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Conformational Aspects of Polypeptide Structure XIV.* Synthesis of L-Glutamate Oligomers via Active Ester and Azide Coupling Reactions†

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Synopsis

In this paper we have once again shown the general utility of the modified active ester synthesis using a dipeptide active ester. We have also demonstrated that the acyl azide coupling method using the modifications of Rudinger and Honzl, and Schwyzer make a good general method for the synthesis of complex oligopeptides. This method employs reactions known to give products of high optical purity in reasonable yields. The use of the *t*-butyloxycarbonyl hydrazyl (CBH) blocking group provides a good route to hydrazides of esters of glutamic acid. The classical route to prepare hydrazides involves the displacement of an ester with hydrazine. Such a pathway is clearly impossible because of the γ-esters. Finally we demonstrated that trifluoroethanol is an excellent solvent for catalytic hydrogenation reactions.

In order to continue our studies on the relationship between primary and secondary structure of peptides in solution, we extended and reinvestigated the synthesis of glutamate oligomers using methods known to avoid racemization. This extension of previous work on glutamate oligomers also serves to reinforce our published data on the optical purity of the oligopeptides prepared by the *p*-nitrophenyl ester technique.[1]

The acylazide method originated by Curtius[2] is the only well-studied amino acid coupling reaction which yields only optically pure products.[3] Unfortunately this reaction is often attended by a variety of side reactions[4] leading to lowered yields and products that are difficult to purify. Curtius rearrangements have rarely been encountered under conditions of peptide syntheses.[5–8] A much more important and less understood side reaction produces amides derived from the starting hydrazide.[9–20] Recently, Rudinger and Honzl[20] reported a modified approach to the azide reaction which involves a homogeneous non-aqueous medium and azide forming

* For previous paper in this series, see M. Goodman and A. M. Felix, *Biochem.*, **3**, 1529 (1964).

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agents such as butylnitrite or nitrosyl chloride. Derivatives of cysteine, a system particularly prone to this side reaction,[12-17] do not yield amide by-products when this modification[20] is employed. We report the successful application of the Rudinger-Honzl method to our oligopeptides in this manuscript.

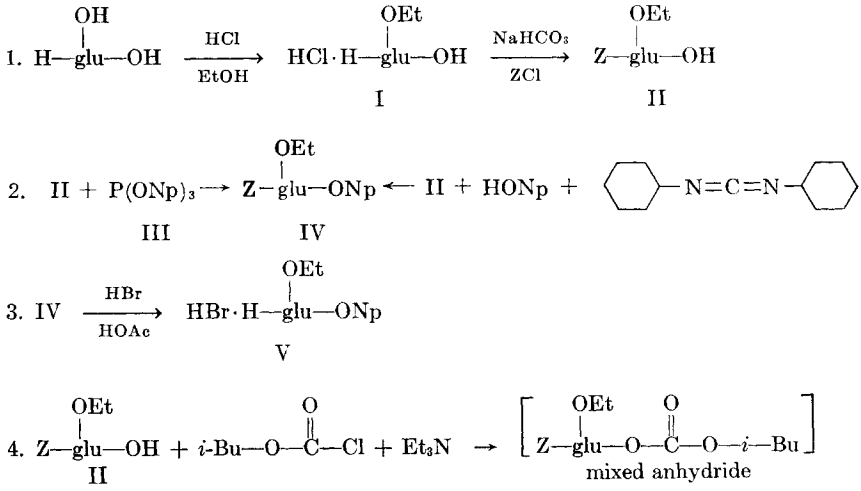
As an additional factor in this new azide coupling technique, we wish to report the extensive use of the *t*-butyloxycarbonyl-hydrazyl blocking group for the protection of the terminal carboxylic acid group.[21] We employ this blocking group in conjunction with the carbobenzoxy group. The latter can be removed by catalytic hydrogenation without affecting the former. Treating the peptide with ethanolic hydrogen chloride or hydrogen chloride in acetic acid, however, will remove the *t*-butyloxycarbonyl group without affecting the carbobenzoxy group yielding a hydrazide which can subsequently be used in an azide reaction. We reported this approach in a partial manner for higher oligopeptides derived from β-methyl-L-aspartate.[22] In this paper we present further examples of the technique as applied to γ-ethyl-L-glutamate oligopeptides. For derivatives of glutamic acid and aspartic acids, this *t*-butyloxycarbonyl-hydrazyl group provides a simple general route to hydrazides. The other approach to hydrazides, namely, displacement by hydrazine on an ester, is impractical because of the side chain ester groups.

