The synthesis of folic acid, multiply labelled with stable isotopes, for bio-availability studies in human nutrition

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Two different methods for the synthesis of folic acid, which are suitable for the incorporation of compounds multiply labelled with stable isotopes, are described. The first method is based on the use of a novel reductive amination to link 2-acetylamino-4-hydroxy-6-formylpteridine with *p*-aminobenzoyl-L-glutamic acid. The second method is based on the penultimate formation of an amide bond between *N*-2-acetyl-*N*-10-trifluoroacetylpteroic acid and dimethyl L-glutamate. Both methods have been used to transform [$^{13}C_6$]aniline into folic acid, labelled with [$^{13}C_6$] in the *p*-aminobenzoate moiety, and [3,3,4,4-$^{2}H_4$]-L-glutamic acid into folic acid, labelled with [$^{2}H_4$] in the glutamate moiety. Doubly labelled [$^{13}C_6$, $^{2}H_4$]-*p*-aminobenzoyl-L-glutamate has also been prepared by the former method.

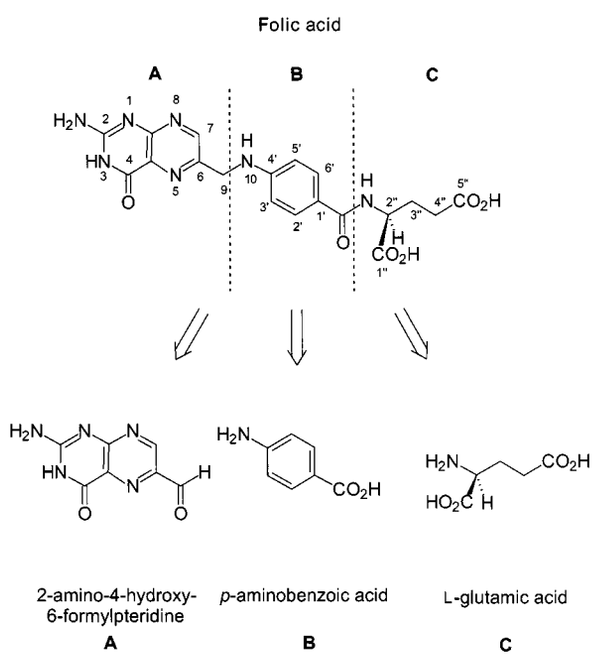
Introduction

The development of sensitive techniques for the study of folate bio-availability in human nutrition is important. The key issues which need to be addressed are the relative absorption of folic acid (as dietary supplements or used to fortify certain foodstuffs) compared to naturally-occurring folates, the efficacy of fortification procedures and the effect of various genetic, age or disease states on aspects of folate absorption or metabolism. Such information is essential in evaluating the adequacy of dietary folate intakes for reducing the incidence of neural tube defects,[1] in reducing plasma homocysteine and hence possible risks of various forms of vascular disease[2] and in reducing the risk of certain cancers.[3]

In order to study the bio-availability of folic acid, stable isotopes must be incorporated into ingested folic acid, so that the material can be identified and quantified in biological fluids. The difference in molar mass between labelled and unlabelled material allows the distribution of the exogenous material to be distinguished from endogenous material by sensitive mass spectrometric techniques. The recent availability of stable isotopically labelled folates[4] and the development of sensitive mass spectrometric techniques for the determination of folates[5] permit the study of folate absorption and metabolism in humans with high specificity and safety.[4]

Nutritional studies of folate bio-availability can be conducted using a dual-label protocol design.[6,7] Recently, a dual-label protocol involving an oral dose of [$^{13}C_5$]folic acid in aqueous solution or in fortified food and a simultaneous intravenous reference dose of [$^{2}H_2$]folic acid, has been reported.[8] The relative absorption between trials and between individuals can be assessed, even if minor variation in urinary excretion occurs, by use of the second labelled folate administered intravenously. The absolute recovery of label is not a major consideration because absorption is determined relative to the urinary excretion of the reference dose injected immediately after consumption of the oral dose. The relative absorption of the oral dose can be calculated from isotope concentration measurements with time of the two labelled folates in plasma or urine using GC-MS procedures.[9]

This paper is concerned with the synthesis of folic acid multi-labelled with stable isotopes for bio-availability studies in human nutrition. The objectives are to synthesise folic acid labelled with six ^{13}C atoms in the aromatic ring of the *p*-aminobenzoate moiety and with four deuterium atoms in the glutamate moiety.† The two coherent retro-synthetic disconnections which are normally employed for the synthesis of folic acid are shown in Scheme 1. Thus fragments **B** and **C** are the candidates for the introduction of isotopic labels and would have to be synthesised from fairly simple labelled starting materials.[10,11]



Scheme 1 Retro-synthetic analysis of folic acid.

† Chosen to ensure the absence of interference between the molecular ion cluster of endogenous folate and administered labelled folate.

From the limited number of labelled compounds available, [$^{13}C_6$]aniline was selected as the starting material for **B** and [3,3,4,4-$^4H_2$]-L-glutamic acid for **C**.[12]

The labelled material should be introduced into the synthesis at a late stage. Ultimately the three components **A**, **B** and **C**, would have to be combined in the most efficient way possible to conserve valuable labelled material. Strategies involving closure of the pteridine ring at a late stage in the synthesis[13] were rejected. The global strategies available are [(**A** + **B**) + **C**] and [**A** + (**B** + **C**)]. There are literature precedents for both combinations.[12,14–17] Although the latter sequence appeared to be the method of choice, as it uses marginally fewer synthetic steps, which is an important consideration when expensive labelled chemicals are being used, it was decided to investigate both approaches.

In this paper we report a novel procedure using the [**A** + (**B** + **C**)] strategy together with an alternative procedure using the [(**A** + **B**) + **C**] strategy for the synthesis of unlabelled and labelled folic acid.

In the [**A** + (**B** + **C**)] strategy,[15] an amide bond is formed between suitably protected **B** and **C** components, the resulting protected *p*-aminobenzoyl-L-glutamate is deprotected and used in a novel ‡ reductive amination reaction with suitably protected pteridine carbaldehyde **A** to yield the protected folic acid which is itself deprotected.

In the [(**A** + **B**) + **C**]-strategy,[12,14,16,17] in the penultimate step, an amide bond is formed between a pteroic acid derivative and dimethyl L-glutamate. This approach has been reported previously for the synthesis of [$^{13}C_1$]folic acid[17] and [$^2H_4$]folic acid.[12]

Both strategies have been used for the synthesis of folic acid and subsequently applied to the synthesis of [$^{13}C_6$]folic acid labelled with [$^{13}C_6$] in the *p*-aminobenzoate moiety **B**, and [$^2H_4$]folic acid labelled with [$^2H_4$] in the glutamate moiety **C**. In the case of the known [$^2H_4$]folic acid significant new characterisation data has been provided. In addition, doubly labelled [$^{13}C_6$,$^2H_4$]-*p*-aminobenzoyl-L-glutamate has been synthesised. A preliminary account of some of this work has appeared.[18]

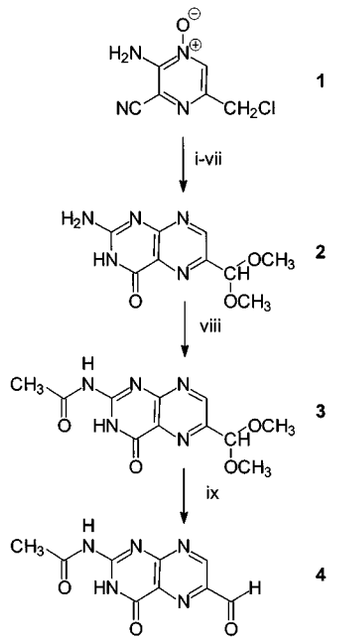
Alkali-labile protecting groups were chosen for masking reactive functional groups: acetyl for the 2-amino group of the pteridine component **A**, trifluoroacetyl[19] for the *para*-amino group of the aminobenzoate component **B**, trifluoroacetyl[19] for the secondary amino group of the pteroic acid portion (**A** + **B**) and methyl ester[12,20] for the twin carboxy groups of the L-glutamate portion **C**.

