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Research Article

Microscale synthesis of isotopically labeled 6*R*-N^5, N^{10} methylene-5, 6, 7, 8-tetrahydrofolate

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Summary

A one-pot chemo-enzymatic microscale synthesis of isotopically labeled *R*-[6-^{Y}H; 11-^{X}H] N^5, N^{10} methylene-5, 6, 7, 8-tetrahydrofolate (CH_2H_4folate) is presented, where Y = 1 or 2 represents protium or deuterium, and X = 1, 2 or 3 represents protium, deuterium or tritium, respectively. In this procedure, *Thermoanaerobium brockii* alcohol dehydrogenase (*tb*ADH) and *Escherichia coli* dihydrofolate reductase (*ec*DHFR) were used simultaneously in the reaction mixture. First, *tb*ADH stereospecifically catalyzes a hydride transfer from [2-^{Y}H] iPrOH to the *re* face of C-4 $NADP^+$. The *ec*DHFR then reduced 7, 8-dihydrofolate (H_2folate) to form (6*S*)-H_4folate. Finally, the enzymatic reactions were followed by chemical trapping with isotopically labeled formaldehyde ([^{X}H]-HCHO) to form the final product. The preparation of deuterium- and tritium-labeled formaldehyde is also presented. Two reverse phase HPLC methods were developed for analysis and purification of product *R*-[6-^{Y}H; 11-^{X}H] CH_2H_4folate. This isotopically labeled cofactor can be used to study 1° and 2° kinetic isotope effects (KIEs) with any CH_2H_4folate dependent enzyme as demonstrated by studies with *E. coli* thymidylate synthase (TS). Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: reverse phase HPLC; labeled folate; labeled formaldehyde; kinetic isotope effect; thymidylate synthase

Introduction

N^5, N^{10}-methylene-5, 6, 7, 8 tetrahydrofolate (CH_2H_4folate) (Scheme 1) is a ubiquitous cofactor that functions in the biosynthesis of purines and pyrimidines.[1] For example, Jaffe and Chrin[2] reported the presence and properties of four folate-related enzymes that are associated with this cofactor,

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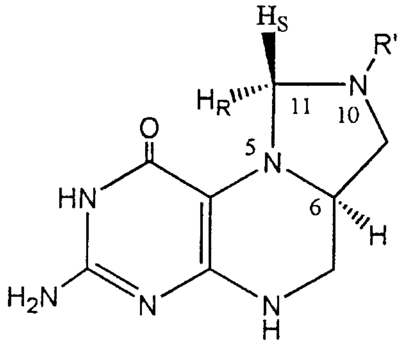
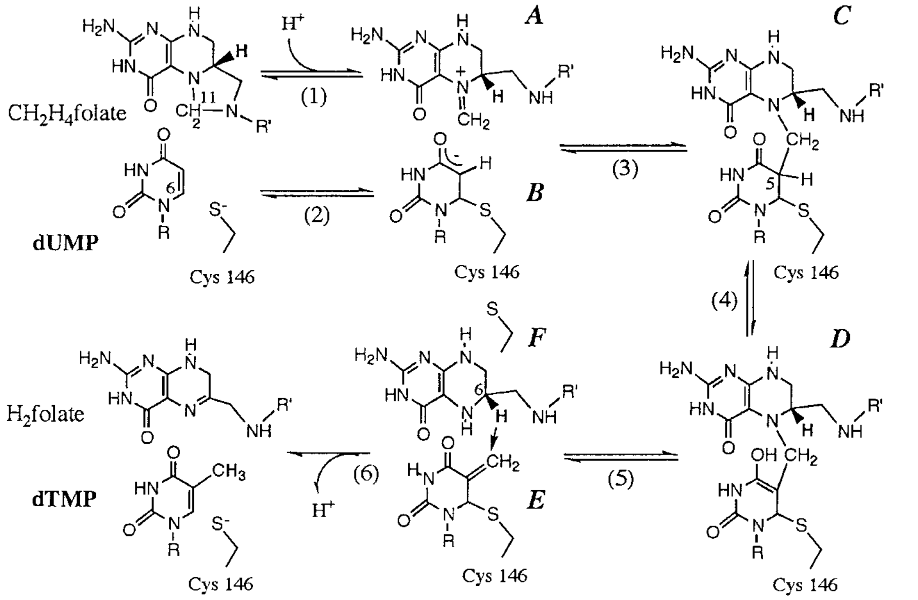