Synthesis of Peptides*

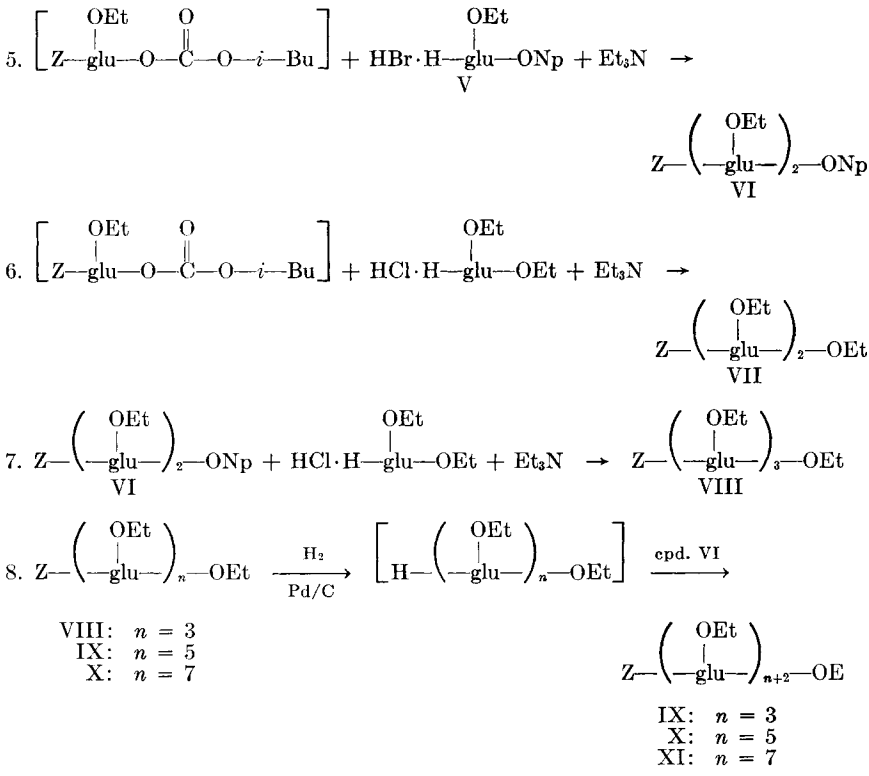
In the following schemes we illustrate our synthetic approach. Scheme I demonstrates the use of a combination of mixed anhydride,[25-27] and active

Scheme I

Preparation of Z—(OEt / glu)$_n$—OEt, n = 2,3,5,7,9 (Compounds I–XI)



* Standard peptide abbreviations and designations are used.[23,24]



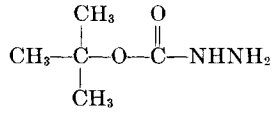
ester coupling reactions.[28–32] This approach utilizes the techniques we previously published for γ-methyl-L-glutamate oligomers.[1,30] Dipeptide derivatives (VI and VII) were prepared via mixed anhydride reactions. The dipeptide active ester (VI) is the key compound used to add two residues to the free amino group of specific peptide esters (reaction 8). By this manner we prepared the blocked trimer (VIII), pentamer (IX), heptamer (X) and nonamer (XI).

Several departures from our previous work[1] are noteworthy: we employed catalytic hydrogenation instead of acidic solvolysis for decarbobenzoxylations (reaction 8).

In order to obtain pure γ-ethyl-L-glutamate, we followed the suggestion of Pravda[33] who found that the use of finely divided L-glutamic acid is essential. Any diethyl-L-glutamate formed is removed by ether extraction.[34]

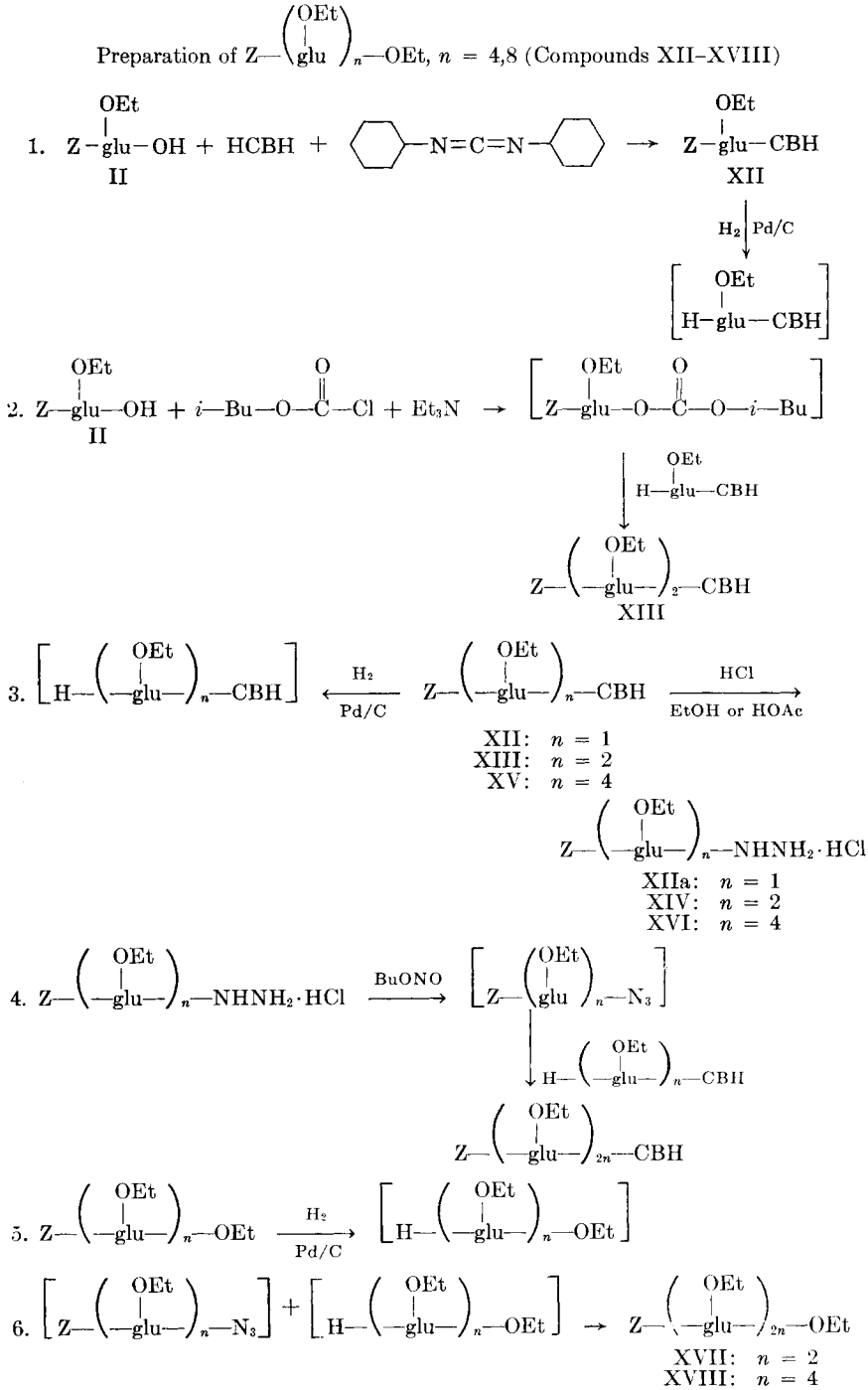
We prepared carbobenzoxy-α-*p*-nitrophenyl-γ-ethyl-L-glutamate by the carbodiimide method[33] which obviated the necessity to synthesize tris (*p*-nitrophenyl) phosphite (III).