Results and discussion

Fragment A

There are several reported syntheses of the **A** component of folic acid.[21–23] We chose to use the Taylor[21] approach (Scheme 2) and 2-amino-3-cyano-5-chloromethylpyrazine *N*-oxide **1** was converted to 2-amino-4-hydroxy-6-formylpteridine dimethyl acetal **2**[23] using standard literature protocols.

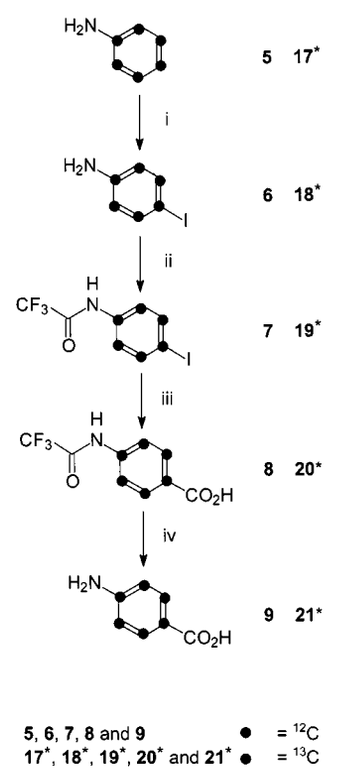
It was argued that the *N*-2-acetylpteridine-6-carbaldehyde **4** could be best prepared in the protected form as the dimethyl acetal **3**, on account of the more favourable solubility properties of the precursor **2**. This proved to be the case and 2-acetyl-4-hydroxy-6-formylpteridine dimethyl acetal **3** was formed in good yield, using the method which had been applied to the corresponding diethyl acetal.[22] Smooth hydrolysis with aqueous formic acid gave the N-protected aldehyde **4** as a pale yellow solid, which on recrystallisation from DMF gave 2-acetylamino-4-hydroxy-6-formylpteridine **4**§ in 15% overall yield over 9 reactions from the original starting material, 2-amino-3-cyano-5-chloromethylpyrazine *N*-oxide **1**.



Scheme 2 Synthesis of the **A**-component. *Reagents and conditions*: i, PCl_3; ii, C_5H_5N; iii, K_2CO_3, *p*-$Me_2NC_6H_4NO$; iv, 6 M aq. HCl; v, MeOH, H^+; vi, MeONa, $(H_2N)_2C{=}NH{\cdot}HCl$ (reflux); vii, aq. NaOH (reflux); viii, Ac_2O (heat); ix, aq. HCO_2H.

Fragment B

The key step in the synthesis of *p*-aminobenzoic acid is the carboxylation[24,25] of a metallated arene produced by the action of *n*-butyllithium on a haloarene (Scheme 3). Iodo was chosen



Scheme 3 Synthesis of the **B**-component. *Reagents and conditions*: i, I_2, $NaHCO_3$; ii, $(CF_3CO)_2O$, DCM; iii, (a) n-BuLi (2 equiv.), (b) solid CO_2, (c) dil. HCl; iv, 0.1 M NaOH (23 °C for 4 h).

as the halo-substituent since it can be introduced into the arene precursor more regioselectively than with the other halogens. Accordingly, aniline **5** was converted to *p*-iodoaniline **6** in 70% yield, using iodine and sodium carbonate as described in Vogel.[26] The amino group in **6** was protected as *N*-trifluoro-

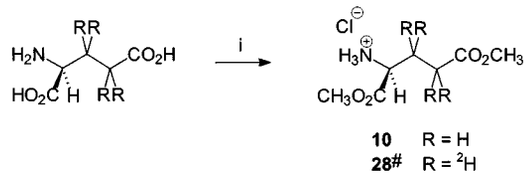
‡ The novelty lies in the fact that the reductive amination is carried out with fully unprotected *p*-aminobenzoyl-L-glutamate.
§ Compound **4** is obtained as the DMF monosolvate.

acetyl using trifluoroacetic anhydride in dichloromethane.[27] The resulting *p*-iodotrifluoroacetanilide **7** was converted to the dianion by treatment at −78 °C with at least two equivalents of *n*-butyllithium and quenched with carbon dioxide in ether. On acidification, the resulting carbamic acid underwent decarboxylation and gave the target *p*-trifluoroacetylaminobenzoic acid **8** in approximately 60% yield.[28,29] The overall yield of **8** from aniline was 34%.

Removal of the trifluoroacetyl protecting group from the acid **8** proceeded smoothly by treatment with 0.1 M sodium hydroxide at ambient temperature for four hours. The resulting *p*-aminobenzoic acid **9** obtained on acidification was readily purified by recrystallisation from hot water. The overall yield of *p*-aminobenzoic acid from aniline was 21%.

Fragment C

The α and γ carboxy groups of L-glutamic acid require protection as methyl esters. The esterification was accomplished using thionyl chloride in methanol[12,20] and gave the hydrochloride of dimethyl L-glutamate **10** in good yield (Scheme 4).



Scheme 4 Synthesis of fragment **C**. *Reagents and conditions*: i, $SOCl_2$, MeOH.

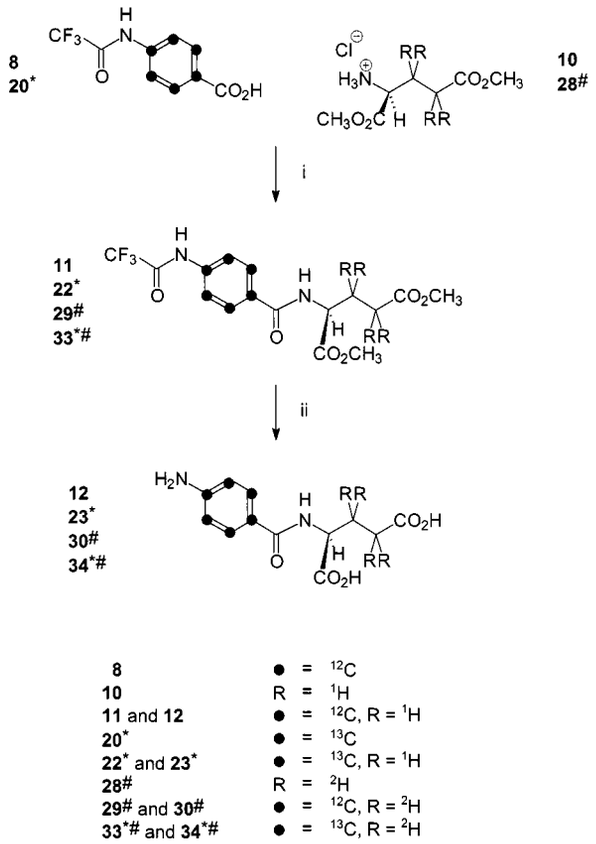
p-Aminobenzoyl-L-glutamate 11

p-Trifluoroacetylaminobenzoic acid **8** and dimethyl L-glutamate hydrochloride **10** were subjected to a DCC mediated coupling reaction in anhydrous THF in the presence of HOBT and diisopropylethylamine as outlined in Scheme 5. The crude amide was purified by column chromatography to give dimethyl *p*-trifluoroacetylaminobenzoyl-L-glutamate **11** in 60–70% yield. Removal of all three protecting groups from **11** was achieved with a four-hour treatment with 0.1 M sodium hydroxide solution at ambient temperature. The target *p*-aminobenzoyl-L-glutamate **12** was isolated in 50% yield by multiple extraction with ethyl acetate of a solution at pH 3, which had been saturated with salt.

