Anal. Calcd. for $C_{13}H_{13}N$: C, 85.21; H, 7.15; N, 7.64. Found: C, 85.47; H, 7.06; N, 7.65.

The nitrile was alkylated in the usual way with β-dimethylaminoethyl chloride. The crude alkylated nitrile was converted directly to the hydrochloride without distillation.

For conversion to the cleavage product, XI, the nitrile, X, was treated in the manner described above. From 29 g. of Δ^2-cyclopentenylphenylacetonitrile there was obtained 23.5 g. of dimethyl-γ-Δ^2-cyclopentenyl-γ-phenylpropylamine, b. p. 116–117° (1.0 mm.).

Oxidation of Dimethyl-γ-cyclopentylidene-γ-phenylpropylamine Hydrochloride.—Two grams of the salt dissolved in 30 ml. of water was stirred as a solution of 6.5 g. of potassium permanganate in 200 ml. of water was added in a thin stream. After one-half hour of stirring the mixture was steam-distilled. To the first 20 ml. of distillate there was added 10 ml. of ethanol, six drops of benzaldehyde and two drops of 10% sodium hydroxide solution. The mixture was shaken vigorously by hand for a few minutes and the yellow solid which formed was collected on a filter. After crystallization from ethanol the yellow needles of dibenzalcyclopentanone melted at 185–186° (uncor.) undepressed by admixture with an authentic specimen.

Summary

1. Condensation of cyclopentane with phenylacetonitrile, *p*-methoxyphenylacetonitrile and 2-thienylacetonitrile gave the corresponding cyclopentylidene arylacetonitriles.

2. Alkylation of the above nitriles with dimethylaminoethyl chloride, followed by removal of the cyano group with sodium amide yielded dimethyl - γ - cyclopentylidene - γ - arylpropylamines.

3. Cleavage of γ-dimethylamino-α-Δ^1-cyclopentenyl-α-phenylbutyronitrile gave a mixture of cyclopentylidene and cyclopentenyl bases. The latter was isomerized to the former with the aid of hydrobromic acid.

4. Alkylation of cyclopentylidenephenylacetonitrile with β-piperidylethyl chloride gave a basic nitrile which was cleaved to the non-conjugated γ-Δ^1-cyclopentenyl-γ-phenylpropylpiperidine.

5. Alkylation of Δ^2-cyclopentenylphenylacetonitrile with β-dimethylaminoethyl chloride gave α-Δ^2-cyclopentenyl-γ-dimethylamino - α - phenylbutyronitrile which suffered cleavage of the cyano group when treated with sodium amide to give dimethyl - γ - Δ^2 - cyclopentenyl - γ - phenylpropylamine.

6. The ultraviolet absorption spectra of some cyclopentylidenearylacetonitriles and γ-cyclopentylidene-γ-arylpropylamines in ethanol solution were recorded.

RENSSELAER, N. Y. RECEIVED OCTOBER 15, 1948

[CONTRIBUTION FROM LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

Pteroic Acid Derivatives. III. Pteroyl-γ-glutamylglutamic Acid and Pteroyl-γ-glutamyl-γ-glutamylglutamic Acid

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

In a previous communication[1] the preparation of pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid was described. The pteroyl tripeptide showed the same biological activity when assayed against *S. faecalis* R. and *L. casei* as did the fermentation *L. casei* factor. Although this first method of synthesis indicated the probable position of the peptide linkages in the fermentation *L. casei* factor, it left much to be desired as a preparative method for large-scale work.

