11 by adding a dilute solution of sodium hydroxide. When the *p*H became constant (about one hour) the solution was heated quickly to 80°, acidified to *p*H 2–3, cooled and filtered. The air-dried product (13 g.) assayed chemically[9] for 56.2% pteroylglutamic acid, bioassay using *S. faecalis* R. 0.064%.

The crude IV was purified by dissolving in 6 *N* hydrochloric acid, diluting to 1 *N*, warming to 60–70°, clarifying with charcoal and cooling. The procedure was repeated several times to obtain a pure sample. This purified material decomposed immediately on heating at 100° in vacuum or at room temperature for 2 to 3 days. PABG was liberated on decomposition. For analysis a sample was filtered from the 1 *N* hydrochloric acid (where it is stable) washed with water, alcohol and ether, dried at room temperature for 20 minutes under high vacuum and then analyzed immediately.

Anal. Calcd. for $C_{19}H_{19}O_6N_5 \cdot 2H_2O$: C, 47.78; H, 4.85; N, 20.53. Found: C, 47.45; H, 4.99; N, 20.37.

One gram of IV was completely oxidized with alkaline permanganate at room temperature in 10 minutes. The product was purified through its insoluble magnesium salt and its slightly soluble sodium salt. The final product was isolated from dilute hydrochloric acid; yield 0.3 g. Its ultraviolet absorption spectrum was identical with that of V.

The chemical assay for purified IV showed 1.4% free PABG and 90% IV. A 2 *N* hydrochloric acid solution of IV was stable for at least 10 days. The residue from a sodium bicarbonate solution of IV which had been frozen and dried under high vacuum was stable at room temperature for at least 10 days. A sample when isolated from 1 *N* hydrochloric acid solution decomposed in 2 days at room temperature to 51.9% of free PABG and 1.16% of IV. A 0.1 *N* sodium hydroxide solution of IV decomposed in a few hours.

The 7-pteroylglutamic acid was not found to be a growth stimulant for *S. faecalis* R. nor antagonistic to pteroylglutamic acid using this same organism.

The ultraviolet absorption spectrum for IV was identical with that for pteroylglutamic acid.[1]

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[CONTRIBUTION FROM THE LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

7-Methylpteroylglutamic Acid and Some Related Compounds

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Three new analogs of pteroylglutamic acid have been prepared, namely, 7-methylpteroylglutamic acid, 7,10-dimethylpteroylglutamic acid and 6-methyl-7-pteroylglutamic acid. These compounds were prepared by treating the corresponding bromomethylpteridine with the requisite *p*-aminobenzoylglutamic acid derivative, and their structures were proved by degradation to known compounds. The first two compounds are biologically active as antagonists of pteroylglutamic acid while the latter compound is inactive as either an antagonist or growth stimulator.

A number of analogs of pteroylglutamic acid have been described in the literature, many of which are antagonists of pteroylglutamic acid in various biological systems. The preparation and properties of pteroylaspartic acid have been described.[1] A derivative of pteroylglutamic acid in which the 4-hydroxyl group is replaced by an amino group has been shown to be a powerful antagonist of pteroylglutamic acid[2a] as has 4-aminopteroylaspartic acid.[2b] Some of the 2,4-diaminopteridines also show some antagonist activity.[3] Alkyl derivatives of pteroylglutamic acid have been prepared including various N^{10}-alkyl analogs[4] as well as C^9-methyl derivatives.[5] The amides of *p*-aminobenzoylglutamic acid and quinoxaline-2-carboxylic acid, xanthopterincarboxylic acid and isoxanthopterincarboxylic acid have been reported[6] to be inhibitors of pteroylglutamic acid. Dibromobutyraldehyde has been substituted for dibromopropionaldehyde and *d*(−)-glutamic acid for *l*(+)-glutamic acid in one of the syntheses of pteroylglutamic acid[7] to yield a product which is an antagonist for pteroylglutamic acid.[8] This product was an impure mixture and the purification or proof of structure has not been reported. However, this crude material was designated "7-methylfolic acid." This same material was also prepared using *l*(+)-glutamic acid and its biochemical properties have been published.[9] The inhibition ratio of this crude antagonist is 20–30 as compared to 7-methylpteroylglutamic which is 252 as indicated later in this paper and 9-methylpteroylglutamic acid[5] which is 2000. Since the inhibition activity of this crude material is approximately 10 times that of the 7-methyl compound and 100 times that of 9-methyl derivative, it is obvious that some other component of this crude material is responsible for its potent antagonist activity.

