147°, was isolated. The infrared spectrum of this solid suggested the presence of III, and microanalyses for three samples from different reactions indicated a varying mixture of III and IV.

Anal. Calcd. for $C_{17}H_{20}N_2O_5$: C, 61.45; H, 6.07. Calcd. for $C_{14}H_{18}N_2$: C, 78.46; H, 8.46. Found: C, 68.24, 71.00, 75.98; H, 7.24, 7.74, 8.21.

There was no depression of the melting point when the various samples were mixed. Also physical mixtures of III and IV over a wide range of composition had melting points of between 147–153°.

A 0.1-g. sample of the constant-melting mixture was heated with 25 ml. of high-boiling petroleum ether. The insoluble crystals, 0.021 g., were isolated and recrystallized from aqueous ethanol, m.p. 178°. There was no depression in a mixed melting point with authentic III. Concentration of the petroleum ether filtrate gave 0.056 g. of light tan crystals. Three recrystallizations from aqueous ethanol gave white plates, m.p. 157°, which did not depress the melting point of authentic IV.

To synthesize this constant-melting mixture, 0.200 g. of III and 0.300 g. of IV were dissolved in 4 ml. of hot xylene. Then this mixture was placed in an ice-bath and seeded with a crystal of the constant-melting mixture. From the cold solution 0.465 g. of white solid was recovered, m.p. 146.5–155.0°. A portion was recrystallized from aqueous ethanol, m.p. 146.0–148.0°.

Reaction of α-(1-Piperidyl)-skatole (IV) with Formamidomalonic Ester.—A solution of 0.214 g. (0.001 mole) of IV and 0.203 g. (0.001 mole) of formamidomalonic ester in 5 ml. of xylene was refluxed for 24 hours and then cooled in the refrigerator. The white solid was collected, washed with petroleum ether and recrystallized twice from aqueous ethanol giving 0.14 g. (42%) of III, m.p. 177–178°.

Reaction of β-Naphthol with Diethyl (1-Piperidylmethyl)-formamidomalonate (II).—A solution of 0.36 g. (0.0025 mole) of β-naphthol, 0.75 g. (0.0025 mole) of II and 0.06 g. (0.0015 mole) of powdered sodium hydroxide in 6 ml. of anhydrous benzene was heated to reflux for 5 hours and then cooled in the refrigerator. The frozen mixture was allowed to melt and the residual slightly yellow solid was collected. After two crystallizations from aqueous ethanol and one from benzene, 0.07 g. of small white crystals was obtained, m.p. 189–191°. The structure of this material has not yet been established. Its infrared spectrum in chloroform indicated the presence of naphthyl, hydroxyl and carbonyl groups.

Concentration of the benzene filtrate *in vacuo* caused the separation of tan crystals. These were recrystallized from aqueous ethanol to give 0.11 g. (18%) of white plates, m.p. 94–95°. There was no depression in a mixed melting point with an authentic sample of α-(1-piperidylmethyl)-β-naphthol, m.p. 94.5°.[15]

This reaction carried out in xylene gave in most cases only an amorphous brown polymeric material. However, in one instance an orange solid was recovered after removing the polymeric material and concentrating the xylene solution. Recrystallization from benzene gave 0.11 g. of white crystals, m.p. 194°, which were soluble in dilute sodium hydroxide, but insoluble in acid. The infrared spectrum in chloroform was identical with that of the unidentified material from the benzene reaction although the spectra in nujol mull were different.

Anal. Found: C, 69.69; H, 6.82; N, 8.45.

(15) J. Decombe, *Compt. rend.*, **197**, 258 (1933).

URBANA, ILLINOIS

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

Analogs of Pteroylglutamic Acid. IX. Derivatives with Substituents on the Benzene Ring[1]

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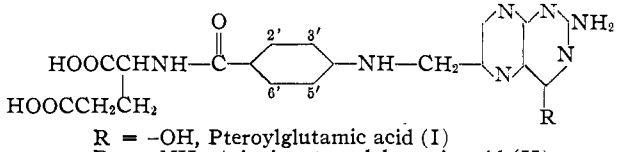
A series of pteroylglutamic acid derivatives has been synthesized with halogen and methyl substituents in the benzene ring moiety. The nitration of pteroylglutamic acid and 4-aminopteroylglutamic acid has been investigated. A number of new nitrobenzoic and nitrobenzoylglutamic acid derivatives have been synthesized as intermediates and reference compounds.

A series of 3′,5′-dihalo analogs of pteroylglutamic acid (PGA, I) was described in a previous publication from this Laboratory.[2] Interesting activity in the inhibition of neoplasms in experimental animals was shown by some of these compounds; therefore, the synthesis of other pteroyl derivatives with substituents on the benzene moiety was undertaken in an effort to enhance this activity. The 3′,5′-dihalo derivatives were readily prepared by the direct halogenation of the appropriate pteroyl derivative in aqueous acid.[2] The nitration of PGA[3a] and 4-amino PGA (II)[3b] was examined briefly, and appeared to yield 3′,5′-dinitro derivatives. For other benzene ring substituents it was necessary to investigate different approaches which involved ultimately the preparation of the 4-aminobenzoylglutamic acids with suitable substituents, and subsequent condensation with a 4,5-diaminopyrimidine and a 3-carbon intermediate to yield the desired pteroyl compounds. The latter were purified in some cases, and in others the crude or partially purified materials were submitted for screening against tumors in experimental animals. Analogs which were obtained in pure form were 3′-chloropteroylglutamic acid, 3′-methylpteroylglutamic acid and the 2′-chloro-, 3′-chloro- and 3′-methyl analogs of 4-aminopteroylglutamic acid. No enhancement of activity in the inhibition of neoplastic disease in experimental animals was observed with any of the compounds described in this paper over that of 4-amino-3′,5′-dichloropteroylglutamic acid.[2]



R = –OH, Pteroylglutamic acid (I)
R = –NH_2, 4-Aminopteroylglutamic acid (II)

(1) For the preceding paper in this series see THIS JOURNAL, **73**, 2869 (1951).

