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PREPARATION OF [2',3',5',6'-$^2H_4$]PTEROYLGLUTAMIC ACID.

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SUMMARY

Folic acid plays a key role in nucleic acid biosynthesis, essential for normal cell proliferation and function. Localized folate deficiencies may be related to changes in cytology associated with cancer development; analogs of folic acid, such as methotrexate, are potent chemotherapeutic agents and are widely used either alone or in combination therapy for many types of cancer. In this report we describe the synthesis of a tetra-deuterated folic acid from perdeuterated toluene. The primary intermediate, N-(4-amino [2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester was coupled to N(2')-acetyl-6-formylpterin to create [2',3',5',6'-$^2H_4$]folic acid. A similar scheme can be used for the preparation of [1',2',3',4',5',6'-$^{13}C_6$]folic acid from [$^{13}C_6$] ring labeled toluene.

Key Words: [2',3',5',6'-$^2H_4$]Folic acid, Isotope, Formylpterin, Aminobenzoylglutamate.

INTRODUCTION:

To facilitate studies of the dynamics of folate metabolism in humans, we developed a scheme (figure 1) for synthesis of [2',3',5',6'-$^2H_4$]pteroylglutamic acid ([2',3',5',6'-$^2H_4$]folic acid) from perdeuterated toluene-d_8 and 6-formylpterin. Toluene-d_8 is an inexpensive and readily available NMR solvent. This [2',3',5',6'-$^2H_4$]folic acid was designed to have 4 deuterons on the benzene ring to minimize proton-deuterium exchange during analysis and/or metabolism. A mass increase of 4 Daltons (Da) would minimize interferences from naturally occurring ^{13}C, ^2H, ^{15}N and ^{18}O during analysis by mass spectrometry (MS). A similar scheme can be used for the preparation of [1',2',3',4',5',6'-$^{13}C_6$]folic acid from [$^{13}C_6$] ring labeled toluene. The magnitude of a biological isotope effect of [2',3',5',6'-$^2H_4$]folic acid could be determined in vivo in humans from the kinetics of an administered mixture of [2',3',5',6'-$^2H_4$]folic acid and [1',2',3',4',5',6'-$^{13}C_6$]folic acid.

Preparation and use of [3',5'-$^2H_2$]folic acid was first described by Rosenberg and colleagues.[1,2] A decade later, Gregory and colleagues[3,4] confirmed and improved the method of preparation and presented an alternate protocol for analysis of the deuterium label

