Synthesis of Hepta-γ-L-glutamic Acid by Conventional and Solid-Phase Techniques[1,2]

JOHANNES MEIENHOFER[3] AND PAULA M. JACOBS

The Children's Cancer Research Foundation and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

HERMAN A. GODWIN AND IRWIN H. ROSENBERG[4]

The Thorndike Memorial Laboratory, Harvard Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

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Syntheses of hepta-γ-L-glutamic acid by the solid-phase method and of the protected peptides of oligo-γ-glutamic acid (two to seven residues) by conventional techniques are described. The solid-phase synthesis of hepta-γ-L-glutamic acid was judged inadequate; electrophoretic analysis showed contaminating lower peptides of glutamic acid which were not removed by any of the column chromatography systems investigated. For the conventional synthesis, the carbonic acid–carboxylic acid mixed anhydride procedure was superior to the pentachlorophenyl ester, the *N*-hydroxysuccinimide ester, and the carbodiimide coupling methods. The crystalline benzyloxycarbonyloligo-γ-L-glutamic acid *tert*-butyl esters were obtained in good yields.

Pteroyl-poly-γ-L-glutamates with up to seven constituent glutamic acid residues are the major forms of folic acid, **1** (vitamin B_c[5,6]), in natural sources. The chemical characterization of isolated folates, which are usually mixtures of several closely related compounds,[7] generally has been insufficient; however, the major folate component in yeast has been reported to be pteroyl-hepta-γ-glutamate.[8]

Studies on the uptake and the nutritional availability of pteroyl-polyglutamates have been impeded by the lack of well-defined compounds for human and *in vitro* investigations.[7,9] We have, therefore, synthesized chemically uniform derivatives of folic acid with different numbers of γ-linked L-glutamic acids. The solid-phase synthesis of polyglutamates of folic acid has been reported recently.[10] This paper describes the preparation of the γ-linked peptides of L-glutamic acid, with two to seven residues, by conventional techniques. The synthesis of folate derivatives will be reported elsewhere.

In the initial attempt to synthesize hepta-γ-L-glutamic acid, the solid-phase method[11] was used because of its speed and convenience. *tert*-Butyloxycarbonyl-L-glutamic acid α-benzyl ester[12,13] was esterified to the polystyrene resin through the γ-carboxyl group. The standard procedure of Marshall and Merrifield[14] was used for the peptide chain elongation, with cycles of washing, deprotection of the amino group, and coupling of another residue to the chain. Removal of the *tert*-butyloxycarbonyl group was accomplished by treatment with 4 *N* hydrogen chloride in dioxane. Subsequent glutamic acid residues were added by coupling *tert*-butyloxycarbonyl-L-glutamic acid α-benzyl ester (threefold excess) with dicyclohexylcarbodiimide (DCCI)[15] to the resin-bound peptide. Treatment of the completed peptide resin with anhydrous hydrogen fluoride[16] gave a quantitative yield of crude peptide.[17]

High voltage paper electrophoresis of the crude hepta-γ-L-glutamic acid showed some contamination by each of the lower homologs.[18] Column chromatography on DEAE–cellulose, CM–cellulose, and CM–Sephadex with a variety of gradients gave only partial separation: for example, removal of glutamic acid, dipeptide, and tripeptide from the unseparated tetra-, penta-, hexa-, and heptapeptides. High voltage electrophoresis of the column eluates (Table I) revealed that pure heptapeptide was not obtained even in single tubes from the extremes of a peak. Chromatography on Sephadex G-10 or G-15 allowed the separation of the smaller components from the remaining mixture of hepta-, hexa-, and pentapeptides. Since high voltage paper electrophoresis remained the sole technique for a complete separation of all components of the mixtures obtained by the solid-phase synthesis,[19] only analytical quantities of purified penta-, hexa-, and heptaglutamates could be isolated. The purpose of this work, however, the preparation of well-defined poly-γ-glu-

(1) Abbreviations follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature, in *Biochemistry*, **5**, 2485 (1966); **7**, 483 (1968); *J. Biol. Chem.*, **241**, 2491, 2991 (1966).

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(3) To whom correspondence should be addressed.

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(17) Krumdieck and Baugh[10] reported unacceptably low yields in this reaction sequence.

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(19) Unless the yield at each step in a solid-phase synthesis is 100%, by-products will be obtained which are closely related to the desired product; purification of a specific peptide may be difficult or impossible with available techniques.

