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Conformational Aspects of Polypeptide Structure XV.* Synthesis of Co-oligomeric Peptides of Glutamic-Aspartic Acids and Glutamic Acid-Glycine†

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Synopsis

We have synthesized three series of co-oligomers of the following type:

1. Z—(—glu(OEt)—asp(OMe)—)$_a$—(—glu(OEt)—)$_b$—OEt
2. Z—(—asp(OMe)—glu(OEt)—)$_n$—(—asp(OEt)—)$_m$—OEt
3. Z—(—gly—)$_w$—(—glu(OEt)—)$_x$—(—gly—)$_y$—(—glu(OEt)—)$_x$—OEt

where Z represents the carbobenzoxy blocking group and the subscripts can assume various small integral values.

The first series includes the dimer ($a = 1, b = 0$), trimer ($a = 1, b = 1$) and pentamers ($a = 2, b = 1$) and was prepared using the *p*-nitrophenyl ester technique. The second series is made up of dimer ($n = 1, m = 0$), trimer ($n = 1, m = 1$), pentamer ($n = 2, m = 1$), heptamer ($n = 3, m = 1$) and undecamer ($n = 5, m = 1$) and was prepared using mixed anhydrides and modified azide reactions. Lastly, the third series is composed of an octamer ($u = 0, x = 3, y = 2$) a decamer ($w = 0, x = 3, y = 4$) and an undecamer ($w = 2, x = 3, y = 2$). This group of compounds was synthesized by using *p*-nitrophenyl ester and modified azide reactions.

As an extension of our work on the secondary structure of peptides in solution, we wish to report the synthesis of a number of co-oligomeric peptides derived from γ-ethyl-L-glutamate, β-methyl-L-aspartate and glycine. Each reaction used in our synthetic scheme is known to yield optically pure peptides. Our previous paper[1] containing descriptions of the azide

* For previous paper n this series, see M. Goodman, Ira G. Rosen and Max Safdy, *Biopolymers* **2**, 503 (1964).

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method[2] coupled with the modifications of Rudinger[3] and Schwyzer[4] proved to be a general method for the joining of large peptide fragments.

Most stereochemical studies in the past have been carried out on polypeptides prepared through the *N*-carboxyanhydride route from a single amino acid.[5-8] Random copolymers or copolymers with unknown sequences can be made by suitably combining *N*-carboxyanhydrides derived from different amino acids.[9] A general approach to the formation of high polymers with known sequences remains a difficult task. Some progress has recently been reported on controlled sequence polymers by employing *p*-nitrothiophenyl ester condensations,[10] acyl hydrazide reactions,[11] and *p*-nitrophenyl ester displacements[12] on peptide derivatives. Because these techniques involve problems of molecular weight distributions and degrees of polymerization, we chose to prepare a group of co-oligomeric peptides via step-wise reactions in order to study the relationship between primary and secondary structure. In addition, by synthesizing specific compounds we avoid the effects of polydispersity on the physical and stereochemical properties we wish to study.[13-15]

SYNTHESIS OF PEPTIDES*

In this paper we extend the approach of the previous manuscript[1] to co-oligopeptide synthesis. Di- and tripeptides were prepared by mixed anhydride reactions. We have also employed active esters and have made extensive use of the modified azide reaction together with the *t*-butoxycarbonyl hydrazyl (CBH) blocking group.

Scheme I describes the route for glu-asp co-oligomers. It is noteworthy that we were able to utilize the *p*-nitrophenyl ester method which we published for oligomers derived from γ-methyl-L-glutamate.[18] This method was very poor for β-methyl-L-aspartate oligomers.[15] Through this route, however, we were only able to reach the pentamer stage.

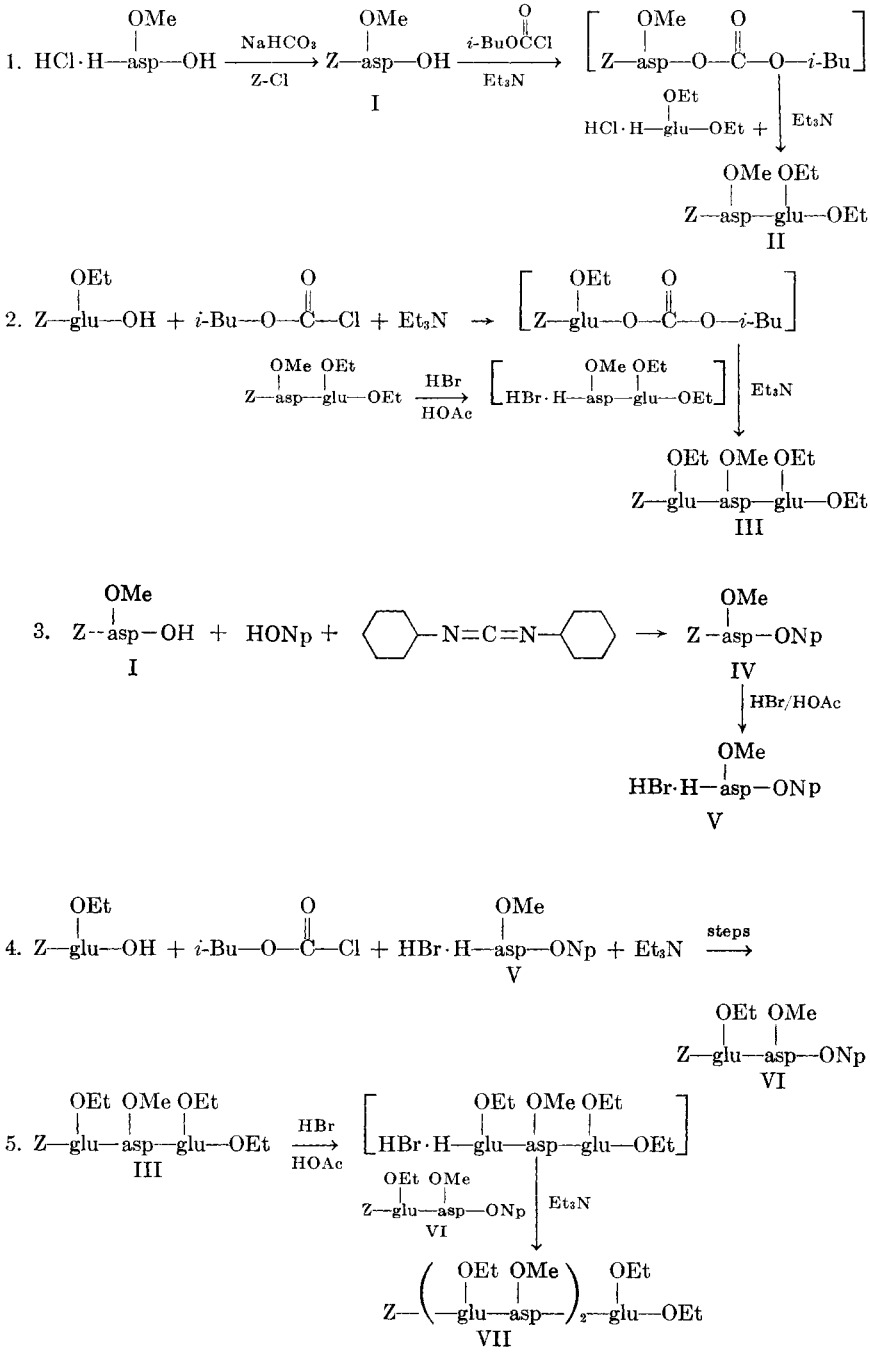
To prepare the asp-glu and glu-gly co-oligomer series, we relied primarily on the azide coupling reaction. Scheme 2 presents the reactions used to prepare the various peptides of the asp-glu type. The synthesis of the trimer (compound IX) proceeded smoothly via the mixed anhydride technique (reactions 1 and 2). Formation of the heptamer (compound XIV) and the undecamer (compound XV) was accomplished with the use of the CBH blocking group[2] and the modified azide coupling reactions[3] described in our previous paper.[1]