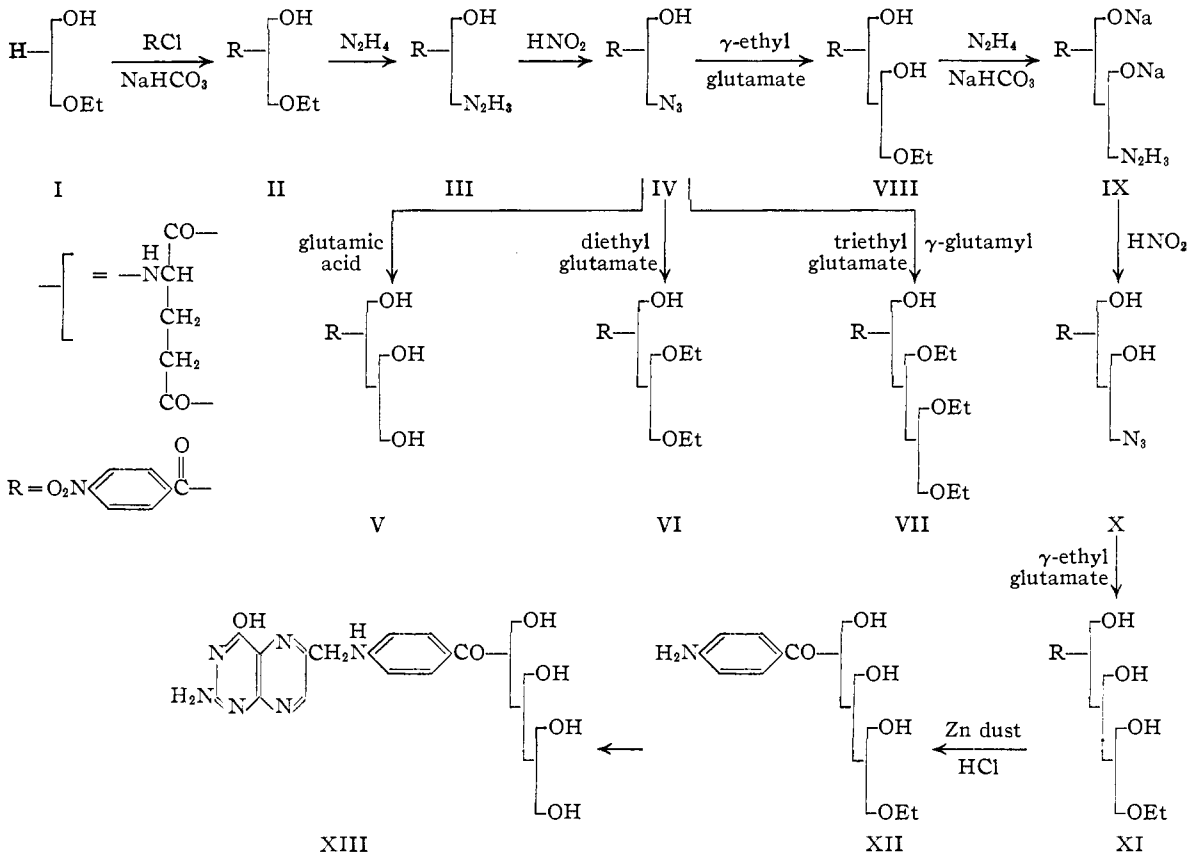
It was thought that it would be desirable to find a method in which the *p*-nitrobenzoyl group could be used instead of the carbobenzoxy group to protect the amino group of glutamic acid. When attempts were made to form the anhydride of *p*-nitrobenzoylglutamic acid analogous to carbobenzoxyglutamic anhydride,[2] the product was found to be completely racemized. Therefore, other methods were investigated.

The method reported here utilizes the γ-monoester of glutamic acid which was prepared by the method of Bergmann and Zervas.[3] The γ-ethyl glutamate (I) was *p*-nitrobenzoylated in a solution of sodium bicarbonate by treating with *p*-nitrobenzoyl chloride. The γ-ethyl *p*-nitrobenzoylglutamate (II) was converted to the hydrazide (III) by use of hydrazine hydrate and then to the azide (IV) by treating with nitrous acid. This azide then reacted with diethyl glutamate in ethyl acetate according to previously described methods[4,5,6] to yield the diester of the *p*-nitrobenzoyl dipeptide (VI).

It was later found that the azide would also react with γ-ethyl glutamate or glutamic acid in an aqueous sodium bicarbonate solution to yield the *p*-nitrobenzoyl dipeptide as the monoester (VIII) or as the triacid (V). The *p*-nitrobenzoyl-γ-glutamylglutamic acid (V) was reduced to the corresponding *p*-amino compound and converted to the pteroyl derivative as previously described.[1,7] In order to characterize the *p*-aminobenzoyl dipeptide, it was esterified and isolated as the triethyl ester.

