Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

Formaldehyde regulates tetrahydrofolate stability and thymidylate synthase catalysis

Xiaolei Chen,[a] Sara Y. Chothia[a], Jaswir Basran[b] and Richard J. Hopkinson*[a]

[a]*Leicester Institute of Structural and Chemical Biology and School of Chemistry, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester, LE1 7RH, UK. E-mail: richard.hopkinson@leicester.ac.uk*

[b]*Department of Molecular and Cell Biology, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester, LE1 7RH, UK.*

Reagents

The pRSETA plasmid containing the thymidylate synthase gene (from Geneart) was a kind gift from Christopher Schofield (University of Oxford). The HEK293F cell pellet was a gift from Louise Fairall (University of Leicester). Other reagents, including media, buffers, folate derivatives, glutathione, nucleotide monophosphates, formaldehyde (HCHO) and D_2O, were purchased from Avocado Research Chemicals, Fisher Scientific, Melford Biolaboratories, Qiagen, Santa Cruz Biotechnology and Sigma Aldrich.

Tetrahydrofolic acid (THF) was purchased from Santa Cruz Biotechnology. To determine the purity and to assign any contaminants, we conducted NMR analyses on a portion of the batch, which was pre-dissolved in 1:1 v/v DMSO-d_6:H_2O (50 µL) and then diluted in ammonium formate buffer pH 7.5 (total sample volume = 600 µL). The major species in the sample (excluding buffer/solvent) after 10 minutes was THF; however, 2-mercaptoethanol, 2-mercaptoethanol disulphide, and three other folate derivatives were observed (Fig. S1). The three folate derivatives were assigned as 5,10-methylenetetrahydrofolic acid (CH_2-THF), N-(4-aminobenzoyl)-L-glutamic acid (ABG) and dihydrofolic acid (DHF) respectively. These assignments were determined on the basis of ^{1}H NMR, ^{1}H-^{1}H-COSY and ^{1}H-^{13}C-HSQC analyses, as well as by comparison with ABG and DHF standards (Fig. S6. Note: signal overlap precluded unequivocal assignment of DHF, which was present at low levels). Assignment of CH_2-THF resonances was also supported by reported studies.[1] The sample

was then analysed after 21 hours, which revealed changes in the concentrations of the folate derivatives. The concentration of THF markedly decreased, while the concentrations of ABG and CH_2-THF increased (Fig. S2). Given the *in situ* formation of ABG and CH_2-THF under the tested conditions, as well as their low initial concentrations after 10 minutes incubation, it is considered likely that most, if not all, the ABG and CH_2-THF in the mixture derives from THF degradation. However, the concentration of DHF (tentatively assigned) remained unchanged during the analysis, suggesting it is a low-level contaminant in the THF stock (<15 %). Overall, the combined THF, CH_2-THF and ABG concentrations in the sample suggest an initial THF:DHF ratio of 4.5:1 (>80 %), which was considered suitable for NMR analyses. Note: 2-mercaptoethanol also oxidised to 2-mercaptoethanol disulphide during the analysis.

Thymidylate synthase (TS) expression and purification

Expression: TS/pRSETA DNA was transformed into BL21 (DE3) cells and plated out onto LB/ampicillin plates. Colonies were taken from this plate and used to inoculate 100 mL of LB media containing ampicillin (50 µg/mL); the culture was grown overnight at 37 ^{o}C. 2YT media (in 2 L baffled culture flasks each containing 500 mL of media) was inoculated with 10 mL of the overnight culture and ampicillin (50 µg/mL). The cells were then grown at 37 ^{o}C (with shaking at 180 rpm) until OD_{600} = 0.8 (ca. 3.5 hours). The flask were then removed from the incubator and left at room temperature for 10 minutes before protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (at a final concentration of 200 µM). Cells were then returned to the incubator and grown for a further 24 hours at 20 ^{o}C. The cells were then harvested by centrifugation at 5500 g for 20 minutes.

Purification: A cell pellet from a 2 L culture was re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). One protease inhibitor tablet (Roche) was added and the suspension was sonicated (on ice, 6 x 30 s pulses with 30 s intervals at 12 microns). DNaseI, lysozyme and $MgCl_2$ (final concentration = 20 mM) were added to the sonicated cell suspension and stirred at 4 ^{o}C for 30 min and then centrifuged at 30000 g for 50 minutes. Clarified cell extract was loaded onto a 5 mL Ni-NTA affinity column (equilibrated with lysis

buffer) and washed with 100 mL of lysis buffer followed by 200 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8). TS was eluted from the column with a gradient (over 20 x CV) made from 50 mL of wash buffer and 50 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). 5 mL fractions were collected and those containing pure TS were pooled, concentrated to ca. 400 µL using a 10 K cut-off Millipore filtration unit, and dialysed overnight against 20 mM HEPES, 500 mM NaCl buffer, pH 7.5. Protein was then aliquoted, snap-frozen in liquid nitrogen and stored at -80 ^{o}C. SDS-PAGE analysis indicated a sample purity of >90 %. Production of TS was supported by whole protein mass spectrometry analysis. All buffers were pre-calibrated to the required pH using either HCl or NaOH.

Nucleotide sequence of insert: ATGCCGGTTGCAGGTAGCGAACTGCCTCGTCGTCCG
CTGCCTCCGGCAGCACAAGAACGTGATGCAGAACCGCGTCCGCCTCATGGTGAACTG
CAGTATCTGGGTCAGATTCAGCATATTCTGCGTTGTGGTGTTCGTAAAGATGATCGCAC
CGGTACAGGCACCCTGAGCGTTTTTGGTATGCAGGCACGTTATAGCCTGCGTGATGAA
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GAGCTGTCAGCTGTATCAGCGTAGCGGTGATATGGGTCTGGGTGTTCCGTTTAACATT
GCAAGCTATGCACTGCTGACCTATATGATTGCACACATTACCGGTCTGAAACCGGGTGA
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CATCAAAATGGAAATGGCAGTTTA

Amino acid sequence (including N-terminal His-tag): MRGSHHHHHHGMASMTGGQQ MGRDLYDDDDKDRWGSEMPVAGSELPRRPLPPAAQERDAEPRPPHGELQYLGQIQHILR CGVRKDDRTGTGTLSVFGMQARYSLRDEFPLLTTKRVFWKGVLEELLWFIKGSTNAKELSS KGVKIWDANGSRDFLDSLGFSTREEGDLGPVYGFQWRHFGAEYRDMESDYSGQGVDQL QRVIDTIKTNPDDRRIIMCAWNPRDLPLMALPPCHALCQFYVVNSELSCQLYQRSGDMGLG VPFNIASYALLTYMIAHITGLKPGDFIHTLGDAHIYLNHIEPLKIQLQREPRPFPKLRILRKVEKI DDFKAEDFQIEGYNPHPTIKMEMAV

NMR Experiments

General: Characterisation of degradation products were conducted on either a Bruker AVIII 500 MHz NMR spectrometer equipped with a 5 mm BBO probe with z-Gradients and installed with TopSpin 3.5 software, or a Bruker AVIII HD NanoBay 400 MHz NMR spectrometer equipped with a 5 mm BBFO probe with z-Gradients and installed with TopSpin 3.6 software. Time-course experiments were conducted on a Bruker AV 500 MHz NMR spectrometer equipped with a 5 mm TXI probe with z-Gradients and installed with TopSpin 2.1pl8 software. All experiments used 5 mm diameter NMR tubes (GPE Scientific) and samples were pre-mixed in microcentrifuge tubes before transfer to NMR tubes and NMR analysis at 298 K or 295 K. ^{1}H NMR resonances are reported relative to the residual solvent water resonance (δ_H 4.7 ppm). Data analysis was conducted using TopSpin 4 software.