Finally, scheme III describes the pathways for the preparation of co-oligomers derived from γ-ethyl-L-glutamate and glycine. In this series we employed the *p*-nitrophenyl ester technique to prepare the tripeptide CBH derivative (compound XVI) and to add the two *N*-terminal glycine units of compounds XVIII, XIX, and XX. All other coupling reactions involved the modified azide approach which we describe in our previous paper.[1]

* Standard peptide abbreviations are used throughout this paper.[16,17]

Scheme I

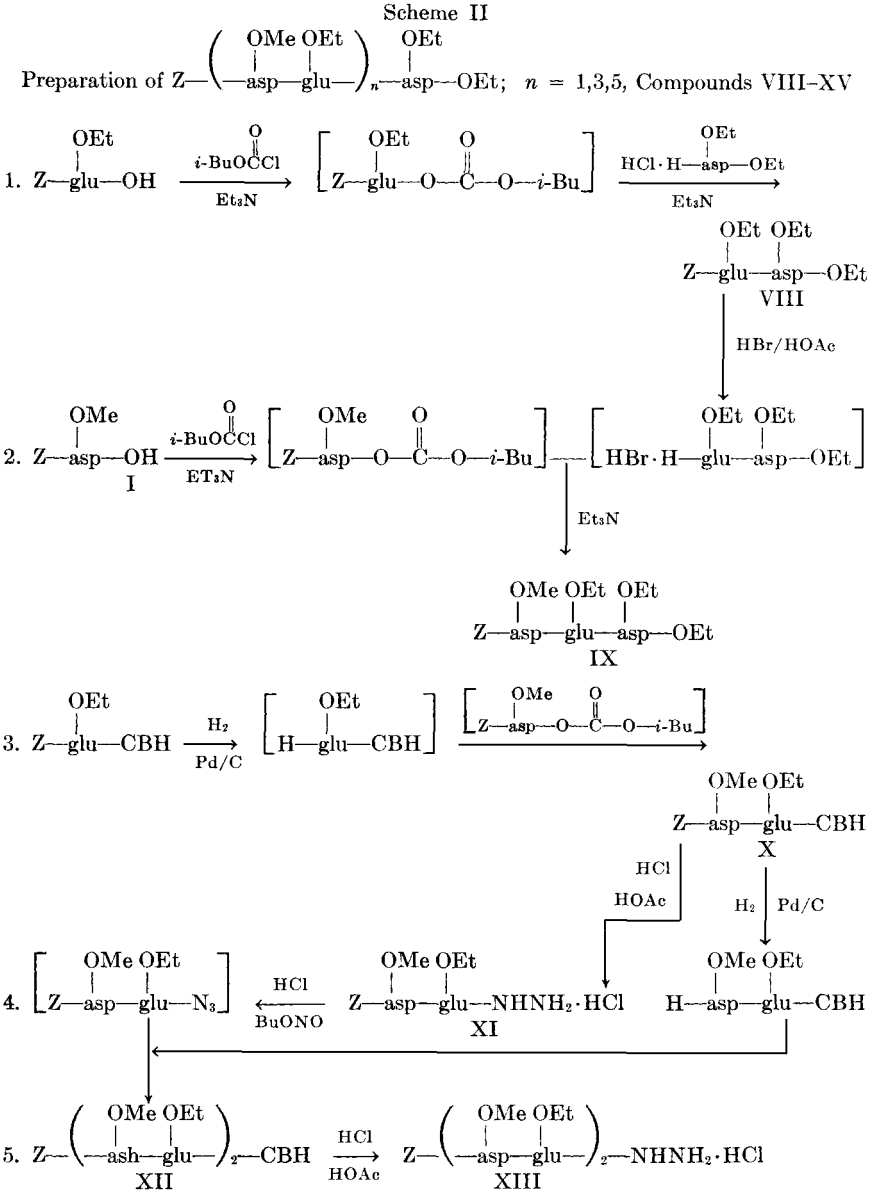
Preparation of Z—(glu(OEt)—asp(OMe))$_n$—glu(OEt)—OEt, n = 1 and 2, Compounds I–VII



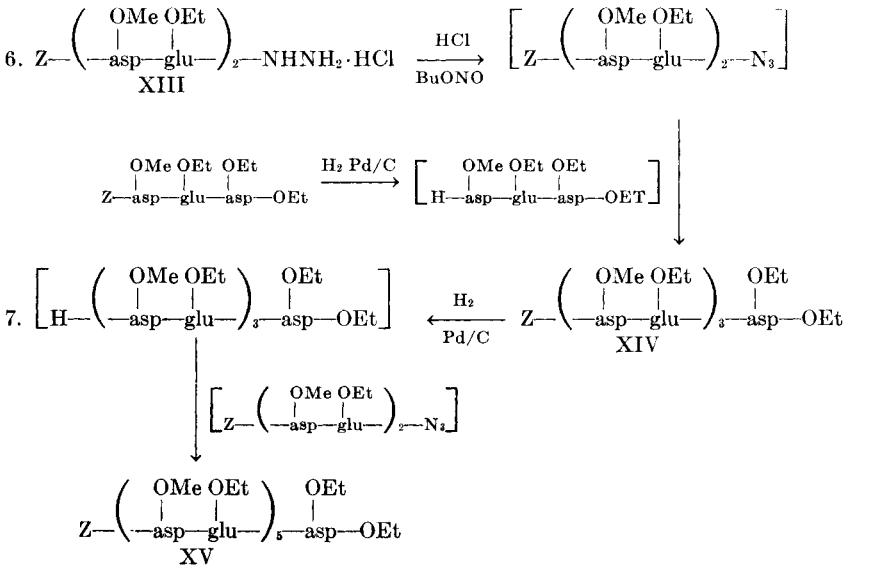
EXPERIMENTAL*

Benzyloxycarbonyl-β-methyl-L-aspartate(I). Sodium bicarbonate (33.6 g.; 0.400 mole) was dissolved in 500 ml. of water, then cooled to 0°. β-Methyl-L-aspartate (26.0 g.; 0.200 mole) was slowly added with stirring.