The *p*-nitrobenzoyl tripeptide was prepared by

(1) Boothe, *et al.*, THIS JOURNAL, **70**, 1099 (1948).
(2) Bergmann and Zervas, *Ber.*, **65**, 1192 (1932).
(3) Bergmann and Zervas, *Z. physik. Chem.*, **221**, 53 (1933).
(4) Fruton and Bergmann, *J. Biol. Chem.*, **127**, 637 (1939).
(5) Fruton, *ibid.*, **146**, 463 (1942).
(6) Plentl and Page, *ibid.*, **163**, 59 (1946).
(7) Waller, *et al.*, THIS JOURNAL, **70**, 19 (1948).



two different methods. It was first prepared as the triethyl ester (VII) by the reaction of the γ-azide of *p*-nitrobenzoylglutamic acid (IV) with triethyl γ-glutamylglutamate. The second method utilized the γ-monoester of *p*-nitrobenzoyl-γ-glutamylglutamic acid (VIII) mentioned above from which was prepared successively the disodium salt, the hydrazide of the disodium salt and the azide (X). This γ-azide of *p*-nitrobenzoyl-γ-glutamylglutamic acid was then treated with γ-ethyl glutamate to yield the *p*-nitrobenzoyl tripeptide as the monoester (XI). Both the monoethyl and the triethyl ester of the *p*-nitro benzoyl tripeptide were completely esterified to tetraethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate and were found to be identical with the compound prepared by the acid chloride method of synthesis.[1] This tetraester was reduced to the *p*-amino derivative as described in the previous communication.[1]

For the preparation of the pteroyl tripeptide, γ-ethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate (XI) was reduced with zinc dust and the solution was condensed directly with 2,3-dibromopropionaldehyde and 2,4,5-triamino-6-hydroxypyrimidine using a modification of the method of Waller, *et al.*[7] The ester group was hydrolyzed off during the purification to yield pteroyl-γ-glutamyl-γ-glutamylglutamic acid (XIII).

Experimental[8]

γ-Ethyl *p*-Nitrobenzoylglutamate (II).—A solution of 2.12 g. of γ-ethyl glutamate hydrochloride, 30 cc. of water and 5 g. of sodium bicarbonate was stirred with 2 g. of *p*-nitrobenzoyl chloride for three hours. A deep violet color developed which faded somewhat after two and one-half hours. The solution was filtered and acidified to below *p*H 2 which precipitated an oil. This oil soon solidified to a white crystalline material which was filtered off and dried; yield, 2.8 g. This was crystallized from 65 cc. of benzene and melted at 113–115°. The m. p. is very near *p*-nitrobenzoylglutamic acid but a mixture of the two melted at 99–101°; $[\alpha]^{25}D$ −10.8 (*c* 4 in ethanol), $[\alpha]^{25}D$ −15.06 (*c* 2 in 1 *N* sodium hydroxide).

Anal. Calcd. for $C_{14}H_{16}O_7N_2$: C, 51.8; H, 4.9; N, 8.6. Found: C, 51.94; H, 5.06; N, 8.69.

***p*-Nitrobenzoylglutamic Acid γ-Hydrazide** (III).—(a) One gram of γ-ethyl *p*-nitrobenzoylglutamate was dissolved in 1 cc. of 66% hydrazine hydrate in water and left at room temperature overnight. The solution was diluted with 10 cc. of water and acidified with hydrochloric acid to *p*H 1 to 1.5. It was then filtered and the *p*H was adjusted to 3.2 at which point the product crystallized out. It was removed by filtration and dried; yield, 0.70 g. The product was again crystallized by dissolving in dilute hydrochloric acid and adjusting to *p*H 3.2; m. p. 186–187° with decomposition; $[\alpha]^{25}D$ −22.8 (*c* 4 in 1 *N* hydrochloric acid).

Anal. Calcd. for $C_{12}H_{14}O_5N_4$: C, 46.45; H, 4.51; N, 18.06. Found: C, 46.37; H, 4.60; N, 17.81.

(b) A crude wet filter cake of γ-ethyl *p*-nitrobenzoylglutamate containing 385 g. of solid and 235 cc. of water

(8) All melting points were taken according to U. S. P. specified conditions and are corrected.

was added slowly to a stirred mixture of 99 g. of sodium bicarbonate and 100 cc. of water. To this solution was added 60 cc. of hydrazine hydrate and allowed to stand for two days at room temperature. The solution was diluted to 1 liter and acidified with concentrated hydrochloric acid to *p*H 1. After filtering and adjusting to *p*H 3.2 the solution was cooled overnight and the product was filtered off; yield, 239 g.; m. p., 181° with decomposition.