(2) D. B. Cosulich, *et al.*, *ibid.*, **73**, 2554 (1951).

(3) (a) R. B. Angier, *et al.*, *Science*, **103**, 667 (1946); (b) D. R. Seeger, J. M. Smith, Jr., and M. E. Hultquist, THIS JOURNAL, **69**, 2567 (1947).

Subsequent to the completion of our work, Backer and Houtman[4] described the synthesis of six analogs of pteroylglutamic acid, the 2'-methyl, 3'-methyl, 2'-methoxy, 3'-methoxy, 2'-chloro and 2'-fluoro compounds.

The direct halogenation of 4-aminobenzoylglutamic acid to give 4-amino-3,5-dihalo-4-aminobenzoylglutamic acid has been reported.[2] Low yields were obtained in some cases. It was thought that protection of the amino group might give better yields in the chlorination; therefore, 4-carbethoxaminobenzoylglutamic acid was prepared by the reaction of ethyl chlorocarbonate with 4-aminobenzoylglutamic acid. Attempts at dichlorination yielded mostly non-crystalline material, but with one mole of chlorine the 3-chloro compound crystallized readily on adding water to the acetic acid–hydrochloric acid reaction mixture. The carbethoxyl group of the 3-chloro compound required a relatively drastic procedure for hydrolysis, 5 *N* sodium hydroxide at 60°, but fortunately the glutamic acid portion of the molecule was not removed or racemized. The position of the chlorine atom was determined by hydrolysis to 4-amino-3-chlorobenzoic acid and comparison with a sample prepared by known methods.[5] 3'-Chloropteroylglutamic acid and 4-amino-3'-chloropteroylglutamic acid were prepared by reaction of 4-amino-3-chlorobenzoylglutamic acid with 2,4,5-triamino-6-hydroxypyrimidine[6] and 2,4,5,6-tetraaminopyrimidine,[7] respectively, and 1,1,3-tribromoacetone by the method of Hultquist and Dreisbach.[8]

4-Nitrotoluenes bearing chloro and methyl substituents are readily available and in most cases the oxidation to the 4-nitrobenzoic acids had been described using either alkaline permanganate, chromic acid or dilute nitric acid. The 2-chloro-, 2,3-dichloro-, 2,5-dichloro-, 2,6-dichloro-, 3-methyl- and 3,5-dimethyl-4-nitrobenzoic acids were prepared utilizing these procedures.

The 4-nitrobenzoylglutamic acid derivatives were prepared either by conversion of the benzoic acids to the acid chlorides, followed by reaction with glutamic acid in aqueous alkali, or by the azide synthesis *via* the methyl ester, acyl hydrazide and reaction of the azide with diethyl glutamate. The latter method proved satisfactory in some cases where the acid chlorides failed to give the desired nitrobenzoylglutamic acids.

Usually the 4-aminobenzoylglutamic acids were not isolated, but the corresponding 4-nitro compounds were reduced in aqueous medium by the zinc–copper couple,[8a] and the solution of the 4-amino compound used directly in the preparation of the pteroylglutamic acid analogs by the method of Hultquist and Dreisbach.[8]

Pteroylglutamic acid and 4-aminopteroylglutamic acid were nitrated in cold concentrated sulfuric acid, and although the products obtained were not entirely pure, the analytical data on partially purified material and the ultraviolet absorption spectra indicated that the 3',5'-dinitropteroyl derivative had been prepared. Acid cleavage or alkaline aerobic oxidation gave recognizable 6-substituted pteridine fragments, but unfortunately the identification of 4-amino-3,5-dinitrobenzoylglutamic acid as the amine fragment was not established unequivocally. 4-(N-Carbethoxyamino)-benzoylglutamic acid was nitrated successfully to give the 3,5-dinitro compound, but attempts to hydrolyze it to 4-amino-3,5-dinitrobenzoylglutamic acid in either alkali or acid were unsuccessful; in the latter case 4-amino-3,5-dinitrobenzoic acid was obtained. For ultraviolet absorption spectra comparisons, 4-amino-3-nitrobenzoic acid was prepared by nitration of 4-acetamidotoluene, followed by oxidation with alkaline permanganate.

Although direct chlorination or bromination of pteroyl and 4-aminopteroyl compounds yielded 3',5'-dihalo derivatives in good yield,[2] the iodination with iodine monochloride yielded a product which after purification had an analysis indicating that the monoiodo compound was the product isolated in the purification procedure.[9] Treatment of 4-aminobenzoylglutamic acid with iodine monochloride under similar conditions yielded a di-iodo compound, probably 4-amino-3,5-diiodobenzoylglutamic acid.

Experimental

Iodination of Pteroylglutamic Acid.—A solution of 4.9 g. of pteroylglutamic acid in 44 ml. of concentrated hydrochloric acid and 44 ml. of water was cooled to 15° and 3.26 g. of iodine monochloride[10] was added. The mixture was warmed gently on the steam-bath to 30° and a precipitate began to appear. The flask was then removed from the steam-bath and allowed to cool and stand overnight. The precipitate was filtered and washed with 5 *N* hydrochloric acid and dissolved in 30 ml. of concentrated hydrochloric acid, then diluted with 30 ml. of water. The thick slurry was cooled, filtered and washed with 5 *N* hydrochloric acid, then reprecipitated again in the same manner. The precipitate was slurried in 250 ml. of water, sodium hydroxide was added to adjust the solution to about *p*H 2.5, and the somewhat gelatinous precipitate filtered and washed with water. On drying it weighed 3.2 g. In a larger run starting with 49 g. of pteroylglutamic acid, 48.5 g. of material was obtained at this point. Of this, 45 g. was dissolved in 500 ml. of water with sodium hydroxide to give *p*H 11 and on gradual acidification with acetic acid a gel was obtained. When a small portion of this gel was treated with 5 *N* hydrochloric acid, a purplish-brown mass was obtained with liberation of iodine. To another portion was added an equal volume of acetone, then enough 5 *N* hydrochloric acid to yield a clear, dark yellow solution, which was adjusted to *p*H 3 to 4 with 5 *N* sodium hydroxide. A yellow precipitate was obtained which was filtered, washed with water and acetone, and dried.