Scheme II

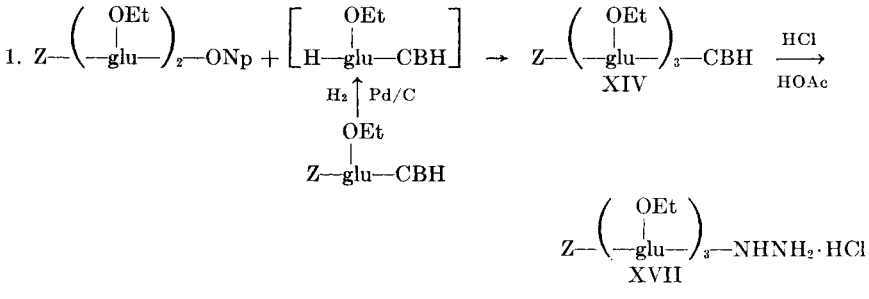


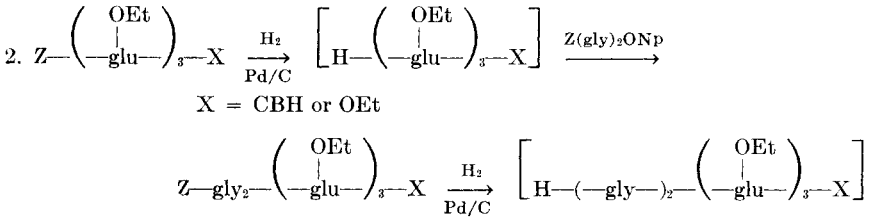
* All melting points are corrected and microchemical analyses were carried out by the Bernhardt Laboratories, Mulheim, Germany.

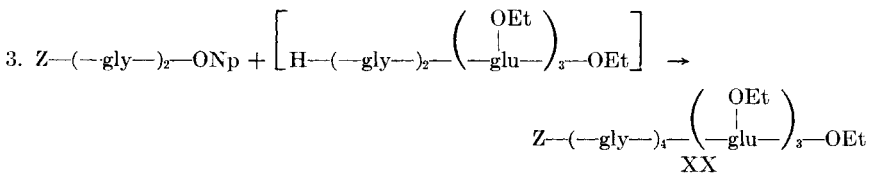


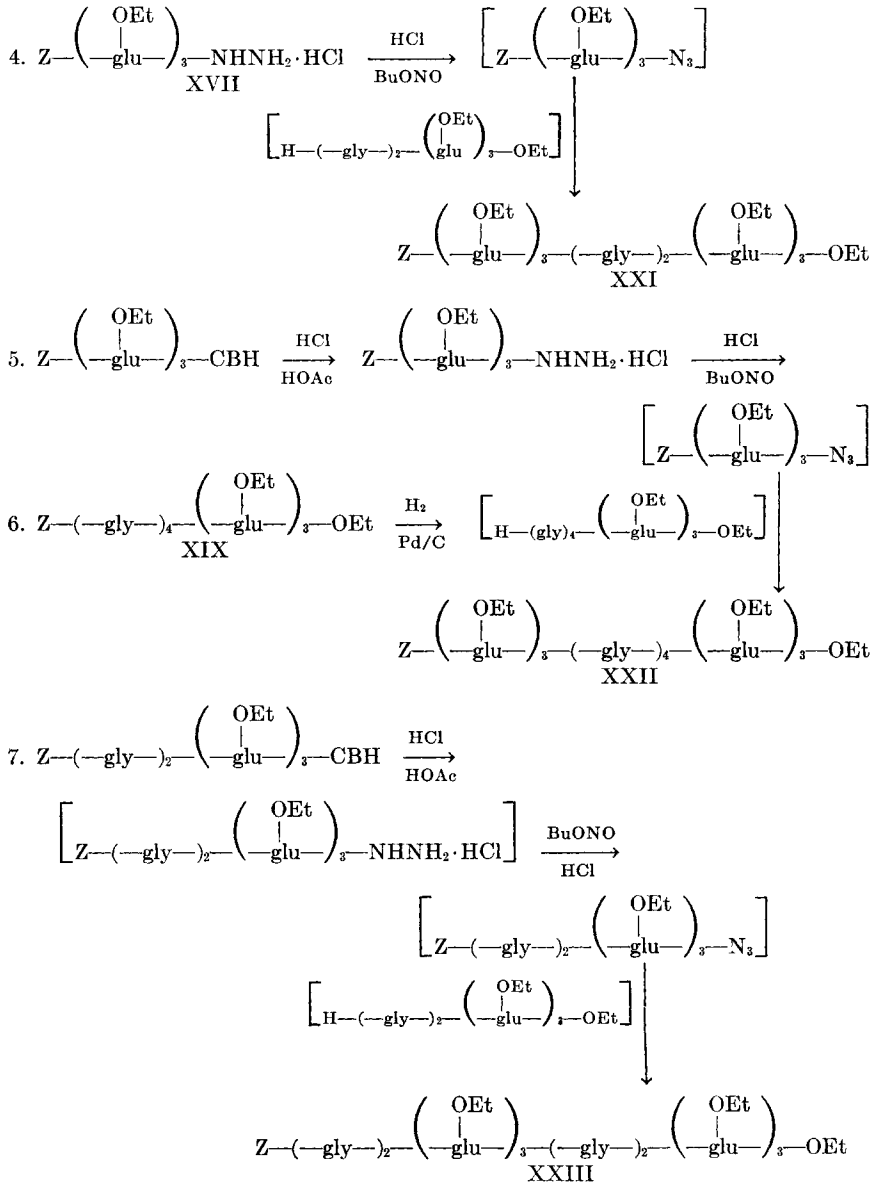
Scheme III

Preparation of Co-oligomers of γ-Ethyl-L-Glutamate and Glycine Compounds XVI–XXIII









It dissolved with evolution of carbon dioxide. Benzyloxycarbonyl chloride (29.0 g.; 0.200 mole) was then added dropwise with vigorous stirring over a period of thirty minutes. The reaction was allowed to slowly warm to room temperature over a period of two hours while the vigorous stirring was continued.