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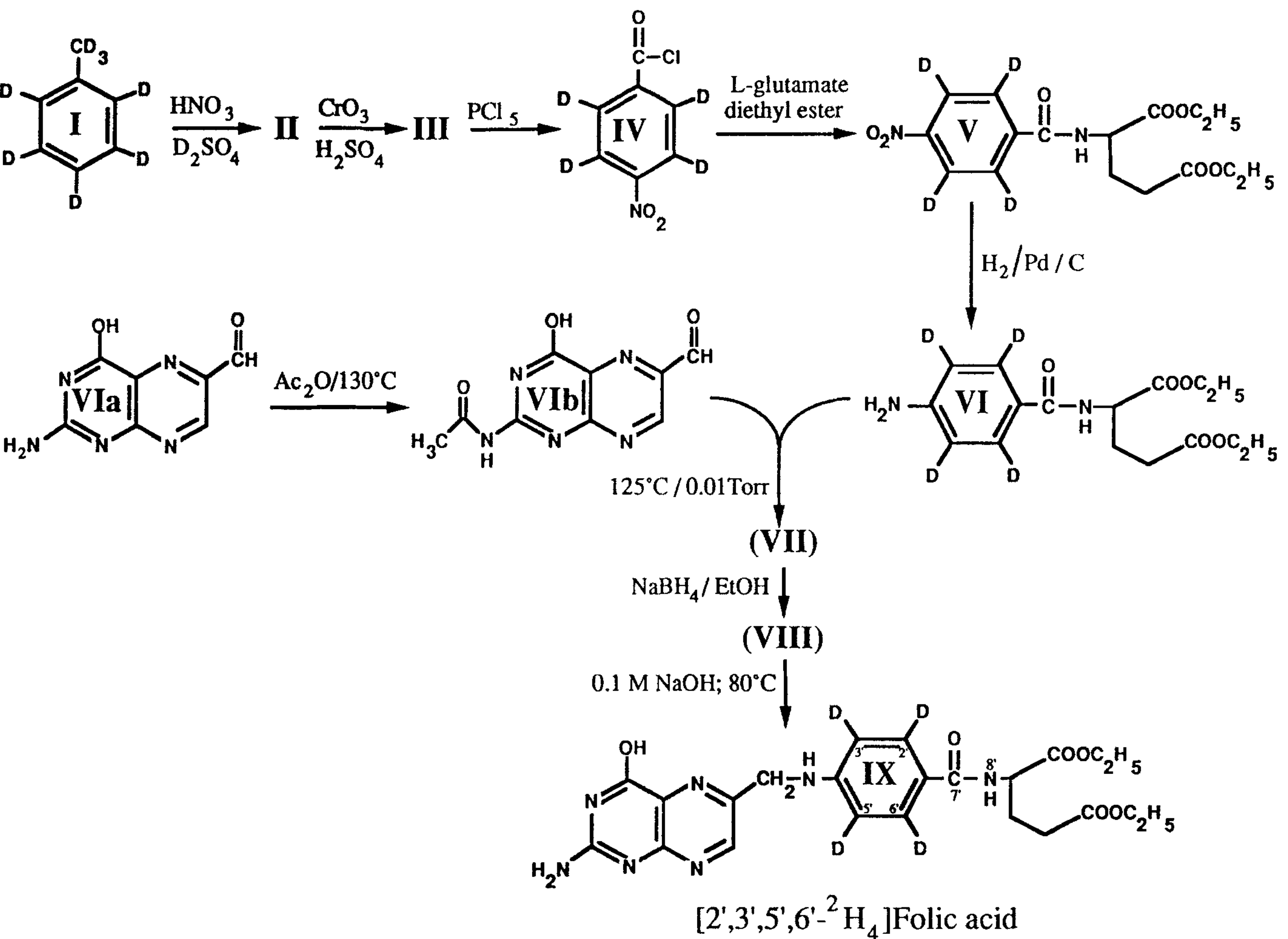

Figure 1. Scheme for synthesis of [2',3',5',6'-$^2H_4$]folic acid. The key intermediate, **VI**, was prepared from toluene-d_8 and coupled to **VIb**.

in biologic specimens. Other stable isotope forms of folic acid that have been made include [α,γ-$^2H_3$]folic acid[1], [7-$^2H_1$]folic acid[5], [6-$^2H_1$]formyltetrahydrofolic acid[6], [ß,γ-$^2H_4$]folic acid[7] and [7'-$^{13}C_1$] folic acid.[8]

Folic acids with only 1 or 2 deuteriums are difficult to analyze by MS at low levels of enrichment because of interferences from natural abundances of ^{13}C, ^2H, and ^{15}N in unlabeled folic acid. Also, folic acids labeled with deuterons in the α, ß, and γ positions of the glutamate moiety are likely to undergo substantial exchange during metabolism. Use of [2',3',5',6'-$^2H_4$]folic acid avoids these difficulties. Our scheme is presented in figure 1.

RESULTS AND DISCUSSION

The source of the deuterium was toluene-d_8 (I). Nitration of toluene-d_8 was performed as previously described[9] and resulted in a mixture of *o*- , *m*- , and *p*-nitrotoluenes-d_7 (II). This mixture of nitrotoluene isomers (without purification) was refluxed for 5 h with chromic acid because superior isolation of individual nitrobenzoic acid isomers (rather than nitrotoluene isomers) could be achieved. The reaction proceeded much slower than with protio species[10], exhibiting a primary kinetic isotope effect. Advantage was taken of the relatively high aqueous solubility of the *o*-, and *m*- isomers (6.85 mg/L and 3.13 mg/L, respectively) compared to the *p*- isomer (0.42 mg/L) to remove these impurities (*o*-, and *m*- isomers) from the final product (III) in a series of acid/base digests. Pure 4-nitro[2,3,5,6-$^2H_4$]benzoic acid (III) was recrystallized from acetic acid, m.p. was 239 - 240°C.

