110° for 24 hours. After cooling, the contents of the tube were rinsed into a flask with distilled water and evaporated to dryness *in vacuo*. The contents were redissolved in water and evaporated a second time. The residue was then taken up in a small amount of water and treated with an excess of dinitrofluorobenzene reagent (prepared by mixing a solution of 0.4 g. of sodium bicarbonate in 5 ml. of water with a solution of 0.4 g. of dinitrofluorobenzene in 10 ml. of ethanol[31] for 2.5 hours with vigorous stirring). After removing most of the ethanol under reduced pressure, the solution was diluted with 5% bicarbonate solution and extracted repeatedly with ether to remove excess reagent. Acidification of the aqueous layer followed by ether extraction yielded an ether solution of the dinitrophenylene derivatives which, after concentration, was used to spot the paper for the chromatographic separation. The paper was eluted for 15 hours with a solvent system of toluene, chloroethanol, pyridine and 0.8 *M* ammonia (5:3:1.5:3).[31] Only two spots were evident; the faster one was the larger. The paper was dried and the spots cut out and extracted separately with 4.0 ml. of water. After heating in a 55–60° water-bath for 15 minutes after a fivefold dilution, the optical density at 360 mμ was determined. The solution from the fastest spot (phenylalanine) had an optical density of 0.407 while the other (glycine) had 0.172, corresponding to a glycine:phenylalanine ratio of 1:2.36.

Attempts to Synthesize 1-(N-Benzyloxycarbonylglycyl)-2,5-dibenzyl-3,6-dioxopiperazine.—A number of unsuccessful attempts was made to monoacylate L-phenylalanine anhydride ("L-diketopiperazine") with benzyloxycarbonylglycine. It is felt that many of these reactions failed because of the insolubility of the diketopiperazine. The following combinations and conditions were tried: (1) Z-glyOH + "L-diketopiperazine" + carbodiimide in acetonitrile at room temperature; (2) above reactants in dimethyl sulfoxide at 45–50° for 2 hours; (3) Z-glyOH + "L-diketopiperazine" + tetraethyl pyrophosphite in dioxane at reflux, 3 hours; the dioxane was then replaced with higher boiling chlorobenzene (b.p. 132°) and refluxed one additional hour; (4) Z-gly acid chloride + product of "L-diketopiperazine" with one part of sodium hydride; ether–benzene solvent at 0° for 0.5 hour, then room temperature for 3 hours; (5) Z-gly acid chloride + "L-diketopiperazine" in pyridine at 0° for 0.5 hour, then room temperature for 5 hours.

Preparation of Benzyloxycarbonylglycylglycine Ethyl Ester *via* the Hypothetical Intermediate Diacylamide.—Using the procedure previously described for the preparation of tripeptide derivatives,[1] benzyloxycarbonylglycine (0.63 g., 0.0030 mole), glycine *p*-nitrophenyl ester hydrobromide (0.83 g., 0.0030 mole) and glycine ethyl ester hydrochloride (0.42 g., 0.0030 mole) (added after 16 hours) were allowed to react. The reaction mixture was worked up in the usual way.[1] Evaporation of the neutral extract gave a residue which was fractionally crystallized from aqueous ethanol and ethyl acetate–petroleum ether (b.p. 30–60°) solvent combinations. The initial crops consisted of 0.16 g. of high and broad range melting materials. After these, a crop of 0.18 g. (17%) of benzyloxycarbonylglycyl diglycine ethyl ester was obtained, m.p. 143–149°. Finally, crops amounting to 0.19 g. (22%), m.p. 64–77°, were obtained. Recrystallization of these final crops gave product, m.p. 77–79° (lit.[32] m.p. 82.5 or 86–87°), of benzyloxycarbonylglycylglycine ethyl ester. Mixed melting point determinations, microchemical analysis and the infrared spectrum confirmed the structure of this material.

Acknowledgment.—We gratefully acknowledge the support of this research by the National Science Foundation Grant G-8614.

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(32) O. Süs, *Ann.*, **572**, 105 (1951), reports 82.5–83° while G. W. Anderson and R. W. Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952), give 86–87°.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN 1, N. Y., AND THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

Conformational Aspects of Polypeptides. III.[1] Synthesis of Oligomeric Peptides Derived from γ-Methyl Glutamate

BY MURRAY GOODMAN, EDWARD E. SCHMITT[2] AND DAVID A. YPHANTIS[3]

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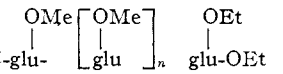
The syntheses of optically pure oligomeric peptides derived from γ-methyl glutamate containing between two and eleven residues are described. The evidence to date relating the physical properties of the oligomeric peptides to conformational structure is presented.

Introduction

In order to study the critical range for intramolecular hydrogen bonding in polypeptides,[1,4] it was necessary to synthesize oligomeric peptides using the most applicable methods available for coupling amino acid and peptide derivatives. Each peptide derivative was prepared in a high degree of chemical and optical purity since conformations of low molecular weight peptides described in this and earlier papers[1,4] are probably sensitive to small irregularities of the configuration of amino acids, and size of the peptide chain. In this paper we wish to describe the synthesis and techniques employed for the preparation of a homologous series of oligomeric peptides derived from γ-methyl L-glutamate using unequivocal methods. A general representation[5] is



where *n* is an integer between zero and nine inclusively.

