Attempts to isolate pure glutamine benzyl ester hydrochloride from this reaction mixture have been unsuccessful. The most serious side reaction appears to be alcoholysis on the *gamma* carbon. Transesterification and hydrolysis at the *alpha* carbon seem to be of secondary importance.

Comparable results were obtained with *N*-formylasparagine benzyl ester where again at least six spots were detected chromatographically. Numerous attempts to make the deformylation reactions more selective by changes in the solvents, acid concentrations, reaction temperature, and time were unsuccessfuls. Other solvent systems that were tried were water, *t*-butyl alcohol, aqueous tetrahydrofuran, aqueous acetic acid, aqueous acetonitrile, and aqueous trifluoroethanol. In these solvents the reaction rate was drastically reduced without apparent increase in the selectivity.

Carbobenzoxy-L-glutamine benzyl ester. To 500 mg. of carbobenzoxy-L-glutamine in 2 ml. of dimethylformamide a solution of crude diazotoluene in petroleum ether was added until a reddish brown color persisted for 30 min. The excess diazotoluene was decomposed with acetic acid and the petroleum ether removed under reduced pressure. The resulting solution was treated with 45 ml. of ether and 40 ml. of water and the mixture stored overnight at 0°. Filtration, washing of the precipitate with ether, and two recrystallizations from aqueous ethanol yielded 320 mg. of ester, 49%, m.p. 130–131°, reported[10] m.p. 123°.

Anal. Calcd. for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.57. Found: C, 64.69; H, 6.07; N, 7.44.

Carbobenzoxy-L-asparagine benzyl ester. This compound was prepared from 650 mg. of carbobenzoxy-L-asparagine by the same procedure as that used for the glutamine derivative. A gel was obtained which crystallized slowly at 50° from ethyl acetate; yield 0.391 g., 45%, m.p. 128.5°, reported[10] m.p. 132°.

Anal. Calcd. for $C_{19}H_{20}N_2O_5$; C, 64.03; H, 5.66; N, 7.86. Found C, 64.03; H, 5.81; N, 7.81.

Carbobenzoxy-glycyl-L-glutamine benzyl ester. To 0.74 g., 2 mmoles, of carbobenzoxy-L-glutamine benzyl ester was added 3 ml. of 30% hydrogen bromide in acetic acid. After storage at room temperature for 15 min. the mixture was cooled in a Dry Ice-acetone bath and freeze-dried. The residue was washed with 30 ml. of ether and then dissolved in a minimum of dimethylformamide and reprecipitated with ether. The residue dissolved in ethanol was treated with 0.28 ml. of triethylamine and the mixture filtered. Evaporation of the filtrate yielded an oil and a further quantity of triethylamine hydrobromide. This residue was treated with 2 ml. of dimethylformamide, the mixture filtered, the precipitate washed with an additional 1 ml. of dimethylformamide and the combined solution stored at 0° for coupling with the azide.

An ethereal solution of carbobenzoxy-glycylazide was prepared in the usual manner from 223 mg., 1 mmole, of carbobenzoxy-glycyl hydrazide. The azide was combined with the benzyl ester and the solution stored overnight at 0°, then 4 hr. at room temperature. The addition of water caused precipitation of an oil which crystallized on storage at 0°. The precipitate was collected, washed with dilute hydrochloric acid, sodium bicarbonate and water, dried *in vacuo* over phosphorus pentoxide; yield 0.28 g., 67% based on the quantity of azide, m.p. 134–135°. Recrystallization from aqueous ethanol yielded 0.20 g. of product, m.p. unchanged.

Anal. Calcd. for $C_{22}H_{25}N_3O_6$: C, 61.80; H, 5.89; N, 9.83. Found: C, 61.73; H, 5.87; N, 10.10.

Carbobenzoxy-glycyl-L-asparagine benzyl ester. This compound was prepared by the same procedure as the glutamine analog in 58% yield, m.p. 135–136°.

Anal. Calcd. for $C_{21}H_{23}N_3O_6$: C, 61.00; H, 5.62; N, 10.19. Found: C, 60.87; H, 5.64; N, 10.46.

Test for racemization and chromatographic purity of the peptides. Hydrogenolysis of the carbobenzoxy-benzyl esters in the presence of palladium black at room temperature and atmospheric pressure yielded gycyl-L-glutamine, m.p. 202–203° reported[16] m.p. 199–200°, R_f 0.055, 1-butanol, acetic acid, water (4:1:5); and glycyl-L-asparagine, m.p. 214°, reported[17] m.p. 216°, R_f 0.052 in 1-butanol, acetic acid and water (4:1:5) and R_f 0.32 in water saturated phenol. Each peptide gave only one spot with ninhydrin. To check for possible racemization the peptides were refluxed 2 hr. in 6*N* hydrochloric acid and $[\alpha]^{25}_D$ of the hydrolyzate compared with $[\alpha]^{25}_D$ for L-glutamic acid and L-aspartic acid containing equivalent quantities of glycine and ammonium chloride. The hydrolyzate from glycylglutamine gave $[\alpha]^{25}_D$ +28.9° (*c* 3.02, 6*N* hydrochloric acid); L-glutamic acid, $[\alpha]^{25}_D$ +28.9°. The hydrolyzate from glycyl-L-asparagine gave $[\alpha]^{25}_D$ +22.2° (*c* 1.57, 6*N* hydrochloric acid); L-aspartic acid $[\alpha]^{25}_D$ +22.4°.

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(16) H. Thierfelder and E. von Cramm, *Z. Physiol. Chem.*, **105**, 58 (1919).

