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Synthesis and physicochemical characterization of (6*S*)-5-formiminotetrahydrofolate; a reference standard for metabolomics†

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The one-carbon carrier of the formate oxidation level derived from the interaction of tetrahydrofolate and formiminoglutamate, which has been tentatively identified as 5-formiminoltetrahydrofolate, has been prepared by chemical synthesis. Treatment of a solution of (6*S*)-tetrahydrofolate in aqueous base with excess ethyl formimidate in the presence of anti-oxidant under anaerobic conditions afforded a gummy solid which, based on mass spectral analysis, conformed to a monoformimino derivative of tetrahydrofolate. Further physicochemical characterization by validated methods strongly suggested that the product of chemical synthesis was identical to the enzymatically produced material and that it was, in fact, (6*S*)-5-formiminotetrahydrofolate. Conditions and handling methods toward maintaining the integrity of this highly sensitive compound were identified and are described, as is analytical methodology, useful for research studies using it.

Introduction

Formiminotetrahydrofolate, formiminoglycine and formiminoglutamic acid, are among the few natural compounds containing the formimino functionality; all three metabolites are said to be short-lived.[1] This one-carbon carrier of carbon at the formate oxidation level is derived from the interaction of tetrahydrofolate and formiminoglutamate;[2] it is converted to 5,10-methenyltetrahydrofolate, which is involved in the purine and methionine cycles,[3] by formiminotransferase cyclodeaminase.[2] As such, it is an important tool in pharmacological and metabolomics studies. Along with the one-carbon carriers 5,10-methenyl- and 10-formyltetrahydrofolate, formiminotetrahydrofolate has been identified as being unstable in solution over the physiological pH range.[4] On the other hand, enzymatically prepared formiminotetrahydrofolate has been reported to be relatively stable when stored in the cold.[2] Using the Cahn–Ingold rules to define the stereochemistry at C-6, one-carbon carriers derived from (6*S*)-tetrahydrofolate that are substituted at the 5-position retain the (6*S*)-designation (*e.g.*, (6*S*)-5-formyltetrahydrofolate) but substitution at the 10-position alters the substituent hierarchy leading to (6*R*)-designation (*e.g.*, (6*R*)-5,10-methenyltetrahydrofolate). Hence the enzymatically prepared product could be (6*S*)-5-formiminotetrahydrofolate or (6*R*)-10-formiminotetrahydrofolate.

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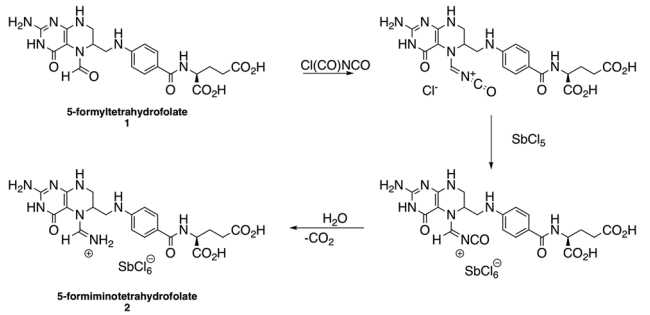
Results and discussion

Synthesis

The literature report wherein a formyl group was aminated to the corresponding formimino group by the action of *N*-(chlorocarbonyl)isocyanate in the presence of antimony pentachloride[5] was attractive as the most unambiguous and straightforward way to approach the chemical synthesis of (6*S*)-5-formiminotetrahydrofolate (**2**). Starting from commercially available (6*S*)-5-formyltetrahydrofolate (**1**) the product **2** would be isolated as a hexachloroantimonate (Scheme 1).

However, considering the difficulties associated with the preparation and handling of *N*-(chlorocarbonyl)isocyanate, this approach was not pursued. Instead, the preparation of **2** by treatment of tetrahydrofolate (**3**) with ethyl formimidate[1,6] was explored. Based on the regeneration of the 1,4-bisformimino grouping in acanthoine[1] by treatment of the 1,4-diamine obtained by hydrolysis of acanthoine with boiling 10% sodium hydroxide with ethyl formimidate (ESI Scheme 1†), it was anticipated that the reaction of a large excess of ethyl formimidate with tetrahydrofolate (**3**) could produce 5-formiminotetrahydrofolate (**2**), 10-formiminoltetrahydrofolate (**4**), or 5,10-bisformiminotetrahydrofolate (**5**), but not the cyclic



