Synthesis of D- δ -Amino- δ -carboxyvaleryl-glycine (a Degradation Product of Cephalosporin N) and of DL- δ -Amino- δ -carboxyvaleramide

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Newton & Abraham (1954) reported that cephalosporin N was probably (D-4-amino-4-carboxy-*n*-butyl)penicillin. On hydrolysis with *N*-hydrochloric acid at 100° for 6 hr., preparations of cephalosporin N yielded D- α -aminoadipic acid. Less vigorous hydrolysis converted them into a penilloic acid, which gave penicillaminic acid and a neutral aldehyde on treatment with bromine water. The aldehyde was oxidized by silver oxide to an α -aminoadipylmonoglycine. The latter was thought to be δ -amino- δ -carboxyvaleryl-glycine, because the *pK* of its amino group was very close to the *pK* of the α -amino group of α -aminoadipic acid. D- δ -Amino- δ -carboxyvaleryl-glycine (I) has now been synthesized and appears to be identical with the natural product.

The synthesis was effected without difficulty by a procedure similar to that used by Hegedüs (1948) and by LeQuesne & Young (1950) for the synthesis of γ -glutamyl peptides. Although Sachs & Brand (1954) have reported that this procedure yields a mixture of α - and γ -glutamyl peptides, no α -peptide of α -aminoadipic acid appeared to be present in the recrystallized product.

Treatment of D- α -aminoadipic acid (Greenstein, Birnbaum & Otey, 1953) with ethanol containing hydrochloric acid at room temperature yielded the hydrochloride of ethyl D- δ -amino- δ -carboxyvalerate (δ -ethyl D- α -aminoadipate), which was converted into the free ester (m.p. 201–202°) by neutralization with ammonia in methanol. The ester reacted with benzyloxycarbonyl chloride to form ethyl D- δ -benzyloxycarbonylamino- δ -carboxyvalerate. The latter reacted with hydrazine hydrate in ethanol to yield crystalline D- δ -benzyloxycarbonylamino- δ -carboxyvalerylhydrazide (II) (m.p. 132–133°).

The azide formed from (II) was coupled with glycine ethyl ester in chloroform solution. The resulting D- δ -benzyloxycarbonylamino- δ -carboxyvaleryl-glycine ester was hydrolysed by *N* sodium hydroxide to the corresponding acid, and the latter was converted into D- δ -amino- δ -carboxyvaleryl-glycine (m.p. 218–219°) by hydrogenation in the presence of palladium black.

D- δ -Amino- δ -carboxyvaleryl-glycine yielded one molecular proportion of carbon dioxide in the ninhydrin-carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for the determination of α -amino acids. Electrometric titration at 20° showed that it contained a basic group with a *pK* of 9.8 and two acidic groups with *pK* values of <3.0 and 3.8 respectively. Similar values had been found for the natural product (Newton & Abraham, 1954). On paper chromatograms run with butanol-acetic acid (Woiwod, 1949) the synthetic and natural products showed the same *R_F* value, which was close to that of glycine. When subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954) they migrated towards the anode at the same rate. Dr Dorothy Hodgkin and Mrs M. Mackay reported that 'X-ray powder photographs of the two products were identical, apart from one or two small details of line structure which might be due to traces of impurity in the natural preparation'.

An attempt to prepare DL- δ -amino- δ -carboxyvaleramide by the action of aqueous ammonia on ethyl DL- δ -amino- δ -carboxyvalerate led to the formation of material that gave no colour with ninhydrin and consisted mainly of 6-oxopiperidine-2-carboxylic acid. The desired compound was obtained by conversion of ethyl DL- δ -benzyloxycarbonylamino- δ -carboxyvalerate into the corresponding amide and removal of the benzyloxycarbonyl group. Electrometric titration showed that it contained an acidic group with a *pK* of 2.6 and a basic group with a *pK* of 9.7. These values indicated that the δ -carboxyl group of α -aminoadipic acid was involved in amide formation.