(17) E. Fischer and E. Koenigs, *Ber.*, **37**, 4585 (1904).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Synthesis of L-Leucyl-L-alanyl-L-valyl-L-glutamic Acid and Intermediate Peptides[1]

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The syntheses of the tetrapeptide, L-leucyl-L-alanyl-L-valyl-L-glutamic acid, the tripeptide, L-leucyl-L-alanyl-L-valine and the dipeptides, L-leucyl-L-alanine and L-valyl-L-glutamic acid, are described. The *p*-nitrobenzyloxycarbonyl group was employed to protect the amino groups during peptide bond formation by either the azide, acid chloride, or acid anhydride methods.

In connection with studies being performed in this laboratory on the oxidative cleavage of peptide bonds[1b,3] some model compounds were needed. It was desirable to obtain a series of peptides up to a tetrapeptide sequence in which the constituent

(1)(a) This work was supported in part by grant A-608 of the National Institute for Arthritis and Metabolic Diseases of the National Institutes of Health. (b) Abstracted from the thesis submitted by W. H. McGregor in partial fulfillment of the requirements for the Ph.D. degree at the University of California, Berkeley, September, 1959.

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(3) F. H. Carpenter and W. H. McGregor, *Fed. Proc.*, **19**, 344 (1960).

di- and tripeptides as well as the amino acids contained therein could all be readily separated from one another by paper chromatography. Furthermore, we preferred to avoid using amino acids containing side chains that are easily oxidizable. The sequence, leucyl-alanyl-valyl-glutamic acid was selected as one which would very likely fullfil these conditions. This paper describes the synthesis of this tetrapeptide as well as the syntheses of two of the constituent dipeptides and one tripeptide. The syntheses were performed using the *p*-nitrobenzyloxycarbonyl (abbreviated as "P-NBC") group[4] to block the amino function during formation of the peptide bond. The dipeptides were prepared by use of the mixed anhydride, azide, or acid chloride methods.[5] Tripeptide and tetrapeptide derivatives were made exclusively by means of the azide method. The use of these procedures should largely precluded racemization during peptide bond formation.[5] Frequent recourse was made in these syntheses to the use of benzyl esters prepared according to a procedure recently published from this laboratory.[6] The peptide sequences effected, as well as the methods used, are outlined in Fig. 1.

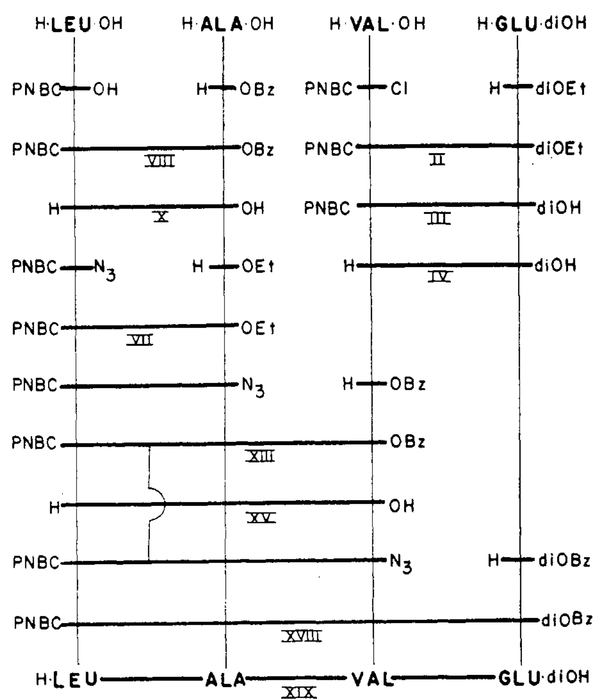

Fig. 1. Diagram of the syntheses of the various peptides; DNBC = *p*-nitrobenzyloxycarbonyl-, Bz = benzyl, and Et = ethyl.

In the course of this work several observations of general interest in peptide synthesis were made. One of these was the superiority of the benzyl over the methyl ester for use in the preparation of the hydrazide of *p*-nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine. The hydrazide of this tripeptide was prepared by treatment of the benzyl ester with hydrazine at 37° for several days. Under similar or even more vigorous conditions, the methyl ester failed to give the product desired.[7] A second observation was that diketopiperazines were formed during the reduction of *p*-nitrobenzyloxycarbonyl protected dipeptide esters. One approach to the synthesis of the tetrapeptide which we explored involved the coupling of two suitably protected dipeptides. When the *p*-nitrobenzyloxycarbonyl group was removed from *p*-nitrobenzyloxycarbonyl-L-valyl-L-glutamic acid diethyl ester by hydrogenation over palladium in methanol-acetic acid, a neutral compound with the properties of the diketopiperazine was the only product isolated. A similar result was obtained when the *p*-nitrobenzyloxycarbonyl group was reduced from *p*-nitrobenzyloxycarbonyl-L-leucyl-L-alanine ethyl ester. Formation of diketopiperazines during the hydrogenation of dipeptide derivatives is not a new observation,[8] but one that has not been generally encountered.[9] Another difficulty experienced in the syntheses was in the saponification of the ester group from *p*-nitrobenzyloxycarbonyl-protected di- and tripeptides. Yields of the free acids of below 70%, in several cases as low as 40%, were encountered after saponification with either one or several equivalents of alkali. It is possible that these low yields were a result of competing reactions involving the formation of substituted hydantoins or ureas.[10] In an effort to circumvent these low yields on saponification, several of the protected peptide esters were subjected to acid hydrolysis in dioxane-water mixtures ($0.04M$ solutions of the esters in 70% dioxane in water which was $0.4N$ in hydrochloric acid were refluxed for one hour). The yields and purity of the products obtained from acid hydrolysis were not appreciably better than those obtained by saponi-

(4) F. H. Carpenter and D. T. Gish, *J. Am. Chem. Soc.*, 74, 3818 (1952).

