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Biosynthesis of Tetrahydrofolate

STEREOCHEMISTRY OF DIHYDRONEOPTERIN ALDOLASE*

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Victoria Illarionova‡, Wolfgang Eisenreich§, Markus Fischer, Christoph Haußmann¶, Werner Römisch, Gerald Richter‖, and Adelbert Bacher

From the Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany

7,8-Dihydroneopterin aldolase catalyzes the formation of the tetrahydrofolate precursor, 6-hydroxymethyl-7,8-dihydropterin, and is a potential target for antimicrobial and anti-parasite chemotherapy. The last step of the enzyme-catalyzed reaction is believed to involve the protonation of an enol type intermediate. In order to study the stereochemical course of that reaction step, [1′,2′,3′,6,7-$^{13}C_5$]dihydroneopterin was treated with aldolase in deuterated buffer. The resulting, partially deuterated [6α,6,7-$^{13}C_3$]6-hydroxymethyl-7,8-dihydropterin was converted to partially deuterated 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate by a sequence of three enzyme-catalyzed reactions followed by treatment with [^{13}C]formaldehyde. The product was analyzed by multinuclear NMR spectroscopy. The data show that the carbinol group of enzymatically formed 6-hydroxymethyl-dihydropterin contained ^{2}H predominantly in the pro-*S* position.

Tetrahydrofolate and its derivatives are essential cofactors of one-carbon metabolism. Although plants and many microorganisms obtain folate coenzymes by *de novo* synthesis, vertebrates are absolutely dependent on nutritional sources (1). Insufficient supply of the vitamin is conducive to anemia in adults and to neural tube malformation in human embryos (2).

The biosynthesis of tetrahydrofolate has been studied in some detail (for review see Ref. 3). The first committed step catalyzed by GTP cyclohydrolase I converts GTP into dihydroneopterin triphosphate (**1**, Fig. 1). The triphosphate motif is removed by a previously unknown process, and the resulting 7,8-dihydro-D-neopterin (**2**) is converted to 6-hydroxymethyl-dihydropterin (**3**) by dihydroneopterin aldolase (FolB) (4). The consecutive action of FolK, FolP, FolC, and FolA enzymes finally affords 6-(*S*)-tetrahydrofolate via the intermediates **4–6**.

The folate biosynthetic pathway is a well established drug target for antimicrobial as well as antiparasite therapy. Sulfonamides, the first synthetic antimicrobial and antiparasitic drugs with a broad action spectrum, act via inhibition of dihydropteroate synthase, the penultimate enzyme of the dihydrofolate biosynthetic pathway (Fig. 1, *step E*) (5–7), and trimethoprim acts against a variety of bacterial pathogens via inhibition of dihydrofolate reductase (Fig. 1, *step G*) (8–10).

The rapid development of microbial resistance against all antibiotics in current use has generated an urgent requirement for novel anti-infective agents. Because the folate pathway is already a well established drug target, it appears worthwhile to explore other folate biosynthetic enzymes besides dihydropteroate synthase and dihydrofolate reductase. This paper describes studies on the mechanism of dihydroneopterin aldolase.

EXPERIMENTAL PROCEDURES

Materials—7,8-Dihydro-D-neopterin, 6-hydroxymethyl-7,8-dihydropterin hydrochloride, pteroic acid, and 7,8-dihydropteroic acid were purchased from Schircks Laboratories, Jona, Switzerland. [U-$^{13}C_6$]Glucose was obtained from Cambridge Isotope Laboratories, Miamisburg, OH. [1′,2′,3′,4′,5′-$^{13}C_5$]GTP was prepared as described (11). All other reagents used were of the highest purity available. A Nucleosil C18 HPLC[1] column (4 × 250 mm) was from Schambeck, Bad Honnef, Germany. Superdex 75, Superdex 200, Q-Sepharose Fast Flow, and DEAE-Sepharose Fast Flow were purchased from Amersham Biosciences. Nanosep 30K Omega Ultrafilter and Omega membrane 10K were purchased from Pall-Gelman (Dreieich, Germany).

Enzymes—Alkaline phosphatase was from Roche Molecular Biochemicals. Recombinant GTP cyclohydrolase I and dihydroneopterin aldolase of *Escherichia coli* were prepared as described (11, 12).

Construction of Expression Plasmids—The *folK* gene and *folP* genes of *Haemophilus influenzae* were amplified by PCR using the plasmids GHIHC06 and GHIDF84 (Table I) as templates. The oligonucleotides HKsense and HKanti (Table II) served as primers for amplification of *folK*, and the oligonucleotides HPsense and HPanti served as primers for amplification of *folP*. The resulting DNA fragments served again as templates for PCR with the primer kEcoRI for both reactions and the oligonucleotides HKanti and HPanti for amplification of *folK* and *folP*, respectively. After restriction with *Eco*RI and *Bam*HI, the amplificates were ligated into the vector pNCO113 affording the plasmids pHPPK and pDHPS comprising *folK* and *folP* gene, respectively. The expression plasmids were transformed into *E. coli* M15[pRep4]. Kanamycin (20 mg/liter) and ampicillin (50 mg/liter) were added for the maintenance of the plasmids in the host strain.

The *folA* gene of *E. coli* was amplified by PCR using chromosomal DNA as template and the oligonucleotides EC-FolA-*Nde*I-sense and EC-FolA-*BamHI*-anti (Table II) as primers. The amplificate was cleaved with *Nde*I and *Bam*HI and was then ligated into the expression vector pET-5a that had been digested with the same enzymes. The resulting expression plasmid designated pDHFR was transformed into *E. coli* BL21(DE3)[pLysS]. Chloramphenicol (20 mg/liter) and ampicillin (50 mg/liter) were added for the maintenance of the plasmids in the host strains.

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‡ On leave of absence from the Institute for Biophysics, Krasnoyarsk 660036, Russia.

§ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13043; Fax: 49-89-289-13363; E-mail: wolfgang.eisenreich@ch.tum.de.