The acyl chloride (IV) was prepared using equal molar ratios of III and phosphorous pentachloride. The acyl chloride (IV) was coupled with L-glutamic acid ethyl ester in benzene and triethylamine to give N-(4-nitro[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester (V). Reduction of the nitro function over 10% Pd/C with hydrogen gas was performed in absolute EtOH to give N-(4-amino[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester (VI). Under these conditions some of the deuterium can exchange with the EtOH, therefore, the use EtOD rather than EtOH and deuterium gas rather than hydrogen is recommended.

The remaining steps of the synthesis follow closely a previous method[11] with few modifications. N-(4-amino[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester (VI) was condensed with N(2')-acetyl-6-formylpterin (VIb) as a solid mixture to give the Schiff base (VII), which was reduced with sodium borohydride to yield N(2')-acetyl-6-[N-(4-amino[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester]-pterin (VIII). The diethyl ester

was hydrolyzed in aqueous base to give the final product, [2',3',5',6'-$^2H_4$]folic acid (**IX**).

The chemical and isotopic purity of the [2',3',5',6'-$^2H_4$]folic acid was confirmed with HPLC (trace not shown), MS (figure 2), and ^1H NMR (figures 3 and 4). The HPLC trace of the [2',3',5',6'-$^2H_4$]folic acid showed a single symmetrical peak with a retention time of 8.97 min . The peak had a absorbance spectrum identical with that of folic acid.

The negative ion mode electrospray mass spectrum exhibited a peak (base) at m/z 444 corresponding to $[M\text{-}H]^-$ for [$^2H_4$]folic acid and at m/z 443 corresponding to $[M\text{-}H]^-$ for [$^2H_3$]folic acid (Figure 2). The peak at m/z 445 corresponds to the naturally abundant ^{13}C in folic acid. Isotopic purity was calculated from the heights of the m/z 444 and m/z 443 peaks. This [2',3',5',6'-$^2H_4$]folic acid was 83% [$^2H_4$] folic acid and 17% [$^2H_3$] folic acid.

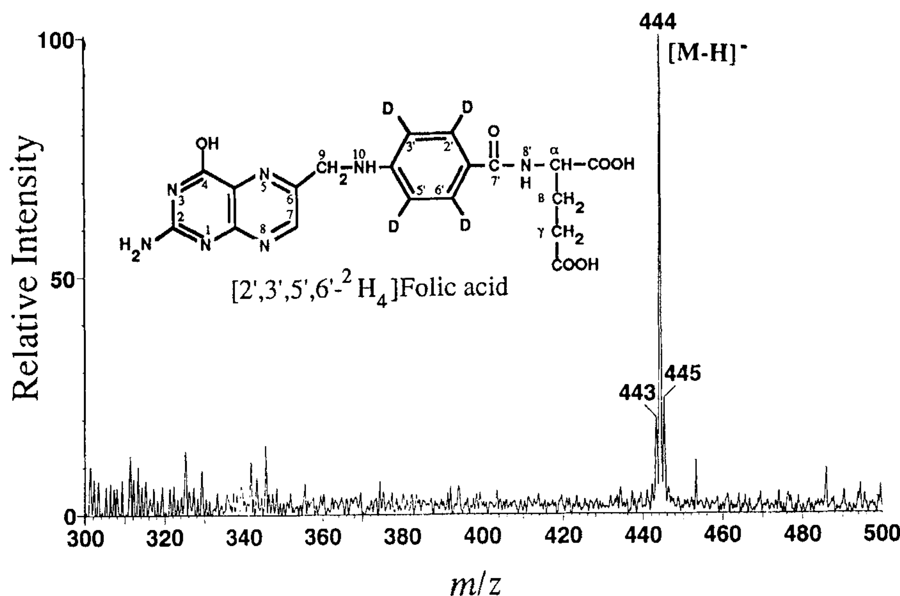

Figure 2. Negative ion mode electrospray ionization mass spectrum of [2',3',5',6'-$^2H_4$]folic acid.