Diethyl *p*-Nitrobenzoyl-γ-glutamylglutamate (VI).—A solution of 5 g. of *p*-nitrobenzoylglutamic acid γ-hydrazide in 50 cc. of water and 7.5 cc. of concentrated hydrochloric acid was stirred with 40 cc. of ethyl acetate and cooled to 0°. With vigorous stirring and cooling, 1.75 g. of sodium nitrite in 10 cc. of water was added dropwise. The solution was stirred fifteen minutes and the ethyl acetate layer was separated off. The water was washed with 25 cc. of cold ethyl acetate and the combined ethyl acetate solutions were washed three times with 50-cc. portions of ice-water. The ethyl acetate solution was then dried over magnesium sulfate while cold and added to 10 g. of diethyl glutamate in 50 cc. of ethyl acetate. After standing overnight a large amount of solid material had precipitated. The ethyl acetate suspension was extracted with 80 cc. of water containing 5 g. of sodium bicarbonate. This aqueous solution was acidified[9] to precipitate the product which was collected and dried; yield, 5.1 g. One gram of this product was crystallized twice from 35 cc. of ethyl acetate; m. p., 161–162°.

Anal. Calcd. for $C_{21}H_{27}O_{10}N_3$: C, 52.4; H, 5.61; N, 8.73. Found: C, 52.7; H, 5.67; N, 8.33.

One gram of this diester was hydrolyzed to *p*-nitrobenzoyl-γ-glutamylglutamic acid by dissolving in 20 cc. of 0.5 *N* sodium hydroxide. After forty-five minutes at room temperature the solution was acidified and the product crystallized. The product was recrystallized from water and melted at 194–195°. There was no m. p. depression when this was mixed with a sample from the previously described synthesis.[1]

γ-Ethyl *p*-Nitrobenzoyl-γ-glutamylglutamate (VIII).—A suspension of 150 g. of γ-ethyl glutamate and 150 g. of sodium bicarbonate in 1000 cc. of water was cooled to 5° and an ethereal solution of *p*-nitrobenzoylglutamic acid γ-azide (from 200 g. of hydrazide) was added. The mixture was warmed to 24° and stirred until the evolution of gas had ceased. The ether layer was discarded and the aqueous solution was made strongly acid[9] with concentrated hydrochloric acid after cooling to 0°. The precipitate was oily at first but soon crystallized; yield, 197 g. A portion was crystallized twice from ethanol and melted at 139°; $[\alpha]^{26}D$ −3.75° (*c* 4 in 0.5 *N* sodium bicarbonate), $[\alpha]^{26}D$ −8.75° (*c* 4 in ethanol).

Anal. Calcd. for $C_{19}H_{23}O_{10}N_3$: C, 50.3; H, 5.09; N, 9.28. Found: C, 50.3; H, 5.73; N, 9.24.

***p*-Nitrobenzoyl-γ-glutamylglutamic Acid (V).**—A solution of *p*-nitrobenzoylglutamic acid γ-azide (from 660 g. of the hydrazide) in 5 liters of ethyl acetate was added to a mixture of 625 g. of glutamic acid and 1200 g. of sodium bicarbonate in 3300 cc. of water. The mixture was stirred three and one-half hours after warming up to room temperature. The layers were separated and the ethyl acetate layer was washed with water. The combined aqueous solutions were treated with charcoal, filtered and acidified to *p*H 1.5 with concentrated hydrochloric acid.[9] The product first came out rather gummy but soon solidified. It was filtered off and recrystallized by dissolving in 2.5 liters of boiling ethanol and diluting to 15 liters with warm water. After cooling the solution, the product was filtered off and dried; yield, 571 g.; m. p., 190–191°. There was no m. p. depression when this material was mixed with samples prepared by other methods.