¶ Present address: Bogenstr. 2, D-40724 Hilden, Germany.

‖ To whom correspondence may be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13336; Fax: 49-89-289-13363; E-mail: gerald.richter@ch.tum.de.

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

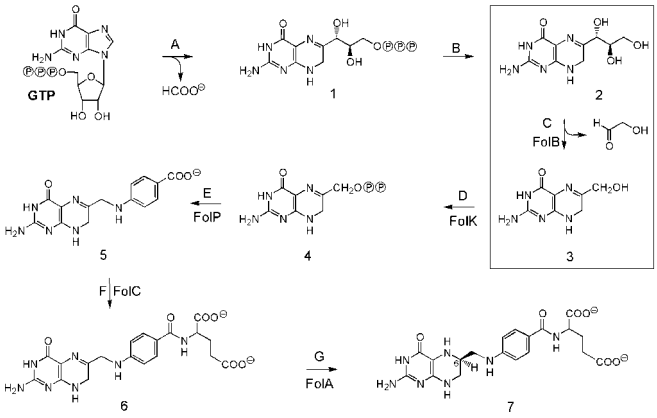

FIG. 1. **Biosynthesis of tetrahydrofolate (compound 7).** The reaction catalyzed by dihydroneopterin aldolase (FolB, reaction C) is shown in a *box*.

Preparation of Recombinant 6-Hydroxymethyl-7,8-dihydropterin Pyrophosphokinase—The recombinant *E. coli* strain M15[pRep4, pHPPK] directing the expression of the *folK* gene of *H. influenzae* was grown aerobically at 37 °C in LB medium containing kanamycin (20 mg/liter) and ampicillin (50 mg/liter) to an optical density of 0.7 (600 nm). IPTG was added to a concentration of 1 mM, and incubation was continued for a period of 4 h. Cells were harvested by centrifugation, washed with 0.9% sodium chloride, and stored at −20 °C.

Frozen cell mass (5 g) was suspended in 35 ml of 50 mM Tris hydrochloride, pH 8.0. The suspension was subjected to ultrasonic treatment and centrifuged. The supernatant was applied to a Q-Sepharose Fast Flow column (2 × 15 cm) that had been equilibrated with 20 mM Tris hydrochloride, pH 8.0. The column was washed with 150 ml of 20 mM Tris hydrochloride, pH 8.0, and developed with a linear gradient of 0–1 M sodium chloride containing 20 mM Tris hydrochloride, pH 8.0 (total volume, 300 ml; flow rate, 1 ml min^{-1}). 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase was eluted from 100 to 130 ml. Fractions were combined and concentrated by ultrafiltration (Omega membrane 10K). The solution was placed on a column of Superdex 75 (2.6 × 60 cm) which was then developed with 20 mM Tris hydrochloride, pH 8.0, containing 100 mM sodium chloride at a flow rate of 3 ml min^{-1}. Fractions were combined and concentrated by ultrafiltration. The concentration of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase was monitored photometrically using an absorbance coefficient of 12,090 M^{-1} cm^{-1} (280 nm). The specific activity was 395 mol mg^{-1} h^{-1}. The enzyme was stored at −70 °C in 60 mM potassium phosphate, pH 7.6.

Purification of Recombinant Dihydropteroate Synthase—E. coli strain M15[pRep4,pDHPS] directing the expression of the *folP* gene of *H. influenzae* was grown in LB medium containing 20 mg of kanamycin and 50 mg of ampicillin per liter. The cultures were incubated with shaking at 37 °C to an optical density of 0.7 (600 nm). IPTG was added to a concentration of 2 mM, and incubation was continued for a period of 3 h. Cells were harvested by centrifugation, washed with 0.9% sodium chloride, and stored at −20 °C.

Frozen cell mass (4 g) was thawed in 30 ml of 50 mM Tris hydrochloride, pH 8.0. The suspension was subjected to ultrasonic treatment and centrifuged. The supernatant was loaded on a Q-Sepharose Fast Flow column (2 × 15 cm) that had been equilibrated with 20 mM Tris hydrochloride, pH 8.0, containing 1 mM EDTA. The column was washed with 200 ml of 20 mM Tris hydrochloride, pH 8.0, containing 1 mM EDTA, and developed with a linear gradient of 0–1 M sodium chloride in 20 mM Tris hydrochloride, pH 8.0, containing 1 mM EDTA (flow rate, 1 ml min^{-1}; total volume, 400 ml). Dihydropteroate synthase was eluted from 120 to 140 ml. Fractions were combined and concentrated by ultrafiltration (Omega membrane 10K). The solution was placed on top of a Superdex 200 column (2.6 × 60 cm) that was developed with 60 mM potassium phosphate, pH 7.8, at a flow rate of 3 ml min^{-1}. The concentration of dihydropteroate synthase was monitored photometrically using an absorbance coefficient of 24,070 M^{-1} cm^{-1} (280 nm). The specific activity was 4.1 mmol mg^{-1} h^{-1}. The enzyme was stored at −70 °C in 60 mM potassium phosphate, pH 7.6.

Purification of Recombinant Dihydrofolate Reductase—The recombinant *E. coli* strain BL21(DE3)[pLysS,pDHFR] directing the expression of the *folA* gene of *E. coli* was grown in LB medium containing chloramphenicol (20 mg/liter) and ampicillin (50 mg/liter) at 37 °C to an optical density of 0.8 (600 nm). IPTG was added to a concentration of 1 mM, and incubation was continued for a period of 4 h. Cells were harvested by centrifugation, washed with 0.9% sodium chloride, and stored at −20 °C.