HCBH:



Scheme II demonstrates the use of the modified azide reaction[20] and *t*-butoxycarbonyl hydrazide (HCBH).

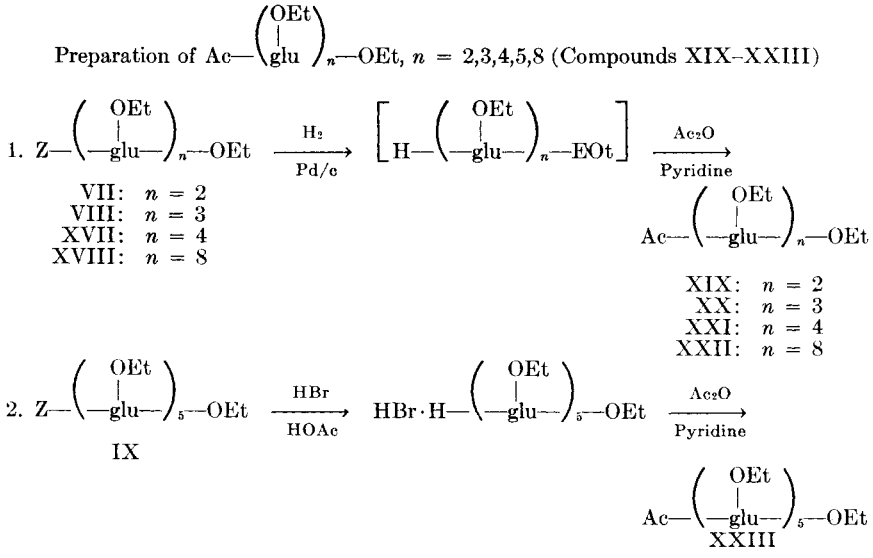
Scheme II



Reaction of HCBH with carbobenzoxy-γ-ethyl-L-glutamate via a carbodiimide coupling introduces the group into the scheme (reaction 1). Catalytic hydrogenation and suitable reaction with a mixed anhydride leads to the carbobenzoxy dimer (cpd. XIII), the generalized picture of the removal of the blocking group is shown in reaction 3. After cleavage of the *t*-butyloxycarbonyl group, the Rudinger-Honzyl modified azide coupling was carried out using butyl nitrite as the nitrosation agent and solvent systems of tetrahydrofuran subsequently diluted with ethyl acetate. Reactions (4–6) demonstrate this general approach to the stepwise oligomer syntheses.

In scheme III we indicate the preparation of a number of acetyl derivatives. It was essential for us to prepare the acetyl dimer by reaction 1

Scheme III



since the dimer is very water soluble and cannot be separated easily from its hydrobromide precursor.

Two features of these reactions are noteworthy: all acetyl derivatives melt higher than the corresponding carbobenzoxy derivatives. Trifluoroethanol (TFE) is superior as a hydrogenation solvent than methanol or highly acidic systems. As with methanol, hydrogenation in TFE proceeds at a rapid rate and the solvent is easily removed. Contrary to methanol, TFE dissolves the entire oligomer series.

EXPERIMENTAL*

Materials

The pure amino acids were obtained from Cyclo Chemical Corp., California, and Mann Research Corporation, New York. Reagent grade

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

chemicals were used for most reactions without further purification. Carbobenzoxy chloride and dicyclohexyl carbodiimide were obtained from the Mann Research Corporation. The trifluoroethanol was purchased from Columbia Organic of South Carolina, and the hexafluoroacetone was obtained from the Allied Chemical Corporation. Dimethylformamide, reagent grade, was vacuum distilled before use and the odorless middle fraction was used. Those reagents for which no source is listed were obtained through standard channels.

Preparation of Compounds

γ-Ethyl-L-glutamate Hydrochloride (I). This compound was prepared following the procedure described by Pravda.[33] The product was recrystallized from ethanol-ether to give a 49% yield of white crystals melting at 136–137° (lit.[33] m.p. 134–136°).