Complete ^1H NMR spectra of folic acid and [2',3',5',6'-$^2H_4$]folic acid are shown in figure 3. Relevant assignments are indicated. All resonances (except those around 8 ppm) of [2',3',5',6'-$^2H_4$]folic acid are indistinguishable from those of unlabeled folic acid. Assignments are from previously published data.[11,12,13] Integrals were normalized to

resonances of the N_8H at 7.95 ppm (nonlabeled) and at 7.92 ppm (deuterated), each of these resonances was assigned a value of 1 proton. Intensities of the 2',6' and 3',5' proton resonances correspond to 0.12 and 0.05 protons, respectively, showing ~17% residual ^{1}H in the ring positions of this batch of [2',3',5',6'-$^2H_4$]folic acid. The 17% value is in close agreement with the MS data in figure 2.

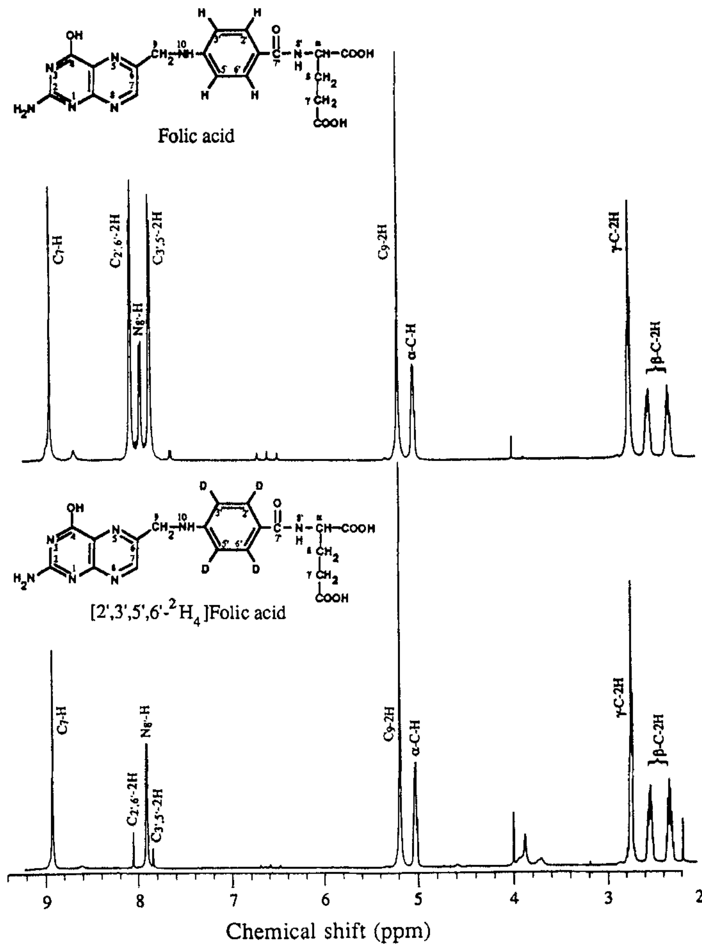

Figure 3. ^{1}H-NMR spectra of folic acid and of [2',3',5',6'-$^2H_4$]folic acid acquired at 25°C in TFA.

A detailed section (7.5 to 8.5 ppm region) of the ^{1}H NMR spectra of folic acid and [2',3',5',6'-$^2H_4$]folic acid are shown in figure 4. The 2',6' and 3',5' protons of non labeled

folic acid in TFA appear at 8.06 and 7.85 ppm, respectively,[11,12,13] flanking the resonance of the acid $N_{8'}$ proton at 7.95 ppm for (nonlabeled) and at 7.92 for labeled folic acid. The ring proton resonances appear as doublets due to mutual coupling. Each peak accordingly exhibits an area corresponding to twice that of the $N_{8'}H$ resonance. In the spectrum of the putative [2',3',5',6'-$^2H_4$]folic acid, resonances of residual ring protons appear as singlets, because each is coupled to a deuterium atom. ^2H-^1H coupling is much smaller than ^1H-^1H, and any splitting resulting from the coupling is removed by quadrupole relaxation of the ^2H. The $N_{8'}H$ resonance is shifted ~0.03 ppm upfield, presumably because of a greater steric interaction with the ring deuterons which have a slightly greater bond length than the ring protons.