Triethyl *p*-Aminobenzoyl-γ-glutamylglutamate.—A suspension of 20 g. of γ-ethyl *p*-nitrobenzoyl-γ-glutamylglutamate in 190 cc. of water and 20 cc. of ethanol was stirred and adjusted to *p*H 3.5. Seventeen grams of zinc dust was added in portions and concentrated hydrochloric acid was added as necessary to maintain *p*H 3.5. After one hour the excess zinc was filtered off, ammonium hydroxide was added until the filtrate was distinctly alkaline, and hydrogen sulfide was bubbled in for thirty minutes. After removal of the zinc sulfide the solution was adjusted to *p*H 3 and evaporated to complete dryness *in vacuo*. The residue was taken up in 80 cc. of absolute ethanol and the ammonium chloride was filtered out. This filtrate was evaporated to dryness *in vacuo* and left a flaky solid weighing 17.4 g. Because of the difficulties encountered in purifying this material it was esterified by dissolving it in 100 cc. of 10% hydrogen chloride in absolute ethanol at 50–60°. After standing overnight at room temperature and then cooling for several hours, the product was filtered off and dried; yield, 15.8 g.; m. p., 186–188°. This hydrochloride was suspended in 150 cc. of water at 60–70° and 10 cc. of pyridine was added. Upon shaking, the hydrochloride dissolved and the free amine crystallized out; yield, 13.7 g. A portion of this material was recrystallized from ethanol; m. p. 131–133°; $[\alpha]^{28}D$ −20.3° (*c* 2 in ethanol).

Anal. Calcd. for $C_{23}H_{33}O_8N_3$: C, 57.6; H, 6.89; N, 8.77. Found: C, 57.42; H, 7.01; N, 9.05.

Pteroyl-γ-glutamylglutamic Acid.—A suspension of 21.25 g. of *p*-nitrobenzoyl-γ-glutamylglutamic acid in 375 cc. of water was stirred and adjusted to *p*H 3.5. This *p*H was maintained with hydrochloric acid while 16 g. of zinc dust was added over fifteen minutes. After stirring one hour the excess zinc was filtered out, and the solution of *p*-aminobenzoyl-γ-glutamylglutamic acid was condensed with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde as described previously.[1,7] After isolation the product showed the same physical and biological properties as already described.

Triethyl *p*-Nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate (VII).—An ethyl acetate solution of *p*-nitrobenzoylglutamic acid γ-azide, prepared as described above from 99 g. of the corresponding hydrazide, was added to a cold, well-stirred solution of 103 g. of triethyl γ-glutamylglutamate hydrochloride[1] and 135 g. of sodium bicarbonate in 500 cc. of water. The mixture was stirred at room temperature for five hours and 300 cc. of water was added during this time. After filtering the mixture, the ethyl acetate layer was drawn off and extracted with 250 cc. of sodium bicarbonate solution and 200 cc. of water. The combined aqueous solutions were washed three times with ether and then acidified to *p*H 1.5.[9] The precipitate was filtered, washed and dried; yield, 142 g. A sample of this material was crystallized first as a pyridine salt from ethanol, and then after acidification as the corresponding free acid from aqueous ethanol; m. p., 155–157°; $[\alpha]^{29}D$ −26.5° (*c* 4 in 0.5 *N* sodium bicarbonate).

Anal. Calcd. for $C_{28}H_{38}O_{13}N_4$: C, 52.7; H, 6.00; N, 8.78. Found: C, 53.02; H, 6.18; N, 8.90.

Fifteen grams of this product was esterified by dissolving in warm 10% ethanolic hydrogen chloride and allowing to stand at room temperature overnight. The fully esterified product crystallized and was found to be identical by m. p. and mixed m. p. with the product described in the previous communication.[1] The same m. p. behavior was noted here, *i.e.*, sometimes a form was obtained melting at 158–159° and sometimes at 173–174°.

Disodium Salt of *p*-Nitrobenzoyl-γ-glutamylglutamic Acid γ-Hydrazide (IX).—A solution of 22.5 g. of γ-ethyl *p*-nitrobenzoyl-γ-glutamylglutamate and 8.4 g. of sodium bicarbonate in 25 cc. of water was filtered and diluted with 250 cc. of ethanol. After cooling, the disodium salt crystallized and was filtered off and dried; yield, 14.5 g. A portion of the product was dissolved in water and acidified, and the starting material was obtained back unchanged.