D- δ -Amino- δ -carboxyvaleryl-glycine, like γ -glutamylpeptides, is readily hydrolysed by acid. It appeared to be largely split into α -aminoadipic acid and glycine by treatment with *N* hydrochloric acid at 105° for 6 hr. When heated in water alone it

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yielded glycine but very little α -amino adipic acid. Presumably autohydrolysis had occurred in a manner similar to that observed by LeQuenesne & Young (1952) with γ -glutamylpeptides, leading in the present case to the formation of glycine and 6-oxopiperidine-2-carboxylic acid. DL- δ -Amino- δ -carboxyvaleramide also appeared to yield 6-oxopiperidine-2-carboxylic acid on heating in aqueous solution. Under similar conditions glutamine has been reported to yield mainly pyrrolidonecarboxylic acid (Chibnall & Westall, 1932; Melville, 1935).

EXPERIMENTAL

Ethyl D- δ -amino- δ -carboxyvalerate. D- α -Amino adipic acid (360 mg.) (kindly supplied by Dr J. P. Greenstein) was dissolved in 1.33N ethanolic HCl (5 ml.). After being kept at 20° for 30 min., the solution was evaporated to dryness *in vacuo*. The residual ester hydrochloride was washed with ether, dissolved in methanol (2.5 ml.) and the solution neutralized with 35% (w/v) NH_3 . The ester formed a crystalline precipitate (316 mg.) which was filtered and washed with methanol (1 ml.). It had m.p. 201–202°, unchanged by recrystallization from aqueous ethanol. (Found: C, 50.7; H, 8.0. $\text{C}_8\text{H}_{15}\text{O}_4\text{N}$ requires C, 50.8; H, 7.9%.)

Ethyl D- δ -amino- δ -carboxyvalerate behaved as a neutral substance when subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). On paper chromatograms run with butanol-acetic acid (Woiwod, 1949) it showed an R_F value of 0.58.

Ethyl DL- δ -amino- δ -carboxyvalerate. DL- α -Amino adipic acid (348 mg.) (kindly supplied by Dr R. Gaudry) was esterified under the conditions described in the preceding experiment. The product (290 mg.) had m.p. 190°, raised by recrystallization from aqueous ethanol to 194–195°. (Found: C, 50.8; H, 7.7. $\text{C}_8\text{H}_{15}\text{O}_4\text{N}$ requires C, 50.8; H, 7.9%.)

Ethyl D- δ -benzyloxycarbonylamino- δ -carboxyvalerate. Ethyl D- δ -amino- δ -carboxyvalerate (290 mg.) was dissolved in water (2 ml.) containing NaHCO_3 (150 mg.). The solution was stirred at 0° while benzyloxycarbonyl chloride (280 mg.) and N-NaOH (1.6 ml.) were added slowly during 20 min. The mixture was stirred for a further 30 min. at room temperature and then extracted with 1 vol. of ether. The aqueous solution was brought to pH 2 with 5N-HCl and extracted twice with 1 vol. of ether. The ether extract was dried over Na_2SO_4 . On evaporating the ether, the benzyloxycarbonyl derivative remained as a syrup. It was precipitated from ether solution by light petroleum as an oil (350 mg.).

Ethyl DL- δ -benzyloxycarbonylamino- δ -carboxyvalerate. Ethyl DL- δ -amino- δ -carboxyvalerate (190 mg.) was allowed to react with benzyloxycarbonyl chloride under the conditions described in the preceding experiment. The product (271 mg.) was obtained as a crystalline mass. It had m.p. 89–90°, unchanged by recrystallization from a mixture of ether and light petroleum. (Found: C, 59.5; H, 6.4. $\text{C}_{16}\text{H}_{21}\text{O}_6\text{N}$ requires C, 59.5; H, 6.5%.)

D- δ -Benzyloxycarbonylamino- δ -carboxyvalerylhydrazide. Ethyl D- δ -benzyloxycarbonylamino- δ -carboxyvalerate (350 mg.) was dissolved in ethanol (0.9 ml.) and the solution added drop by drop to 0.38 ml. of 100% (w/w) hydrazine hydrate. The mixture was left at room temperature for

48 hr. and most of the ethanol was removed in a stream of air at 30°. The remaining solution was adjusted to pH 2 with 2N-HCl and a saturated aqueous solution of sodium acetate was then added until the pH rose to 4.7. The product began to crystallize. After the mixture had been kept for 4 hr. at 0°, the hydrazide was filtered, washed with water, and dried (190 mg.). It had m.p. 128–130°, raised by recrystallization from hot water to 132–133°. (Found: C, 54.0; H, 6.2; N, 13.7. $\text{C}_{14}\text{H}_{19}\text{O}_5\text{N}_3$ requires C, 54.3; H, 6.2; N, 13.6%.)

