PREPARATION OF PTEROYLGLUTAMIC ACID-3',5'-$^2H_2$ BY ACID CATALYZED EXCHANGE WITH DEUTERIUM OXIDE

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SUMMARY

Pteroylglutamic acid was labeled with deuterium by trifluoroacetic acid-catalyzed exchange with deuterium oxide. The product, pteroylglutamic acid-3',5'-$^2H_2$, was selectively labeled with deuterium in the 4-aminobenzoyl portion of the molecule; there was no evidence for isotope incorporation at C_7 or C_9 of the pteridine ring or at the glutamate residue. Isotope composition of the pteroylglutamates was determined by chemical ionization mass spectrometry following a base-catalyzed, oxidative cleavage to either 4-aminobenzoic acid or 4-aminobenzoyl-glutamic acid. Products from the exchange reaction typically contained 1% $^2H_0$, 9% $^2H_1$, and 90% $^2H_2$ isotopic species.

Key Words: Pteroylglutamic Acid, Deuterium, Acid-catalyzed exchange, Chemical ionization mass spectrometry.

INTRODUCTION

In the course of clinical studies on absorption, utilization, and turnover of pteroylglutamic acids (Pte-Glu, 1), we required gram quantities of this compound labeled with deuterium in the 3',5' positions (2). Tritium-labeled Pte-Glu has been prepared by catalytic hydrogenolysis of the corresponding 3',5'-dibromo compound over platinum on charcoal.[1] These preparations generally result in a product that contains as much as 60% of the isotope on

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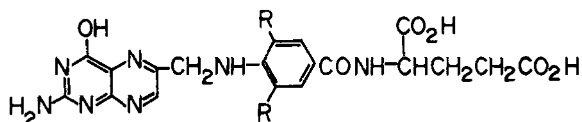
C_9.[1] Attempts to prepare 2 in a manner analogous to the radiochemical synthesis were frustrated by incomplete bromination of 1, incomplete hydrogenolysis of the intermediate 3',5'-dibromide, and competing reduction of the pteridine ring. The result was a complex mixture of products with a variable isotopic purity.[2]



1 R = H
2 R = ^{2}H

Aromatic protons may be substituted for deuterium by acid-catalyzed exchange with deuterium oxide.[3] This procedure, however, is seldom used to label aromatic protons of complex biological molecules because the drastic conditions required for the exchange reaction generally result in decomposition of the sample. It is possible to exchange-label aromatic protons under mild conditions where substituents impart a favorable directing influence on electrophilic substitution of the aromatic ring. This procedure has been used to exchange-label substituted aniline derivatives in good yield.[4] We have extended this method to prepare 1 deuterated at the 3',5' positions of the 4-aminobenzoyl moiety.

EXPERIMENTAL

Pteroylglutamic acid (Sigma Chemical Co.) was purified by crystallization from water. Paper chromatography (ascending) was carried out on Whatman No. 1 paper in solvent systems (A) 5% ammonium carbonate in 0.2% mercaptoethanol or (B) n-butanol:pyridine:water (1:1:1). Preparative TLC was carried out with Merck silica gel H (0.5 mm thickness) developing with (C) ether:methanol:formic acid (98:1:1). Nuclear magnetic resonance spectra were obtained at 220 mHz using a Varian HR-220 instrument. Chemical ionization mass spectra (CI/MS) were obtained with isobutane reagent gas using a Biospect CI/MS spectrometer.