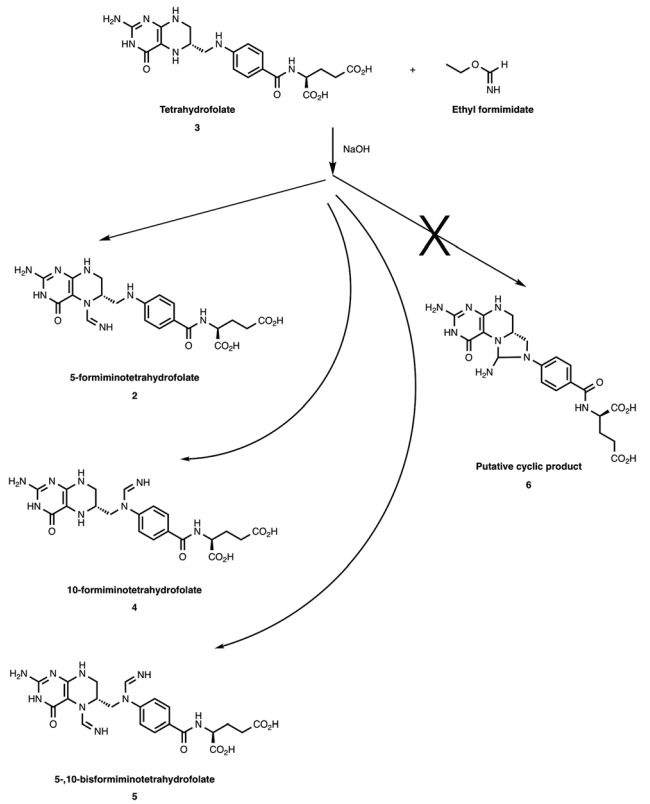
Scheme 1 Unambiguous chemical synthetic route to (6*S*)-5-formiminotetrahydrofolate (**2**).

material (**6**) considered as a possible structure of the enzymatic product (Scheme 2).[2]

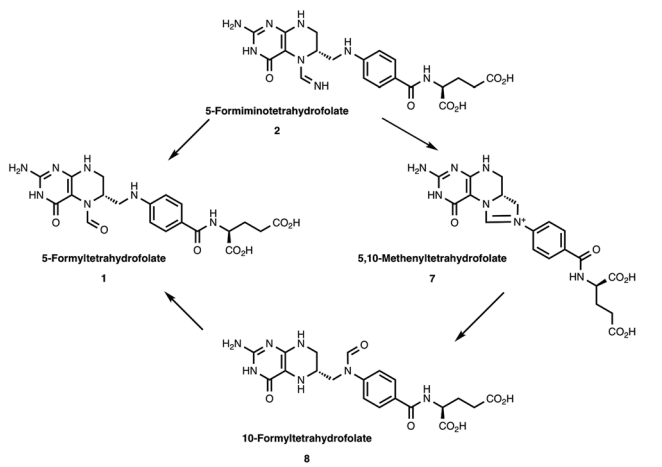
Based on cost considerations probe experiments were carried out using (6*RS*)-tetrahydrofolate (*RS*-**3**), as a model for the more expensive (6*S*)-tetrahydrofolate (**3**). Treatment of a solution of *RS*-**3** in aqueous sodium hydroxide with ethyl formimidate, under strictly anaerobic conditions and in the presence of the oxidation inhibitor β-mercaptoethanol (BME), gave a product with mass consistent with a formimino derivative of tetrahydrofolate (*m*/*z* $[M + H]^+$ calcd for $C_{20}H_{24}N_8O_6$: 473.1892; found: 473.1926). Monitoring the reaction progress by HPLC showed the concentration of this product to increase for the first 30 minutes of the reaction, and then to decrease, with concomitant appearance of a product with *m*/*z* 456 and two products with *m*/*z* 474. This suggested that 5-formiminotetrahydrofolate (*RS*-**2**) was formed, hydrolyzed to 5-formyltetrahydrofolate (*RS*-**1**) (*m*/*z* $[M + H]^+$ calcd for $C_{20}H_{23}N_7O_7$: 474.14) under the basic conditions, and also cyclized to 5,10-methenyltetrahydrofolate (*RS*-**7**) (*m*/*z* $[M]^+$ calcd for $C_{20}H_{22}N_7O_6$: 456.16). The latter is known to rapidly react with base[7,8] to form 10-formylterahydrofolate (*RS*-**8**) (*m*/*z* $[M + H]^+$ calcd for $C_{20}H_{23}N_7O_7$: 474.14), which would convert to its thermodynamically favored isomer, 5-formyltetrahydrofolate (*RS*-**1**) as shown in Scheme 3.