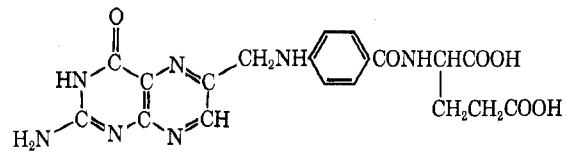
TABLE I
ELECTROPHORETIC ANALYSES OF COLUMN ELUATES

Peptide synthesis method	Column type (no. of runs)	Maximum separation achieved (measured by high voltage electrophoresis)
Merrifield	DEAE–cellulose (5)	Mixture of penta-, hexa-, and heptapeptide
Merrifield	CM–cellulose (3)	Mixture of hexa- and heptapeptide
Merrifield	CM–Sephadex G-25 (1)	Mixture of hexa- and heptapeptide
Merrifield	Sephadex G-15 (2)	Mixture of hexa- and heptapeptide
Merrifield	Sephadex G-10 (10)[a]	Mixture of penta-, hexa-, and heptapeptide
Conventional	Sephadex G-10	Heptapeptide

[a] Sephadex G-10 was often employed as an initial step to eliminate mono-, di-, tri-, and tetrapeptide before the remaining mixture was applied to either DEAE- or CM–cellulose.

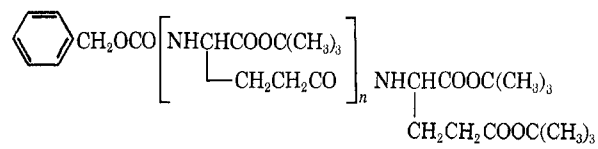
tamates in large enough quantities for extensive biological testing of their pteroyl derivatives, had not been accomplished. We decided, therefore, to use conventional peptide synthesis in solution. For the actual synthesis, more time was required, but well-characterized, homogeneous, crystalline protected intermediates were obtained after each step of this linear chain elongation procedure.

In the synthesis of the peptides **2** (n = 1–6), the *tert*-butyl ester group[20] was chosen for carboxyl protection, rather than the methyl or benzyl esters, to avoid the danger of γ to α transpeptidation.[21,22] The benzyloxycarbonyl group[23] was used for temporary amine protection and was removed by catalytic hydrogenation before chain extension. For the formation of the peptide bond, the active ester and mixed anhydride methods were successfully used; the dicyclohexylcarbodiimide method was abandoned when considerable amounts of the *N*-acyl-*N*,*N*′-dicyclohexylurea of benzyloxycarbonylglutamic acid α-*tert*-butyl ester (**3**) were formed. The active esters of **3** were more successful but not without disadvantages. Benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester γ-pentachlorophenyl ester required 2-day reaction periods at 45–50°. The protected peptides crystallized only after the complete removal of pentachlorophenol by column chromatography on Sephadex LH-20. Benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester γ-*N*-hydroxysuccinimide ester (**4**) was not stable for long periods of time, making it somewhat inconvenient for a continuing synthesis with the same intermediate. The best results were obtained with the mixed carboxylic acid–carbonic acid anhydride between **3** and isobutyl chloroformate:[24] it was fast, the protected peptides generally crystallized after a simple work-up, and the yields were higher. The yields and physical constants of the peptides prepared by the active ester and the mixed anhydride methods are given in Table II.



1

folic acid, pteroylglutamic acid



2

A sample of the protected heptapeptide was deprotected by treatment with anhydrous trifluoroacetic acid for 15 min, followed by catalytic hydrogenation. After desalting on Sephadex G-10, the peptide was homogeneous by high voltage electrophoresis (Table I) and gave a correct elemental analysis.

Materials and Methods.—Chloromethylated copolystyrene–2% divinylbenzene was obtained from Cyclo Chemical Corp., Los Angeles, Calif. Hydrogen fluoride was purchased from Matheson Gases, East Rutherford, N. J. DEAE–cellulose Whatman 32 and CM–cellulose Whatman 23 were purchased from Reeve Angel, Clifton, N. J., and precycled as recommended by the company. CM–Sephadex and Sephadex G-10, G-15, and LH-20 were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Absolute ethanol was purchased from U. S. Industrial Chemicals, New York, N. Y. Triethylamine (TEA) was distilled from and stored over sodium wire. Dioxane was distilled from sodium and then from lithium aluminum hydride and stored over sodium wire. Dimethylformamide (DMF) was distilled under vacuum and stored over Linde Molecular Sieve 4A. Methylene chloride was distilled and stored over Linde Molecular Sieve 4A. Dicyclohexylcarbodiimide (DCCI) was distilled under vacuum and stored at 4°. *N*-Methylmorpholine was distilled from ninhydrin, then from sodium, and stored over sodium. Amino acid analyses were done on a Phoenix analyzer Model M-6800. The high voltage electrophoresis apparatus was Model D, Gilson Medical Electronics, Middleton, Wis. Melting points were taken on a Mel-Temp apparatus from Laboratory Devices, Cambridge, Mass., and are corrected. Optical rotations were taken on a Bellingham & Stanley Ltd., London, England, manual polarimeter. Microanalyses were done by Werby Laboratories, Boston, Mass.