Degradation of tetrahydrofolic acid (THF) and derivatives: Samples were prepared by adding 6 µL of a stock solution containing the folate derivative (50 mM in 50% v/v H_2O 50% v/v DMSO) to a pre-prepared mixture of ammonium formate buffer pH 7.5 (511 µL), H_2O (18 µL), D_2O (60 µL) and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP, 5 µL of a 10 mg/mL stock in D_2O). The mixtures were then monitored by ^{1}H NMR with water suppression immediately after mixing, and after 20 hours. The sample monitoring degradation of 5,10-methylenetetrahydrofolic acid (CH_2-THF) was prepared by combining THF and HCHO (833 µM final concentration), which induced formation of CH_2-THF *in situ*. For detection of HCHO production during THF degradation, a sample was prepared containing THF and a 10-fold

excess of 5,5-dimethylcyclohexane-1,3-dione (dimedone, added from a stock of 500 mM in DMSO-d_6).

Time-course experiments monitoring THF and HCHO: Samples were prepared containing THF (6 µL from a stock of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6), ammonium formate buffer pH 7.5 (524 µL), HCHO (from stocks of differing concentrations in H_2O, 5 µL), TSP (5 µL of a 10 mg/mL stock in D_2O) and D_2O (60 µL). The mixtures were then immediately transferred to NMR tubes and monitored by ^{1}H NMR with water suppression over 10-12 hours; each ^{1}H NMR experiment consisted of 32 transients, corresponding to 222 seconds total acquisition time. The first ^{1}H NMR experiment was completed between 516 and 905 seconds after mixing.

Competition experiments with THF and glutathione: Firstly, a stock solution was prepared by mixing the following stock samples: 10 µL of 1 M HCHO in H_2O, 2560 µL of 50 mM ammonium formate buffer pH 7.5, 10 µL of 10 mg/mL TSP in D_2O, and 300 µL of D_2O. Then, 20 µL of 500 mM glutathione in H_2O was added to initiate formation of *S*-hydroxymethylglutathione (HMG). Immediately after addition of glutathione, 582 µL of the solution was extracted and added to 9 µL of DMSO-d_6 and 9 µL of H_2O in a microcentrifuge tube. This sample was then transferred to an NMR tube and monitored by ^{1}H NMR with water suppression over 65 minutes (556 seconds to first time-point). Then, another 582 µL of the stock solution was extracted and added to 6 µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6 in a microcentrifuge tube. DMSO-d_6 (6 µL) and H_2O (6 µL) were then added, and the sample was transferred to an NMR tube and monitored by ^{1}H NMR over 35 minutes (first time-point at 548 seconds after addition of THF, and 5166 seconds after start of experiment). After analysis of this sample, another 582 µL of the original solution was extracted and added to 12 µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6 in a microcentrifuge tube. DMSO-d_6 (3 µL) and H_2O (3 µL) were then added, and the sample was again transferred to an NMR tube and monitored by ^{1}H NMR over 35 minutes (first time-point at 554 seconds after addition of THF, and 7388 seconds after start of experiment). Finally, another 582 µL of the stock solution was extracted and added to 18

µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6 in a microcentrifuge tube. The mixture was then transferred to an NMR tube and monitored by ^1H NMR over 35 minutes (first time-point at 548 seconds after addition of THF, and 9965 seconds after start of experiment).

Experiments in HEK293F cell lysate: 1.06 g of HEK293F cells were suspended in ammonium formate buffer pH 7.5 (5 mL) and then subjected to sonication using a microsonicator (18 x 10 s pulses with 10 s intervals at 12 microns). The suspension was then centrifuged at 8000 g for 5 min before the lysate was separated from the solid fraction and stored in a microcentrifuge tube on ice. Aliquots of lysate (504 µL each) were then transferred to microcentrifuge tubes and were mixed with D_2O (60 µL). The first aliquot was then mixed with H_2O (27 µL) and DMSO-d_6 (9 µL), while the second aliquot was mixed with H_2O (18 µL) and 18 µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6. The third aliquot was combined with 18 µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO-d_6 and 18 µL of 1 M ^{13}C-labelled HCHO (^{13}C-HCHO) in H_2O. Immediately after mixing, each sample was transferred to an NMR tube and was subjected to NMR analysis.

TS activity assays: For analyses with added HCHO, 40 µL of a stock sample of TS in 20 mM HEPES 500 mM NaCl buffer pH 7.5 was added to a reaction mixture prepared from the following stock samples: 1 µL of 60 mM $MgCl_2$ in H_2O, 3 µL of 500 mM deoxyuridine monophosphate (dUMP) in H_2O, 6 µL of 50 mM THF in 50% v/v H_2O 50% v/v DMSO, 2 µL of 1 M HCHO in H_2O, 483 µL of 20 mM HEPES 500 mM NaCl buffer pH 7.5, 5 µL of 10 mg/mL TSP in D_2O, and 60 µL of D_2O. After mixing, the sample was immediately transferred to an NMR tube and monitored by ^1H NMR with water suppression. Analyses without added HCHO replaced the 1 M HCHO solution with H_2O (the THF stock also contained DMSO-d_6 in place of protiated DMSO).