HPLC and UPLC/MS analysis (Fig. 1) of the reaction mixture confirmed the presence of 5,10-methenyltetrahydrofolate (**7**), as well as of 5- and 10-formyltetrahydrofolate (**1** and **8**, respectively), consistent with this explanation (Scheme 3). Isolation of a small amount of the product with *m*/*z* $[M + H]^+$ 473 by preparative HPLC gave a gummy solid that, based on HPLC analysis, appeared to be stable for at least a week, when kept refrigerated in neutral buffer.

To isolate the product obtained by chemical synthesis on a preparative scale, the conditions utilized in the enzymatic synthesis were optimized. Thus, to mimic the literature conditions,[2] the reaction mixture obtained by treatment of (6*RS*)-tetrahydrofolate (*RS*-**3**) with excess ethyl fomimidate in aqueous base containing BME for 30 minutes (maximum product) was diluted with 0.2 M potassium phosphate buffer (pH 6.8), applied directly to a Dowex column, and eluted with a gradient of 0.3 to 1 M acetic acid at 2 °C. Although the collected fractions were highly enriched in the desired product assumed to be *RS*-**2**, these conditions were deemed inappropriate for preparative scale. A modified protocol in which the reaction mixture was rapidly diluted with cold (0 °C) water and, working rapidly at 2 °C, directly chromatographed on a solid phase extraction cartridge, eluting with 0.1 M acetic acid, afforded pure *RS*-**2**. These conditions were used to prepare the desired diastereoisomer, (6*S*)-5-formiminotetrahydrofolate (**2**). Because isolation of **2** had given a gummy solid, the pure product was formulated in 0.1 M acetic acid solution. Aliquots



Scheme 2 Putative reaction products of treatment of tetrahydrofolate (**3**) with excess ethyl formimidate.



Scheme 3 Degradation of 5-formimnotetrahydrofolate (**2**).

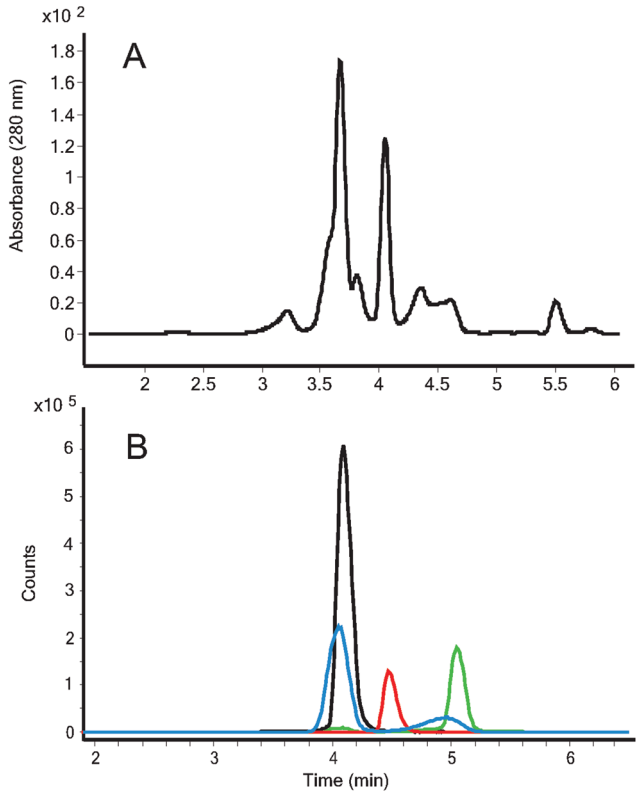


Fig. 1 LC-MS analysis of (6*S*)-5-formiminotetrahydrofolate. (A) UV trace at 280 nm. (B) Extracted ion chromatograms are shown for the expected ions (*m*/*z*) for 5-formiminotetrahydrofoate (**2**) (black, 473), tetrahydrofolate (**1**) (red, 446), 5-formyl/10-formyltetrahydrofoate (**3**/**5**) (blue, 474) and 5,10-methenyltetrahydrofolate (**4**) (green, 456).

(10 mL) of the solution of **2** were dispensed into vials (20 mL), and argon gas was bubbled into the solution before the vials were crimp-sealed and stored at −80 °C.