Peptides derived from glutamic acid were prepared because of the fact that many correlations between conformational structure and physical

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(2) Submitted by Edward E. Schmitt to the faculty of the Polytechnic Institute of Brooklyn, 1961, in partial fulfillment of the requirements for the Ph.D. degree.

(3) The Rockefeller Institute, New York, N. Y.

(4) M. Goodman and E. E. Schmitt, *J. Am. Chem. Soc.*, **81**, 5507 (1959).

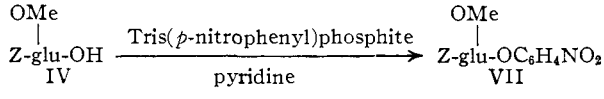
(5) The abbreviations used follow a modified Brand–Edsall scheme [*cf.* E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 223 (1947)]. Z refers to the benzyloxycarbonyl group. The symbols above the "glu" term denote the methyl or ethyl esters of the γ-position [*cf.* M. Goodman and G. W. Kenner, *Adv. in Protein Chem.*, **12**, 465 (1957)].

properties have already been determined for polymers of this amino acid and its esters.[6-12]

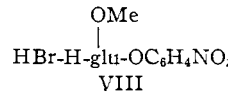
The benzyl esters of the glutamic acid peptides were originally considered as the preferred model oligomeric peptides because of the extensive studies made on the high molecular weight polymers[6,8] and the ease of conversion by solvolysis using hydrogen bromide in glacial acetic acid to the corresponding water-soluble polyglutamic acids.[13] A problem arose in the course of the synthesis of these oligomeric peptides which prevented their successful preparation. Since the preparative scheme involved building up of the chain from the N-terminal end, it was necessary to remove the amine blocking group before each coupling reaction. The conditions for removal of the benzyloxycarbonyl group and a benzyl ester are similar, differing only in rate.[14] Consequently it proved impossible to remove the benzyloxycarbonyl group in the presence of benzyl esters with complete selectivity. The γ-methyl ester compounds prepared were more suitable since methyl esters are not cleaved during the removal of the amine blocking group.

Results and Discussion

Synthesis of Peptides.—Both D- and L-glutamic acid were converted to γ-methyl glutamate or its hydrochloride by Fisher esterification under dilute conditions.[15,16] The diethyl ester III[17,18] was easily synthesized by using more concentrated solutions of hydrogen chloride. The γ-methyl glutamate was blocked with the benzyloxycarbonyl group before it was converted to the active *p*-nitrophenyl ester by the technique of Schwyzer.[19] Removal

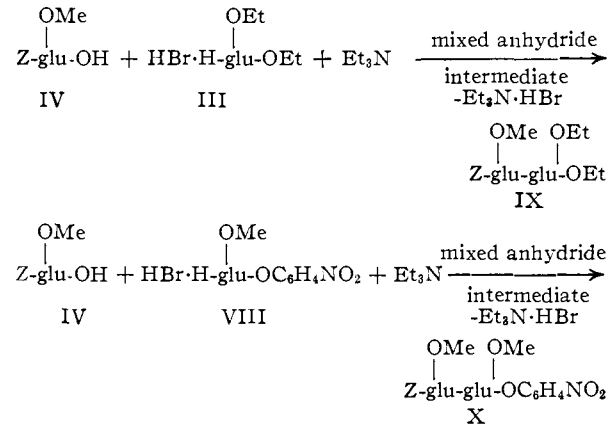


of the N-terminal blocking group from VII afforded the active ester hydrobromide VIII.



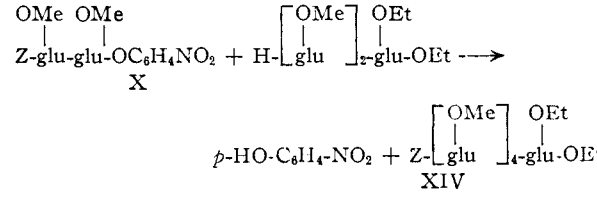
Three general coupling methods were employed to prepare further intermediates and members of the peptide series. They involve the mixed anhydride reactions,[20-22] the active ester procedure[19,23,24] and combinations of the two.[25]

The first coupling technique (referred to in this paper as method A) was employed to synthesize the completely blocked dipeptide and the amine-blocked dipeptide active ester. The latter is an essential intermediate for the preparation of higher peptides of the series



The product in each case was completely blocked, *i.e.*, it contained no free acidic or basic groups and was freed from unreacted starting materials by extraction with dilute acid and aqueous sodium bicarbonate.

