[CONTRIBUTION FROM THE PHARMACEUTICAL RESEARCH SECTION, CALCO CHEMICAL DIVISION, AMERICAN CYANAMID COMPANY]

Analogs of Pteroylglutamic Acid. VI. 3′,5′-Dihalopteroyl Derivatives

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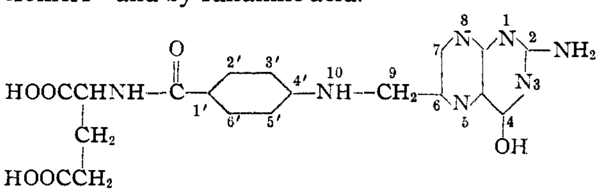
Analogs of pteroylglutamic acid and related compounds have been synthesized having halogens in the 3′,5′-positions on the benzene ring. These were prepared by direct halogenation of the parent compounds; proof of structure resulted from both degradative and synthetic methods. The microbiological activity of the various compounds against *S. faecalis* R varied from slight growth promotion to strong inhibition.

Structural modifications[2] in the pteroylglutamic acid molecule[3] have led to compounds with interesting biological properties. The extensive literature reporting investigations with many of the analogs of pteroylglutamic acid in leukemia and neoplasms both in experimental animals and in man has been summarized in an excellent review.[4a] Numerous analogs have been synthesized,[4b] and in general the variations fall into the following classes: (1) changes in the substituents in the 2- and 4-positions,[2d,g]; (2) replacement of the pteridine moiety by other cyclic systems[5]; (3) alteration of the 9,10-configuration[2b,c,f,5b]; (4) replacement of glutamic acid by other amino acids[2a,e,h]; and (5) replacement of *p*-aminobenzoic acid by position isomers[6a] and by sulfanilic acid.[6]

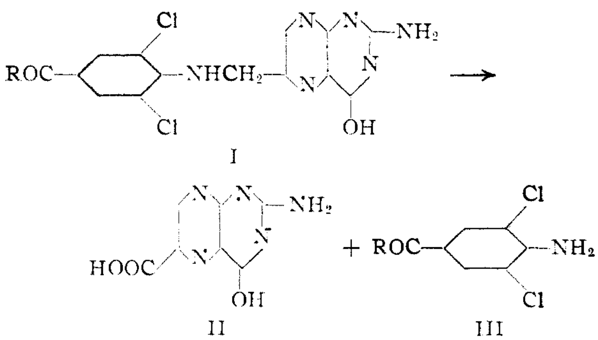


Notably absent in this classification are analogs having substituents in the benzene ring portion of the pteroylglutamic acid molecule, and it appeared to be of interest to synthesize such compounds. In the course of a program designed to study the influence of substitution in the benzene nucleus upon the biological properties, a number of compounds of this type have been prepared. The present paper describes some of these, namely, the 3′,5′-dihalo derivatives, which have been obtained by the direct halogenation of the appropriate pteroyl compounds.

The action of chlorine on simpler pteridines such as leucopterin and desiminoleucopterin resulted either in glycol formation or in cleavage of the pteridine ring system.[7] Treatment of pteroylglutamic acid with sodium chlorate and hydrochloric acid or sodium bromate and hydrobromic acid caused cleavage of the molecule to give a pteridine and the corresponding 4-amino-3,5-dihalobenzoylglutamic acid.[8]

It has been found in this investigation that pteroylglutamic acid in cold aqueous acid solution reacts smoothly with two moles of chlorine or bromine to give 3′,5′-dihalopteroylglutamic acid in good yield. The reaction appears to be general for analogs of pteroylglutamic acid. Absorption of more than the theoretical two moles of halogen was deleterious and caused deep-seated changes in the molecule, which were probably allied to those described by earlier workers.[7] The chloro and bromo derivatives were prepared by introducing the halogen in gaseous form to the pteroyl compound dissolved in hydrochloric acid.

Proof of structure of the 3′,5′-dihalo compounds was obtained by a degradation reaction and by an alternative synthesis. The alkaline aerobic oxidation of 3′,5′-dichloropteroylglutamic acid (I) yielded 2-amino-4-hydroxypteridine-6-carboxylic acid[3] (II) and 4-amino-3,5-dichlorobenzoylglutamic acid[8] (III), which located the positions of the chlorine atoms.