Anal. Calcd. for $C_{19}H_{17}N_7O_6I_2$: N, 14.1; I, 36.6. Calcd. for $C_{19}H_{18}N_7O_6I$: N, 17.3; I, 22.4. Found (cor. for 11.3% ash): N, 17.92; I, 22.75.

The substance obtained is probably 3'-iodopteroylglutamic acid. Its ultraviolet absorption spectra agreed with the 3'-chloro derivative, in that the maximum at 280 mμ was present, thus being intermediate between the pteroyl derivatives, which have strong absorption in this region, and the 3',5'-dihalopteroyl compounds which show no maximum in this region.[2] The biological data are shown in Table I.

(4) H. J. Backer and A. C. Houtman, *Rec. trav. chim.*, **70**, 738 (1951).

(5) F. C. Schmelkes and M. Rubin, THIS JOURNAL, **66**, 1632 (1944).

(6) W. Traube, *Ber.*, **33**, 1372 (1900).

(7) W. Traube, *ibid.*, **37**, 4545 (1904).

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

(8a) J. H. Mowat and C. W. Waller, U. S. Patent 2,537,366 (January 9, 1951).

(9) Compare H. E. Skipper, *et al.*, *Nucleonics*, **7**, 57 (1950).

(10) "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 197.

TABLE I

SUBSTITUTED PTEROYLGLUTAMIC ACIDS

Substituents	Purity, %	Biological[b] activity	Ultraviolet absorption[c] Maxima, mμ	Minima, mμ
3',5'-Dinitro	*ca.* 80[a]	+0.22	258, 367	312
3'-Iodo	Analytical	−0.3	255, 280, 365	243, 270, 321
3'-Chloro	79.6[a]	−0.8	255, 278, 365	236, 270, 328
3'-Methyl	Analytical	−1.5	255, 285, 365	236, 267, 330
4-Amino-3',5'-dinitro	Analytical	0	256, 375	315
4-Amino-2'-chloro	82[a]	−11.2	260, 370	238, 325
4-Amino-3'-chloro	85.4[a]	−80	260, 280, 369	240, 270, 330
4-Amino-3'-methyl	Analytical	−160	260, 280, 370	240, 274, 330
4-Amino-3',5'-dimethyl	Analytical	−2.4	259, 370	238, 325

[a] By the modified chemical assay procedure.[2] [b] Determined for *S. faecalis* R; + = growth activity as compared to pteroylglutamic acid with an arbitrary value of 100 for half-maximum inhibition of the growth of *S. faecalis* R, D. B. Cosulich and J. M. Smith, Jr., THIS JOURNAL, **70**, 1922 (1948). [c] 10 mg./l. in 0.1 *N* sodium hydroxide.

4-Amino-3,5-diiodobenzoylglutamic Acid.—To a rapidly stirred slurry of 26.6 g. of 4-aminobenzoylglutamic acid[11] in 250 ml. of water was added 32.48 g. of iodine monochloride over a ten-minute period. The product separated as a purple solid, which was filtered, reslurried in 500 ml. of water containing 25 g. of potassium iodide, filtered, and washed well with water. It was dissolved in 500 ml. of dilute sodium hydroxide at *p*H 11.5, and the solution was decolorized with a little sodium hydrosulfite, stirred with activated carbon, and filtered. On acidification with hydrochloric acid a yellow gum separated which quickly solidified. It was filtered, washed well, and recrystallized from dilute alcohol three times. There was obtained 25 g. of material with a neutralization equivalent of 261 (theoretical value, 259) and m.p. 224.5° (dec.). For analysis a small sample was dried at 100° and 2–3 mm. for four hours.

Anal. Calcd. for $C_{12}H_{12}N_2O_5I_2$: C, 27.8; H, 2.32; N, 5.41; I, 49.0. Found: C, 27.7; H, 2.5; N, 5.38; I, 49.1.

4-Carbethoxaminobenzoylglutamic Acid.—To 532 g. of 4-aminobenzoylglutamic acid[11] in 3 liters of hot water was added 240 ml. of 50% sodium hydroxide solution, cooled to 10 to 20°, and 262 g. of ethyl chlorocarbonate was added slowly, together with sufficient sodium hydroxide to keep the solution alkaline to phenolphthalein. When the reaction was complete, as indicated by no more alkali consumption, the solution was acidified with concentrated hydrochloric acid to about *p*H 2.5 to 3.0. After cooling to 15° and filtering, the monosodium salt of 4-carbethoxyaminobenzoylglutamic acid was obtained. It was redissolved in 1 liter of hot water, decolorized with activated carbon, and acidified to *p*H 1. After standing overnight the crystalline product was collected on the filter, washed with water, alcohol and benzene, and dried at 50°. The yield was 390 g., m.p. 173.5–175.0°, corresponding to 57.5% of theoretical. A small sample was recrystallized several times from water to a constant melting point at 175.4–177.5°.

Anal. Calcd. for $C_{16}H_{18}O_7N_2$: C, 53.2; H, 5.36; N, 8.28. Found: C, 53.0; H, 5.38; N, 8.27.

In another experiment using 7.5 times the above quantities, the yield was 72% of the theoretical.

4-Carbethoxamino-3,5-dinitrobenzoylglutamic Acid.—To a mixture of 7.48 ml. of 70% nitric acid and 13.52 ml. of concentrated sulfuric acid at 0 to 5° was added slowly 3.38 g. of 4-carbethoxyaminobenzoylglutamic acid. After one hour, the solution was poured into an ice and water mixture, to give a white, gelatinous precipitate that was filtered, washed and dried to give 5 g., which was purified by five crystallizations from water. There was obtained 1.5 g. of 4-carbethoxamino-3,5-dinitrobenzoylglutamic acid, m.p. 203.5–204°.

Anal. Calcd. for $C_{15}H_{16}N_4O_{11}$: C, 42.0; H, 3.73; N, 13.1. Found: C, 41.9; H, 3.73; N, 12.9.