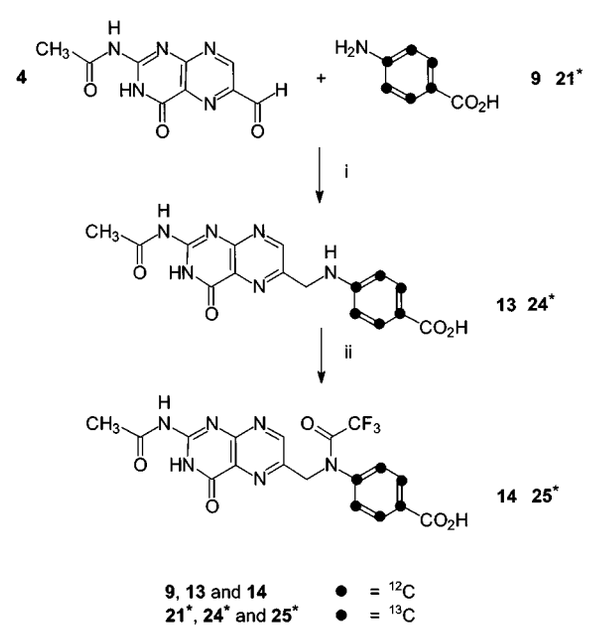
Pteroic acid derivatives

2-Acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** and *p*-aminobenzoic acid **9** were suspended in glacial acetic acid and treated with dimethylamine–borane complex in glacial acetic acid as described by Plante.[14,17] The resulting secondary amine, *N*-2-acetylpteroic acid **13**, was obtained in yields of up to 80% after recrystallisation from hot water, (Scheme 6).

Trifluoroacetylation of *N*-2-acetylpteroic acid **13** with trifluoroacetic anhydride gave the diacylated pteroic acid **14**.[17,30,31] Interestingly, although the compound showed the expected molar mass in the mass spectrum (EI), the ^1H and ^{19}F NMR spectra were consistent with the existence of several forms in solution (d^6-dimethyl sulfoxide).¶ This behaviour was confirmed when the corresponding [$^{13}C_6$]-diacylated pteroic acid **25*** was prepared (see later).‖ Furthermore, a temperature dependence study of the ^1H chemical shifts of a solution of **25*** between 30 and 140 °C indicated an absence of conformer interconversion



Scheme 5 Synthesis of *p*-aminobenzoyl-L-glutamates. *Reagents and conditions*: i, DCC, HOBT, Pr^i_2NEt, THF; ii, 0.1 M NaOH (23 °C for 4 h).



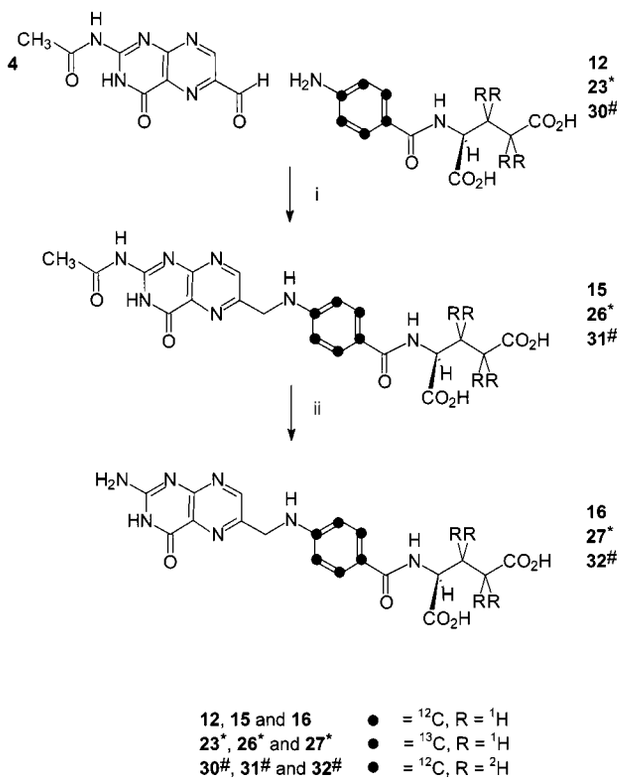
Scheme 6 Synthesis of pteroic acids. *Reagents and conditions*: i, AcOH, $Me_2NH–BH_3$; ii, $CF_3CO_2H–(CF_3CO)_2O$ (reflux for 90 min).

on the NMR time scale. The data showed that **14** exists in d^6-dimethyl sulfoxide at 30 °C in three distinct forms in the ratio 50:25:25 and likewise **25*** in the ratio 50:25:25. An explanation for the interesting behaviour of **14** and **25*** awaits further study: it may be due to *cis–trans* isomerism about the *N*(10) amide bond or acyl-migration. Nevertheless, the diacylated pteroic acids **14** and **35*** proved to be effective components for the synthesis of the folic acids by the **[(A + B) + C]** route.

¶ In conformity with our observation, Plante, Crawford and Friedkin[30] observed that their sample of **14** was chromatographically inhomogeneous, although it was an effective component for further coupling.
‖ In the numeration of [$^{13}C_6$]-compounds an * is used as an aid to identification.

Folic acid 16 by the [A + (B + C)] route

Of the methods available in the literature for carrying out the reductive amination between 2-acetylamino-4-hydroxy-6-formylpteridine **4** and *p*-aminobenzoyl-L-glutamate **12**, that of Sletzinger[22] proved ineffective, confirming the observations of Plante,[14,17] that of Bieri and Viscontini[15] proved unsuitable for our purpose and so the method of Plante which had originally been devised for an [(**A** + **B**) + **C**] synthesis was adopted.[14] 2-Acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** and *p*-aminobenzoyl-L-glutamate **12** were suspended in glacial acetic acid and treated with dimethylamine–borane complex in glacial acetic acid.†† The resulting *N*-2-acetylfolic acid **15** was obtained in yields of up to 60% after recrystallisation from hot water (Scheme 7). A high state of purity of *p*-aminobenzoyl-L-glutamate **12** is crucial to the success of the reductive amination.

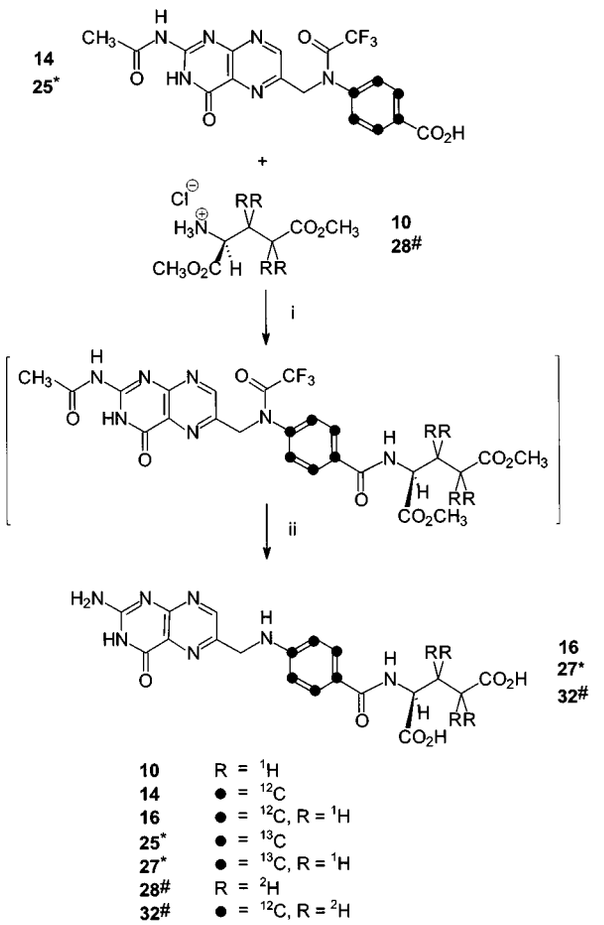


Scheme 7 Synthesis of folic acids using the [**A** + (**B** + **C**)] route. *Reagents and conditions*: i, AcOH, Me_2NH–BH_3; ii, 0.1 M NaOH (reflux for 90 min).