Frozen cell mass (5.5 g) was suspended in 40 ml of 20 mM potassium phosphate, pH 7.0, containing 100 mM potassium chloride and 1 mM EDTA. The suspension was subjected to ultrasonic treatment and centrifuged. The supernatant was applied to a DEAE-Sepharose FF column (2 × 15 cm) that had been equilibrated with 20 mM potassium phosphate, pH 7.0, containing 100 mM potassium chloride and 1 mM EDTA. The column was developed with a linear gradient of 0–0.5 M potassium chloride (flow rate, 1 ml min^{-1}; total volume, 400 ml). Dihydrofolate reductase was eluted from 240 to 290 ml. The concentration of dihydrofolate reductase was monitored photometrically using an absorbance coefficient of 31,100 M^{-1} cm^{-1} (280 nm) (17). The specific activity was 17 mol mg^{-1} h^{-1}. The enzyme was stored at −70 °C in 60 mM potassium phosphate, pH 7.6.

Assay of 6-Hydroxymethyl-7,8-dihydropterin Pyrophosphokinase—Assay mixtures containing 50 mM Tris hydrochloride, pH 8.0, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 10 mM ATP, 100 μM 6-hydroxymethyl-7,8-dihydropterin hydrochloride, and protein in a total volume of 100 μl were incubated at 37 °C (12, 18). The reaction was terminated by the addition of 50 μl of 1 M hydrochloric acid containing 1% (w/v) iodine and 2% (w/v) potassium iodide. 6-Hydroxymethyl-7,8-dihydropterin diphosphate was determined by reversed phase HPLC using a column of Nucleosil C18. The eluent contained 7% methanol and 30 mM formic acid. The flow rate was 1 ml min^{-1}. The effluent was monitored fluorimetrically (excitation, 350 nm; emission, 450 nm). The retention time of 6-hydroxymethyl-7,8-dihydropterin diphosphate was 3.2 min.

Assay of Dihydropteroate Synthase Activity—Assay mixtures containing 60 mM potassium phosphate, pH 7.6, 5 mM $MgCl_2$, 10 mM ATP, 100 μM 6-hydroxymethyl-7,8-dihydropterin hydrochloride, 10 mM 4-aminobenzoate, 10 μM 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, and protein in a total volume of 0.3 ml were incubated at 37 °C. Aliquots (100 μl) were analyzed by HPLC using a reversed phase column of Nucleosil C18 (4 × 250 mm) and an eluent containing 5 mM tetra-*n*-butylammonium phosphate, pH 6.8, 25 mM sodium chloride, 0.5 mM dithioerythritol, and 10% acetonitrile. The flow rate was 1 ml min^{-1}. The effluent was monitored spectrophotometrically using a TIDAS diode array photometer (J & M Analytische Mess und Regeltechnik GmbH, Aalen, Germany). The retention volume of 7,8-dihydropteroic acid was 9 ml.

Assay of Dihydrofolate Reductase Activity—Assay mixtures containing 60 mM potassium phosphate, pH 7.8, 5 mM mercaptoethanol, 0.25 mM NADPH, 0.12 mM 7,8-dihydropteroate, and protein (19) were incubated at 37 °C under an atmosphere of nitrogen. The conversion of di-hydropteroate to tetrahydropteroate was analyzed by HPLC.

Ion Pair HPLC Analysis of GTP, 7,8-Dihydroneopterin, and 7,8-Dihydroneopterin Triphosphate—Experiments were performed using a reversed phase column of Nucleosil C18 (4 × 250 mm) that was developed with a mixture of isopropyl alcohol, triethylamine, 85% phosphoric acid, water (3:10:3:984, v/v) (20). The flow rate was 1.5 ml min^{-1}. The effluent was monitored photometrically using a TIDAS diode array photometer (retention times: GTP, 14 min; 7,8-dihydroneopterin, 3.5 min; and 7,8-dihydroneopterin triphosphate, 8.9 min).

Ion Pair HPLC Analysis of Pteroate Derivatives—Experiments were performed using a column of Nucleosil C18 (4 × 250 mm) and an eluent containing 5 mM tetra-*n*-butylammonium phosphate, pH 6.8, 25 mM sodium chloride, 0.5 mM dithioerythritol, and 10% acetonitrile (21). The flow rate was 1 ml min^{-1}. The effluent was monitored spectrophotometrically using a TIDAS diode array photometer (retention volumes: 7,8-dihydropteroic acid, 9 ml; tetrahydropteroic acid, 6.7 ml; and 5,10-methylenetetrahydropteroic acid, 11.5 ml).

Synthesis of [11-$^{13}C_1$]6-(RS)-5,10-Methylenetetrahydropteroate—To a reaction mixture containing 60 mM potassium phosphate, pH 7.6, and 1 mM dihydropteroic acid in a total volume of 1 ml, sodium borohydride was added to a final concentration of 10 mM under a stream of nitrogen (22). After 20 min, the excess of sodium borohydride was destroyed by the addition of 20 μl of 17 M acetic acid, and the solution was adjusted to pH 7.1 with 10 N NaOH. Mercaptoethanol was added to a final concentration of 2 mM, and [^{13}C]formaldehyde was added to a concentration of 3 mM. The formation of 6-(*R,S*)-[11-$^{13}C_1$]5,10-methylenetetrahydropteroate was monitored by HPLC and by NMR spectrometry.

Preparation of [1′,2′,3′,6,7-$^{13}C_5$]7,8-Dihydroneopterin—All enzymes used were dialyzed against 60 mM potassium phosphate, pH 7.6. For experiments to be performed in D_2O, enzyme solutions were lyophilized and subsequently dissolved in D_2O. Reactions were performed in a sealed vial under an atmosphere of nitrogen. Reagents were injected through a rubber septum.

