\alpha} L\text{-Ileu}$, in which $\rightarrow$ denotes a C-N bond (Lockhart & Abraham, 1954*a, b*, 1956; Craig, Hausmann & Weisiger, 1954; Theodoropoulos & Craig, 1956). Part of the evidence for this sequence is based on the analysis of two products of hydrolysis of bacitracin A which are strikingly resistant to acid. One of these products appeared to consist only of lysine and aspartic acid residues and the other of lysine, aspartic acid and isoleucine residues (Lockhart & Abraham, 1956). Nevertheless, both products were more basic than was to be expected if they were peptides containing two amino groups and two carboxyl groups. Further information about their structures was therefore desirable.

Experiments recorded in this paper provide an explanation of the anomalous behaviour of the products containing lysine and aspartic acid which are formed from bacitracin A. Synthetic ϵ -(α -L-aspartyl)-L-lysine and ϵ -(β -L-aspartyl)-L-lysine (Swallow, Lockhart & Abraham, 1958) are both different from the 'Asp.Lys' found in hydrolysates of bacitracin A. However, both synthetic peptides yield a derivative of aminosuccinimide, ϵ -(aminosuccinyl)-lysine (I), which appears to be identical with the product from the antibiotic, on treatment with concentrated hydrochloric acid at 80°. A similar type of cyclization has been found to occur with the α - and β -isohexylamides of aspartic acid (Swallow *et al.* 1958) and (less readily) with α -(α -L-aspartyl)-L-lysine and α -(β -L-aspartyl)-L-lysine which have been synthesized by Pimlott & Young (unpublished work).

After this work had begun Cummins & Harris (1956) reported that hydrolysis of the cell walls of certain lactobacilli yielded a substance which was composed largely of lysine and aspartic acid but which resisted treatment with 6*N*-hydrochloric acid at 100°. The properties of this substance raised the question whether it was related to the cyclic compound from bacitracin A and from the ϵ -aspartyl-lysines (Swallow & Abraham, 1957). A direct comparison of the two products has now indicated that they are identical or differ only in optical configuration.

Brief accounts of some of the work described here have already been given (Swallow & Abraham, 1957; Abraham, 1957).

METHODS

Materials

ϵ -Aspartyl-lysines. The α - and β -isomers were prepared as described by Swallow *et al.* (1958).

α -Aspartyl-lysines. The two isomers were kindly provided by Miss P. Pimlott and Dr G. T. Young.

N-Benzoyloxycarbonyl-L-aspartic anhydride. This was prepared by the method of John & Young (1954).

α - and β -isohexylamides of aspartic acid. These were prepared by the method of Swallow *et al.* (1958).

Bacitracin. The bacitracin used (lot B 55-13, 68 i.u./mg.) was supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

Partial hydrolysate of bacitracin. A solution of bacitracin in 11*N*-HCl (40 ml./g.) was kept at 80° for 43 hr. in a sealed flask. The hydrolysate was evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950). The residue was dissolved in water and evaporation repeated twice to remove HCl. The pattern obtained when