(5) See the reviews by J. S. Fruton [*Adv. in Protein Chem.*, 5, 1 (1949)] and M. Goodman and G. W. Kenner [*Adv. in Protein Chem.*, 12, 465 (1957)] for a general description of the methods and references to the original literature.

(6) J. E. Shields, W. H. McGregor, and F. H. Carpenter, *J. Org. Chem.*, 26, 141 (1961).

(7) J. A. Maclaren, W. E. Savige, and J. M. Swan [*Australian J. Chem.*, 11, 345 (1958)] have reported on the difficulties encountered in the preparation of hydrazides from methyl and ethyl esters of some peptides. They described an improvement in yield when *n*-butyl alcohol was used as a solvent. With *p*-nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine methyl ester the use of *n*-butyl alcohol as a solvent did not improve the yield of the hydrazide.

(8) E. L. Smith and M. Bergmann [*J. Biol. Chem.*, 153, 627 (1944)] made a similar observation during the hydrogenation of carbobenzoxyglycyl-L-prolinamide.

(9) Neither one of the two review articles cited above (Ref. 5) makes a point of this problem in the synthesis of dipepetide esters.

(10) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, 145, 253 (1942); C. A. Dekker, S. P. Taylor, and J. S. Fruton, *J. Biol. Chem.*, 180, 155 (1949); J. A. Maclaren, *Australian J. Chem.*, 11, 360 (1958).

fication. The difficulties experienced with hydrolysis of the methyl and ethyl esters stimulated the use of benzyl esters which were cleaved by hydrogenolysis.[11]

A rather low yield (45-50%) was obtained upon the synthesis of the protected tetrapeptide (XVIII) from the tripeptide hydrazide (XVI) and glutamic acid dibenzyl ester. During the conversion of the hydrazide (XVI) to the azide, part of the product was found to be insoluble in ethyl acetate and was removed before going on to the coupling step with glutamic acid dibenzyl ester. The removal of this ethyl acetate-insoluble product accounts in good measure for the low yield experienced in preparing the protected tetrapeptide (XVIII). With the exceptions noted above the other steps in the syntheses, including the hydrogenations, proceeded smoothly in good yield. However, in the case of the final tetrapeptide (XIX) recourse was had to purification by countercurrent distribution in order to obtain a crystalline product.

EXPERIMENTAL[12]

p-Nitrobenzyloxycarbonyl-L-valine (I). The procedure used by Gish and Carpenter[13] for DL-valine was applied to the L-isomer. The product was crystallized from *n*-butyl ether in 50% yield, m.p. 72-74°, $[\alpha]_D^{24}$ +5.5° (*c* 3.16, ethanol).

Anal. Calcd. for $C_{13}H_{16}N_2O_6$: C, 52.71; H, 5.44; N, 9.45. Found: C, 52.86; H, 5.58; N, 9.52.

p-Nitrobenzyloxycarbonyl-L-valyl-L-glutamic acid diethyl ester (II). To 3 g. (0.01 mole) of *p*-nitrobenzyloxycarbonyl-L-valine (I) in 75 ml. of cold diethyl ether, 3 g. (0.014 mole) of phosphorus pentachloride was added and allowed to react in an ice salt bath for 1 hr. with occasional swirling. After the mixture had warmed to room temperature, it was filtered through a sintered glass filter to remove any unchanged phosphorus pentachloride and the solvent was removed under reduced pressure. The oily residue was washed several times with petroleum ether that had been dried over phosphorus pentoxide and the oil was dissolved in 50 ml. of diethyl ether. This solution was added to 100 ml. of an ethereal solution of L-glutamic acid diethyl ester, obtained from 5 g. (0.021 mole) of L-glutamic acid diethyl ester hydrochloride[14] by dissolving it in 15 ml. of methanol and 3.3 ml. (0.024 mole) of triethylamine followed by precipitation of the triethylamine hydrochloride by the addition of ether. The reaction was allowed to proceed for 2 hr. at room temperature when the crystals which had formed (6.7 g.) were filtered, triturated with water, and collected. For analysis, the product was dried over phosphorus pentoxide *in vacuo* at room temperature, yield 4.1 g. (84%), m.p. 138-141°, $[\alpha]_D^{24}$ −7.9° (*c* 2.8, dioxane).

Anal. Calcd. for $C_{22}H_{31}N_3O_9$: C, 54.89; H, 6.49; N, 8.73. Found: C, 54.87; H, 5.96; N, 8.78.

p-Nitrobenzyloxycarbonyl-L-valyl-L-glutamic acid (III). Ester II (0.5 g., 1 mmole) was dissolved in 20 ml. of ethanol, and 10 ml. of *N* potassium hydroxide was added. The mixture was allowed to react at room temperature for 30 min. and then acidified with 6*N* hydrochloric acid. The solution was concentrated to a small volume under reduced pressure and placed in the refrigerator overnight giving 294 mg. (67%) of the desired product. For analysis it was recrystallized from aqueous ethanol and dried over phosphorus pentoxide *in vacuo* at room temperature, m.p. 162-163°, $[\alpha]_D^{24}$ −7.8° (*c* 1.1, ethanol).

Anal. Calcd. for $C_{18}H_{23}N_3O_9$: C, 50.83; H, 5.45; N, 9.88. Found: C, 50.70; H, 5.45; N, 9.70.

The same compound was also prepared by saponification in which only two equivalents of base were used. The product was obtained in similar yield to the previous case.