Quantitation

To accurately determine the concentration of **2** we followed the literature approach[2] wherein **2** was quantitated by converting it to **7**. To determine the optimal conditions for the conversion of **2** to **7** the reaction progress at room temperature was monitored by HPLC using UV/vis absorbance at 368 nm (λ_{max} of **7**) and 280 nm (λ_{max} of **2**). It was found that – after addition of a drop of hydrochloric acid to a solution of **2** in 0.1 M acetic acid – the amount of **2** (280 nm) decreased over a 3-hour period, with a concomitant increase in the amount of **7** (368 nm) (ESI Fig. 1†). Separately, a Beer's Law[9,10] dose–response curve was prepared (ESI Fig. 2†) using commercially procured (6*RS*)-5,10-methenyltetrahydrofolate (*RS*-**7**) chloride. The ampouled solution of **2** in 0.1 M acetic acid was quantitated by conversion to **7** (under the optimized conditions), and determining the concentration of **7** in the resulting solution by HPLC from the Beer's Law dose–response curve for *RS*-**7**. The converted sample was found to contain 0.000145 mmol mL^{-1} of **7**, corresponding to 0.069 mg mL^{-1} of **2** in the ampouled sample.

Physicochemical characterization

Purified (6*S*)-5-formiminotetrahydrofolate (**2**) that had been stored ampouled in 0.1 M acetic acid at −80 °C for two weeks was characterized using a combination of UPLC/UV and LC-MS (Fig. 2). As part of the characterization, a gradient RP-UPLC/UV method for determination of related organic impurities was developed and qualified for use in determining the chromatographic purity of **2**. The UPLC/UV impurity method was found to be acceptable for analysis of **2** in the presence of related organic impurities **6**. The UPLC analysis (Fig. 2) revealed that **2** eluted as a single peak with a chromatographic purity of 94.2% (280 nm). The peak corresponding to **2** was well-resolved from (6*R*)-5,10-methenyltetrahydrofolate (**7**), which was present at 1.8%. The presence of a UV-transparent dimer related to BME was also confirmed as a late-eluting peak in the chromatogram (*m*/*z* $[M + NH_4]^+$ and $[M + Na]^+$ calcd for $C_4H_{10}O_2S_2$:172 and 177; found: 172, 177).

The high-resolution ESI^+ mass spectrum (Fig. 2, inset) of **2** exhibited the $(M + H)^+$ ion (*m*/*z* 473.1926) as the base peak, which is within 7.2 ppm of the theoretical value for $C_{20}H_{24}N_8O_6$, obviating the possibility of the product being a bis-formimino adduct, but leaving open the question of whether the chemically synthesized product is one of the expected monosubstituted products, 5-formiminotetrahydrofolate (**2**), or 10-formiminoltetrahydrofolate, or the cyclic material **6** considered as a possibility by Tabor.[2]

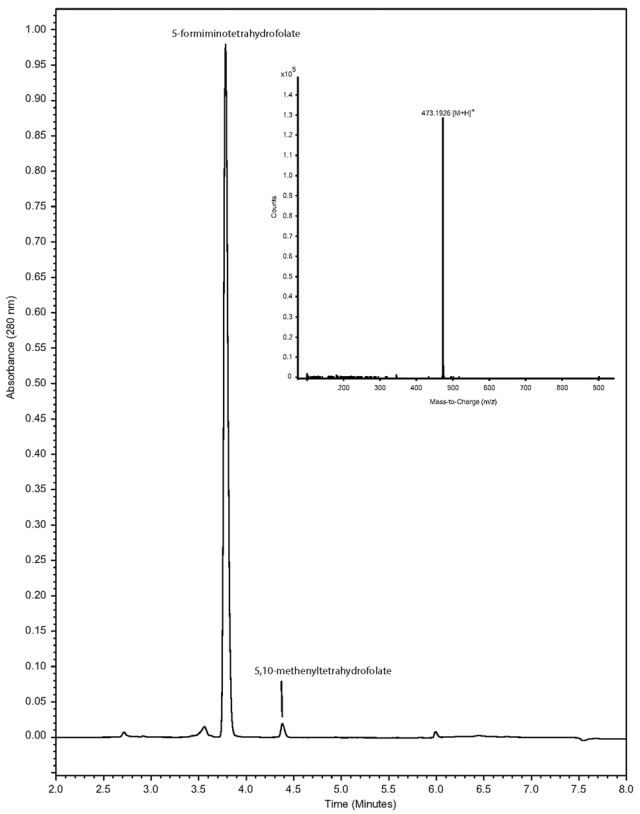


Fig. 2 Analysis of (6*S*)-5-formiminotetrahydrofolate (**2**) by UPLC/UV and LC-MS.

The UV/vis spectrum (ESI Fig. 3,† Panel A) corresponding to **2** exhibited two maxima at 222 nm and 286 nm, consistent with the reported UV for the enzymatically produced product.[2]

The ^{1}HNMR spectrum of **2** was consistent but not confirmatory of the structure (data not shown) and the ^{13}C NMR spectrum could not be obtained due to the limited solubility of **2**.

