it was composed of four units of the dipeptide on the average.

*Anal.* Calcd. for $C_{32}H_{59}O_9N_8Cl$ ($n$ = 4): C, 52.2; H, 8.0; N, 15.2; Van Slyke nitrogen, 1.92; Cl, 4.8. Found: C, 51.5; H, 8.1; N, 14.9; Van Slyke nitrogen, 1.96; Cl, 4.5; amino nitrogen after hydrolysis, 14.5.

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[CONTRIBUTION FROM THE NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, CORNELL UNIVERSITY]

# The Synthesis of Peptides of L-Glutamine by the Carbobenzoxy Azide Method[1]

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The carbobenzoxy azide method has been adapted to the synthesis of peptides of L-glutamine. Using this method L-glutaminylglycine, L-glutaminyl-L-leucine, L-glutaminyl-L-valine, L-glutaminyl-L-alanine, carbobenzoxy-L-glutaminyl-L-serine and carbobenzoxy-L-glutaminyl-L-tyrosine have been prepared. Carbobenzoxy-L-alanyl-L-glutamine methyl ester was obtained by coupling carbobenzoxy-L-alanyl azide with L-glutamine methyl ester.

The number of glutaminyl peptides that has been reported is rather small. Melville[2] prepared L-glutaminylglycine and L-glutaminyl-L-glutamic acid by converting the carbobenzoxy-L-glutamyl derivatives to the $\gamma$-acid chlorides and treating these with ammonia. This method also has been used by Harington and Mead[3] to synthesize L-glutaminyl-L-cysteine and bis-(L-glutaminyl)-L-cystine and by Fruton and Bergmann[4] to prepare carbobenzoxy-L-glutaminyl-L-phenylalanine. Treatment of the $\gamma$-ethyl esters of $\alpha$-L-glutamic acid derivatives with ammonia was employed by Fruton and Bergmann[4] to synthesize carbobenzoxy-L-glutaminyl-L-tyrosinamide and by Miller and Waelsch[5] to prepare L-glutaminylglycine. More recently, du Vigneaud and his co-workers[6] reported the use of 1-tosylpyrrolid-5-one-2-carboxyl chloride in the preparation of L-glutaminyl-L-asparagine.

None of these methods combines the general applicability and ease of manipulation usually encountered with peptide syntheses involving the use of the carbobenzoxy azide procedure. It therefore seemed desirable to investigate the use of this method in the synthesis of peptides of L-glutamine. The adaptation of the carbobenzoxy azide procedure depends on the success with which side reactions, introduced by the presence of the $\gamma$-amide group, can be prevented.

The starting compound for this reaction sequence, carbobenzoxy-L-glutamine methyl ester, could be prepared smoothly from the previously described carbobenzoxy-L-glutamine by reaction with diazomethane. Difficulties were expected in the conversion of this ester to the hydrazide and in the reaction of nitrous acid with the hydrazide to give the desired azide.

Esters are generally considered to show greater reactivity than amides toward hydrazine hydrate. It was hoped that this generalization would hold for the methyl ester of carbobenzoxy-L-glutamine and that reaction conditions could be found where nearly quantitative conversion of the ester group to the hydrazide would not be accompanied by significant replacement of the $\gamma$-amide group. This goal was realized by carrying out the reaction at 0°. Using a 10% solution of the ester in methanol and excess hydrazine hydrate, the reaction appears to be complete in less than four hours and no replacement of the $\gamma$-amide group was observed.[7]

Although there are reports in the literature that the amide nitrogen of L-glutamine is abnormally susceptible to attack by nitrous acid[8] this anomaly is apparently not observed with those derivatives in which the $\alpha$-amino group is substituted.[9] Using two molar equivalents of hydrochloric acid and equimolar amounts of sodium nitrite and carbobenzoxy-L-glutamine hydrazide, no evidence of amide hydrolysis was observed. Carbobenzoxy-L-glutaminyl azide precipitates from the aqueous solution at 0° as an oil which crystallizes readily. There is therefore a very marked tendency for it to reprecipitate from solution in organic solvents since the crystalline form is less soluble than the oil. This difficulty has been overcome by filtering off the crystalline azide, washing it and drying *in vacuo*. All these operations are carried out at 0°. The azide is then dissolved in cold dimethylformamide, the amino acid ester added in ether or ethyl acetate, and the low boiling solvent is removed by evaporation. The carbobenzoxypeptide esters are precipitated, usually as crystals, by the careful addition of water. The yields in the coupling reaction varied from 55 to 81%. The conversion of the carbobenzoxy-L-glutaminyl peptide esters to L-glutaminyl peptides is carried out by the usual methods.