I   II   III

R = glutamic acid

The addition of bromine to an aqueous suspension of *p*-aminobenzoylglutamic acid[9] resulted in the formation of 4-amino-3,5-dibromobenzoylglutamic acid (IV) which had been prepared by Wittle, *et al.*, by another route.[8] This compound when condensed with 2,4,5-triamino-6-hydroxypyrimidine sulfate[10] (V) and 1,1,3-tribromoacetone[11] (VI)

(1) Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

(2) (a) B. L. Hutchings, *et al.*, *J. Biol. Chem.*, **170**, 323 (1947); (b) D. B. Cosulich and J. M. Smith, Jr., THIS JOURNAL, **70**, 1922 (1948); (c) M. E. Hultquist, *et al.*, *ibid.*, **71**, 619 (1949); (d) D. R. Seeger, *et al.*, *ibid.*, **71**, 1753 (1949); (e) W. B. Wright, Jr., *et al.*, *ibid.*, **71**, 3014 (1949); (f) D. B. Cosulich and J. M. Smith, Jr., *ibid.*, **71**, 3574 (1949); (g) B. Roth, J. M. Smith, Jr., and M. E. Hultquist, *ibid.*, **72**, 1914 (1950); (h) B. L. Hutchings, *et al.*, *J. Biol. Chem.*, **180**, 857 (1949); (i) B. Roth, J. M. Smith, Jr., and M. E. Hultquist, in press.

(3) R. B. Angier, *et al.*, *Science*, **103**, 667 (1946).

(4) (a) J. B. Thiersch and F. S. Philips, *Am. J. Med. Sciences*, **217**, 575 (1949); (b) J. M. Smith, Jr., *New Jersey J. Pharm.*, **22**, No. 8, 15 (1949).

(5) (a) F. E. King, *et al.*, *Nature*, **162**, 153 (1948); F. E. King, R. M. Acheson and P. C. Spensley, *J. Chem. Soc.*, 1401 (1949); (b) D. W. Woolley and A. Pringle, *J. Biol. Chem.*, **174**, 327 (1948).

(6) (a) Progress Report, Southern Research Institute, Birmingham, Alabama, August, 1949; (b) M. Viscontini and J. Meier, *Helv. Chim. Acta*, **32**, 877 (1949).

(7) M. Gates, *Chem. Revs.*, **41**, 75 (1947).

(8) E. L. Wittle, *et al.*, THIS JOURNAL, **69**, 1789 (1947).

(9) J. Van der Scheer and K. Landsteiner, *J. Immunol.*, **29**, 373 (1937).

(10) W. Traube, *Ber.*, **33**, 1371 (1900).

(11) H. B. Watson and E. D. Yates, *J. Chem. Soc.*, 1207 (1932).