The second general coupling procedure (method B) involved the displacement of the dipeptide active ester X by the amine group of an amino acid ester. This procedure was employed to prepare higher molecular weight peptides by first removing[26] the benzyloxycarbonyl group from the completely blocked peptide prepared as above and then utilizing the resulting amino peptide ester as a reagent. This approach has also been employed with excellent results by Bodansky.[24]



Again the final material could be purified by simple extraction techniques. The above procedure was applicable only when the peptide formed was readily soluble in an organic solvent which was immiscible with water. When the peptide was not soluble in the usual extraction solvents such as chloroform, ether or ethyl acetate, another modification, method B′, was used. The

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(7) E. J. Ambrose and A. Elliott, *ibid.*, **A208**, 75 (1951).
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(15) W. E. Hanby, S. G. Waley, J. Watson and E. J. Ambrose, *J. Chem. Soc.*, 3239 (1950).
(16) D. Coleman, *ibid.*, 2294 (1951).
(17) The diethyl ester was obtained and recrystallized to yield a material of higher purity than the corresponding dimethyl ester.
(18) F. Knoop and H. Oesterlin, *Z. physiol. Chem.*, **170**, 186 (1927).
(19) B. Iselin, W. Rittel, P. Sieber and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).
(20) R. A. Boissonnas, *ibid.*, **34**, 874 (1951).
(21) J. R. Vaughan, *J. Am. Chem. Soc.*, **73**, 3547 (1951); J. R. Vaughan and R. L. Osato, *ibid.*, **74**, 676 (1952).
(22) T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).
(23) M. Bodansky, *Nature*, **175**, 685 (1955).
(24) M. Bodansky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(25) M. Goodman and K. C. Stueben, *ibid.*, **81**, 3980 (1959).
(26) The oligomeric peptide hydrobromides were not characterized since they were precipitated as hygroscopic semi-crystalline masses. They were best handled by allowing them to react with the dipeptide active ester X in the presence of an equivalent of triethylamine as soon as they were prepared.

procedure was similar to method B except for the workup. Fortunately, it was found that those peptides which were not soluble in the general extraction solvents were also insoluble in warm ethanol, while all the unreacted starting materials and side products were soluble in this solvent. Thus these higher peptides were obtained in a relatively pure state by precipitation from the reaction mixture by the addition of excess ethanol, filtration and repeated washing with warm ethanol.

The third method of coupling, method C, is actually a combination of method A and method B carried out sequentially in the same reaction flask[25] and was generally used to prepare tripeptides. For higher peptides it was more convenient to isolate a large amount of the dipeptide active ester and proceed according to method B or its modification B′.

The higher peptides were synthesized according to Charts I and II. A large quantity of blocked dipeptide and tripeptide was prepared as already described by methods A and C, respectively. Removal of the benzyloxycarbonyl blocking group from each of these peptides by treatment with hydrogen bromide in glacial acetic acid afforded the hydrobromide which was then allowed to react with the dipeptide active ester X in the presence of an equivalent of triethylamine according to method B. The process could then be repeated utilizing the newly-formed tetra- and pentapeptide yielding the hexa- and heptapeptide derivatives, respectively. Hence, two series of peptides were obtained, each one differing from its closest member by two residues. The series commencing with the dipeptide included the tetramer and hexamer (Chart I) while peptides derived from the tripeptide contained the pentamer, heptamer, nonamer and undecamer (Chart II).

CHART I

SYNTHETIC APPROACH TO THE TETRAMER AND HEXAMER

Z-dipeptide ester IX —(HBr–HOAc)→

dipeptide ester hydrobromide + Z-dipeptide active ester X —(method B)→ Z-tetrapeptide ester XIII

Z-tetrapeptide ester XIII —(HBr–HOAc)→ tetrapeptide ester hydrobromide +

Z-dipeptide active ester X —(method B)→ Z-hexapeptide ester XVI

CHART II

SYNTHETIC APPROACH TO THE PENTAMER, HEPTAMER, ETC.

Z-tripeptide ester XI —(HBr–HOAc)→ tripeptide ester hydrobromide +

Z-dipeptide active ester X —(method B)→ Z-pentapeptide ester XIV —(HBr–HOAc)→

pentapeptide ester hydrobromide + Z-dipeptide active ester X —(method B′)→ Z-heptapeptide ester XVII etc.

Z represents the carbobenzoxy group and "ester" denotes that all carboxyl groups are esterified

During the course of our studies on conformation, it became desirable to obtain the pentapeptide possessing a centrally located D-residue. A tripeptide was synthesized which had a D-L-L configuration by substituting benzyloxycarbonyl-γ-methyl-D-glutamate (V) in place of the "L"-isomer IV. In the subsequent reaction of this tripeptide with the dipeptide active ester X a pentapeptide was produced with a L-L-D-L-L configuration.

A complete list of all the peptides prepared may be found in Table I. Microchemical elemental analyses were carried out on all of the blocked oligomeric peptides and were found to agree with the theoretical values within the experimental limits. Molecular weight determinations of these compounds were carried out in two solvents in which the peptides showed no association. The values are found in Table II, and appear in good agreement with the theoretical and experimental values.

Although the reaction between amine and active ester has been shown to proceed without racemization,[19,24,25] a spot check was made to confirm the optical purity of this series of compounds. The pentamer was hydrolyzed in 6 *N* hydrochloric acid. Under the same conditions, a standard, composed of an equivalent weight of L-glutamic acid, was also prepared. The two resulting solutions were subjected to optical rotation analysis and were found to be essentially identical, indicating that the peptides had maintained over 98% of the L-configuration of glutamic acid during the synthetic process.