Pteroylglutamic Acid-3',5'-$^2H_2$(2). Pteroylglutamic acid (1) 10.0 g (22.7 mmoles), was stirred at 0°C in 270 ml deuterium oxide (99.8 atom % ^2H) containing 1.35 g ascorbic acid (7.7 mmoles). Sufficient trifluoracetic anhydride was carefully added to effect solution of 1 (this amount should be experimentally determined, but is generally about 30% by weight of the amount of deuterium oxide used). The reaction was carried out under nitrogen. The solution was heated at 42° for 8 days in the dark. At the end of this time, solvents were evaporated in vacuo. The crude product was treated with fresh deuterium oxide, ascorbic acid, and trifluoroacetic anhydride for an additional 7 days. Solvents were evaporated and the product was isolated by precipitation from water at pH 5. The crude yield was 7.41 g (74% yield) of a dark brown powder. The crude product was purified by chromatography on DEAE cellulose using a convex gradient of 0.02 M diethylammonium acetate to 1.20 M deithylammonium acetate containing 0.3% mercaptoethanol at pH 6.2. The fractions containing 2 were pooled and concentrated, and the product was recrystallized from water. Two additional purifications on DEAE cellulose, followed by crystallization from water, yielded 3.38 g (34%) pteroylglutamic acid-3',5'-$^2H_2$ (2) as pale lemon-yellow crystals. The product showed a single UV-absorbing spot on ascending paper chromatography in both solvent systems A and B. The NMR spectrum (10^{-2} M in DMSO-$^2H_6$) exhibited peaks at 1.8-2.9 ppm (alphatic CH), 4.43 ppm (d, 2H, H_9), 7.62 ppm (s, 2H, $H_{2',6'}$), and 8.61 ppm (s, 1H, H_7). Analysis by chemical ionization mass spectrometry showed that the product contained 2.2% $^2H_0$, 5.8% $^2H_1$ and 92.0% $^2H_2$ species. Microbiological assay (L. casei) of 2 indicated greater than 95% of the biological activity of pteroylglutamic acid.

Analysis by Chemical Ionization Mass Spectrometry (CI/MS). A. Pteroylglutamic acid, 50 μg - 5 mg, was dissolved in 5 ml 1 N NaOH and heated at 125° in a teflon-lined reaction bomb for 5 hours. The bomb was cooled and the

contents evaporated to dryness under a stream of compressed air. The semisolid mass was acidified with acetic acid, evaporated almost to dryness, and then dissolved in a small volume of methanol. The solution was applied to a preparative TLC plate and eluted with solvent system C. The R_f zone corresponding to 4-aminobenzoic acid (PABA, R_f = 0.62) was collected and the amino acid was eluted with ether-methanol (90:10) and evaporated to dryness. PABA was methylated with diazomethane and evaporated to dryness. The methyl esters were taken up in 100 µl trifluoroacetic anhydride and heated to 40° for 10 minutes. The volume was adjusted with benzene to give a nominal concentration of 1 µg/µl of PABA methylester, N-TFA derivative. The product recovery of PABA for an 8 hour hydrolysis of 2 was about 40% of the theoretical value. The sample was analyzed by GC-CI-MS on a 1 mm ID x 3.7 m glass column packed with 0.25% EGGS-X and 0.75% DEGS on Chromosorb W at 155° at a helium flow rate of 6.8 ml/min. Under these conditions the PABA methylester, N-TFA derivative had a retention time of 290 seconds. The CI mass spectrometer was operated at a source temperature of 180° and a source pressure of 0.5 torr isobutane. Isotope ratios were measured using a stable isotope ratiometer-multiple ion detection system described in a previous communication.[5] For deuterated compounds, isotope ratios were measured using the ions [MH^+] at m/e 248 ($^2H_0$), m/e 249 ($^2H_1$), and m/e 250 ($^2H_2$).

B. Pteroylglutamic acid, 10-500 µg, was dissolved in 1 ml of 0.1 N NaOH. The solution was treated with 1 ml of 0.03 M potassium permanganate at room temperature for 15 minutes. The oxidation reaction was quenched with 1 ml ethanol for 30 minutes. The sample was centrifuged and the oxidation products were decanted from the manganese dioxide. The solution was acidified with acetic acid and concentrated to 200 µl under a stream of nitrogen. The product, 4-aminobenzoylglutamic acid (3), was isolated by high pressure liquid chromatography (HPLC) on a Vydac™ anion exchange column using a 0.25 M acetate buffer at pH 4.05. The fraction containing 3 was evaporated to dryness, then dissolved

in 200 μl methanol and 20 μl acetic acid, and the methyl ester was obtained by treatment with freshly distilled diazomethane. The solution was concentrated to dryness and redissolved in 100 μl methanol. The products were analyzed by CI/MS using the direct insertion probe and the conditions described above. Isotope ratios were measured using the protonated molecular ions [MH^{+}] at m/e 295 ($^{2}H_{0}$), m/e 296 ($^{2}H_{1}$) and m/e 297 ($^{2}H_{2}$).