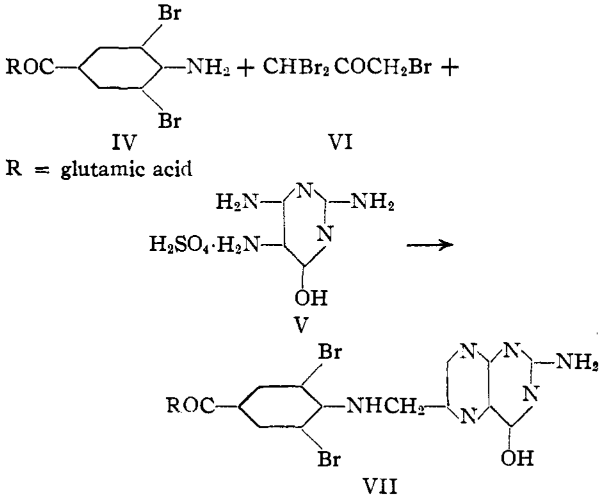
TABLE I

3',5'-DIHALOPTEROYL DERIVATIVES

No.	Compound[a] 3',5'-Dichloro acids	Chem. assay, %	Microbiological activity[b]	Ultraviolet absorption, mμ 0.1 N NaOH Max.	0.1 N NaOH Min.	0.1 N HCl Max.	0.1 N HCl Min.
I	Pteroylglutamic[3]	95.6	+0.61	256 365	239 325	280	262
X	Pteroyl-γ-glutamyl-γ-glutamylglutamic[c]	86.8	0	257 365	239 326	283	262
XI	Pteroyl-α-glutamylglutamic[d]	56.4	0	257 365	238 329	280	261
XII	9-Methylpteroylglutamic[3c]	82.5	−0.029	255 363	238 324	280	265
XIII	10-Methylpteroylglutamic[2b]	..	−0.5	255 362	236 327	313	270
XIV	9,10-Dimethylpteroylglutamic[2c]	..	0	254 363	235 327	310	271
IX	4-Aminopteroylglutamic[2d]	89.5	−19.0	259 370	241 325	280 335	259 321
XV	4-Amino-10-nitrosopteroylglutamic[2f]	..	−10	.....	.....	.....	.....
XVI	4-Amino-10-methylpteroylglutamic[2d]	..	−230	259 370	241 334	285 333	267 317
XVII	4-Amino-9,10-dimethylpteroylglutamic[2c]	..	−340	258 368	241 331	287	268
XVIII	2-Dimethylamino-4-aminopteroylglutamic[2i]	90.1	0	277 395	252 335	253 347	244 324
XIX	4-(1-Piperidyl)-pteroylglutamic[2g]	66.6	+0.002	278 386	254 358	290	252
XX	4-Aminopteramidomalonic[2e]	..	−0.3	259 371	241 328	279 335	259 322
XXI	4-Aminopteroyl-*dl*-isoleucine[2e]	..	−0.3	260 371	242 325	278 337	258 319
XXII	4-Aminopteroyl-*dl*-valine[2e]	..	−0.4	260 370	.....	279 337	257 321
XXIII	4-Aminopteroyl-*l*-aspartic[2h]	..	−4.7	260 372	241 326	281 338	260 321
	3',5'-Dibromo acids						
VII	Pteroylglutamic	..	+0.39	255 365	240 324	280	267
XXIV	10-Nitrosopteroylglutamic	..	−0.05	.....	.....	.....	.....
VIII	4-Aminopteroylglutamic	..	−7.1	259 273	243 327	283 338	260 320

[a] The superscripts containing a numeral indicate the reference in which the parent compound is described. [b] (+) values indicate growth promotion of *S. faecalis* R with pteroylglutamic acid as the reference compound. (−) values indicate half-maximum inhibition of *S. faecalis* R as compared to an arbitrary value of 100 assigned for the antagonist activity of 10-Methylpteroylglutamic acid.[2b] [c] Boothe, *et al.*, THIS JOURNAL, **70**, 1099 (1948). [d] Mowat, *et al.*, *ibid.*, **70**, 1096 (1948).

by the method of Hultquist and Dreisbach[12] yielded a crude material which was purified in a



manner similar to that described for pteroylglutamic acid and its derivatives. The material prepared in this way was identical with the 3',5'-dibromopteroylglutamic acid (VII) obtained by direct bromination of pteroylglutamic acid.

In Table I are summarized the chemical assays, the ultraviolet absorption data and the microbiological activities of the 3',5'-dihalo analogs. It is noteworthy that the ultraviolet absorption curves of the 3',5'-dihalo pteroyl compounds show only two maxima in alkaline solutions in the regions at 265 mμ and 365 mμ, rather than the three maxima exhibited by pteroylglutamic acid at 256, 283 and 365 mμ.

The chemical assay method used for the dichloro compounds was essentially that of Hutchings, *et al.*,[13] with a few modifications. The concentrations for reduction and the absorption maximum for the azo dye arising from 4-amino-3,5-dichlorobenzoylglutamic acid (III) were different from those utilized by Hutchings.

Preliminary experiments, using crude 4-amino-3',5'-dibromopteroylglutamic acid (VIII) prepared in this Laboratory, indicated a moderate chemotherapeutic effect in the treatment of transplanted mouse leukemia.[14] In independent studies using the pure substances, McKenzie, *et al.*, of the Lederle Laboratories Division, American Cyanamid Company,[15] have shown that 4-amino-3',5'-dichloropteroylglutamic acid (IX) ("Dichloroaminopterin") and certain related analogs have interesting effects in the treatment of leukemia and neoplastic disease in experimental animals. Similar results have been obtained in clinical studies by Farber[16] at the Children's Medical Center, Boston. Detailed reports by these investigators are being published elsewhere.

The microbiological properties of these compounds have been examined by Dr. B. L. Hutchings and Mr. A. C. Dornbush, of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. The inhibition index of 4-amino-3',5'-dichloropteroylglutamic acid (IX) for half-maximum growth of *S. faecalis* R is 5.4 at a concentration of 0.1 microgram of pteroylglutamic acid per 10 ml.