Scheme 1. Structure of *R*-N[5], N[10]-methylene 5, 6, 7, 8-tetrahydrofolate. R' = (*p*-amino-benzoyl) glutamate

namely serine hydroxymethyltransferase, CH_2H_4folate dehydrogenase, CH_2H_4folate reductase, and thymidylate synthase (TS). In the TS-catalyzed reaction (Scheme 2),[3] CH_2H_4folate serves as the donor for both a hydride (C6) and methylene (C11) to the substrate 2′-deoxyuridine 5′-monophosphate (dUMP), leading to the formation of 2′-deoxythymidine 5′-monophosphate (dTMP) and dihydrofolate (H_2folate).

Labeling substrates or cofactors at primary (1°) hydrogen (hydrogen that is involved in C–H bond cleavage) or secondary (2°) hydrogen (another hydrogen on the same carbon) with multiple hydrogen isotopes is of general interest. Such labeling has been used to measure competitive KIEs in many biological systems.[4,5] These studies could reveal important mechanistic features of the enzymic H-transfers including H-tunneling, coupled motion and more.[6–8] Comparison of the relationship between the 2° KIEs of H, D, and T (e.g. ln(H/T)/ln(D/T), denoted as the Swain–Schaad relationship) with H or D at the 1° position has been shown to be a most sensitive probe of H-tunneling and coupled motion.[9] For example, measurement of H/T 2° KIE (k_H/k_T) with H transfer from 1° position and H/T 2° KIE (k_H/k_T) with D transfer can directly examine possible coupling between the 1° and the 2° hydrogens along the reaction coordinate.[10,11] In short, this type of multiple isotopic labeling can expose the nature of H-transfer in complex systems like enzymes.

Isotopologues (Molecules that differ only in their isotopic composition) of CH_2H_4folate can be used in mechanistic studies of any CH_2H_4folate-dependent enzyme. The large-scale nonradioactive synthesis of CH_2H_4folate by the condensation of formaldehyde with tetrahydrofolate has been reported previously.[12,13] The *in situ* enzymatic synthesis of (6*R*, 11*R*)-and (6*R*, 11*S*)-5, 10-methylene [11-^{1}H, ^{2}H] tetrahydrofolate by Slieker and Benkovic[1] was also reported. Multiple isotopic labeling of the C6 hydrogen of 6*R*-CH_2H_4folate was used previously to study the nature of the hydride transfer step in the TS



Scheme 2. Reaction pathway of thymidylate synthase (step 6 is target for the 2° KIE study)

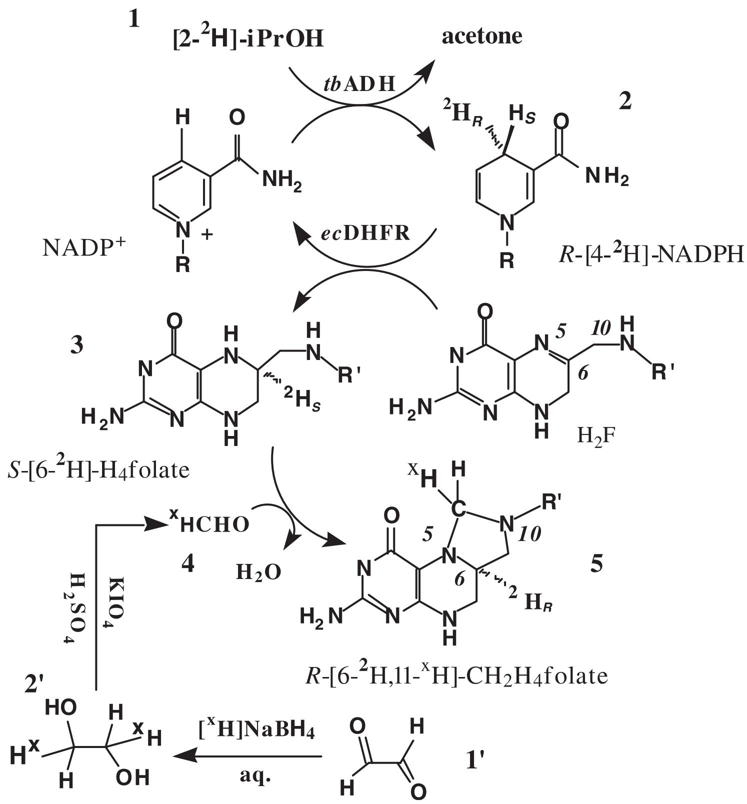
reaction through the measurement of 1° KIEs and their temperature dependence.[14,15]