The solution was then extracted three times with ether. The aqueous layer was acidified to the congo red point with 6 N hydrochloric acid. The precipitated oil was extracted with ether and the ether solution was dried

over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under reduced pressure to yield a clear oil. The oil was crystallized from ethyl acetate-hexane and recrystallized from the same solvent system to yield 18 g. (32%) of white solid melting at 96–98° (lit.[19] m.p. 97–98°).

Benzyloxycarbonyl-β-methyl-L-aspartyl-diethyl-L-glutamate (II). Benzyloxycarbonyl-β-methyl-L-aspartate (I) (2.80 g.; 10.0 mmole) was dissolved in 10 ml. of dimethylformamide and cooled to −10° in an ice-salt bath. Isobutylchloroformate (1.30 ml.; 10 mmole) was added followed by the dropwise addition of triethylamine (1.50 ml.; 10 mmole). After twenty minutes, diethyl-L-glutamate hydrochloride (2.40 g.; 10 mmole) was added, followed by the dropwise addition of triethylamine (1.50 ml.; 10 mmole) and the solution was allowed to stir at 0° for five hours.

The reaction mixture was then diluted with 150 ml. of chloroform and extracted with saturated sodium bicarbonate, 10% hydrochloric acid, water, then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under reduced pressure to yield a clear oil. The oil was crystallized from ethyl acetate-hexane and recrystallized three times from the same solvent system to yield 2.16 g. (46%) of white solid melting at 80°.

ANAL. Calcd. for $C_{22}H_{30}N_2O_9$: C, 56.64; H, 6.48; N, 6.01. Found: C, 57.00; H, 6.66; N, 5.86.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-diethyl-L-glutamate (III). Benzyloxycarbonyl-β-methyl-L-aspartyl-diethyl-L-glutamate (II) (2.00 g.; 4.29 mmole) was dissolved in 2 ml. of glacial acetic acid saturated with hydrogen bromide. After standing at room temperature for five hours, excess ether was added and a brown oil formed. The supernatant liquid was removed by decantation and the oil was washed with ether several times. The oil was dissolved in 10 ml. of methanol, carbon black was added and the solution was boiled for two minutes. The suspension was filtered and the solvent was removed under reduced pressure to yield 1.16 g. (65%) of a brown oil.

Benzyloxycarbonyl-γ-ethyl-L-glutamate (0.865 g.; 2.80 mmole) was dissolved in 25 ml. of ethyl acetate and cooled to −10° in an ice-salt bath. Isobutylchloroformate (0.36 ml.; 2.80 mmole) was added, followed by the dropwise addition of triethylamine (0.42 ml.; 2.80 mmole) and the mixture stirred at −10° for thirty minutes. The oil prepared in the preceding paragraph was added in 10 ml. of cold dimethylacetamide, followed by the dropwise addition of triethylamine (0.42 ml.; 2.80 mmole) and the reaction mixture was stirred at 0° for four hours.

The reaction mixture was diluted with 250 ml. of ethyl acetate, extracted with 10% hydrochloric acid, saturated sodium bicarbonate, water, then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under reduced pressure to yield a

white solid. The product was recrystallized twice from ethyl acetate-hexane, yielding 1.10 g. (63%) of white solid melting at 142–143°.

ANAL. Calcd. for $C_{29}H_{41}N_3O_{12}$: C, 55.85; H, 6.63; N, 6.74. Found: C, 55.78; H, 6.43; N, 6.93.

Benzyloxycarbonyl-α-*p*-nitrophenyl-β-methyl-L-aspartate (IV). Benzyloxycarbonyl-β-methyl-L-aspartate (I) (10.0 g.; 35.4 mmole) and *p*-nitrophenol (5.90 g.; 42.5 mmole) were dissolved in 50 ml. of ethyl acetate. The mixture was cooled to 0° in an ice bath and dicyclohexylcarbodiimide (7.30 g.; 35.4 mmole) was added with stirring. The solution was stirred for two hours at 0° and two hours at room temperature.

The solution was diluted with 50 ml. of ethyl acetate and filtered. The precipitate was washed with 100 ml. of ethyl acetate. The combined filtrate was extracted with a saturated sodium bicarbonate, 10% hydrochloric acid, water, and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent removed under reduced pressure to yield a white solid. After two recrystallizations from hot ethanol, a product of 10.5 g. (73%) of a white solid melting at 106° was obtained.

α-*p*-Nitrophenyl-β-methyl-L-aspartate Hydrobromide (V). This compound was prepared by the method of Goodman and Boardman[19] in 75% yield, m.p. 116–117° d. (lit. 118° d.).

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-*p*-nitrophenyl-β-methyl-L-aspartate (VI). Benzyloxycarbonyl-γ-ethyl-L-glutamate (5.32 g.; 1.72 mmole) was dissolved in 20 ml. of dimethylformamide and cooled to −10° in an ice-salt bath. Isobutylchloroformate (2.24 ml.; 1.72 mmole) was added followed by the dropwise addition of triethylamine (2.58 ml.; 1.72 mmole) with stirring. After twenty minutes, α-*p*-nitrophenyl-β-methyl-L-aspartate hydrobromide (V) (6.00 g.; 1.72 mmole) was added followed by the dropwise addition of triethylamine (2.18 ml.; 1.46 mmole). The triethylamine addition was stopped before one equivalent was added because the solution turned a bright yellow signifying that some *p*-nitrophenyl ester was hydrolyzing. The reaction was carried out for two hours at 0° and one and a half hours at room temperature.

Chloroform (250 ml.) was added and the solution was extracted with 5% sodium bicarbonate, 10% hydrochloric acid, water, and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under reduced pressure to yield a yellow-white solid. This material was twice recrystallized from hot ethyl acetate to yield 6.65 g. (81%) of a white solid melting at 162.5–63°.

ANAL. Calcd. for $C_{26}H_{29}N_3O_{11}$: C, 55.80; H, 5.23; N, 7.51. Found: C, 56.00; H, 5.24; N, 7.36.

Benzyloxycarbonyl-di-(γ-ethyl-L-glutamyl-β-methyl-L-aspartyl)-diethyl-L-glutamate (VII). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-β-methyl-L-as-

partyl-diethyl-L-glutamate (III) (530 mg.; 0.850 mmole) was dissolved in 1 ml. of glacial acetic acid saturated with hydrogen bromide. In one hour all of the compound dissolved and evolution of carbon dioxide ceased. Excess ether was added to the reaction mixture causing an oil to separate. The oil was washed with ether, then dissolved in 1 ml. of absolute methanol and precipitated with ether. The oil was again separated by decantation and dried overnight in vacuum yielding 403 mg. (83%) of a clear oil.