This communication deals with the preparation, purification and proof of structure of 7-methylpteroylglutamic acid, 6-methyl-7-pteroylglutamic acid and 7,10-dimethylpteroylglutamic acid.

The first attempt to prepare 7-methylpteroylglutamic acid yielded the isomeric 6-methyl-7-pteroylglutamic acid exclusively. This was done through bromination of 2-amino-4-hydroxy-6,7-dimethylpteridine,[10] using one mole of bromine in a manner similar to that used for the preparation of

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(7) C. W. Waller, *et al.*, THIS JOURNAL, **70**, 19 (1948).

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(10) F. Sachs and G. Meyerheim, *Ber.*, **41**, 3965 (1908).

pteroylglutamic acid.[11] The dimethylpteridine brominated much more readily than did the monomethylpteridine. The crude bromomethylpteridine was condensed with *p*-aminobenzoylglutamic acid in an aqueous solution at *p*H 10–11.[12] The product crystallized upon acidification in good yield and in a high state of purity. This product was shown to be very unstable in dilute sodium hydroxide solution especially in the presence of air, releasing *p*-aminobenzoylglutamic acid. The isolated free acid is also somewhat unstable, darkening when dried. The sodium salt prepared at *p*H 7 is however, quite stable. These same properties were noted with 7-pteroylglutamic acid.[12]

The structure of the compound was proved by first decomposing it in sodium hydroxide solution and oxidizing the resulting crude pteridine with potassium permanganate. The product was identical with 2-amino-4-hydroxy-6-methylpteridine-7-carboxylic acid as determined by ultraviolet absorption spectra.[13] This was confirmed by decarboxylation and oxidation of this product to yield 2-amino-4-hydroxypteridine-6-carboxylic acid.

Since in the bromination of the dimethylpteridine the bromine substituted exclusively in the 7-methyl group, another method was devised for the preparation of 7-methylpteroylglutamic acid. Dibromodiacetyl[14] for which an improved method of preparation is described, was condensed with 2,4,5-triamino-6-hydroxypyrimidine to yield 2-amino-4-hydroxy-6,7-bis-(bromomethyl)-pteridine. This compound was dissolved in dilute hydrobromic acid and partially reduced by adding one molecular equivalent of potassium iodide. The crude product from this reduction was then condensed with *p*-aminobenzoylglutamic acid in aqueous solution at *p*H 10–11. The product of this reaction was apparently a mixture of 7-methylpteroylglutamic acid and a small amount of 6-methyl-7-pteroylglutamic acid. The latter compound was decomposed by heating in dilute sodium hydroxide during the purification.

For proof of structure, the purified compound was oxidized with potassium permanganate. The resulting pteridine had ultraviolet absorption spectra identical with 2-amino-4-hydroxy-7-methylpteridine-6-carboxylic acid.[15] As a confirmation of the identity of this degradation product, it was decarboxylated by heating at 300° in vacuum and the product was oxidized to 2-amino-4-hydroxypteridine-7-carboxylic acid.

7,10-Dimethylpteroylglutamic acid was also prepared as described above for 7-methylpteroylglutamic acid using *p*-methylaminobenzoylglutamic acid[4] instead of *p*-aminobenzoylglutamic acid.

