dium hydroxide at 365 mμ; $E^{1\%}_{1cm.}$ calcd., 134; $E^{1\%}_{1cm.}$ for pteroylglutamic acid at 365 mμ, 212; found, 134.

Acknowledgment.—The authors wish to acknowledge the assistance of Mr. William Kinley in preparing various intermediates, Mr. Louis Brancone and his associates for the microanalysis, Miss Eleanora Boggiano for the microbiological assays and Mrs. Anna deGrunigen for the chemical assays.

Summary

An improved synthesis of *p*-nitrobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester has been described and pure pteroyl-α,γ-glutamyldiglutamic acid has been prepared.

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[CONTRIBUTION FROM LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

Pteroic Acid Derivatives. V. Pteroyl-α-glutamyl-α-glutamylglutamic Acid, Pteroyl-γ-glutamyl-α-glutamylglutamic Acid, Pteroyl-α-glutamyl-γ-glutamylglutamic Acid

BY J. SEMB, J. H. BOOTHE, R. B. ANGIER, C. W. WALLER, J. H. MOWAT, B. L. HUTCHINGS AND Y. SUBBAROW

In previous communications from this Laboratory the syntheses of two of the five possible isomers of pteroyltriglutamic acid were described.[1,2] The purpose of this communication is to present the synthesis of the remaining isomers and to ascertain their biological activities in order to prove conclusively the structure of the fermentation *L. casei* factor.

It seemed desirable to present the syntheses of these three compounds in one communication for the following reasons: first, the glutamic acid peptides were in part or wholly prepared by the use of carbobenzoxy glutamic anhydride; second, γ-ethyl carbobenzoxy-α-glutamylglutamate (II) was the key intermediate in all three syntheses; third, the proof of structure of pteroyl-α-glutamyl-α-glutamylglutamic acid (XII) was dependent on the proof of structure of pteroyl-γ-glutamyl-α-glutamylglutamic acid (XX) or more precisely on the proof of structure of the intermediate tetraethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (XVI).

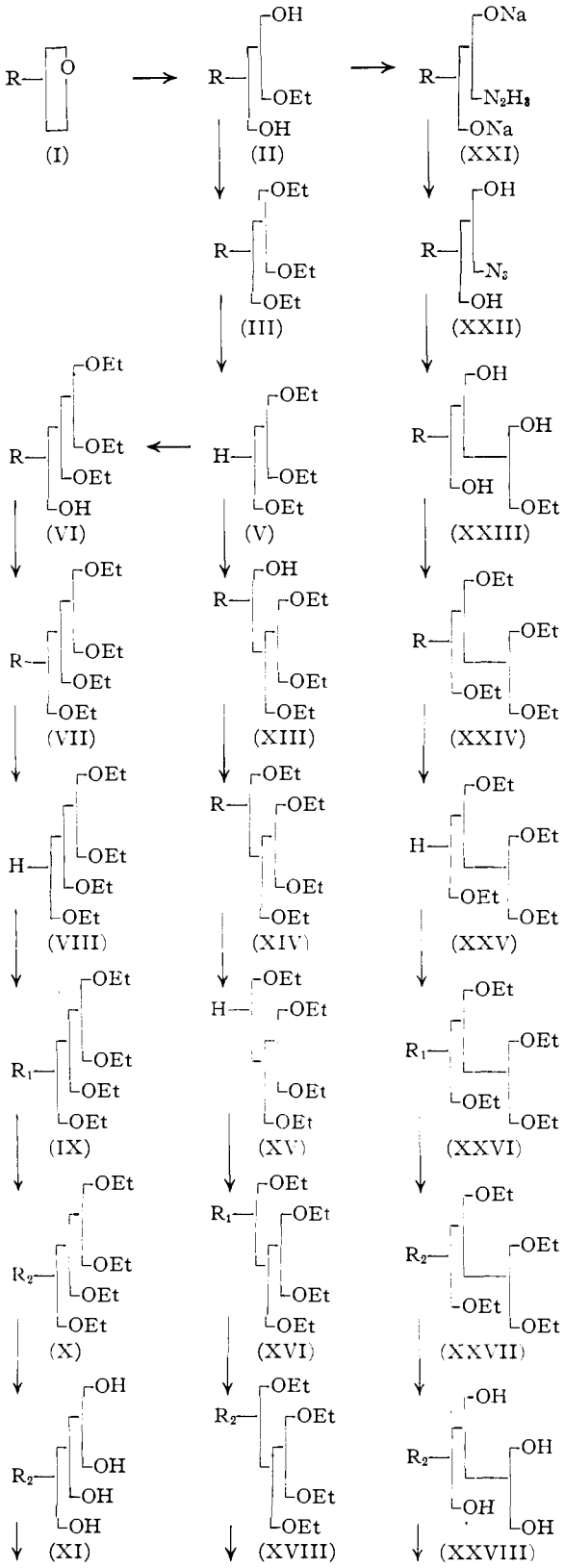
The key compound, γ-ethyl carbobenzoxyl-α-glutamylglutamate (II) was prepared by condensing carbobenzoxyglutamic anhydride with γ-ethyl glutamate in water. The structure of this compound was proved by hydrolyzing to carbobenzoxy-α-glutamylglutamic acid which was shown to be identical with that prepared by the method of Bergmann and Zervas.[3] It was also completely esterified and shown to have different properties from its isomer, triethyl carbobenzoxy-γ-glutamylglutamate.[4]