D- δ -Benzyloxycarbonylamino- δ -carboxyvalerylglycine. The hydrazide from the preceding experiment (168 mg.) was dissolved in water (4 ml.) containing 11N-HCl (0.193 ml.). CHCl_3 (3 ml.) was added and the mixture stirred at 0°. A solution of NaNO_2 (60 mg.) in water (0.5 ml.) was added dropwise during 5 min. and stirring continued for a further 10 min. The CHCl_3 solution of the azide was separated, washed twice with ice-cold water and dried for a few seconds over Na_2SO_4 . It was then added to a solution of glycine ethyl ester (prepared from 660 mg. of glycine ethyl ester hydrochloride by the method of Hillmann, 1946) in CHCl_3 at 0°. The mixture was kept for 30 min. at 0° and then for 18 hr. at room temperature. It was then washed successively with 2N-HCl and with water and dried over Na_2SO_4 . Evaporation of the CHCl_3 *in vacuo* left the ethyl ester of D- δ -benzyloxycarbonylamino- δ -carboxyvalerylglycine as a syrup (200 mg.). It was converted into the corresponding acid by dissolving in N-NaOH (1.7 ml.) and keeping the solution at room temperature for 2 hr. The solution was acidified with 5N-HCl and extracted 4 times with ethyl acetate (3.5 ml.). The ethyl acetate was dried over Na_2SO_4 and evaporated *in vacuo*. The product remained as an oil (127 mg.). It was precipitated as an oil from ethyl acetate by light petroleum.

D- δ -Amino- δ -carboxyvalerylglycine. The product from the previous experiment (126 mg.) was dissolved in 50% (v/v) methanol (2.5 ml.) containing glacial acetic acid (0.06 ml.). Palladium black (30 mg.) was added and H_2 passed through the mixture. The evolution of CO_2 ceased in 2 hr. The solution was separated from the catalyst and evaporated *in vacuo*, leaving a crystalline residue (76 mg.). After recrystallization from aqueous ethanol, D- δ -amino- δ -carboxyvalerylglycine (60 mg.) had m.p. 218–219°; $[\alpha]_D^{20} - 6.0^\circ$ in water (c , 3.4). (Found: C, 44.0; H, 6.3; N, 13.1. $\text{C}_8\text{H}_{14}\text{O}_5\text{N}_2$ requires C, 44.0; H, 6.4; N, 12.9%.) On mixing with the corresponding compound (m.p. 213–214°) from cephalosporin N, the m.p. was 214–215°.

DL- δ -Benzyloxycarbonylamino- δ -carboxyvaleramide. Ethyl DL- δ -benzyloxycarbonylamino- δ -carboxyvalerate (200 mg.) was dissolved in 35% (w/w) NH_3 (2.5 ml.) and the solution kept at 18° for 48 hr. The solution was then evaporated to dryness *in vacuo*, the residue dissolved in 0.5 ml. of water, and glacial acetic acid gradually added. A crystalline precipitate began to form when the pH of the solution had fallen to 3.7. After 1 hr. the crystals were filtered, yielding 88 mg. of product which had m.p. 168–170°. A second crop (62 mg.), which had m.p. 160–170°, was obtained by acidifying the filtrate to pH 3.0. The two crops were combined and recrystallized from 50% (w/w) ethanol, yielding 135 mg. of fine needles which had m.p. 172–173°. (Found: C, 57.0; H, 6.1; N, 9.5. $\text{C}_{14}\text{H}_{18}\text{O}_5\text{N}_2$ requires C, 57.1; H, 6.1; N, 9.5%.)

