Acknowledgment.—The authors wish to thank both the University of Texas Research Institute and the Research Corporation for their support of these and related researches on allylic chlorides.

Summary

The reaction of allylic chlorides with potassium iodide in acetone has been used to elucidate the influence of substitutents on both the number 1 and number 2 carbons and also of geometrical isomerism on the relative reactivity of the allylic chlorine.

The greater the relative electronegativity of the group on the number 2 carbon, the less is the activity of the allylic chlorine toward iodide ion. The differences in reactivity, however, are small.

Because the reaction of potassium iodide with allylic chlorides is an S_N2 type reaction, either a relatively negative group or a relatively large group, or both, in the *cis* position on the number 1 carbon would inhibit the reaction.

Since the low boiling geometrical isomers of both 1,3-dichloropropene and 1,3-dichloro-2-methyl-1-propene react more readily than the high boiling isomers, the low boiling isomers have tentatively been assigned the *trans* structure.

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Pteroic Acid Derivatives. I. Pteroyl-α-glutamylglutamic Acid and Pteroyl-α,γ-glutamyldiglutamic Acid

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

In the course of the degradation of the fermentation *L. casei* factor[1] this compound was found to contain three molecules of glutamic acid in contrast to the liver *L. casei* factor (pteroylglutamic acid) which contained only one molecule of glutamic acid.

The synthesis of the fermentation *L. casei* factor became of particular interest when Lewisohn and his associates[2] reported that the substance caused regression of spontaneous breast tumors in mice.

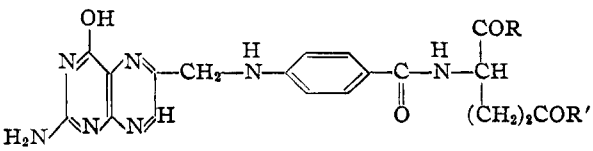
This communication describes the synthesis of one of the two possible isomers of pteroyldiglutamic acid and one of the five possible isomers of pteroyltriglutamic acid. These compounds were prepared during the course of our work on the structure and synthesis of the fermentation *L. casei* factor.

In the preparation of pteroyl-α-glutamylglutamic acid (I) the dipeptide α-glutamylglutamic acid[3] was treated with *p*-nitrobenzoyl chloride and after reduction of the nitro group the resulting *p*-aminobenzoyl-α-glutamylglutamic acid was condensed with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde by the procedure of Waller, *et al.*[4]

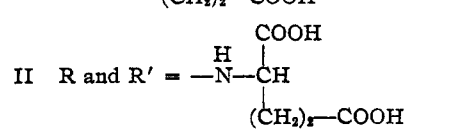
The purified product was only slightly active when assayed with *Lactobacillus casei* or *Streptococcus faecalis R.* The low activity with *Lactobacillus casei* indicated that the fermentation *L. casei* factor probably contained at least one gamma-linkage in the peptide side chain. It therefore seemed desirable to prepare pteroyl-α,γ-glutamyldiglutamic acid (II).

This substance was prepared by condensing diethyl glutamate[5] with the γ-acid chloride of carbobenzoxy-α-glutamyldiethylglutamate[3] to give carbobenzoxy-α,γ-glutamyldiglutamic acid tetraethyl ester. The carbobenzoxy group was removed in the usual manner with hydrogen and palladium charcoal catalyst in the presence of acetic acid, giving the acetate of α,γ-glutamyldiglutamic acid tetraethyl ester which was then converted to the *p*-nitrobenzoyl derivative. After reduction of the nitro group, the resulting *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid was condensed with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde by the procedure of Waller, *et al.*[4]

The crude product, pteroyl-α,γ-glutamyldiglutamic acid, was not available in quantities sufficient for a satisfactory purification but the biological assay of both the crude and the partially purified material did not differ appreciably from that of pteroyl-α-glutamylglutamic acid and it



I R = —N(H)—CH(COOH)—$(CH_2)_2$—COOH R′ = —OH



II R and R′ = —N(H)—CH(COOH)—$(CH_2)_2$—COOH

(1) Hutchings, *et al.*, THIS JOURNAL, **70**, 10 (1948).

(2) Lewisohn, C. Leuchtenberger, R. Leuchtenberger and Kereszstesy, *Science*, **104**, 436 (1946).

(3) Bergmann and Zervas, *Ber.*, **65**, 1192 (1932).

(4) Waller, *et al.*, THIS JOURNAL, **70**, 19 (1948).

(5) Chiles and Noyes, *ibid.*, **44**, 1798 (1922).

was evident that this α,γ-isomer was not identical with the fermentation *L. casei* factor.