In the preparation of the first two pteroyltriglutamic acids, (XII) and (XX), the key compound (II) was first esterified to the triester (III) which was decarbobenzoxylated by means of palladium and hydrogen. The resulting dipeptide (V) was not isolated but was condensed with carbobenzoxyglutamic anhydride. The product of this reaction was fully esterified and was found to consist of two compounds having the same analyses but different properties. These two isomeric tetraethyl carbobenzoxytriglutamates, (VII) and (XIV), were separated by fractional crystallization from alcohol and both were converted from the carbobenzoxy derivatives to the *p*-nitrobenzoyl derivatives by reduction and then *p*-nitrobenzoylation. The properties of one of these isomers showed it to be identical with tetraethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamyl-α-glutamylglutamate (XVI) prepared by the following independent synthesis: the γ-azide of *p*-nitrobenzoylglutamic acid[1] was condensed with triethyl α-glutamylglutamate (V) to yield triethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (XVII) which was esterified to the desired tetraester (XVI). Since the structure of one of the isomers from the above reaction was proved, it became obvious that the other isomer was tetraethyl *p*-nitrobenzoyl-α-glutamyl-α-glutamylglutamate (IX).

The third isomer, tetraethyl *p*-nitrobenzoyl-α-glutamyl-γ-glutamylglutamate (XXVI), was synthesized by the following series of reactions in which several of the intermediate products were not isolated and characterized. It is quite obvious however that the three glutamic acids must be joined in the positions indicated. γ-Ethyl carbobenzoxy-α-glutamylglutamate was first converted to the disodium salt, then to the hydrazide (XXI) and to the azide (XXII). This azide was condensed with γ-ethyl glutamate to yield the monoester of carbobenzoxy-α-glutamyl-γ-glutamylglutamic acid (XXIII). This was esterified to the tetraester (XXIV) and then decarbobenzoxylated with palladium and hydrogen. The resulting peptide was *p*-nitrobenzoylated and the tetraethyl *p*-nitrobenzoyl-α-glutamyl-γ-glutamylglutamate isolated (XXVI).

All three of these isomeric tetraethyl *p*-nitrobenzoyltripeptides were reduced with zinc dust to the corresponding *p*-amino derivatives. However, in the preparation of the pteroyl derivatives

(1) Boothe, *et al.*, THIS JOURNAL, **71**, 2304 (1949).
(2) Mowat, *et al.*, *ibid.*, **71**, 2308 (1949).
(3) Bergmann and Zervas, *Ber.*, **65**, 1192 (1932).
(4) Boothe, *et al.*, THIS JOURNAL, **70**, 1099 (1948).



(*Continued in next column*)

the *p*-amino compounds were not isolated but their solutions were used directly to condense with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde using a modification of the procedure of Waller, *et al.*[5] These three pteroyltriglutamic acids all showed much lower activity than the fermentation *L. casei* factor when assayed with *Lactobacillus casei*.

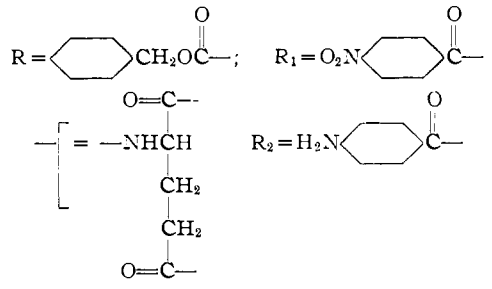
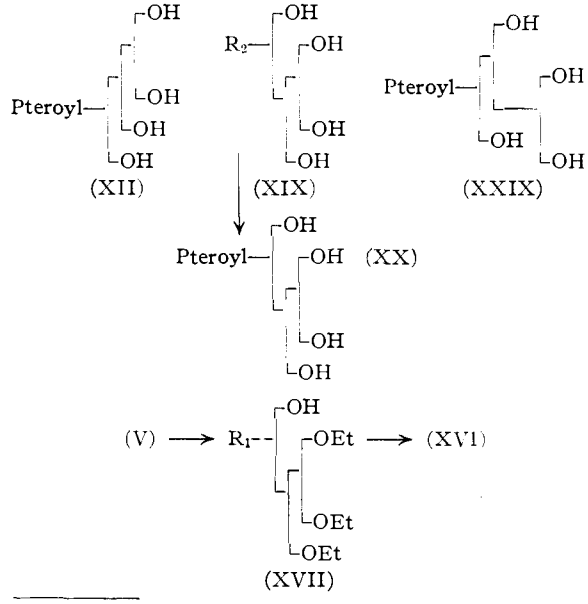


TABLE I

	% *L. casei*	% *S. faecalis R*
Pteroylglutamic acid	100	100
Pteroyl-γ-glutamylglutamic acid[1]	62.9	70.9
Pteroyl-α-glutamylglutamic acid[6]	0.8	0.5
Pteroyl-γ-glutamyl-γ-glutamyl-glutamic acid[1]	64	2.4
Pteroyl-γ-glutamyl-α-glutamyl-glutamic acid	4.4	3.6
Pteroyl-α-glutamyl-γ-glutamyl-glutamic acid	1.4	0.5
Pteroyl-α-glutamyl-α-glutamyl-glutamic acid	5.0	0.59
Pteroyl-α,γ-glutamyldiglutamic acid[2]	1.2	0.9
Fermentation *L. casei* factor[7]	60-80	4-6



(5) Waller, *et al.*, THIS JOURNAL, **70**, 19 (1948).
(6) Mowat, *et al.*, *ibid.*, **70**, 1096 (1948).
(7) Hutchings, *et al.*, *ibid.*, **70**, 1 (1948).