Conformation of Peptides.—The specific rotations of the L-configurational oligomeric peptides measured in helix-forming solvents display shifts toward a positive direction after the number of residues exceeds a certain minimum size.[1,4] This shift is expected in light of the positive specific rotations of the high molecular weight analogs in the same solvents. Positive specific rotations begin abruptly with the pentapeptide in dioxane solutions,[4] while positive contributions to the specific rotation gradually appear in the range between the penta- and nonapeptide in *m*-cresol and dimethylformamide.[1] It has also been determined that these peptides aggregate in dioxane solutions but in *m*-cresol and dimethylformamide they are completely unassociated.[1] The b_0 values obtained from the Moffitt plot of rotatory dispersion data measured in dimethylformamide also exhibit a change of sign and magnitude as the size of the oligomeric peptides increases.

Conclusions

The methods described for the preparation of these oligomeric peptides result both in good yields and high chemical and optical purity. The physical data obtained from these peptides[1,4,27] show definite conformational trends at chain lengths consistent with the onset of intramolecular hydrogen bonding. Peptides with four or fewer residues exist only in a random state. Higher membered peptides beginning with the pentapeptide exist in *m*-cresol and dimethylformamide partially in folded forms. In dioxane these folded forms associate. In *m*-cresol and dimethylformamide the folded forms gradually increase in importance as the number of residues in the peptide

(27) M. Goodman, E. E. Schmitt and D. A. Yphantis, *J. Am. Chem. Soc.*, **84**, 1288 (1962).

increases.[1,27] At the nonapeptide, a stabilizing force for the folded forms manifests itself, which we interpret as being caused by the appearance of residues which are doubly intramolecularly hydrogen bonded in the folded form. In a nonapeptide, for example, the fifth residue is hydrogen bonded to both the first and ninth unit. Additional conformational studies on these compounds are contained in the next paper in this series.[27]

Experimental[28]

Materials.—The glutamic acid used in the preparation of these compounds was purchased from Mann Research Laboratories, Inc., New York, N. Y., and was used without further purification. The triethylamine (obtained from Eastman Kodak Co.) was distilled from barium oxide prior to use. Eastman Kodak Co. reagent grade isobutyl chloroformate was employed directly without distillations. All organic solvents were purified by fractional distillation, with the exception of Mallinckrodt anhydrous ether, which was used without further purification or drying.

Preparation of Compounds. γ-Methyl L-glutamate (I) was prepared by the method reported by Hanby, *et al.*[15] Recrystallization was carried out by dissolving the crude material in 3 parts of hot water and adding slowly 7 parts of boiling methanol. The product was obtained in a 33% yield, m.p. 175–176° (lit.[15] m.p. 182°), $[\alpha]^{24.5}D$ +31.87° (*c* 2.0, 0.5 *N* HCl).

γ-Methyl D-glutamate (II) was prepared in a 29% yield exactly as the L-isomer I above; m.p. 175–176°, $[\alpha]^{25}D$ −32.0° (*c* 2.0, 0.5 *N* HCl).

Diethyl L-Glutamate Hydrochloride (III).—Into a suspension of glutamic acid (100 g.) in 1 liter of absolute ethyl alcohol at 0° was bubbled anhydrous hydrogen chloride until all the reagents were in solution. This solution was then reduced to 100 ml. under reduced pressure. Upon addition of 250 ml. of anhydrous ether, the product crystallized from solution. Recrystallization of the diester hydrochloride from methanol–ether afforded 40 g. (34%) of desired product, m.p. 109–110° (lit.[29] m.p. 96–98°, lit.[18] m.p. 107–108°), $[\alpha]^{25.5}D$ +22.5° (*c* 2.0, ethanol) (lit.[29] $[\alpha]^{25}D$ +22.8° (*c* 7, ethanol)).

Anal. Calcd. for $C_9H_{18}NO_4Br$: C, 45.09; H, 7.62; N, 5.85. Found: C, 45.15; H, 7.62; N, 5.97.

Benzyloxycarbonyl-γ-methyl L-glutamate (IV) was prepared according to the procedure of Hanby, *et al.*[15] The crude reaction product, obtained in 61% yield as an oil, was purified by converting the free acid to the piperazonium salt, m.p. 212°, by the method of Prigot and Pollard.[30] The salt (22 g.), after recrystallization from methanol–ether, was suspended in 200 ml. of a 50% mixture of 1 *N* HCl–ether and shaken until the salt dissolved. The aqueous layer was extracted with ether. The ether washings were dried over magnesium sulfate and evaporated leaving 19.6 g. (95%) of a thick oil. Upon standing, the product crystallized, m.p. 76° (lit.[15] m.p. 72–73°), $[\alpha]^{25}D$ −16.23° (*c* 3.0, 1 *N* $KHCO_3$) (lit.[15] $[\alpha]^{25}D$ −15.3° (*c* 7.5, 1.4 *N* $KHCO_3$)).

Benzyloxycarbonyl-γ-methyl D-glutamate (V) was prepared by a similar procedure as for the L-isomer IV. The piperazonium salt, m.p. 205–209°, was decomposed and worked up as above to leave an oil (69% yield) which slowly crystallized in the form of needles, m.p. 65–70° (lit.[15] m.p. 72–73°), $[\alpha]^{25}D$ +15.7° (*c* 2.0, 1 *N* $KHCO_3$) (lit.[15] $[\alpha]^{25}D$ +15.3° (*c* 7.5, 1.4 *N* $KHCO_3$)).

