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Chemo-Enzymatic Assembly of Isotopically Labeled Folates

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ABSTRACT: Pterin-containing natural products have diverse functions in life but an efficient and easy scheme for their *in vitro* synthesis is not available. Here, we report a chemo-enzymatic 14-step, one-pot synthesis that can be used to generate ^{13}C- and ^{15}N-labeled dihydrofolates (H_2F) from glucose, guanine and p-aminobenzoyl-L-glutamic acid. This synthesis stands out from previous approaches to produce H_2F in that the average yield of each step is >91% and it requires only one single purification step. The use of a one-pot reaction allowed us to overcome potential problems with individual steps during the synthesis. The availability of labeled dihydrofolates allowed the measurement of heavy atom isotope effects for the reactions catalyzed by the drug target dihydrofolate reductase and established that protonation at the N5 position of H_2F and hydride transfer to the C4 position occur in a stepwise mechanism. This chemo-enzymatic pterin synthesis can be applied to the efficient production of other folates and a range of other natural compounds with applications in nutritional, medical and cell biological research.

INTRODUCTION

Pterin is a common motif found in natural products. Folate, the essential vitamin that fuels the one carbon cycle for the biosynthesis of nucleotide and amino acid building blocks, was one of first natural products found to contain pterin.[1, 2] The metabolic importance of pterins is illustrated by their integration into enzyme cofactors such as molybdopterin and tetrahydrobiopterin.[3-5] Pterin natural products are also used as pigments in the butterflies *Catopsilia argante* and *Appias nero*,[6] whereas biopterin-α-glucoside serves as a natural sunscreen that protects cellular contents from photo-induced damages in photosynthetic cyanobacteria.[7]

5,6,7,8-Tetrahydrofolic acid (H_4F), which in addition to the pterin ring system contains *p*-aminobenzoic acid (*p*ABA) and L-glutamic acid (Glu), is required for the biosynthesis of metabolites that are key for cell survival and replication.[2] A one-carbon unit in different oxidation states can be added to the N5 and/or N10 position of H_4F and used to produce metabolites such as thymidylate, purines, glycine, serine and S-adenosylmethionine (SAM) (Figure 1).[8] Given the central importance of folate biochemistry for cell replication and survival, dihydrofolate reductase (DHFR), thymidylate synthase (TS) and serine hydroxymethyltransferase (SHMT) have long been exploited as important drug targets in the treatment of bacterial infections,[9] malaria[10, 11] and cancer,[12, 13] and the DHFR-targeting drugs trimethoprim, proguanil, pyrimethamine and methotrexate are listed as essential medicines by the World Health Organization (WHO).[14] Nevertheless, as with many clinically used drugs, resistance to antifolates has begun to emerge[15-17] and the investigation of the enzymes of the one-carbon cycle is an important part of inhibitor design strategies.[18]

Detailed mechanistic insight into enzyme-catalyzed reactions is often obtained by isotopic labeling and measurement of kinetic isotope effects (KIE)[19-22] or spectroscopic analysis.[23,24] Information derived from regio- and stereospecific substrate labeling has been used to design inhibitors with dissociation constants in the micro- to picomolar range.[25] However, the use of these techniques to investigate folate dependent enzymes is hindered by the absence of a general and efficient method to specifically label atoms of the pterin ring system, particularly at the N5, C6, C7 and C9 positions, which are directly linked to the chemistry of the catalyzed reactions. Folate and its derivatives can be synthesized by connecting the pterin, *p*ABA and glutamate groups in sequential order,[26] and several synthetic strategies to incorporate an isotopic label into pterin in a regiospecific manner have been reported.[27-30] Pterins have been synthesized by condensing guanidine or dihydroxyacetone with the respective heterocyclic starting materials, and N5, C2 and C6 labeled folates have been made previously.[27, 28] However, because symmetric reagents are used in these syntheses, regio-selective isotope labeling of C7 and C9 positions cannot easily be achieved.[28, 30] In all cases, the yields of labeled folate or derivatives are low (<5% overall yield) and the procedures depend on multiple purification steps. Chemo-enzymatic strategies have also been described,[31-33] whereby H_4F was condensed with ^{11}C-formaldehyde or ^{14}C-formic acid to yield the corresponding isotopically labeled [^{11}C]-5,10-methylene-H_4F, [^{14}C]-5-formyl-H_4F and [^{14}C]-10-formyl-H_4F. However, a general and efficient method to label the pterin ring in folates has never been developed.