Table I shows the relative microbiological activities of various glutamic acid peptide derivatives of pteroic acid.

Experimental[8]

γ-Ethyl Carbobenzoxy-α-glutamylglutamate (II).—A solution of 875 g. of γ-ethyl glutamate and 420 g. of sodium bicarbonate in 2500 ml. of water was heated to about 30° with stirring. The solution was cooled to 10° and the *p*H adjusted to 9.0. To this was added 1042 g. of carbobenzoxyglutamic anhydride over a period of one and one-half hours holding the *p*H at 8.9 with sodium hydroxide. This was diluted to 9 liters with water, the *p*H was lowered to about 1.5 with concd. hydrochloric acid and an oil settled out. Upon further stirring the oil solidified, and the solid matter was filtered off and suspended in 5 liters of water at 55°. The insoluble material was filtered off and resuspended in 5 liters of water at 55° and filtered; weight of crude γ-ethyl carbobenzoxy-α-glutamylglutamate 814 g.; m. p., 149°. Forty grams of crude γ-ethyl carbobenzoxy-α-glutamylglutamate was purified further by dissolving in 800 ml. of water heated to 90° and filtering. The filtrate was cooled to 60° and filtered. The white crystalline precipitate had a m. p. of 157-158°.

Anal. Calcd. for $C_{20}H_{26}O_9N_2$: C, 54.79; H, 5.93; N, 6.39. Found: C, 54.45; H, 5.9; N, 6.63.

Carbobenzoxy-α-glutamylglutamic Acid (IV).—Ninety-three grams of γ-ethyl carbobenzoxy-α-glutamylglutamate was saponified with 750 ml. of 1.0 *N* sodium hydroxide by allowing to stand at room temperature for two hours. After filtering, the solution was adjusted to *p*H of 3.9 and allowed to stand overnight in a cool place; 72.3 g. of product (IV) was filtered off, m. p., 165-169°. This was dissolved in 750 ml. of warm water, cooled a little and filtered. The filtrate was cooled in an ice-bath and 60 g. of crystalline material was filtered off, m. p., 172-173°. Bergmann and Zervas[3] report 176° (cor.).

Triethyl Carbobenzoxy-α-glutamylglutamate (III).—One hundred grams of air-dried γ-ethyl carbobenzoxy-α-glutamylglutamate with a m. p. of 155-156° was further dried on a steam-bath under reduced pressure. To this dried material was added 1000 ml. of anhydrous alcohol (2B) and 42 g. of *p*-toluenesulfonic acid monohydrate. The solution was filtered and allowed to stand for two days at room temperature. Upon cooling, 95 g. of the triester was obtained; m. p., 104-106°. Upon concentrating the filtrate to 325 ml., 7 g. of material with a m. p. of 103-104° was obtained; total yield, 102 g. (The pure isomer, triethyl carbobenzoxy-γ-glutamylglutamate,[4] has a m. p. of 91-92° (cor.).)

Anal. Calcd. for $C_{24}H_{34}O_9N_2$: C, 58.40; H, 6.94; N, 5.67. Found: C, 58.17; H, 6.89; N, 5.96.

Triethyl Carbobenzoxy-α-glutamyl-α-glutamylglutamate (VI).—A solution of 49.4 g. of triethyl carbobenzoxy-α-glutamylglutamate (III) and 19 g. of *p*-toluenesulfonic acid monohydrate in 1000 ml. of anhydrous alcohol (2B) was stirred with 2.5 g. of palladium-on-charcoal and hydrogen was passed in. At one hour and twenty minutes the reduction was complete. The catalyst was filtered off and the filtrate concentrated under reduced pressure to a thick sirup. This was washed with a little petroleum ether and again evacuated. The sirupy residue was made up to a volume of 125 ml. with water and adjusted to *p*H 8.5. To this was added quite rapidly 28.9 g. of carbobenzoxyglutamic anhydride. The *p*H was held at 8.5 and the temperature at 15 to 20°. When no more alkali was used up the reaction mixture was diluted to 400 ml. and concentrated hydrochloric acid added to *p*H 3. A gummy precipitate formed, the mixture was warmed to 60° and the water phase poured off. The gummy precipitate was added to 400 ml. of water, heated to 90° and stirred thoroughly. The water phase was poured off and the oily phase poured into a graduate; volume of oily phase, 30 ml. This was dissolved in 170 ml. of (2B) anhydrous alcohol and after chilling 12.5 g. of white crystalline material was obtained, m. p., 143-144°. This was further purified by crystallizing 2.5 g. from 2B alcohol, m. p., 146°.

Anal. Calcd. for $C_{29}H_{41}O_{12}N_3$: C, 55.86; H, 6.58; N, 6.74. Found: C, 55.76; H, 6.86; N, 6.78.