Since the above procedure was not entirely satisfactory for the preparation of useful quantities of the pure α,γ-compound, other methods of synthesis have been investigated and will be described in a later communication.

Experimental

p-Nitrobenzoyl-α-glutamylglutamic Acid.—A mixture of α-glutamylglutamic acid[5,6] (17.0 g.), 43 cc. of water, and 111 cc. of 2 *N* sodium hydroxide solution was treated with 22.88 g. of *p*-nitrobenzoyl chloride and 104.8 cc. of 2 *N* sodium hydroxide solution. These reagents were added in several portions during about twenty minutes with vigorous stirring. After about forty-five minutes the mixture was acidified by the addition of 51 cc. of 6 *N* hydrochloric acid, cooled and filtered. The filter-cake was then extracted several times with a total volume of 200 cc. of hot water. The combined aqueous extracts were filtered while hot and then allowed to cool. The crystalline precipitate was collected, washed with cold water and dried; yield, 17.8 g. (68%). A sample of the material was recrystallized from hot water and dried at 60° *in vacuo* for analysis; m. p. 146–148°, cor.; $[\alpha]^{29}D$ +8.12° (*C* 4, acetone).

Anal. Calcd. for $C_{17}H_{19}O_{10}N_3$: C, 48.00; H, 4.50; N, 9.88. Found: C, 47.71; H, 4.86; N, 9.77.

p-Aminobenzoyl-α-glutamylglutamic Acid Triethyl Ester Hydrochloride.—A mixture of *p*-nitrobenzoyl-α-glutamylglutamic acid (35.0 g.) glacial acetic acid (32 cc.), water (750 cc.), and platinum oxide catalyst (0.6 g.) was shaken with hydrogen at room temperature and atmospheric pressure until 6.57 l. of hydrogen had been absorbed. After filtering off the catalyst, the solution was adjusted to *p*H 3.0 and extracted several times with ether to remove any traces of *p*-aminobenzoic acid which might be present. The aqueous solution contained 28.8 g. of *p*-aminobenzoyl-α-glutamylglutamic acid as determined by the method of Bratton and Marshall.[7] After evaporation to dryness *in vacuo* 21.6 g. of the non-crystalline residue was esterified at room temperature for eight hours with absolute ethanol containing 0.1 g. of hydrogen chloride per cc. of solution. After cooling overnight the crystalline precipitate was collected, washed with ethanol and dried; wt. 16.9 g., m. p. 164–167°. This was recrystallized from 140 cc. of ethanol for analysis; wt. 12.6 g., m. p. 166–168°, cor.

Anal. Calcd. for $C_{23}H_{33}O_8N_3 \cdot HCl$: C, 53.54; H, 6.64; N, 8.14. Found: C, 53.34; H, 6.63; N, 8.19.

p-Aminobenzoyl-α-glutamylglutamic Acid Triethyl Ester.—A suspension of the above hydrochloride (12 g.) in 120 cc. of water at 60° was treated with 8 cc. of pyridine. The oily precipitate crystallized upon cooling; wt. 10.7 g., m. p. 113–115°. This material was recrystallized from 75 cc. of ethanol; wt. 6.8 g., m. p. 114–115°, cor.; $[\alpha]^{28}D$ −8.75° (*C* 2, ethanol).

Anal. Calcd. for $C_{23}H_{33}O_8N_3$: C, 57.61; H, 6.94; N, 8.76. Found: C, 57.99; H, 7.32; N, 8.69.

Pteroyl-α-glutamylglutamic Acid.—*p*-Nitrobenzoyl-α-glutamylglutamic acid (35 g.) was reduced as previously described, and the solution, containing about 29 g. of *p*-aminobenzoyl-α-glutamylglutamic acid, was diluted to a volume of 2 liters. To this solution was added 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride (31.2 g.) and sufficient sodium hydroxide to bring the mixture to *p*H 4.0. Then with vigorous stirring, a solution of 2,3-dibromopropionaldehyde (31.5 g.) in 750 cc. of ethanol was added during about thirty minutes, together with sufficient sodium hydroxide solution to maintain the reaction mixture at *p*H 4.0. After adding all of the reagents, the mixture was stirred for about thirty minutes. The precipitate was collected on the filter, washed with water, ethanol, acetone and ether and dried. The crude product weighed 36.8 g. Chemical assay[8] indicated that the crude material contained 22.2% by weight of pteroyl-α-glutamylglutamic acid.