Structural characterization

Of the four possible products of the treatment of (6*S*)-tetrahydrofolate (**3**) with excess ethyl formimidate in the presence of base, the bis-adduct can be eliminated based on the molecular formula determined mass spectrometrically. To unambiguously determine the site of attachment of the formimino group NMR correlation studies (COSY, ASQC, HMBC) were carried out; unfortunately, these studies, performed at room temperature to minimize sample degradation, proved inconclusive. In fact, the ^{1}HNMR of the synthetic product was difficult to interpret, possibly due to conformational and configurational heterogeneity. If the chemically produced product were 5-substituted, two rotamers may be detected by NMR: one rotamer with the formimino nitrogen *anti* to the isocytosine ring and *syn* to the 9-methylene (**A**, R = NH, Fig. 3), and a second rotamer with the formimino nitrogen *syn* to the isocytosine ring and *anti* to the 9-methylene (**B**, R = NH); these rotamers may, or may not, be present in equal concentrations. Likewise, if 10-formiminotetrahydrofolate (**4**) were the chemically produced product it could exist as a mixture of the rotamers **C** (R = NH) and **D** (R = NH) (Fig. 3) where the imino nitrogen would be *syn* to the 9-methylene and *anti* to the phenyl ring in **C** and *vice versa* for **D**. This situation, resulting from restricted rotation around the carbon–nitrogen bond in amides, has been described for the analogous 5- and 10-formyltetrahydrofolates (**1** and **8**, resp)[11–13] and can be observed in the ^{1}H NMR spectrum of 5-formyltetrahydrofolate (**1**) shown in ESI Fig. 8.† At the same time, comparison of the chemical shifts of the aromatic protons of the synthetic product (at 6.6 and 7.6 ppm, ESI Fig. 4†) to the shifts reported[11–13] and observed (6.4–6.8 and 7.6–7.9 ppm) for 5-formyltetrahydrofolate (**1**, ESI Fig. 5†) and not those reported[8,9] and observed (7.5 and 7.9 ppm) for 10-formyltetrahydrofolate (**8**, ESI Fig. 6†), strongly supports attachment of the formimino group at N-5 and not at N-10 in the chemically synthesized product.

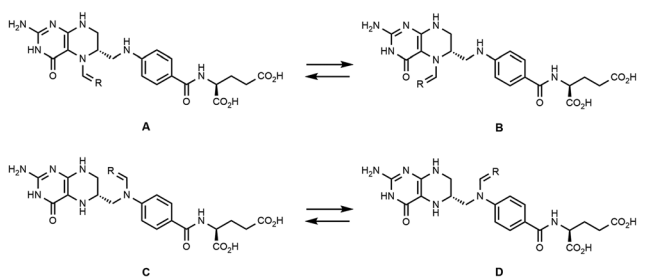


Fig. 3 Conformational heterogeneity of (6*S*)-5-formyl-/(6*S*)-5-formiminotetrahydrofolate (**1**/**2**, resp) and (6*R*)-10-formyl-/(6*R*)-10-formiminotetrahydrofolate (**8**/**4**, resp).

However, it could be argued that this comparison may be invalid since, due to the extreme insolubility of **2** in water, the ^{1}H NMR spectrum was recorded in deuterated dimethylsulfoxide, whereas the ^{1}H NMR spectra of **1** and **8**, which as the calcium salt and the potassium salt, respectively, are insoluble in dimethyl sulfoxide, were recorded (on the same instrument) in deuterated water. In an attempt to address this concern, the ^{1}H NMR spectrum of a saturated solution of **1** was recorded in deuterated dimethylsulfoxide. The noisy spectrum (ESI Fig. 7†) showed the aromatic resonances to occur at 6.60 and 7.59 ppm; addition of 5 drops of deuterated water significantly improved the appearance of the spectrum (ESI Fig. 8†) but did not change the chemical shifts of the aromatic protons, which were 6.60 and 7.62 ppm, in good agreement with the chemical shifts observed by us for **1** in deuterated water, 6.73 and 7.66 ppm. The noisy spectrum of 5-formyltetrahydrofolate (**1**) in dimethyl sulfoxide (ESI Fig. 7†) also showed multiple downfield resonances that disappeared upon addition of 5 drops of water (ESI Fig. 8†) consistent with the observation of exchangeable protons in dimethyl sulfoxide but not in water and suggesting that, similarly, the downfield resonances observed in the spectrum of **2** are associated with exchangeable protons. In summary, we therefore conclude that the chemically prepared product is, indeed, 5-formiminotetrahydrofolate **2**.

