The latter compound was purified and found to have the same microbiological activity and the same ultraviolet absorption spectrum as does the fermentation *L. casei* factor. The synthesis presented here has the advantages of better yields, fewer steps, and easier operating conditions over the synthesis previously reported.

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Pteroic Acid Derivatives. IV. Pteroyl-α,γ-glutamyldiglutamic Acid

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

The synthesis of small amounts of pteroyl-α,γ-glutamyldiglutamic acid (VII) has been described in a previous publication of this series.[1] This procedure was found to be impractical for the preparation of large amounts of the pteroyl compound and other methods of synthesis were investigated.

It has been shown[2,3,4] that acid azides react with esters of amino acids to form N-acyl derivatives, and, using an analogous method, Boothe, *et al.*,[5] have prepared *p*-nitrobenzoyl-γ-glutamylglutamic acid and *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamic acid, and from these intermediates the corresponding pteroyl compounds.

The present communication describes a convenient and satisfactory synthesis of *p*-nitrobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester (V) and the conversion of this substance to the corresponding pteroyl derivative. *p*-Nitrobenzoylglutamic acid was esterified and the diester was treated with hydrazine hydrate to form the dihydrazide. Treatment of the dihydrazide with nitrous acid gave the diazide which was taken up in ethyl acetate and condensed with excess diethylglutamate in the presence of water and sodium bicarbonate. The crude *p*-nitrobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester, after recrystallization and hydrolysis, was reduced to give the corresponding *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid. This product was condensed with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde by a modification of the procedure of Waller, *et al.*[6] The crude pteroyl compound was collected and purified. Biological assay of the purified product showed that pteroyl-α,γ-glutamyldiglutamic acid was not identical with the fermentation *L. casei* factor.

Experimental

Diethyl *p*-Nitrobenzoylglutamate (II).—A mixture of *p*-nitrobenzoylglutamic acid (I)[7] (1000 g.), 2B absolute ethanol (7.0 l.), and concentrated sulfuric acid (50 cc.) was refluxed on the steam-bath for eighteen hours. About 3.0 l. of alcohol was then removed by distillation and the residue was added to a mixture of 2.0 l. of water and 300 g. of sodium bicarbonate. This solution was diluted with about 3.0 l. of water and stirred until the product crystallized. The mixture was then diluted with water to a volume of 12.0 l. and the product was collected on the filter, washed thoroughly with water and dried; yield, 1000 g., or 84.3%. This material was satisfactory for the preparation of the dihydrazide. A sample was crystallized from chloroform and petroleum ether for analysis; m. p., 96–98° cor.

Anal. Calcd. for $C_{16}H_{20}O_7N_2$: C, 54.54; H, 5.72; N, 7.95. Found: C, 54.95; H, 6.01; N, 7.95.

***p*-Nitrobenzoylglutamic Acid Dihydrazide (III).**—A solution of diethyl-*p*-nitrobenzoylglutamate (1880 g.) in 6.0 l. of warm 3A ethanol was filtered and cooled to 50°. Then, with good stirring, 950 cc. of hydrazine hydrate (100%) was added and the mixture was allowed to stand at room temperature. In a short time the mixture crystallized to a solid mass. After standing overnight, the precipitate was slurried with several liters of 3A ethanol, filtered and washed well with alcohol. The yield of air-dried product was 1700 g. or 98%; m. p. 207–209° cor. This material was satisfactory for the preparation of the diazide without further purification. For analysis, a sample of the crude product was recrystallized (with some difficulty) from aqueous alcohol; m. p., 221–222°, cor.

Anal. Calcd. for $C_{12}H_{16}O_5N_6$: C, 44.44; H, 4.97; N, 25.92. Found: C, 44.48; H, 5.07; N, 26.10.

Diethylglutamate Hydrochloride.—Glutamic acid (1250 g.) was esterified in 2B absolute ethanol in the presence of hydrogen chloride by substantially the procedure of Chiles and Noyes.[8] After concentrating the mixture *in vacuo* until no more alcohol would distil over, the viscous residue was freed of alcohol and water by azeotropic distillation of the well stirred mixture with petroleum ether (b. p. 90–100°) until the vapor temperature reached 90°. This product was dissolved in water just before use, and condensed with *p*-nitrobenzoylglutamic acid diazide as described below.

