PTEROYLASPARTIC ACID, AN ANTAGONIST FOR PTEROYLGLUTAMIC ACID

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

(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York)

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Pteroylaspartic acid, N-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino}benzoyl]aspartic acid, has been found to be an antagonist of pteroylglutamic acid and certain of its derivatives for several species. The present communication reports the preparation of the compound and summarizes the experiments demonstrating its antipteroylglutamic acid activity.

EXPERIMENTAL

Preparation of Pteroylaspartic Acid. Aspartic Acid—A mixture of Bacto-asparagine (250 gm.), water (740 ml.), and concentrated hydrochloric acid (270 ml.) was refluxed for 3 hours, and then cooled to 30°. To this solution were added 112 ml. of 28 per cent ammonium hydroxide solution with good stirring. The precipitate of aspartic acid was collected and recrystallized from 3800 ml. of water. Yield, 169 gm.

p-Nitrobenzoylaspartic Acid—To a well stirred soution of aspartic acid (80 gm.) and sodium hydroxide (60 gm.) in 600 ml. of water were added 140 gm. of *p*-nitrobenzoyl chloride and 600 ml. of 2 N sodium hydroxide solution during about 45 minutes. The temperature of the reaction mixture was not allowed to rise above 35°. After being stirred for 1 hour the solution was acidified with 164 ml. of concentrated hydrochloric acid. The precipitate of *p*-nitrobenzoic acid (48 gm.) was separated by filtration, and the filtrate (1700 ml.) was adjusted to pH 1.5, seeded, and chilled for several hours. The crystalline product was collected, washed with water, and dried. Weight 110 gm.; m.p. 145-147°.

$$[\alpha]_D^{30} = +26.3°$$ (2% solution in 1 N sodium hydroxide)

p-Aminobenzoylaspartic Acid—A mixture of *p*-nitrobenzoylaspartic acid (100 gm.), glacial acetic acid (700 ml.), water (300 ml.), and platinum oxide catalyst (1.5 gm.) was heated to 45° and shaken with hydrogen at atmospheric pressure until the theoretical amount of hydrogen had been absorbed. The catalyst was removed by filtration and the filtrate evaporated nearly to dryness *in vacuo*. The residue was then suspended in glacial acetic acid,

collected, and washed with acetic acid, acetone, and ether. The air-dried product weighed 71 gm. This crude product was extracted with 500 ml. of boiling acetone and then collected on the filter and washed with acetone, ether, and petroleum ether. Yield, 45 gm.; m.p. 171–173°. The acetone extraction resulted in considerable loss and is probably unnecessary.

$[\alpha]_D^{25} = -10.7°$ (2% solution in 1 N hydrochloric acid)

A sample was recrystallized from glacial acetic acid for analysis.

$C_{11}H_{12}O_5N_2$.	Calculated.	C 52.35, H 4.76, N 11.11
	Found.	" 52.11, " 5.29, " 11.04

Pteroylaspartic Acid—A solution of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride[1] (68.5 gm.) and *p*-aminobenzoylaspartic acid (40 gm.) in 4 liters of water was adjusted to pH 4.0 by the addition of sodium hydroxide solution. Then with good stirring, a solution of 2,3-dibromopropionaldehyde (69 gm.) in 1500 ml. of ethanol was added during about 2 hours. The pH was maintained at 4.0 by the addition of sodium hydroxide solution. After the addition of the dibromopropionaldehyde, the solution was stirred for an additional 45 minutes and the precipitate was then collected on the filter, washed with water, alcohol, acetone, and ether, and dried. Weight, 71 gm.

Chemical assay (2) of the product was 19.2 per cent. Yield, 13.65 gm. of pteroylaspartic acid.

Purification of Pteroylaspartic Acid. Precipitation of Impurities with Barium-Ethanol—71 gm. of the crude pteroylaspartic acid were dissolved in 50 liters of 0.2 N sodium hydroxide solution. Barium chloride was added to 0.2 N and ethanol to a concentration of 2 per cent. Filter-Cel was added and the solution clarified. The excess barium was removed from the filtrate by the addition of sulfuric acid and the precipitated barium sulfate removed by filtration. There were 9.068 gm. of pteroylaspartic acid by chemical assay in the filtrate.

Precipitation at pH 3.0—The solution was neutralized to pH 7.0 and concentrated under nitrogen to 20 liters. The precipitate that formed was removed by filtration and discarded. The solution was adjusted to pH 3.0, thoroughly chilled, and collected with the aid of Filter-Cel. The precipitate was extracted with 12 liters of 0.2 N sodium hydroxide solution. Yield, 5.92 gm. by chemical assay.