But are the chemically and enzymatically prepared formiminotetrahydrofolates one and the same? The fact that the product obtained by formimidation of tetrahydrofolate, and the enzymatically produced formiminotetrahydrofolate possess very similar properties suggests that they are the same. In particular, the UV spectrum (λ_{max} 286.3 nm) for the chemically synthesized compound matches the reported value,[2] as does the overall behavior of the two compounds. Our study, therefore, confirms the structural assignment of the enzymatic product as (6*S*)-5-formiminotetrahydrofolate, as proposed by Tabor.[2]

Stability

Overall, chemically prepared (6*S*)-5-formiminotetrahydrofolate (**2**) has been found to be reasonably stable. Lyophilization of high purity chromatographic fractions affords a gummy solid that is relatively stable in the cold (−20 to −80 °C), in agreement with the literature.[2] However, handling of this material is extremely difficult, particularly for quantitation and physicochemical characterization, with anaerobic conditions being essential to maintain the integrity of this material. At room temperature solutions of **2** were found to degrade at 15% per day, even under anaerobic conditions but brief (2-hour) exposure to room temperature conditions led to minimal (*ca.* 2%) degradation. Solutions in dilute acetic acid afford good compound stability at −80 °C, but not at −20 °C.

The observed decomposition of **2** in base is worthy of some discussion. It has been shown that treatment of formimidated amines with strong base while heating, removes the formimino functionality, generating the corresponding amine.[1] For **2**, this would regenerate the starting (6*S*)-tetrahydrofolate (**3**). While

this study did not explore this reaction, the fact that the conversion of **3** to **2** (which is carried out in basic medium) never went to completion despite the use of a large excess of ethyl formimidate, is consistent with this possibility. On the other hand, since the conversion of **3** to **2** was carried out at low temperature, deformylation may not take place although ammonia may be stripped from the formimino functionality, leaving behind a formyl group, thereby accounting for the formation of 5-formyltetrahydrofolate (**1**) even before 5,10-methenyltetrahydrofolate (**7**) is detected. The observed decomposition products of **2** in dilute acid are consistent with slow cyclization and hydrolysis to **7** and **1**, respectively.

Experimental

Instrumentation

^{1}HNMR spectra were recorded on a Bruker Avance 300 MHz spectrometer or a 500 MHz NMR spectrometer. Low-resolution LC-MS data were obtained using a PerkinElmer API 150 EX mass spectrometer outfitted with an ESI (turbospray) source, coupled to a PerkinElmer 200 Series liquid chromatography system. High resolution LC/MS analysis was performed on an Agilent system consisting of a 1290 Infinity UPLC coupled to a 6230 accurate-mass time-of-flight mass spectrometer with a Dual AJS ESI source. Mass spectral data were acquired in positive-ion mode over the range of 100–1700 *m*/*z* using a gas temperature of 350 °C, a nozzle potential of 1000 V, and a capillary potential of 3500 V. HPLC analyses were performed either on a dual pump system consisting of two HPLC pumps (Varian Prostar 210 solvent system delivery system), a Rheodyne injector and a Varian ProStar 335 DAD UV detector (or a Varian ProStar 320 UV detector) controlled by Varian Star Workstation software or on Agilent HPLC 1100 system (two HPLC pumps, an autosampler, and a diode-array detector) controlled by ChemStation HPLC software.

Materials

The chemicals, reagents and solvents used in the synthesis were inspected and released for use based on visual inspection and conformance of the individual lot with the manufacturer's Certificate of Analysis. (6*S*)-Tetrahydrofolate and 5,10-methenyltetrahydrofolate chloride were purchased from Schircks Laboratories; (6*RS*)-tetrahydrofolate and ethyl formimidate were from Sigma; solvents were from Sigma Aldrich. UPLC analyses were performed using HPLC-grade acetonitrile (Fisher), HPLC-grade ammonium acetate (Fisher), and in-house de-ionized water. For high-resolution LC-MS, water and acetonitrile were obtained from Fisher (Optima LC/MS grade), and ammonium acetate was obtained from Fluka (LC-MS Ultra grade). All other buffers and reagents were reagent grade or better.

Synthesis

All solvents and solutions were purged with argon immediately before use and were kept ice-cold.