TABLE I
Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Ref.
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lac1^q Δ(lacZ)M15 Tn109*]	13
M15[pREP4]	*lac ara gal mtl recA$^+$ uvr$^+$* [pREP4, lacI, kana^r]	14
BL21(DE3)[pLysS]	F$^-$*ompT gal dcm hsdS$_B$ ($r_B^- m_B^-$)* (DE3) [pLysS,Cm^R]	Novagen
Plasmids		
pNCO113	High copy number plasmid vector	15, 16
pET-5a	High copy number plasmid vector	Novagen
GHIHC06	pUC18 plasmid containing the *folK* gene of *H. influenzae*	ATCC
GHIDF84	pUC18 plasmid containing the *folP* gene of *H. influenzae*	ATCC
pHPPK	pNCO113 plasmid containing the *folK* gene of *H. influenzae*	This study
pDHPS	pNCO113 plasmid containing the *folP* gene of *H. influenzae*	This study
pDHFR	pET-5a plasmid containing the *folA* gene of *E. coli*	This study

TABLE II
Oligonucleotides used for PCR amplification
Restriction sites are shown in boldface type.

EC-FolA-*Nde*I-sense	5′-ataataata**catatg**atcagtctgattgcggc-3′
EC-FolA-*Bam*HI-anti	5′-tattat**ggatcc**ttaccgccgctccagaatctc-3′
HKsense	5′-ggagaaattaaccatgattacggcatatattgc-3′
Hkanti-*Bam*HI	5′-cat**ggatcc**ctagggatttaattttatcattttc-3′
HPsense	5′-ggagaaattaaccatgaaactttacgcaaataataaatg-3′
HPanti-*Bam*HI	5′-tcgttat**ggatcc**atcaggcatttgc-3′
k*Eco*RI	5′-acaca**gaattc**attaaagaggagaaattaaccatg-3′

A mixture containing 60 mM potassium phosphate, pH 7.6, 10 mM [1′,2′,3′,4′,5′-$^{13}C_5$]GTP, and 20 mg of GTP cyclohydrolase I of *E. coli* in a total volume of 1 ml was incubated at 37 °C for 5 h. A solution (161 μl) made up from 100 μl of 60 mM potassium phosphate, pH 7.6, 10 μl of 100 mM magnesium chloride, 1 μl of 100 mM zinc chloride, and 50 μl of alkaline phosphatase solution (50 units) was added, and incubation at 37 °C was continued for 2 h. The solution was passed through an ultrafilter (Nanosep 30K Omega) by centrifugation at 20,000 × *g*. The ultrafiltrate was collected under a stream of nitrogen.

Preparation of [6,7,9,11-$^{13}C_4$]6-(R)-5,10-Methylenetetrahydropteroate—To a solution of [1′,2′,3′,6,7-$^{13}C_5$]7,8-dihydroneopterin (800 μl), a mixture made up of 40 μl of 250 mM ATP, 50 μl of 100 mM magnesium chloride, 100 μl of 100 mM 4-aminobenzoate, 4 μl of 1.4 M mercaptoethanol, 40 μl of 0.25 mM dihydroneopterin aldolase, 20 μl of 1 mM 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, and 16 μl of 0.15 mM dihydropteroate synthase was added, and the resulting mixture was incubated for 4 h at 37 °C. A solution (152 μl) made up of 10 mg of NADPH, 2 μl of 1.4 M mercaptoethanol, and 150 μl of 0.25 mM dihydrofolate reductase was added, and the mixture was incubated for 1 h at 37 °C. [^{13}C]Formaldehyde was added at a molar ratio of 2:1 based on the amount of tetrahydropteroic acid present in the reaction mixture (23). The mixture was kept at room temperature and was then analyzed by NMR spectroscopy without further treatment.

NMR Spectroscopy—NMR measurements were performed at 27 °C using a Bruker DRX 500 spectrometer operating at 500.13 and 125.7 MHz for ^{1}H and ^{13}C NMR experiments, respectively. The spectrometer was equipped with a lock-switch unit for ^{2}H-decoupling experiments using the lock channel.

One-dimensional ^{1}H and ^{13}C NMR experiments and two-dimensional COSY, NOESY, ROESY, HMQC, and HMQC-TOCSY experiments were performed with standard Bruker software (XWIN-NMR 3.0). ^{13}C spectra were obtained using a dual ^{13}C/^{1}H probe head, and proton detection experiments were performed using a ^{1}H/^{13}C/^{15}N inverse triple resonance probe head. The mixing time was 500 ms for NOESY, 300 ms for ROESY, and 60 ms for TOCSY transfer.

RESULTS

Dihydroneopterine aldolase had been proposed to cleave 7,8-dihydro-D-neopterin (**2**, Fig. 2) by a retroaldol-type mechanism, although no experimental evidence has been reported (24). The hypothetical reaction sequence requires the protonation of carbon 6α of the postulated intermediate (**8**). The proton for that reaction step could be supplied by an acidic group of the protein, by the solvent, or by internal return of a proton abstracted from the substrate in an earlier reaction step. In order to determine the stereochemical features of the protonation, we decided to study the incorporation of deuterium from solvent D_2O into the heterocyclic reaction product by multinuclear NMR spectroscopy.

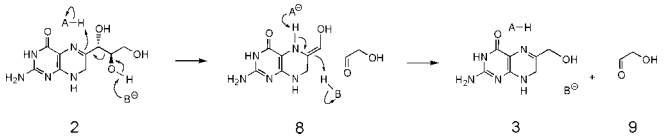

FIG. 2. **Hypothetical reaction mechanism of dihydroneopterin aldolase.**

For the purpose of NMR analysis, we designed a reaction sequence in order to convert the enzyme product, 6-hydroxymethyl-7,8-dihydropterin (**3**), into 5,10-methylenetetrahydropteroate (**11,** Fig. 3). In that tricyclic compound, carbon atom 9 which is equivalent to the 6α carbinol group of 6-hydroxymethyl-7,8-dihydropterin (**3**) is embedded into a five-membered, relatively rigid ring system, which was expected to enable the stereospecific assignment of the diastereotopic protons of the position 7 and 9 methylene groups of **11** by NMR spectroscopy using carbon 6 as a chiral reference center.

The reaction steps designated *A*, *C*, *D*, and *E* in Fig. 1 are catalyzed by enzymes of the tetrahydrofolate pathway and all were documented in the literature (4, 6, 25–30). The details of removal of the triphosphate motif under physiological conditions in the biosynthetic pathway (Fig. 1, *reaction B*) are still not known with certainty; however, alkaline phosphatase could be used for the dephosphorylation of **1**.