Tetraethyl Carbobenzoxy-α-glutamyl-α-glutamylglutamate (VII). **A.**—One gram of triethyl carbobenzoxy-α-glutamyl-α-glutamylglutamate (VI) was dissolved in 20 ml. of 2B alcohol containing 3.04 g. of *p*-toluenesulfonic acid monohydrate. This was heated at 55-60° for one hour and then allowed to stand at room temperature overnight. The resultant thick slurry was filtered and the precipitate recrystallized from 2B alcohol, m. p., 128°.

Anal. Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.14; H, 6.91; N, 6.45. Found: C, 57.60; H, 6.67; N, 6.53.

B.—A solution of 1238 g. of triethyl carbobenzoxy-α-glutamylglutamate (III) in 6500 ml. of anhydrous alcohol (2B) containing 475 g. of *p*-toluenesulfonic acid monohydrate was heated to 50-60°, stirred, and 55 g. of palladium (10%)-on-charcoal in 550 ml. of ethanol was added. This was stirred and hydrogen passed in. When the decarbobenzoxylation was complete, the catalyst was filtered off, and the filtrate was concentrated to a sirup under reduced pressure. The sirup, triethyl α-glutamylglutamate (V), was dissolved in 3000 ml. of water and adjusted to *p*H 8. To this was added 1005 g. of carbobenzoxyglutamic anhydride (1.5 moles) with the *p*H held between 7.5 and 8.0 and the temperature at about 30°. This was stirred for thirty minutes. Concentrated hydrochloric acid was added dropwise but after the addition of a small amount, the solution turned to a gel. The gel was diluted with 15 liters of warm water and the *p*H brought down to 1.0. A curdy precipitate was collected and air dried; weight, 1618 g. This was dissolved in 6000 ml. of anhydrous alcohol (2B) containing 250 g. of *p*-toluenesulfonic acid monohydrate and heated at 55-60° for two hours. Upon cooling to room temperature a thick slurry formed which could not be filtered. Therefore, the mass was diluted to 15 liters with alcohol and warmed to dissolve the insoluble material. After cooling overnight at about 0°, 603 g. of white crystalline material was filtered off; m. p. 126°, yield, 37%. The filtrate was kept for the preparation of tetraethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (XVI).

Tetraethyl *p*-Nitrobenzoyl-α-glutamyl-α-glutamylglutamate (IX). **A.**—Nine grams of pure triethyl carbobenzoxy-α-glutamyl-α-glutamylglutamate (VI) was esterified as above, the solution of the tetraethyl ester was set in a water-bath held at 50° and 0.5 g. of palladium-on-charcoal was added. Hydrogen was passed in and after twenty minutes the reduction was complete. The catalyst was filtered off and the solvent removed under reduced pressure. The residue was dissolved in 45 ml. of water. Ninety ml. of ether and 3.6 g. of sodium bicarbonate were added. With stirring 3.6 g. of *p*-nitrobenzoyl chloride was added rapidly and almost immediately a white slurry appeared. The mixture was stirred for about twenty minutes and filtered. The precipitate was dissolved in 100 ml. of ethanol, 100 ml. of water was added, heated and filtered. After standing at room temperature for two hours 6.3 g. of light-yellow crystalline material was filtered off; yield, 66%, m. p., 143-144°.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.05; H, 6.31; N, 8.41. Found: C, 53.74; H, 6.46; N, 8.36.

B.—One hundred fifty-six grams of triethyl carbobenzoxy-α-glutamyl-α-glutamylglutamate (VI) was esterified and reduced as described above. The reduction was completed in two hours, the catalyst was filtered off and the solvent removed under reduced pressure. To the residue 600 ml. of water and 100 g. of sodium bicarbonate was added. To this was added 58 g. of *p*-nitrobenzoyl chloride in 500 ml. of chloroform; temperature, 18°. This mixture was gradually heated to 38° and stirred for forty-five minutes at this temperature. The chloroform phase was separated and washed once with a small quantity of sodium bicarbonate solution. The chloroform

(8) Melting points uncorrected.

was removed under reduced pressure and the residue was dissolved in 1000 ml. of alcohol and placed in a refrigerator held at −5° overnight. The resultant white crystalline product was collected; yield, 169 g.; m. p., fused at 114° and collapsed indistinctly. This is approximately a 100% yield. Since the melting point of this material did not check with material prepared by the first method, it was further purified as follows without appreciably altering the melting point; it was crystallized from 1000 ml. of warm alcohol by adding 1000 ml. of water. The melting point was essentially the same as the original. It was then dissolved in 1000 ml. of ethyl acetate and stirred with 300 ml. of water containing sodium bicarbonate in suspension at 70°. The ethyl acetate phase was concentrated to a solid under reduced pressure and the residue was crystallized from 1000 ml. of alcohol; weight of white crystalline material, 113 g.; m. p., fused at 115°.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.05; H, 6.31; N, 8.41. Found: C, 54.05; H, 6.52; N, 8.54.

C.—In another preparation 716 g. of tetraethylcarbobenzoxy-α-glutamyl-α-glutamylglutamate (VII) was reduced and the resulting tetraethyl α-glutamyl-α-glutamylglutamate was *p*-nitrobenzoylated by the same method as is outlined in procedure B above. The white crystalline product weighed 750 g., m. p. 114-115°. However, a second recrystallization from 7 liters of ethanol yielded 625 g. (86%) of material which had a m. p. of 144° corresponding to the product from procedure A. This phenomenon of two different melting points for the same compound has been noted in the preparation of two of the other isomeric tetraethyl *p*-nitrobenzoyltriglutamates.[1,2,4] In this case samples having the different melting points were reduced to the same *p*-amino derivatives.