(6*S*)-5-Formiminotetrahydrofolate (2). To (6*S*)-tetrahydrofolate (**3**, Scheme 2) (140 mg, 0.31 mmol) and ethyl formimidate hydrochloride (679 mg, 6.2 mmol) in a centrifuge tube (50 mL) was added BME (1.6 mL), followed by aqueous NaOH (2 N, 3.4 mL, 6.8 mmol). Argon gas was bubbled into the mixture for 1 min. All the solids dissolved. After stirring at RT under argon for 30 min the yellow solution was mixed with cold (0 °C) H_2O (400 mL). The resulting slightly yellow solution was passed through a Phenomenex Strata-XL-A (strong anion) solid phase extraction (SPE) tube (10 g per 60 mL), activated with CH_3OH (60 mL) and equilibrated with H_2O (60 mL, 0 °C) before use, under N_2 pressure. The SPE tube was washed with H_2O (50 mL, 0 °C), CH_3OH (30 mL) then with 0.1 M HOAc (4 × 100 mL, 0 °C); the eluent was collected into Erlenmeyer flasks that were cooled in an ice bath during and after elution. The combined eluent, containing 95% pure **2**, (HPLC: Phenomenex Kinetex EVO C18, 150 × 4.6 mm, 2.6 μm: 25 mM Na_2HPO_4/H_2O, 0.8 mL min^{-1}, λ 280 nm, λ 298 nm) was divided into two portions that were lyophilized overnight to give pale brown solids (16 mg and 15 mg respectively). Repeat of the preparation on the 140 mg scale of **3** gave consistent results but attempted scale-up to 280 mg of **3** led to a significantly lower yield of **2**.

Purification. Batches of **2** with different purities, obtained from several preparations and stored at −80 °C, were allowed to warm to RT, and then combined in 0.1% NH_4OH (392 mL, ice-cold). HPLC analysis (*vide infra*) showed the resulting solution to contain 90% pure **2**. The solution was passed through a Phenomenex Strata-XL-A (polymer strong anion) solid exchange Giga tube (10 g per 60 mL), activated with CH_3OH (50 mL) and equilibrated with H_2O (50 mL) before use dropwise (under nitrogen pressure). The SPE tube was washed with H_2O (50 mL, ice-cold), and then eluted with 0.1 M HOAc–H_2O (ice-cold) to give several fractions containing **2** with different purities. Highest purity fractions were combined to give **2** with 96.3% purity (λ 280 nm) in 0.1 M HOAc solution. MS *m*/*z* calcd for $C_{20}H_{24}N_8O_8$: 472; found: 473 $(M + H)^+$; 471 $(M - H)^-$. Aliquots (10 mL) of the solution of **2** were dispensed into vials (20 mL); argon gas was bubbled into the solution before the vials were crimp-sealed and stored at −80 °C.

Determination of the solution concentration of (6*S*)-5-formiminotetrahydrofolate (2). A solution of **2** in 0.1 M HOAc containing 1 M BME was de-aerated with argon gas and then treated with HCl. Aliquots were analyzed by HPLC (Phenomenex Kinetex EVO C18 150 × 4.6 mm, 2.6 μm, A: [25 mM Na_2HPO_4/H_2O], B: CH_3CN, 2% B, 0.7 mL min^{-1}, λ 368 nm, λ 280 nm) (ESI Fig. 1†). Separately, authentic (6*RS*)-5,10-methenyltetrahydrofolate (*RS*-**7**) chloride (2.494 mg, 5.07×10^{-3} mmol) was weighed in an aluminum weighing boat that was then placed into a volumetric flask (25 mL). Methanol (3 mL) and 0.1 M HOAc were added to volume and the sample was sonicated to achieve solution (2.028×10^{-4} M). A Beer's Law dose–response curve (ESI Fig. 2†) was generated by HPLC (*vide infra*) of aliquots (0.5, 1, 2, 3, 4, and 5 μL) of the solution. Plotting the injected amount (μmol, *Y*) against the UV response (integrated area, *X*) gave a straight line ($Y = 10^7X + 0.0394$, $R^2 = 0.9995$).

The concentration of the purified sample of (6*S*)-5-formiminotetrahydrofolate (**2**) in 0.1 M HOAc solution was determined by adding BME (70 μL) to an aliquot (0.93 mL), bubbling argon gas into the solution and adding concentrated HCl (20 μL). Bubbling with argon gas and stirring at RT was continued for 4 hours. To determine the concentration of **7** generated from **2** an aliquot (5 μL) of the reaction mixture was quantitated by HPLC under the conditions utilized to generate the Beer's Law dose–response curve and the peak area was used to calculate the molar amount of the injected material. After correcting for the initial amount of **7** in **2** the concentration of **2** was calculated to be 0.069 mg mL^{-1} (0.145 mM).