Direct nitration of 4-aminobenzoylglutamic acid by a similar procedure was unsuccessful. Attempts to hydrolyze the 4-carbethoxamino compound in dilute sodium hydroxide solution resulted in low yields of a product which melted over a wide range (196–218°) and was not further identified. Hydrolysis in hot 2 *N* sulfuric acid yielded 4-amino-3,5-dinitrobenzoic acid, m.p. 255–256°, which showed no depression in a mixture melting point with an authentic sample.[12]

Nitration of Pteroylglutamic Acid.—A solution of 4.9 g. of pteroylglutamic acid in 30 ml. of concentrated sulfuric acid at 0° was treated with 1.87 ml. of 70% nitric acid. The temperature rose rapidly to 6° and after two or three minutes the mixture was poured onto flaked ice. A yellow precipitate appeared which redissolved on warming to 40° and adding a small amount of water. After clarification with activated carbon, the solution was adjusted to *p*H 1 with sodium hydroxide and at 8° the precipitate was filtered, washed with water, and dried to give 3.3 g. of yellow material. It had 0.22% of pteroylglutamic acid activity as a growth factor for *S. faecalis*. For further purification, 0.773 g. was slurried in 530 ml. of water and dissolved with the minimum amount of sodium hydroxide, while warming the solution on the steam-bath. The hot solution was added slowly to 265 ml. of 30% acetic acid, clarified and cooled. The product separated as small spherulites, and was filtered and dried to give 0.46 g.

Anal. Calcd. for $C_{19}H_{17}N_9O_{10}\cdot H_2O$: C, 41.5; H, 3.46; N, 22.9. Found: C, 42.1; H, 3.84; N, 22.1.

These results with the exception of the nitrogen values are in fair agreement for the theoretical values for 3',5'-dinitropteroylglutamic acid. The ultraviolet absorption data and biological properties are shown in Table I.

Cleavage of 3',5'-Dinitropteroylglutamic Acid.—Oxygen was passed through a solution of 2 g. of 3',5'-dinitropteroylglutamic acid in 200 ml. of 1 *N* sodium hydroxide at 95–100° for 16 hours. The cooled solution was adjusted to *p*H 3, and the precipitate centrifuged, washed three times with water and once with acetone. The dried solid weighed 0.52 g. and was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid.[13]

The supernatant from the centrifugation and the washes were combined and evaporated *in vacuo*. On cooling the concentrate (50 ml.), a white precipitate appeared which was isolated and extracted with cold acetone. Evaporation of the acetone yielded 0.185 g. of needles with a melting point of 140–175°. Since no 4-amino-3,5-dinitrobenzoylglutamic acid was available for comparison, the ultraviolet absorption spectrum was studied and found to resemble the curve expected for 4-amino-3,5-dinitrobenzoylglutamic acid.

A suspension of 2 g. of 3',5'-dinitropteroylglutamic acid in 200 ml. of 2 *N* sulfuric acid was refluxed overnight under an atmosphere of nitrogen. The insoluble material was filtered, washed with water and acetone and dried to give 0.842 g. of 2-amino-4-hydroxypteridine-6-carboxaldehyde which was identified by comparison of its ultraviolet absorption spectrum with that of an authentic sample.[14] Evaporation of the acetone wash gave a very small amount of gummy material. The main acid filtrate was adjusted to about *p*H 3 and cooled, the precipitate was collected on the filter, washed with acetone, and dried to give 0.18 g. of material showing a maximum at 368 mμ in the ultraviolet absorption spectrum. It was not otherwise identified. The filtrate was evaporated to dryness *in vacuo*, and the residue was extracted with acetone. The insoluble residue was then extracted with boiling alcohol, and from the alcohol extracts, on concentration to a small volume and dilution with water, 0.355 g. of solid melting over a wide range was obtained. It was recrystallized twice from dilute alcohol to give 0.19 g. of white, feathery needles, m.p. 205.5–207°.

Anal. Found: C, 49.8; H, 5.22; N, 20.6.

A marked depression was noted in the mixture melting point of this material and the alkaline hydrolytic product from 4-carbethoxamino-3,5-dinitrobenzoylglutamic acid.

Nitration of 4-Aminopteroylglutamic Acid.—In a similar experiment to the one described for pteroylglutamic acid above, 4-aminopteroylglutamic acid was nitrated. From 6.9

(11) J. Van der Scheer and K. Landsteiner, *J. Immunology*, **29**, 373 (1953.)

(12) T. Friederici, *Ber.*, **11**, 1977 (1878).

(13) J. H. Mowat, *et al.*, THIS JOURNAL, **70**, 17 (1948).

(14) C. W. Waller, *et al.*, *ibid.*, **72**, 4632 (1950).

g. of 4-aminopteroylglutamic acid was obtained 0.1374 g. of material, purified by crystallization from water. The properties are shown in Table I.

Anal. Calcd. for $C_{19}H_{18}N_{10}O_6 \cdot 3H_2O$: C, 39.0; H, 4.11; N, 24.0. Found: C, 39.1; H, 3.54; N, 24.6.

3-Chloro-4-carbethoxaminobenzoylglutamic Acid.—To a slurry of 476 g. of 4-carbethoxaminobenzoylglutamic acid in 1400 ml. of glacial acetic acid and 140 ml. of concentrated hydrochloric acid, was added 112 g. of chlorine which was bubbled in slowly at 20–30°. A clear solution was obtained. Excess chlorine, hydrochloric acid and some acetic acid were removed by distillation under water-pump vacuum at 30 to 40° for one-half hour, and the remaining solution was diluted to 6 l. with water. After standing a short time the product crystallized, and after several days at 0–5° it was filtered, washed with water and a little ethyl acetate, and dried at 40°. There was obtained 425 g. of material, m.p. 158.5–161.0°, corresponding to an 81.5% yield. A small sample, recrystallized twice from water, melted at 165.0–166.2°.

Anal. Calcd. for $C_{15}H_{17}O_7N_2Cl$: C, 48.33; H, 4.60; N, 7.52; Cl, 9.51. Found: C, 48.4; H, 4.51; N, 7.46; Cl, 9.63.