The *N*-2-acetyl protecting group was removed from acetylfolic acid **15** by refluxing with 0.1 M sodium hydroxide for 90 minutes.[22] The final product, folic acid **16**, was isolated by centrifugation at pH 3.5 and purified by recrystallisation from hot water containing ascorbic acid. Pure folic acid **16** was obtained by this method in an overall yield, based on the starting *p*-trifluoroacetylaminobenzoic acid **8**, of 12%.

Folic acid 16 by the [(A + B) + C] route

The diacylated pteroic acid **14** was coupled with dimethyl L-glutamate hydrochloride **10** by the mixed anhydride method using isobutyl chloroformate and triethylamine in dimethylformamide (Scheme 8).[12,17] The crude dimethyl *N*-2-acetyl-*N*-10-trifluoroacetylfolate, obtained by evaporation of the solvent *in vacuo*, was purified by repeated extraction with aqueous



Scheme 8 Synthesis of folic acids using the [(**A** + **B**) + **C**] route. *Reagents and conditions*: i, Bu^iOCOCl, NEt_3, DMF; ii, 0.1 M NaOH (reflux for 90 min).

ammonium bicarbonate and the resulting gelatinous material deprotected by refluxing with 0.1 M sodium hydroxide for 90 minutes.[12,17] The final product, folic acid **16**, was isolated by centrifugation after acidification to pH 3.5 and purified by anion-exchange chromatography (eluting with an aqueous ammonium hydrogen carbonate gradient) and semi-preparative HPLC using a reversed phase ion pairing technique.‡‡ The overall yield of folic acid produced by this method, based on the starting *p*-aminobenzoic acid was 11%.§§

Stability of glutamate containing precursors to alkali

The severe alkaline hydrolysis conditions required to remove the protecting groups posed a threat to the integrity of the glutamate moiety both as regards its chiral homogeneity and also for the retention of the deuterium label in the 3″ and 4″ positions.[12] The literature indicated these fears were groundless.[15] Nevertheless, the specific optical rotation of the folic acid **16** prepared by both routes was carefully monitored and found to be identical within the accuracy of the method with the literature value.[15,33] Moreover, a ^1H NMR study of *p*-aminobenzoyl-L-glutamate **12**, incubated with NaOD in D_2O, showed no diminution in the intensity of the relevant proton signals. Conversely, subsequent studies on the incubation of the deuterium labelled precursor **30**$^{\#}$ with NaOH in H_2O showed no

†† Recently, a parallel procedure has been reported for the synthesis of *N*-(fervenulin-3-ylmethyl)-4-aminobenzoyl-L-glutamic acid (a folic acid analogue with a modified heterocyclic moiety).[32]

‡‡ If the contamination of the folic acids with pteroic acids was low, the HPLC step could be omitted and recrystallisation from water sufficed; if however the contamination was greater than *ca.* 5% then the HPLC purification step was included.

§§ The yield of folic acid based on *N*-trifluoroacetamidobenzoic acid is 12% for the [**A** + (**B** + **C**)] route and 7% for the [(**A** + **B**) + **C**] route.

detectable appearance of the relevant proton signals and the expected specific optical rotation was observed.[34]¶¶

[$^{13}C_6$]-Fragment B

The starting material used was commercial [$^{13}C_6$]aniline **17***.||||

The [$^{13}C_6$]-*p*-trifluoroacetylaminobenzoic acid **20*** and the [$^{13}C_6$]-*p*-aminobenzoic acid **21*** were prepared in exactly the same way[24–27,35] as the unlabelled materials **8** and **9** with comparable results (Scheme 3).‖

[$^{13}C_6$]-*p*-Aminobenzoyl-L-glutamate 23*

The acid **20*** was coupled with the hydrochloride of dimethyl L-glutamate **10** to yield [$^{13}C_6$]dimethyl *p*-trifluoroacetylaminobenzoyl-L-glutamate **22*** and deprotected by alkaline hydrolysis to yield [$^{13}C_6$]-*p*-aminobenzoyl-L-glutamate **23*** (Scheme 5) essentially as previously described for the unlabelled series of compounds.

[$^{13}C_6$]Pteroic acid derivatives

The reductive amination of 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** and the [$^{13}C_6$]-*p*-aminobenzoic acid **21*** gave the [$^{13}C_6$]-*N*-2-acetylpteroic acid **24*** directly, in good yield (Scheme 6).[14,17]

Subsequent protection of the *N*-10 secondary amino group by trifluoroacetylation with trifluoroacetic anhydride gave the [$^{13}C_6$]-*N*-2-acetyl-*N*-10-trifluoroacetylpteroic acid **25***,[17,30,31] which, as discussed earlier, exists in solution in three distinct forms.

Synthesis of [$^{13}C_6$]folic acid 27* by the [A + (B + C)] route

The reductive amination of 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** and the [$^{13}C_6$]glutamate derivative **23*** gave [$^{13}C_6$]-*N*-2-acetylfolic acid **26*** which was deprotected by refluxing with aqueous sodium hydroxide (Scheme 7).[22] The resulting labelled folic acid **27*** was obtained in an overall yield, based on the starting [$^{13}C_6$]-*p*-*N*-trifluoroacetylaminobenzoic acid **20***, of 11% in a high state of purity.

Synthesis of [$^{13}C_6$]folic acid 27* by the [(A + B) + C] route

The diacylated pteroic acid **25*** was coupled with dimethyl L-glutamate hydrochloride **10** by the mixed anhydride method (Scheme 8).[12,17] The fully protected crude [$^{13}C_6$]dimethyl *N*-2-acetyl-*N*-10-trifluoroacetyl folate was deprotected by refluxing with aqueous sodium hydroxide to yield [$^{13}C_6$]folic acid **27*** which was purified by anion-exchange chromatography and semi-preparative HPLC, using a reversed phase ion pairing technique. Based on the starting [$^{13}C_6$]-*p*-aminobenzoic acid, [$^{13}C_6$]folic acid was produced in a high state of purity in an overall yield of 14%.†††

Synthesis of [$^2H_4$]folic acid 32# by the [A + (B + C)] route

The starting material used was commercial [3,3,4,4-$^2H_4$]-L-glutamic acid.|||| The amino acid was esterified with thionyl chloride in methanol[12,20] under the conditions used earlier for the unlabelled L-glutamic acid to give the hydrochloride of [$^2H_4$]dimethyl L-glutamate **28**# in very good yield (Scheme 4).‡‡‡

p-*N*-Trifluoroacetylaminobenzoic acid **8** was coupled with the hydrochloride of [$^2H_4$]dimethyl L-glutamate **28**# to yield [$^2H_4$]dimethyl *p*-*N*-trifluoroacetylaminobenzoyl-L-glutamate **29**# and deprotected by alkaline hydrolysis to yield [$^2H_4$]-*p*-aminobenzoyl-L-glutamate **30**# essentially as previously described for the unlabelled and [$^{13}C_6$] series of compounds (Scheme 5). The reductive amination of 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** and the [$^2H_4$]aminobenzoyl-L-glutamate derivative **30**# gave [$^2H_4$]-*N*-2-acetylfolic acid **31**# which was converted to [$^2H_4$]-labelled folic acid **32**# by alkaline hydrolysis (Scheme 7).[22] The labelled folic acid **32**# was obtained in a high state of purity in an overall yield, based on the starting *p*-*N*-trifluoroacetylaminobenzoic acid **8**, of 6%.