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(2) J. Melville, *Biochem. J.*, **29**, 179 (1935).

(3) C. R. Harington and T. H. Mead, *ibid.*, **30**, 1598 (1936).

(4) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939).

(5) H. K. Miller and H. Waelsch, *Arch. Biochem. Biophys.*, **35**, 176 (1952).

(6) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953).

(7) In order to facilitate characterization of compounds containing both hydrazide and primary amide groups, a method was developed for the differentiation of the hydrazide and amide nitrogen. The procedure consists of acid hydrolysis of the compound followed by an iodometric titration of the liberated hydrazine (S. Siggia, "Quantitative Organic Analysis *via* Functional Groups," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 75) and determination of ammonia by distillation of the residual solution. The acid hydrolysis was found to be necessary since direct iodometric titration gave low results for the hydrazine nitrogen. This is probably due to the formation of disubstituted hydrazides which can be obtained by oxidizing hydrazides with iodine (N. V. Sidgwick, "The Organic Chemistry of Nitrogen," Oxford University Press, Oxford, England, 1942, p. 398).

(8) A. C. Chibnall and R. G. Westall, *Biochem. J.*, **26**, 122 (1932).

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

TABLE I

| Carbobenzoxy-L-glutaminyl derivative | Yield, % | Solvent for recryst.,[a] ml./100 mg. | M.p., °C. | Nitrogen, % Calcd. | Found |
|---|---|---|---|---|---|
| -L-alanine ethyl ester | 55 | 0.9 dimethylformamide + 0.6 water | 195–197 | 11.3 | 11.2 |
| -L-valine methyl ester[b] | 59 | 1 dimethylformamide + 2 water | 173–175 | 10.7 | 11.2 |
| -L-serine methyl ester[c] | 81 | 0.7 dimethylformamide | 182–185[d] | 11.0 | 11.1 |
| -L-tyrosine methyl ester | 64 | 0.7 dimethylformamide + 1 water | 198–201 | 9.2 | 9.5 |
| -glycylglycine ethyl ester[e] | 55 | 1.5 ethanol 2X | 172–180[f] | 13.3 | 13.2 |
| -L-alanine[g] | 86 | 3 80% acetone | 209–213 | 12.0 | 12.1 |
| -L-valine | 88 | 2 80% acetone | 176–183 | 11.1 | 11.1 |
| -L-serine[h] | 78 | 2 65% ethanol | 199–202 | 11.4 | 11.4 |
| -L-tyrosine | 89 | 3 33% acetone | 183–185 | 9.5 | 9.5 |

[a] Recovery from recrystallizations averaged 80%. [b] Nice needles were obtained when the crystallizations were carried out at 45°. At lower temperatures gels formed. [c] The compound is rather insoluble in cold dimethylformamide and no water was added in order to precipitate the crystalline coupling product. [d] Transition at 167°. [e] Prepared from carbobenzoxy-L-glutaminyl azide and glycylglycine ethyl ester. [f] Transition at 140°. Melville[2] reports a melting point of 167° for this compound. [g] Calcd.: neut. equiv., 351. Found: neut. equiv., 352. [h] This compound tended to form a gel but crystallized on scratching. Poor yields were obtained on recrystallization. Calcd.: neut. equiv., 367. Found: neut. equiv., 366.

L-Glutaminylglycine has been synthesized by this method and the physical constants of our preparation are in good agreement with those recorded for this compound in the literature. We also prepared the following new peptides and carbobenzoxy derivatives: L-glutaminyl-L-leucine, L-glutaminyl-L-valine, L-glutaminyl-L-alanine, carbobenzoxy-L-glutaminyl-L-serine and carbobenzoxy-L-glutaminyl-L-tyrosine. Thus far no attempt has been made to convert a carbobenzoxy-L-glutaminylamino acid ester to the corresponding hydrazide and azide for the preparation of larger glutaminyl peptides.