Purification.—The above crude product, containing 8.17 g. of activity, was dissolved in 22.8 l. of 0.2 *N* sodium hydroxide solution and treated with 557 g. of barium chloride dihydrate. Alcohol was then added to 2.5% by volume and the mixture was filtered. The filtrate, containing 6.45 g. of activity, was diluted with water to a volume of 75 liters and the barium was removed as barium sulfate. The filtrate was treated with 18.75 liters of alcohol and diluted with water to a volume of 125 liters. After adjusting the solution to *p*H 6.0, 225 g. of zinc acetate was added and the mixture was then adjusted to *p*H 6.9, heated to 70° and filtered. The filter cake, containing the active material, was suspended in about 125 liters of water, treated with sodium hydroxide to 0.05 *N* and the active material was then precipitated with zinc at *p*H 6.9 as described above. After again precipitating with zinc, the zinc salt precipitate was extracted with 7 liters of 0.1 *N* sodium hydroxide solution. The alkaline extract, containing 4.46 g. of activity, was decolorized with 5 g. of Norite A, filtered and made up to 0.2 *N* with sodium hydroxide. The solution was treated with barium chloride to 0.2 *N* and then alcohol was added to 2% by volume. After filtering off the precipitate, the filtrate was acidified to *p*H 2.8, cooled and filtered. The filter-cake was dissolved in hot water (8 liters) and on cooling, the active material precipitated. After repeating this precipitation from hot water, 2.11 g. of material was obtained; chemical assay,[8] 93.9%. Extinction coefficient: calcd. 164. Found: 151–152; 151/164 = 92.2% which compares favorably with the above chemical assay. After further purification from hot water a sample was obtained for analysis.

Anal. Calcd. for $C_{24}H_{26}O_9N_8$: C, 50.52; H, 4.59; N, 19.64. Found (corrected for 1.1% ash): C, 50.40; H, 5.11; N, 19.86. Biological assay: *S. faecalis R.* 0.5%; *L. casei* 0.8%; (pteroylglutamic acid, 100%). The compound was fully active (on a molar basis) in the chick assay.[9]

Carbobenzoxy-α,γ-glutamyldiglutamic Acid Tetraethyl Ester.—To a cold (−10°) well-stirred solution of diethyl carbobenzoxy-α-glutamylglutamate[3] (43 g.) in 140 cc. of dry, alcohol-free chloroform was added 29.5 g. of phosphorous pentachloride in several portions during thirty minutes. The mixture was stirred at −10° for an additional forty-five minutes and the solution was then decanted from a small amount of residual solid matter and evaporated *in vacuo* (below 0°) to remove most of the chloroform. The residue was quickly washed several times by decantation with 400 cc. of cold, dry heptane and then dissolved in 90 cc. of cold, dry chloroform. This solution was added immediately to a solution of 47 g. of diethyl glutamate in 66 cc. of cold (−30°) dry chloroform. After about ten minutes, the reaction mixture was removed from the cooling bath and allowed to stand at room temperature for about three hours.

The reaction mixture was then extracted several times with each of the following, 0.2 *N* hydrochloric acid, water, 10% sodium bicarbonate solution, water, 0.1 *N* hydrochloric acid, and water. The chloroform solution was then dried over magnesium sulfate, clarified with charcoal, filtered and evaporated to dryness *in vacuo*. The waxy residue was triturated with ether and the crystalline product was collected, washed with ether and petroleum ether and dried; wt. 27.5 g. This material was recrystallized from 28 cc. of warm, dry chloroform by the addition of ether; wt. 22 g. A sample of the material was recrystallized several times from chloroform and ether for analysis; m. p. 140.5–142.0°, cor. (sintering 105–107°).

(6) The melting point of this peptide was found to be 184–185°, cor. instead of 205°, cor. All other properties of this material and of its precursors agreed with the values reported by Bergmann and Zervas.[3]

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

(8) Hutchings, *et al.*, *ibid.*, **168**, 705 (1947).

(9) J. J. Oleson, unpublished data.

Anal. Calcd. for $C_{31}H_{46}O_{12}N_2$: C, 57.13; H, 6.96; N, 6.45. Found: C, 57.13; H, 7.56; N, 6.52.