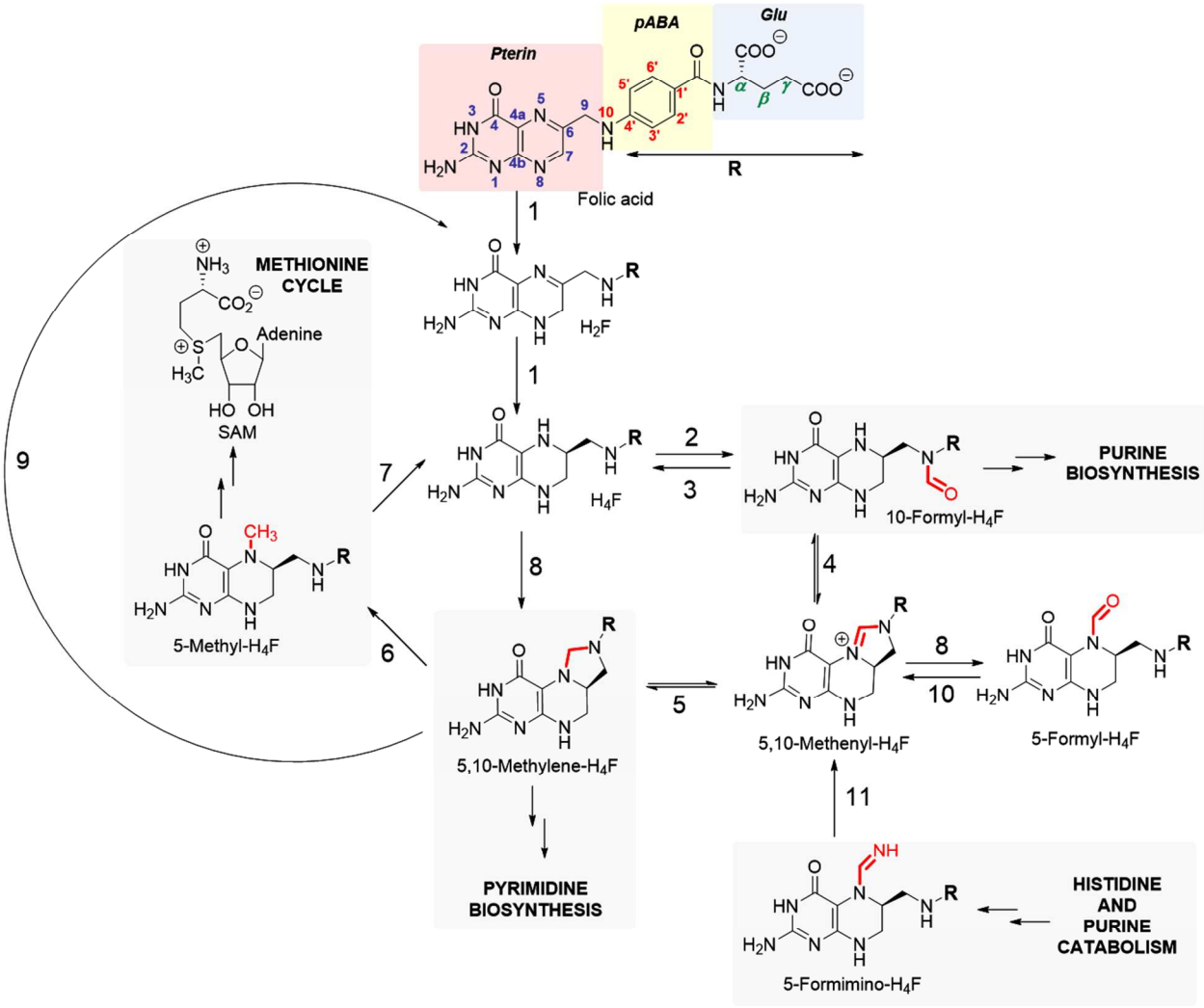

Figure 1. Folate coenzymes in one-carbon metabolism. Folic acid is converted to 7,8-dihydrofolic acid (H_2F) and 5,6,7,8-tetrahydrofolic acid (H_4F). One-carbon units attached to H_4F are highlighted. **1**: dihydrofolate reductase (DHFR); **2**: 10-formyl-H_4F synthetase (FTHFS); **3**: 10-formyl-H_4F dehydrogenase (FDH); **4**: 5,10-methenyl-H_4F cyclohydrolase (MTHFC); **5**: 5,10-methylene-H_4F dehydrogenase (MTHFD); **6**: 5,10-methylene-H_4F reductase (MTHFR); **7**: methionine synthase (MS); **8**: serine hydroxymethyl-transferase (SHMT); **9**: thymidylate synthase (TS); **10**: 5,10-methenyl-H_4F synthetase (MTHFS); **11**: 5-formimino-H_4F cyclodeaminase (FTCD); S-adenosylmethionine (SAM).