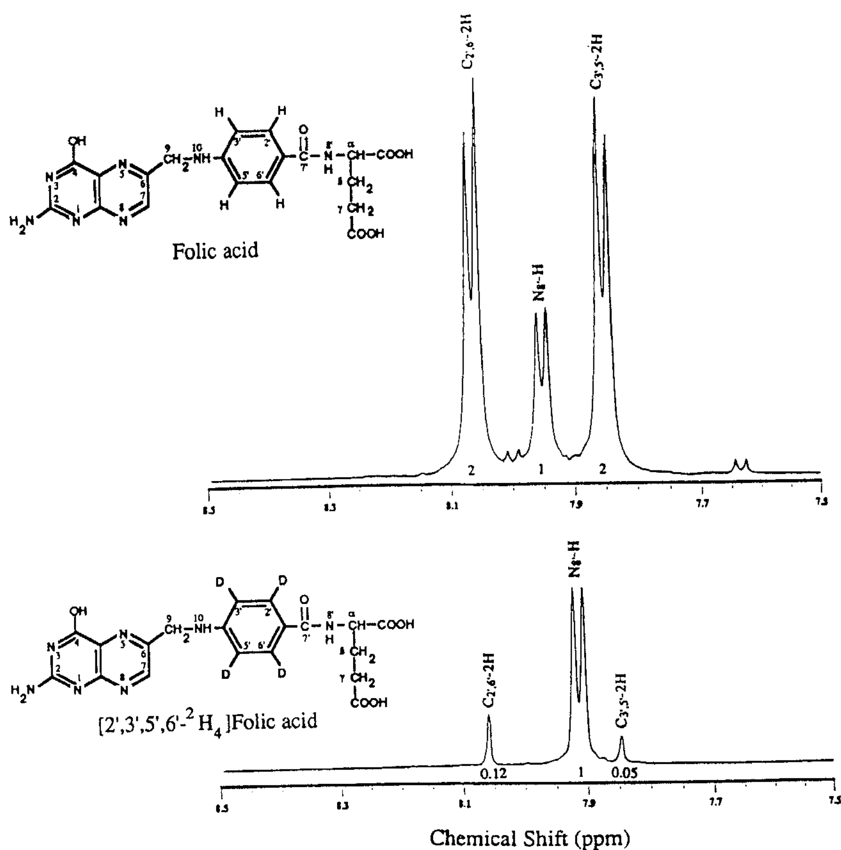

Figure 4. The 8 ppm region of the ^1H NMR spectra in figure 3 showing residual ring proton resonances.

The integrated intensities of the 2',6' and 3',5' proton resonances correspond to 0.12 and 0.05 protons, respectively, indicating ~17% residual ^1H in the ring positions in this [2',3',5',6'-$^2H_4$]folic acid (which used EtOH and hydrogen gas to make **IV**). Thus, the NMR spectrum is consistent both with the structure of [2',3',5',6'-$^2H_4$]folic acid and with the mass spectrum.

EXPERIMENTAL

4-Nitro[2,3,5,6-$^2H_4$]toluene (II). To an ice cold flask with 70% HNO_3 (45.7 g; 0.52 mol; d = 1.40 g/mL) was added [$^2H_2$]sulfuric acid (26.5 g; 0.26 mol, 96-98% w/w in D_2O; Cambridge Isotope Labs). The mixture was allowed to warm to room temperature (RT, ~23°C). While maintaining the flask temperature below 40°C, perdeuterated toluene (**I**) (0.2 mol; 20 g; 99+% isotopic purity; Cambridge Isotope Labs) was added dropwise over 1 hour. The reaction continued for an additional 1.5 h. Two volumes H_2O were added to the reaction mixture and the product was extracted with CH_2Cl_2 and dried over sodium sulfate. The extract was concentrated under reduced pressure to 26.7 g of an oil which was a mixture of the *o*- (~57%), *p*- (~39%) and *m*- (~4%) isomers of nitro[$^2H_7$]toluene. The product was used without purification.