RESULTS AND DISCUSSION

Exchange of the 3',5' aromatic protons in 1 was accomplished by careful addition of sufficient trifluoroacetic anhydride to a stirred suspension of 1 in deuterium oxide to effect dissolution (3 - 10 M trifluoroacetic acid). This solution was then heated in the dark for 168 hours at 42°C. Since pteroylglutamates are sensitive to decomposition by light and oxygen, the reaction was carried out under nitrogen, and ascorbic acid was added to scavenge any oxygen dissolved in the solvents. When products from the exchange were examined by NMR, integration of the aromatic proton region indicated about 90% deuteration. Solvents were removed under vacuum and replaced with fresh solvents and the exchange was carried out a second time. The product was isolated and purified by column chromatography on DEAE-cellulose followed by crystallization from water. The pure compound exhibited the UV spectral properties and biological activity (*L. casei* assay, > 95% active) of pteroylglutamic acid. The 220 MHz NMR spectrum (10^{-2} M in DMSO-$^{2}H_{6}$) indicated almost complete exchange of the 3', 5' aromatic protons (Figure 1). The doublet at 6.67 ppm (J = 8.6 Hz) in 1 disappeared and the doublet at 7.62 ppm (J = 8.6 Hz) collapsed to a singlet for 2. The aromatic proton on C_7 of the pteridine ring and the benzylic protons on C_9 remain unchanged. The α protons on the glutamic acid moiety were not exchanged with deuterium. Under similar conditions Bak *et al*. found that the aromatic protons of tryptophan exchanged at different rates, but that the aliphatic positions were not deuterated.[6]