The Acetate of α,γ-Glutamyldiglutamic Acid Tetraethyl Ester.—Carbon dioxide-free hydrogen was passed into a well-stirred mixture of carbobenzoxy-α,γ-glutamyldiglutamic acid tetraethyl ester (10 g.), ethanol (150 cc.), water (50 cc.), glacial acetic acid (3.0 cc.), and 10% palladium–charcoal catalyst (0.7 g.) for two hours. An additional 0.6 g. of catalyst was added and the reduction was continued until carbon dioxide was no longer evolved. The carbon dioxide liberated in the reaction was absorbed in potassium hydroxide solution, and then precipitated as barium carbonate which was collected and weighed. Nearly the theoretical amount of barium carbonate was obtained. The reduction mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was shaken with dry ether, giving a white, crystalline precipitate which was collected, washed with dry ether and petroleum ether and dried; wt. 7.05 g. This material was satisfactory for the subsequent *p*-nitrobenzoylation. Some difficulty was experienced in recrystallizing the material directly, and finally an analytical sample was prepared as follows: The above crude product (0.222 g.) was suspended in about 5 cc. of dry ether and a small excess of diethylamine was added. The mixture was filtered to remove diethylamine acetate and the filtrate was evaporated to dryness *in vacuo* several times with small portions of ether to remove excess diethylamine. The final residue was taken up in a little dry ether, filtered and treated with a few drops of acetic acid. The crystalline acetate precipitated, and after washing with ether and petroleum ether it was dried *in vacuo*.

Anal. Calcd. for $C_{35}H_{48}O_{12}N_2$: C, 51.98; H, 7.50; N, 7.28. Found: C, 52.30; H, 7.40; N, 7.20.

No sharp melting point was obtained. The sample softened between 71.5 and 78.0° and melted at 78.0–81.0°, cor.

***p*-Nitrobenzoyl-α,γ-glutamyldiglutamic Acid Tetraethyl Ester.**—A stirred solution of the acetate of α,γ-glutamyldiglutamic acid tetraethyl ester (3.0 g.) in 50 cc. of dry ether was treated with 1 cc. of diethylamine. The ether solution was decanted and the gummy residue was washed twice with dry ether. The combined ether solution was filtered and treated with a solution of *p*-nitrobenzoyl chloride (1.0 g.) in 10 cc. of dry ether. After thirty minutes the precipitate was collected and washed with ether and then with water. The residue was dried *in vacuo*; wt. 1.72 g. For analysis a sample (0.070 g.) was twice recrystallized from 2 cc. of alcohol and 3 volumes of water; m. p. 144.0–145.0° cor. (sintering about 130°).

Anal. Calcd. for $C_{30}H_{42}O_{13}N_4$: C, 54.04; H, 6.35; N, 8.40. Found: C, 53.88; H, 6.75; N, 8.15.

***p*-Aminobenzoyl-α,γ-glutamyldiglutamic Acid Tetraethyl Ester.**—A well-stirred mixture of *p*-nitrobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester (0.666 g.), ethanol (20 cc.), water (10 cc.), concentrated hydrochloric acid (0.25 cc.), and iron dust (0.333 g.) was warmed to 50° for about thirty minutes and then allowed to react at room temperature for an additional four hours. The reduction of the nitro group was then 95% complete as determined by the method of Bratton and Marshall.[7] The mixture was filtered and the filtrate was adjusted to *p*H 8.2. The precipitate was removed and washed with a little ethanol and the combined filtrates were acidified to *p*H 6.5. After evaporation to dryness *in vacuo*, the residue was suspended in a little water, collected on the filter, washed with water and dried; wt. 0.435 g. The material was recrystallized from aqueous alcohol and dried *in vacuo*; wt. 0.344 g.; m. p. melted to a semi-solid at 146.5–147.5° and became completely liquid at 149.0–150.0°, cor.

Anal. Calcd. for $C_{30}H_{44}O_{11}N_4$: C, 56.59; H, 6.97; N, 8.81. Found: C, 56.76; H, 7.19; N, 9.00.

Pteroyl-α,γ-glutamyldiglutamic Acid.—A mixture of *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid tetraethyl ester (0.158 g.), ethanol (1.0 cc.), and 0.1 *N* sodium hydroxide solution (10 cc.) was warmed to about 60° for a few minutes and then allowed to stand at room temperature for about ninety minutes. The solution was filtered to remove a trace of insoluble material. The filtrate was acidified with 1 cc. of acetic acid and treated with 0.107 g. of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride. The mixture was then adjusted to *p*H 4.0 and reacted at *p*H 4.0 with 1.5 cc. of an acetic acid solution containing 0.108 g. of 2,3-dibromopropionaldehyde during twenty minutes. The precipitated crude product was collected, washed with ethanol, acetone, and ether and air dried; wt. 0.064 g.; chemical assay,[8] 26.8%. Microbiological assay: *S. faecalis R.* 0.14%; *L. casei* 0.17% (pteroylglutamic acid, 100%).

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Summary

Pteroyl-α-glutamylglutamic acid and pteroyl-α,γ-glutamyldiglutamic acid have been prepared. The latter compound was not identical with the fermentation *L. casei* factor.

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