L-Valyl-L-glutamic acid (IV). *p*-Nitrobenzyloxycarbonyldipeptide III (0.5 g., 1.2 mmoles) dissolved in 10 ml. of methanol containing 0.1 ml. of glacial acetic acid was hydrogenated with 70 mg. of palladium oxide[15] as catalyst by bubbling hydrogen through the solution while stirring for 1.5 hr. The catalyst was removed and the solvent was evaporated under reduced pressure. The residue was dried to constant weight *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets. The peptide was crystallized from aqueous acetone to yield 230 mg. (90%) of product, m.p. 220-222° dec., $[\alpha]_D^{24}$ +23° (*c* 1.0, water).

Anal. Calcd. for $C_{10}H_{18}N_2O_5$: C, 48.78; H, 7.37; N, 11.37. Found: C, 49.16; H, 7.52; N, 11.29.

3-[2-(Carboxy)ethyl]-6-isopropyl-2,5-piperazinedione ethyl ester (V). Ester II (1 g., 2 mmoles) in 15 ml. of methanol and 0.2 ml. of glacial acetic acid was hydrogenated using 120 mg. of palladium oxide as catalyst for 1 hr. The catalyst was removed, the solvent was evaporated under reduced pressure, and the residue was dessicated *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets. The residue was crystallized from aqueous ethanol giving 460 mg. of product with m.p. 185-187°. The compound was ninhydrin negative, had no ultraviolet absorption, and behaved as a neutral compound upon electrometric titration. Acid hydrolysis and paper chromatography of the hydrolysate showed the compound contained valine and glutamic acid.

Anal. Calcd. for $C_{12}H_{20}N_2O_4$: N, 10.93; sapon. equiv., 256. Found: N, 11.12; sapon. equiv., 261.

When this compound was saponified and the saponification mixture was acidified, a product crystallized which melted at 207° dec. When this product was titrated, it was found to contain an acidic group with a p*Ka* of approximately 4.1 which is attributed to the γ-carboxyl group of glutamic acid and which indicates that this carboxyl was the one esterified. From this evidence the structure was assigned.

When *p*-nitrobenzyloxycarbonyl-L-leucyl-L-alanine ethyl ester was hydrogenated under similar conditions, the only product isolated was a substance melting at 261-262° which behaved as a neutral compound upon electrometric titration. It was not characterized further, but was presumed to be the corresponding diketopiperazine.

p-Nitrobenzyloxycarbonyl-L-leucyl-hydrazide (VI). L-Leucine methyl ester hydrochloride[16] (5 g., 0.028 mole) was dissolved in 15 ml. of methanol and triethylamine (3.8 ml., 0.028 mole) was added followed by 200 ml. of ether. The mixture was stirred in an ice bath and the triethylamine hydrochloride was removed by filtration. An additional 3.8 ml. (0.028 mole) of triethylamine was added to the cold filtrate followed by the addition of 100 ml. of an ethereal solution of *p*-nitrobenzyl chloroformate (6 g., 0.028 mole). The mixture was stirred for 30 min. in an ice bath and allowed to stand for 2 hr. at room temperature. The precipitate was removed by filtration; the filtrate was washed with

(11) M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, **66**, 1288 (1933); M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **117**, 189 (1937).

(12) All melting points are uncorrected. Analyses were performed by the Microchemical Laboratory, Department of Chemistry, University of California, Berkeley.

(13) D. T. Gish and F. H. Carpenter, *J. Am. Chem. Soc.*, **75**, 950 (1953).

(14) H. M. Chiles and W. A. Noyes, *J. Am. Chem. Soc.*, **44**, 1798 (1922).

(15) R. L. Shriner and R. Adams, *J. Am. Chem. Soc.*, **46**, 1683 (1924).

(16) The procedure used by C. S. Smith and A. E. Brown [*J. Am. Chem. Soc.*, **63**, 2605 (1941)] for the preparation of D-leucine methyl ester hydrochloride was applied to the L-compound.

0.1*N* hydrochloric acid, 0.1*N* sodium bicarbonate, and water and then dried over anhydrous sodium sulfate. Evaporation of the ether gave *p*-nitrobenzyloxycarbonyl-L-leucine methyl ester as an oil (7.5 g., 84%). This material (0.023 mole) was dissolved in 25 ml. of ethanol and 2 ml. of 95+ % hydrazine was added and allowed to react for 48 hr. at room temperature. The solvent was removed *in vacuo* and the residue was crystallized from 50 ml. of ethanol to give 6 g. of the hydrazide (80% from *p*-nitrobenzyloxycarbonyl-L-leucine methyl ester), m.p. 133–134°, $[\alpha]^{24}_D$ −10° (*c* 1.21, ethanol).

Anal. Calcd. for $C_{14}H_{20}N_4O_5$: C, 51.85; H, 6.21; N, 17.27. Found: C, 51.80; H, 6.10; N, 17.35.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanine ethyl ester (VII). *By the mixed anhydride method. p*-Nitrobenzyloxycarbonyl-L-leucine monohydrate[4] (2 g., 6.4 mmoles), 1 ml. (6.4 mmoles) triethylamine and 6 ml. of tetrahydrofuran were cooled to −10° in an ice salt bath and 1 ml. of cold isobutyl chloroformate was added. After the mixture had remained for 10 min. at this temperature, 1 g. (0.0065 mole) of L-alanine ethyl ester hydrochloride[17] in 5 ml. of purified dioxane containing 1 ml. of triethylamine and 1 ml. of water was added, and the reaction mixture was stirred for 2 hr. at room temperature. Ethyl acetate (10 ml.) and 5 ml. of water were added to give two phases. The aqueous phase was extracted with two 5-ml. portions of ethyl acetate. The ethyl acetate extracts were combined and washed with *N* hydrochloric acid and saturated sodium bicarbonate. The ethyl acetate solution was dried over anhydrous magnesium sulfate and the solvent was removed *in vacuo*. The product was crystallized from aqueous ethanol to yield 1.8 g. (77%), m.p. 131–133°, $[\alpha]^{24}_D$ −29° (*c* 1.2, ethanol).