Figures

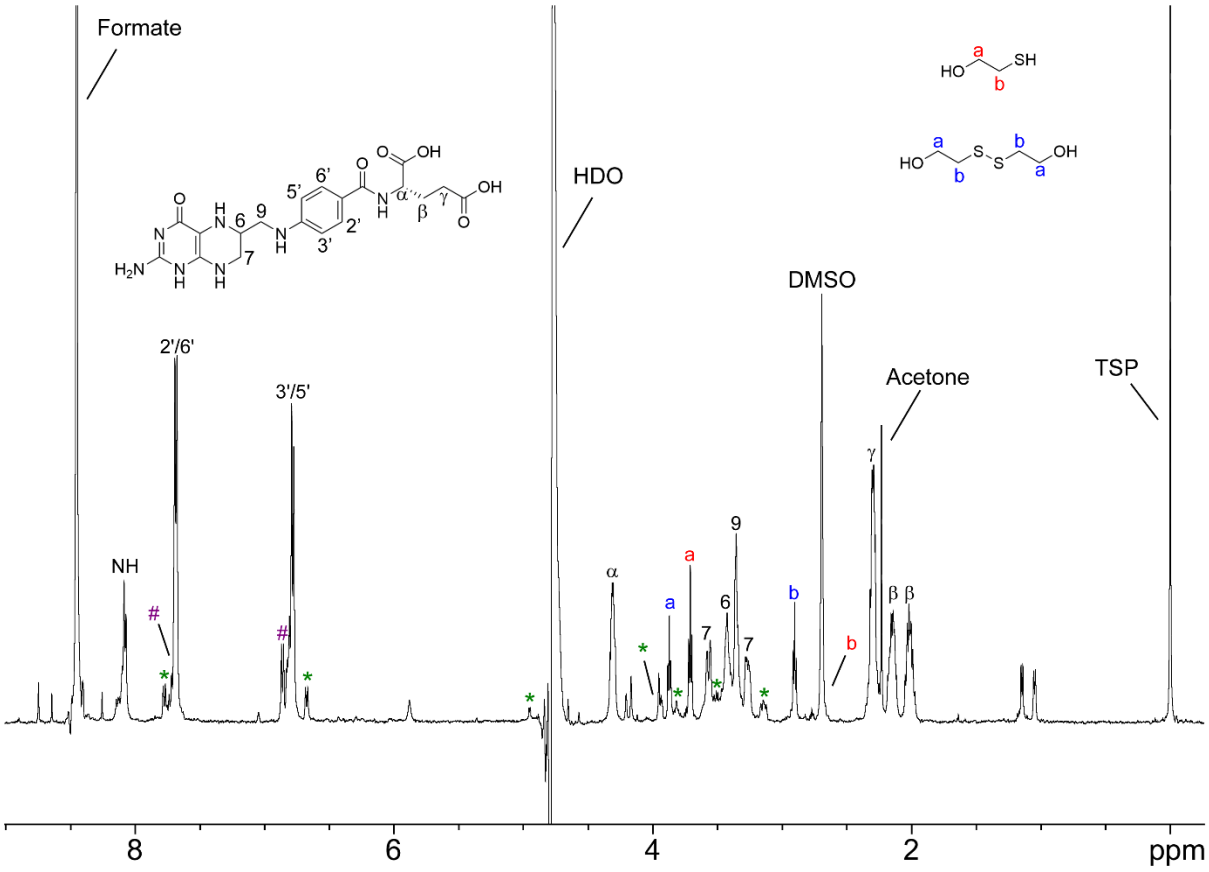

Figure S1. A ^{1}H NMR spectrum of commercially sourced THF in ammonium formate buffer (10 minutes after mixing). ^{1}H resonances corresponding to THF are highlighted. Low-level ^{1}H resonances corresponding to CH_2-THF (green asterisks) and ABG (purple hashtags) are also observed (note: other ^{1}H resonances for these species are obscured by THF and solvent resonances). While small amounts of CH_2-THF and ABG might be present in the stock solution, they are also products of THF degradation under the sample conditions. The sample also contained low levels of reduced and oxidised 2-mercaptoethanol (red and blue lettering respectively), which derives from the THF stock. ^{1}H resonances for residual solvents, formate buffer and TSP are also highlighted. Note: other ^{1}H resonances were observed but their intensities were too low for assignment.

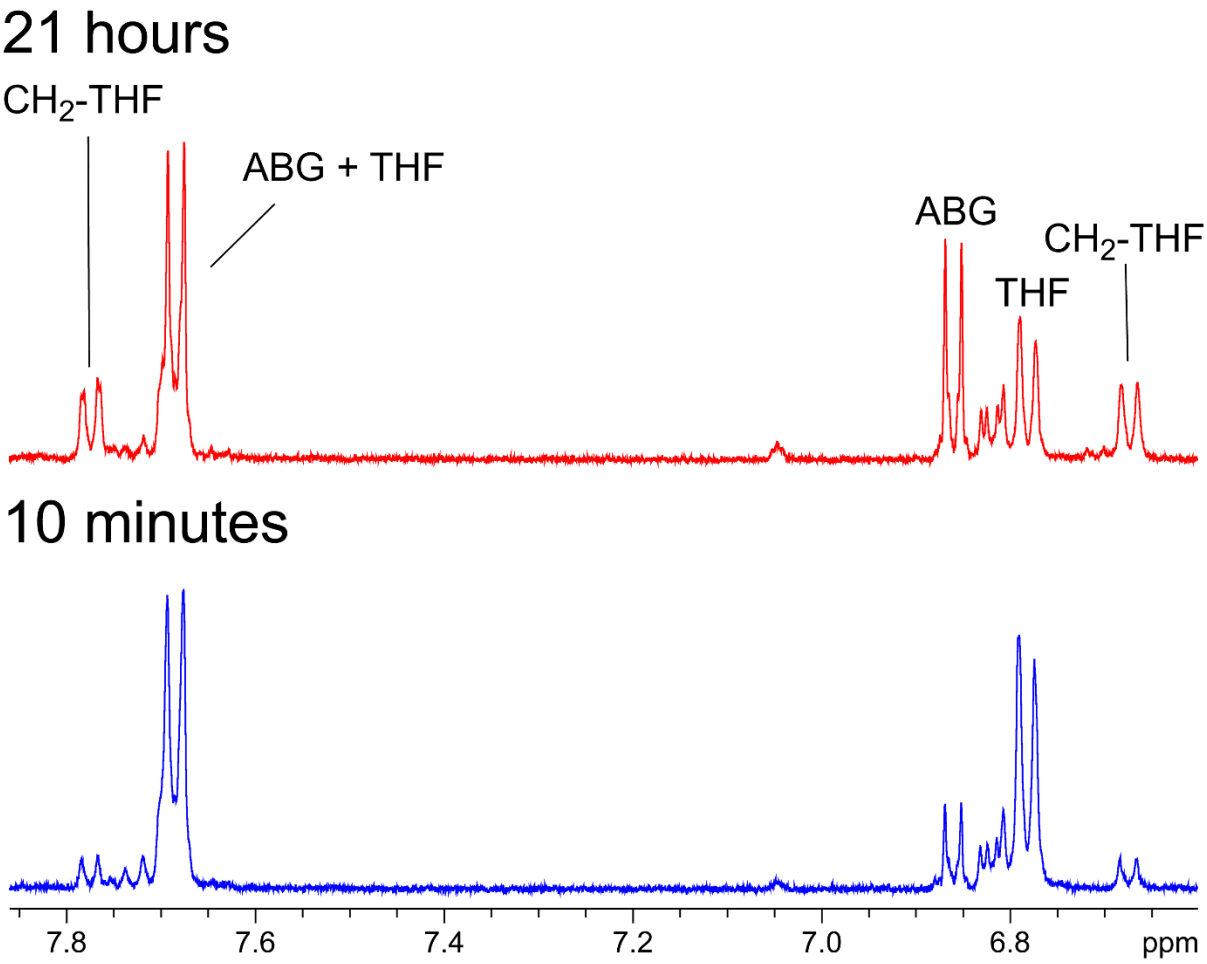

Figure S2. ^{1}H NMR spectra showing time-dependent formation of CH_2-THF and ABG in a sample of THF in ammonium formate buffer. ^{1}H resonances corresponding to the aromatic protons of THF, CH_2-THF and ABG are highlighted. The ^{1}H resonance at δ_H 6.82 ppm is tentatively assigned to DHF.