In nature, the pterin ring in folate is formed from guanosine triphosphate (GTP) in one biochemical step catalyzed by GTP cyclohydrolase (GTP-CH),[34, 35] an enzyme found in all kingdoms of life ranging from archaebacteria, insects and plants to humans. In all GTP-CH-catalyzed reactions, GTP is converted to a pterin *via* a set of tandem reactions that have no equivalent in organic chemistry. GTP cyclohydrolase I (GTP-CH-I) catalyzes the formation of 7,8-dihydroneopterin triphosphate (DHNTP) from GTP by mediating four distinct chemical reactions (Figure S1): hydrolysis of the purine ring yielding a N-formyl intermediate, N-deformylation, a stereospecific Amadori rearrangement of the ribose moiety and ring closure to form the pterin.[36-38] Because no symmetric reagent is used in this reaction, GTP-CH-I can be used to synthesize the pterin ring system of folate with heavy isotopes incorporated regio- and stereo-selectively.[39] It is therefore surprising that GTP-CH-I has not been used in any *in vitro* enzymatic pathway to synthesize folates. Perhaps, the low catalytic turnover (k_{cat} = 0.05 s^{-1})[40] and the rather low stability of the product 7,8-dihyropterin[41] toward oxygen and light have limited the use of GTP-CH-I in synthesis.

Here we report a 14-step one-pot chemo-enzymatic synthesis of 7,8-dihydrofolic acid (H_2F) that exploits the well-established procedures to isotopically label GTP[39, 42, 43] by using GTP-CH-I to site-specifically isotope label pterins. The low enzymatic activity and product instability of GTP-CH-I were addressed by enzymatic coupling. Using our methodology, H_2F enriched with stable isotopes at the N5 and C6 positions could be synthesized efficiently in pure form in >30% yield from isotopically enriched D-glucose and guanine. Given the high degree of purity and isotopic enrichment (>97%, supplementary information), heavy-atom KIEs could be measured to investigate the mechanism of the *E. coli* DHFR (EcDHFR) catalyzed reduction of H_2F to H_4F.

RESULTS AND DISCUSSION

In vitro synthesis of folate

The biosynthetic pathway to folate in *E. coli* uses the building blocks D-glucose, guanine and *p*-aminobenzoyl-L-glutamate (*p*ABA-Glu). In the biosynthetic scheme of H_2F (Figure 2), the C2, C3 and C4 positions of glucose correspond to the C7, C6 and C9 positions of H_2F and N1, C2, N3, C4, C5, C6, N7 and N9 of guanine correspond to N3, C2, N1, C4b, C4a, C4, N5 and N8 in H_2F. GTP is the key intermediate in this synthetic pathway, connecting the purine salvage pathway to *de novo* folate biosynthesis. Accordingly, a minimum of 14 enzymes need to be assembled *in vitro* to produce H_2F.

The GTP biosynthetic pathway is composed of enzymes from the pentose phosphate and purine salvage pathways (Figure 2 and S2). D-Glucose serves as the starting material, which is transformed into phosphoribose pyrophosphate (PRPP) in five steps that are catalyzed by hexokinase (HK), glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), phosphoriboisomerase (PRI) and ribose-phosphate pyrophosphokinase (phosphoribosylpyrophosphate synthetase; PRS).[44] PRPP is then combined with guanine to form GMP under xanthine guanine phosphoribosyl transferase (XGPRT) catalysis. The resulting GMP is converted to the corresponding nucleotide triphosphate, in reactions catalyzed by guanylate kinase (GK) and pyruvate kinase (PK). Since HK and GK use ATP as the phosphate source, PK can also function as the recycling enzyme. On the other hand, PRS uses ATP as the pyrophosphate source and so myokinase (MK) was included to regenerate ATP from AMP.[45] A significant amount of $NADP^+$ is also needed for GTP biosynthesis. Hence, the recently developed glutathione reductase (GR)/glutaredoxin 2 (GRX2) recycling system was used for the regeneration of the oxidized cofactor.[44] The GR/GRX2 system uses disulfides like 2-hydroxyethyl disulfide (HED or oxidized β-mercaptoethanol) or cystine as regenerating reagents and produces thiols as useful byproducts, which protect the enzymes and intermediates from oxidative damage.

The conversion of GTP to DHNTP by GTP-CH-I marks the entry point of the folate *de novo* pathway. It has been reported that potassium and magnesium ions are positive allosteric effectors that can increase the rate of the GTP-CH-I reaction up to 5-fold.[46] The addition of these cations, however, was insufficient, as the reaction was found to be incomplete giving a poor yield of DHNTP (Figure 3a). In folate *de novo* biosynthesis, dephosphorylation of DHNTP to 7,8-dihydroneopterin monophosphate (DHNMP) by DHNTP pyrophosphohydrolase (DHNTPase) is the biochemical step followed by the GTP-CH-I reaction (Figure 2).[47, 48] Knockout of the DHNTPase gene significantly impairs folate metabolism in *E. coli*,[48] which suggests that DHNTP phosphohydrolysis is a key regulatory step in folate metabolism. In other words, the activity of GTP-CH-I is most likely inhibited by its own product, DHNTP, which therefore needs to be immediately converted to DHNMP in order to sustain the activity of GTP-CH-I. In the presence of DHNTPase, GTP-CH-I showed a marked rate enhancement (Figure S3), with near complete conversion of GTP and a high yield of DHNMP (Figure 3b).