Benzyloxycarbonyl-γ-ethyl-L-glutamate (II). This compound was prepared by the general procedure of Greenstein and Winitz.[35] γ-Ethyl-L-glutamate hydrochloride (I) (21.1 g.; 0.100 mole) was dissolved in 500 ml. of water in a one-liter three-necked flask equipped with a strong mechanical stirrer and a dropping funnel and cooled in an ice-salt bath. Sodium bicarbonate (26.0 g.; 0.306 mole) was slowly added with stirring. When all the sodium bicarbonate dissolved the stirring rate was increased greatly and benzyloxycarbonyl chloride (17.4 g.; 0.102 mole) was added dropwise from the dropping funnel over a period of twenty minutes. The reaction mixture was maintained at 0° for one hour and then allowed to warm to room temperature. After two hours the stirring was halted and the mixture extracted three times with 100 ml. portions of ether. The aqueous layer was acidified with 6*N* hydrochloric acid to the congo red point. The resulting oil was extracted with ether, the ether extracts were combined, dried over magnesium sulfate, and filtered, and the ether removed under reduced pressure. The resulting oil soon crystallized on standing in an open flask at room temperature. The crude product was recrystallized from ethyl acetate-hexane to yield 14.0 g. (45%) of white crystals, m.p. 88.5–89° (lit.[36] 87°).

Tris-(*p*-nitrophenyl)phosphite (III). This compound was prepared by the method of Strecker and Grossman[37] using the modification of Goodman and Boardman.[22] The yield was 72% of grey powder, m.p. 167–170° (lit.[37] m.p. 170–1°).

Benzoyloxycarbonyl-α-*p*-nitrophenyl-γ-ethyl-L-glutamate (IV): *Method 1.* The general method of Schwyzer[30] was employed. To 10 ml. of pyridine was added with stirring benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (6.8 g.; 22 mmole) and tris-(*p*-nitrophenyl)phosphite (III) (6.0 g.; 14 mmole). The reaction was allowed to proceed at room temperature for three hours. The reaction mixture was diluted with 200 ml. of chloroform and extracted with 1% hydrochloric acid (three times), saturated aqueous potassium chloride, saturated sodium bicarbonate (until the yellow color of the chloroform layer no longer changed), and one time more with saturated

aqueous potassium chloride. The chloroform layer was dried over magnesium sulfate. The suspension was filtered and the filtrate evaporated under reduced pressure to yield a yellow solid. This solid was recrystallized from boiling ethanol yielding 6.1 g. (62%) of white powder. m.p. 77°, $[\alpha]_D = -34.1°$ ($c = 1$, DMF).

ANAL. Calcd. for $C_{21}H_{22}N_2O_8$: C, 58.60; H, 5.15; N, 6.51. Found: C, 59.04; H, 5.43; N, 6.69.

Method 2. The general method of Bodanzky and du Vigneaud[31] was employed. To a solution of benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (6.00 g.; 19.0 mmoles) and *p*-nitrophenol (3.24 g.; 23.0 mmoles) in 50 ml. of ethyl acetate, at 0°, dicyclohexylcarbodiimide (4.00 g.; 19.4 mmole) was added with stirring. After 30 minutes the reaction was allowed to warm to room temperature and proceed for an additional hour. The white precipitate was filtered and washed with ethyl acetate. The combined filtrate was evaporated under reduced pressure at 35° to yield a brown oil. The oil was crystallized from ethanol, washed with cold ethanol, and recrystallized from hot ethanol. The resulting white solid weighed 4.7 g. (56%), m.p. 76–77°, $[\alpha]_D^{25} = -34.1$ ($c = 1$, DMF).

α-*p*-Nitrophenyl-γ-ethyl-L-glutamate Hydrobromide (V). To benzyloxycarbonyl-α-(*p*-nitrophenyl)-γ-ethyl-L-glutamate (IV) (20 g.; 0.46 mole) was added 24 ml. of a saturated acetic acid solution of hydrogen bromide. The solid dissolved with evolution of gas. After ten minutes it formed a solid mass. The reaction mixture was allowed to stand for 30 minutes. The solid was pressed out on a sintered glass funnel, then washed with ether. The crude product was triturated with hot ethyl acetate and filtered through a sintered glass funnel. Recrystallization from hot methanol and ether gave 12.0 g. (69%) of white crystalline material melting at 174°.

Benzyloxycarbonyl - γ - ethyl - L - glutamyl - α - *p* - nitrophenyl-γ-ethyl-L-glutamate (VI). Benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (1.55 g.; 5.00 mmole) and *i*-butylchloroformate (0.65 ml.; 5.00 mmole) were dissolved in 25 ml. of dimethylformamide which had been cooled to −10° in an ice-salt bath. Triethylamine (0.75 ml.; 5.00 mmole) was added dropwise with stirring. After twenty minutes, γ-ethyl-α-(*p*-nitrophenyl)-L-glutamate hydrobromide (V) (1.89 g.; 5.00 mmole) was added followed by the dropwise addition of triethylamine (0.75 ml.; 5.00 mmole). The solution was allowed to stir for 4.5 hours at 0°. The reaction mixture was then diluted with 250 ml. of chloroform, extracted with 4% sodium bicarbonate (until the organic layer was colorless), water, 2*N* hydrochloric acid, and water (a second time), and finally dried over magnesium sulfate. The dried solution was filtered and the solvent removed under reduced pressure, yielding a white solid. The product was recrystallized three times from hot ethyl acetate to yield 1.84 g. (62%) melting at 154–155°.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-diethyl-L-glutamate (VII). Benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (1.54 g.; 5.00 mmole) and

i-butylchloroformate (0.65 ml.; 5.00 mmole) in 50 ml. of ethyl acetate was cooled to −10° in an ice-salt bath and triethylamine (0.70 ml.; 5.00 mmole) was added with stirring. After 30 minutes a dimethylformamide solution (5 ml.) of diethyl-L-glutamate hydrochloride (1.20 g.; 5.00 mmole) and triethylamine (0.70 ml.; 5.00 mmole) was added and the solution stirred at 0° for six hours, continuing overnight at room temperature.