Solid-Phase Synthesis. *tert*-Butyloxycarbonyl-γ-L-glutamyl Resin α-Benzyl Ester.—Chloromethylated polymer (10 g, 1.04 mequiv of Cl/g), *tert*-butyloxycarbonyl-L-glutamic acid α-benzyl ester[12] (7 g, 20.8 mmol), and triethylamine (2.1 g, 20.8 mmol) were stirred mechanically in refluxing benzene for 24 hr. The resin was collected by filtration and washed successively with ethyl acetate, ethanol, methanol, water, methanol, ethanol, and ether (two 30-ml portions each), and dried overnight under high vacuum, yield 10.9 g.

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TABLE II

PROPERTIES OF *N*-BENZYLOXYCARBONYL OLIGOGLUTAMIC ACID α-*tert*-BUTYL ESTERS

Compd	Registry no.	Method	% yield	Mp, °C	[α]D (temp),[a] °C	% C Calcd	% C Found	% H Calcd	% H Found	% N Calcd	% N Found
Z-Glu$_2$(O-*tert*-Bu)$_3$	26308-92-9	OPcp[b]	66	Oil	−26.7 (23)	62.27	62.12	8.01	8.13	4.84	4.67
		MA[c]	82	83–84	−25.4 (22)	62.27	62.08	8.01	7.89	4.84	4.88
Z-Glu$_3$(O-*tert*-Bu)$_4$	26308-93-0	OPcp	71	79.5–81	−28.9 (23)	61.32	61.59	8.05	8.05	5.50	5.51
		MA	75	77.5–79	−30.2 (21)	61.32	61.29	8.05	8.08	5.50	5.63
Z-Glu$_4$(O-*tert*-Bu)$_5$	26308-94-1	OSu[d]	59	84.5–85	−31.3 (23)	60.74	61.13	8.07	8.22	5.90	5.80
		MA	75	87–89	−33.2 (22)	60.74	60.62	8.07	8.31	5.90	6.02
Z-Glu$_5$(O-*tert*-Bu)$_6$	26358-48-5	OSu	70	102–103.5	−36.8 (23) −17.5 (21)	60.35	60.64	8.08	8.20	6.17	6.08
		MA	95	75–100	−37.5 (21)	60.35	60.54	8.08	8.05	6.17	6.27
Z-Glu$_6$(O-*tert*-Bu)$_7$	26308-95-2	OPcp	67	115–118	−39.5 (23)	60.07	59.88	8.09	8.17	6.37	6.35
		MA	71	123–125	−39.9 (22.5)	60.07	59.97	8.09	8.34	6.37	6.42
Z-Glu$_7$(O-*tert*-Bu)$_8$	26358-49-6	OPcp	32	95–100	−42.0 (23)	59.86	59.56	8.10	8.08	6.52	6.60
		MA	70	(90) 130–132	−43.7 (21)	59.86	59.53	8.10	7.98	6.52	6.55

[a] *c* 1, in methanol. [b] Pentachlorophenyl ester. [c] Mixed carbonic acid–carboxylic acid anhydride. [d] *N*-Hydroxysuccinimide ester.

Hepta-γ-L-glutamyl Resin Hepta-α-benzyl Ester.—*tert*-Butyloxycarbonyl-γ-L-glutamyl resin α-benzyl ester (10.9 g) was placed in a custom-built glass apparatus and preswollen with 70 ml of dioxane for 30 min. The vessel was mechanically rocked to suspend the resin. A cycle for the incorporation of one amino acid in the growing peptide chain consisted of (1) two 50-ml portions of dioxane, (2) 50 ml of 4 *N* hydrogen chloride in dioxane, 30 min, (3) three 50-ml portions of dioxane, (4) three 50-ml portions of ethanol, (5) three 50-ml portions of dioxane, (6) one 50-ml portion of DMF, (7) 50-ml of DMF and 5 ml of TEA, 10 min, (8) four 50-ml portions of DMF, (9) 3.37 g of Boc-Glu-OBzl in 40 ml of DMF, 10 min, followed by 2.06 g of DCCI in 10 ml of methylene chloride, 4 hr, (10) three 50-ml portions of methylene chloride. In the first cycle the washings from steps 7 and 8 were reserved, diluted with water, and titrated[25] with 0.1 *N* silver nitrate (dichlorofluorescein indicator) to give 3.88 mequiv of chloride (0.361 mequiv/g), equal to the amount of free amine on the resin.