4-Nitro[2,3,5,6-$^2H_4$]benzoic acid (III). To a flask with 190 mL H_2O is added 86.8 g sodium dichromate (0.29 mol) and 26 g (0.19 mol) of **II**. By means of a dropping funnel, 217 g H_2SO_4 (2.1 mol) was added over a 30 min period with constant stirring. After the spontaneous heating had ended, the solution was heated under reflux to a gentle boil for 5 h. After cooling to RT, the solution was poured into a beaker containing 300 mL H_2O. Crude nitrobenzoic acids precipitated and were collected on a filter. They were transferred to a flask with 75 mL of a 5% solution of H_2SO_4, stirred for 1 h at 50°C, and allowed to cool to RT. The crystals were again collected by filtration and treated with NaOH (5% solution) until the liquid remained alkaline. Decolorizing carbon was added and the solution warmed to 50°C for 10 min. The solids were removed by filtration, and the alkaline solution was poured into 100 mL H_2SO_4 (5% solution) with vigorous stirring. The product was collected by filtration, washed with cold water and dried overnight to open air. The product was crystallized from glacial acetic acid and the pale yellow needles collected and dried under high pressure for 24 h to give 4.1 g of **III** (24 mmol), m.p. 239-240°C.

4-Nitro[2,3,5,6-$^2H_4$]benzoyl chloride (IV). A mixture of 2.00 g of **III** (0.0117 mol) and 2.43 g PCl_5 (17 mmol) were slowly heated to 60°C. Vigorous gas evolution (HCl) began while the mixture liquefied. The reaction was over in 15 min. Upon completion of the reaction, 15 mL hot ligroin (anhydrous) was added and the solution filtered to remove insoluble compounds. The solution was allowed to crystallize on ice for 2 h and yellow crystals were collected and placed under strong vacuum for 4 h to give 2.43 g of **IV** (12.8 mmol) as yellow needles, m.p. 72-73°C.

N-(4-Nitro[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester (V). A flask containing 5 mL benzene (anhydrous), 0.50 g triethylamine, and 2.73 g (11.4 mmol) L-glutamic acid diethyl ester (hydrochloride salt from Aldrich Chemical Co., Milwaukee, WI) was shaken in an ice-water bath under argon while 1.00 g of **IV** (5.27 mmol) in 5 mL benzene was slowly added over a 20 min period using a dropping funnel. Upon addition of **IV** the solution was allowed to warm to RT (~15 min). The solids were removed by filtration and the excess glutamic acid was removed by partitioning against H_2O (2 x 10 mL). The benzene layer was concentrated to dryness under reduced pressure and placed under high vacuum for 4 h to yield 2.39 g (7.11 mmol) of a light yellow oil. This was used without further purification.

N-(4-Amino[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester (VI). To a two-necked flask outfitted with a septum and a gas inlet tube was added 300 mg Pd/C (10%, 50% wet weight) in 2O mL EtOH (absolute) and the flask was evacuated and filled with H_2 three times with vigorous stirring. To this was added 1 g of **V** (2.97 mmol) in 5 mL EtOH through the septum, and the system was attached to a hydrogen filled balloon and stirred for 1 h. The solution was concentrated to 15 mL (with precipitation of some white solid) and the solution heated to boiling to solubilized the precipitate. The mixture was allowed to cool at room temperature for 1 h then placed on ice for an additional hour. The light, white precipitate was collected by filtration and washed with an ice-cooled 50/50 ethanol/diethyl ether mixture. The product, **VI**, was dried under high vacuum for 4 h to yield 0.862 g (2.64 mmol) of **VI**, m.p. 138 - 140°C.

N(2')-Acetyl-6-formylpterin (VIb). Following the methods of Bieri and Viscontini,[11] 400 mg of **VIa** (2.09 mmol) was reacted with 540 mL acetic anhydride under nitrogen with stirring. The reaction was conducted at 130°C for 4 h, until the solution

cleared. After cooling to RT, the yellow solution was dried under vacuum and the solid residue was crystallized from boiling water following hot filtration with a small amount of decolorizing carbon. Pale yellow platelets crystallized after slow cooling and sitting at 5°C for 24 h. The product was washed with cold water and dried at 0.01 torr for 6 h. Yield was 404 mg (1.73 mmol), 83% of **VIb**.