Synthesis of [$^2H_4$]folic acid 32# by the [(A + B) + C] route

N-2-Acetyl-*N*-10-trifluoroacetylpteroic acid **14** was coupled with [$^2H_4$]dimethyl L-glutamate hydrochloride **28**# by the mixed anhydride method (Scheme 8). The fully protected crude [$^2H_4$]dimethyl *N*-2-acetyl-*N*-10-trifluoroacetylfolate was deprotected by refluxing with aqueous sodium hydroxide to yield [$^2H_4$]folic acid **32**# which was contaminated with pteroic acid. Pure [$^2H_4$]folic acid was obtained by anion exchange chromatography followed by high performance semi-preparative liquid chromatography using a reversed phase ion pairing technique. The labelled folic acid **32**# was obtained in a high state of purity in an overall yield, based on the starting *p*-*N*-trifluoroacetylaminobenzoic acid **8**, of 11%.§§§

Synthesis of [$^2H_4$,$^{13}C_6$]-*p*-aminobenzoyl-L-glutamate 34*#

[$^{13}C_6$]-*p*-*N*-Trifluoroacetylaminobenzoic acid **20*** was coupled with the hydrochloride of [$^2H_4$]dimethyl L-glutamate **28**# to yield [$^{13}C_6$,$^2H_4$]dimethyl *p*-*N*-trifluoroacetylaminobenzoyl-L-glutamate **33***# and deprotected by alkaline hydrolysis to yield [$^{13}C_6$,$^2H_4$]-*p*-aminobenzoyl-L-glutamate **34***# (Scheme 5) essentially as previously described for the unlabelled and singly labelled series of compounds.¶¶¶, ||||||

Conclusion

Two methods have been developed for the synthesis of folic acid, which allows for the incorporation of precursors, multiply labelled with stable isotopes, at a late stage in the synthesis, which minimises the loss of expensive labelled precursors. The methods allow the two forms of isotopic labelling required, namely [$^{13}C_6$] and [$^2H_4$], to be introduced at a relatively late stage in the synthesis, where the yields of the remaining synthetic steps are tolerable. The procedures have been shown to retain the isotopic content of the labelled starting materials.

The [A + (B + C)] method is flexible and yields folic acid which requires little subsequent purification. The [(A + B) + C] route has the advantage that the polar dimethyl glutamate moiety is introduced at a late stage in the synthesis. On the other hand, the folic acid produced is often contaminated with varying amounts of pteroic acid and this necessitates lengthy chromatographic purification steps. Indeed, this problem can be ameliorated by using a larger excess of the hydrochloride of dimethyl L-glutamate **10** in the coupling step. However, this is not a viable option with the hydrochloride of [$^2H_4$]dimethyl L-glutamate **28**# due to the high cost of labelled intermediates. Thus on balance the former method is preferred but its success depends crucially on the high state of purity of the *p*-amino-

¶¶ The deuterium content of [$^2H_4$]-*p*-aminobenzoyl-L-glutamate **30**# was found to be 90% against an expected value of 92%. Since the deuterium content of [$^2H_4$]folic acid **32**# was 94% against an expected value of 92%, it is unlikely that a slight leakage of deuterium has occurred in the former case, rather the figures just represent experimental variation.

|||| The actual composition of the labelled starting material is reported in the experimental section.

††† The yield of [$^{13}C_6$]folic acid based on [$^{13}C_6$]-trifluoroacetamidobenzoic acid is 8% for the [(A + B) + C] route and 11% for the [A + (B + C)] route.

‡‡‡ In the numeration of [$^2H_4$]-compounds a # is used as an aid to identification.

§§§ The yield of [$^2H_4$]folic acid based on trifluoroacetamidobenzoic acid is 6% for the [A + (B + C)] route and 7% for the [(A + B) + C] route.

¶¶¶ In the numeration of [$^{13}C_6$][$^2H_4$]-compounds an *# is used as an aid to identification.

|||||| [$^{13}C_6$,$^2H_4$]-*p*-Aminobenzoyl-L-glutamate **34***# is a suitable doubly labelled standard for MS calibration; it was therefore unnecessary to synthesise the doubly labelled folic acid.

Table 1 Summary of the isotopic purity of the labelled compounds and their destination in the human nutrition studies

Sample[a]	Isotopic purity (%) Found[b]/Expected[c]	Destination
[$^{13}C_6$]Folic acid (**27***)	96/94	Capsules and foods
[$^{2}H_4$]Folic acid (**32**$^{\#}$)	94/92[d]	IV injections
[$^{13}C_6$],[$^{2}H_4$]-PABG (**34***$^{\#}$)	82/86	Internal standard
[$^{2}H_4$]-PABG (**30**$^{\#}$)	90/92	MS calibrant
[$^{13}C_6$]-PABG (**23***)	96/94	MS calibrant

[a] PABG refers to *p*-aminobenzoyl-L-glutamate. [b] The measured isotopic purity was obtained from electrospray negative ion mass spectrometry by comparing the intensities of the isotope cluster corresponding to $[M - H]^-$. [c] The expected minimum isotopic purity was calculated from the isotopic purity of the two labelled starting materials, [$^{13}C_6$]aniline and [3,3,4,4-$^{2}H_4$]-L-glutamic acid. [d] Gregory and Toth[12] prepared [$^{2}H_4$]folic acid **32**$^{\#}$ with an isotopic purity of 88% (measured as [$^{2}H_4$]-*p*-aminobenzoyl-L-glutamate **30**$^{\#}$) from [3,3,4,4-$^{2}H_4$]-L-glutamic acid with an isotopic enrichment of 98 atom percent.

Table 2 Summary of the isotopic composition of the labelled compounds

Sample **27***	Isotopic composition (%)[a]	Sample **32**$^{\#}$	Isotopic composition (%)[a]
[$^{13}C_6$]Folic acid	95.7	[$^{2}H_4$]Folic acid[b]	93.7
[$^{13}C_5$]Folic acid	3.9	[$^{2}H_3$]Folic acid	5.4
[$^{13}C_4$]Folic acid	0.4	[$^{2}H_2$]Folic acid	0.9

[a] The measured isotopic purity was obtained from electrospray negative ion mass spectrometry by comparing the intensities of the isotope cluster corresponding to $[M - H]^-$. [b] Gregory and Toth[12] prepared [$^{2}H_4$]-folic acid **32**$^{\#}$ with an isotopic purity of 88% (measured as [$^{2}H_4$]-*p*-aminobenzoyl-L-glutamate) from [3,3,4,4-$^{2}H_4$]-L-glutamic acid with an isotopic enrichment of 98 atom percent.

benzoyl-L-glutamate **12, 23***, **30**$^{\#}$ and **34***$^{\#}$ used in the reductive amination step.

The conversion of the labelled folic acid to the various reduced forms is currently under investigation.

The isotopic purity of each labelled compound prepared and their destination in human nutrition studies is summarised in Table 1. The isotopic composition is summarised in Table 2. The labelled compounds are currently being used in single dose, dual-labelled isotope protocols with human volunteers to study folate bio-availability from food extrinsically labelled with these folates. Isotope ratios of plasma folate are determined by GC-MS–MS following HPLC fractionation, chemical cleavage to a *p*-aminobenzoyl-L-glutamate fragment and derivatisation.

Folic acid and its derivatives are difficult targets to synthesise on account of their pronounced lack of stability to heat, light and water. New methods need to be investigated which use milder reagents. Methods based on the protective influence of a solid phase and the use of solid-phase biotransformations are currently under investigation. The field of combinatorial chemistry can offer some useful insights in this context.