Benzyloxycarbonyl - γ - ethyl - L - glutamyl - α - p - nitrophenyl - β-methyl-L-aspartate (VI) (395 mg.; 0.706 mmole) was added to the clear oil prepared above and 1 ml. of dimethylformamide was added, followed by triethylamine (0.105 ml.; 0.706 mmole). The yellow solution was allowed to stir at room temperature for twelve hours.

The mixture was then diluted with 25 ml. of chloroform and extracted with 10% hydrochloric acid, saturated aqueous sodium bicarbonate until it was colorless, water, then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the chloroform was evaporated under reduced pressure yielding a white solid. Two recrystallizations from hot ethyl acetate gave 343 mg. (53%) of white solid melting at 149°.

ANAL. Calcd. for $C_{41}H_{59}N_5O_{18}$: C, 54.12; H, 6.54; N, 7.70. Found: C, 54.04; H, 6.46; N, 7.57.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-diethyl-L-aspartate (VIII). Benzyloxycarbonyl-γ-ethyl-L-glutamate (3.09 g.; 10.0 mmole) was dissolved in 10 ml. of dimethylformamide and cooled to $-10°$ in an ice-salt bath. Isobutylchloroformate (1.30 ml.; 10.0 mmole) was added, followed by the dropwise addition of triethylamine (1.50 ml.; 10 mmole). After twenty minutes, diethyl-L-aspartate hydrochloride (2.26 g.; 10.0 mmole) was added, followed by the dropwise addition of triethylamine (1.50 ml.; 10.0 mmole) and the solution was allowed to stir at 0° for five hours.

The reaction mixture was then diluted with 150 ml. of chloroform and extracted with saturated sodium bicarbonate, 10% hydrochloric acid, water, then dried over magnesium sulfate. The drying agent was removed by filtration and the solvent evaporated under reduced pressure to yield a clear oil. The oil was crystallized from ethyl acetate-hexane, then recrystallized from the same solvent system to yield 3.05 g. (64%) of white solid melting at 111–112°.

ANAL. Calcd. for $C_{23}H_{32}N_2O_9$: C, 57.49; H, 6.71; N, 5.83. Found: C, 57.48; H, 6.80; N, 5.90.

Benzyloxycarbonyl-β-methyl-L-aspartyl-γ-ethyl-L-glutamyl-diethyl-L-aspartate (IX). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-diethyl-L-aspartate (VIII) (700 mg.; 1.65 mmole) was dissolved in 1 ml. of glacial acetic acid saturated with hydrogen bromide. After standing for five hours, excess ether was added and a brown oil formed. The oil was washed with ether several times. The oil dissolved in 5 ml. of methanol was decolorized with carbon, filtered, and precipitated with ether. The

supernatant liquid was removed by decantation and the oil dried under vacuum to yield 0.528 g. (75%) of hydrobromide.

Benzyloxycarbonyl-β-methyl-L-aspartate (I) (350 mg.; 1.24 mmole) dissolved in 25 ml. of ethyl acetate was cooled to −10° in an ice-salt bath and isobutylchloroformate (0.160 ml.; 1.24 mmole) was added, followed by the dropwise addition of triethylamine (0.190 ml.; 1.24 mmole). The mixture was allowed to stir for thirty minutes, then the hydrobromide prepared above was added in 10 ml. of dimethylacetamide, followed by the dropwise addition of triethylamine (0.190 ml.; 1.24 mmole). The reaction was allowed to run for four hours at 0°.

The reaction mixture was diluted with 250 ml. of ethyl acetate, extracted with 10% hydrochloric acid, saturated sodium bicarbonate, water, then dried over magnesium sulfate. The drying agent was removed by filtration and the solvent evaporated under reduced pressure, yielding a white solid. The product was recrystallized from ethyl acetate-hexane to yield 390 mg. (52%) of material melting at 135–136°.

ANAL. Calcd. for $C_{28}H_{39}N_3O_{12}$: C, 55.17; H, 6.45; N, 6.89. Found: C, 55.15; H, 6.42; N, 7.00.

Benzyloxycarbonyl-β-methyl-L-aspartyl-α-*t*-butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (X). Benzyloxycarbonyl - α - *t* - butyloxycarbonylhydrazidyl - γ - ethyl - L - glutamate (10.0 g.; 23.6 mmole) dissolved in 90 ml. of methanol containing 150 mg. of 30% palladium on carbon was hydrogenated for two hours at atmospheric pressure. The catalyst was removed by filtration and the solvent evaporated under reduced pressure. The catalyst was removed by filtration and the solvent evaporated under reduced pressure to yield an oil. This oil was dissolved in 10 ml. of ethyl acetate and stored at −20° until used.

Benzyloxycarbonyl-β-methyl-L-aspartate (I) (6.65 g.; 23.6 mmole) was dissolved in 50 ml. of ethyl acetate, cooled to −20° in an ice-salt bath and isobutylchloroformate (3.07 ml.; 23.6 mmole) was added, followed by the dropwise addition of triethylamine (3.53 ml.; 23.6 mmole) with stirring. After twenty minutes the solution of amine prepared by hydrogenation was filtered into the reaction mixture to remove a small amount of colorless needles that formed upon standing. (This side product was not analyzed.) The reaction was allowed to run for five hours at 0°.

The solution was diluted to 150 ml. with ethyl acetate, extracted with saturated aqueous sodium bicarbonate, 10% hydrochloric acid, water, then dried over magnesium sulfate. The drying agent was removed by filtration and the solvent evaporated to dryness under reduced pressure to yield a light yellow oil. The oil was crystallized by repeatedly heating and cooling an ethyl acetate-hexane solution which was held near the cloud point. Recrystallization from the same solvent system gave 8.10 g. (62%) of white solid melting at 92–94°.

Benzyloxycarbonyl-β-methyl-L-aspartyl-α-hydrazidyl-γ-ethyl-L-glutamate Hydrochloride (XI). To benzyloxycarbonyl-β-methyl-L-aspartyl-α-

t-butyloxy-carbonylhydrazidyl-γ-ethyl-L-glutamate (X) (5.00 g.; 1.05 mmole) was added 10 ml. of glacial acetic acid saturated with hydrogen chloride. The solid dissolved with evolution of gas. After standing one and a half hours at room temperature, the product was freeze-dried and washed with ether to yield 4.20 g. (94%) of product melting at 149–151°.

Benzyloxycarbonyl-β-methyl-L-aspartyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-α-*t*-butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (XII). Benzyloxycarbonyl - β - methyl - L - aspartyl - α - *t* - butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (X) (790 mg.; 1.43 mmole) was dissolved in 1 ml. of dimethylformamide containing 80 mg. of 30% palladium on carbon and 1 ml. of methanol and the system hydrogenated for one hour at atmospheric pressure. The catalyst was removed by filtration and the methanol removed under reduced pressure to yield a dimethylformamide solution of amine.