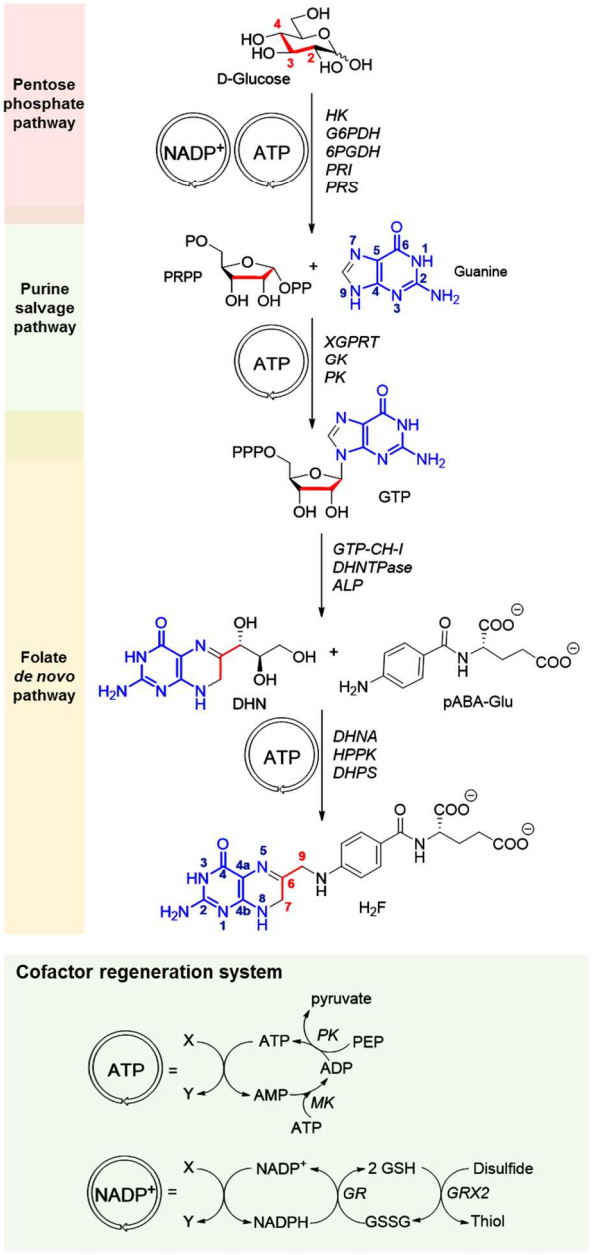

Figure 2. Strategy for the synthesis of 7,8-dihydrofolate (H_2F). Guanosine triphosphate (GTP) was made *via* the pentose phosphate and purine salvage pathways under hexokinase (HK), glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), phosphoriboisomerase (PRI), ribose-phosphate pyrophosphokinase (PRS), xanthine guanine phosphoribosyl transferase (XGPRT), guanylate kinase (GK) and pyruvate kinase (PK) catalysis. In the folate *de novo* pathway, GTP is converted to 7,8-dihydroneopterin (DHN), catalyzed by GTP cyclohydrolase I (GTP-CH-I), 7,8-dihydroneopterin pyrophosphatase (DHNTPase) and alkaline phosphatase (ALP); DHN is then converted to H_2F, catalyzed by dihydroneopterin aldolase (DHNA), 6-hydroxymethyl 7,8-dihydropterin pyrophosphokinase (HPPK) and dihydropteroate synthase (DHPS). The additional enzymes myokinase (MK), glutathione reductase (GR) and glutaredoxin 2 (GRX2) are used for the regeneration of ATP and $NADP^+$.[44, 45] The pterin atoms of H_2F derived from glucose (red) and guanine (blue) are highlighted. Details for each biosynthetic step are described in Figure S2.