Anal. Calcd. for $C_{19}H_{27}N_3O_7$: C, 55.74; H, 6.65; N, 10.26. Found: C, 55.84; H, 6.47; N, 10.22.

By the azide method. To hydrazide VI (1 g., 3 mmoles) in 8 ml. of cold 1.2*N* hydrochloric acid, 210 mg. (3 mmoles) of sodium nitrite in 2 ml. of cold water was added over a period of 3 min. while the solution was stirred in an ice bath. Cold saturated potassium carbonate (3.6 ml.) was added and the mixture was extracted with cold ethyl acetate. A second extraction with 15 ml. of cold ethyl acetate was made and the ethyl acetate extracts were combined and dried over magnesium sulfate. It was convenient to carry out these steps in the 4° room. The dry ethyl acetate solution of the azide was added to a cold ethyl acetate solution of L-alanine ethyl ester which was obtained from 0.6 ml. (4.3 mmole) of triethylamine and 0.67 g. (4.3 mmole) of L-alanine ethyl ester hydrochloride[17] by a procedure similar to that described above for the preparation of leucine methyl ester. The reaction was allowed to proceed at 4° for 48 hr. after which time it was extracted with *N* hydrochloric acid and sodium bicarbonate. The product obtained upon removal of solvent was crystallized from aqueous ethanol to give 1.65 g. (66%), m.p. 126–128°, $[\alpha]^{24}_D$ −25° (*c* 1.11, ethanol).

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanine benzyl ester (VIII). *p*-Nitrobenzyloxycarbonyl-L-leucine monohydrate[4] (2 g., 6 mmoles) and 2.5 g. (8 mmoles) of L-alanine benzyl ester benzenesulfonate[6] were allowed to react following the procedure outlined for the preparation of ethyl ester VII by the mixed anhydride method. The product was crystallized from aqueous ethanol to yield 2.4 g. (83%). For analysis, it was recrystallized from aqueous ethanol and dried over phosphorus pentoxide *in vacuo*, m.p. 105.5–107.5°, $[\alpha]^{24}_D$ −36° (*c* 1.1, ethanol).

Anal. Calcd. for $C_{24}H_{29}N_3O_7$: C, 61.14; H, 6.20; N, 8.91. Found: C, 61.57; H, 6.27; N, 8.90.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanine (IX). Ethyl ester VII (0.5 g., 1 mmole) dissolved in 20 ml. of ethanol was added to 10 ml. of *N* potassium hydroxide and the mixture was allowed to stand at room temperature for 40 min. At the end of this time the solution was acidified, and the solvent was removed under reduced pressure to give an oil which was extracted several times with 20 ml. of ethyl acetate. The ethyl acetate layers were extracted with three 20-ml. portions of *N* potassium bicarbonate. The combined bicarbonate extracts were acidified and placed in the refrigerator to give 350 mg. (72%), m.p. 126.5–127.5°. Recrystallization from aqueous ethanol did not improve the melting point nor remove a slight yellow color from the product. The compound was dried over phosphorus pentoxide *in vacuo* for analysis, $[\alpha]^{24}_D$ −14° (*c* 1.1, ethanol).

Anal. Calcd. for $C_{17}H_{23}N_3O_7$: C, 53.55; H, 6.08; N, 11.02. Found: C, 53.65; H, 6.36; N, 11.37.

The ester was also saponified under conditions where only one equivalent of base was employed to give the desired product in similar yield to the previous case where excess of alkali was used.

L-Leucyl-L-alanine (X) by catalytic hydrogenation of IX. *p*-Nitrobenzyloxycarbonyldipeptide IX (0.5 g., 1.3 mmoles) was hydrogenated as previously described for compound IV. The residue remaining after removal of the catalyst and solvent was washed with acetone and crystallized from methanol by the addition of ethyl acetate, 130 mg. (50%), m.p. 255–256° dec., $[\alpha]^{24}_D$ +23° (*c* 5, methanol); lit.,[18] m.p. 257°, $[\alpha]^{20}_D$ +22.9 to +23.5° (*c* 5, methanol). The compound was dried at 78° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_9H_{18}N_2O_3$: C, 53.45; H, 8.97; N, 13.85. Found: C, 52.84; H, 8.84; N, 13.73.

By catalytic hydrogenation of benzyl ester VIII. Benzyl ester VIII (0.5 g., 1.06 mmoles) in 20 ml. of methanol containing 0.2 ml. of glacial acetic acid was hydrogenated over 90 mg. of palladium oxide and the peptide was isolated and crystallized as described above; yield 175 mg. (80%), m.p. 258–259° dec.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanine hydrazide (XI). To a solution of ethyl ester VII (15 g., 37 mmoles) in 120 ml. of methanol, 10 ml. of 95+ % hydrazine was added and allowed to react for 48 hr. at room temperature. The product, which had crystallized (12.3 g., 85%), was collected and recrystallized from methanol and dried over phosphorus pentoxide *in vacuo* at room temperature for analysis, m.p. 194–196°, $[\alpha]^{24}_D$ −24° (*c* 1.1, acetic acid).

Anal. Calcd. for $C_{17}H_{25}N_5O_6$: C, 51.65; H, 6.37; N, 17.79. Found: C, 51.86; H, 6.29; N, 17.75.