Benzyloxycarbonyl - β - methyl - L - aspartyl - α - hydrazidyl - γ - ethyl-L-glutamate hydrochloride (XI) (699 mg.; 143 mmole) was suspended in 3 ml. of a saturated solution of hydrogen chloride in tetrahydrofuran and cooled to −20° in a Dry Ice-ethanol bath. Butyl nitrite (0.324 ml.; 2.86 mmole) was added with stirring and the solution allowed to warm to room temperature. The hydrazide dissolved and the solution was immediately cooled to −20°, diluted with 40 ml. of pre-cooled ethyl acetate and extracted three times with 25 ml. of pre-cooled 1% sodium bicarbonate, then dried over magnesium sulfate. This solution was filtered into the solution of amine prepared above and the reaction mixture was stored at −5° for twelve hours.

The solute was precipitated with hexane and the product was twice recrystallized from hot ethyl acetate to yield 864 mg. (72%) of white solid melting at 156–158°.

ANAL. Calcd. for $C_{37}H_{54}N_6O_{16}$: C, 52.97; H, 6.50; N, 10.02. Found: C, 53.09; H, 6.61; N, 9.86.

Benzyloxycarbonyl-β-methyl-L-aspartyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-α-hydrazidyl-γ-ethyl-L-glutamate-Hydrochloride (XIII). Benzyloxycarbonyl - β - methyl - L - aspartyl - γ - ethyl - L - glutamyl - β-methyl - L - aspartyl - α - *t* - butyloxycarbonylhydrazidyl - γ - ethyl - L-glutamate (XII) (311 mg.; 0.371 mmole) was mixed with 10 ml. of a saturated solution of hydrogen chloride in glacial acetic acid and the mixture allowed to stand for seventeen hours at room temperature. The product was precipitated with ether, filtered on a sintered glass funnel and washed on the funnel with ether, yielding 236 mg. (82%) of white powder decomposing at 173–177°.

Benzyloxycarbonyl - tri - (β - methyl - L - aspartyl - γ - ethyl - L - glutamyl)-diethyl-L-aspartate (XIV). Benzyloxycarbonyl-β-methyl-L-aspartyl-γ-ethyl-L-glutamyl-diethyl-L-aspartate (IX) (2.12 g.; 3.08 mmole) was dissolved in 75 ml. of methanol containing 212 mg. of 30% palladium on

carbon and hydrogenated at atmospheric pressure for two hours. The catalyst was removed by filtration and the solvent evaporated under reduced pressure to yield a clear colorless oil of amine.

The azide was prepared by adding butyl nitrite (0.07 ml.; 6.16 mmole) to a suspension of benzyloxycarbonyl-β-methyl-L-aspartyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-α-hydrazidyl-γ-ethyl-L-glutamate hydrochloride (XIII) (2.38 g.; 3.08 mmole) in 27 ml. of tetrahydrofuran saturated with hydrogen chloride and cooled to −20°. On warming to room temperature, some hydrazide which had dissolved precipitated out on cooling. Pre-cooled ethyl acetate (150 ml.) was added and a clear solution was obtained. This solution was extracted in a pre-cooled separatory funnel with pre-cooled 1% sodium bicarbonate and dried over magnesium sulfate. The dry solution was filtered into the hydrogenation product obtained above and the clear solution was allowed to stand for two days at −5°.

The white precipitate which formed was filtered, washed with ether, dissolved in 3 ml. of dimethylformamide and diluted with 200 ml. of chloroform. The solution was extracted with 10% hydrochloric acid, saturated sodium bicarbonate, and water, then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent evaporated under reduced pressure on a rotatory evaporator. The solution was viscous and great care was needed to prevent excessive bumping. The resulting film was triturated with boiling ether, washed into a sintered glass funnel and washed with ethyl acetate, yielding 1.56 g. (43%) of white film melting at 219–221°.

ANAL. Calcd. for $C_{52}H_{75}N_7O_{24}$: C, 52.82; H, 5.60; N, 8.37. Found: C, 52.92; H, 6.42; N, 8.42.

Benzoylcarbonyl - penta - (β - methyl - L - aspartyl - γ - ethyl - L-glutamyl)-diethyl-L-aspartate (XV). Benzyloxycarbonyl-tri-(β-methyl-L-aspartyl-γ-ethyl-L-glutamyl)-diethyl-L-aspartate (XIV) (500 mg.; 0.423 mmole) was dissolved in 15 ml. of dimethylformamide and 75 ml. of methanol was added. Palladium on carbon (50 mg.) was added and the solution was hydrogenated for two hours at atmospheric pressure. The catalyst was removed by filtration and the methanol removed by evaporation under reduced pressure to yield a dimethylformamide solution of the amine which was stored at 0°.

Benzyloxycarbonyl - β - methyl - L - aspartyl - γ - ethyl - L - glutamyl - β - methyl - L - aspartyl - α - hydrazidyl - γ - ethyl - L - glutamate hydrochloride (XIII) (328 mg.; 0.423 mmole) was suspended in 5 ml. of tetrahydrofuran saturated with hydrogen chloride and cooled to −20°. Butyl nitrite (0.100 ml.; 0.846 mmole) was added with strong stirring and the solution allowed to slowly warm to room temperature. In ten minutes all the hydrazide dissolved and the solution was quickly cooled to −20° in a Dry Ice-ethanol bath. The solution was diluted with 75 ml. of pre-cooled ethyl acetate, extracted in a pre-cooled separatory funnel with cold 1%

sodium bicarbonate, then dried over magnesium sulfate in a pre-cooled flask. The magnesium sulfate was removed by filtration and the ethyl acetate solution of azide was added to the dimethylformamide solution of amine prepared above. The reaction mixture was allowed to stand at −5° for three days.

The product was precipitated by the addition of an equal volume of ether. The product was filtered, washed with ether and twice recrystallized from trifluoroethanol-ether to yield 424 mg. (57%) of white solid which did not melt under 230°.

ANAL. Calcd. for $C_{76}H_{111}N_{11}O_{36}$: C, 52.01; H, 6.39; N, 8.78. Found: C, 52.21; H, 6.50; N, 8.92.