In another experiment using six times the above quantities, the yield was 84.6%.

4-Amino-3-chlorobenzoylglutamic Acid.—A solution of 410 g. of 3-chloro-4-carbethoxaminobenzoylglutamic acid in 1760 ml. of 5 *N* sodium hydroxide was heated at 50 to 60° for three hours, then neutralized with hydrochloric acid, clarified with activated carbon and acidified. An oily layer separated which on standing overnight crystallized. It was recrystallized from hot water containing a little acetic acid to give 123 g. (41%) of a product melting at 142–145°. A small portion, recrystallized three times more from water, melted at 150.0–151.0°.

Anal. Calcd. for $C_{12}H_{13}O_5N_2Cl$: C, 47.9; H, 4.36; N, 9.32; Cl, 11.8. Found: C, 47.7; H, 4.29; N, 9.22; Cl, 11.9.

The hydrolysis step did not racemize the L-glutamic acid, since the material showed $[\alpha]^{25}D$ −11.3° (*c* 2, 0.1 *N* hydrochloric acid).

4-Amino-3-chlorobenzoic Acid by Hydrolysis of 4-Amino-3-chlorobenzoylglutamic Acid.—Two grams of 4-amino-3-chlorobenzoylglutamic acid was boiled in 20 g. of 40% sodium hydroxide solution for ten minutes. After cooling and diluting with 25 ml. of water, the solution was neutralized to *p*H 7, decolorized with activated carbon, and acidified. The precipitate which separated on cooling was filtered and recrystallized from 50% alcohol. There was obtained 0.2 g., melting point 225–227°. A mixture melting point with 4-amino-3-chlorobenzoic acid prepared by the method of Schmelkes and Rubin[5] showed no depression.

3′-Chloropteroylglutamic Acid.—From 15 g. of 4-amino-3-chlorobenzoylglutamic acid, 25.4 g. of 2,4,5-triamino-6-hydroxypyrimidine sulfate and 36.8 g. of 1,1,3-tribromoacetone was obtained 43 g. of crude[6] 3′-chloropteroylglutamic acid, purity 10% by chemical assay.[2] It showed a low level of antagonist activity to folic acid in the microbiological assay.

For purification the crude material containing 9.5 g. of 3′-chloropteroylglutamic acid was dissolved in five liters of 0.1 *N* sodium hydroxide by heating at 70–75° for one-half hour. After treatment with 30% calcium chloride the mixture was filtered and to the filtrate was added 10% zinc chloride to adjust to *p*H 10.84. Following clarification, the solution was adjusted to *p*H 2.5 and cooled. The precipitate after filtration was dissolved in four liters of water containing sodium hydroxide to give *ca.* *p*H 11. The solution was heated to 70° and then adjusted to *p*H 7 while cooling to 20°. After clarification, the solution was brought to *p*H 2.56 and cooled. The precipitate after filtration was again dissolved in dilute sodium hydroxide and the treatment with calcium chloride and zinc chloride and adjustment to *p*H 2.5 repeated. The dried material weighed 5.1 g. and was 79.6% 3′-chloropteroylglutamic acid by chemical assay.[2] The ultraviolet absorption spectrum and biological data are recorded in Table I.

4-Amino-3′-chloropteroylglutamic Acid.—From 150 g. of 4-amino-3-chlorobenzoylglutamic acid, 274 g. of 2,4,5,6-tetraaminopyrimidine sulfate and 442 g. of 1,1,3-tribromoacetone condensed in the usual manner[8] there was obtained 1200 g. of wet crude (containing a filter aid, Hyflo Super-Cel) 4-amino-3′-chloropteroylglutamic acid which had a chemical assay of 4.58%. It was purified by a process similar to that described above to give 17.5 g. of 4-amino-3′-chloropteroylglutamic acid, 85.4% pure by chemical assay. In Table I are shown the ultraviolet and biological data.

2-Chloro-4-nitrobenzoic Acid.[4]—By the oxidation of 2-chloro-4-nitrotoluene[15] with alkaline permanganate as described for *o*-nitrobenzoic acid,[16] 2-chloro-4-nitrobenzoic acid was prepared in 53% yield; it was purified by crystallization from water and then melted at 142–143°. Backer and Houtman reported the same melting point.[4]

2-Chloro-4-nitrobenzoyl Chloride.—Attempts to repeat the work of Grohmann,[17] who describes the preparation of the acid chloride from the acid by the action of phosphorus pentachloride, yielded only an oily product which, however, did contain some of the desired acid chloride since it yielded the amide, m.p. 166°, on treatment with aqueous ammonia. Attempts to combine the crude oil with glutamic acid were unsuccessful. Backer and Houtman[4] reported a b.p. of 158–160° at 13 mm. for the acid chloride.

Methyl 2-Chloro-4-nitrobenzoate.[18]—A mixture of 80 g. of 2-chloro-4-nitrobenzoic acid and 2400 ml. of absolute methanol was treated cautiously with 32 ml. of 100% sulfuric acid and then heated under reflux for 18 hours. After removal of methanol by distillation, the concentrate was allowed to crystallize, slurried in ice-water and neutralized with sodium bicarbonate. Filtration gave the ester in 91–96% yield; m.p. 75.5–76.7°.

2-Chloro-4-nitrobenzhydrazide.—To a solution of 78.4 g. of methyl 2-chloro-4-nitrobenzoate in 2400 ml. of absolute methanol, 59.2 ml. of hydrazine hydrate was added slowly. The flask was then swept with dry nitrogen and held at room temperature for 18 hours. Most of the methanol was distilled off under reduced pressure, the residue poured into water, and then acidified with hydrochloric acid. The insolubles were removed by clarification and the filtrate neutralized to yield the yellow hydrazide, which was washed well, dried and used without purification. It melted at 156–157°; the yield was 61.8 g. (79%).

Anal. Calcd. for $C_7H_6O_3N_3Cl$: C, 39.0; H, 2.81; N, 19.5; Cl, 16.4. Found: C, 39.0; H, 2.91; N, 19.4; Cl, 16.6.