***p*-Nitrobenzoyl-α,γ-glutamyldiglutamic Acid Tetraethyl Ester (V).**—*p*-Nitrobenzoylglutamic acid dihydrazide (550 g.) was dissolved in a mixture of 2500 cc. of water and 850 cc. of concentrated hydrochloric acid. Ethyl acetate (3500 cc.) was added and the mixture was cooled to −5° in an efficient cooling bath. Then, with vigorous stirring, a solution of 340 g. of sodium nitrite in 1100 cc. of cold water was added during twenty-five minutes, keeping the temperature of the reaction mixture below 5°. After stirring for an additional forty minutes, the water layer was separated and extracted once with 1500 cc. of ethyl acetate. The combined ethyl acetate solution of the diazide was washed once with about 2000 cc. of ice-water and the water washings were discarded. The cold ethyl acetate solution of the diazide was then added during ten minutes to a well-stirred solution of diethylglutamate hydrochloride (prepared from 1250 g. of glutamic acid as previously described) in a mixture of

(1) Mowat, *et al.*, THIS JOURNAL, **70**, 1096 (1948).
(2) Fruton and Bergmann, *J. Biol. Chem.*, **127**, 637 (1939).
(3) Fruton, *ibid.*, **146**, 463 (1942).
(4) Plentl and Page, *ibid.*, **163**, 59 (1946).
(5) Boothe, *et al.*, THIS JOURNAL, **71**, 2304 (1949).
(6) Waller, *et al.*, *ibid.*, **70**, 19 (1948).
(7) Van Der Scheer and Landsteiner, *J. Immunology*, **29**, 371 (1935).
(8) Chiles and Noyes, THIS JOURNAL, **44**, 1798 (1922).

2500 cc. of water and 1600 g. of sodium bicarbonate. After stirring at room temperature for about one hour the product began to crystallize from the reaction mixture. Stirring was continued for about four hours and the mixture was chilled overnight in the ice-box. The precipitate was then collected by filtration, washed with about 1.0 l. of ethyl acetate, then with water and air-dried, at room temperature. This slightly gummy crude product was stirred with 3000 cc. of hot (70°) 3A ethanol and the alcoholic solution was decanted into another vessel. The residue was then extracted once more with 1000 cc. of hot alcohol, filtered and the insoluble material washed with about 250 cc. of hot alcohol. The combined alcoholic extracts were heated to 70°, clarified with charcoal and filtered. The filtrate (5.0 l.) was then well stirred and diluted with hot (80°) water to a volume of 11.0 l. After further stirring, seeding, and cooling, the mixture crystallized to a solid mass. This mixture was diluted with hot water to a volume of about 20 l., stirred for several hours and then chilled overnight. The product (fine, hair-like crystals) was collected, washed thoroughly with water and air-dried at room temperature; yield of dry product, 690 g. or 61%; m. p. sintering at 131-133° and melting at 139-141°, cor. This material was used without further purification for the preparation of pteroyl-α,γ-glutamyldiglutamic acid.

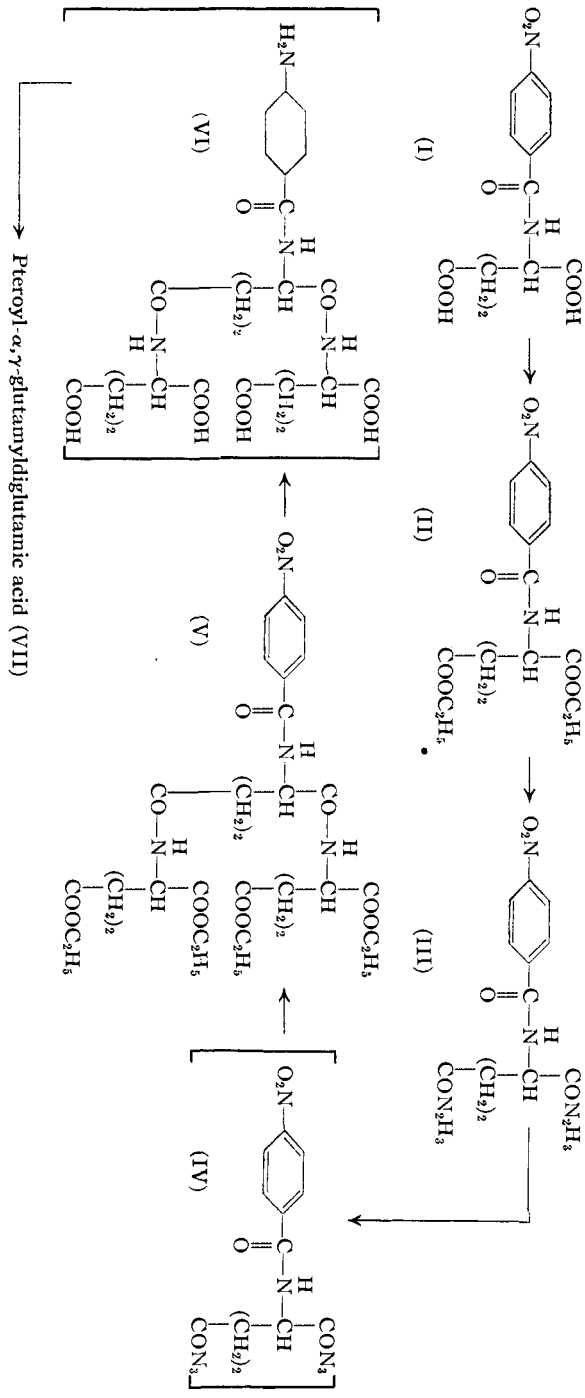
For analysis, a sample of the above material was recrystallized from aqueous alcohol.