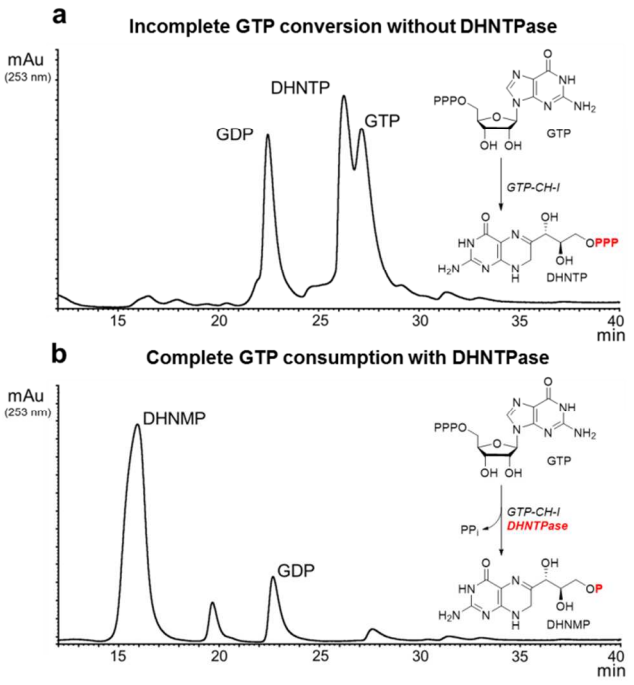

Figure 3. Conversion of guanosine triphosphate (GTP) into 7,8-dihydroneopterin triphosphate (DHNTP) and monophosphate (DHNMP). Anion exchange chromatographic analyses of GTP incubated for 2 h at 37 °C with (**a**) GTP cyclohydrolase I (GTP-CH-I) which shows partial conversion to DHNTP, and (**b**) both GTP-CH-I and 7,8-dihydroneopterin triphosphate pyrophosphohydrolase (DHNTPase) for essentially complete conversion to DHNMP. Production of guanosine diphosphate (GDP) is most likely due to the non-enzymatic hydrolysis of GTP during incubation.

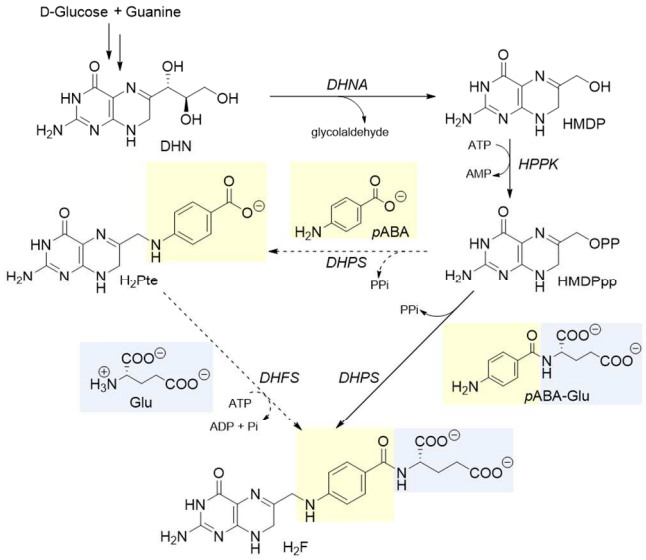

Figure 4. Conversion of 7,8-dihydroneopterin (DHN) to dihydrofolate (H_2F). Dihydroneopterin aldolase (DHNA) catalyzes the transformation of DHN to 6-hydroxymethyl-7,8-dihydropterin (HMDP), which is pyrophosphorylated by 6-hydroxymethyl 7,8-dihydropterin pyrophosphokinase (HPPK). The resulting 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (HMDPpp) is converted to H_2F by dihydropteroate synthase (DHPS), whose promiscuity allows the use of pre-assembled *p*ABA-Glu as substrate and the omission of dihydrofolate synthase (DHFS) in the *in vitro* synthetic pathway.

Additional enzymes are needed to convert DHNMP into folate. To the best of our knowledge, the natural enzyme responsible for the conversion of DHNMP to 7,8-dihydroneopterin (DHN) is unknown[37, 48] and alkaline phosphatase (ALP) was used instead as a surrogate. DHN is subjected to a retro-aldol reaction catalyzed by dihydroneopterin aldolase (DHNA) to yield 6-hydroxymethyl-7,8-dihydropterin (HMDP),[49, 50] which then reacts with ATP in the presence of 6-hydroxymethyl 7,8-dihydropterin pyrophosphokinase (HPPK) (Figure 4).[51] In *E. coli*, the resulting intermediate 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (HMDPpp) is first condensed with *p*ABA and then with glutamate, catalyzed by dihydropteroate synthase (DHPS)[52] and dihydrofolate synthase (DHFS)[53] respectively, to finally generate dihydrofolate (H_2F). However, DHPS accepts pre-assembled *p*ABA-Glu as a substrate,[54] so DHFS is not required in the *in vitro* reaction. The entire H_2F synthetic pathway only requires one purification step of the product, but ALP needs to be removed by ultrafiltration prior to the addition of DHNA, HPPK and DHPS as the phosphatase can also catalyze the phosphorolysis of ATP and HMDPpp. Two additional modifications were made to further improve the overall yield. A N_2-filled glove box system was used, because all reduced pterin-containing compounds, DHNTP, DHNMP, DHN, HMDP, HMDPpp and the final product H_2F, are oxygen-sensitive.[41] Also, cystine was found to be the preferred reagent over HED for the $NADP^+$ regeneration system operated by GR and GRX2. Perhaps, β-mercaptoethanol made from the reduction of HED interferes with other enzymatic reactions, such as the chelation of Zn^{2+} in GTP-CH-I. The total turnover numbers for the regeneration of ATP from ADP by pyruvate kinase or of ATP from AMP by pyruvate kinase/myokinase are both ~100,[55] while the total turnover number for our GR/GRX2-based $NADP^+$ recycling system can reach 5 x 10^5.[44] In general, a typical biosynthetic cascade produced 6.6 mg of H_2F from 9 mg of glucose with an overall yield of 30%, *i.e.* the yield of each chemical transformation is in excess of 91%.