Tris-(*p*-nitrophenyl)-phosphite (VI) was prepared according to the procedure of Strecker and Grossman[31] by the reaction of *p*-nitrophenol with phosphorus trichloride. The product was prepared in 44% yield and recrystallized from hot toluene; m.p. 167–169° (lit.[31] m.p. 170–171°).

Benzyloxycarbonyl-α-*p*-nitrophenyl-γ-methyl L-Glutamate (VII).—To 10 ml. of pyridine was added with stirring benzyloxycarbonyl-γ-methyl L-glutamate (IV) (6.6 g., 0.0223 mole) and tris-(*p*-nitrophenyl)-phosphite (VI) (6.0 g., 0.0135 mole). The reaction was allowed to proceed at room temperature for 3 hours. The reaction mixture was diluted with 200 ml. of chloroform and washed three times each with 1 *N* hydrochloric acid, saturated sodium chloride and 10% sodium bicarbonate. After the chloroform solution was dried over magnesium sulfate, it was evaporated to dryness. The solid residue was crystallized from hot ethanol furnishing 5.6 g. (63%) of product in the form of white needles, m.p. 108°, $[\alpha]^{25.5}D$ −33.8° (*c* 2.0, dimethylformamide).

Anal. Calcd. for $C_{20}H_{20}N_2O_8$: C, 57.69; H, 4.84; N, 6.73. Found: C, 57.75; H, 4.97; N, 6.73.

α-*p*-Nitrophenyl-γ-methyl L-Glutamate Hydrobromide (VIII).—To 10 ml. of an anhydrous saturated solution of hydrogen bromide in glacial acetic acid was added benzyloxycarbonyl-α-*p*-nitrophenyl-γ-methyl L-glutamate (VII) (8.0 g., 0.00199 mole). After standing for 20 minutes the product began to precipitate from the reaction mixture. The solid was washed with ether and filtered. The hydrobromide was recrystallized from hot chloroform–ether and then from methanol–ether, giving white needles weighing 5.5 g. (79%), m.p. 179°, $[\alpha]^{25}D$ +23.8° (*c* 1.7, ethanol).

Anal. Calcd. for $C_{12}H_{15}N_2O_6Br$: C, 39.80; H, 4.16; N, 7.73. Found: C, 40.03; H, 4.23; N, 7.57.

Benzyloxycarbonyl-γ-methyl-L-glutamyl Diethyl L-Glutamate (IX). **Method A.**—To a solution of benzyloxycarbonyl-γ-methyl L-glutamate (IV) (1.22 g., 0.0041 mole) in 20 ml. of chloroform chilled to 0° was added in succession triethylamine (0.56 ml., 0.0041 mole) and isobutyl chloroformate (0.56 ml., 0.0041 mole). After 20 minutes diethyl L-glutamate hydrochloride (III) (0.868 g., 0.0036 mole) was added followed by the dropwise addition of triethylamine (0.56 ml., 0.0041 mole). After 5 hours the reaction mixture was washed three times each with 1 *N* hydrochloric acid, saturated potassium chloride and 10% sodium carbonate. After drying over magnesium sulfate and evaporation, the product was recrystallized from hot ethanol affording a material which gave, after drying *in vacuo*, 1.2 g. (69%) of dipeptide, m.p. 86°, $[\alpha]^{25}D$ −5.1° (*c* 2.0, dioxane).

Anal. Calcd. for $C_{23}H_{32}O_9N_2$: C, 57.49; H, 6.71; N, 5.83. Found: C, 57.84; H, 6.67; N, 5.99.

Benzyloxycarbonyl-γ-methyl-L-glutamyl-α-*p*-nitrophenyl-γ-methyl L-Glutamate (X).—To a chilled solution of benzyloxycarbonyl-γ-methyl L-glutamate (IV) (4.43 g., 0.015 mole) in 50 ml. of dimethylformamide was added triethylamine (2.05 ml., 0.015 mole) followed by isobutyl chloroformate (2.05 ml., 0.015 mole). After 20 minutes, the temperature being maintained at 0°, α-*p*-nitrophenyl-γ-methyl L-glutamate hydrobromide (VIII) (5.42 g., 0.015 mole) was added together with triethylamine (2.05 ml., 0.015 mole). The reaction was allowed to proceed for 5 hours in which time the ambient temperature was reached. The dimethylformamide solution was diluted with chloroform and washed with 2 *N* hydrochloric acid, saturated potassium chloride, and then extracted repeatedly with 10% sodium bicarbonate until the aqueous wash was almost colorless. After drying the organic layer over magnesium sulfate, it was evaporated to dryness, giving 8 g. of a solid mass. Recrystallizing from hot chloroform–ethanol gave 3.80 g. (45%) of product in the form of needles, m.p. 161°, $[\alpha]^{25.5}D$ −32.6° (*c* 2.0, dimethylformamide).

Anal. Calcd. for $C_{26}H_{29}N_3O_{11}$: C, 55.82; H, 5.22; N, 7.51. Found: C, 55.99; H, 5.23; N, 7.25.