The hydrazide (XI) was also prepared from benzyl ester VIII (0.5 g.) dissolved in 5 ml. of ethanol and 0.33 ml. of 95+ % hydrazine. After 3 days at room temperature, 0.39 g. (94%) of hydrazide (m.p. 194–195°) was obtained.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine methyl ester (XII). To a suspension of hydrazide XI (12 g., 0.03 mole) in 240 ml. of cold 1.2*N* hydrochloric acid, was added 2.4 g. (0.035 mole) of sodium nitrite in 5 ml. of cold water; and the mixture was stirred for 3 min. at 4°. The insoluble azide which formed was extracted into ice cold ethyl acetate and dried over magnesium sulfate. The cold solution of the azide was added to 50 ml. of cold ethyl acetate solution of L-valine methyl ester prepared from 5.5 g. (0.033 mole) of L-valine methyl ester hydrochloride[19] and 4.9 ml. (0.035 mole) of triethylamine in the usual manner. The reaction was allowed to proceed at 4° for 2 days during which time the product had crystallized (11.4 g.). The mother liquor yielded another 1.51 g. of product, giving a combined yield of 12.9 g. (86%). For analysis, the product was recrystallized from methanol and dried *in vacuo* over phosphorus pentoxide at room temperature, m.p. 199–200°, $[\alpha]^{24}_D$ −37° (*c* 1.2, dioxane).

(17) D. A. Rowlands and G. T. Young, *J. Chem. Soc.*, 3937 (1952).

(18) E. Fischer, *Ber.*, **39**, 2893 (1906); M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider, and H. Schleich, *J. Biol. Chem.*, **109**, 325 (1935); W. J. Polglase and E. L. Smith, *J. Am. Chem. Soc.*, **71**, 3081 (1949).

(19) R. A. Boissonnas, St. Guttmann, P. A. Jacquenoud, and J. P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956).

Anal. Calcd. for $C_{23}H_{34}N_4O_8$: C, 55.87; H, 6.93; N, 11.33. Found: C, 56.02; H, 6.89; N, 11.29.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine benzyl ester (XIII). Hydrazide XI (12 g., 0.03 mole) and 12 g. (0.033 mole) of L-valine benzyl ester benzenesulfonate[6] were allowed to react following the procedure described for the preparation of the methyl ester. The product was recrystallized from aqueous ethanol and dried over phosphorus pentoxide *in vacuo* at room temperature to give 13.6 g. (79%), m.p. 162–165°, $[\alpha]_D^{24}$ −37° (*c* 1.1, acetic acid).

Anal. Calcd. for $C_{29}H_{38}N_4O_8$: C, 61.05; H, 6.71; N, 9.82. Found: C, 60.43; H, 6.62; N, 9.60.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine (XIV). Ester XII (0.5 g., 1 mmole) dissolved in 7.5 ml. of purified dioxane was mixed with 0.5 ml. of *N* potassium hydroxide. After 30 min. an additional 0.6 ml. of *N* potassium hydroxide was added, and the mixture was stirred for 3 hr. at room temperature at which time the solution had become clear. The product was isolated in the manner previously described for the other saponification products; yield, 230 mg. (47%). For analysis, the product was recrystallized from aqueous ethanol and dried over phosphorus pentoxide *in vacuo* at room temperature, m.p. 160–161°, $[\alpha]_D^{24}$ −31° (*c* 1.0, ethanol).

Anal. Calcd. for $C_{22}H_{32}N_4O_8$: C, 55.00; H, 6.71; N, 11.66. Found: C, 55.33; H, 6.89; N, 11.66.

The use of an excess of alkali in the saponification procedure did not improve the yield of the desired product.

L-Leucyl-L-alanyl-L-valine (XV). *By catalytic hydrogenation of* XIV. *p*-Nitrobenzyloxycarbonyltripeptide XIV (2.06 g., 4.3 mmoles) in 40 ml. of methanol containing 0.4 ml. of glacial acetic acid was hydrogenated with 300 mg. of palladium oxide[15] as catalyst by bubbling hydrogen through the solution for 2 hr. while stirring. During this time, the peptide precipitated. Water was added at the end to redissolve the peptide and the catalyst was removed by filtration. The solvent was removed *in vacuo* and the residue was dried to constant weight over phosphorus pentoxide and sodium hydroxide pellets. The product was crystallized from aqueous ethanol to give 1.12 g. (78%) of the dihydrate, m.p. 243–247° dec. with previous sintering.

Anal. Calcd. for $C_{14}H_{27}N_3O_4$: $2H_2O$: C, 49.84; H, 9.26; N, 12.46. Found: C, 50.41; H, 9.22; N, 12.17.

The material was dried *in vacuo* over phosphorus pentoxide at 100° and lost 11.6% of its weight (theory for dihydrate is 10.7%). The dried material is hydroscopic, $[\alpha]_D^{29}$ −31° (*c* 1.0, water). The peptide gave a single ninhydrin positive spot after chromatography in *n*-butyl alcohol–acetic acid–water (4:1:1). Acid hydrolysis of the peptide in constant boiling hydrochloric acid and paper chromatography of the hydrolysate showed the presence of all three of the expected amino acids in approximately equal amounts.

By catalytic hydrogenation of benzyl ester XIII. Benzyl ester XIII (0.5 g., 0.88 mmole) in 20 ml. of methanol, containing 0.2 ml. of glacial acetic acid was hydrogenated with 80 mg. of palladium oxide[15] as catalyst for 2 hr. in the usual way. The peptide was isolated and crystallized as described above to give 230 mg. (85%), m.p. 240–242° dec.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valine hydrazide (XVI). To a warm solution of benzyl ester XIII (5 g., 9 mmoles) in 100 ml. of absolute ethanol, 4 ml. of 95+% hydrazide was added and the mixture was allowed to react for 2 days at 37°. The crystalline product was collected on a filter, washed with acetone and ether, and dried over phosphorus pentoxide *in vacuo* at room temperature for analysis, yield, 3.7 g. (85%), m.p. 249.5–251.5°, $[\alpha]_D^{24}$ −42° (*c* 0.81, acetic acid).