Synthesis of selectively labeled folate

Five isotopically labeled H_2Fs were synthesized using the newly developed *in vitro* pathway (Figure 5a). Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis of [6-^{13}C]-H_2F, produced from [3-^{13}C]-D-glucose, showed an increase of ~1 amu; ^{13}C-NMR spectroscopy revealed a singlet peak at 152 ppm. Additionally, long-range coupling between 6-^{13}C and protons on C7 and C9 of [6-^{13}C]-H_2F were observed in the ^{1}H,^{13}C-HMBC spectrum (Figure 5b).

To incorporate a ^{15}N label into the pterin moiety at N5, [7-^{15}N]-guanine was used. The resulting product showed ~1 amu increase in LC-HRMS analysis and a ^{15}N signal at 285 ppm coupling to C7 and C9 protons in the ^{1}H,^{15}N-HMBC spectrum (Figure 5c).

When [3-^{13}C]-D-glucose and [7-^{15}N]-guanine were combined to produce [5-^{15}N][6-^{13}C]-H_2F, a mass increase of ~2

amu was measured. The ^{13}C-NMR spectrum showed a doublet at 152 ppm with a coupling constant $^{1}J_{CN}$ of 7.5 Hz; long range coupling between the protons on C7 and C9 was observed in the ^{1}H,^{13}C-HMBC and ^{1}H,^{15}N-HMBC spectra (Supplementary Information) indicating that both the N5 and C6 positions of H_2F were isotopically enriched.