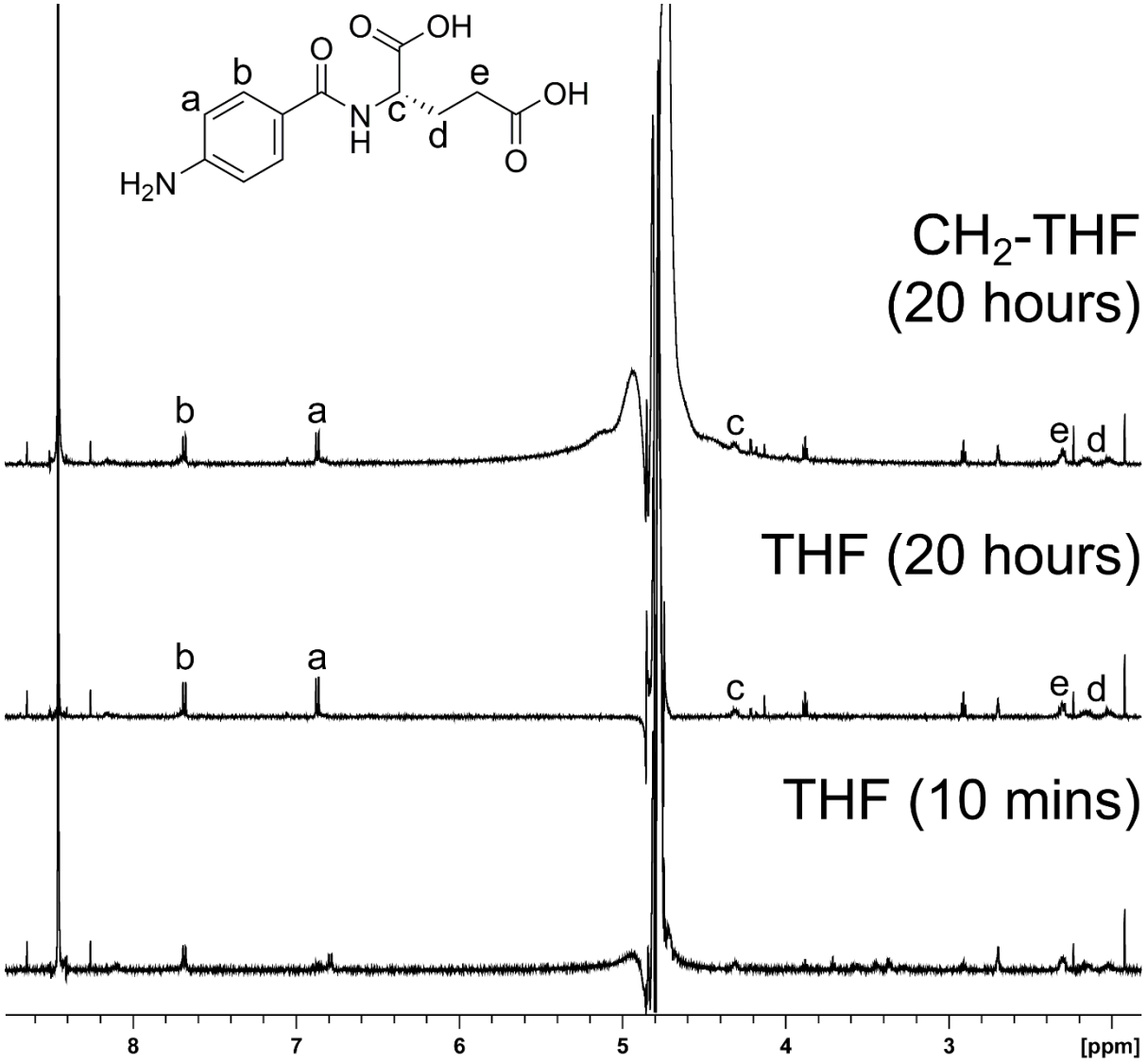

Figure S3. ^{1}H NMR spectra showing time-dependent formation of ABG during degradation of THF (bottom and middle) and CH_2-THF (top). ^{1}H resonances corresponding to ABG are highlighted. The triplet ^{1}H resonances at δ_H 2.9 ppm and δ_H 3.9 ppm correspond to 2-mercaptoethanol disulfide, which derives from 2-mercaptoethanol present in the THF stock.

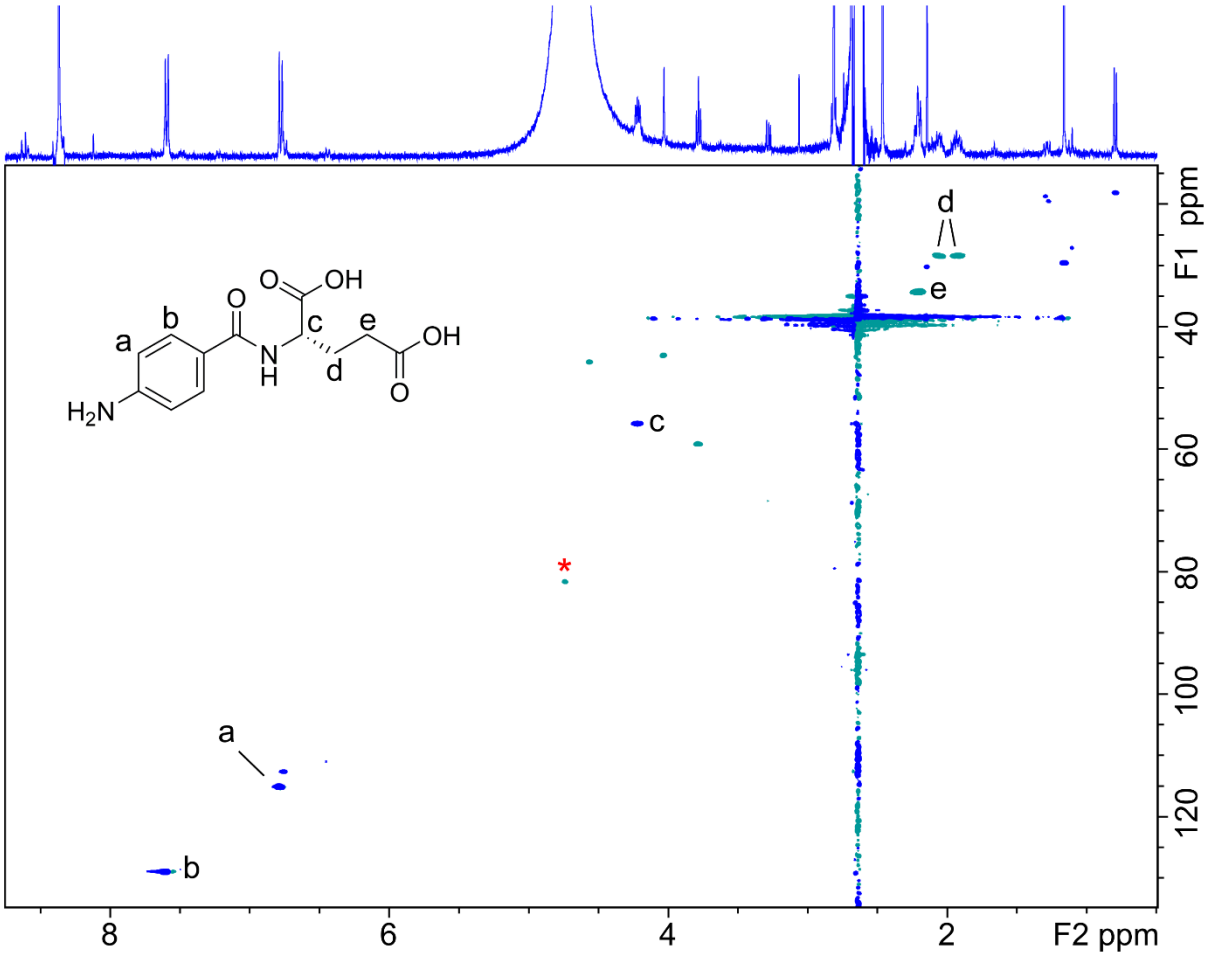

Figure S4. A 2D ^{1}H-^{13}C-HSQC spectrum of a sample of THF in ammonium formate buffer after 20 hours. HSQC correlations corresponding to ABG are highlighted. The correlation tentatively assigned to hydrated HCHO is also highlighted (red asterisk).