Treatment with Charcoal—The alkaline extract was stirred with 7 gm. of norit A[2] for 15 minutes and the norit A removed by filtration. The

[1] 2,4,5-Triamino-6-hydroxypyrimidine (1) was dissolved in dilute hydrochloric acid and crystallized by the addition of concentrated hydrochloric acid to 6 N.

[2] Activated charcoal; Pfanstiehl Chemical Company, Waukegan, Illinois.

filtrate was adjusted to pH 3.0, chilled thoroughly, and the precipitate collected on a Sharples centrifuge.

Formation of Magnesium Salt—The precipitate was suspended in 1 liter of water and excess magnesium oxide added. The solution was heated to 65° for 15 minutes and filtered. The hot solution was treated with norit A until the brownish pigments were removed. The light yellow solution was chilled and the needles of magnesium pteroylaspartate were collected and dried. Yield, 1.133 gm.

$C_{18}H_{14}N_7O_6Mg_{1.5}\cdot H_2O$.	Calculated.	C 45.14, H 3.34, N 20.48, Mg 7.62
	Found.	" 44.93, " 3.89, " 20.30, " 7.66

The compound is quite stable as the magnesium salt. Attempts to crystallize the free acid out of hot water previously adjusted to pH 3.0 caused the compound to break down partially into pteroic acid and aspartic acid.

Bacterial Inhibition—The antipteroylglutamic acid activity of pteroylaspartic acid was determined with *Lactobacillus casei* and *Streptococcus faecalis* R. *S. faecalis* R was grown on the medium of Tepley and Elvehjem (3). For the experiments with *L. casei*, alanine and asparagine were omitted from the medium. The incubation times for *L. casei* and *S. faecalis* R were 16 and 72 hours, respectively, at 32–33°.

The inhibition index is defined as the ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition. The general procedure is to add a given amount of the metabolite to a set of tubes and to add varying amounts of the inhibitor. An inhibition curve is plotted and from this the point of half maximum inhibition is obtained.

As an example of an organism that does not require preformed pteroylglutamic acid, *Escherichia coli* was used. The bacterium was grown on a medium containing 0.5 per cent peptone, 0.3 per cent beef extract, and 0.1 per cent dextrose.

Chick and Rat Experiments—For the inhibition studies with the chick, day-old New Hampshire red chicks were placed on the basal diet of Hutchings *et al.* (4). All preparations of the metabolite and inhibitor were injected intramuscularly daily. For maximum stability of the inhibitor, the magnesium salt was dissolved by gentle heating in water containing a small amount of sodium bicarbonate. After cooling to room temperature, the compound was injected immediately.

In the rat experiments the basal diet of Spicer *et al.* (5) was used. The effect of the inhibitor was also studied on the above diet supplemented with 0.5 per cent carboxysulfathiazole. The general procedure was to place 21 day-old rats on the diets and inject intramuscularly varying amounts of the inhibitor daily. Growth and complete blood counts were followed.

Results

Lactobacillus casei—The inhibitory characteristics of pteroylaspartic acid were studied with pteroylglutamic acid and thymine as the metabolites. The data are summarized in Table I. The constancy of the inhibition index at the various levels indicates that the phenomenon is a typical example of competitive inhibition. The highest level of metabolite used is approximately 7 times the amount necessary to produce maximum growth.

When thymine at a concentration of 7.5 γ was used in lieu of pteroylglutamic acid, no inhibition was apparent when the inhibitor was used in amounts of 1 mg. per tube. The inhibition seems to be specific for the system utilizing pteroylglutamic acid.

Streptococcus faecalis R—The effectiveness of pteroylaspartic acid against a number of metabolites was tested with *Streptococcus faecalis* R. The data are summarized in Table II.

TABLE I

Pteroylaspartic Acid Inhibition of Metabolites for Lactobacillus casei

Metabolite	Concentration of metabolite	Inhibition index*
	γ per 10 ml.	
Pteroylglutamic acid	0.00015	2166
" "	0.0003	1667
" "	0.003	1466
" "	0.015	1550
Thymine	7.5	Slight stimulation

* The inhibition index is the ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition.

With each metabolite the inhibition index remains constant within experimental error, thus demonstrating that the inhibition is of a competitive nature.