Anal. Calcd. for $C_{22}H_{34}N_6O_7$: C, 53.44; H, 6.93; N, 16.99. Found: C, 53.89; H, 6.81; N, 17.27.

This compound could not be prepared from the methyl ester under similar conditions. Almost quantitative recovery of starting material was obtained in several attempts using ethanol, methanol or *n*-butyl alcohol as solvents. A crude product (m.p. 233–235°) was obtained by refluxing methyl ester XII in methanol with hydrazine for 1 hr.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valyl-L-glutamic acid diethyl ester (XVII). To hydrazide XVI (4 g., 8 mmoles) dissolved in 100 ml. of cold 1.2*N* hydrochloric acid was added 800 mg. (12 mmoles) of sodium nitrite in 3 ml. of cold water and the mixture was stirred for 3 min. Cold ethyl acetate (50 ml.) was added followed by cold saturated potassium carbonate in the manner previously described for the other azides. A product (1.3 g.) which was insoluble in either phase appeared at the azide extraction step and was filtered off. The ethyl acetate soluble material was added to a cold ethyl acetate solution of L-glutamate diethyl ester, obtained from 2.5 g. (0.01 mole) of L-glutamic acid diethyl ester hydrochloride[14] in the usual manner. The reaction was allowed to proceed in the cold room for 2 days during which time a gel had formed. The product was collected on a filter, washed with ethyl acetate, and dried over phosphorus pentoxide to give 2.12 g. An additional 300 mg. of product was obtained from the mother liquor. The combined yield was 2.42 g. (45%). After the product had been reprecipitated several times from ethanol, it attained a constant melting point of 220–223°. This material was dried over phosphorus pentoxide *in vacuo* at room temperature for analysis, $[\alpha]_D^{24}$ −46° (*c* 1.0, acetic acid).

Anal. Calcd. for $C_{31}H_{47}N_5O_{11}$: C, 55.94; H, 7.11; N, 10.52. Found: C, 56.07; H, 6.92; N, 10.57.

p-Nitrobenzyloxycarbonyl-L-leucyl-L-alanyl-L-valyl-L-glutamic acid dibenzyl ester (XVIII). Hydrazide XVI (4 g., 8 mmoles) and 6 g. (12 mmoles) of L-glutamic acid dibenzyl ester benzenesulfonate[6] were allowed to react following the procedure already described for the diethyl ester. Insoluble material (1.5 g.) was removed at the azide stage. The reaction mixture was kept in the cold room for 2 days at which time a gel had formed which was collected and washed with ethyl acetate to give 2.9 g. An additional 350 mg. of product was obtained from the mother liquor; combined yield was 3.25 g. (50%). For analysis the product was precipitated twice from ethanol and dried over phosphorus pentoxide *in vacuo* at room temperature, m.p. 178–186°, $[\alpha]_D^{24}$ −35° (*c* 1.0, acetic acid).

Anal. Calcd. for $C_{41}H_{51}N_5O_{11}$: C, 62.35; H, 6.51; N, 8.86. Found: C, 61.87; H, 6.80; N, 8.65.

L-Leucyl-L-alanyl-L-valyl-L-glutamic acid hydrochloride (XIX). Benzyl ester XVIII (10.4 g., 13.2 mmoles) was dissolved in 500 ml. of 80% acetic acid and hydrogenated over 1.6 g. of palladium oxide[15] as catalyst for 3 hr. The catalyst was filtered and the solvent was removed *in vacuo* to give a residue which weighed 6.5 g. after it had been dried over phosphorus pentoxide and sodium hydroxide (theory is 5.7 g.). Six grams of this residue was dissolved in 150 ml. of the lower phase of the system obtained by equilibrating equal volumes of 2-butanol with 0.1*N* hydrochloric acid. The solution was placed in the first fifteen tubes of a 200-tube, all glass countercurrent distribution apparatus and 200 transfers were performed. Aliquots were removed from the lower phase of every fifth tube for analysis by ninhydrin[20] and copper-Folin[21] methods and for solids. As a result of these analyses, the solvent was removed from all the tubes except tubes 60–100 and replaced with fresh upper and lower layers. The last tube was connected to the zero tube and the solvent was recycled for 1507 transfers. Aliquots were removed from every fifth tube for ninhydrin analysis. The contents of tubes 500 to 630 were combined and evaporated to a small volume under reduced pressure. The concentrated solution was lyophilized to give a solid product (3.7 g.). The peptide hydrochloride was crystallized by dissolving the lyophilate (0.5 g.) in ethanol (10 ml.) and

(20) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(21) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

adding ethyl acetate (45 ml.) until a permanent turbidity appeared. After the mixture had remained overnight at 4°, the crystals were collected, 0.19 g. A second crop, 0.13 g., was obtained from the mother liquor. The product was dried over phosphorus pentoxide *in vacuo* at 100° for analysis, m.p. 194.5–196° dec., $[\alpha]_D^{24}$ −62° (*c* 1.0, water).

Anal. Calcd. for $C_{19}H_{34}N_4O_7.HCl$: C, 48.87; H, 7.55; N, 12.00; Cl, 7.59. Found: C, 47.92; H, 7.71; N, 11.95; Cl, 7.59.

The expected amino acids were detected in approximately equal molar amounts upon paper chromatography of an acid hydrolysate of the tetrapeptide.