Tetraethyl *p*-Aminobenzoyl-α-glutamyl-α-glutamylglutamate (X).—A suspension of 6.66 g. of tetraethyl *p*-nitrobenzoyl-α-glutamyl-α-glutamylglutamate in 100 ml. of water and 100 ml. of alcohol was stirred and the *p*H was brought down to 1.0 with concd. hydrochloric acid. Seven grams of zinc dust was added slowly over a period of thirty minutes, while holding the *p*H at 1.0. The excess zinc was filtered off and to the filtrate 10 g. of sodium acetate was added. The amine started to precipitate immediately. This was chilled and the product collected; weight, 5.3 g.; m. p., 147°. This was recrystallized from alcohol; m. p., 148°.

Anal. Calcd. for $C_{30}H_{44}O_{11}N_4$: C, 56.60; H, 6.93; N, 8.80. Found: C, 56.27; H, 6.93; N, 8.88.

This gave a Bratton-Marshall[9] amine assay of 102% based on a molecular weight of 636 and using *p*-aminobenzoic acid as a standard.

Triethyl *p*-Nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (XVII).—Triethyl carbobenzoxy-α-glutamylglutamate (15.3 g.) was reduced as already described to remove the carbobenzoxy group. The alcoholic solution was concentrated to dryness *in vacuo*, and the residue was dissolved in 100 cc. of water containing 10 g. of sodium bicarbonate. To this solution was added a solution of *p*-nitrobenzoylglutamic acid γ-azide (prepared from 10 g. of the corresponding hydrazide as described in a previous communication[1]) in 50 cc. of ethyl acetate. The mixture was stirred three hours at room temperature. The aqueous layer was drawn off and acidified with hydrochloric acid. An oil separated which solidified on cooling overnight and was filtered off and dried; yield, 13.5 g. A portion of this material was crystallized once from an alcohol-water mixture and three times from alcohol; m. p., if put in the bath below 115° and heated slowly, it melts quite sharply at 146-147°. If put in the bath at 120° or above, it melts immediately.

Anal. Calcd. for $C_{29}H_{38}O_{13}N_4$: C, 52.7; H, 6.00; N, 8.78. Found: C, 52.53; H, 6.28; N, 9.08.

Tetraethyl *p*-Nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (XVI). A.—The triethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate prepared from 36.4 g. of triethyl carbobenzoxy-α-glutamylglutamate (III) as described above was dissolved in 250 cc. of absolute alcohol containing 15 g. of *p*-toluenesulfonic acid and heated to boiling. After standing at room temperature for one hour the solution had solidified with crystals. It was again heated to boiling, cooled to 30° and the product filtered, washed with alcohol, and dried; yield, 23 g. A portion was crystallized twice from alcohol, m. p., 183-184°.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.05; H, 6.31; N, 8.41. Found: C, 54.1; H, 7.05; N, 8.67.

B.—Approximately 4/5 of the filtrate obtained from procedure B used in preparation of tetraethyl carbobenzoxy-α-glutamyl-α-glutamylglutamate (VII) was diluted with 2 volumes of water which precipitated 500 g. of a sticky material. The solvent was decanted and to the solid was added 1000 ml. of anhydrous alcohol (2B) containing 150 g. of *p*-toluenesulfonic acid monohydrate and 35 g. of palladium-on-charcoal. The mixture was heated to 50-55° and with stirring hydrogen was passed in until decarbobenzoxylation was complete. The catalyst was filtered off and the filtrate concentrated to a sirup under reduced pressure. The sirup was dissolved in 1000 ml. of water and 160 g. of sodium bicarbonate was added. To this was added, at room temperature, and with stirring, 150 g. of *p*-nitrobenzoyl chloride in 1000 ml. of chloroform. The mixture was heated to 40° and stirred for one and one-half hours. The chloroform phase was drawn off and most of the chloroform was removed under reduced pressure. The residue solidified before all the chloroform had been removed. The residue was crystallized from 2 liters and then from 4.5 liters of anhydrous alcohol (2B) yielding 177 g. of almost white crystalline precipitate, m. p., 184-185°. A mixed melting point with the compound prepared by the previous procedure gave no depression.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.05; H, 6.31; N, 8.41. Found: C, 54.09; H, 6.25; N, 8.75.

Tetraethyl *p*-Aminobenzoyl-γ-glutamyl-α-glutamylglutamate (XVIII).—Tetraethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate (6.66 g.) was added to a solution of 100 ml. of water and 100 ml. of ethanol, warmed to 50° and the *p*H adjusted to 0.6. Over a period of thirty minutes, 8 g. of zinc dust was added. Very little of the ester had gone into solution. Therefore, 10 ml. of acetic acid was added, the temperature was raised to 75° and 4 g. more of zinc dust was added. The ester went into solution rapidly and after a short period the excess zinc dust was filtered off. To the filtrate 10 g. of sodium acetate was added and after standing overnight in a cool place 5.5 g. of crystalline material was filtered off, m. p., 166°. It was recrystallized from alcohol and water; m. p., 168°.

Anal. Calcd. for $C_{30}H_{44}O_{11}N_4$: C, 56.60; H, 6.93; N, 8.80. Found: C, 56.82; H, 7.51; N, 9.04.