This communication describes a new microscale chemo-enzymatic synthesis of isotopic labeled CH_2H_4folate at positions most relevant to its role as methylene and hydride donor in many enzymatic reactions. The usage of the labeled cofactor in studies of the enzyme TS is demonstrated.

Results and discussion

***R*-[6-^{2}H; 11-^{X}H] CH_2H_4folate 5** was synthesized in two steps, preparation of *S*-[6-^{2}H] H_4folate and trapping with [^{X}H]-HCHO (Scheme 3).† To synthesize the H_4folate with stereospecifically labeled 6C position, two enzymes were used simultaneously in the reaction mixture. *tb*ADH, stereospecifically catalyzed a deuteride transfer from uniformly deuderated isopropanol **1** to the *re* face of oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) to form *R*-[4-^{2}H]-labeled reduced nicotinamide adenine dinucleotide phosphate (NADPD) **2**. Then, *ec*DHFR was used to catalyze the transfer of the deuteride from **2** to the *si* face of 7, 8-dihydrofolate (H_2folate), forming *S*-[6-^{2}H] H_4folate **3**. The enzymatic reactions were followed by chemical trapping of **3** with labeled formaldehyde **4**.

†Please note that the change from *S* to *R* in the 6C position is only due to change in the nomenclature and not due to inversion of configuration.



Scheme 3. Chemoenzymatic synthesis of isotopically labeled (*R*)-[6-^{Y}H], [11-^{X}H] CH_2H_4folate, where Y = 1, 2, X = 1, 2, or 3 represents H, D, or T, respectively. *R* **= 2′-deoxyribose-5′-phosphate and** *R*′ **= (*p*-aminobenzoyl) glutamate**

The deuterium and/or tritium-labeled formaldehyde ([^{X}H]-HCHO) **4** used in the above procedure were prepared by a series of redox reactions initiated with [^{X}H]-$NaBH_4$. The synthetic route is shown in Scheme 3. In this process, glyoxal **1**′ was first reduced to ethylene glycol **2**′ by [^{X}H] $NaBH_4$.[16] When using $NaBD_4$ (>99% D) the solvent was D_2O (99.96% D) which increased the deuterium content of the labeled formaldehyde. Then, the solution was acidified and the [1, 2-$^{X}H_2$] ethylene glycol was oxidized by potassium periodate (KIO_4) to form the product **4** [^{X}H] HCHO. The product described in the current procedure is labeled with trace tritium or >99% protium or deuterium on one of the formaldehyde's hydrogens, while the other is always protium. This is aimed at measuring 2° KIE of only one hydrogen and not of doubly labeled methylene of TS intermediate as described below. Alternative labeling patterns of formaldehyde, for other applications, can be made by usage of labeled glyoxal **1**′.

***R*-[6-^{1}H; 11-^{X}H] CH_2H_4folate** was synthesized by incubation of *S*-[6-^{1}H] H_4folate with [^{X}H] HCHO **4**.

In all the tritiated CH_2H_4folate syntheses, four fold excess of [^{3}H] HCHO over CH_2H_4folate was used to form 3/1 mixture of [^{3}H] HCHO/[11-^{X}H] CH_2H_4folate. This led to complete capture of the H_4folate as labeled CH_2H_4folate. The HPLC radiogram of the tritiated CH_2H_4folate with 3:1 excess of tritiated formaldehyde is shown in Figure 1. Such practice was necessary due to the dynamic equilibrium between the reactants HCHO and H_4folate and the product CH_2H_4folate. Pure CH_2H_4folate (collected from HPLC separation) dissociated to free formaldehyde and H_4folate in equilibrium in about 120 min at 25°C. Our goal in the procedure described here was to use the labeled cofactor for measuring the 2° KIEs of the TS reaction. Thus, the limiting reagent was H_4folate that was fully (within the analytical limits) converted to the cofactor of interest (CH_2H_4folate). Alternatively, in the case that the yield of labeled HCHO is important, an excess of H_4folate should be used under strict anaerobic conditions (H_4folate is much more sensitive to O_2 than CH_2H_4folate). While using the CH_2H_4folate in enzyme kinetic experiments, the stability of the cofactor under aerobic conditions was tested in a control experiment. In that control experiment, the reaction mixture containing all the reagents and labeled CH_2H_4folate, was incubated at the experimental temperature for 40 min with no detectable degradation. The product 5 might be stable for even longer period of time, but