The mixture was extracted with 10% hydrochloric acid, saturated potassium chloride, 10% sodium bicarbonate, and saturated potassium chloride, dried over magnesium sulfate, filtered and the filtrate evaporated under reduced pressure to yield a white solid. The product was recrystallized from ethyl acetate-hexane to yield 2.02 g. (82%), m.p. 104° (lit.[38] m.p. 104–105°).

Benzyloxycarbonyl-di-(γ-ethyl-L-glutamyl) diethyl-L-glutamate (VIII). To benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*p*-nitro-phenyl)-γ-ethyl-L-glutamate (VI) (0.59 g.; 1.0 mmole) and diethyl-L-glutamate hydrochloride (0.29 g.; 1.2 mmole) dissolved in 5 ml. of dimethylformamide was added triethylamine (0.15 ml.; 1.0 mmole) with stirring. The reaction progressed for twelve hours at room temperature. The mixture was diluted with 50 ml. of chloroform and extracted with 10% potassium carbonate solution until the extracts were colorless. The chloroform layer was then extracted twice with 2*N* hydrochloric acid and once with water. The chloroform solution was dried over magnesium sulfate, then filtered and the solvent removed under reduced pressure. The resulting white solid was recrystallized twice from ethyl acetate-hexane to yield 0.48 g. (74%) of product melting at 127–128°, $[\alpha]_D^{25} = -14.6°$ (c = 2, DMF) (lit.[38] m.p. 114–116°) (lit.[39] m.p. 128°).

Benzyloxycarbonyl-tetra-(γ-ethyl-L-glutamyl) diethyl-L-glutamate (IX). Benzyloxycarbonyl-di-(γ-ethyl-L-glutamyl) diethyl-L-glutamate (VIII) (0.652 g.; 1.00 mmole) and 0.13 g. of 10% palladium on carbon was hydrogenated for two hours at atmospheric pressure in 10 ml. of dimethylformamide and 15 ml. of methanol. The catalyst was removed by filtration and the methanol removed under reduced pressure to yield a greenish-brown solution. Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*p*-nitrophenyl)-γ-ethyl-L-glutamate (VI) (0.588 g.; 1.00 mmole) was added to the dimethylformamide solution and the reaction mixture was stirred for twelve hours at room temperature. The reaction mixture was then diluted with 100 ml. of chloroform and extracted with 10% potassium carbonate until the aqueous extracts were colorless. The chloroform solution was extracted once with water, twice with 2*N* hydrochloric acid, and once more with water, and then dried over magnesium sulfate. The solution was filtered and the solvent removed under reduced pressure to yield a clear oil. The oil was crystallized from ethyl acetate-hexane to yield 0.567 g. (58%) of a white solid melting at 177–178.5°.

Benzyloxycarbonyl-hexa-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (X). Benzyloxycarbonyl-tetra-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (IX)

(0.715 g.; 0.740 mmole) was dissolved in dimethylformamide (20 ml.) and 10% palladium on carbon (0.10 g.) was added. The mixture was hydrogenated for two hours at room temperature and atmospheric pressure, filtered to remove catalyst, and added to benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-*p*-nitrophenyl-γ-ethyl-L-glutamate (VI) (0.435 g.; 0.740 mmole). The reaction mixture was stirred at room temperature for twelve hours. The reaction mixture was diluted with 500 ml. of chloroform, extracted with saturated sodium bicarbonate (until the aqueous layer was clear), water, 10% hydrochloric acid, and water again, and then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the chloroform solution evaporated to dryness. The resulting white solid was recrystallized three times from boiling ethanol to yield 0.534 g. (56%) of product which did not melt below 235°. $[\alpha]_D^{25} = -15.8°$ ($c = 1$, DMF), $[\alpha]_D^{25} = -20.5°$ ($c = 1$, DCA).

Benzyloxycarbonyl-octa-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XI). Benzyloxycarbonyl-hexa-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (X) (0.200 g.; 0.156 mmole) was dissolved in dimethylformamide (20 ml.) and 10% palladium on carbon (0.020 g.) was added. The mixture was hydrogenated for two hours at room temperature and atmospheric pressure, filtered to remove the catalyst, and added to benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-*p*-nitrophenyl-γ-ethyl-L-glutamate (VI) (0.0917 g.; 0.156 mmole). The reaction mixture was diluted with 500 ml. of chloroform, extracted with saturated sodium bicarbonate solution (until the aqueous extracts were colorless), water, 10% hydrochloric acid, and water again, and then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the chloroform filtrate was evaporated under reduced pressure leaving a colorless film on the sides of the flask. The film was triturated with boiling ether to remove it from the flask walls and filtered. The solid was then dissolved in 1 ml. of dimethylformamide, and filtered through a fine sintered glass funnel. The solute was precipitated with water. The product, the white solid obtained by filtration, was again dissolved in dimethylformamide, diluted with 25 ml. of chloroform, extracted with water, and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the chloroform filtrate evaporated under vacuum to yield a clear film. The film was triturated with hot ether and filtered to yield 98 mg. (39%) of product which did not melt below 235°. $[\alpha]_D^{25} = -10.8°$ ($c = 1$, DMF).

Benzyloxycarbonyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XII). To a solution of benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (3.1 g.; 0.010 mole) and *t*-butyl carbazate (1.6 g.; 0.012 mole) in 40 ml. of ethyl acetate was added dicyclohexylcarbodiimide (2.10 g.; 0.010 mole) with stirring at 0°. A white precipitate formed almost immediately. The solution was allowed to warm to room temperature over a period of two hours. Five drops of acetic acid were added and the stirring was continued for fifteen minutes. The reaction mixture was filtered and the solid washed with 50 ml. of ethyl acetate. The ethyl acetate filtrates were combined,

extracted with 10% hydrochloric acid in saturated potassium chloride, 10% sodium bicarbonate, and water, and dried over magnesium sulfate. The magnesium sulfate was filtered from the solution; the filtrate was evaporated under reduced pressure leaving a white solid. This material was recrystallized from hot ethyl acetate to yield 3.8 g. (90%) of white powder, m.p. 114°.

ANAL. Calcd. for $C_{20}H_{29}O_7N_3$: C, 56.72; H, 6.90; N, 9.90. Found: C, 56.75; H, 6.75; N, 9.91.

Benzyloxycarbonyl-α-hydrazidyl-γ-ethyl-L-glutamate Hydrochloride (XIIa). To finely powdered benzyloxycarbonyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XII) (0.50 g.; 1.2 mmole) was added 5 ml. of a saturated ethanolic solution of hydrogen chloride. After 90 minutes, 30 ml. of ethyl acetate and 160 ml. of hexane were added to the solution causing a white precipitate to form. The white powder obtained was filtered, and triturated with ether yielding 0.40 g. (94%) of white powder, m.p. 136–8°. After recrystallization from hot ethanol m.p. 138–9°.

ANAL. Calcd. for $C_{15}H_{22}N_3O_5Cl$: C, 50.01; H, 6.16; N, 11.68. Found: C, 50.28; H, 6.27; N, 12.02.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XIII). Benzyloxycarbonyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XII) (2.0 g.; 4.7 mmole) in 50 ml. of methanol with 0.2 g. of 10% palladium on carbon was hydrogenated for three hours under 40 pounds of hydrogen using a Paar apparatus. The solution was filtered to remove the catalyst and the solvent was removed under reduced pressure to yield a clear oil.

Benzyloxycarbonyl-γ-ethyl-L-glutamate (II) (1.45 g.; 4.70 mmole) and *i*-butylchloroformate (0.61 ml.; 4.70 mmole) were dissolved in 20 ml. of dimethylformamide cooled to −10° in an ice-salt bath. Triethylamine (0.66 ml.; 4.70 mmole) was added slowly with stirring. A white precipitate formed. The solution was allowed to warm to 0° after 45 minutes and the oil of decarbobenzoxylated product was washed into the reaction mixture with 20 ml. of cold dimethylformamide. The reaction was stirred for two hours at 0° and allowed to warm to room temperature. After twelve hours, the reaction mixture was diluted ten-fold with ethyl acetate, extracted in turn with 1*N* hydrochloric acid, saturated potassium chloride, 10% sodium carbonate, and saturated potassium chloride, and dried over magnesium sulfate. The filtered solution was then evaporated under reduced pressure yielding a yellow oil which solidified under high vacuum. Recrystallization from ethyl acetate-hexane yielded 1.0 g. (37%) of product, m.p. 90–92° $[\alpha]_D = -37.5°$ ($c = 1$, Dioxane).

This procedure was later modified. The hydrogenation was carried out at atmospheric pressure using 30% palladium on carbon. The mixed anhydride reaction was carried out in ethyl acetate. The product was isolated in a standard manner giving a material melting at 124–125° in 72% yield.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-hydrazidyl-γ-ethyl-L-glutamate Hydrochloride (XIV). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XIII) (1.9 g.; 3.3 mmole) was allowed to react with 5 ml. of saturated ethanolic hydrogen chloride. When the evolution of carbon dioxide ceased, the ethanol was removed under reduced pressure yielding 1.29 g. (76%) of white solid, m.p. 140–144°.

This procedure was modified by using acetic acid as the solvent. The solution was allowed to stand for twenty hours at room temperature, whereupon the product precipitated out as a white solid. Recrystallization from ethanol-ether yielded a white solid in 76% yield, m.p. 143–145°.

Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-α-*t*-butyloxycarbonyl hydrazidyl-γ-ethyl-L-glutamate (XV). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-hydrazidyl-γ-ethyl-L-glutamate hydrochloride (XIV) (1.70 g.; 3.30 mole) in 150 ml. of water, containing 30 ml. of 1*N* hydrochloric acid (30 mmole), and 40 ml. of ethanol was cooled in an ice-salt bath to −10° and sodium nitrite (0.570 g.; 8.30 mmole) in 25 ml. of water was added. The precipitated azide was extracted into cold ethyl acetate which was washed with 1*N* sodium bicarbonate in saturated potassium chloride solution, dried over magnesium sulfate, and filtered. The filtrate was added to an ethyl acetate solution of γ-ethyl-L-glutamyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate. This solution had been prepared by hydrogenating benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XIII) (1.9 g.; 3.3 mmole) in 30 ml. of methanol containing 0.19 g. of 10% palladium on carbon in a Paar apparatus for three hours under 40 pounds of hydrogen pressure. The mixture was then filtered, evaporated under vacuum, and the resulting oil dissolved in ethyl acetate and cooled, before being used in the above reaction.