The product gave a Bratton-Marshall amine assay[9] of 97% based on a molecular weight of 636 and using *p*-aminobenzoic acid as a standard.

Tetraethyl *p*-Nitrobenzoyl-α-glutamyl-γ-glutamylglutamate (XXVI).—A suspension of 87.6 g. of γ-ethyl carbobenzoxy-α-glutamylglutamate (II) in 250 ml. of methanol was placed in an ice-bath. To this was added dropwise 22.7 g. of sodium methoxide dissolved in 100 ml. of methanol. Forty ml. of hydrazine hydrate was added and the solution was boiled for five minutes. The solution was concentrated to a thin sirup under reduced pressure and 300 ml. of anhydrous alcohol (2B) was added. A gummy precipitate came down. The supernatant liquid was poured off and the residue was dissolved in 100 ml. of methanol. To this solution 300 ml. of anhydrous alcohol (2B) was added and the mixture was chilled overnight. The supernatant liquor was poured off and the residue was broken up in 300 ml. of ethyl acetate and filtered; weight of crude disodium monohydrazide of carbobenzoxy-α-glutamylglutamic acid (XXI), 82 g.

The above crude hydrazide was dissolved in 300 ml. of water and cooled in an ice-bath. To this was added 300 ml. of ethyl acetate and 150 ml. of concd. hydrochloric acid and after thorough mixing the ethyl acetate phase was separated and discarded. Ethyl acetate (300 ml.) was added and the mixture was cooled to −5°. With

(9) Bratton and Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

vigorous stirring 18 g. of sodium nitrite, dissolved in 75 ml. of water, was added slowly keeping the temperature at −2 to −5°. The ethyl acetate phase was separated and a small aliquot evaporated to dryness. The solid matter so obtained indicated that there was 51 g. of azide (XXII) present.

A suspension of 30 g. of γ-ethyl glutamate and 50 g. of sodium bicarbonate in 150 ml. of water was stirred with the above ice-cold solution of azide in ethyl acetate. The mixture was warmed to 28° and stirred for one and one-half hours, then heated to 40° and stirred for twenty minutes more. The ethyl acetate phase was separated and discarded. The water phase was treated with Norite and filtered. The filtrate was made acid with concd. hydrochloric acid whereupon an oily-solid precipitate appeared. After standing overnight in a refrigerator the precipitate had not solidified. Therefore, the oily material was separated and dried under high vacuum; weight of crude γ-ethyl carbobenzoxy-α-glutamyl-γ-glutamylglutamate (XXIII), 66 g.

To the above dried material was added 300 ml. of anhydrous alcohol and 25 g. of *p*-toluenesulfonic acid monohydrate and the mixture brought to a boil. This was allowed to stand several days at 0°. A few gelatinous balls had settled which were filtered off and discarded. The filtrate was heated to 50–55° and decarbobenzoxylated as described above. The resulting sirup of tetraethyl α-glutamyl-γ-glutamylglutamate (XXV) was dissolved in 125 ml. of water to which was added 25 g. of sodium bicarbonate and 22 g. of *p*-nitrobenzoyl chloride dissolved in 100 ml. of chloroform. This was stirred vigorously for one hour at room temperature, then warmed up to 40° and stirred for thirty minutes more. The chloroform phase was drawn off and concentrated to a sirup which was dissolved in 250 ml. of anhydrous alcohol (2B). Five grams of *p*-toluenesulfonic acid monohydrate was added, the solution heated to 60° and then cooled overnight at 0°. The resulting white crystalline material was filtered off and weighed 22 g., m. p., 144°. This was recrystallized from (2B) anhydrous alcohol; m. p., 154°.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.05; H, 6.31; N, 8.41. Found: C, 54.05; H, 6.70; N, 8.35.

Tetraethyl *p*-Aminobenzoyl-α-glutamyl-γ-glutamylglutamate (XXVII).—A suspension of 6.66 g. of tetraethyl *p*-nitrobenzoyl-α-glutamyl-γ-glutamylglutamate in 100 ml. of water and 100 ml. of ethanol was adjusted to a *p*H of 4.0 with acetic acid. With stirring 4 g. of zinc dust was added slowly with the *p*H held at 4.0. The *p*H was lowered to 3.0 with concd. hydrochloric acid and 2 g. of zinc dust added. After thirty minutes, the excess zinc dust was filtered off. The filtrate was diluted with 100 ml. of water and extracted with chloroform. The chloroform phase was dried over sodium sulfate and then reduced to a thin sirup under reduced pressure. Ether was added and a crystalline product came out which was filtered off; weight, 5.2 g.; m. p., indistinct, started to fuse at 100°. The product was recrystallized from alcohol; m. p., 117°.

Anal. Calcd. for $C_{30}H_{44}O_{11}N_4$: C, 56.60; H, 6.93; N, 8.80. Found: C, 56.61; H, 7.15; N, 9.16.

This gave a Bratton-Marshall amine assay[9] of 103% based on a molecular weight of 636 and using *p*-aminobenzoic acid as a standard.