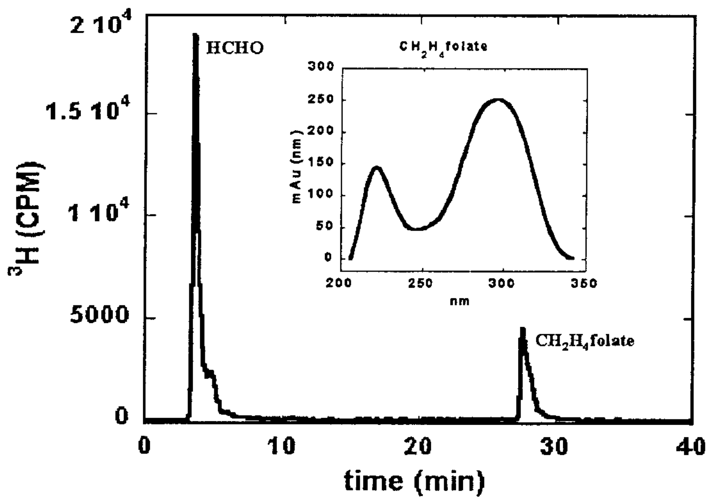


Figure 1. HPLC tritium radiogram of the reaction mixture of C11 labeled CH_2H_4folate in excess of [^{3}H]-HCHO under equilibrium condition. The peak of CH_2H_4folate eluted at 26 min contained 25% of the total radioactivity. The inserted UV spectrum of this peak confirms its identity as CH_2H_4folate

since all the kinetic measurements were much faster (less than a few minutes), this has not been tested here.

As a result of one-hydrogen labeling of formaldehyde ([^{X}H]-HCHO), trapping of formaldehyde resulted in a mixture of (6*R*, 11*S*) and (6*R*, 11*R*) CH_2H_4folate diastereoisomers. The distribution between the two diastereoisomers was verified by NMR to be 1:1 after 120 min even when a pure diasteromer was synthesized due to epimerization at C11.[1] The libel methylene (11C) imposes an inherent limitation on the synthesis of 5. Stereospecifically labeled 5 at 11C epimerize to racemic mixture and cannot be preserved as is. Down-stream products using 5 as cofactor can be synthesized only by using coupled enzymatic mixtures that will capture stereospecifically labeled 5 as it is formed.[1]

During the TS catalyzed reaction, the 1:1 *R*:*S* labeled 11C led to 1:1 ratio of *E* and *Z* intermediates of methylene-dUMP (*E* in Scheme 2). Importantly, step 6 that reduces this intermediate is the rate limiting step in the while TS reaction.[14] 2°KIEs on the *E* and *Z* intermediates are expected to be very similar or identical because they both involve similar change in vibrational states along the reduction coordinate. Using these radiolabeled cofactors, competitive measurements on the second order rate constant (V/K) were performed with TS. Inverse 2° KIEs (e.g. 0.76 for $^{T}V/K_{H}$) were observed for all the labeled cofactors whose synthesis is described here. The in-depth investigation of their contribution to understanding the TS mechanism will be described elsewhere.

Materials

H_4folate was a gift from Eprova Inc., Switzerland. H_2folate was synthesized according to the procedure of Blakley and its purity examined by NMR and UV as described elsewhere.[17] Potassium periodate was from Alfa Aesar. D_2O (99.96% isotopic purity), $NaBD_4$ (>99% D), [U-^{2}H] isopropanol (>99% D) were from Cambridge Isotope Laboratories Inc. [^{3}H] formaldehyde (HCTO 10 Ci/mmol) and [^{3}H] sodium borohydride ($NaBT_4$ 80 Ci/mmol) were from American Radiolabeled Chemicals Inc. [2-^{14}C] dUMP (60 Ci/mol) was from Moravek Biochemicals. The expression system for *E. coli* TS was a generous gift from R. Stroud, University of California at San Francisco. The enzyme was purified following the procedure of Changchien *et al.*[18] All other materials were purchased from Sigma.