Hydrogenolysis of carbobenzoxy-L-glutamine methyl ester in the presence of hydrochloric acid yielded L-glutamine methyl ester hydrochloride. Attempts to use this compound in the preparation of L-glutamine peptides were not satisfactory, largely due to difficulties encountered in the preparation of the free ester. However, by the hydrogenolysis of carbobenzoxy-L-glutamine methyl ester in the absence of acids, a solution of L-glutamine methyl ester in dimethylformamide could be prepared. By coupling this solution with carbobenzoxy-L-alanyl azide, carbobenzoxy-L-alanyl-L-glutamine methyl ester was obtained. Conditions necessary for the conversion of this ester to carbobenzoxy L-alanyl-L-glutamine remain to be determined. Difficulties are expected with the usual alkaline saponification procedures since under these conditions some carbobenzoxy-L-alanyl-L-isoglutamine might form.[10]

## Experimental[11]

**Carbobenzoxy-L-glutamine Hydrazide.**—A solution of 5.6 g. (0.019 mole) of carbobenzoxy-L-glutamine methyl ester[10] in 60 ml. of warm methanol was cooled rapidly to 0° and 6.0 ml. of hydrazine hydrate (99–100%) was added.[12] After storage at 0° for 4 hours, the crystals were filtered off. A second crop was obtained by concentrating the filtrate to 45 ml. and adding 160 ml. of water. The combined precipitates were washed twice with 10-ml. portions of methanol and then with water, yielding, after drying in a vacuum desiccator over phosphorus pentoxide, 5.6 g. of product (94%), m.p. 173–176° (transition at 147°). Recrystallization by dissolving in 27 ml. of dimethylformamide, filtering and adding 30 ml. of water, yielded 5.24 g. of carbobenzoxy-L-glutamine hydrazide hydrate, m.p. 174–176° (transition at 147°). Heating at 100° *in vacuo* for 4 hours was necessary to remove the water of hydration. Since this caused some decomposition, the hydrate was analyzed after drying *in vacuo* at room temperature.

*Anal.* Calcd. for $C_{13}H_{18}N_4O_4 \cdot H_2O$: C, 49.99; H, 6.46; N, 17.9; hydrazide-N, 9.0; amide-N, 4.5; weight loss, 5.77. Found: C, 50.20; H, 6.48; N, 18.1; hydrazide-N, 8.8; amide-N, 4.6; weight loss, 6.05.

The estimation of the hydrazide and amide nitrogen was carried out with a weighed amount of approximately 20 mg. of the hydrazide in 2 ml. of 2 *N* hydrochloric acid. This solution was refluxed for two hours. After cooling to room temperature 1.8 ml. of 2 *N* sodium hydroxide was added followed by the addition of excess solid sodium bicarbonate. This solution was then titrated with standardized 0.1 *N* iodine to the starch end-point. The titrated mixture was transferred to a semi-micro Kjeldahl flask and after the addition of 20 ml. of 2 *N* sodium hydroxide, the ammonia was distilled and trapped in 10 ml. of 1% boric acid. Ammonia was determined by titrating with standardized 0.1 *N* hydrochloric acid to the brom cresol green–chlor phenol red transition point.

**Carbobenzoxy-L-glutaminyl-L-leucine Methyl Ester.**—The preparation of the azide was carried out at 0°. To a solution of 156 mg. (0.5 millimole) carbobenzoxy-L-glutamine hydrazide hydrate in 2 ml. of 0.5 *N* hydrochloric acid 35 mg. of sodium nitrite in 0.25 ml. of water was added with stirring. After storage for 10 minutes, the crystals were filtered off, washed with 1 ml. of 3% sodium bicarbonate and 2 ml. of water. The material was dried to constant weight *in vacuo* and then dissolved in 1 ml. of dimethylformamide. In all cases this solution was used at once for the coupling reaction. However, no adverse effects were noted when the dried, crystalline azide, which decomposes around 90° on heating, was held overnight at 0° before use.

An ethereal solution of L-leucine methyl ester was prepared by equilibrating at 0° 110 mg. (0.6 millimole) of the hydrochloride with 0.5 ml. of aqueous potassium carbonate (50% wt./vol.) and 3 ml. of ether. The supernatant was dried over sodium sulfate and added to the azide solution. Most of the ether was removed by evaporation at 0° and the solution was then stored overnight at 0° and 24 hours at room temperature. On the addition of 1.8 ml. of water the product started to crystallize. After holding this mixture at room temperature for 3 hours another 2.2 ml. of water was added and storage was continued 3 hours at 0°. The crystals were filtered off, washed with water and dried *in vacuo;* yield 146 mg. (71%), m.p. 162–164°. Recrystallization by dissolving the product in 1 ml. of dimethylformamide, filtering, and adding 1 ml. of water raised the melting point to 163–164°.