Benzyloxycarbonyl - di - (γ - ethyl - L - glutamyl) - α - (*t* - butyloxycarbonylhydrazidyl)-γ-ethyl-L-glutamate (XVI). Benzyloxycarbonyl-α-(*t*-butyloxycarbonylhydrazidyl)-γ-ethyl-L-glutamate (0.846 g.; 2.00 mmole) was hydrogenated in methanol with 0.1 g. of 10% palladium on carbon for one and a half hours at atmospheric pressure. The solution was filtered and the methanol evaporated under reduced pressure. The resulting oil was dissolved in 5 ml. of dimethylformamide to which benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*p*-nitrophenyl)-γ-ethyl-L-glutamate (0.588 g.; 1.00 mmole) was added and the reaction mixture stirred for twelve hours at room temperature. The reaction mixture was then diluted with 50 ml. of chloroform and extracted with 10% potassium carbonate until the extracts were colorless. The chloroform solution was extracted twice with 2*N* hydrochloric acid, once with water, then dried over magnesium sulfate. The suspension was filtered and the solvent was removed under reduced pressure to yield a white solid. Two recrystallizations from hot ethyl acetate-hexane and one recrystallization from hot ethyl acetate yielded 0.5 g. (67%) of white solid melting at 132–134°.

Benzyloxycarbonyl - di - (γ - ethyl - L - glutamyl) - α - hydrazidyl - γ-ethyl-L-glutamate hydrochloride (XVII). To benzyloxycarbonyl-di-(γ-ethyl - L - glutamyl) - α - *t* - butyloxycarbonylhydrazidyl - γ - ethyl - L-glutamate (XVI) (115 mg.; 0.200 mmole) was added 0.25 ml. of a saturated solution of hydrogen chloride in glacial acetic acid. In two hours an equal volume of ether was added and the product was precipitated with hexane. After filtration and washing with ether on a sintered glass funnel, the product was recrystallized from methanol-ether-hexane to yield 84 mg. (63%) of product decomposing at 175°.

Benzyloxycarbonyl - di - glycyl - di - (γ - ethyl - L - glutamyl) - α - *t*-butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (XVIII). Benzyloxycarbonyl - di - (γ - ethyl - L - glutamyl) - α - *t* - butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (XVI) (1.90 g.; 2.58 mmole) was dissolved in 5 ml. of dimethylformamide and 50 ml. of methanol was added. Palladium on carbon (30%) (0.220 g.) was added and the mixture hydrogenated for three hours at atmospheric pressure. The catalyst was removed by filtration

and the filtrate evaporated under vacuum to remove the methanol, leaving a dimethylformamide solution of the amine. Benzyloxycarbonyl-glycyl-*p*-nitrophenyl glycinate (1.00 g.; 2.58 mmole) was added and the reaction allowed to stir for twelve hours at room temperature.

The reaction mixture was diluted with 75 ml. of chloroform and extracted with saturated sodium bicarbonate solution until the organic layer was colorless. It was then extracted with hydrochloric acid, water, and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was evaporated under reduced pressure to yield a colorless oil. The oil was crystallized from ethyl acetate-hexane and recrystallized twice from the same solvent system to yield 1.83 g. (83%) of product melting at 155–155.5°.

ANAL. Calcd. for $C_{38}H_{57}N_7O_{15}$: C, 53.56; H, 6.76; N, 11.51. Found: C, 53.50; H, 6.81; N, 11.48.

Benzyloxycarbonyl - di - glycyl - di - (γ - ethyl - L - glutamyl) - diethyl-L-glutamate (XIX). Benzyloxycarbonyl-di-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (1.68 g.; 2.58 mmole) was dissolved in 5 ml. of dimethylformamide and 50 ml. of methanol was added, followed by 170 mg. of 30% palladium of carbon. The mixture was hydrogenated for three hours at atmospheric pressure. The catalyst was removed by filtration and the methanol evaporated under reduced pressure, leaving a dimethylformamide solution of the amine. Benzyloxycarbonyl-glycyl-*p*-nitrophenyl glycinate (1.00 g.; 2.58 mmole) was added and the reaction allowed to stir for twelve hours at room temperature.

The reaction mixture was diluted with 75 ml. of chloroform and extracted with saturated sodium bicarbonate solution until the organic layer was colorless. It was then extracted with hydrochloric acid, water, then dried over magnesium sulfate. The drying agent was removed by filtration and the solvent was removed under reduced pressure to yield a colorless oil. The oil was crystallized from ethyl acetate-hexane and recrystallized twice from the same solvent system to yield 1.34 g. (68%) of product melting at 163.5–164°.

ANAL. Calcd. for $C_{35}H_{51}N_5O_4$: C, 54.89; H, 6.71; N, 9.15. Found: C, 54.82; H, 6.68; N, 9.34.

Benzyloxycarbonyl - tetra - glycyl - di - (γ - ethyl - L - glutamyl) - diethyl-L-glutamate (XX). Benzyloxycarbonyl-di-glycyl-di-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XIX) (1.36 g.; 1.78 mmole) was dissolved in 25 ml. of trifluoroethanol and 150 mg. of 30% palladium on carbon was added. The mixture was hydrogenated for three hours at atmospheric pressure. The catalyst was removed by filtration and the solvent evaporated under reduced pressure to yield a clear oil. Benzyloxycarbonyl-glycyl-*p*-nitrophenyl glycinate (695 mg.; 1.78 mmole) was added, followed by 1 ml. of dimethylformamide and the yellow solution was stirred for twelve hours at room temperature.

The reaction mixture was diluted with 50 ml. of chloroform and extracted with saturated sodium bicarbonate solution until the organic layer was colorless. It was then extracted with hydrochloric acid, water, then dried over magnesium sulfate. The drying agent was removed by filtration and the solvent was evaporated under reduced pressure to yield a yellow solid. This solid was extracted in a Soxhlet extractor with ethanol to yield 1.03 g. (68%) of product melting at 189.5–190°.

Benzyloxycarbonyl - tri - (γ - ethyl - L - glutamyl) - di - glycyl - di - (γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XXI). Benzyloxycarbonyl-diglycyl-di-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XIX) (1.20 g.; 1.57 mmole) was dissolved in 25 ml. of trifluoroethanol and 120 mg. of 30% palladium on carbon was added. The reaction mixture was hydrogenated for one hour at atmospheric pressure. The catalyst was removed by filtration and the solvent evaporated under reduced pressure to yield a clear oil which was stored at −5° until used.

Benzyloxycarbonyl - di - (γ - ethyl - L - glutamyl) - α - hydrazidyl - γ-ethyl-L-glutamate (XVII) (1.06 g.; 1.57 mmole) was dissolved in 5 ml. of a 1:1 solution by volume of trifluoroethanol-glacial acetic acid, saturated with hydrogen chloride and cooled to 0°. Butyl nitrite (1.76 ml.; 15.7 mmole) was added with stirring and the reaction allowed to proceed for ten minutes. The solution was diluted with 50 ml. of pre-cooled ethyl acetate and extracted with pre-cooled 1% sodium bicarbonate solution in a pre-cooled separatory funnel, then dried over magnesium sulfate in a pre-cooled flask. The drying agent was removed by filtration and the ethyl acetate solution of azide was added to the oil of amine prepared in the hydrogenation reaction and the reaction run for twelve hours.