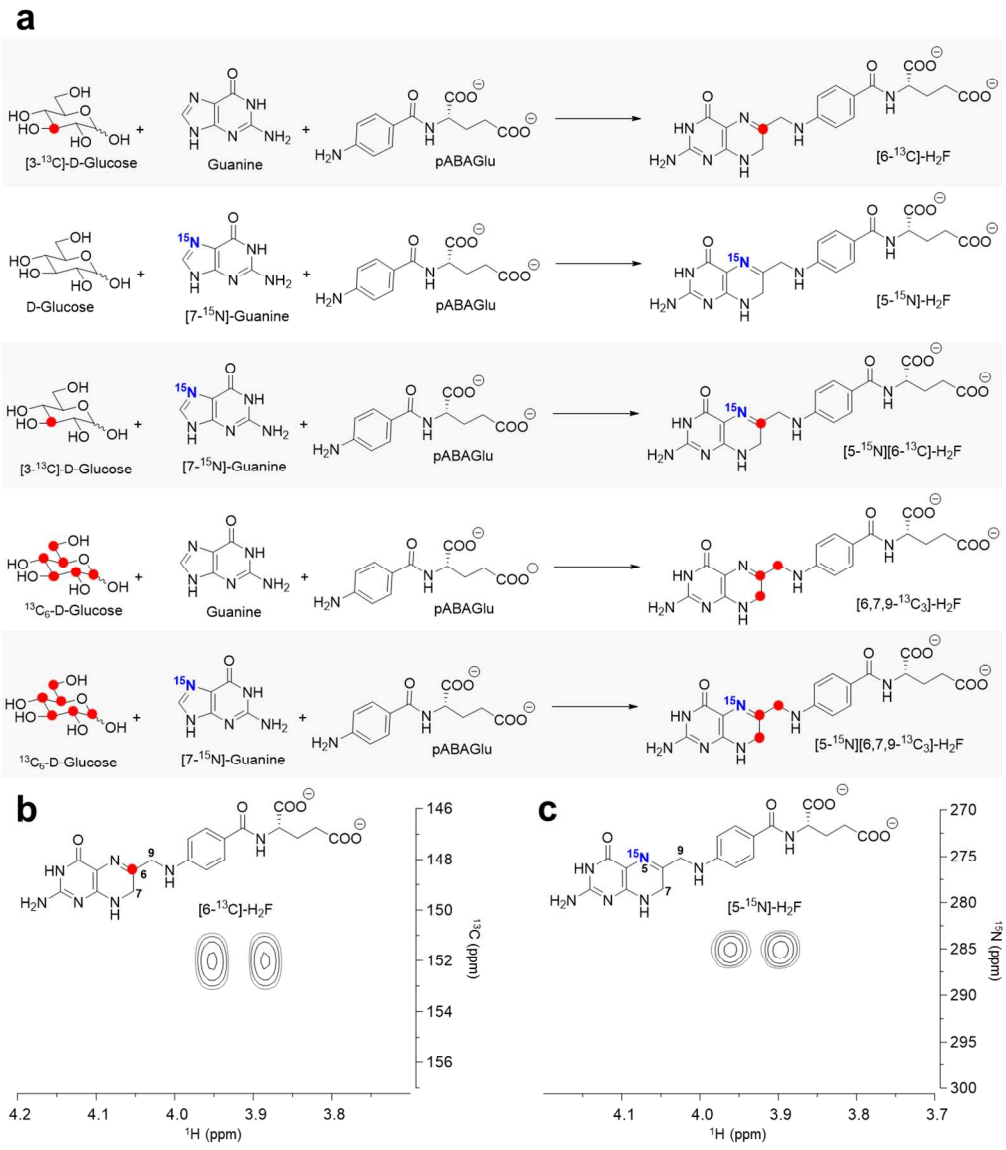

Figure 5. H_2Fs labeled with stable isotopes on the pterin ring system. (**a**) Patterns of isotopic distribution are determined by the starting material isotopic enrichment used during H_2F biosynthetic assembly. (**b**) ^{1}H,^{13}C-HMBC and (**c**) ^{1}H,^{15}N-HMBC of [6-^{13}C]-H_2F and [5-^{15}N]-H_2F. Protons attached to C7 (3.95 ppm) and C9 (3.87 ppm) correlate to C6 (152 ppm) or N5 (285 ppm). ^{1}H-NMR spectra of all compounds and additional 2D-NMR characterization of [5-^{15}N][6-^{13}C]-H_2F, [6,7,9-$^{13}C_3$]-H_2F and [5-^{15}N][6,7,9-$^{13}C_3$]-H_2F are reported in Figures S12-S15. For HRMS data of all compounds see Table S3 and Figures S16-S21. Isotopic enrichment was calculated to be at least 97% from the mass spectrometric data (illustrated in Figures S22-24).

Similarly, [6,7,9-$^{13}C_3$]-H_2F and [5-^{15}N][6,7,9-$^{13}C_3$]-H_2F were synthesized from $^{13}C_6$-D-glucose and [7-^{15}N]-guanine, and their identity confirmed by HRMS and NMR spectroscopy (Supporting Information).

Heavy-atom kinetic isotope effects on the reaction catalyzed by EcDHFR

The preparation of ^{13}C- and ^{15}N-labeled dihydrofolates allowed the measurement of heavy atom isotope effects for the reactions catalyzed by dihydrofolate reductase (DHFR), a key enzyme in the one-carbon metabolism and validated target for the treatment of bacterial infections, malaria and cancer.[56] DHFR catalyzes the reduction of H_2F to H_4F *via* transfer of the pro-*R* hydride from C4 of NADPH to the *Re*-face on C-6 accompanied with protonation of N5 of H_2F (Figure 6).[57, 58] Several aspects of the reaction mechanism warrant additional investigation. In particular, the transition state structure and the order of chemical transformation events have not been fully determined.[59, 60] The active site of DHFR provides a favorable environment for N5 protonation by elevating the pK_a from 2.6 to 6.5 and using an active site water as the proton source.[61, 62] Solvent and hydrogen KIE measurements combined with site-

directed mutagenesis have suggested a stepwise mechanism, in which protonation precedes hydride transfer.[63, 64] However, D_2O increases the viscosity of the reaction buffer relative to H_2O[65, 66] and site-directed modification can alter the catalytic behavior of an enzyme[67] and additional mechanistic investigations are needed to establish the order of events. Since isotopic substitution does not alter the chemistry of the reaction but only the kinetics, [6-^{13}C]-H_2F and [5-^{15}N]-H_2F were used to measure the ^{15}N- and ^{13}C-heavy atom kinetic isotope effects. Pre-steady-state kinetic measurements at 15 °C, by fluorescence resonance energy transfer from the active site tryptophan in DHFR to the reduced cofactor, yield first-order hydride transfer rate constants with an accuracy up to 0.7% (Figure S25-27, Table S4). While a ^{13}C-KIE of 1.015 ± 0.006 was observed for the reduction of [6-^{13}C]-H_2F, the corresponding ^{15}N-KIE for [5-^{15}N]-H_2F was essentially unity (0.999 ± 0.006) under the same conditions. To confirm this finding, [5-^{15}N][6-^{13}C]-H_2F was used to probe both positions at the same time and the measured value for the corresponding multiple heavy-atom KIE was 1.014 ± 0.008, statistically identical to that obtained when the substrate was labeled with ^{13}C only.