Microbiological Activity.—6-Methyl-7-pteroylglutamic acid was inactive as a growth stimulant and practically inactive as an antagonist, using *S. faecalis* R. as the test organism. 7-Methylpteroylglutamic acid was an antagonist for pteroylglutamic acid, having an inhibition ratio of 242. When expressed as a percentage using N^{10}-methylpteroylglutamic as 100%,[4,5] the inhibition is 0.62%. On the same basis 7,10-dimethylpteroylglutamic acid has in inhibition ratio of 3.0 and a percentage inhibition of 49.3%.

Ultraviolet Absorption.—These compounds show the same general ultraviolet absorption characteristics as other pteroylglutamic acid analogs of this type.[4,5] These are summarized in Table I.

TABLE I

ULTRAVIOLET ABSORPTION DATA

Compound	Maxima 0.1 *N* NaOH mμ	$E^{1\%}_{1\text{ cm.}}$	0.1 *N* HCl mμ	$E^{1\%}_{1\text{ cm.}}$
7-Methylpteroylglutamic acid	252	513	252–255	242
	285	540	297–300	394
	357	225		
6-Methyl-7-pteroylglutamic acid hydrate	252	491	255	261
	280	459	300	361
	360–362	224		
7,10-Dimethylpteroylglutamic acid	252	493	252	251
	305	523	310–312	440
	357	219		

Experimental

6-Methyl-7-pteroylglutamic Acid.—A suspension of 5 g. of 2-amino-4-hydroxy-6,7-dimethylpteridine in 250 cc. of 48% hydrobromic acid was stirred and heated to 95°. A solution of 1.34 cc. of bromine in 10 cc. of 48% hydrobromic acid was added. A clear solution formed and the bromine color disappeared in a few minutes. After 10 minutes the solution was cooled in ice and the crystalline precipitate was filtered off, washed with 48% hydrobromic acid, alcohol, and ether and dried; yield 3.5 g. This product was added in portions to a solution of 20 g. of *p*-aminobenzoylglutamic acid in 350 cc. of water at *p*H 11. The *p*H was maintained at 10–11 by additions of 10 *N* sodium hydroxide. After all was in solution, it was clarified with Norite and the *p*H was adjusted to 1.3 with hydrochloric acid. An amorphous precipitate formed as the *p*H was lowered which changed to crystalline on stirring. The solid was removed by filtration and washed well with water. It was then dissolved in 100 cc. of water by adding sodium hydroxide to *p*H 7.4 and lyophilized. The dried product weighed 5.2 g. and the chemical assay showed it to be 93.5% pure as the disodium salt.

Two hundred mg. of this sodium salt was dissolved in 40 cc. of water, heated to 50–60°, and 2 cc. of concentrated hydrochloric acid was added. A clear solution resulted which crystallized on cooling. This purification was repeated once more and the product was dried 30 minutes at 60° *in vacuo* which darkened it slightly.

Anal. Calcd. for $C_{20}H_{21}N_7O_6 \cdot H_2O$: C, 50.74; H, 4.86; N, 20.72. Found: C, 50.65; H, 5.71; N, 20.49.

Proof of Structure.—A solution of 500 mg. of this compound in 20 cc. of 0.2 *N* sodium hydroxide was warmed to 50–60° and air was bubbled through for five hours. After standing overnight at room temperature the chemical assay showed that all the compound had been decomposed. The solution was diluted to 40 cc. and acidified to *p*H 1.8. The precipitate was collected and dried; yield 250 mg. This was dissolved in 30 cc. of 0.2 *N* sodium hydroxide and heated for 10 minutes at 95° with a slight excess of potassium permanganate. The solution was filtered and acidified and the precipitate was collected and dried. The ultraviolet absorption spectra were identical to 2-amino-4-hydroxy-6-methylpteridine-7-carboxylic acid.[13] It was decarboxylated and oxidized as described later for its isomer and the ultraviolet absorption spectra were identical with 2-amino-4-hydroxypteridine-6-carboxylic acid and showed no indication of any 2-amino-4-hydroxypteridine-7-carboxylic acid.