A solution of 35 g. of this crystalline disodium salt in 10.5 cc. of hydrazine hydrate and 70 cc. of methanol was refluxed one and one-half hours. After cooling and filtering, the methanol solution was poured slowly into 700 cc. of well-stirred absolute ethanol. The granular precipitate

(9) In all reactions involving an azide, hydrazoic acid is a by-product. These reaction mixtures should be acidified in a well-ventilated hood because of the toxicity of this gaseous by-product.

was filtered off, washed well with ethanol and dried; yield, 30 g. This material is hygroscopic.

Anal. Calcd. for $C_{17}H_{19}O_9N_5Na_2$: C, 39.75; (assuming the ash is sodium carbonate); H, 3.93; N, 14.49. Found: C, 39.44; H, 4.85; N, 14.63.

γ-Ethyl *p*-Nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate (XI).—A solution of 30 g. of the disodium salt of *p*-nitrobenzoyl-γ-glutamylglutamic acid γ-hydrazide in 150 cc. of water and 45 cc. of concentrated hydrochloric acid was washed twice with 150-cc. portions of ethyl acetate which were discarded. Another 150 cc. of ethyl acetate was added to the aqueous solution and the mixture was stirred and cooled to 0°. With continued stirring and cooling, 6 g. of sodium nitrite in 20 cc. of water was dropped in over a fifteen-to-twenty-minute period. The mixture was stirred an additional fifteen minutes and the layers were separated. The aqueous phase was washed twice with ethyl acetate and the combined ethyl acetate solutions were washed with water. The ethyl acetate solution of the azide was then added to a solution of 23.4 g. of γ-ethyl glutamate and 28 g. of sodium bicarbonate in 100 cc. of water and this mixture was stirred at 30° for three hours. The aqueous phase was treated with charcoal and filtered, and upon slow acidification[9] with hydrochloric acid, the product crystallized. It was removed by filtration and dried; yield, 19 g. A portion of the product was crystallized twice from water; m. p., 171–172°; $[\alpha]^{28}D$ −13.0° (*c* 6 in 2 parts ethanol and 1 part water); $[\alpha]^{26}D$ +5.0° (*c* 6 in 0.5 *N* sodium hydroxide).

Anal. Calcd. for $C_{24}H_{30}N_4O_{13}$: C, 49.5; H, 5.16; N, 9.62. Found: C, 49.9; H, 5.55; N, 9.94.

A portion of this material was fully esterified by the same method as was used for triethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate and the products were identical by m. p. and mixed m. p.

Tetraethyl *p*-Aminobenzoyl-γ-glutamyl-γ-glutamylglutamate.—A suspension of 15 g. of γ-ethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate in 150 cc. of water was reduced with zinc dust and hydrochloric acid as described for the *p*-nitrobenzoyl dipeptide. After removal of the zinc salts as the sulfide and the ammonium chloride by alcohol extraction as described above, the flaky, solid residue weighed 11.4 g. This was esterified as described above using 60 cc. of 10% alcoholic hydrogen chloride. The gelatinous product after drying (9.7 g.) was suspended in 75 cc. of water at 60° and 3.5 cc. of pyridine was added. The amine hydrochloride dissolved and the free amine separated as an oil which solidified on cooling. This product was crystallized after treating with charcoal from 20 cc. of absolute ethanol; yield, 5.7 g.; m. p., 146–148°; $[\alpha]^{26}D$ −27.2° (*c* 4 in ethanol). This product showed no depression of m. p. when mixed with a sample of the material described in the previous communication.[1]

Pteroyl-γ-glutamyl-γ-glutamylglutamic Acid (XIII).—A suspension of 29.15 g. of γ-ethyl *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamate in 200 cc. of water was adjusted to *p*H 3.5. The suspension was stirred and 20 g. of zinc dust was added in portions over twenty minutes. Hydrochloric acid was added as necessary to maintain the *p*H at 3 to 3.5 and external cooling was used to keep the temperature below 40°. After stirring an additional twenty minutes the solution was clarified by filtering and used in the next reaction.