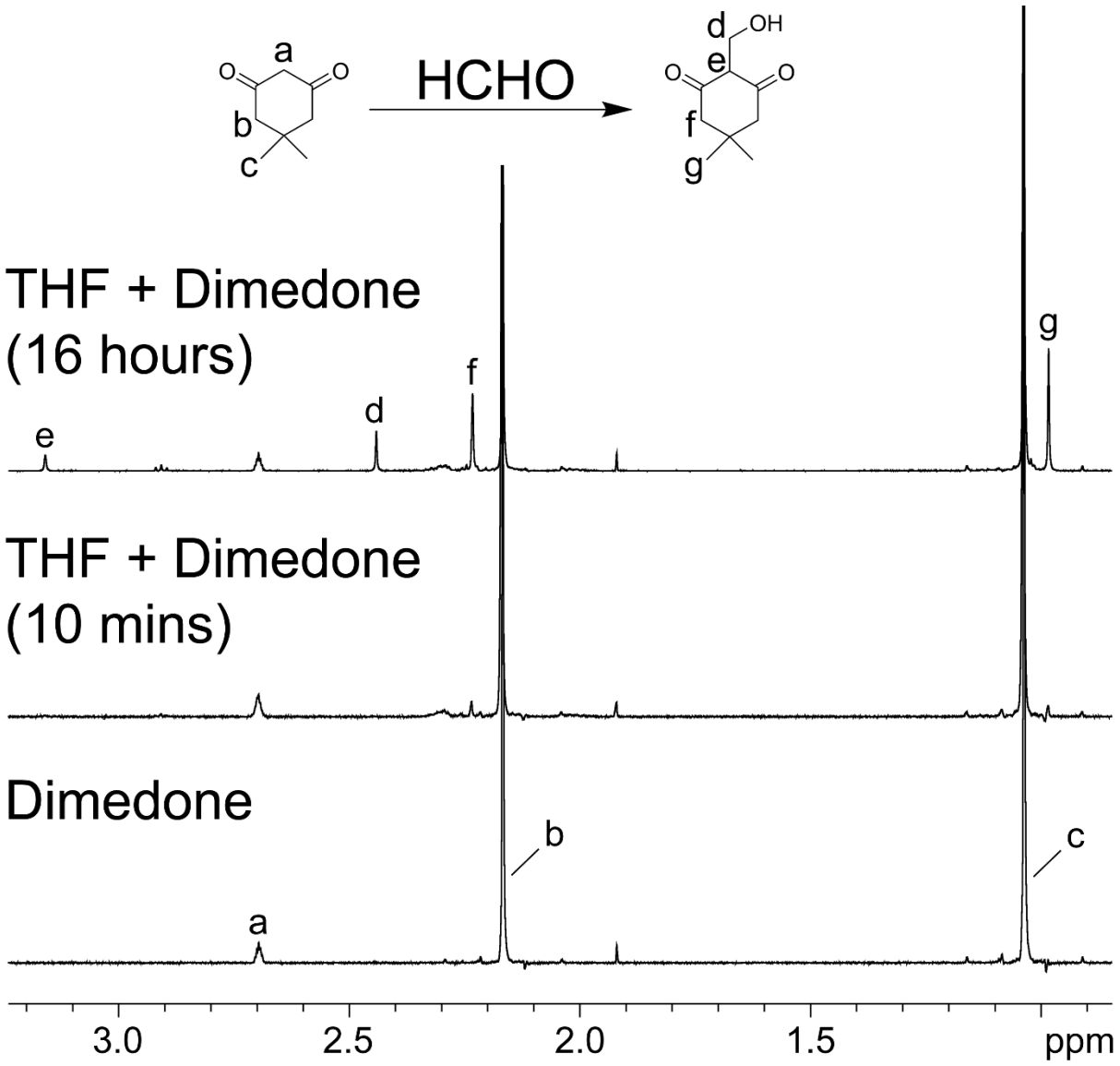

Figure S5. ^{1}H NMR spectra showing time-dependent formation of HCHO during degradation of THF. HCHO was detected by monitoring formation of its hydroxymethyl adduct with dimedone (top). The ^{1}H NMR spectrum of authentic dimedone is shown below.