Methods

HPLC analysis and separation

Analysis of R-[6-^{Y}H; 11-^{X}H] CH_2H_4folate and its precursors. The HPLC separation and analysis system has been described elsewhere.[19] In short, the

HPLC system consisted of an online degasser, quaternary pump, temperature-controlled column chamber, and a UV/VIS diode array detector (Agilent 1100 series). The column (C 18, 250 mm × 4.6 mm, 5 μm, Discovery series) was from Supelco. Following the UV detector, a flow scintillation analyzer (Model RT505 from Packard, now Perkin Elmer Biosciences) was used to analyze the radioactivity eluted from the column. The liquid scintillation flow rate was 2.4 ml/min and the HPLC flow rate was 1.0 ml/min. For purification, a splitter was set to divert 97% of the eluent to a fraction collector and 3% to the radioactivity analyzer.

For purification of labeled molecules, the separation system[19] was used where eluent A was a mixture of 32 mM Na_2HPO_4 and 3.7 mM KH_2PO_4, pH 7.8 (Buffer A) and eluent B was a 1:4 mixture of MeOH: Buffer A at pH 7.8 (Buffer B). The column was preequilibrated for 5 min in Buffer A at a flow rate of 1.0 ml/min. After the injection of the sample the following gradient was applied: 0–13% B for 0–14 min, 13–85% B for 14–28 min, and 85% B for 28–30 min. The column temperature was maintained at 25°C and the retention times were: HCTO (5 min, $\lambda_{max} = 210$ nm), $NADP^+$ (6–7 min, $\lambda_{max} = 258$ nm), iPrOH (11.50 min), H_4folate (20 min, $\lambda_{max} = 298$ nm), H_2folate (23–24 min, $\lambda_{max} = 284$ nm) and CH_2H_4folate (26–28 min, $\lambda_{max} = 294$ nm).

Analysis of the TS catalyzed reaction

For products and reactants analysis of the TS catalyzed reaction, the previously published procedure[19] was used. In short, eluent A, 20 mM triethyl ammonium acetate (TEAA), pH 6.6; Eluent B, 5:95 mixture of 20 mM TEAA (pH 5.1): acetonitrile. The column was preequilibrated for 5 min in Buffer A at a flow rate of 0.8 ml/min. After injection of the sample the following gradient was applied: 0–0.5% B for 0–10 min, 0.5–1% B for 10–30 min, 1.0–100% B for 30–35 min, and 100% B for 35–40 min and analyzed by RP HPLC. The retention times were: dUMP (20 min, $\lambda_{max} = 263$ nm), dTMP (28–29 min, $\lambda_{max} = 269$ nm) and CH_2H_4folate (37 min, $\lambda_{max} = 294$ nm).

Synthetic procedures

Synthesis of the mixture of [^{2}H and ^{3}H]-HCHO, **4**

The synthesis of **4** is depicted in Scheme 3. First, 200 μl glyoxal solution (**1'**) (83 mM in D_2O) was cooled to 0°C, and neutralized with NaOD (final pD = 8). This solution was then added dropwise into a mixture of 240 μl $NaBT_4$ (80 Ci/mmol, 25 mCi) and $NaBD_4$ (33 mM, >99% D) in D_2O. The solution was rapidly stirred at 0°C for 30 min. The reaction mixture was then acidified by sulfuric acid and KIO_4 (166 mM as final) was added to it. This oxidation step was carried out at room temperature for another half an hour.

Finally, the pH of the product mixture solution was adjusted to 7.4 and store at 4°C before use.

Synthesis of R-[6-^{1}H; 11-^{X}H] CH_2H_4folate

R-[6-^{1}H; 11-^{X}H] CH_2H_4folate was prepared by incubation of H_4folate with H/T or D/T formaldehyde (mixtures of trace HCTO in HCHO or HCTO in HCDO, respectively) under strict anaerobic conditions. The reaction mixture of trace T labeled CH_2H_4folate contained 500 mM H_4folate, 2.93 mM [^{3}H] HCHO (final specific activity was 1.5 Ci/mmol), and 280 mM *Tris*/HCl buffer. The reaction was kept at room temperature in an argon-filled glove bag for 30 min in the dark and the product was used without further purification. The synthesis of the trace C11 T in D substitute CH_2H_4folate was performed by a similar procedure.