DL- δ -Amino- δ -carboxyvaleramide. DL- δ -Benzyloxycarbonylamino- δ -carboxyvaleramide (113 mg.) was dissolved in 50% (v/v) methanol (4.5 ml.) containing glacial acetic acid (0.06 ml.) and hydrogenated in the presence of

palladium black (30 mg.). The product (56 mg.) was recrystallized from aqueous ethanol. It had m.p. 195–196°. (Found: C, 44.6; H, 7.4; N, 17.9. $C_6H_{12}O_5N_2$ requires C, 45.0; H, 7.5; N, 17.5%.)

This product behaved as a neutral compound when subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). It appeared, however, to contain a trace of α -aminoadipic acid. On paper chromatograms run with butanol-acetic acid (Woiwod, 1949) it occupied the same position as aspartic acid ($R_F=0.18$) and on chromatograms run with phenol-HCN it showed an R_F value of 0.67. In both cases the R_F values were slightly greater than those for glutamine.

Autohydrolysis. D- δ -Amino- δ -carboxyvaleryl-glycine (1 mg.) was dissolved in water (1 ml.) and the solution heated in a sealed tube at 105° for 24 hr. Samples of the product (20 μ g.) were subjected to ionophoresis on paper in a collidine acetate buffer, pH 7.0 (Newton & Abraham, 1954), and to chromatography on paper in butanol-acetic acid (Woiwod, 1949). When developed with ninhydrin, both papers showed a strong spot in the position occupied by glycine, but only a very weak spot in the position occupied by α -aminoadipic acid. No spot corresponding to the original peptide was visible. When the peptide was hydrolysed in 6N-HCl at 105° for 20 hr. the product showed two strong spots corresponding to glycine and α -aminoadipic acid in approximately equimolecular proportions. The spots were only slightly less intense when the same amount of peptide was hydrolysed in N-HCl at 105° for 6 hr.

DL- δ -Amino- δ -carboxyvaleramide (1 mg.) was dissolved in water (0.25 ml.), and the solution heated at 105° in a sealed tube for 18 hr. A sample (20 μ g.) was subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). Development with ninhydrin showed no spot corresponding to the neutral amide and only a trace of material in the position occupied by α -aminoadipic acid. When the amide was heated in N-HCl at 105° for 6 hr. the product showed a single strong spot in the position occupied by α -aminoadipic acid.

SUMMARY

1. D- δ -Amino- δ -carboxyvaleryl-glycine has been synthesized by a process in which D- α -aminoadipic

acid was converted, through its δ -ester, into the azide of D- δ -benzyloxycarbonylamino- δ -carboxyvaleric acid and the latter coupled with glycine ethyl ester. The synthetic substance appears to be identical with a product obtained by degradation of cephalosporin N.

2. DL- δ -Amino- δ -carboxyvaleramide has been synthesized by conversion of the ethyl ester of DL- δ -benzyloxycarbonylamino- δ -carboxyvaleric acid into the corresponding amide and removal of the benzyloxycarbonyl group.

3. D- δ -Amino- δ -carboxyvaleryl-glycine and DL- δ -amino- δ -carboxyvaleramide are readily hydrolysed by hot dilute hydrochloric acid with the liberation of α -aminoadipic acid. When heated in water alone they are hydrolysed without the formation of α -aminoadipic acid. In the latter case 6-oxopiperidine-2-carboxylic acid is presumably one of the main products of hydrolysis.

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The Formation of Urocanic Acid and Glutamic Acid in the Fermentation of Histidine by *Clostridium tetanomorphum*

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Woods & Clifton (1937) showed that washed-cell suspensions of *Clostridium tetanomorphum* ferment various single amino acids, and that L-glutamic acid and L-histidine each yield hydrogen, carbon dioxide and ammonia, together with steam-volatile fatty acids. In the case of glutamic acid, these last consisted of butyric and acetic acids in the molar ratio of 1:3; the fatty acids arising from histidine

were not identified. The production of more than 2 moles NH_3 /mole histidine decomposed indicated that disruption of the glyoxaline ring of histidine had occurred at some stage in the fermentation. Some years previously, Edlbacher & Kraus (1930) had obtained evidence that glutamic acid was an intermediate in the catabolism of histidine by mammalian liver, and these observations, taken in