Experimental

General

Melting points were determined with a Gallenkamp digital capillary apparatus and are uncorrected. Optical rotations (given in units of 10^{-1} deg cm^{-2} g^{-1}) were measured on an Optical Activity AA-1000 polarimeter using a 1 dm path length micro cell. Mass spectra were recorded on a Kratos Profile HV3 instrument (EI or [LSIMS, using *m*-nitrobenzyl alcohol as the matrix]) and a Micromass Platform instrument in ES ionisation mode [samples were dissolved in acetonitrile–water (1:1 by volume), containing 0.1% acetic acid, to give a concentration of 20–250 pmol μl^{-1} and injected into a mobile phase of the same solvent composition, flowing at 10 μl min^{-1}]. NMR spectra were recorded on an Avance DRX Bruker 400 MHz machine, with tetramethylsilane as the reference: ^{1}H at 400 MHz, ^{13}C at 100 MHz with broad band decoupling, and with hexafluorobenzene as reference: ^{19}F at 376 MHz. *J* Values are given in Hz.

Thin layer chromatography was carried out on Merck Kieselgel GF_{254} or cellulose plates. Column chromatography was carried out using Merck silica gel 60H. All solvents were purified following standard literature methods. Light petroleum refers to the fraction boiling in the range 60–80 °C. Analytical reversed phase HPLC was carried out using a Lichrosphere 100 RP-18 (5 μm), 125 × 4 mm column and a Lichrosphere 100 RP-18, 4 × 4 mm guard column and using as mobile phase 5.0 mM aqueous tetrabutylammonium phosphate containing 14% acetonitrile and 2.5% methanol. The test sample was injected in 100 μl of the eluant, eluted isocratically at a flow rate of 0.9 cm^3 min^{-1} and the absorbance at 280 nm monitored: under these conditions the following retention times were recorded; pteroic acid 4.1 min, *p*-aminobenzoyl-L-glutamic acid 6.8 min, folic acid 11.2 min. Semi-preparative reversed phase HPLC was carried out using a Vydac C_{18} column (Phenomenex 201TP510), 250 × 10 mm and using as mobile phase 5.0 mM aqueous tetrabutylammonium phosphate containing 14% acetonitrile and 2.5% methanol. The sample of folic acid to be purified (5 mg) was dissolved in water (2 cm^3), 1 drop of 2 M NaOH was added to clarify the solution which was then filtered through a nylon net and injected in 500 μl portions, eluting isocratically at a flow rate of 2.5 cm^3 min^{-1} and monitoring the absorbance at 280 nm: under these conditions the impurities emerged between a retention of 5.5–15.5 min leaving a clear window region before pure folic acid was collected as a single peak with a retention time of 18–20.5 min.

[$^{13}C_6$]Aniline with an isotopic enrichment of 99.1 atom% was obtained from Europa Scientific. This corresponds to a quoted specification of 94.8% of [$^{13}C_6$]aniline and 5.2% of [$^{13}C_5$]aniline. Our own mass spectrometric estimation (EI) of the distribution of ^{13}C proved difficult because the $[M - 1]^+$ ion contains contributions from [$^{13}C_5$]aniline and a tropylium-type species derived from [$^{13}C_6$]aniline. It is estimated that the content of [$^{13}C_5$]aniline is much less than 9% and that of [$^{13}C_4$] and less substituted materials is negligible. [3,3,4,4-$^{2}H_4$]-L-Glutamic acid was obtained from Fluorochem with an isotopic enrichment of >98 atom%. This corresponds to a minimum content of 92.2% of [3,3,4,4-$^{2}H_4$]-L-glutamic acid. Our own mass spectrometric estimation (EI) of the distribution of ^{2}H was accomplished by derivatisation as the *n*-propyloxycarbonyl-L-glutamic acid di-*n*-propyl ester and monitoring the principal fragment ion, $[M - Pr^nOCO]^+$ at *m*/*z* 226–230 (in the case of unlabelled glutamic acid) and 230–234 (for the labelled material). It is estimated that the content of [$^{2}H_3$]-L-glutamic acid is 6% and that of [$^{2}H_2$] and less substituted materials is negligible. The isotopic purity of the target compounds was estimated from the relative abundances within the molecular ion cluster of the negative ion ES mass spectrum, after correcting for proton losses (using the data for the corresponding unlabelled compounds) and for the contribution of natural abundance ^{13}C.

Pteridine precursors

2-Amino-4-hydroxy-6-formylpteridine dimethyl acetal 2.[23] This compound was prepared from 2-amino-3-cyano-5-chloromethylpyrazine *N*-oxide **1** by a series of 7 reactions essentially according to the literature[23] in an overall yield of 34%. 2-Amino-4-hydroxy-6-formylpteridine dimethyl acetal **2** was obtained as a yellow solid, mp >310 °C (decomp.) (lit.,[23] mp >330 °C); δ_H(400 MHz; d^6-DMSO) 3.33 (6 H, s, 2 × OCH_3), 5.38 [1 H, s, C*H*(OCH_3)$_2$], 6.95–7.10 (2 H, br s, 2-NH_2), 8.70 (1 H, s, 7-H); δ_C(100 MHz; d^6-DMSO) 54.1 (OCH_3), 103.5 (*C*H(OCH_3)$_2$), 128.3 (C 10), 146.5 (C 6), 147.9 (C 7), 154.8

(C 9), 157.7 (C 2) and 161.5 (C 4); *m/z* (LSIMS) 238 (2%, M + 1), 260 (6, M + 23[Na]), 176 (33), 154 (100) and 136 (79).

2-Acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal 3.[22] A suspension of 2-amino-4-hydroxy-6-formylpteridine dimethyl acetal **2** (2.97 g, 12.5 mmol) in acetic anhydride (30 cm^3) was heated at 100 °C for 4 h, during which time the bulk of the starting material dissolved. A small quantity of residual amorphous material was removed by hot filtration through sintered glass. The solution was cooled slowly to 25 °C and finally to 0 °C. The resulting precipitate was collected by suction filtration and washed with cold acetic anhydride, cold toluene and dried *in vacuo* to give *2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal* **3** (from ethanol) as a white microcrystalline solid (2.57 g, 74%), mp 195–197 °C. Found *m/z* (LSIMS, mNBA): MH^+, 280.10481. Calc. for $C_{11}H_{14}N_5O_4$: 280.10458 (*MH*), deviation 0.8 ppm; δ_H(400 MHz; *d*^{6}-DMSO) 2.21 (3 H, s, $COCH_3$), 3.28 (1.4 H, br s, NH), 3.37 (6 H, s, 2 × OCH_3), 5.48 (1 H, s, C*H*(OCH_3)$_2$), 8.93 (1 H, s, 7-H); δ_C(100 MHz; *d*^{6}-DMSO) 24.5 (CO*C*H_3), 54.4 (OCH_3), 103.3 [*C*H(OCH_3)$_2$], 131.0 (C 10), 148.6 (C 7), 149.8 (C 6), 150.4 (C 9), 159.2 (C 2), 162.3 (C 4) and 174.7 (*C*OCH_3); *m/z* (LSIMS, mNBA) 280 (25%, MH^+), 302 (25, M + 23[Na]), 248 (30, M − OCH_3), 177 (49), 154 (100) and 136 (93).