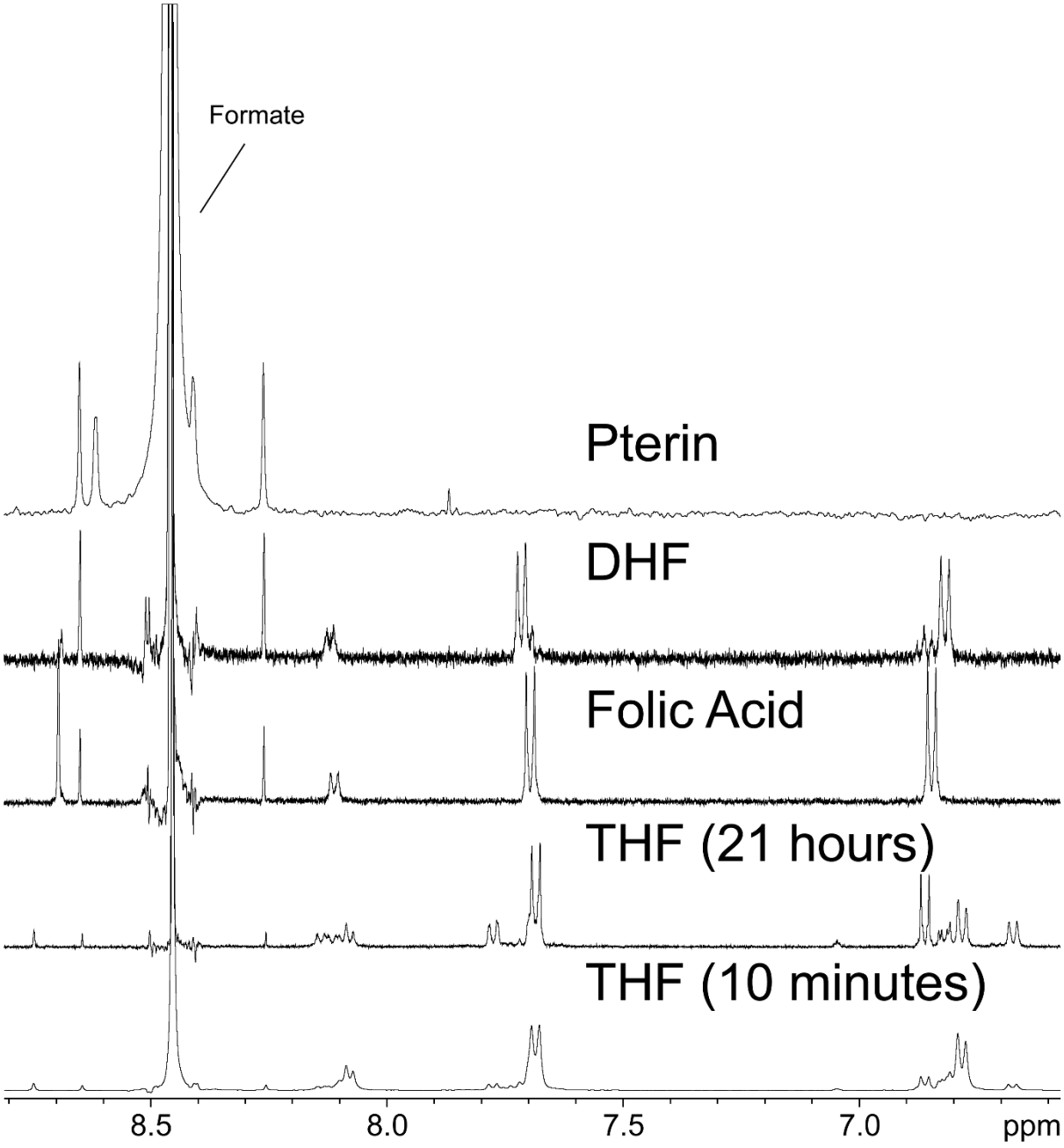

Figure S6. ^{1}H NMR spectra of folic acid, DHF and pterin in ammonium formate buffer. ^{1}H NMR spectra for a sample of THF in ammounium formate buffer (after 10 minutes and 21 hours respectively) are shown below. Note: the ^{1}H resonance at δ_H 6.9 ppm in the THF experiments (bottom) might suggest the presence of DHF, although this resonance does not change in intensity over time. Therefore, it is possible that DHF is a low-level contaminant of the THF stock, although full assignment is not possible due to the low signal intensities.

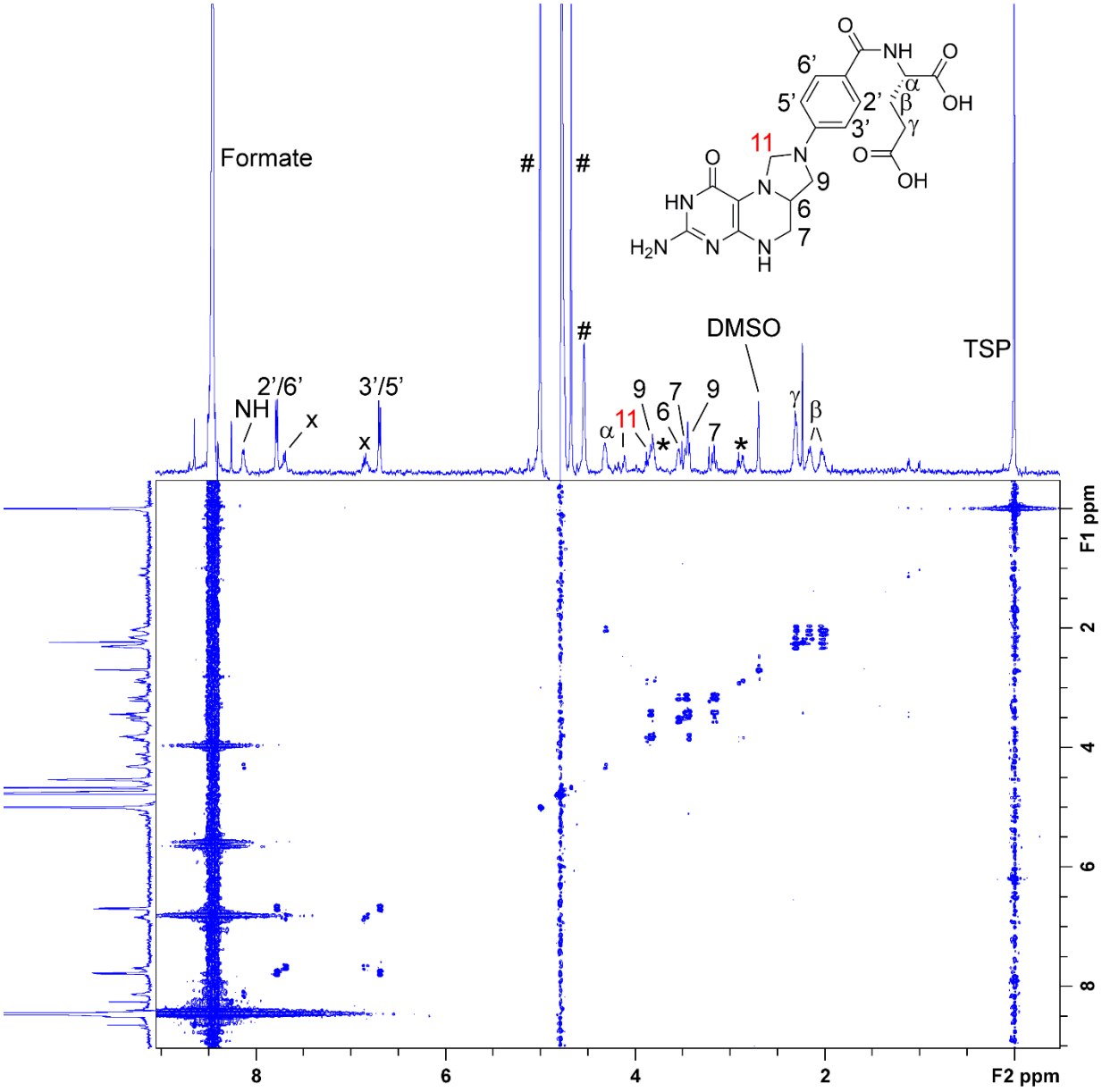

Fig. S7. A 2D ^{1}H-^{1}H-COSY spectrum of a sample containing THF and ^{13}C-HCHO. ^{1}H resonances corresponding to CH_2-THF ^{13}C-labelled at the HCHO-derived methylene are highlighted. Hashtag = ^{1}H resonances derived for the ^{13}C-HCHO stock. X = ABG resonances. Note: some resonances are obscured by the residual solvent resonance.