Diethyl 2-Chloro-4-nitrobenzoylglutamate.—A mixture of 21.5 g. of 2-chloro-4-nitrobenzhydrazide, 300 ml. of cold water, 30 ml. of glacial acetic acid, 20 ml. of concentrated hydrochloric acid and 250 ml. of isopropyl acetate was cooled to 0° and a solution of 9.65 g. of sodium nitrite in 100 ml. of water was added during 10 minutes. After stirring for an additional 20 minutes at 0°, the layers were separated and the aqueous layer was extracted with 250 ml. of isopropyl acetate. The combined isopropyl acetate layers containing the azide were washed with water, and then added to a solution of 130 g. of diethyl glutamate sulfate in 200 ml. of water, previously neutralized with sodium bicarbonate in excess. The mixture was stirred 20 hours at room temperature, and then the isopropyl acetate layer was separated, washed with water, dried, and the solvent removed under reduced pressure. The crude solid was redissolved in 100 ml. of isopropyl acetate, then the solution was diluted with 1000 ml. of carbon tetrachloride and 1000 ml. of petroleum ether. The yield was 22.7 g., m.p. 98–98.5°.

Anal. Calcd. for $C_{16}H_{19}N_2O_7Cl$: C, 49.7; H, 4.95; N, 7.24; Cl, 9.17. Found: C, 49.7; H, 4.94; N, 7.27; Cl, 9.14.

4-Amino-2′-chloropteroylglutamic Acid.—Attempts to isolate diethyl 4-amino-2-chlorobenzoylglutamate in solid form were unsuccessful. Therefore, the 4-nitro compound (4.8 g.) was treated with 100 ml. of 2 *N* sodium hydroxide at room temperature for 24 hours to saponify the ester. The resulting solution was acidified, and a mixture of 4.1 g. of zinc dust, 0.5 g. of cupric sulfate pentahydrate and 10 ml. of water was added during 25 minutes at about 30–35°, with sufficient concentrated hydrochloric acid to maintain *p*H 3 to 4. After stirring for an additional half hour, the mixture was clarified to give 141 ml. of solution. Nitrite titration[11] indicated a 95% yield of 4-amino-2-chlorobenzoyl-

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glutamic acid, which, without isolation was condensed with 7 g. of 2,4,5,6-tetraminopyrimidine sulfate and 9.2 g. of 1,1,3-tribromoacetone, by the method of Hultquist and Dreisbach.[8] There was obtained 7.6 g. of crude 4-amino-2'-chloropteroylglutamic acid, 23.7% pure as indicated by the modified chemical assay procedure.[2] Microbiological assays indicated that it was an antagonist for pteroylglutamic acid of a very low order. The crude material (6.5 g.) was slurried in 700 ml. of water, adjusted to *p*H 12 with sodium hydroxide at 60°, and then treated with calcium chloride to *p*H 11.4. The insolubles were removed by clarification and the filtrate adjusted to *p*H 10.65 with aqueous zinc chloride, clarified and the filtrate adjusted to *p*H 3.1 with hydrochloric acid. The precipitate was filtered and reslurried in 500 ml. of water, and then dissolved with the minimum amount of dilute sodium hydroxide to give *p*H 11 to 12, heated at 60° for one-half hour, then adjusted to *p*H 7 and cooled to 20°. After filtration to remove the precipitated materials, the filtrate was acidified to *p*H 4 and the 4-amino-2'-chloropteroylglutamic acid collected on the filter. It was purified further by solution in sodium hydroxide and successive treatments with calcium chloride and zinc chloride as before, and finally precipitated by pouring the solution into 60 ml. of hot 30% acetic acid. There was obtained 0.413 g. of 4-amino-2'-chloropteroylglutamic acid, 82% pure by the chemical assay method.[2] The biological and ultraviolet data are shown in Table I.

2,3-Dichloro-4-nitrobenzoic Acid.—After trying several methods for the oxidation of 2,3-dichloro-4-nitrotoluene,[19] the following procedure was adopted, which gave yields of 3 to 15% with 35–50% recovery of the starting material. A mixture of 43.3 g. of 2,3-dichloro-4-nitrotoluene in 109 ml. of concentrated sulfuric acid and 20.8 ml. of water was heated to 60–65°. Then 80.5 g. of potassium dichromate (140 mesh) was added gradually over a period of 1.5 hours, with the temperature maintained at 62–65°. Both hydrogen chloride and chlorine were evolved during the reaction. The stiff mass was thinned with 6 ml. of water, and stirred at 60–70° for one hour. After cooling 120 ml. of water was added and an oil separated, which was removed by steam distillation. About nine liters of distillate collected. From the first four liters, 20.5 g. of 2,3-dichloro-4-nitrotoluene was recovered, representing 47.3% of the starting material. The residual hot aqueous liquor was decanted from a green, gummy material which seemed to be largely inorganic, clarified and cooled. The precipitate was filtered, washed with water containing a little hydrochloric acid and dissolved in 200 ml. of water with sodium bicarbonate. The solution was treated with activated carbon, clarified and acidified to about *p*H 1. After cooling, the precipitated material was collected on the filter, washed and dried to yield 5.7 g. of 2,3-dichloro-4-nitrobenzoic acid, melting at 127–146°. It was purified by three reprecipitations from water as described above; and after final drying at 102°, it melted at 135.8–143°.

Anal. Calcd. for $C_7H_3NO_4Cl_2$: Cl, 30.1; N, 5.93. Found: Cl, 30.2; N, 6.04.

Oxidation of 2,3-dichloro-4-nitrotoluene was also carried out by means of dilute nitric acid in a sealed tube at 140° under the conditions used by Cohen and Dakin for the various trichloronitrotoluenes.[19] The yields of the acid were 7 to 16% with 30 to 60% recovery of the starting material.