It is of interest to note that the inhibitory effect of pteroylaspartic acid is not directly related to the activity of the metabolite for *Streptococcus faecalis* R. The inhibitor is least effective against pteroylglutamic acid. Pteroylaspartic acid is more effective as an inhibitor against pteroic acid and pteroyl-γ-glutamylglutamic[3] acid. These compounds are as active on a molar basis in promoting the growth of *Streptococcus faecalis* R as is pteroylglutamic acid. The inhibitor is most effective as a displacing agent for pteroyl-γ-glutamyl-γ-glutamylglutamic acid[3] (the fermentation *Lactobacillus casei* factor). The activity of this compound in promoting the growth of *Streptococcus faecalis* R is 1.0 to 2.0 per cent that of pteroylglutamic acid.

[3] Data on synthesis to be published.

Escherichia coli—The addition of the inhibitor in amounts up to 1.0 mg. per 10 ml. of medium caused no decrease in the rate or extent of growth.

TABLE II

Pteroylaspartic Acid Inhibition of Metabolites for Streptococcus faecalis R

Metabolite	Concentration of metabolite	Inhibition index
	γ per 10 ml.	
Pteroic acid	0.003	2.08
" "	0.006	1.91
" "	0.012	2.39
" "	0.06	3.1
Pteroylglutamic acid	0.0015	53.3
" "	0.003	34.6
" "	0.006	53.3
" "	0.03	47.5
" "	0.15	44.3
Pteroyl-γ-glutamylglutamic acid	0.003	16.7
" "	0.006	4.5
" "	0.03	4.3
" "	0.15	9.46
" "	0.30	6.25
Pteroyl-γ-glutamyl-γ-glutamylglutamic acid	0.12	0.24
" "	0.16	0.31
" "	0.24	0.28
" "	0.50	0.20
" "	1.00	0.20

TABLE III

Pteroylaspartic Acid Inhibition of Pteroylglutamic Acid in Chick

Series No. (6 chicks each)	Supplement	Average weights and No. alive*			Hemoglobin
		1 wk.	2 wks.	3 wks.	
		gm.	*gm.*	*gm.*	*gm. per cent*
1	None	56 (6)	76 (5)	74 (2)	1.8
2	3 γ pteroylglutamic acid per day	77 (6)	132 (6)	191 (5)	4.1
3	Series 2 + 0.75 mg. pteroyl-aspartic acid per day	78 (4)	132 (4)	170 (4)	3.5
4	Series 2 + 1.5 mg. pteroyl-aspartic acid per day	61 (4)	86 (4)	96 (3)	2.8

* Shown by the figures in parentheses. The initial weight was 38 gm. in each series.

Chick—Typical data for the antipteroylglutamic acid activity of pteroyl-aspartic acid for the chick are summarized in Table III. The addition of

the inhibitor caused a marked decrease in the growth rate, and the hemoglobin level was decreased to a value approaching that for the basal diet. It is apparent that the inhibitor antagonizes the growth effects of pteroylglutamic acid in a ratio of 500:1 by weight.

Rat—The addition of the inhibitor in amounts up to 1.5 mg. per day per rat caused no significant decrease in the growth rate or the appearance of any symptoms characteristic of a pteroylglutamic acid deficiency on either of the diets used.

DISCUSSION

From the foregoing experiments it is apparent that pteroylaspartic acid is an effective inhibitor in the utilization of pteroylglutamic acid for growth by *Lactobacillus casei*, *Streptococcus faecalis* R, and the chick. With *Streptococcus faecalis* R the compound was more effective as an inhibitor of the utilization of pteroic acid and the pteroyldiglutamic and pteroyltriglutamic acids. These latter compounds were not tested with *Lactobacillus casei* or the chick.

The inhibitor was not effective in interfering with the metabolism involving pteroylglutamic acid or derivatives in *Escherichia coli*. In the rat the inhibitor was not effective in interfering with the utilization of pteroylglutamic acid.

SUMMARY

Pteroylaspartic acid was synthesized from *p*-aminobenzoylaspartic acid, 2,3-dibromopropionaldehyde, and 2,4,5-triamino-6-hydroxypyrimidine. The compound is an antagonist for pteroylglutamic acid with *Lactobacillus casei* and the chick. With *Streptococcus faecalis* R the inhibitor effectively prevents the utilization of pteroic acid, pteroylglutamic acid, pteroyl-γ-glutamylglutamic acid, and pteroyl-γ-glutamyl-γ-glutamylglutamic acid. In all instances the inhibition is of a competitive nature.

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