Experimental

General Procedure for Halogenation.—One mole of the pteroyl derivative was dissolved in a volume of concen-

(12) M. E. Hultquist and P. F. Dreisbach, U. S. Patent 2,443,165, June 8, 1948.

(13) B. L. Hutchings, *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).

(14) J. H. Burchenal, *et al.*, *Proc. Soc. Exp. Biol. Med.*, **71**, 381 (1949).

(15) D. McKenzie, *et al.*, private communication.

(16) S. Farber, *et al.*, private communication.

trated hydrochloric acid equal to 10 times the weight of compound in grams. The solution was then diluted with an equal volume of water, except in certain cases noted below, in which solubility properties made some adjustments necessary. While maintaining the temperature of the solution at 0–5°, two moles of the halogen were introduced. In the chlorinations, the gas was simply bubbled into the solution; in the brominations, to prevent local bromine concentration, bromine gas was delivered to the solution by bubbling nitrogen through warmed liquid bromine.

In general the pteroylglutamic acids with no substituents in either the 9- or the 10-position precipitated out as the hydrochlorides during the course of the reaction and could be separated by filtration. One or two reprecipitations of the hydrochlorides from solutions of the same concentrations as above usually sufficed to give material about 90% pure. The physical properties of these compounds bear mentioning in that the free compounds showed such pronounced tendencies toward gel formation that their isolation was avoided. Most of these were obtained conveniently by adding the concentrated aqueous solution of the ammonium salt to five to six volumes of acetone.

On the other hand, the 9- and/or 10-substituted pteroylglutamic acids did not form insoluble hydrochlorides. The halogenated solutions were diluted once with water, cooled and slowly adjusted to *p*H 2–4 with sodium hydroxide. The free compounds in this case showed little tendency toward gel formation, most of them being amorphous solids which were easily isolated. Some were purified by reprecipitation from acid solutions.

The yields in all reactions ranged from 60 to 70% with but few exceptions. Compounds in which the general procedure was rigorously followed are not described below, except when analytical samples were prepared, but do appear in Table I. Because of the small amounts of starting materials available, and the difficulties encountered in purification, no attempt was made to prepare some of the dihalopteroyl derivatives in a state of analytical purity, but these substances were characterized by the ultraviolet absorption spectra (see Table I).

Chemical Assay of 3′,5′-Dichloro Derivatives.—In a modification of the method of Hutchings, *et al.*,[13] approximately 0.1 g. of the sample was dissolved in 0.1 *N* sodium hydroxide and the resulting solution diluted to 100 ml. with 0.1 *N* sodium hydroxide. To 10 ml. of this solution was added 50 ml. of 0.1 *N* hydrochloric acid and water to dilute to 100 ml. About 30 ml. of the acid solution was reduced with 5 ml. of zinc amalgam (1% zinc) for 30 minutes. To 10 ml. each of this solution and of the original alkaline solution were added 20 ml. of water, 15 ml. of 6 *N* hydrochloric acid and 15 ml. of 0.2% sodium nitrite. After two minutes 15 ml. of 0.5% ammonium sulfamate and 15 ml. of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride was added. The solutions were allowed to stand 10 minutes and then were diluted to 100 ml. with 0.1 *N* hydrochloric acid. The per cent. transmittancy at 500 mμ was obtained in the Beckman spectrophotometer.

The absorption maximum in two samples was found to be at 500 mμ. A curve plotting concentration of 4-amino-3,5-dichlorobenzoylglutamic acid against the per cent. transmittancy of 500 mμ was determined and used for the calculations.

Obviously the chemical assay procedure is not applicable to N^{10}-substituted pteroylglutamic acids. The values for the chemical assay shown in Table I were obtained from materials which had been partially purified as described above.

3′,5′-Dichloropteroylglutamic Acid (I).—The ammonium salt (2 g.) was dissolved in two liters of hot water, and the free compound precipitated by adjusting to *p*H 3 with dilute hydrochloric acid. The well-cooled mixture was centrifuged and the slimy precipitate filtered to remove more water. The leathery cake was boiled with 200 ml. of glacial acetic acid containing 15 ml. of water. Most of the material dissolved so, after clarification with Darco G-60, the filtrate was diluted with water and cooled. The yellow fluffy precipitate was isolated by filtration, washed well with water, dried and weighed 0.925 g.