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.04; H, 6.35; N, 8.40. Found: C, 54.35; H, 6.49; N, 8.70.

Some discrepancy has been observed in the melting points of different preparations. In some cases the product melted at about 132°, while in other cases the melting point was about 144° with sintering at about 132°. Both products gave satisfactory analytical values. Similar variations in the melting point have been observed with other members of this series of compounds.[9,10]

p-Aminobenzoyl-α,γ-glutamyldiglutamic Acid Tetraethyl Ester (VI).—A mixture of the above nitro compound (1.0 g.), ethanol (10.0 cc.) and water (5.0 cc.) was vigorously stirred while 0.52 g. of zinc dust and 1.2 cc. of concentrated hydrochloric acid were added simultaneously during ten minutes, keeping the reaction mixture at about *p*H 3.0. After stirring for an additional twenty minutes, the mixture was filtered. The filtrate was heated to boiling, diluted with three volumes of hot water, seeded and cooled. After several hours the precipitate was collected and recrystallized from hot aqueous ethanol. The colorless crystalline precipitate was collected, washed with water and dried; m. p., sintered at 147-148° and melted at 150-152°, cor. A mixed melting point with *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester prepared by a different method[1] showed no depression of the melting point.

Pteroyl-α,γ-glutamyldiglutamic Acid (VII).—To a well-stirred solution of *p*-nitrobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester (50 g.) in 300 cc. of warm ethanol was added 300 cc. of 1.0 *N* sodium hydroxide solution. After forty minutes at room temperature the solution was acidified with hydrochloric acid to *p*H 3.3 and reduced with zinc dust and hydrochloric acid at *p*H 3.0-3.5. The filtered solution of *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid was then treated with 2,4,5-triamino-6-hydroxypyrimidine sulfate (38.5 g.), 2,3-dibromopropionaldehyde (33.0 g.) and sodium dichromate dihydrate (7.5 g.) by the method described in communication III[5] of this series. The crude product contained 19.4 g. of activity as shown by chemical assay.[11] The crude material was purified by a previously described method[5] through the second *p*H 0.9 precipitation. The product was then converted to the tetrasodium salt, frozen and dried; yield, 7.75 g.; chemical assay, 91%. Biological assay: *S. faecalis R.*, 0.8%; *L. casei*, 1.14% (pteroylglutamic acid taken as 100%).



An analytical sample was prepared by converting a portion of the above material to the magnesium salt, decolorizing with charcoal and precipitating the free acid at *p*H 2.0. After washing with cold water, the material was twice precipitated by cooling a solution of the compound in hot water. The final product was washed with water, acetone and ether and dried at 110° *in vacuo* over phosphorus pentoxide; chemical assay, 99.5%.

Anal. Calcd. for $C_{29}H_{33}O_{12}N_9$: C, 49.78; H, 4.75; N, 18.02. Found: C, 49.71; H, 4.91; N, 18.05.

The extinction coefficient was determined in 0.1 *N* so-

(9) Boothe, *et al.*, THIS JOURNAL, **70**, 1099 (1948).
(10) Semb, *et al.*, *ibid.*, **71**, 2310 (1949).
(11) Hutchings, *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).

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

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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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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).