Benzyloxycarbonyl-di-(γ-methyl-L-glutamyl) Diethyl L-Glutamate (XI). **Method C.**—To a solution of benzyloxycarbonyl-γ-methyl L-glutamate (IV) (5.70 g., 0.0193 mole) in 60 ml. of dimethylformamide chilled to 0° was added consecutively triethylamine (2.65 ml., 0.0193 mole) and isobutyl chloroformate (2.64 ml., 0.0193 mole). After 20 minutes α-*p*-nitrophenyl-γ-methyl L-glutamate hydrobromide (VIII) (7.0 g., 0.0193 mole) was added followed by the addition of triethylamine (2.65 ml., 0.0193 mole). After 5 hours, diethyl L-glutamate hydrochloride (III) (4.08 g., 0.0171 mole) was added, followed by the addition of triethylamine (2.65 g., 0.0193 mole) in three portions over an hour period. The reaction was allowed to proceed for 12 hours, at which time the reaction mixture was diluted with 200 ml. of ethyl acetate and washed with 2 *N* hydrochloric acid. The ethyl acetate layer was further washed with 10% sodium carbonate until the aqueous layer remained colorless, then was dried over magnesium sulfate,

(28) All melting points are corrected. Analyses are by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

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(30) M. Prigot and C. B. Pollard, *ibid.*, **70**, 2758 (1948).

(31) W. Strecker and C. Grossman, *Ber.*, **49**, 87 (1916).

TABLE I

SUMMARY OF PHYSICAL DATA OF PREPARED OLIGOMERIC PEPTIDES DERIVED FROM γ-METHYL GLUTAMATE[a]

Formula	Peptide	Configuration	M.p., °C.	Prepared by method	Yield, %	Elemental analyses, % Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
IX	Di-	(All L)	86	A	69	57.49	6.71	5.83	57.84	6.67	5.99
XI	Tri-	(All L)	124	C	87	55.85	6.63	6.74	56.04	6.35	6.84
XII	Tri-	(DLL)	101	C	78	55.85	6.63	6.74	56.06	6.35	7.41
XIII	Tetra-	(All L)	139	B	74	54.32	6.49	7.66	54.82	6.57	7.31
XIV	Penta-	(All L)	200	B	78	54.12	6.54	7.70	54.09	6.49	8.07
XV	Penta-	(LLDLL)	161	B	86	54.12	6.54	7.70	54.25	6.27	8.55
XVI	Hexa-	(All L)	250	B	56	53.61	6.51	7.98	53.59	6.50	7.85
XVII	Hepta-	(All L)	259	B′	79	53.20	6.49	8.21	53.57	6.09	8.72
XVIII	Nona-	(All L)	Chars	B′	85	52.66	6.46	8.51	52.18	6.25	8.49
XIX	Undeca	(All L)	Chars	B′	76	52.28	6.44	8.71	51.95	6.42	8.75

[a] For optical activity data see ref. 27.

and then evaporated to a solid residue. The 9.7 g. of crude material was recrystallized from hot ethanol and gave, after drying, 8.7 g. (87%) of tripeptide, m.p. 114°, $[\alpha]^{25}D$ −16.9° (*c* 2.0, dioxane).

Anal. Calcd. for $C_{29}H_{41}N_3O_{12}$: C, 55.85; H, 6.63; N, 6.74. Found: C, 55.67; H, 6.41; N, 7.12.

Benzyloxycarbonyl-γ-methyl-D-glutamyl-γ-methyl-L-glutamyl-diethyl L-glutamate (XII) was also prepared according to method C by coupling the following reactants in the order and manner described above: benzyloxycarbonyl-γ-methyl D-glutamate (V) (0.527 g., 0.00179 mole), α-*p*-nitrophenyl-γ-methyl L-glutamate hydrobromide (VIII) (0.700 g., 0.00179 mole), diethyl L-glutamate hydrochloride (III) (0.428 g., 0.00179 mole). Recrystallization of the crude material from ethanol and then from ethyl acetate–petroleum ether afforded 0.870 g. (78%) of product, m.p. 97–101°, $[\alpha]^{25}D$ −9.9° (*c* 2.0, dioxane).

Anal. Calcd. for $C_{29}H_{41}N_3O_{12}$: C, 55.85; H, 6.63; N, 6.74. Found: C, 56.06; H, 6.35; N, 7.31.

Benzyloxycarbonyl-tetra-(γ-methyl-L-glutamyl)-diethyl L-Glutamate (XIV). Method B.—To 10 ml. of dry dimethylformamide was added with stirring benzyloxycarbonyl-γ-methyl-L-glutamyl-α-*p*-nitrophenyl-γ-methyl L-glutamate (X) (0.860 g., 0.0014 mole), di(-γ-methyl-L-glutamyl)-diethyl L-glutamate hydrobromide[32] (0.780 g., 0.0014 mole) and finally triethylamine (0.20 ml., 0.0014 mole). The reaction was allowed to proceed for 12 hours. The reaction mixture was diluted with 250 ml. of ethyl acetate and extracted three times each with 2 *N* hydrochloric acid, saturated potassium chloride, and then extracted repeatedly with 10% sodium carbonate until the aqueous wash was colorless (as many as twenty washings were often needed). Upon evaporation there was obtained 1.20 g. of neutral product. This material was recrystallized from 10 ml. of ethanol. The precipitated product was filtered, washed with petroleum ether and dried at 56° under vacuum. There was obtained 1.020 g. (78%) of product, m.p. 196–200°, $[\alpha]^{25}D$ −21.3° (*c* 2.0, dichloroacetic acid). Further recrystallizations from ethanol failed to raise the melting point or change the specific rotation in dichloroacetic acid.