Dibromodiacetyl.—A solution of 129 cc. of diacetyl in 400 cc. of carbon tetrachloride was placed in a 3-neck flask equipped with a stirrer, condenser and dropping funnel. The solution was heated to reflux temperature and bromine (160 cc.) was added just fast enough to keep the solution refluxing. The bromine was used up rapidly at first but

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(12) C. W. Waller, *et al.*, *ibid.*, **74**, 5405 (1952).
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(14) R. Fittig, C. Daimler and H. Keller, *Ann.*, **249**, 207 (1888).
(15) E. L. Wittle, *et al.*, THIS JOURNAL, **69**, 1786 (1947).

when about half of it had been added it was necessary to heat the flask in a steam-bath to keep it refluxing. After all the bromine had been used up the solution was cooled well and the yellow crystalline product was filtered off, washed well with low boiling petroleum ether, and dried; yield 225 g., m.p. 115–117°.

2-Amino-4-hydroxy-6,7-bis-(bromomethyl)-pteridine.—A solution of 1.22 g. of dibromodiacetyl in 10 cc. of alcohol was added to a solution of 1.07 g. of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride in 25 cc. of 2.5 *N* hydrobromic acid. After one hour at room temperature the solution was left at 5° overnight. A small amount of precipitate was filtered off and the filtrate was diluted with 100 cc. of water. After cooling in ice several hours the crystalline precipitate was filtered off, washed and dried at 100° for two hours *in vacuo;* yield 0.9 g.

Anal. Calcd. for $C_8H_7ON_5Br_2$: C, 27.5; H, 2.02; N, 20.2; Br, 45.8. Found: C, 27.95; H, 2.51; N, 20.46; Br, 45.64.

7-Methylpteroylglutamic Acid.—A solution of 15 g. of 2-amino-4-hydroxy-6,7-bis-(bromomethyl)-pteridine in 210 cc. of 48% hydrobromic acid was diluted to 750 cc. with water. The clear solution was stirred at 55° while 7.13 g. of potassium iodide in 25 cc. of water was added slowly over one hour. The solution turned dark and a dark precipitate formed. It was stirred one additional hour and then cooled to 15°. Sodium hydrosulfite was added with stirring until the solution turned yellowish and most of the dark precipitate redissolved. The solution was filtered and neutralized to *p*H 1.0 with saturated sodium acetate solution. The precipitate was filtered off, washed well and dried; yield 12.1 g. This material was added in portions to a stirred solution of 50 g. of *p*-aminobenzoylglutamic acid in 800 cc. of water at *p*H 10–11 by additions of sodium hydroxide solution as necessary. When the reaction was over, as indicated by the fact that the *p*H remained constant, 8 cc. of 10 *N* sodium hydroxide was added and the solution was heated to 90° for 15 minutes. To this hot solution was added 60 cc. of concentrated hydrochloric acid (*p*H 1.3) and the temperature was lowered to 15°. The precipitate was centrifuged and washed and then dissolved in 8 l. of 0.1 *N* sodium hydroxide. A chemical assay indicated that there was 10 g. of the desired compound in the solution. It was heated to 95° and stirred for 15 minutes and 148 cc. of 30% calcium chloride solution was added. The solution was filtered hot and the dark precipitate was discarded. To the filtrate was added 5% zinc chloride solution to *p*H 10.9 and the insoluble material was filtered off with Celite and discarded. To the filtrate was added 20% zinc chloride solution to *p*H 6.8 which precipitated most of the desired product. This precipitate containing 6.5 g. of product by chemical assay was filtered off and the wet filter cake was dissolved in 100 cc. of concentrated hydrochloric acid. This solution was clarified with Norite and diluted with 1 l. of water at 35–40°. On cooling in ice the product crystallized and was filtered off and dried; yield 6.0 g. having a chemical assay of 89.2%.