Anal. Calcd. for $C_{19}H_{17}N_7O_6Cl_2 \cdot H_2O$: C, 43.2; H, 3.59; N, 18.58; Cl, 13.44; H_2O, 3.41. Found: C, 42.9; H, 3.48; N, 18.6; Cl, 13.3; H_2O, 3.41.

Aerobic Alkaline Oxidation of I.[3]—A solution of two grams of the ammonium salt of I in 220 ml. of 1.0 *N* sodium hydroxide was heated at 100° for six hours under a continuous stream of oxygen. The solution, which gave a negative test for chloride ion, was adjusted to *p*H 3 with hydrochloric acid and cooled. The precipitate was isolated by filtration and washed well with acetone. The cake when dried weighed 0.713 g. It was, by ultraviolet absorption spectra analysis, a mixture of 2-amino-4-hydroxy-6-methylpteridine (30%) and 2-amino-4-hydroxypteridine-6-carboxylic acid (II) (60%). When oxidized by alkaline permanganate,[2b] 0.7 g. of material was isolated which was 2-amino-4-hydroxypteridine-6-carboxylic acid alone.

The acetone wash was evaporated under a stream of air to yield a residue which weighed 0.538 g. The inorganic salts were removed by extraction of the organic portion with ethyl acetate. The residue from evaporation of the solvent weighed 0.24 g. By ultraviolet analysis it was a mixture of 4-amino-3,5-dichlorobenzoic acid[17] and 4-amino-3,5-dichlorobenzoylglutamic acid (III).[8] It was recrystallized from ethyl alcohol to yield 0.033 g. of material, m.p. 280.5–282.5°, which was identified as 4-amino-3,5-dichlorobenzoic acid by the ultraviolet absorption spectra analysis and by the melting point. The residue obtained from the alcohol mother liquor was still a mixture of the two acids with the benzoylglutamic acid predominating.

3′,5′-Dichloro-10-methylpteroylglutamic Acid (XIII).—Three and a half grams of the material isolated from the halogenated solution by neutralization was dissolved in 150 ml. of acetic acid at the boil. After treating with Darco G-60, the filtrate was diluted with 500 ml. of hot water and cooled. The buff-colored solid, isolated by filtration, was dissolved in 750 ml. of water by the addition of sodium hydroxide. The solution was warmed and treated with Darco G-60. To the hot solution acetic acid was added slowly. The material came out as a gel which was not completely broken by the addition of 14 g. of sodium chloride. However, after standing, it changed to packets of feathery needles. These were centrifuged out and washed with water, after which they were dissolved in a small amount of concentrated hydrochloric acid. The solution was diluted with water, treated with Darco G-60 and the filtrate adjusted to *p*H 3. After cooling, the white needles were filtered off, washed free of chloride ion and dried at 100° and 2–3 mm. for three hours.

Anal. Calcd. for $C_{20}H_{19}N_7O_6Cl_2$: C, 45.8; H, 3.63; N, 18.71; Cl, 13.54. Found: C, 45.8; H, 3.73; N, 18.7; Cl, 13.5.

4-Amino-3′,5′-dichloropteroylglutamic Acid (IX).—The ammonium salt (2 g.) was dissolved in about 350 ml. of hot water, adding enough sodium hydroxide to give *p*H 10. To the solution at 60–70°, was added 6 g. of magnesium sulfate. The precipitate which formed immediately was filtered off. On cooling the filtrate a bright yellow precipitate came down which was filtered, washed with water and dissolved in 22 ml. of concentrated hydrochloric acid. This solution was treated with Darco G-60 and filtered. On diluting the filtrate with 250 ml. of water a slimy precipitate appeared, which was centrifuged and washed twice by reslurrying in water and centrifuging. The wet centrifugate was frozen overnight, causing the physical state of the solid to change from a thick pudding to a heavy amorphous solid, which was washed with water until the washing was chloride-free. The solid was extracted twice with a total of 500 ml. of boiling methanol containing 50 ml. of water. To the combined solutions was added ether to throw out the solid, which was filtered, washed with ether and dried at 100° overnight. The pale yellow solid weighed 0.42 g.

Anal. Calcd. for $C_{19}H_{18}N_8O_5Cl_2$: C, 44.8; H, 3.54; N, 22.01; Cl, 13.94. Found: C, 44.7; H, 3.68; N, 22.0; Cl, 14.0.