The enzymatic reduction of dihydropteroate (**5**) affording 6-(*S*)-tetra-hydropteroate (6-*S*-**10**) (Fig. 3) has not been described to the best of our knowledge, but we could show that the non-physiological reaction can be catalyzed at an appreciable rate by dihydrofolate reductase of *E. coli*. The conversion of 6-(*S*)-tetrahydropteroate into 6-(*R*)-5,10-methylenetetrahydropteroate (6-*R*-**11**) could be performed by treatment with formaldehyde following a suggestion of R. Matthews.[2]

It should be noted that the experimental strategy required the recombinant expression of the *folK, folP, and folA* genes in order to prepare 6-hydroxymethyldihydropterin pyrophosphokinase, dihydropteroate synthase, and dihydrofolate reductase as described under "Experimental Procedures."

In order to check the feasibility of the planned NMR analysis, we prepared racemic 6-(*R,S*)-5,10-methylenetetrahydropteroate (**11**) by sodium borohydride reduction of a commercial sample of 7,8-dihydropteroate (**5**) followed by condensation with formaldehyde (Fig. 3). In order to improve the sensitivity and selectivity of product analysis, we used ^{13}C-labeled formaldehyde in the condensation reaction. ^{1}H and ^{13}C NMR sig-

[2] R. Matthews, personal communication.

nals of the resulting 6-(*R,S*)-[11-$^{13}C_1$]5,10-methylenetetrahydropteroate were assigned by two-dimensional COSY, NOESY, ROESY, and HMQC spectroscopy (Tables III and IV).

The ^{1}H NMR signals for the diastereotopic protons at C-11, C-9, and C-7 of 6-(*R,S*)-[11-^{13}C]5,10-methylenetetrahydropteroate were found to be well separated from each other (Table III and Fig. 4). More specifically, the resonances for H-11 protons and the C-11 carbon atom in 6-(*R,S*)-[11-^{13}C]5,10-methylenetetrahydropteroate were assigned by their enhanced intensities in two-dimensional ^{1}H^{13}C HMQC experiments. The expected ^{1}H^{13}C couplings of the H-11 protons with the attached ^{13}C-11 were also observed in the one-dimensional ^{1}H NMR spectra of 6-(*R,S*)-[11-^{13}C]5,10-methylenetetrahydropteroate (*cf.* Fig. 5*A*). The doublet of triplets centered at 3.20 ppm showed coupling to the NH-8 proton in the two-dimensional COSY experiment. Moreover, the coupling signature of the well resolved signal in the one-dimensional ^{1}H NMR experiment (Table III and Fig. 4) indicated coupling to NH-8 (coupling constant, 4 Hz). Consequently, the signal at 3.20 ppm was assigned as one of the H-7 protons (H-7e). The signal for the second H-atom (H-7d) attached to C-7 was assigned by HMQC spectroscopy where the signals for H-7e and H-7d were both correlated to the same ^{13}C NMR signal at 38.9 ppm. The signals for H-9 methylene protons could then be assigned via the ^{1}H^{1}H correlation pattern gleaned from the COSY experiment as well as from ^{1}H^{13}C correlations in the HMQC experiment (Table III). Stereo-specific assignments were obtained from ROESY and NOESY experiments (Fig. 5 and Table IV). Thus, negative cross-peaks between resonances for H-9b, H-6, and H-7e (Fig. 5*C*), respectively H-9a and H-7d (Fig. 5*B*) indicated *syn* orientation of H-6, H-9b, and H-7e.

The compounds **10** and **11** are oxygen sensitive; partial degradation of 5,10-methylenetetrahydropteroate (**11**) was observed after a few hours even at 5 °C. We therefore decided to perform the reaction steps in Fig. 3 as a one-pot reaction with direct NMR analysis. As a substitute for product purification, we made use of ^{13}C labeling (Fig. 6) in order to enhance the sensitivity and selectivity of the NMR analysis. As described below, this approach enabled the acquisition of ^{13}C-filtered ^{1}H NMR data, thus virtually eliminating the ^{1}H signals of unlabeled reagents.

The [1′,2′,3′,6,7-$^{13}C_5$]7,8-dihydro-D-neopterin (**2**) designed to serve as aldolase substrate was prepared by a complex sequence of enzyme-assisted reactions. Briefly, [1′,2′,3′,4′,5′-$^{13}C_5$]GTP was prepared by enzymatic multistep transformation of [U-$^{13}C_6$]glucose (**12**) as described earlier (11) and was then converted to [1′,2′,3′,6,7-$^{13}C_5$]7,8-dihydroneopterin triphosphate by treatment with GTP cyclohydrolase I (11). The triphosphate was dephosphorylated by treatment with alkaline phosphatase from calf intestine. Enzymes were removed from the reaction mixture by ultrafiltration, and the resulting solution of [1′,2′,3′,6,7-$^{13}C_5$]7,8-dihydroneopterin (**2**) was treated with recombinant dihydroneopterin aldolase from *E. coli* to afford [6α,6,7-$^{13}C_3$]6-hydroxymethyldihydropterin (**3**) which was converted to [6,7,9-$^{13}C_3$]7,8-dihydropteroate by treatment