N(2')-Acetyl-6-[N-(4-Amino[2,3,5,6-$^2H_4$]benzoyl)]-L-glutamic acid diethyl ester-azomethin]-pterin (VII). Following the methods of Bieri and Viscontini[11], 200 mg of **VIb** (0.856 mmol) and 500 mg of **VI** (1.531 mmol) are mixed intensively by pulverization and reacted at 120-125°C under vacuum (0.01 torr). The reaction was allowed to proceed for 15 min. After cooling to RT, the product was resuspended in hot acetonitrile with decolorizing carbon, hot filtered, and allowed to crystallize at -10°C for 24 h. The product was washed with cold acetonitrile and ether and dried under vacuum for 6 h. Yield was 262 mg (0.452 mmol) of **VII** (MW 579.5).

N(2')-Acetyl-6-[N-(4-Amino[2,3,5,6-$^2H_4$]benzoyl)-L-glutamic acid diethyl ester]- pterin (VIII). Eighty mL EtOH was saturated with argon. To this was added 12 mg of sodium borohydride (0.32 mmol) and then 200 mg of finely pulverized **VII** (0.345 mmol). The temperature was increased to 45°C over 10 min. This temperature was maintained at 45°C for an additional 10 min during which 6 mg of sodium borohydride was added in small portions. The pH was monitored and kept below 9 using 10% acetic acid. After this, the pH was brought to 6.0 and the reaction mix quickly cooled to RT and concentrated to dryness under vacuum. The product was brought up in boiling water and allowed to recrystallized overnight at 5°C. Yield was 118 mg of (0.203 mmol) of **VIII** as the dihydrate.

[2',3',5',6'-$^2H_4$]Folic acid (IX). Forty mL of 0.1 N sodium hydroxide is saturated with argon and to this was added 100 mg of **VIII**. The solution was heated to 80°C and the pH was lowered to 3.5 with glacial acetic acid. The solution was allowed to cool slowly to RT and then stored at 5°C for 24 h to obtain crystals of **IX**. The product was collected by filtration and washed with water and acetone and dried at 0.01 torr for 6 h. The yield was 81 mg (0.168 mmol) of [2',3',5',6'-$^2H_4$]folic acid dihydrate ($C_{23}H_{21}[^2H_4]N_7O_7$-$2H_2O$).

HPLC analysis. The [2',3',5',6'-$^2H_4$]folic acid was analyzed on a 250 x 10 mm; 5 micron particle size Ultracarb 5 ODS 30 reverse phase column (Phenomenex, Torrance,

CA) with an isocratic solvent system of 0.5% trifluoroacetic acid/acetonitrile 85/15, flow rate 3 mL/min. The HPLC was a Varian 50600-00 (Varian Associates, Palo Alto, CA). Absorbance was measured at 280 nm and 360 nm using a Lee 501 uv-visible detector (Lee Scientific, Salt Lake City, UT).

^{1}H NMR analysis. ^{1}H-NMR spectra were acquired at 25°C in TFA without a field-frequency lock, using a GE Ω 500 spectrometer, operating at 500 MHz. The resonance of the acidic proton of the solvent was suppressed by preirradiation for 3 s, followed by a 100 ms delay, a 70° observed pulse and a 0.82 s data acquisition time. The 8K data points were treated with a 0.5 Hz exponential multiplication and zero filled to 16k points before Fourier transformation. Chemical shifts are reported relative to external TMS.

MS analysis. MS characterization of [2',3',5',6'-$^2H_4$]folic acid was performed on VG Quattro-BQ mass spectrometer (VG Biotech, Altrincham, UK) using electrospray ionization in negative ion mode. Ionization was effected by application of -3.6 kV at the probe capillary and -500 V at the source counter electrode. The source was held at 70°C and the mass analyzer was tuned to ensure unit mass resolution. Spectra were acquired over the range of m/z 100-700 at 10 s/scan. Ten μL of a methanol solution of [2',3',5',6'-$^2H_4$]folic acid (225 pmol/μL) was injected using a Rheodyne injector, and a 10 μL/min flow of methanol/water (50/50 v/v) delivered the sample into the source.

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