Pteroyl-α-glutamyl-α-glutamylglutamic Acid (XII).—Tetraethyl *p*-nitrobenzoyl-α-glutamyl-α-glutamylglutamate (66.6 g.) was added to 500 ml. of alcohol and 500 ml. of 1.0 *N* sodium hydroxide was added slowly, at room temperature, with stirring. A clear solution resulted. The alcohol was evaporated off under reduced pressure. The remaining solution, about 500 ml., was adjusted to a *p*H of 3.5 and 35 g. of zinc dust was added slowly. The *p*H was held at 3.5 and the temperature at 30–35° during the addition and for an additional fifteen minutes. The mixture was filtered, the filtrate diluted to 1200 ml. and condensed with 51.5 g. of 2,4,5-triamino-6-hydroxypyrimidine sulfate and 43 g. of 2,3-dibromopropionaldehyde as previously described.[1] The weight of crude pteroyl compound was 62 g., which assayed 30.1% pteroyltriglutamic acid by chemical assay.[10] This was purified by the following procedure. The crude material was added to 4000 ml. of water containing 75 ml. of 10 *N* sodium hydroxide until all the material was in solution. To this was added 200 ml. of 20% calcium chloride. This was heated to 60° and adjusted to *p*H 10.8 by adding 20% zinc chloride solution. Celite was added, the mixture was filtered and the precipitate was discarded. The filtrate was lowered to a *p*H of 6.8 by adding 20% zinc chloride; temperature 40°. The zinc salt precipitate was filtered off and washed with water. This precipitate was stirred in 500 ml. of dilute alkali, the *p*H of the solution was lowered to 2.5 with hydrochloric acid and the precipitate was filtered off. The precipitate was redissolved in 1200 ml. of dilute alkali and acidified to a *p*H of 0.6 and cooled overnight. The precipitate weighing 0.7 g. and assaying 70.6% pteroyltriglutamic acid by chemical assay was filtered off. The filtrate was brought to a *p*H of 1.5, cooled overnight and filtered. This precipitate was dissolved in a warm solution adjusted to *p*H 0.9 with hydrochloric acid and chilled. Material weighing 0.65 g. and having an assay of 86.5% pteroyltriglutamic acid was filtered off. This material was dissolved with sodium hydroxide at a *p*H of 7.3 and lyophilized. This dried sodium salt gave a chemical assay of 68.95% pteroyltriglutamic acid. This lyophilized material was further purified by dissolving 117 mg. in 15 ml. of water and acidifying with hydrochloric acid and the mixture heated to 65°. A small amount of Norite was added and the mixture filtered. The filtrate was adjusted to *p*H 0.8 with hydrochloric acid and a clear solution resulted at 47°. Upon cooling, a light yellow material came down which was centrifuged and washed twice with 10 ml. of ice-water; yield, 24 mg.

Anal. Calcd. for $C_{29}H_{33}O_{12}N_9 \cdot H_2O$: C, 48.53; H, 4.88; N, 17.58. Found: C, 48.33; H, 5.28; N, 17.92.

A chemical assay[10] showed this material to be 98% pteroyltriglutamic acid.

Pteroyl-γ-glutamyl-α-glutamylglutamic Acid (XX).—A suspension of 19.1 g. of triethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate in 180 cc. of water was stirred and 12 cc. of 10 *N* sodium hydroxide was added. After thirty minutes the nearly clear solution was filtered and hydrochloric acid was added to *p*H 3.5. This *p*H was maintained with hydrochloric acid while 10 g. of zinc dust was added in portions over a twenty-minute period. After stirring an additional thirty minutes, the excess zinc was filtered out and the filtrate was used to react with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde as previously described.[1] The resulting crude material containing 8.64 g. of the pteroyl derivative was purified by a previously described method,[1] carrying the procedure through the second precipitation at *p*H 0.9. This yielded 3.5 g. of material, 89% pure by chemical assay.[10]

A portion (0.6 g.) of this material was further purified by treating with magnesium oxide and charcoal and an acid precipitation at *p*H 0.9 as described previously. The material was then dissolved in 90 cc. of hot water and 0.5 cc. of concentrated hydrochloric acid was added (without the acid present the material gels). After cooling, the precipitate was centrifuged off, washed and dried at 110° for one hour.

Anal. Calcd. for $C_{29}H_{33}O_{12}N_9 \cdot H_2O$: C, 48.5; H, 4.88; N, 17.58. Found: C, 48.6; H, 5.09; N, 17.68.

Pteroyl-α-glutamyl-γ-glutamylglutamic Acid (XXIX).—Tetraethyl *p*-nitrobenzoyl-α-glutamyl-γ-glutamylglutamate (60.0 g.) was dissolved in 600 cc. of ethanol by heating. Then, while stirring, 600 cc. of water was added causing the product to crystallize out. An additional 200 cc. of ethanol was added to facilitate stirring and the compound was reduced in the usual manner using 36.0 g. of zinc dust and about 60 cc. of concentrated hydrochloric acid. The reduction required forty-five minutes. At the end of this time the zinc was filtered off and a Bratton-Marshall amine determination was run indicating 89% reduction.

(10) Hutchings, *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).

The filtrate was adjusted to *p*H 10.5 using 90 cc. of 10 *N* sodium hydroxide. After stirring for thirty minutes to allow for hydrolysis of the ester groups, the mixture was again adjusted to *p*H 3.5. This solution then reacted in the usual manner[1] with 46.0 g. of 2,4,5-triamino-6-hydroxypyrimidine bisulfate and 43.0 g. of barium chloride dihydrate in 750 cc. of water, 39.0 g. of dibromopropional in 50 cc. of acetic acid and 9.0 g. of sodium dichromate in 75 cc. of water. The yield of crude product was 114.5 g.; chemical assay 19.7%.