Analysis

UPLC/UV analysis was performed using a Waters Acquity UPLC system with a photodiode array detector, Waters Acquity HSS T3, 2.1 × 100 mm, 1.8 μ, A: 10 mM NH_4OAc pH 6.8, B: CH_3OH, 0.2 mL min^{-1}, with a linear gradient of 0–20% B from 0–4 min, 20–90% 4–5 min, hold at 90% B 5–6 min, and re-equilibration to 0% B 6.1–10 min. The column temperature was 25 °C, and the auto-sampler was chilled to 5 °C to minimize sample degradation during analysis. The nominal injection parameters were 2 μL of a 0.1 mg mL^{-1} solution. UV detection was at 280 nm, and the UV spectrum (190–400 nm) was included to support peak identification.

UPLC method qualification

Linearity. To verify that analyzed sample concentrations were well within the linear range of the detector response for the analyte, method linearity was determined by single analysis of three test concentrations of 5-formiminotetrahydrofolate (**2**) over the nominal concentration range of 0.01 mg mL^{-1} to 0.15 mg mL^{-1} (*i.e.*, 10% to 150% of the target analyte concentration of 0.10 mg mL^{-1}). To simulate sample concentrations in the range of 0.05–0.15 mg mL^{-1} (50%, 100% and 150% of target concentration) three separate direct injections of the diluted stock solution at 1, 2 and 3 μL were made. An additional solution was prepared by 10-fold dilution of the stock sample solution (nominally at 0.1 mg mL^{-1} in 0.1 M HOAc) into 0.1 M HOAc to produce a test solution at 0.01 mg mL^{-1} (10% of the target concentration). This solution was also analyzed for linearity using a 2 μL injection. For this qualification, the main peak corresponding to **2** was integrated for each linearity injection, and analyte response (peak area) was plotted *versus* concentration. The data were fit using linear regression techniques and the results showed a best-fit line with r^2 = 0.9999, and % error within 5.0% at all 4 concentrations, establishing a linear range (peak area) for 5-formiminotetrahydrofolate from 0.01–0.15 mg mL^{-1}.

Limit of detection (LOD). The limit of detection for the impurity method was visually confirmed at a level ≥0.2% of the target assay concentration (0.1 mg mL^{-1}), corresponding to ≥0.2 μg mL^{-1}. The analyte peak height at 0.2% of the target assay concentration was measured and the signal-to-noise level was determined to be 22 : 1. Thus, the method is capable of detecting related organic impurities at ≥0.2% of the target assay concentration level for **2**.

Specificity. Specific confirmation of detection and separation of potential impurities was performed for 5,10-methenyltetrahydrofolate (**7**). Authentic (6*RS*)-5,10-methenyltetrahydrofolate (*RS*-**7**) chloride, as a solid, was used as a reasonable surrogate for (6*R*)-5,10-methenyltetrahydrofolate (**7**), which is the expected degradation product of (6*S*)-5-formiminotetrahydrofolate (**2**). A control solution at ~0.1 mg mL^{-1} in CH_3OH was prepared and analyzed (single injection) to determine the retention time of **7** relative to 5-formiminotetrahydrofolate (**2**). UPLC/UV results (Fig. 1) showed that **7** eluted after **2** (RRT 1.16) and was baseline-resolved from **2** by visual inspection. High-resolution LC-MS (Fig. 1 inset) confirmed the identity of both peaks. These results demonstrated the ability to discriminate between these two components.

Solution stability

A vial of **2** in 0.1 M HOAc was removed from storage at −20 °C, placed in an auto-sampler at 5 °C, and analyzed by LC-MS to monitor purity over a 72-h period. Based on peak area at 280 nm, the purity of **2** was found to decrease by *ca.* 15% per day, with the major degradation product being **7**, accompanied by **1**. Analysis of an ampouled solution of **2** in 0.1 M HOAc by LC-MS after 11-month storage at −80 °C showed sample purity of >94%. At 5 °C the sample purity decreased to 91.7% after 2 hours; the largest impurity was 5,10-methenyltetrahydrofolate (**7**) (~5% total area). The IDs of the main component (**2**) and of the known impurity **7** were confirmed by mass spectrometry.

Conclusions

The one-carbon carrier (6*S*)-5-formiminotetrahydrofoalte (**2**) has been prepared by chemical synthesis. Based on its properties, it appears to be the same as the enzymatic product derived from the interaction of tetrahydrofolate and formiminoglutamate, thus confirming the structural assignment of the enzymatic product as (6*S*)-5-formiminotetrahydrofolate, as proposed by Tabor.[2] Conditions for handling this sensitive compound have been determined, facilitating its use as a reference standard as well as in biochemical studies.

Conflicts of interest

There are no conflicts to declare.

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