2,3-Dichloro-4-nitrobenzoylglutamic Acid.—One gram of 2,3-dichloro-4-nitrobenzoic acid was heated with 5 ml. of thionyl chloride until solution occurred (30 minutes). The excess thionyl chloride was distilled under vacuum and the residue was extracted with hot petroleum ether. This solution on evaporation yielded an oily residue, which was added slowly to 0.62 g. of glutamic acid in 8.4 ml. of *N* sodium hydroxide solution with additional sodium hydroxide to maintain *p*H 11. After 1.5 hours the mixture was heated to 70° and filtered to remove some insoluble material, then the solution was acidified to give a waxy precipitate. It was reprecipitated from sodium bicarbonate solution, and eventually the oil crystallized in the form of needles. There was obtained 0.875 g. of material melting over a wide range (70–105°). It was reprecipitated from sodium bicarbonate three times more; each time it separated as an oil which on standing in strongly acid solution eventually crystallized. The purified 2,3-dichloro-4-nitrobenzoylglutamic acid melted at 79–83° (0.340 g.).

Anal. Calcd. for $C_{12}H_{10}N_2O_7Cl_2$: N, 7.68. Found: N, 7.53.

2,5-Dichloro-4-nitrobenzoic Acid.—Five grams of 2,5-dichloro-4-nitrotoluene[19] was slurried in 15 ml. of 80% sulfuric acid and heated to 65°, then oxidized by the addition of 9.3 g. of potassium dichromate added over a 20-minute period. The reaction was slightly exothermic. Subsequently the mixture was heated at 65° for one hour, diluted with an equal volume of water and filtered. The precipitate was treated in 140 ml. of water at 60° with sodium carbonate to an alkaline reaction on phenolphthalein test paper, then cooled to 10° and filtered. By neutralization of the filtrate 2,5-dichloro-4-nitrobenzoic acid was obtained and it was purified by two recrystallizations from 50% alcohol. It then melted at 207–210° (1.2 g.).

Anal. Calcd. for $C_7H_3NO_4Cl_2$: C, 35.6; H, 1.27; N, 5.94; Cl, 30.1. Found: C, 35.5; H, 1.27; N, 5.97; Cl, 30.1.

2,5-Dichloro-4-nitrobenzoyl Chloride.—A mixture of 0.5 g. of 2,5-dichloro-4-nitrobenzoic acid, 1 ml. of thionyl chloride, and one drop of dry pyridine was heated under reflux until all solid dissolved (about 30 minutes). Excess thionyl chloride was removed and the residue solidified; it was recrystallized from about 5 ml. of naphtha (varnish-makers' and painters' naphtha, b.p. 135–145°) to yield 0.284 g. of the acid chloride, m.p. 62–64°. It was used without purification in subsequent experiments.

2,5-Dichloro-4-nitrobenzoylglutamic Acid.—Crude 2,5-dichloro-4-nitrobenzoyl chloride was condensed with glutamic acid in aqueous alkali as described for the 2,3-dichloro derivative to yield 0.22 g. of 2,5-dichloro-4-nitrobenzoylglutamic acid, m.p. 182–185°.

Anal. Calcd. for $C_{12}H_{10}N_2O_7Cl_2$: C, 39.4; H, 2.76; N, 7.67; Cl, 19.5. Found: C, 39.3; H, 3.02; N, 7.67; Cl, 19.5.

In a similar experiment on a somewhat larger scale the yield was 78%.

2,5-Dichloro-4-nitrobenzoylglutamic acid was reduced to the 4-amino compound using the zinc–copper couple in dilute acid medium,[9] and titration with sodium nitrite indicated a 75% yield of the amine. The solution was used in condensation with 2,4,5,6-tetraaminopyrimidine and 1,1,3-tribromoacetone,[8] and with 2,2,3-trichlorobutyraldehyde.[20] The crude reaction products were not purified since they showed no interesting biological activity in preliminary tests.

2,6-Dichloro-4-nitrobenzoic Acid.—2,6-Dichloro-4-nitrotoluene was prepared in 9–17% yields by the method of Davies.[21] A mixture of 5 g. of this product, 8 ml. of 70% nitric acid and 16 ml. of water was heated in a sealed tube at 140° for 16.5 hours.[19] The crude isolated after cooling was partially purified by reprecipitation from its sodium bicarbonate solution by the addition of acid. After recrystallization from water yields of 62–69% of the desired material were obtained, m.p. 175.6–176.4°.

Anal. Calcd. for $C_7H_3NO_4Cl_2$: C, 35.6; H, 1.28; N, 5.94; Cl, 30.0. Found: C, 35.6; H, 1.36; N, 6.20; Cl, 30.0.

2,6-Dichloro-4-nitrobenzoyl Chloride.—By heating the acid with excess thionyl chloride in the presence of a small amount of pyridine, the acid chloride which was obtained was used without further purification in subsequent experiments.

Methyl 2,6-Dichloro-4-nitrobenzoate.—A mixture of 10 g. of 2,6-dichloro-4-nitrobenzoic acid, 50 ml. of thionyl chloride and 20 drops of pyridine was heated under reflux for one hour, excess thionyl chloride was removed under reduced pressure and 200 ml. of methanol was added to the residue. It was heated under reflux for five hours and the methanol distilled. The residue was slurried in ice-water with sufficient sodium bicarbonate to give an alkaline reaction. The yield of dry material was 10.4 g., m.p. 119–121.5°. It was recrystallized from boiling methanol and then melted at 121.5–123.5°.

Anal. Calcd. for $C_8H_5O_4NCl_2$: C, 38.4; H, 2.02; N,

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5.60; Cl, 28.4. Found: C, 38.5; H, 2.11; N, 5.54; Cl, 28.2.

2,6-Dichloro-4-nitrobenzoylglutamic Acid.—Crude 2,6-dichloro-4-nitrobenzoyl chloride (0.4 g.) was treated with glutamic acid (0.235 g.) in aqueous alkali as described for the 2,3-dichloro derivative to give 2,6-dichloro-4-nitrobenzoylglutamic acid, m.p. 215–220° (precipitated from aqueous alkali with acid).

Anal. Calcd. for $C_{12}H_{10}O_7N_2Cl_2$: C, 39.5; H, 2.76; N, 7.68; Cl, 19.4. Found: C, 39.2; H, 2.71; N, 7.77; Cl, 19.4.