The reaction mixture had formed a gel which was triturated with hexane and filtered to yield a yellow solid. The product was dissolved in 2 ml. of dimethylformamide and diluted with 50 ml. of chloroform, then extracted with 10% hydrochloric acid, 10% sodium bicarbonate, water, and dried over magnesium sulfate. The drying agent was removed by filtration and the solvent evaporated under reduced pressure to yield a colorless film. The film was removed from the walls of the flask by trituration with hot ether and filtered to yield 0.943 g. (48%) of white solid not melting under 230°.

ANAL. Calcd. for $C_{56}H_{84}N_8O_{23}$: C, 54.36; H, 6.84; N, 9.06. Found: C, 54.27; H, 6.69; N, 9.15.

Benzyloxycarbonyl - tri - (γ - ethyl - L - glutamyl) - tetra - glycyl - di-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XXII). Benzyloxycarbonyl-tetra-glycyl-di(γ-ethyl-L-glutamyl)-di-ethyl-L-glutamate (XX) (500 mg.; 0.568 mmole) was dissolved in 25 ml. of trifluoroethanol; 50 mg. of 30% palladium on carbon was added and the mixture hydrogenated at atmospheric pressure for two hours. The catalyst was removed by filtration and the solvent evaporated under reduced pressure to yield an oil, which was stored at −5°.

Benzyloxycarbonyl - di - (γ - ethyl - L - glutamyl) - α - *t* - butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (XVI) (1.00 g.; 1.35 mmole) was dissolved in 1 ml. of a saturated hydrogen chloride solution in glacial acetic acid. After standing at room temperature for two hours, a saturated solution of hydrogen chloride (1 ml.) in trifluoroethanol was added and the reaction mixture cooled to $-10°$. Butyl nitrite (0.64 ml.; 5.68 mmole) was added with stirring and the reaction allowed to run for five minutes at $-10°$. The reaction mixture was diluted with 50 ml. of pre-cooled ethyl acetate and extracted with 1% sodium bicarbonate, then dried over magnesium sulfate. The drying agent was removed by filtration and the filtrate added to the amine oil prepared by hydrogenation above. All reagents and equipment used above were pre-cooled to -20–$0°$. The reaction was allowed to run for 48 hours at $-5°$.

The gel that had formed was triturated with hexane and filtered and washed on the funnel with hexane, to yield a greenish yellow solid. This solid was dissolved in 5 ml. of trifluoroethanol and filtered through an ultrafine funnel and the filtrate diluted with 50 ml. of chloroform. This solution was extracted with saturated sodium bicarbonate solution, 10% hydrochloric acid, water, and dried over magnesium sulfate. The drying agent was removed by filtration and the solvent was evaporated to yield a film. The film was triturated with hexane and filtered to yield 293 mg. (38%) of a white solid that did not melt below 220°.

ANAL. Calcd. for $C_{60}H_{90}N_{10}O_{25}$: C, 53.32; H, 6.71; N, 10.37. Found: C, 53.13; H, 6.67; N, 10.44.

Benzyloxycarbonyl - di - glycyl - tri - (γ - ethyl - L - glutamyl) - diglycyl-di-(γ-ethyl - L - glutamyl) - diethyl-L-glutamate (XXIII). Benzyloxycarbonyl - di - glycyl - di - (γ - ethyl - L - glutamyl) - α - *t* - butyloxycarbonylhydrazidyl-γ-ethyl-L-glutamate (XVIII) (750 mg.; 0.888 mmole) was dissolved in 2 ml. of saturated hydrogen chloride in glacial acetic acid solution. After two hours an excess of ether was added and a white solid was obtained. This was recrystallized twice from ethanol ether to yield 440 mg. (63%) of a white solid.

Benzyloxycarbonyl - di - glycyl - di - (γ - ethyl - L - glutamyl) - diethyl-L-glutamate (XIX) (680 mg.; 0.888 mmole) dissolved in 25 ml. of trifluoroethanol with 100 mg. of 30% palladium on carbon was hydrogenated for two hours. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to yield a colorless oil.

The hydrazide hydrochloride prepared above was dissolved in 2 ml. of trifluoroethanol, cooled to $-20°$ and butyl nitrite (0.63 ml.; 5.6 mmole) was added with stirring. After ten minutes, 50 ml. of pre-cooled ethyl acetate was added and the solution extracted with 1% sodium bicarbonate. The solution was dried over magnesium sulfate and then filtered into the solution of the amine prepared in the hydrogenation above. The reaction was allowed to run for 48 hours at $-5°$.

The gel was triturated with hexane and filtered. The product was dissolved in 5 ml. of dimethylformamide and 50 ml. of chloroform was added to yield a gel that could not be extracted. It was precipitated with hexane and filtered to yield a fibrous product. This was dissolved in trifluoroethanol, filtered through an ultra-fine sintered glass funnel and precipitated with ether and hexane. The product was 2.12 mg. (28%) of a white solid which did not melt below 220°.

ANAL. Calcd. for $C_{60}H_{90}N_{10}O_{25}$: C, 53.32; H, 6.71; N, 10.37. Found: C, 51.14; H, 6.38; N, 9.92.

APPARATUS AND MEASUREMENTS

Catalytic Hydrogenations

Catalytic hydrogenations were generally carried out in a 50 ml. one-neck Ehrlenmeyer flask fitted with a ground glass joint having an inlet and outlet tube. The inlet tube was below the level of the solvent. The inlet tube was connected to a hydrogen tank and a nitrogen tank by a "y" tube. The hydrogenation was carried out in a hood.

The compound to be hydrogenated was dissolved in the appropriate solvent and set in the hydrogenating apparatus. The system was flushed with nitrogen for ten minutes. The catalyst, 10% by weight of 30% palladium on carbon, was wrapped in a small piece of filter paper. The joint containing the inlet and outlet tubes was removed and the wrapped catalyst added to the solvent and immediately submerged with a glass rod. The joint was quickly replaced and nitrogen flushing continued for 10 to 15 minutes. Hydrogen was passed through and the nitrogen flushing stopped. The reaction was followed by titrating the carbon dioxide in the outlet gas by the method of Patchornik and Shalitin.[20] The reaction was stopped when complete and the mixture flushed with nitrogen for ten minutes. It was then filtered to remove catalyst and the solvent evaporated under reduced pressure to yield an oil of amine.

Caution: Mixtures of methanol, dry catalyst, and air are explosive, as are mixtures of dry catalyst, hydrogen, and air. Great care must be taken when using methanol to keep the catalyst wet and the air out.

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