At the end of six cycles the resin was washed with three 35-ml portions each of methylene chloride, dioxane, ethanol, methylene chloride, ethanol, and ether, and dried 3 days *in vacuo* to give 15.92 g. Amino acid analysis[26] (8.3 mg, 6 *N* HCl, 110°, 24 hr) gave 1.91 mmol glutamic acid/g resin.

Intermediate Peptide Resins.—Another synthesis was done as above; 1–2 g of resin was removed at the tripeptide, pentapeptide, and hexapeptide stage to provide reference compounds.

Hepta-γ-L-glutamic Acid.—The peptide was cleaved from the resin in 1–2-g batches with anhydrous hydrogen fluoride (7–10 ml) at 0° in the presence of a tenfold molar excess of anisole using a simple polyethylene apparatus.[27] After evaporation of the hydrogen fluoride, the resin was washed with ether to remove the anisole and with water or 1 *N* NH_4OH to remove the peptide. Lyophilization gave a hygroscopic white solid in over 100% yield. High voltage electrophoresis on Whatman 3 MM paper at 3500 V, pH 2.1 (formic acid–acetic acid–water, 25:87:888), for 1 hr showed seven ninhydrin positive spots: R_f (relative to glutamic acid, toward cathode) 0.32, 0.37, 0.41, 0.45, 0.51, 0.72, 1.0. The two slowest moving spots (hepta- and hexaglutamate) were the most intense and slightly overlapping, but were separated after prolonged electrophoresis.

Tri-γ-L-glutamic Acid, Penta-γ-L-glutamic Acid, and Hexa-γ-L-glutamic Acid.—The tri-, penta-, and hexapeptide resins obtained from the second synthesis were cleaved as above and subjected to electrophoresis simultaneously with the heptapeptide. Each contained one major spot and other minor spots. The tripeptide preparation had three spots matching the three fastest spots of the heptapeptide preparation; the pentapeptide spots matched the five fastest spots; the hexapeptide spots matched the six fastest spots.

Chromatography of Hepta-γ-L-glutamic Acid.—The following chromatographic columns failed to separate the desired hepta-γ-L-glutamic acid from the contaminants, as judged by electrophoresis: (1) DEAE–cellulose in column lengths from 30–120 cm with various linear gradients of sodium chloride or ammonium acetate from 0.05 *M* to 1.5 *M* at pH 6.8 and at various flow rates; (2) CM–Sephadex G-25, 80 cm, eluted with 0.1 *M* formic acid pH 2.1 followed by a gradient from 0.1 *M* acetic acid pH 2.8 to 0.1 *M* acetic acid–ammonium acetate pH 4.8 to 0.1 *M* ammonium acetate pH 6.8; (3) CM–cellulose in column lengths from 27–37 cm, same gradient as CM–Sephadex. Sephadex G-10 or G-15 could consistently remove the smaller contaminants from the mixture of hepta-, hexa-, and pentapeptides using column lengths from 37–80 cm and 0.1 *M* ammonium acetate as eluent.

Conventional Synthesis.—Derivatives of glutamic acid used in this synthesis were benzyloxycarbonyl-L-glutamic acid di-*tert*-butyl ester,[20] benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester (**3**),[28] benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester γ-pentachlorophenyl ester,[24] and benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester γ-*N*-hydroxysuccinimide ester (**4**).

Benzyloxycarbonyl-L-glutamic Acid α-*tert*-Butyl Ester γ-*N*-Hydroxysuccinimide Ester (4).—To a solution of **3** (10 g, 19 mmol) in methylene chloride was added *N*-hydroxysuccinimide (2.4 g, 21 mmol) and DCCI (4.35 g, 21 mmol). The mixture was stirred overnight at room temperature. Acetic acid (0.5 ml) was added and the suspension was stirred for 5 min. The solvent was removed *in vacuo* and ethyl acetate was added. The suspension was filtered. The filtrate was washed with saturated $NaHCO_3$, water, 2 *N* H_2SO_4, and saturated

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NaCl and dried ($MgSO_4$). After concentration *in vacuo*, the oil was dissolved in ether and filtered to remove insoluble material. Hexane was added to the solution and it was cooled to yield 4.6 g of a white solid, mp 51–56°. Recrystallization from ether–hexane gave 3.8 g (45%), mp 61–62°, $[\alpha]^{22.5}D$ −18.7° (*c* 1.47, methanol).