3-Methyl-4-nitrobenzoic Acid.[22]—The acid was prepared in 25% yield by oxidation of a 25:75 mixture of 2- and 4-nitroxylenes with chromic acid in glacial acetic acid. It melted at 216–217°, which agrees with the value of 217° reported by Backer and Houtman.[4]

Methyl 3-Methyl-4-nitrobenzoate.[22,23]—The ester was prepared in 95% yield by refluxing a mixture of 18.1 g. of 3-methyl-4-nitrobenzoic acid, 650 ml. of methanol and 11.8 g. of 100% sulfuric acid for 20 hours on the steam-bath. It melted at 80–81°.

3-Methyl-4-nitrobenzhydrazide.—A solution of 270 g. of methyl 3-methyl-4-nitrobenzoate was dissolved in 4200 ml. of methanol, at 40–45°; the temperature was then lowered to 30° and the flask swept with dry nitrogen and 208 g. of hydrazine hydrate was added. The mixture was stirred overnight at room temperature under nitrogen atmosphere, and the solid was filtered, washed with water and dried to yield 135.2 g. of hydrazide, m.p. 149–150°. From the alcoholic liquors an additional 96.5 g. was obtained by partial evaporation of the methanol and dilution with water. The combined yield represented 86% of the theoretical. A small sample was recrystallized from water with no appreciable change in the melting point.

Anal. Calcd. for $C_8H_9O_3N_3$: C, 49.22; H, 4.65; N, 21.54. Found: C, 49.1; H, 4.57; N, 21.5.

Diethyl 3-Methyl-4-nitrobenzoylglutamate.—3-Methyl-4-nitrobenzhydrazide was converted to the azide and condensed with diethyl glutamate by a process similar to that described above for the 2-chloro derivative. The yield was 62.8% of the theoretical amount of material melting at 66.5–67.4°.

Anal. Calcd. for $C_{17}H_{22}N_2O_7$: C, 55.7; H, 6.05; N, 7.65. Found: C, 55.8; H, 5.96; N, 7.58.

3'-Methylpteroylglutamic Acid.[4]—Diethyl 3-methyl-4-nitrobenzoylglutamate was hydrolyzed in dilute sodium hydroxide solution, and then reduced using the zinc–copper couple.[9] Titration of the solution indicated a 100% yield of the amino compound, and it was used without isolation in the condensation with 2,4,5-triamino-6-hydroxypyrimidine and 1,1,3-tribromoacetone.[8] The yield was about 27–36% based on a chemical assay[2] of the crude material. It was purified by a process similar to that indicated for 4-amino-2'-chloropteroylglutamic acid above. The ultraviolet and biological data are shown in Table I.

Anal. Calcd. for $C_{20}H_{21}N_7O_6 \cdot 2.5H_2O$: C, 47.9; H, 5.24; N, 19.6. Found: C, 47.8; H, 5.29; N, 19.6.

4-Amino-3'-methylpteroylglutamic Acid.—By a similar procedure, substituting 2,4,5,6-tetraminopyrimidine for the triamine, this compound was prepared and purified; see Table I.

Anal. Calcd. for $C_{20}H_{22}N_8O_5 \cdot 2H_2O$: C, 48.9; H, 5.34; N, 22.8. Found: C, 48.8; H, 5.02; N, 22.8.

3,5-Dimethyl-4-nitrobenzoyl Chloride.—By reaction of 35.5 g. of 4-nitromesitylenic acid[24] with 118 g. of thionyl chloride and 2–3 ml. of dry pyridine 3,5-dimethyl-4-nitrobenzoyl chloride was obtained on removal of the excess thionyl chloride. It was recrystallized from naphtha (b.p. 135–145°) and then melted at 52–53°.

Anal. Calcd. for $C_9H_8NO_3Cl$: C, 50.8; H, 3.78; N, 6.57; Cl, 16.62. Found: C, 50.9; H, 4.12; N, 6.46; Cl, 16.1.

Attempts to combine the acid chloride with glutamic acid were unsuccessful and, therefore, the azide synthesis was utilized; see below.

Methyl 3,5-Dimethyl-4-nitrobenzoate.—By esterification of the acid in methanol with sulfuric acid catalyst, this compound was prepared, m.p. 107.5–109°, in 97% yield. It was used without further purification.

3,5-Dimethyl-4-nitrobenzhydrazide.—By reaction with hydrazine hydrate in methanol methyl 3,5-dimethyl-4-nitrobenzoate was converted to the hydrazide in 86% yield. It was purified by reprecipitation from dilute acid with ammonia and melted at 172.8–173.7°.

Anal. Calcd. for $C_9H_{11}N_3O_3$: C, 51.7; H, 5.30; N, 20.1. Found: C, 51.5; H, 5.35; N, 20.1.

Diethyl 3,5-Dimethyl-4-nitrobenzoylglutamate.—By the azide procedure similar to that described above this compound was prepared in 68% yield. For analysis a small sample was reprecipitated from alcohol with water several times. It melted at 87.5–89.0°.

Anal. Calcd. for $C_{18}H_{24}N_2O_7$: C, 56.8; H, 6.36; N, 7.37. Found: C, 56.7; H, 6.21; N, 7.32.

Diethyl 3,5-dimethyl-4-nitrobenzoylglutamate was hydrolyzed in dilute alkali, the nitro group reduced[9] and the solution of the 4-amino compound used for the condensation with 2,4,5-triamino-6-hydroxypyrimidine and tribromoacetone.[8] The crude reaction product was not purified since it showed no interesting biological properties in preliminary tests.

4-Amino-3',5'-dimethylpteroylglutamic Acid.—The compound was prepared as described for the 3-methyl derivative above, using 2,4,5,6-tetraaminopyrimidine and a solution of 4-amino-3,5-dimethylbenzoylglutamic acid. It was purified by a process similar to those described before.

Anal. Calcd. for $C_{21}H_{24}N_8O_5$: N, 23.9. Found: N, 23.1.

Its properties are shown in Table I.

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