The reaction mixture was allowed to stand overnight at room temperature; solvent was then removed to yield a yellow oil. The oil was dissolved in hot ethanol and cooled to form a gel. The gel was recrystallized twice from hot ethanol, washed with ether, and dried to yield 1.5 g. (51%) of white solid, m.p. 169–171°, $[\alpha]_D = -37.6$ ($c = 1$, Dioxane).

ANAL. Calcd. for $C_{41}H_{62}N_6O_{16}$: C, 55.02; H, 6.98; N, 9.93. Found: C, 55.13; H, 7.17; N, 9.65.

Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-α-hydrazidyl-γ-ethyl-L-glutamate Hydrochloride (XVI). To benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-α-(*t*-butyloxycarbonyl hydrazidyl)-γ-ethyl-L-glutamate (XV) (1.00 g.; 1.12 mmole) a saturated solution of hydrogen chloride in acetic acid (2 ml.) was added with stirring. The reaction mixture was allowed to stand overnight at room temperature. The mixture was precipitated by the addition of ether, filtered through a sintered glass funnel, and washed with ether. Recrystallization from ethanol-ether yielded 0.80 g. (86%) of white powder melting at 176°.

Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XVII). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-diethyl-L-glutamate (VII) (1.92 g.; 3.88 mmole) was dissolved in 50 ml. of methanol and added to 10% palladium on carbon in a 100 ml. flask. The suspension was hydrogenated for one hour at room temperature. After the catalyst was removed by filtration, the solvent was evaporated under reduced pressure leaving an oil which was stored at 0°.

Benzyloxycarbonyl-γ-ethyl-L-glutamyl-α-hydrazidyl-γ-ethyl-L-glutamate hydrochloride (XIV) (2.00 g.; 3.88 mmole) was suspended in a saturated solution of hydrogen chloride in tetrahydrofuran (10 ml.) and cooled to −20° in an ethanol-Dry Ice bath. Butyl nitrite (4.4 ml.; 39 mmole) was added to the reaction mixture which was vigorously stirred until complete solution was obtained. The resulting red solution was diluted with 100 ml. of pre-cooled ethyl acetate and then extracted in a pre-cooled separatory funnel with pre-cooled 1% sodium bicarbonate solution. The resulting clear straw-colored solution was dried over magnesium sulfate, filtered to remove the drying agent, then added to the pre-cooled hydrogenated material, and allowed to stand at 5° for two days.

The white solid which precipitated from the reaction mixture was separated by filtration, and hexane was added to the filtrate to precipitate more product. Both solids were recrystallized separately from hot ethyl acetate-hexane, yielding 1.1 g. (35%) of white powder melting at 137–139°.

Benzyloxycarbonyl-hepta-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XVIII). Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XVII) (1.10 g.; 1.36 mmole) was dissolved in dimethylformamide (20 ml.) and 30% palladium on carbon was added. When all the catalyst was wet with solution, 20 ml. of methanol was added. The mixture was hydrogenated at atmospheric pressure for two hours, filtered to remove catalyst, and the solvent removed under reduced pressure at 30°. The resulting oil of the amine was stored at 0°.

Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-α-hydrazidyl-γ-ethyl-L-glutamate hydrochloride (XVI) (0.800 g.; 0.970 mmole) was suspended in a saturated solution of hydrogen chloride in tetrahydrofuran (6 ml.) which had been cooled to −20° in a Dry Ice-ethanol bath. Pre-cooled butyl nitrite (1.1 ml.; 9.7 mmole) was added with strong stirring. The solution was allowed to warm to 25°, then cooled to 0°. Some hydrazide still had not dissolved. The mixture was diluted with 60 ml. of pre-cooled ethyl acetate, extracted three times in a pre-cooled separatory funnel with pre-cooled 1% sodium bicarbonate solution, and dried over magnesium sulfate in a pre-cooled flask maintained at 0°. The mixture was filtered into the amine oil obtained from the hydrogenation reaction above and allowed to stand at 5° for twelve hours.

The reaction mixture was precipitated with hexane; it was then filtered and washed on a sintered glass funnel with hexane and ethanol to yield a white solid. The solid was dissolved in 1 ml. of dimethylformamide (diluted with 30 ml. of chloroform) extracted with saturated sodium bicar-

bonate, water, 10% hydrochloric acid, and water again, and then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the filtrate evaporated under reduced pressure leaving a film on the side of the flask. The film was triturated with hot ethanol to remove it from the side of the flask, filtered, and then dried, yielding 280 mg. (20%) of white solid which did not melt below 135°.