Aerobic Alkaline Oxidation of IX.[3]—A solution of 2.5 g. of the ammonium salt of IX in 220 ml. of 1.0 *N* sodium hydroxide was heated at 100° for six hours under a constant stream of oxygen. The cooled solution was neutralized to *p*H 2–3 with hydrochloric acid and cooled several days. The precipitate, when filtered, washed with water and dried, weighed 1.475 g. It was extracted with boiling ethyl acetate several times. The insoluble portion, 0.9172 g., was 60% 2-amino-4-hydroxypteridine-6-carboxylic acid by ultraviolet absorption spectra analysis. The ethyl acetate

(17) M. Schubert, *Ann.*, **558**, 31 (1947).

extract was evaporated by a stream of air, and the residue dried to yield 0.4475 g. of tan-white solid, which was dissolved in hot alcohol and treated with Darco G-60. The filtrate was treated slowly with water to produce white crystals which were isolated, after cooling, by filtration. The washed and dried material weighed 0.2 g., m.p. 289–291° with no depression when mixed with synthetic 4-amino-3,5-dichlorobenzoic acid.

4-Amino-3',5'-dichloro-10-nitrosopteroylglutamic Acid (XV).—A solution of 5.5 g. of the ammonium salt of IX in 50 ml. of concentrated hydrochloric acid and a very small amount of water was cooled to 0–5°. An aqueous solution of 0.8 g. of sodium nitrite was added slowly to the stirred solution. When a slight excess of sodium nitrite was present, the solution was diluted with an equal volume of water to precipitate the hydrochloride of XV which was filtered off and dissolved in warm water. The solution was decolorized with Darco G-60 and adjusted to *p*H 3–4 with sodium hydroxide. The material which was a gum at the start became solid on continued cooling. After isolation by filtration, washing with water and drying, the solid weighed 4.5 g. About 1.6 g. of this material was heated with 350 ml. of boiling water. A small amount of dark gum which did not dissolve was removed by filtration while decolorizing the solution with Darco. Upon cooling slowly microscopic round beads separated, which were filtered, washed with water and redissolved in boiling water. The solution was decolorized with Darco G-60 and cooled. The small beads were recrystallized again from boiling water. The dried material weighed 0.545 g., gradually decomposed between 180–202° and gave positive Beilstein and Liebermann nitroso tests. A small sample was dried for analysis at 100° and 2–3 mm. for three hours.

Anal. Calcd. for $C_{19}H_{17}N_9O_6Cl_2 \cdot H_2O$: C, 40.8; H, 3.42; N, 22.55; Cl, 12.68. Found: C, 41.1; H, 3.37; N, 22.3; Cl, 12.3.

3',5'-Dichloro-2-dimethylamino-4-aminopteroylglutamic Acid (XVIII).—About 1.76 g. of 2-dimethylamino-4-aminopteroylglutamic acid[21] was dissolved in 25–30 ml. of concentrated hydrochloric acid. The solution was diluted with 22–25 ml. of 5 *N* hydrochloric acid. After removal of a small amount of insoluble material, the cooled filtrate was treated with 0.6 g. of chlorine gas. After standing 10–15 minutes, 50 ml. of water was added and cooling continued. The usual yellow slightly stringy hydrochloride of XVIII was isolated and treated in the manner described in the general procedure.

3',5'-Dichloro-4-(1-piperidyl)-pteroylglutamic Acid (XIX).—A solution of 1.47 g. of 4-(1-piperidyl)-pteroylglutamic acid[2e] in 14.7 ml. of concentrated hydrochloric acid was diluted with an equal volume of water and the solution cooled. Chlorine gas was then bubbled in until the weight had increased by 0.4 g. The insoluble hydrochloride which precipitated was then treated as outlined in the general procedure.

4-Amino-3',5'-dichloropteramidomalonic Acid (XX).—A solution of 4.12 g. of 4-aminopteramidomalonic acid[2e] was prepared by heating to 50° with 1500 ml. of 6 *N* hydrochloric acid. A small amount of insoluble material was removed by filtration. After cooling, 1.4 g. of chlorine was bubbled in, and the solution cooled for 20 minutes more. This solution was then neutralized as described above.

4-Amino-3',5'-dichloropteroyl-*dl*-isoleucine (XXI).—A sample of 4.24 g. of 4-aminopteroyl-*dl*-isoleucine[2e] was dissolved in 63 ml. of concentrated hydrochloric acid. The solution was diluted with 42 ml. of water, cooled to 0–5° and treated with 1.4 g. of chlorine. After cooling 20 minutes, the yellow hydrochloride was isolated and treated in the usual manner.