A portion of this product (500 mg.) was purified twice more by dissolving in 15 cc. of 6 *N* hydrochloric acid and diluting with 75 cc. of water. The product crystallized out on cooling. It was filtered, washed and dried at 110° for 2.5 hours *in vacuo*.

Anal. Calcd. for $C_{20}H_{21}N_7O_6$: C, 52.7; H, 4.61; N, 21.54. Found: C, 51.96; H, 4.98; N, 21.53.

Proof of Structure.—A solution of 0.5 g. of the 89.2% material in 50 cc. of 0.2 *N* sodium hydroxide was stirred and solid potassium permanganate (1–1.5 g.) was added until the purple color persisted. The solution was stirred 90 minutes and then the excess permanganate was reduced with a little sodium hydrosulfite. The solution was filtered, acidified to *p*H 1.8, and the precipitate was filtered off and dried; yield 0.25 g. This crude product was crystallized once more from 12.5 cc. of 2.5 *N* sodium hydroxide. The sodium salt was dissolved in 10 cc. of water and added to 20 cc. of hot water containing 1 cc. of concentrated hydrochloric acid. The precipitate was filtered, washed and dried; yield 130 mg. The ultraviolet absorption spectra were identical to 2-amino-4-hydroxy-7-methylpteridine-6-carboxylic acid. The 130 mg. of product was heated at 300° for two hours in vacuum to decarboxylate it and the residue was dissolved in 15 cc. of 0.2 *N* sodium hydroxide and heated for three hours on the steam-bath with excess potassium permanganate. The solution was filtered and acidified and the precipitate was centrifuged and washed. The ultraviolet absorption spectra were identical to 2-amino-4-hydroxypteridine-7-carboxylic acid.

7,10-Dimethylpteroylglutamic Acid.—A solution of 85.6 g. of 2-amino-4-hydroxy-6,7-bis-(bromomethyl)-pteridine in 1200 cc. of 48% hydrobromic acid was diluted to 4300 cc. with water and reduced with 40.8 g. of potassium iodide as described above. The product of this reduction was condensed with 172 g. of *p*-methylaminobenzoylglutamic acid in 3000 cc. of water at about *p*H 11 as described above. When the reaction was complete 200 g. of Celite was added and after the *p*H was adjusted to 2.5 with hydrochloric acid the solid was filtered off. The product was dissolved in 43 l. of 0.1 *N* sodium hydroxide, heated to 92° for 15 minutes, and 774 cc. of 30% calcium chloride was added. The insoluble material was filtered off and discarded. To the filtrate was added 5% zinc chloride solution to *p*H 10.8–11 and the insoluble material was filtered off and discarded. To the filtrate was added 50% zinc chloride solution to *p*H 6.5 and after heating to 94° the zinc salt of the product was removed by filtration. This wet zinc salt was slurried in 7.5 l. of water and 450 cc. of concentrated hydrochloric acid was added. This solution was stirred with 50 g. of charcoal and filtered. The filtrate was adjusted to *p*H 2 and after cooling, the product was filtered off, washed and dried; yield 42.0 g. This material was about 80% pure as estimated from the ultraviolet absorption spectra.

A solution of 0.8 g. of the above material in 20 cc. of dilute sodium hydroxide was treated with charcoal and diluted with 20 cc. of 10 *N* sodium hydroxide. The yellow amorphous precipitate was centrifuged off and the above purification was repeated. The yellow sodium salt was then dissolved in water and precipitated by acidification while hot. The precipitate was centrifuged and dissolved by boiling in 25 cc. of 0.2 *N* hydrochloric acid. The solution was filtered, cooled well and the product was centrifuged, washed well and dried at 110° *in vacuo* for two hours.

Anal. Calcd. for $C_{21}H_{23}O_6N_7$: C, 53.7; H, 4.91; N, 20.9. Found: C, 53.39; H, 4.83; N, 20.88.

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PEARL RIVER, N. Y.