A suspension of 25.7 g. of 2,4,5-triamino-6-hydroxypyrimidine sulfate and 24.4 g. of barium chloride dihydrate in 450 cc. of water was stirred and heated at 60° for ten minutes. This suspension was then added to the reduced solution described above and stirred while 31.5 cc. of a solution of 21.6 g. of 2,3-dibromopropionaldehyde in acetic acid and 4.97 g. of sodium dichromate in 30 cc. of water were added simultaneously over thirty minutes.[7] The *p*H was maintained at 3 by addition of sodium hydroxide solution and the temperature was kept at 35–40°. The mixture was stirred an hour longer and the precipitate was filtered out and dried. The weight was 70 g. and contained 16% of the pteroyl derivative by chemical assay.[10]

This crude reaction product was dissolved in 11 liters of 0.1 *N* sodium hydroxide at 80° and 30 g. of Celite and 198 cc. of 30% calcium chloride were added. The hot solution was filtered and the precipitate was discarded. To the filtrate at 60° was added 30 g. of Celite and enough 5% zinc chloride to bring to *p*H 10.9.[11] The solution was filtered and the precipitate was discarded. Sufficient zinc chloride solution was added to bring to *p*H 6.8. After adding 30 g. of Celite, the suspension was heated to 90° and the precipitate containing 7.8 g. of the desired product was filtered off. This precipitate was slurried in 1560 cc. of 0.1 *N* sodium hydroxide for thirty minutes and hydrochloric acid was added to *p*H 2.5. The slurry was filtered and the filtrate was discarded. The precipitate was stirred in 1400 cc. of 0.1 *N* sodium hydroxide at 45° and then hydrochloric acid was added to *p*H 0.9 and the suspension was cooled to 0° for several hours. The precipitate was filtered out and the filtrate which contained 1.3 g. of product was discarded. The precipitate was dissolved in 1100 cc. of 0.1 *N* sodium hydroxide and heated to 80°. It was then treated with 20 cc. of 30% calcium chloride and the *p*H was brought to 10.9 with zinc chloride as described in the first steps of this purification. The filtrate from the zinc chloride treatment was warmed to 45° and concentrated hydrochloric acid was added to about *p*H 0.9 or until the precipitate which first formed just redissolved. On cooling to 0° for several hours the product precipitated and was removed and washed with water in a centrifuge. After drying, this material weighed 3.8 g. and contained 3.3 g. of the desired pteroyl derivative by chemical assay (87% pure).

To obtain an analytical sample 1.2 g. of this material was further purified. It was suspended in 200 cc. of water, stirred and magnesium oxide was added until the *p*H rose to 9.3. The mixture was heated to 85°, treated with 1 g. of Norite and filtered. Sufficient hydrochloric acid was added to make the filtrate to 0.5 *N* and it was cooled to 0°. Some material precipitated out and was filtered off, which was 92% pure by chemical assay. The filtrate from this was adjusted to *p*H 0.9 and the precipitate was filtered off. This precipitate was dissolved in 80 cc. of very dilute sodium hydroxide and adjusted to *p*H 0.6 with hydrochloric acid. After cooling to 0°, the light yellow precipitate was filtered off and dried at 100° *in vacuo*. The analyses indicated that the compound retained one molecule of water and the chemical assay on this basis was 100.5%.

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, 48.62; H, 5.27; N, 17.69.

This material showed the same ultraviolet absorption spectrum as did the fermentation *L. casei* factor.[12] The microbiological activity was also found to be the same, *i.e.*, the compound was 2–4% as active as pteroylglutamic acid when assayed with *S. faecalis* R. and equally as active on a molar basis as pteroylglutamic acid when assayed with *L. casei*.

Acknowledgments.—The microbiological assays were carried out by Miss Eleanora Boggiano, the chemical assays by Mrs. Anna de Grunigen, and the chemical analyses by Mr. Louis Brancone and staff. Certain intermediates were prepared by Mr. Willard McEwen and Mr. William Kinley. We are especially indebted to Mr. Albert Gazzola for his assistance during this work.

Summary

A new synthesis of intermediates leading to pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid is described.

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

(11) We are indebted to Dr. Erwin Kuh, Calco Chemical Division, American Cyanamid Company, for the development of this step in the purification.

(12) Hutchings, *et al.*, THIS JOURNAL, **70**, 1 (1948).

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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[CONTRIBUTION FROM LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

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

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(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).