Synthesis of R-[6-^{2}H; 11-^{X}H] CH_2H_4folate, **5**

R-[6-^{2}H; 11-^{X}H] CH_2H_4folate was prepared by a modification of the procedure of Agrawal *et al.*[19] The reaction mixture (total volume 700 μl) contained 54 mg $NADP^+$ (final concentration 100 mM), 6 μl [U-^{2}H] iPrOH **1** (final concentration 111 mM), 31 mg H_2folate (final concentration 100 mM), *Tris*/HCl buffer pH 7.5 (final concentration 233 mM), and dithiothreitol (DTT, final concentration 4 mM). The pH was adjusted to 7.7 (at 37°C) with 10 M NaOH and the reaction was initiated by adding 50 units of *ec*DHFR and 25 units of *tb*ADH. The reaction was incubated at 37°C under argon atmosphere and its progress was monitored.‡ Due to the inherent instability of H_4folate, the synthesis was performed under the same strict anaerobic conditions as the *R*-[6-^{1}H; 11-^{X}H] CH_2H_4folate. After H_4folate was obtained, labeled formaldehyde **4** was added into the reaction mixture followed by incubation for half an hour at 4°C. The progress of this reaction was also monitored by analytical HPLC (e.g. Figure 1).

The above reaction mixture was filtered through Centricell 20 (10 000 NMWL) to remove the enzymes. The mixture was then sealed under argon and preserved at −80°C. The identity and purity of the *R*-[6-^{2}H; 11-^{X}H] CH_2H_4folate product were verified by its use as a cofactor in the TS-catalyzed reaction. The labeled cofactor was fully consumed by excess dUMP in the presence of *E. coli* TS.

‡Aliquots (2 μl) from the reaction mixture were diluted with 120 μl Tris buffer (pH 7.5, 400 mM) and analyzed by RP HPLC (see above HPLC analysis and separation). The progress of the reaction was determined by UV chromatogram at 298 nm.

Kinetic measurements

R-[6-^{Y}H; 11-^{X}H] CH_2H_4folate **5** was used as a cofactor in the measurement of 2° KIE with *ec*TS. 2° KIEs were measured competitively on the second-order rate constant (V/K). All the measurements of H/T KIE ($^{T}(V/K)_{H}$ the enzymologist's nomenclature) and D/T KIE ($^{T}(V/K)_{D}$) were performed in 100 mM *Tris*-buffer (pH 7.5), 50 mM β-mercaptoethanol and 1 mM EDTA. Prior to the kinetic experiments, the tritiated cofactor (trace *R*-[6-^{Y}H; 11-^{3}H] CH_2H_4folate in C11 protiated or deuterated CH_2H_4folate for H/T or D/T KIE experiments, respectively) and ^{14}C-labeled substrate ([2-^{14}C] dUMP) were mixed (typically 2 Mdpm ^{3}H with 0.5 Mdpm of ^{14}C). To enable measurement of the fractional conversion of CH_2H_4folate to dTMP, the [2-^{14}C] dUMP was in 40–50% molar excess over the CH_2H_4folate as described in more detail elsewhere.[14,19] The reaction mixture (final volume 1.1 ml) was preequilibrated at the experimental temperature. An aliquot of 100 µl was removed and quenched in 30 µM (stock solution) 5-fluoro-2′-deoxyuridine 5′-monophosphate (F-dUMP, a specific inhibitor of TS with $K_i = 1$ nM) and used to test the radio purity of the reactants. The reaction was then initiated by addition of the enzyme and five 100 µl aliquots were removed at 2 min intervals and quenched in 30 µM F-dUMP. Finally, a concentrated enzyme was added to the rest of the reaction mixture and incubated at the same temperature for an additional 10 min to achieve complete conversion (infinity time points, R_∞). For each experiment, three infinity points were removed and quenched as described above. After quenching, all of the samples were frozen and stored at −80°C prior to RP HPLC analysis. The ratio of ^{3}H/^{14}C in the product dTMP and the fractional conversion (f) was determined by RP HPLC separation, followed by fraction collection and liquid scintillation counting (LSC) analysis (Figure 2). To calculate a 2° KIE, three values were measured: the ratio of ^{3}H/^{14}C in the product at each time point (R_t), the ratio of ^{3}H/^{14}C at the infinity time points (R_∞), and the fractional conversion (f). The KIE was then calculated using the equation[20]

$$\mathrm{KIE} = \frac{\ln(1-f)}{\ln\left(1 - f\dfrac{R_t}{R_\infty}\right)} \tag{1}$$