2-Acetylamino-4-hydroxy-6-formylpteridine 4.[22] 2-Acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal **3** (2.56 g, 9.2 mmol) was suspended in aqueous formic acid (88%, 12 cm^3) at 25 °C. The suspension was stirred for 2 h during which time the solid dissolved. The solution was cooled to 5 °C for 3 h to allow a fine precipitate to form. The precipitate was collected by suction filtration, washed with cold formic acid and cold ether and dried *in vacuo*. The resulting solid was recrystallised from hot DMF with addition of ether to give pale yellow crystals of 2-acetylamino-4-hydroxy-6-formylpteridine **4** as its DMF monosolvate (1.75 g, 61%). Found *m/z* (EI): M^+, 233.05484, $C_9H_7N_5O_3$ requires 233.05489, deviation 0.1 ppm; δ_H(400 MHz; CF_3CO_2D) 2.58 (3 H, s, $COCH_3$), 3.27 (3 H, s, NCH_3 [DMF solvate]), 3.39 (3 H, s, NCH_3 [DMF solvate]), 8.44 (1 H, s, 7-H), 9.58 (1 H, s, CHO) and 10.25 (1 H, s, CHO [DMF solvate]); *m/z* (EI) 233 (61%, M^+), 218 (43, M − CH_3), 191 (100, M − C_2H_2O), 163 (15) and 136 (45).

p-Aminobenzoic acid precursors

***p*-Iodoaniline 6.**[26] A mixture of redistilled aniline **5** (10.05 g, 0.11 mol), sodium hydrogen carbonate (13.52 g, 0.16 mol) and water (95 cm^3) was cooled to 10 °C by the addition of crushed ice. The reaction mixture was vigorously stirred and iodine (23 g, 0.09 mol) was added in small portions at 2–3 min intervals over 30 min. The suspension was stirred for 60 min and the precipitated product was recovered by suction filtration washed with cold water and dried in air. The crude product was digested with light petroleum (90 cm^3) and the hot solution was decanted into a beaker, at 0 °C, with constant stirring. The product crystallised immediately as colourless needles which were isolated by suction filtration to give *p*-iodoaniline **6** (15.5 g, 65%), mp 61.0–61.5 °C (lit.,[26] mp 62–63 °C). Found *m/z* (EI): M^+, 218.95442, C_6H_6IN requires 218.95450, deviation 0.3 ppm; δ_H(400 MHz; $CDCl_3$) 3.62–3.72 (2 H, br s, NH_2), 6.47 and 7.41 (2 H, d, *J* 7.5, 2Ar*H*[2 and 6]) and 2 H, d, *J* 7.5, 2Ar*H*[3 and 5], AA′BB′); δ_C(100 MHz; $CDCl_3$) 79.4 (*C*–I), 117.3 (C 2 and 6), 137.9 (C 3 and 5) and 146.0 (*C*–NH_2); *m/z* (EI) 219 (100%, M^+) and 92 (27, M − I).

***p*-Iodotrifluoroacetanilide 7.**[27,36] Trifluoroacetic anhydride (1.95 cm^3, 13.8 mmol) was added dropwise over a period of 30 min to a cold, 0 °C, stirred solution of *p*-iodoaniline, **6** (0.99 g, 4.5 mmol) in dichloromethane (10 cm^3). The reaction mixture was allowed to warm to room temperature and was stirred for a further 60 min. On completion, the reaction mixture was concentrated on a rotary evaporator to give a solid residue. Residual trifluoroacetic anhydride was removed by repeated evaporation with carbon tetrachloride. The solid product was recrystallised from ether–light petroleum to give *p*-iodotrifluoroacetanilide **7** (1.11 g, 78%) as colourless needles, mp 147.2–147.5 °C (lit.,[36] mp 141–142 °C). Found *m/z* (EI): M^+, 314.93572, $C_8H_5F_3INO$ requires 314.93680, deviation 3.4 ppm; δ_H(400 MHz; CD_3OD) 7.45 and 7.70 (2 H, d, *J* 6.8, 2Ar*H*[2 and 6] and 2 H, d, *J* 6.8, 2Ar*H*[3 and 5], AA′BB′); δ_C(100 MHz; CD_3OD); 88.8 (*C*–I), 116.0 (q, *J* 286, *C*F_3), 122.6 (C 2 and 6), 136.3 (*C*–NHR), 137.8 (C 3 and 5) and 155.4 (q, *J* 37, *C*OCF_3); δ_F(376 MHz; CD_3OD); 86.9 (s, C*F*$_3$); *m/z* (EI) 315 (75%, M^+), 226 (52), 210 (24), 201 (46), 180 (13), 164 (18), 153 (21), 136 (18), 120(12), 107 (37) and 91 (100).

***p*-Trifluoroacetamidobenzoic acid 8.**[24,25,28,29] A solution of *p*-iodotrifluoroacetanilide **7** (2.03 g, 6.4 mmol) in anhydrous ether (63 cm^3) was cooled to −78 °C in an acetone–"Drikold" bath and treated dropwise with stirring with *n*-butyllithium (1.6 M in hexane, 8.75 cm^3, 14 mmol) under nitrogen. The reaction mixture was stirred for 2 h at −78 °C and poured onto a slurry of ether and solid carbon dioxide, with vigorous stirring. The pale yellow reaction mixture was left to warm to room temperature and acidified with dilute hydrochloric acid until the evolution of gas had ceased. The organic phase was separated and the aqueous phase extracted with ether (3 × 50 cm^3). The combined organic phases were washed with saturated sodium hydrogen carbonate solution (3 × 23 cm^3) and the combined aqueous phases were neutralised with hydrochloric acid. The resulting precipitate was collected by suction filtration to give *p*-trifluoroacetamidobenzoic acid **8** as a white powder (1.01 g, 67%), mp 297–298 °C (decomp.) [lit.,[28] 285 °C, lit.,[29] 274 °C (sublim.)]. Found: *m/z* (EI): M^+, 233.02989, $C_9H_6F_3NO_3$ requires 233.02998, deviation 0.3 ppm; δ_H(400 MHz; CD_3OD) 7.78 and 8.03 (2 H, d, *J* 8.9, Ar*H*[3 and 5] and 2 H, d, *J* 8.9, Ar*H*[2 and 6], AA′BB′); δ_C(100 MHz; CD_3OD) 113.9 (q, *J* 286, *C*F_3), 120.1 (C 3 and 5), 127.6 (C 1), 130.4 (C 2 and 6), 140.6 (C 4), 156.9 (q, *J* 33, *C*OCF_3) and 167.7 (*C*O_2H); δ_F(376 MHz; CD_3OD) 86.8 (s, C*F*$_3$); *m/z* (EI) 233 (100%, M^+), 216 (18, M − OH), 188 (2, M − CO_2H), 164 (47, M − CF_3), 146 (9), 136 (32, M − CF_3CO), 121 (30, M − CF_3CONH) and 108 (10).††††

***p*-Aminobenzoic acid 9.**[35] *p*-Trifluoroacetamidobenzoic acid **8** (0.71 g, 3.05 mmol) was suspended in aqueous sodium hydroxide (0.1 M, 100 cm^3) and stirred for 4 h at ambient temperature. TLC analysis (EtOAc) revealed that the starting material had been completely consumed and a slower moving, more polar species, had been formed. The reaction was quenched by the addition of dilute hydrochloric acid to pH 4. The reaction mixture was extracted with ethyl acetate (3 × 20 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and concentrated on a rotary evaporator to give an off white solid. Recrystallisation from hot water gave *p*-aminobenzoic acid **9** (0.26 g, 62%) as colourless needles mp 187–188 °C (lit.,[35] mp 186–187 °C). Found: *m/z* (EI): M^+, 137.04784, $C_7H_7NO_2$ requires 137.04768, deviation 1.1 ppm; δ_H(400 MHz; $CDCl_3$) 6.60 (2 H, d, *J* 8.0, Ar*H*[3 and 5]) and 7.82 (2 H, d, *J* 8.0, Ar*H*[2 and 6], AA′BB′) and 6.60–6.68 (2 H, br s, NH_2); *m/z* (EI) 137 (100%, M^+), 120 (94, M − OH), 92 (44, M − CO_2H) and 69 (74).