The observed ^{15}N-KIE on hydride transfer indicates that N5 protonation is not isotopically sensitive, likely because it is not rate-limiting under pre-steady state conditions (as the reaction is essentially irreversible,[58] the observed KIE will tend to unity rather than to the equilibrium isotope effect).[22] On the other hand, the measured ^{13}C-KIE indicates that the hydride transfer step is rate-determining. This strongly suggests a stepwise mechanism. If the protonation and hydride transfer steps were concerted, ^{15}N- and ^{13}C-labeled H_2Fs should both yield measurable KIEs;[19, 21, 22] this interdependency may also lead to an additive effect in the multiple heavy-atom isotope effect measurement with the double-labeled substrate.[19, 21, 22] In other words, our results suggest that the pre-steady state kinetic measurement at pH 7.0 reveals only the step of hydride transfer, because protonation of N5 is in rapid equilibrium and the ensemble of reaction-ready conformations is mostly populated with protonated H_2F. Importantly, these results confirm the validity of previous solvent KIE and site-directed mutagenesis studies, which also concluded that the sequence of chemical events (protonation and hydride transfer) is distinct and strictly ordered.[64, 68] Overall, the results provided here strongly support a mechanism where protonation and hydride transfer are independent of each other and occur in a stepwise fashion.

CONCLUSIONS

Dihydrofolate was produced enzymatically in an easy one-pot, high yielding reaction sequence from glucose, guanine and *p*ABA-Glu that required only a single purification step. Potential problems with individual steps during the synthesis could be overcome through the use of a one-pot reaction. This methodology can be used to generate dihydrofolates labeled in specific positions with stable isotopes with average overall yields of >30%, facilitating many applications in cell biology and mechanistic enzymology.[27-29, 61, 69, 70] For the first time, heavy-atom KIEs for the DHFR-catalyzed reduction of H_2F could be measured to provide strong support for a stepwise reduction of the substrate, in which protonation at N5 and hydride transfer from C4 of the NADPH to C6 of protonated dihydrofolate proceed independently. This chemo-enzymatic pterin synthesis can be integrated into other enzymatic procedures to generate folate derivatives[31, 32] and other high-value natural products that are not easily accessible by conventional synthesis.[71] It can be applied to nutritional, medical and cell biological research to address questions of *in vivo* bioavailability and to explore the kinetics of folate metabolism in intact cells and organisms.[70, 72-76]

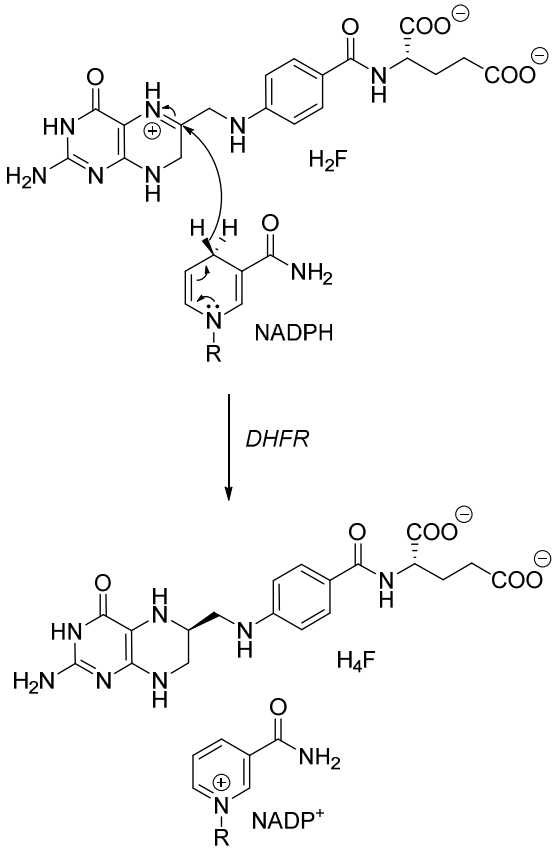

Figure 6. Reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) catalyzed by dihydrofolate reductase (DHFR).

ASSOCIATED CONTENT

Supporting Information. Full experimental procedures, supplementary figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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