Figure 3 presents a typical plot of 2° H/T KIE vs fractional conversion. The fact that KIEs are f independent within experimental error serves as a good indication that no experimental artifact has affected the measurement.[20,21] The value of the observed KIE was the average of at least three independent experiments with five time points and three infinity points each.

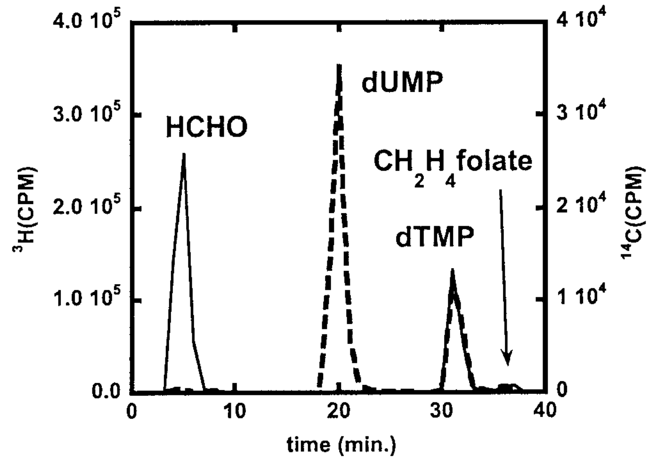


Figure 2. HPLC ^{3}H (solid trace) and ^{14}C (dashed trace) radiograms of *R*-[6-^{1}H; 11-^{3}H] CH_2H_4folate and [2-^{14}C] dUMP mixture 15 min after adding 0.014 units of TS. The fraction conversion (*f*) is 95%

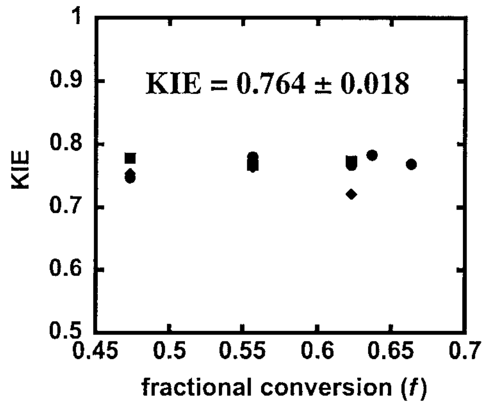


Figure 3. Competitive KIEs at 20°C: 2° $^{T}V/K_H$ are plotted vs fractional conversion (*f*). Different shapes indicate independent experiments (triplicate)

Conclusion

In this communication, we describe a chemo-enzymatic synthesis of *R*-[6-^{Y}H; 11-^{X}H] CH_2H_4folate that results in a short, microscale, and rather simple procedure. We also demonstrated the utility of this cofactor in measuring the 2° KIEs of the TS catalyzed reaction. The synthetic procedure described here, led to four mixtures of isotopically labeled *R*-[6-^{1}H; 11-^{X}H] CH_2H_4folate cofactors. These mixtures were then used for 2° H/T or D/T KIE measurements with H-transfer or D-transfer from the 6*R* position of

CH_2H_4folate (step 6 in Scheme 2) with the enzyme *ec*TS. These studies examine the possible contribution of H-tunneling from the breakdown of the Swain–Schaad exponential relationship as well as coupled motion[9] between 1° and 2° hydrogens as described elsewhere.[22] This labeling pattern may also be applied to the kinetic study of other folate-dependent enzymes.[2] The procedure described here can easily be modified to synthesize other labeling patterns of *R*-[6-^{1}H; 11-^{X}H] CH_2H_4folates for a wide variety of experiments.

Acknowledgements

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