*Anal.* Calcd. for $C_{20}H_{29}N_3O_6$: N, 10.3. Found: N, 10.2.

Other carbobenzoxy-L-glutaminyl peptide esters are described in Table I.

**Carbobenzoxy-L-glutaminyl-L-leucine.**—To a solution of the ester in 10 ml. of 80% aqueous ethanol, 0.76 ml. of 1.27 *N* sodium hydroxide was added and the solution held at room

(10) E. Sondheimer and R. W. Holley, THIS JOURNAL, **76**, 2467 (1954).

(11) All melting points were determined on a microscope hot-stage and are corrected. Analyses are by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England. Unless otherwise noted compounds were dried at 100° before analysis.

(12) The solubility of the ester is increased by the addition of the hydrazine hydrate.

temperature for 2 hours. The solution was then acidified with 1 *N* hydrochloric acid and concentrated *in vacuo* to the point where crystallization started. After storage overnight the mixture was filtered, the crystals washed with water and dried *in vacuo*; yield 293 mg. (85%), m.p. 178–180°, after recrystallization from 30% aqueous ethanol, m.p. 178–181°.

*Anal.* Calcd. for $C_{19}H_{27}N_3O_6$: N, 10.7. Found: N, 10.9.

Other carbobenzoxy-L-glutaminyl peptides are described in Table I.

**L-Glutaminyl-L-leucine.**—To a solution of 293 mg. of carbobenzoxy-L-glutaminyl-L-leucine in 5 ml. of 95% ethanol and 0.75 ml. of 0.99 *N* hydrochloric acid, 50 mg. of palladium black was added and the mixture was shaken mechanically. Hydrogen was bubbled through the system for 2 hours at room temperature. After filtration, the filtrate was concentrated *in vacuo* to one-half its original volume and neutralized with 0.59 ml. of 1.27 *N* sodium hydroxide. The product crystallized out of solution immediately. The mixture was filtered and the crystals washed with water and dried *in vacuo*, yielding 136 mg. (70%) of product, m.p. 186–187°, $[\alpha]^{22}D$ +11.5° (*c* 1, 1 *N* hydrochloric acid). The compound is insoluble in water but dissolves readily in 1 *N* hydrochloric acid or 1 *N* sodium hydroxide. The ninhydrin test is positive.

*Anal.*[13] Calcd. for $C_{11}H_{21}N_3O_4$: N, 16.2. Found: N, 15.9.

**L-Glutaminyl-L-alanine.**—The hydrogenolysis was carried out by the same procedure as that used for L-glutaminyl-L-leucine using 4 ml. of 95% ethanol per millimole carbobenzoxy-L-glutaminyl-L-alanine. After hydrogenolysis, the mixture was filtered and the filtrate neutralized with one equivalent of 1.27 *N* sodium hydroxide. After storage at 0° for 1 hour the crystals were filtered off, washed with 80% aqueous ethanol and dried *in vacuo*. Recrystallization by dissolving the product in 1.8 ml. of water, filtering and adding 4 ml. of absolute ethanol yielded 126 mg. (78%) of L-glutaminyl-L-alanine, m.p. 174–177°, $[\alpha]^{24}D$ +8° (*c* 1, 1 *N* hydrochloric acid). The compound is soluble in water and gives a positive ninhydrin test. Nitrogen analyses for this compound have been consistently low.

*Anal.*[13] Calcd. for $C_8H_{15}N_3O_4$: C, 44.23; H, 6.91; N, 19.35. Found: C, 44.22; H, 6.87; N, 18.4.

**L-Glutaminyl-L-valine.**—The hydrogenolysis of carbobenzoxy-L-glutaminyl-L-valine methyl ester by the procedure outlined for L-glutaminyl-L-alanine gave a 46% yield of L-glutaminyl-L-valine, m.p. 178–180°, $[\alpha]^{22}D$ +22.5° (*c* 1, 1 *N* hydrochloric acid). The dipeptide is readily soluble in warm water and gives a positive ninhydrin test.