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

A New Synthesis of DL-Glutamine

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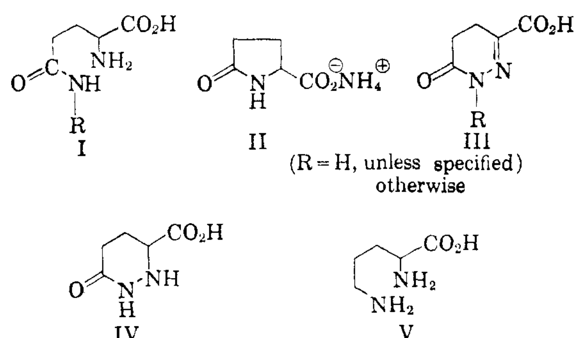
1,4,5,6-Tetrahydro-6-oxo-3-pyridazinecarboxylic acid has been converted to DL-glutamine by hydrogenation in water using palladium-on-carbon as the catalyst, and to DL-ornithine by hydrogenation in acetic acid using platinum oxide as the catalyst. A convenient preparation of benzylhydrazine is described.

Although glutamine (I. R = H),[1] the naturally occurring monoamide of glutamic acid, has shown promise as a therapeutic agent in several important medicinal areas,[2] extensive clinical investigation of this substance has been retarded by the prohibitive cost of both the natural L-isomer and the racemate. The tendency of glutamine to undergo cyclization to ammonium pyroglutamate (II) under rather mild conditions,[3] necessitates special precautions in the isolation of glutamine from natural sources and the use of blocking groups in synthetic procedures.[4] We wish to report here a simple synthetic sequence which obviates this inherent difficulty and makes DL-glutamine readily accessible.



The arrangement of atoms in 1,4,5,6-tetrahydro-6-oxo-3-pyridazinecarboxylic acid (III), readily prepared from α-ketoglutaric acid and hydrazine, made this compound appear particularly attractive as a potential intermediate for the desired synthesis. The conversion of III to glutamine requires only the saturation of the carbon-nitrogen double bond and the reductive cleavage of the nitrogen-nitrogen single bond. The possibility that both of these steps might be accomplished in one operation led us to undertake a study of the catalytic hydrogenation of III.[5,6]

Ethyl oxalosuccinate was prepared by the method of Friedman and Kosower[7] and hydrolyzed to α-ketoglutaric acid by the method of Blaise and

(1) For a review on the chemical characteristics and physiological roles of glutamine, see R. M. Archibald, *Chem. Rev.*, **37**, 161 (1945); for metabolism studies, see A. Meister, *Physiol. Rev.*, **36**, 103 (1956).

(2) D. B. Tower, *Neurology*, **5**, 113 (1955); L. L. Rogers and R. B. Pelton, *Texas Repts. Biol. Med.*, **15**, 84 (1957): J. M. Ravel, B. Felsing, E. M. Lansford, R. H. Trubey, and W. Shive, *J. Biol. Chem.*, **214**, 497 (1955); L. L. Rogers, R. B. Pelton, and R. J. Williams, *J. Biol. Chem.*, **214**, 503 (1955); **220**, 321 (1956); W. Shive and J. M. Ravel, *U.S. Pat.* **2,868,693**; E. A. Swinyard, L. Chin, F. R. Cole, and L. S. Goodman, *Proc. Soc. Exptl. Biol. Med.*, **94**, 12 (1957).

(3) The complete conversion of glutamine to ammonium pyroglutamate can be accomplished by heating the solid to its melting point or by holding an aqueous solution at 100° for one hour.

(4) (a) F. E. King and D. A. Kidd, *J. Chem. Soc.*, 3315 (1949). (b) M. Bergmann, L. Zervas, and L. Salzman, *Ber.*, **66B**, 1288 (1933); H. Nienberg, *Ber.*, **68B**, 2232 (1935); B. Vassel, *U.S. Pat.* **2,762,841**; R. M. Joyce and B. Vassel, *U.S. Pat.* **2,798,092**.

(5) Although there is ample precedent for the catalytic reduction of a carbon-nitrogen double bond to a carbon-nitrogen single bond, the catalytic reductive cleavage of a nitrogen-nitrogen single bond by hydrogen has seen only limited application. W. F. Whitmore and A. J. Revukas, *J. Am. Chem. Soc.*, **59**, 1500 (1937); **62**, 1687 (1940), reported the cleavage of azo compounds using Raney nickel catalyst and hydrogen. The presence of a small amount of isopropylamine as a by-product in the catalytic hydrogenation (platinum catalyst) of acetone semicarbazone to isopropylsemicarbazide was observed by D. W. Neighbors, A. L. Foster, S. M. Clark, J. E. Miller, and J. R. Bailey, *J. Am. Chem. Soc.*, **44**, 1557 (1922). The chemical reductive cleavage of substituted hydrazines to amines using sodium amalgam and acetic acid in alcohol has been reported by J. Tafel, *Ber.*, **19**, 1924 (1886).

(6) The cleavage of nitrogen-nitrogen bonds by the use of excess Raney nickel has received some application in recent years. In addition to work reported by C. Ainsworth, *J. Am. Chem. Soc.*, **76**, 5774 (1954); **78**, 1636 (1956); and R. L. Hinman, *J. Org. Chem.*, **22**, 148 (1957), there is the synthesis of L-glutamine from L-glutamic acid γ-hydrazide, S. Akabori and K. Narita, *Proc. Japan Acad.*, **29**, 264 (1953); S. Rath, U.S. Pat. **2,788,370**.

(7) L. Friedman and E. Kosower, *Org. Syntheses*, **Coll. Vol. III**, 510 (1955).