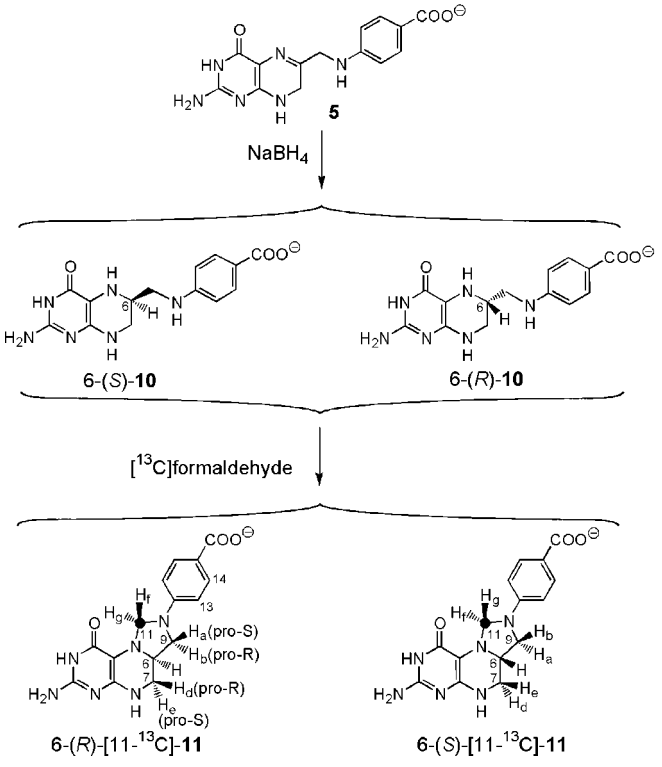

FIG. 3. **Preparation of [11-$^{13}C_1$]5,10-methylenetetrahydropteroate.** ^{13}C labeling in position 11 is indicated by *filled circles*. The enzymatic reduction of dihydropteroate (**5**) affords 6-(*S*)-tetrahydropteroate (6-*S*-**10**).

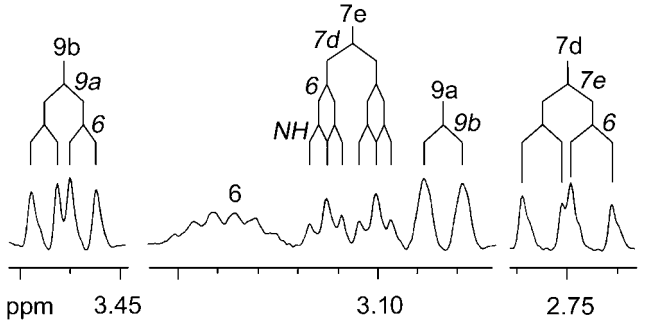

FIG. 4. **Partial ^{1}H NMR spectrum of 6-(*RS*)-[11-$^{13}C_1$]5,10-methylenetetrahydropteroate.** Coupling patterns are indicated.

TABLE III
NMR data of 6-(RS)-[11-^{13}C]5,10-methylenetetrahydropteroate

Position[a]	Chemical shifts, ppm		Coupling constants, Hz, J_{HH}	Coupling patterns			
	δ ^{1}H	δ ^{13}C		HMQC	HMQC-TOCSY	COSY	NOESY/ROESY[b]
9a	3.16 (d)	48.7	9.6 (9)	9a, 9b	9, 6, 7 (w)	9b	13, 9b, 7d
9b	3.56 (dd)		9.4 (9), 6.6 (6)			9a, 6	13, 9a, 7e, 6
6	3.26 (m)	52.8		6	6, 9, 7, NH (w)	9a, 7d	9b
7d	2.84 (dd)	38.9	12.3 (7), 10.3 (6)	7d, 7e	7, NH, 6, 9 (w)	7e, 6	7e, 9a
7e	3.20 (dt)		12.3 (7), 4 (6), 4 (NH)			7d, NH(8)	7d, NH(8) (w), 9b
NH(8)	6.29 (d)		4 (7)				7e (w)
11f	3.67 (d)	67.8	4.8	11f, 11g	nd	11g	13, 7d
11g	4.71 (d)		4.8			11f	13
13	6.40 (d)	111.4	8.5 (14)	13	nd	14	11g, 11f, 9a, 9b
14	7.58 (d)	131.4	8.5 (13)	14	nd	13	

[a] For atom numbers, see Fig. 3.
[b] For a quantitative analysis, see Table IV.

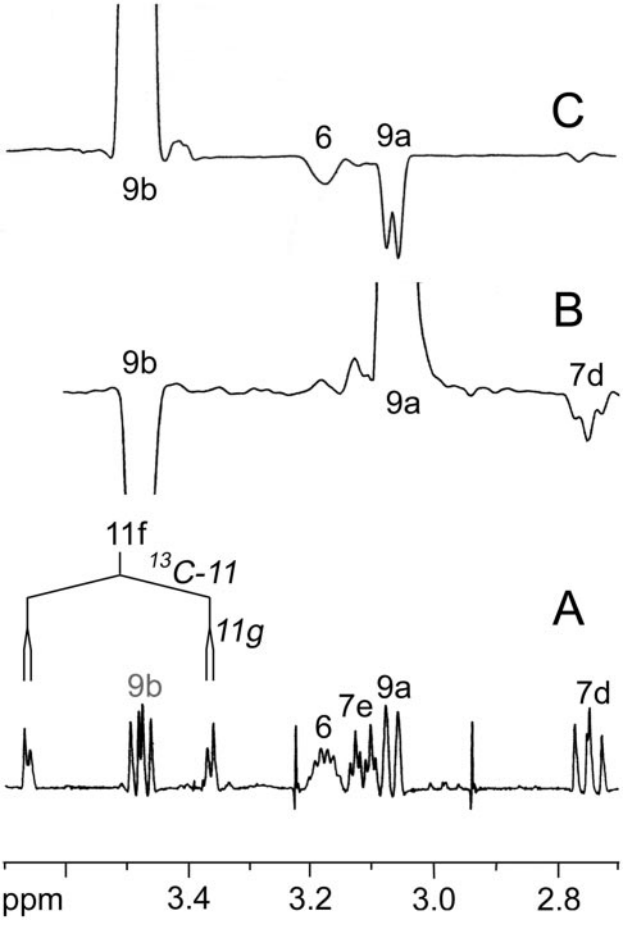

FIG. 5. **Nuclear Overhauser analysis of 6-(*RS*)-[11-$^{13}C_1$]5,10-methylenetetrahydropteroate.** *A*, section of the one-dimensional ^{1}H NMR spectrum. Coupling patterns are indicated. *B*, trace from a two-dimensional NOESY spectrum with the signal for H-9a (truncated positive signal) in the diagonal of the two-dimensional matrix; *C*, trace from a two-dimensional ROESY spectrum with the signal for H-9b (truncated positive signal) in the diagonal of the two-dimensional matrix.