Anal. Calcd for $C_{21}H_{26}N_2O_8$ (434.5): C, 58.10; H, 6.03; N, 6.45. Found: C, 58.40; H, 6.33; N, 6.29.

After a 1-month storage in a desiccator, the material decomposed.

Removal of *N*-Benzyloxycarbonyl Group by Catalytic Hydrogenation.—Each derivative was hydrogenated in methanol at room temperature using freshly prepared palladium black catalyst[29] (*ca.* 1 g/10 g of peptide). The solution was stirred by a vibromixer[30] (Rainin, Boston, Mass.); hydrogen gas was passed through until evolution of CO_2 ceased, determined by passing the exit gases through $Ba(OH)_2$ solution. Complete hydrogenolysis required 0.5–1.5 hr. The catalyst was removed by filtration and the filtrate concentrated *in vacuo*. Completeness of hydrogenation was verified by tlc (ethyl acetate); the products were used directly.

Elongation of the Peptide Chain. A. By Pentachlorophenyl Ester.—The hydrogenated peptide (1–100 mmol) was dissolved in ethyl acetate and a 10% molar excess of benzyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester γ-pentachlorophenyl ester was added. The solution was heated to 45–50° and stirred until all free amine had disappeared (determined by ninhydrin spray on tlc plates), usually 1–2 days. The solution was washed with 0.5 *N* H_2SO_4, water, saturated $NaHCO_3$, saturated NaCl, dried ($MgSO_4$), and concentrated *in vacuo*. The resulting oil was dissolved in 95% ethanol and chromatographed on a 190 × 5 cm column of Sephadex LH-20 with ethanol. The first large peak was product; the second, excess active ester; and the third, pentachlorophenol. The fractions from the first peak were concentrated to an oil which crystallized slowly from ether–hexane. Recrystallization was effected from the same solvent mixture. The hexa- and heptapeptides were extracted with boiling isopropyl ether before recrystallization.

B. By *N*-Hydroxysuccinimide Ester.—The hydrogenated peptide (0.1–1 mmol) was dissolved in ethyl acetate and a 10% excess of **4** was added. The solution was stirred at room temperature until no free amine was present, ~24 hr. The mixture was washed several times with water, then with 0.5 *N* H_2SO_4, water, saturated $NaHCO_3$, saturated NaCl, dried ($MgSO_4$), and concentrated *in vacuo*. The oil was dissolved in 95% ethanol and chromatographed on a 45 × 2.5 cm column of LH-20. The fractions from the first peak were concentrated to an oil which crystallized from ether–hexane. Recrystallization was effected from the same solvent system.

C. By Mixed Anhydride.—Reactants, solvent, and glassware were dried prior to use *in vacuo* or over drying agents. A solution of **3** (10% excess over amine component, 1–100 mmol) in ethyl acetate was cooled to −15° in a flask equipped with a drying tube. An equimolar amount of *N*-methylmorpholine was added, followed by an equimolar amount of isobutyl chloroformate. After stirring for 2–4 min at −15°, a precooled solution of the amine in ethyl acetate was added. The cooling bath was replaced with ice. The solution was stirred for 4 hr at 0° and 8 hr at room temperature. The reaction mixture was then washed with 2 *N* H_2SO_4, water, saturated $NaHCO_3$, saturated NaCl, dried ($MgSO_4$), and concentrated *in vacuo*. With the exception of the heptapeptide, the oil was immediately crystallized from ether–hexane and recrystallized from the same solvent mixture. The heptapeptide was chromatographed on a column (190 × 5 cm) of Sephadex LH-20 in ethanol and then crystallized.

Hepta-γ-L-glutamic Acid.—The protected heptapeptide (150 mg, 0.1 mmol) was treated with 1 ml of anhydrous trifluoroacetic acid for 15 min. Addition of ether precipitated the N-protected acid, which was collected by filtration. It was hydrogenated in methanol with 0.1 g of palladium catalyst for 1 hr. The suspension was filtered and the catalyst washed several times with water. Lyophilization gave a white hygroscopic solid. Chromatography on Sephadex G-10 with water as eluent and lyophilization gave 68.7 mg (75%) of a white powder. High voltage electrophoresis for 1 hr at pH 2.1 and 4000 V showed only one spot, corresponding to the heptapeptide, $[\alpha]^{24}D$ −15.5° (*c* 0.76, H_2O).

Anal. Calcd for $C_{35}H_{51}N_7O_{22}\cdot 6H_2O$: C, 40.81; H, 6.16; N, 9.52. Found: C, 40.60; H, 5.57; N, 10.20.

Registry No.—Hepta-γ-L-glutamic acid, 21919-07-3; **4**, 26308-97-4.

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