*Anal.*[13] Calcd. for $C_{10}H_{19}N_3O_4$: N, 17.1. Found: N, 17.1.

**L-Glutaminylglycine.**—Hydrogen was bubbled at room temperature into a mixture of 128 mg. of carbobenzoxy-L-glutaminylglycine benzyl ester and 20 mg. of palladium black suspended in 15 ml. of 80% aqueous methanol and 0.2 ml. of glacial acetic acid. After 15 minutes of mechanical shaking the ester had gone into solution. The hydrogenolysis was stopped after 90 minutes. After filtration, the filtrate was concentrated *in vacuo* to a sirup from which 55 mg. (90%) of L-glutaminylglycine, m.p. 161–164°, crystallized on the slow addition of 15 ml. of absolute ethanol. Recrystallization by the addition of 3 ml. of absolute ethanol to a filtered solution of the crude material in 0.3 ml. of water gave 49 mg. of product, m.p. 163–165°, $[\alpha]^{22}D$ +78° (*c* 1.7, water). Additional recrystallization did not raise the melting point. The compound gives a positive ninhydrin test. Miller and Waelsch[5] reported a melting point of 166°, $[\alpha]^{27}D$ +77° (*c* 2.6, water), and Melville,[2] who did not report a melting point gives $[\alpha]^{18}D$ +76° (*c* 4.8, water) for L-glutaminylglycine.

**L-Glutamine Methyl Ester Hydrochloride.**—To a solution of 0.83 g. (2.83 millimoles) of carbobenzoxy-L-glutamine methyl ester in 27 ml. of methanol and 3 ml. of 0.99 *N* hydrochloric acid, 150 mg. of palladium black was added and the mixture shaken mechanically at room temperature. Hydrogen was bubbled through the mixture for 2 hours. After filtration the filtrate was concentrated *in vacuo* to a sirup. Overnight storage of this sirup in a vacuum desiccator over phosphorus pentoxide caused partial crystallization. This crude product was crystallized by dissolving it in 2 ml. of methanol, filtering and slowly adding 3.5 ml. of ethyl acetate. After 30 minutes another 0.5 ml. of ethyl acetate was added and the mixture was stored at 0° for 3 hours before filtration; yield 0.436 g. (78%), m.p. 144–147°, raised to 145–147° by another recrystallization.[14]

*Anal.* Calcd. for $C_6H_{13}ClN_2O_3$: N, 14.25; $OCH_3$, 15.77. Found: N, 13.95; $OCH_3$, 15.5.

**Carbobenzoxy-L-alanyl-L-glutamine Methyl Ester.**—Hydrogen was bubbled through a mixture of 600 mg. (2.04 millimoles) of carbobenzoxy-L-glutamine methyl ester, dissolved in 3 ml. of dimethylformamide and 200 mg. of palladium black for 30 minutes. The mixture was filtered, the catalyst washed twice with 0.5 ml. of dimethylformamide and the filtrate and washings combined.

Carbobenzoxy-L-alanyl azide was prepared from 475 mg. (2 millimoles) of carbobenzoxy-L-alanine hydrazide in 4 ml. of 1 *N* hydrochloric acid by the addition of 140 mg. of sodium nitrite in 0.5 ml. of water and equilibrating with 12 ml. of ether at 0°. The ether extract was washed with cold 3% sodium bicarbonate and dried over sodium sulfate. This solution was then added to the glutamine methyl ester in dimethylformamide, the ether was evaporated at 0° and the solution was stored at 0° overnight followed by storage at room temperature for 8 hours. The solution was then concentrated to a sirup by vacuum distillation at a bath temperature not exceeding 60°. The product crystallized slowly from this sirup on the addition of 4 ml. of water and storage at 0° for 3 days. Filtration and drying yielded 269 mg. (37%) of crude product, which after recrystallization from water gave 230 mg., m.p. 153–155°. The melting point of this material was raised to 154–156° by dissolving it in 2 ml. of 95% ethanol and adding enough ether to start crystallization; yield 155 mg.

*Anal.* Calcd. for $C_{17}H_{23}N_3O_6$: N, 11.5. Found: N, 11.5.

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(13) The preparation was dried at room temperature *in vacuo* prior to analysis.

(14) When the melt was held at 150° for a short time, new crystals appeared which did not melt below 250°. They are believed to be ammonium chloride, which may result from the formation of 5-carbomethoxy-L-pyrrolidone.