Anal. Calcd. for $C_{41}H_{59}N_5O_{18}$: C, 54.12; H, 6.54; N, 7.70. Found: C, 54.09; H, 6.49; N, 8.07.

Benzyloxycarbonyl-di(-γ-methyl-L-glutamyl)-γ-methyl-D-glutamyl-γ-methyl-L-glutamyl-dimethyl L-glutamate (XV) was prepared by a procedure similar to that described for the preparation of the all-L isomer XIV. The tripeptide hydrobromide used in the preparation was obtained from the benzyloxycarbonyl tripeptide XI in a similar manner to that described above. Recrystallization from ethyl alcohol and ethyl acetate–petroleum ether afforded the product in 86% yield, m.p. 161–162°, $[\alpha]^{25}D$ −13.93° (*c* 1.7, dioxane).

Anal. Calcd. for $C_{41}H_{59}N_5O_{18}$: C, 54.12; H, 6.54; N, 7.70. Found: C, 54.25; H, 6.27; N, 8.55.

It was found that this material could be recrystallized from a minimum of hot ethyl acetate to yield a crystalline material, m.p. 188–190°, $[\alpha]^{25}D$ −13.82° (*c* 1.00, dioxane), which had an infrared absorption pattern (both KBr and solution spectra) identical with the above material.

Anal. Calcd. for $C_{41}H_{59}N_5O_{18}$: C, 54.12; H, 6.54; N, 7.70. Found: C, 53.91; H, 6.35; N, 8.41.

Benzyloxycarbonyl-octa(-γ-methyl-L-glutamyl)-diethyl L-Glutamate (XVIII). Method B′.—To 3 ml. of dimethylformamide was added benzyloxycarbonyl-γ-methyl-L-glutamyl-α-*p*-nitrophenyl-γ-methyl L-glutamate (X) (0.327 g., 0.00055 mole), hexa(-γ-methyl-L-glutamyl)-diethyl L-glutamate hydrobromide[32] (0.662 g., 0.00055 mole) and triethylamine (0.077 ml. 0.00055 mole) in the order and manner described previously. During a 15-hour interval, the reaction mixture slowly became more and more viscous and eventually formed a gel. The gel was broken up mechanically in warm ethanol. The product was then filtered and ground up in petroleum ether in order to make the particle size as small as possible. The remaining *p*-nitrophenol and triethylamine hydrobromide was removed by triturating the product with warm ethanol. The product was filtered, washed with ether and dried, affording 0.80 g. (85%) of the nonapeptide, $[\alpha]^{25}D$ −32.4° (*c* 2.0, dichloroacetic acid). This material was dissolved in 100 ml. of warm glacial acetic acid. The resulting solution was filtered through a Celite filter bed and lyophilized. The process was repeated using dioxane as a solvent. After lyophilization, 0.704 g. (75%) of product, $[\alpha]^{25}D$ −32.06° (*c* 3.0, dichloroacetic acid), was obtained.

Anal. Calcd. for $C_{65}H_{95}N_9O_{30}$: C, 52.66; H, 6.46; N, 8.51. Found: C, 52.18; H, 6.25; N, 8.49.

Check of Optical Purity of Compounds. Degradation of Benzyloxycarbonyl-tetra-(γ-methyl L-glutamyl)-diethyl L-Glutamate (XIV).—A suspension of the pentapeptide (XIV) (0.091 g., 0.0001 mole) in 7.00 ml. of 6 *N* hydrochloric acid was sealed in a glass tube under nitrogen. A standard solution consisting of L-glutamic acid (0.0736 g., 0.0005 mole) in 7.00 ml. of 6 *N* hydrochloric acid also was enclosed in a similar manner. Both tubes were heated for 14 hours at 100° in a water-bath. After this interval both tubes were cooled and opened. The optical rotations of both solutions were measured at the sodium D line (589 mμ). The tube which originally contained the pentapeptide (0.0001 mole) and which was converted to a solution of glutamic acid hydrochloride (0.0005 mole) had an observed rotation of +0.658°, while the standard solution of L-glutamic acid hydrochloride (0.0005 mole) had an observed rotation of +0.684°. The specific rotations of these solutions were found to be +31.39° and +32.53°, respectively (lit.[33] +30.36° (aq. HCl)).

Molecular Weight Determinations.—Weight average molecular weights were determined using a short column

(32) Both the tetramer and hexamer were prepared by similar procedures from the di- and tetrapeptide hydrobromides, respectively. Pertinent data are recorded in Table I. In all these cases the hydrobromide was prepared just prior to use by treating the benzyloxycarbonyl derivative with an equal weight of saturated acetic acid solution of hydrogen bromide for 30 minutes. The peptide hydrobromide was precipitated by adding an excess of ether. It was precipitated from chloroform–ether and then twice from methanol–ether. The hydrobromide was once more dissolved in methanol, filtered through an activated charcoal–Celite filter bed into a tared flask. The solution was stripped of its solvent leaving the hydrobromide which was further dried *in vacuo* for 1 hour before its weight was determined. This weighing determined the proportional quantities of the other reagents employed in the reaction.