Acetyl-γ-ethyl-L-glutamyl-diethyl-L-glutamate (XIX). Benzyloxycarbonyl-γ-ethyl-L-glutamyl-diethyl-L-glutamate (VII) (0.543 g.; 1.21 mmole) in 25 ml. of methanol was hydrogenated at atmospheric pressure in the presence of 54 mg. of 30% palladium on carbon. The solution was filtered to remove the catalyst and the filtrate evaporated under reduced pressure to yield a clear oil. To this oil was added 10 ml. of a 3:1 pyridine–acetic anhydride solution (93.0 mmole pyridine; 26.3 mmole acetic anhydride) and the reaction mixture was allowed to stand for one hour at room temperature.

The reaction mixture was evaporated *in vacuo* at room temperature and the resulting solid recrystallized twice from ethyl acetate-hexane. The product was dissolved in trifluoroethanol, the solution filtered through a fine sintered glass funnel and evaporated to dryness, yielding 200 mg. (46%) of a white solid melting at 94–95°.

ANAL. Calcd. for $C_{18}H_{30}N_2O_8$: C, 53.72; H, 7.51; N, 6.96. Found: C, 53.68; H, 7.36; N, 7.08.

Acetyl di-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XX). Benzyloxycarbonyl-di-(γ-ethyl-L-glutamate)-diethyl-L-glutamate (VIII) (500 mg.; 0.824 mmole) in 25 ml. of trifluoroethanol containing 50 mg. of 30% palladium on carbon was hydrogenated at atmospheric pressure for two hours. The solution was filtered to remove the catalyst and the solvent was removed under reduced pressure to yield a clear oil. Pyridine-acetic anhydride (3:1) solution (10 ml.) (93.0 mmole pyridine; 26.3 mmole acetic anhydride) was added to the oil. After standing for one hour at room temperature, the reaction mixture was evaporated under reduced pressure at room temperature. The resulting white solid was twice recrystallized from ethyl acetate-hexane, dissolved in trifluoroethanol, and filtered through a fine sintered glass funnel. The solvent was removed under reduced pressure, yielding 324 mg. (76%) of white solid melting at 150–151°.

ANAL. Calcd. for $C_{25}H_{41}N_3O_{11}$: C, 53.65; H, 7.39; N, 7.51. Found: C, 51.44; H, 6.97; N, 7.62.

Acetyl-tri-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XXI). Benzyloxycarbonyl-tri-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (VXII) (522 mg.; 0.684 mmole) in 25 ml. of trifluoroethanol containing 52 mg. of 30% palladium on carbon was hydrogenated for two hours at atmospheric pressure. The reaction mixture was filtered to remove the catalyst, and the solvent was removed under reduced pressure to yield a clear yellow oil. Pyridine-acetic anhydride (3:1) solution (10 ml.) (93.0 mmole pyridine; 26.3 mmole acetic anhydride) was added to the oil and the reaction mixture

was allowed to stand for one hour at room temperature. The solvent was removed under reduced pressure at room temperature. The resulting white solid was dissolved in 25 ml. of chloroform, extracted with saturated aqueous sodium bicarbonate solution, saturated potassium chloride solution, 10% hydrochloric acid, and saturated potassium chloride again, and then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under reduced pressure to yield a white crystalline solid. The solid was recrystallized twice from hot ethyl acetate yielding 210 mg. (46%) of white solid melting at 171°.

ANAL. Calcd. for $C_{32}H_{62}N_4O_{14}$: C, 53.62; H, 7.31; N, 7.82. Found: C, 53.62; H, 7.15; N, 7.77.

Acetyl-hepta-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XXII). Benzyloxycarbonyl-hepta-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XVIII) (253 mg.; 0.176 mmole) dissolved in 25 ml. of trifluoroethanol containing 25 mg. of 30% palladium on carbon was hydrogenated at atmospheric pressure for two hours. The reaction mixture was filtered to remove the catalyst and the solvent was removed under reduced pressure to yield a clear oil. To this oil, 10 ml. of 3:1 pyridine-acetic anhydride (93.0 mmole pyridine; 26.3 mmole acetic anhydride) was added and the reaction mixture was allowed to stand for one hour at room temperature. The solvent was then removed under reduced pressure at room temperature to yield a white solid. After the solid was recrystallized from boiling ethanol twice, it was dissolved in trifluoroethanol and the solution filtered through a fine sintered glass funnel. The solvent was removed under reduced pressure leaving 193 mg. (81%) of white solid which did not melt under 230°.

ANAL. Calcd. for $C_{60}H_{96}N_8O_{26}$: C, 53.56; H, 7.19; N, 8.33. Found: C, 53.56; H, 6.97; N, 8.23.

Acetyl-tetra-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (XXIII). To benzyloxycarbonyl-tetra-(γ-ethyl-L-glutamyl)-diethyl-L-glutamate (IX) (1.00 g.; 0.109 mmole) was added 2 ml. of a saturated solution of hydrogen bromide in glacial acetic acid. The reaction mixture was allowed to stand at room temperature for twelve hours; upon addition of ether a brown oil was formed. The supernatant liquid was decanted and the oil was washed several times with ether. The oil was crystallized from methanol-ether to yield a white solid. The solid was dissolved in 10 ml. of 3:1 pyridine-acetic anhydride (93.0 mmole pyridine; 26.3 mmole acetic anhydride) and allowed to stand for one hour at room temperature. The mixture was precipitated with ice cold water and filtered. The resulting white solid was washed on the funnel with cold water, dissolved in trifluoroethanol, and filtered through a fine sintered glass funnel. The product was precipitated with water, then recrystallized from hot ethanol to yield 552 mg. (46%) of white solid which did not melt under 230°.

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