4-Amino-3',5'-dichloropteroyl-*dl*-valine (XXII).—A solution of 4.1 g. of 4-aminopteroyl-*dl*-valine[2e] in 168 ml. of concentrated hydrochloric acid was diluted with 20 ml. of water. The resulting solution was cooled to 0–5° and 1.4 g. of chlorine absorbed. After standing cold for 20 minutes, 147 ml. of water was added and the precipitated hydrochloride treated as outlined in the general procedure.

3',5'-Dibromopteroylglutamic Acid (VII) (A).—The hydrochloride of this compound, prepared as described in the general procedure, was heated at 80° in one liter of water with enough magnesium oxide to give a pink spot on phenolphthalein test paper. After stirring to dissolve, the solution was decolorized with Darco G-60 and filtered. On neutralization with acetic acid and cooling, a fluffy yellow precipitate separated. It was treated a second time as described with magnesium oxide and Darco G-60. Since drying at 100° tended to bring about some decomposition, a sample dried at 50° overnight was used for microanalysis.

Anal. Calcd. for $C_{19}H_{17}N_7O_6Br_2 \cdot 2H_2O$: C, 35.95; H, 3.31. Found (cor. for 0.68% ash): C, 36.36; H, 3.64.

(B).—This compound was also synthesized by the method of Hultquist and Dreisbach[12] except that 4-amino-3,5-dibromobenzoylglutamic acid (IV) (see below) was substituted for *p*-aminobenzoylglutamic acid. A mixture of 24 g. of the crude condensation product in two liters of water was heated at 60° for one-half hour with enough sodium hydroxide to give *p*H 11.8–12.0. Then a 30% solution of calcium chloride was added to give *p*H 11.26, and the resulting mixture filtered with Hyflo Super-Cel. The cake was washed with 700 ml. of water at 60°. The filtrate and wash were combined and treated with a 10% solution of zinc chloride to adjust to *p*H 10.65. This mixture was filtered with Hyflo Super-Cel, and the filtrate adjusted to *p*H 3 with hydrochloric acid. The cake was slurried in 1.5 liters of water and heated to 80° with enough sodium hydroxide to give about *p*H 11.2. After stirring 20 minutes under these conditions, the *p*H was adjusted to 7 while cooling to 20°. After clarification with Hyflo Super-Cel, the filtrate was adjusted to *p*H 3 with hydrochloric acid. The solid isolated by filtration was treated with magnesium oxide and Darco G-60 as described above. By ultraviolet absorption spectra analysis the material isolated was identical with VII prepared in A above.

4-Amino-3,5-dibromobenzoylglutamic Acid (IV).[8]—A slurry of 26.6 g. of *p*-aminobenzoylglutamic acid[9] in 250 ml. of water was treated at room temperature with 32 g. of bromine, added during a 10-minute period with rapid stirring. Decolorization of the bromine was practically instantaneous. The mixture was stirred for an additional 10 minutes, then filtered and washed with water. This wet cake was recrystallized from 3.5 liters of hot water, and the product was obtained as 31.4 g. of fine white needles, melting at 202–203°. The ultraviolet absorption maximum in 0.1 *N* sodium hydroxide was 276 mμ. This compound seemed to be identical with that obtained by bromic acid oxidation of pteroylglutamic acid.[8]

Anal. Calcd. for $C_{12}H_{12}O_5N_2Br_2$: N, 6.6; Br, 37.7. Found: N, 6.7; Br, 37.8.

3',5'-Dibromo-10-nitrosopteroylglutamic Acid (XXIV).—The method of preparation of the partially purified material was the same as that described above for XV. At this point, 4.0 g. of material was obtained, which was dissolved in 75 ml. of boiling glacial acetic acid. The solution was decolorized with Darco G-60 and diluted slowly with water to a final volume of 550 ml. The material which separated was white; the liquor was yellow. This procedure was repeated twice more using 150 ml. of boiling glacial acetic acid, and diluting to 900 ml. with hot water. The material isolated gave strong positive Beilstein and Liebermann nitroso tests. A small sample was dried at 100° and 2–3 mm. for three hours for microanalysis.

Anal. Calcd. for $C_{19}H_{16}N_8O_7Br_2 \cdot H_2O$: C, 35.3; H, 2.78; N, 17.35; Br, 24.75. Found: C, 35.2; H, 2.77; N, 16.9; Br, 24.8.

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