(33) M. Bergmann and H. Ensslin, *Ber.*, **58**, 1042 (1925).

TABLE II

WEIGHT AVERAGE MOLECULAR WEIGHTS BY EQUILIBRIUM ULTRACENTRIFUGATION OF OLIGOMERIC PEPTIDES DERIVED FROM γ-METHYL-L-GLUTAMATE IN DICHLOROACETIC ACID AND DIMETHYLFORMAMIDE

Peptide	Formula weight	Dichloroacetic acid Concn. range, % w./v.	Dichloroacetic acid Mol. wt.	Dimethylformamide Concn. range, % w./v.	Dimethylformamide Mol. wt.
Tri-	624				
Tetra-	767	3	760 ± 50		
Penta-	910	1.5–3	930 ± 85		
Hexa-	1053				
Hepta-	1196	0.5–3	1070 ± 95	0.5–2	1240 ± 70
				.8	1142 ± 70*
Nona-	1483	0.75–1.5	1440 ± 200	.3–1.4	1480 ± 70
				.3–1.0	1460 ± 130*
Undeca-	1769			.8–1.5	1890 ± 190

equilibrium ultracentrifugation technique.[34] A model E ultracentrifuge was used, equipped with phase plate, Rayleigh interference optics and temperature control. The multichannel short column cells were machined of Kel-F, as were the single channel synthetic boundary cells of the Kegeles type.[35] With dichloroacetic acid (DCA) as solvent the term $(1 - \bar{V}\rho)$, where $\bar{V}$ is the partial specific volume of the solute and ρ is the density of the solution, was negative. Accordingly, the solutions were less dense than solvent and it was essential to layer solution on top of solvent in these synthetic boundary runs. Also, the oligomers flotated rather than sedimented; the obvious modifications in technique were made. Fluorocarbon FC-43[36] was used as base fluid since it was only sparingly soluble in the solvents. A few runs were made using long columns (~ 3 mm. column height); in these runs three-place double channel cells of Kel-F[37] were used and the interferometric techniques of Richards and Schachman[38] were employed for the measurements. All equilibrium runs were performed at room temperature and at 39,460 r.p.m.; at this speed it was found helpful in obtaining good interference records to use fine aperture masks (0.020″) and either very fine interference slits (0.007″) or light source slits (<0.001″). Equilibrium was attained within 2 hours with the short (~0.7 mm.) columns even with DCA. The longer (~3 mm.) columns were run for about 18 hours. Solvent densities were interpolated from data in the "International Critical Tables." An estimate of the partial specific volumes of the oligomers in water by the methods of Traube[39] and Cohn, *et al.*,[40,41] gave values ranging from 0.72 to 0.74. In the less polar solvents used here it is expected that the volumes would probably be somewhat higher. Accordingly, the value 0.75 was used; this value provided good agreement between the values of the molecular weights in the two solvents. The values of the molecular weights are quite sensitive to a choice of $\bar{V}$, particularly with DCA as solvent: the molecular weights using $\bar{V} = 0.72$ were approximately 38% higher in DCA and about 10% lower in dimethylformamide (DMF) than the values presented. The possible differences in compressibilities between solvents and solutes were neglected since at the middle of the short columns the pressure was less than 8 atmospheres.

No regular concentration dependence was observed; therefore the molecular weights found for the various concentrations in a given solvent were averaged. The results are given in Table II where the second column lists the formula weights, the third column the concentration ranges used in DCA and the fourth column the average values of the molecular weights found in DCA and their standard deviations. Similarly, columns five and six give the concentration ranges and molecular weights in DMF. The starred values were obtained using the longer columns of solution.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN, N. Y., AND THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

Conformational Aspects of Polypeptides. IV.[1] Folded and Associated Forms of Oligomeric Peptides Derived from γ-Methyl Glutamate

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This report presents the results of studies on the conformational characteristics of oligomers based on γ-methyl glutamate in solution. Dichloroacetic acid solvates the peptide chain yielding a "random coil." Dimethylformamide and *m*-cresol allow intramolecular hydrogen bonds to form, beginning in the range of the pentamer through the nonamer. The results for the pentamer and higher in dioxane are interpreted as a combination of intramolecular hydrogen bonding and association. The temperature dependence of the optical activity for the entire oligomeric series in dimethylformamide is explained by a helix–random coil transition. The differences between oligomers and high polymers are discussed as are association phenomena and infrared spectra of the peptides.

Introduction

In the past decade much has been learned about the conformations[4] of native proteins and synthetic polypeptides. The conformational structures were studied *via* a number of techniques which include optical rotatory properties,[5–18] infrared spectros-

(1) Previous paper in this series, M. Goodman, E. E. Schmitt and D. A. Yphantis, *J. Am. Chem. Soc.*, **84**, 1283 (1962).

(2) Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the Polytechnic Institute of Brooklyn.

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(4) The terms *configuration* and *conformation* have been used interchangeably by workers in this field for the description of the secondary structures possible for peptide chains. We have adopted a convention which has received support elsewhere; namely, to reserve the use of *configuration* to its original sense, *i.e.*, the spatial relationship of the asymmetric carbon atoms, and to let the word *conformation* refer only to the secondary structure (see for example, ref. 11, p. 239).

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