This crude pteroyl derivative was purified by a previously described method[1] carrying the procedure through the second precipitation at *p*H 0.9. This yielded 5.9 g. of material, 69% pure by chemical assay.[10] This material seems to be soluble in wet acetone or wet alcohol; therefore, it was never washed with organic solvents. A portion of this (1.4 g.) was dissolved in 190 cc. of dilute sodium hydroxide at *p*H 11.0 and the resulting solution was acidified to *p*H 6.0. This was filtered through Celite; 4 g. of Celite was added to the filtrate and it was acidified to *p*H 2.1 at 50°. After cooling this was filtered and the solid dissolved in 130 cc. of a dilute sodium hydroxide solution. The material was then further purified by repeating the previously described[1] purification treatment. The resulting free acid was collected by centrifuging. It was washed twice with water, mixed with a little Norite and enough magnesium oxide to bring to *p*H 9.0 in 8 cc. of water. After heating to 80° it was filtered, cooled, and the magnesium salt of the pteroyl-α-glutamyl-γ-glutamylglutamic acid was thrown out by adding alcohol; yield 100 mg. The chemical assay was 83%. However, the ultraviolet absorption spectra were almost identical with those of pteroylglutamic acid and the other pteroylpolyglutamic acids except in regard to the extinction coefficient.

Ultraviolet Absorption Spectra.—The 5 isomeric pteroyltriglutamic acids had essentially the same ultraviolet absorption spectra which were very similar to pteroylglutamic acid. In 0.1 *N* sodium hydroxide the compounds had maxima of about equal magnitude at 257 and 286 mμ and another at 365 mμ. There were minima at 235, 265 and 334 mμ. In 0.1 *N* hydrochloric acid there was a plateau at 240 to 248 mμ, a maximum at 290 mμ, and a minimum at 262.5 mμ.

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Summary

The synthesis of tetraethyl *p*-nitrobenzoyl-α-glutamyl-α-glutamylglutamate, tetraethyl *p*-nitrobenzoyl - α - glutamyl - γ - glutamylglutamate, tetraethyl *p*-nitrobenzoyl-γ-glutamyl-α-glutamylglutamate and their corresponding *p*-amino derivatives have been described. The three corresponding isomeric pteroylglutamylglutamylglutamic acids have been prepared and purified, and have been shown to be different from the fermentation *L. casei* factor.

A comparison of the five isomers of pteroylglutamylglutamylglutamic acid (Table I) has shown conclusively that the fermentation *L. casei* factor is pteroyl-γ-glutamyl-γ-glutamylglutamic acid.

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β,β-Diarylacrylic Acids. III. Esters with Diethylaminoethanol as Anesthetics[1]

BY MOSHE WEIZMANN, SAUL PATAI, ELCHANAN DIMANT AND FELIX BERGMANN

The anesthetic activity of esters of benzoic acid is usually ascribed to the presence of the resonating aromatic system, because intercalation of a methylene group between the benzene ring and the carboxyl group destroys this effect.[2] On the other hand, an extension of the resonating system, as *e. g.*, in cinnamic acid should be expected to increase the pharmacological activity. In a few examples this seems to be the case—*e. g.* cinnamamide is a much stronger hypnotic than benzamide[3]—although in the majority of esters of cinnamic acid no improvement over the corresponding derivatives of benzoic acid is found.[4] However, the strong anesthetic activity of the diethylaminoethyl ester of β,β-diphenylacrylic acid (I) has been established,[5] and it seemed of interest to study the influence of substituents in the aromatic rings of (I), since a large number of substituted diarylacrylic acids has recently become available.[1]

It was especially attractive to study the *p*-amino derivatives of (I). A monoamino derivative could not be prepared so far, because *p*-nitrobenzophenone resisted both reaction with a Grignard reagent and Reformatsky condensation with ethyl halogenoacetates. The same inactivity was observed for *p,p'*-dinitrobenzophenone. However, direct nitration of (I) was found to introduce two nitro groups in the *p,p'*-positions (II, R = H), since oxidation of (II) with potassium permanganate yielded *p,p'*-dinitrobenzophenone.[6] Conditions of nitration were rather drastic and resulted in a partial decarboxylation of the acid, thus producing a considerable amount of tarry by-products. Nitration of the methyl ester of (I) was a more convenient procedure[7] and gave the crystalline ester (II, R = CH_3) in 52% yield.

(1) For Part I see F. Bergmann, *et al.*, THIS JOURNAL, **70**, 1612 (1948).

(2) Pyman, *J. Chem. Soc.*, **111**, 167 (1917).

(3) S. Fränkel, "Die Arzneimittel Synthese," Julius Springer, Berlin, 1927, p. 536.

(4) Pyman, *J. Chem. Soc.*, **111**, 1119 (1917).

(5) Burtner and Cusic, THIS JOURNAL, **65**, 262 (1943); Lehmann and Knoefel, *J. Pharm. Exp. Therap.*, **74**, 274 (1942).

(6) Actually isomeric dinitro derivatives may be present in the non-crystalline by-products, which were not investigated further.

(7) Johnson and Offenhauer, THIS JOURNAL, **67**, 1045 (1945).