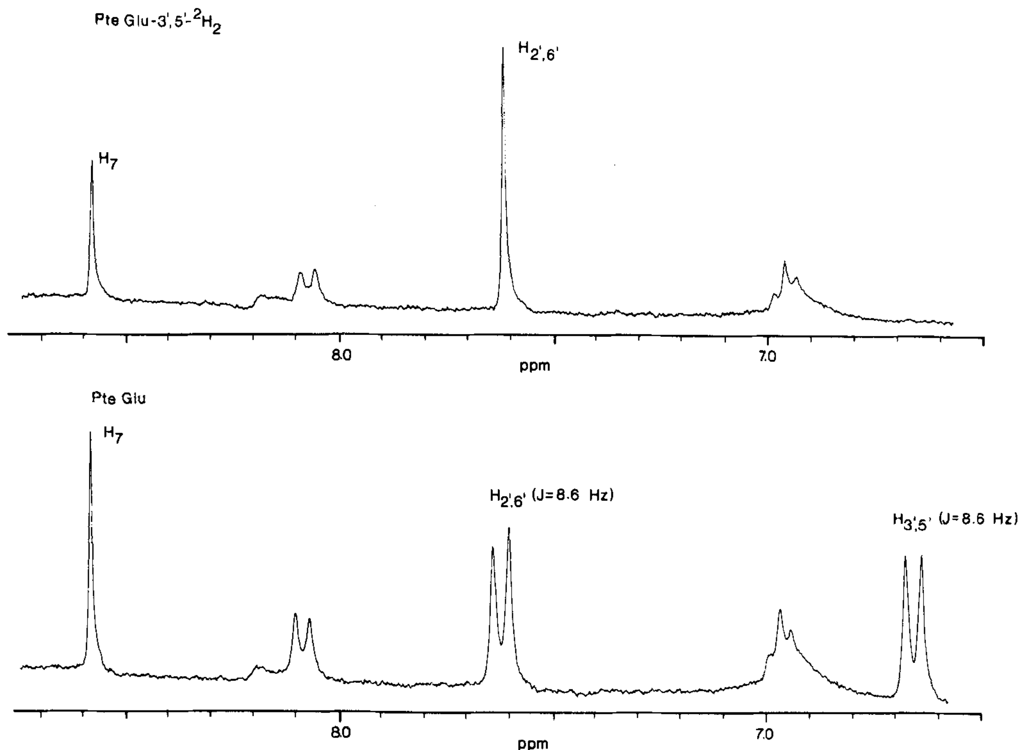


Figure 1. NMR spectra for the aromatic proton region of pteroylglutamic acid (1) (Bottom) and pteroylglutamic acid-3',5'-$^2H_2$ (2) (Top).

In order to obtain a more accurate estimate of the deuterium content for various preparations of 2, we developed a gas chromatographic-mass spectrometric (GC-MS) assay for pteroylglutamic acids based on alkaline hydrolysis of pteroylglutamates in the presence of oxygen to give 4-aminobenzoic acid (PABA). PABA was analyzed as the methyl ester, N-trifluoroacetate derivative using isobutane chemical ionization GC-MS. Isotope ratios were measured on the protonated molecular ion (MH^+) at m/e 248 for 1 and m/e 250 for 2 using techniques previously described.[5] Alternatively, pteroylglutamic acid was oxidized to 4-aminobenzoylglutamic acid (3) by alkaline potassium permanganate. Microgram quantities of 3 were isolated by high pressure liquid chromatography on an anion exchange column. The methyl esters were analyzed by CI/MS using the direct insertion probe. Isotope ratios were measured on the protonated molecular ions at m/e 295 ($^2H_0$), m/e 296 ($^2H_1$) and m/e 297 ($^2H_2$). For a small scale (1.5 g)

deuteration of 1 carried out at 55° for 48 hours, the product contained 0.6% d_0, 11.6% d_1 and 87.8% d_2 species. When the product was recovered and deuterated a second time, the deuterium content of 2 was only slightly changed ($^2H_0$ = 1.5%, $^2H_1$ = 10.2%, $^2H_2$ = 88.3%). The results for several preparations of 2 are summarized in Table I.

Table I. Deuterium content for typical preparations of pteroylglutamic acid-3',5'-$^2H_2$ (2).

Run	Yield[a]	Deuterium Content[b]		
		d_0	d_1	d_2
1[c]	35%	0.6%	11.6%	87.8%
2[d]	34%	2.2%	5.8%	92.0%
3[e]	--	1.8%	12.1%	86.1%

[a]For final crystalline products. [b]Estimated by CI/MS and corrected for natural ^{13}C content. [c]1.5 g 1 deuterated in 45 ml ^2H_2O using 33 g TFA anhydride. [d]See example in Experimental Section. [e]Measured on 4-aminobenzoylglutamate fragment of 2.

The present method offers a significant improvement for the preparation of deuterated pteroylglutamates over existing procedures. This procedure should also be applicable for preparation of tritiated pteroylglutamates as well as other folic acid analogs containing the 4-aminobenzoyl function.

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REFERENCES

1. Zakrzewski, S. F., Evans, E. A., and Phillips, R. F. - Anal. Biochem. 36: 197 (1970)

2. Rosenberg, I. H., Hachey, D. L., Beer, D. E., and Klein, P. D. - Proceedings of the First International Conference on Stable Isotopes in Chemistry, Biology and Medicine, Klein, P. D. and Peterson, S. V. Editors, USAEC CONF-730525, 1973, p. 421.

3. Thomas, A. F. - Deuterium Labeling in Organic Chemistry, Appleton-Century-Crofts, New York, NY, 1971, Chapter 3.

4. Blackborow, J. R. and Ridd, J. H. - Chem. Commun. 132 (1967)

5. Klein, P. D., Haumann, J. R., and Hachey, D. L. - Clin. Chem. 21: 1253 (1975)

6. Bak, B., Dambmann, C., and Nicolaisen, F. - Acta Chem. Scan. 21: 1647 (1967)