TABLE IV
Nuclear Overhauser spectroscopy of 6-(RS)-[11-^{13}C]5,10-methylenetetrahydropteroate

Position	NOE intensity[a]	ROE intensity[a]
9a	85 (9b), 9.4 (7d)	70 (9b), 15 (7d)
9b	85 (9a)	70 (9a), 18 (7e), 30 (6)
6		30 (9b)
7d	100 (7e), 9.4 (9a)	100 (7e), 15 (9a)
7e	100 (7d)	100 (7d), 18 (9b)
11f	5.5 (7d)	

[a] Arbitrary units relative to a intensity of 100 for cross-signals between the geminal protons at C-7.

with 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and dihydropteroate synthase. The product was then converted to 6-(*S*)-[6,7,9-$^{13}C_3$]tetrahydropteroate by catalytic action of dihydrofolate reductase. Treatment of the reaction mixture with [^{13}C]formaldehyde afforded 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate (**11**). These procedures could be all performed as a one-pot reaction, and the reaction mixture was immediately analyzed by NMR spectroscopy. As described under "Experimental Procedures," all solutions were made up in D_2O when required.

The ^{13}C NMR spectrum of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate (**11**) showed intense ^{13}C-coupled signals at 38.9 ppm (doublet, 35 Hz, C-7), 48.7 ppm (doublet, 36 Hz, C-9), and 52.8 ppm (triplet, 36 Hz, C-6) (see projection in Fig. 7*A*). The ^{13}C NMR triplet at 52.8 ppm signaled two adjacent ^{13}C atoms, thus confirming its assignment as C-6 (Table III). The protons connected to the ^{13}C-enriched carbon atom resonating at 48.7 ppm showed NOESY cross-peaks with the aromatic protons of the phenyl ring; hence, the carbon resonating at 48.7 ppm is C-9. The ^{13}C-enriched carbon atom resonating at 67.8 ppm does not show ^{13}C^{13}C coupling and is therefore assigned as the solitary C-11. Hence, the ^{13}C-enriched C-atom resonating at 38.9 ppm can be assigned as C-7 confirming the assignments obtained with racemic [11-$^{13}C_1$]5,10-methylenetetrahydropteroate (Table III).

6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-Methylenetetrahydropteroate obtained in D_2O solution was characterized by broadened ^{13}C signals for C-7 and C-9 signaling incorporation of deuterium (see projection in Fig. 7*B*).

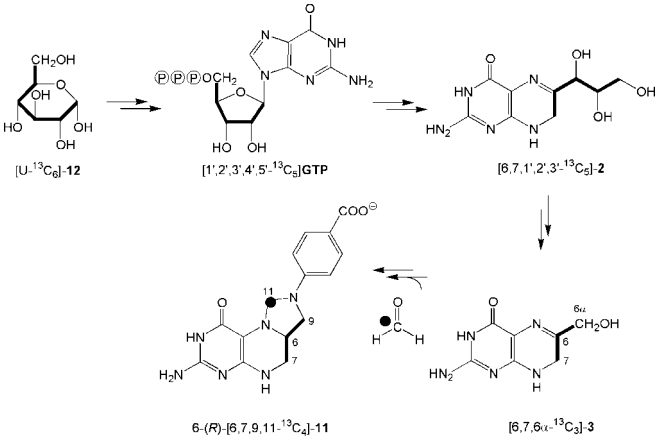

FIG. 6. **Preparation of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate starting from [U-$^{13}C_6$]glucose.** *Bold bonds* connect ^{13}C-labeled atoms. ^{13}C labeling arising from ^{13}C-labeled formaldehyde is indicated by *filled circles*.

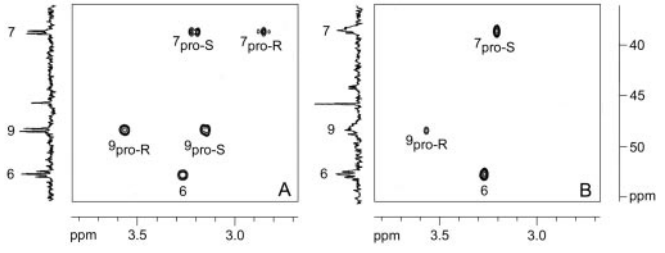

FIG. 7. **Two-dimensional HMQC spectra of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate.** *A*, prepared in buffer containing ^1H_2O; *B*, prepared in buffer containing ^2H_2O. The corresponding one-dimensional ^{13}C NMR spectra are shown as projections.

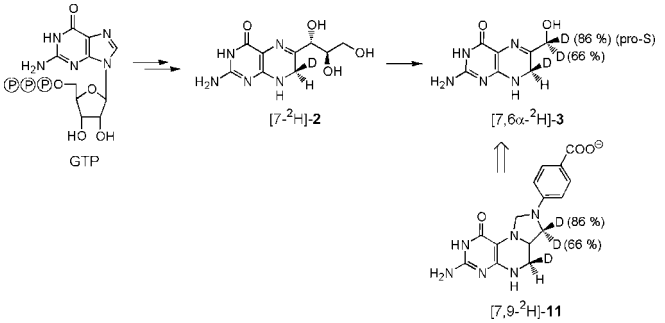

FIG. 8. **Incorporation of ^{2}H from ^2H_2O into 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate.** The labeling pattern of **3** was reconstructed from the observed pattern in **11**.