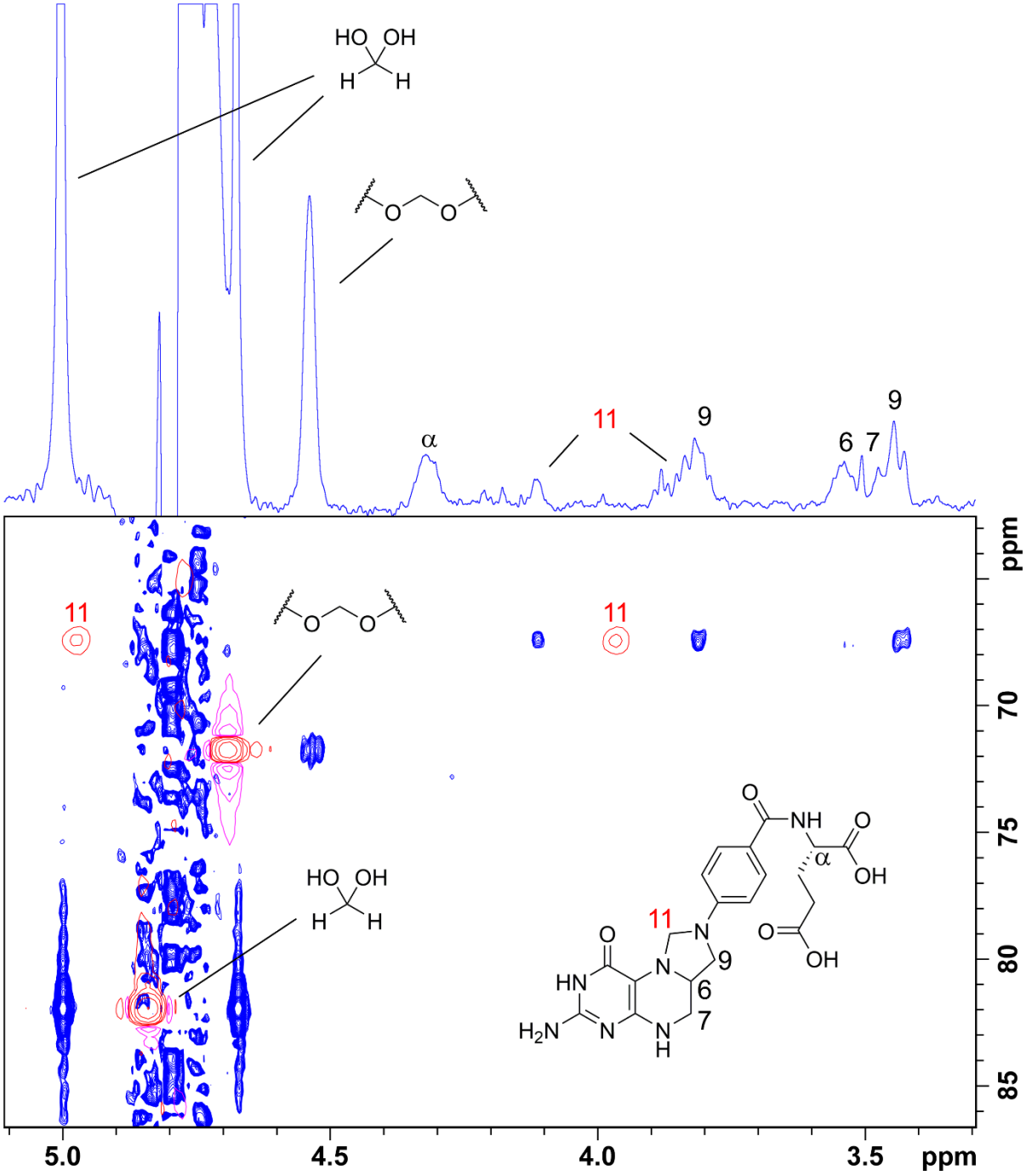

Fig. S8. Overlay of 2D ^{1}H-^{13}C-HSQC (red) and ^{1}H-^{13}C-HMBC (blue) spectra of a sample containing THF and ^{13}C-HCHO. HSQC correlations for the ^{13}C-labelled methylene of CH_2-THF are highlighted.

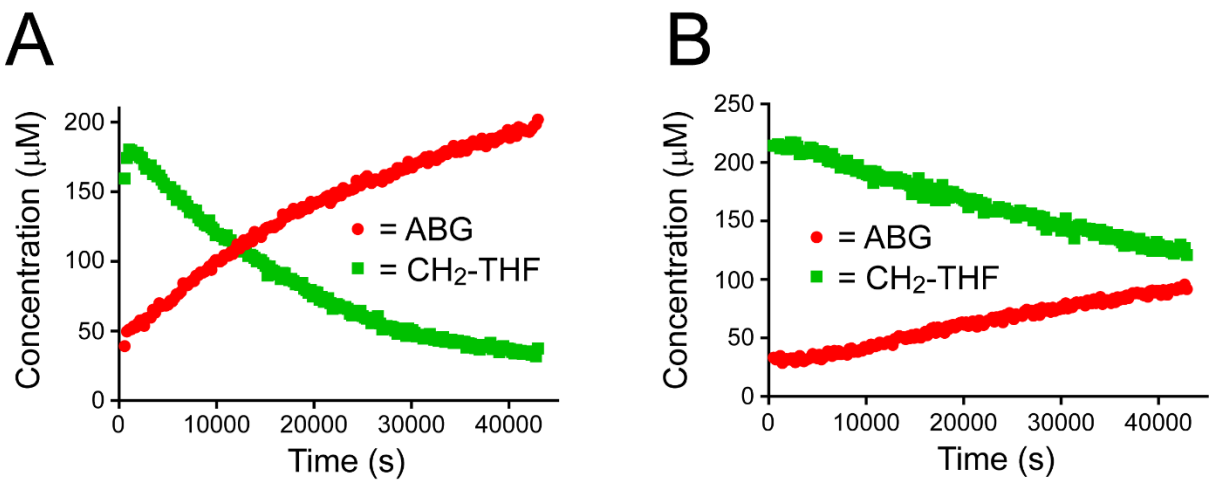

Figure S9. Time-courses showing concentrations of CH_2-THF (green) and ABG (red) over time in mixtures of THF and HCHO. (A) THF + 1.67 equivalents of HCHO, (B) THF + 16.7 equivalents of HCHO.

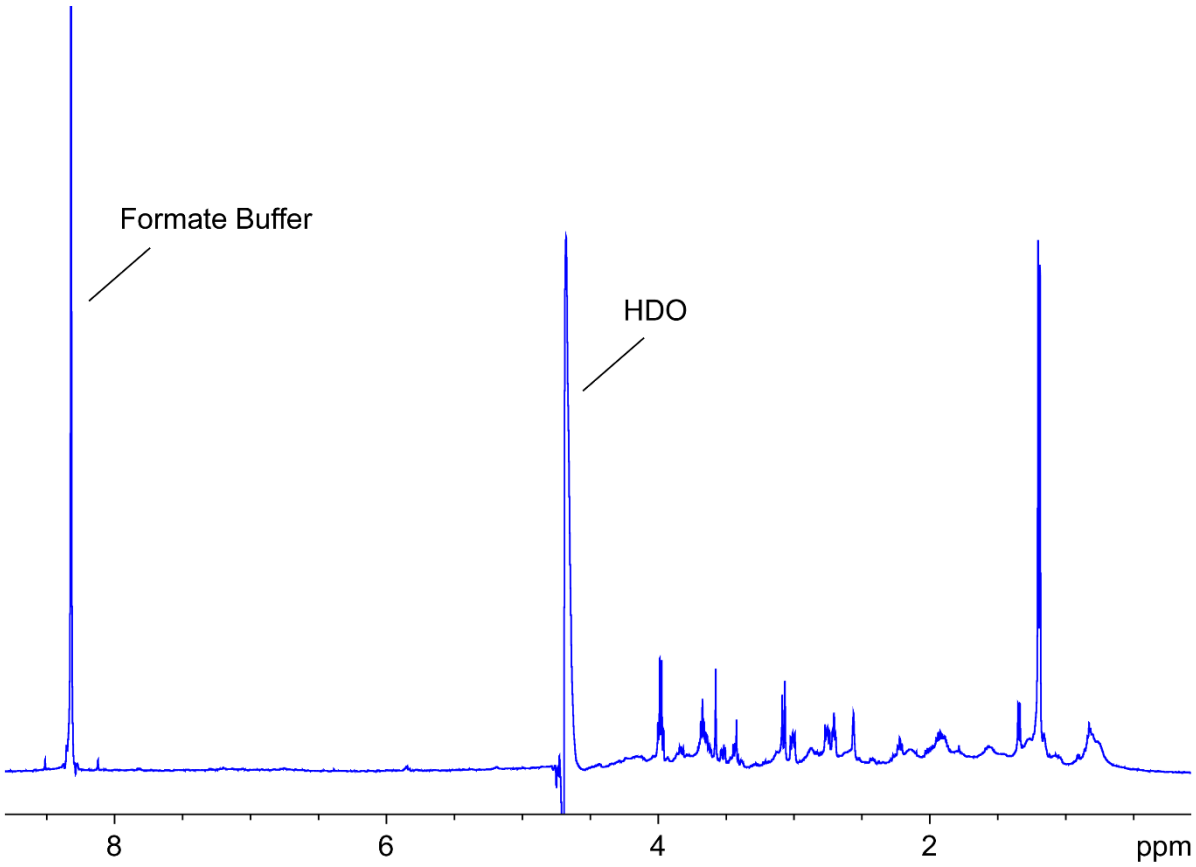

Figure S10. ^{1}H NMR spectrum of HEK293F cell lysate in ammonium formate buffer. ^{1}H resonances corresponding to THF are not observed, which suggests the THF concentration is low.

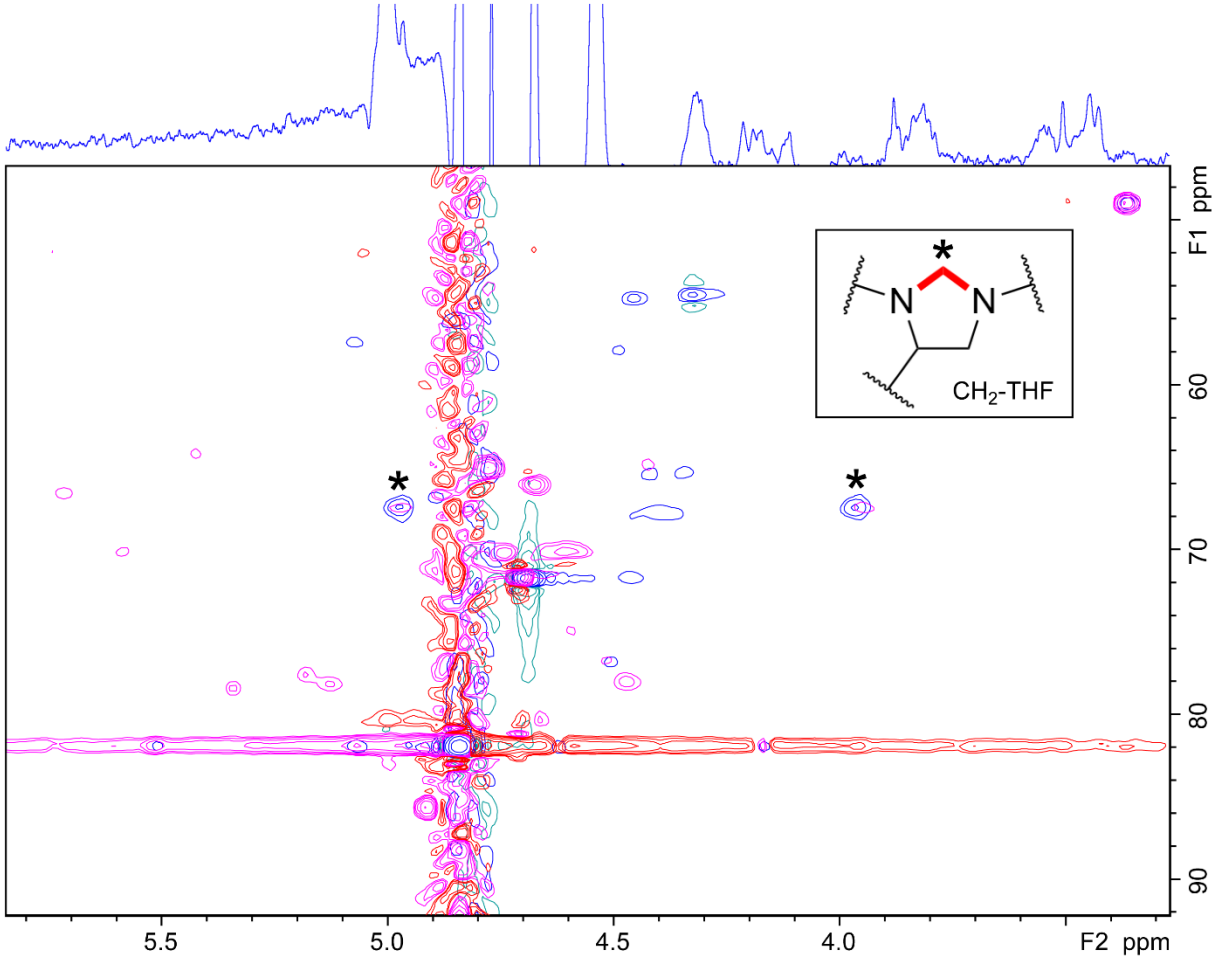

Figure S11. View from a 2D ^{1}H-^{13}C-HSQC spectrum of HEK293F cell lysate supplemented with THF and ^{13}C-HCHO (red and pink, conducted at 295 K). Resonances tentatively assigned to the HCHO-derived methylene of CH_2-THF are highlighted (asterisks). A 2D ^{1}H-^{13}C-HSQC spectrum of CH_2-THF ^{13}C-labelled at the HCHO-derived methylene is overlaid for comparison (blue and green, conducted at 298 K, realigned in the F2 channel).

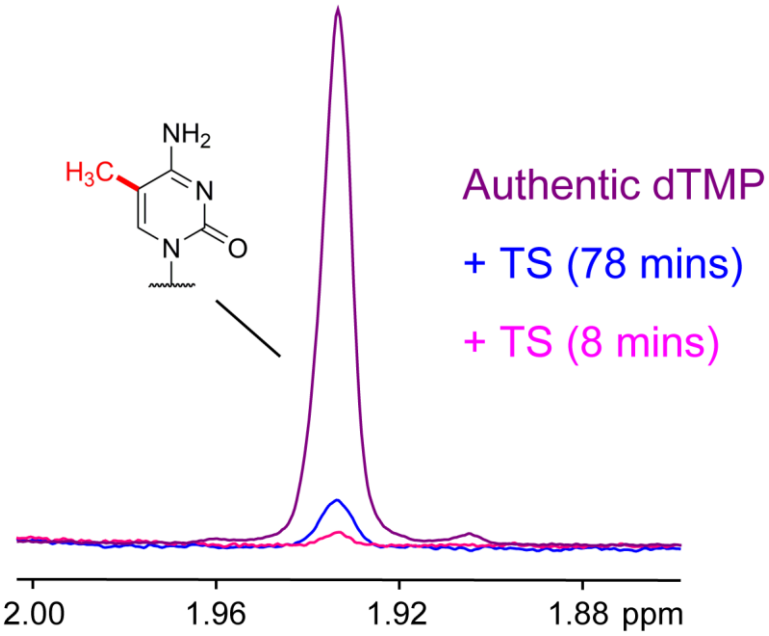

Figure S12. Overlay of ^{1}H NMR spectra of a reaction mixture containing TS, dUMP, THF and HCHO. Time-dependent TS-catalysed formation of dTMP is observed.

References

1. M. Poe, L. M. Jackman, S. J. Benkovic, *Biochemistry*, 1979, **18**, 24, 5527.