TABLE V
HMQC spectroscopy of 6-(R)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate

Position	^{1}H signal intensity[a]			^{2}H incorporation[b]
	δ ^{1}H	H_2O buffer	D_2O buffer	
				%
$9_{pro\text{-}S}$	3.16	1.18	0.17	86
$9_{pro\text{-}R}$	3.56	1.06	0.36	66
6	3.26	1.00	1.00	0
$7_{pro\text{-}R}$	2.84	0.90	0.21	77
$7_{pro\text{-}S}$	3.20	0.94	0.95	0

[a] Intensities of $^1H^{13}C$ correlation signals from HMQC spectra in arbitrary units relative to signal intensities of 1.00 for H-6.
[b] Deuterium content of product obtained in deuterium-enriched solvent as determined from the $^1H^{13}C$ HMQC signals.

It is well established that dihydrofolate reductase affords *S*-configuration at the chiral center of C-6 in tetrahydrofolate (Fig. 1) (31). Under the assumption (which is addressed in more detail under "Discussion") that the reaction of dihydrofolate reductase with dihydropteroate follows the same stereochemical course, 6-(*S*)-tetrahydropteroate (6-*S*-**10**, Fig. 3) is the expected reaction product. Reaction of 6-(*S*)-tetrahydropteroate with formaldehyde leads to 6-(*R*)-5,10-methylenetetrahydropteroate (6-*R*-**11**) (the configuration at C-6 is not affected by the reaction with formaldehyde; the introduction of C-11 does, however, change the stereodescriptor from *S* to *R*).

The ^{1}H NMR signals for the methylene protons at C-7, C-9, and C-11 of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate can all be assigned using C-6 as a chiral reference center. Notably, the methylene protons at C-7 and C-9 with *syn* orientation to H-6 (*i.e.* H-7e and H-9b, respectively; see assignments for the enantiomeric 6-(*R,S*)-mixture) can then be assigned as H-$7_{pro\text{-}S}$ and H-$9_{pro\text{-}R}$, respectively (Fig. 3).

Two-dimensional HMQC spectra of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate obtained in water resp. in D_2O are shown in Fig. 7. In the experiment with deuterium-enriched buffer, the intensities of the signals assigned as H-$9_{pro\text{-}S}$ and H-$9_{pro\text{-}R}$ are reduced to relative values of 14 and 34% as compared with product formed in experiments without deuterium enrichment (Table V). Thus, H-$9_{pro\text{-}R}$ contained 66% deuterium, and H-$9_{pro\text{-}S}$ contained 86% deuterium. These data are discussed in more detail below. In line with earlier work (11), 77% deuterium introduced by the action of GTP cyclohydrolase I was found at H-$7_{pro\text{-}R}$; no significant amount of deuterium was detected in the H-$7_{pro\text{-}S}$ position.

DISCUSSION

In an earlier study (11), we showed that the action of GTP cyclohydrolase in D_2O afforded [7-$^2H_1$]dihydroneopterin triphosphate with ^{2}H located at the 7-pro-*R* position (Fig. 8). In that study, the stereochemistry of deuterium incorporation was determined by transformation of the product into tetrahydrobiopterin by the catalytic action of 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase. The stereochemical assignments of the protons at the position 7 methylene group could then be determined using C-6 of tetrahydrobiopterin as a chiral reference.

In the present study, the deuterium found at C-7 of 6-(*R*)-[6,7,9,11-$^{13}C_4$]5,10-methylenetetrahydropteroate (**11**) has been incorporated from solvent by GTP cyclohydrolase I. Hence, the deuterium at C-7 of compound **11** must be located in the pro-*R* position (*i.e.* H-7d in Fig. 3). Because we could show that H-7d and H-6 in **11** have *anti* orientation (Table III), the findings confirm that dihydrofolate reductase converts dihydropteroate into 6-(*S*)-tetrahydropteroate. Thus, our working hypothesis that the reduction of dihydropteroate and dihydrofolate by dihydrofolate reductase affords products with the same stereochemical features (see above) is now confirmed by direct evidence.

The residual ^{1}H in the H-$7_{pro\text{-}R}$ position (23%) of **11** indicates that the solvent had not been completely deuterated. This finding was not unexpected because H_2O/D_2O exchange had been performed by lyophilization of the multienzyme mixture. The impact of incomplete deuteration of the solvent may have been enhanced by a deuterium isotope effect because release of a proton from bulk water or from an acidic group in exchange with the bulk solvent would occur preferentially by comparison with the release of a deuteron.

Whereas the hypothetical reaction mechanism in Fig. 2 suggests the incorporation of precisely one deuterium equivalent at C-9 of **11** by protonation of the hypothetical intermediate **8** (Fig. 2), the data in Table V show a considerable excess of ^{2}H. Specifically, 86% deuterium was found in the pro-*S* position and 66% in the pro-*R* position at C-9 of **11**. This could be due to stereospecific protonation of the enol **8** in the pro-*S* position in conjunction with wash-in of additional deuterium by spontaneous or enzyme-mediated exchange processes.

The 6α hydrogen atoms of compound **3** have increased CH acidity due to activation by the aminal motif in the dihydropyrazine ring. This activation should also extend to the adjacent ring atom, C-7, but the acidity of the C-6α and C-7 protons may well differ quantitatively, and this could explain the absence of excess deuterium in the position 7 methylene group of compound **11**.

The data leave no doubt that the protonation of the intermediate **8** by dihydroneopterin aldolase occurs preferentially in the pro-*S* position (Fig. 8). However, due to the excess deuterium at the position 6α carbon of compound **3**, the degree of stereospecificity of the protonation of the intermediate **8** cannot be determined unequivocally.

The present data do not answer the question whether the donor for proton transfer to the 6α carbon of the enol **8** is an acidic group of the protein or a solvent water. However, it is clear that the protonation of the enol does not occur by return of the proton that has been abstracted from the substrate. Thus, if the protonation of **8** occurs by an amino acid residue involved in the formation of an anion from the substrate, **2**, that residue would have to be water-accessible, and the abstracted proton would have to be exchanged with bulk solvent.

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