Glutamic acid precursor

Dimethyl L-glutamate hydrochloride 10.[12,20] Freshly distilled thionyl chloride (6.5 cm^3, 75 mmol) was added dropwise with

†††† Note the change of numbering of the aromatic system at this point.

stirring to cold, 0 °C, anhydrous methanol (20 cm^3). Dry L-glutamic acid (2.04 g, 14 mmol) was added, in portions, to this mixture and stirred for a 18 h at ambient temperature. On completion, confirmed by TLC analysis (*n*-butanol–acetic acid–water, 6:2:2), the reaction mixture was concentrated on a rotary evaporator to give a heavy syrup which was crystallised by the addition of dry diethyl ether to give a fine white powder, mp 75–77 °C. Recrystallisation from acetone–diethyl ether gave *dimethyl L-glutamate hydrochloride* **10** (2.32 g, 79%) as fine white crystals, mp 78–80 °C, $[\alpha]_D^{23}$ +25.5 (*c* 5.0 in H_2O). Found: *m/z* (EI): M^+, 175.08456. Calc. for $C_7H_{13}NO_4$ (free base): 175.08446, deviation 0.6 ppm and 176.09198. Calc. for $C_7H_{14}NO_4$ (protonated species): 176.09228, deviation 1.7 ppm; δ_H(400 MHz; CD_3OD) 2.16–2.27 (2 H, c m, 2 × β-H), 2.56–2.61 (2 H, c m, 2 × γ-H), 3.70 (3 H, s, γ-CO_2CH_3), 3.84 (3 H, s, α-CO_2CH_3), 4.13 (1 H, t, *J* 6.7, α-H) and 4.76–4.84 (3 H, br s, $^+NH_3$); δ_C(100 MHz; CD_3OD), 25.2 (γ-CO_2CH_3), 28.8 (α-CO_2CH_3), 51.0 (β-CH_2), 51.9 (α-CH), 52.4 (γ-CH_2), 169.1 (γ-CO_2CH_3), 172.7, (α-CO_2CH_3); *m/z* (EI), 176 (15%, MH^+), 144 (51, $M^+ - [OCH_3+H^+]$), 116 (100), 100 (24), 88 (61), 84 (98), 71 (9) and 56 (87).

p-Aminobenzoyl-L-glutamate precursors

Dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate 11. A suspension of *p*-trifluoroacetamidobenzoic acid **8** (1.00 g, 4.29 mmol), dimethyl L-glutamate hydrochloride **10** (0.95 g, 4.50 mmol) and HOBT (1.16 g, 8.59 mmol) in anhydrous THF (20 cm^3) was cooled to −5 °C in an ice salt bath and treated with diisopropylethylamine (1.47 cm^3, 8.60 mmol) followed by DCC (0.88 g, 4.27 mmol). The mixture was stirred under an atmosphere of dry nitrogen until TLC (light petroleum–ethyl acetate, 1:1) indicated completion (48 h). The reaction mixture was then suction filtered and the filtrate concentrated *in vacuo* to give a heavy syrup which was purified by flash chromatography (light petroleum–ethyl acetate, 1:1). Combination and concentration of the appropriate product fractions gave the desired *p-trifluoroacetamidobenzoyl-L-glutamic acid dimethyl ester* **11** (1.17 g, 70%) as a pale yellow solid, mp 135–136 °C. Found: *m/z* (EI): M^+ 390.10410. Calc. for $C_{16}H_{17}F_3N_2O_6$: 390.10387, deviation 0.5 ppm; δ_H(400 MHz; CD_3OD) 2.05–2.15 (1 H, c m, β-H_a), 2.25–2.35 (1H, c m, β-H_b), 2.50 (2 H, t, *J* 6.4, 2 × γ-H), 3.65 (3 H, s, γ-CO_2CH_3), 3.75 (3 H, s, α-CO_2CH_3), 4.63 (1 H, m, α-H), 7.79 and 7.88 (2 H, d, *J* 8, Ar*H*[3 and 5] and 2 H, d, *J* 8, Ar*H*[2 and 6], AA′BB′); δ_C(100 MHz; CD_3OD), 26.0 (β-CH_2), 29.9 (γ-CH_2), 50.8 (γ-CO_2CH_3), 51.5 (α-CO_2CH_3), 52.4 (α-CH), 115.9 (q, *J* 285, CF_3), 120.2 (C 2 and 6), 128.2 (C 3 and 5), 130.6 (C-1), 139.7 (C-4), 155.5 (q, *J* 38, $COCF_3$), 168.0 (CONH), 172.3 (γ-CO_2CH_3) and 173.5 (α-CO_2CH_3); δ_F(376 MHz; CD_3OD), 86.83 (s, CF_3); *m/z* (EI), 390 (1%, M^+), 331 (6, $M - CO_2CH_3$) and 216 (100, $M - C_7H_{12}NO_4$ [*i.e.*, M − dimethyl glutamate moiety]).

***p*-Aminobenzoyl-L-glutamic acid 12.**[34] *p*-Trifluoroacetamidobenzoyl-L-glutamic acid dimethyl ester **11** (0.108 g, 0.28 mmol) was suspended in aqueous sodium hydroxide (0.1 M, 12 cm^3) containing methanol (1 cm^3) and stirred at ambient temperature for 4 h. TLC (light petroleum–ethyl acetate, 1:3) showed that the starting material had been completely consumed and a slower moving, more polar species had been formed. The mixture was extracted with ether (1 × 20 cm^3). The reaction was quenched by the addition of dilute hydrochloric acid to pH 4. The reaction mixture was saturated with sodium chloride and extracted with ethyl acetate (5 × 20 cm^3). The organic fractions were pooled, dried ($MgSO_4$) and evaporated on a rotary evaporator to give *p*-aminobenzoyl-L-glutamic acid **12** (45 mg, 61%) as an off white solid, mp 173–174 °C (decomp.) [lit.,[34] mp 174.5–175 °C (decomp.)], $[\alpha]_D^{23}$ −15 (*c* 2.0 in 0.1 M HCl) [lit.,[34] $[\alpha]_D^{20}$ −17.0 (*c* 8.7 in aq. HCl)]. Found: *m/z* (EI): M^+ 266.09036, $C_{12}H_{14}N_2O_5$ requires 266.09027, deviation 0.3 ppm; δ_H(400 MHz; CD_3OD) 2.06–2.14 (1 H, c m, β-H_a), 2.25–2.33 (1H, c m, β-H_b), 2.47 (2 H, t, *J* 7.6, 2 × γ-H), 4.61 (1 H, m, α-H), 6.91 and 7.75 (2 H, d, *J* 8.8 Ar*H*[3 and 5] and 2 H, d, *J* 8.8 Ar*H*[2 and 6], AA′BB′); δ_C(100 MHz; CD_3OD) 26.3 (β-CH_2), 30.1 (γ-CH_2), 52.3 (α-CH), 116.2 (C 2 and 6), 125.3 (C-4), 128.9 (C 3 and 5), 146.5 (C-1), 168.6 (CONH), 173.8 (γ-CO_2H) and 175.2, (α-CO_2H); *m/z* (EI), 266 (3%, M^+), 248 (6, $M - H_2O$), 222 (3, $M - CO_2$), 137 (45) and 120 (100, $M - C_5H_8NO_4$[glutamate moiety]); *m/z* (ES), 797 (*ca*. 10%, $[3M - H]^-$), 531.1 (100, $[2M - H]^-$) and 265.1 (95, $[M - H]^-$).

Pteroic acid derivatives

***N*(2)-Acetylpteroic acid (*p*-[(2-acetamido-4-hydroxypteridin-6-yl)methylamino]benzoic acid) 13.**[17] Glacial acetic acid (5 cm^3) was added to a finely powdered mixture of *p*-aminobenzoic acid **9** (137 mg, 1 mmol) and 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** (306 mg, 1 mmol) and the resulting paste was stirred for 15 min at ambient temperature. As far as possible the rest of the experiment was conducted in the dark. Dimethylamine–borane complex (100 mg, 1.7 mmol) in glacial acetic acid (1.5 cm^3) was added to the paste and the reaction mixture was stirred for a further 20 min and heated for 10 min at 60 °C. The solution was allowed to cool slowly in the dark to 25 °C and left at 4 °C overnight. The precipitated solid was recovered by centrifugation, washed with water (3 × 2 cm^3) and acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give *N*(2)-acetylpteroic acid **13**. The product was recrystallised from hot water as a bright yellow powder (275 mg, 78%). Found *m/z* (EI): M^+, 354.10615, $C_{16}H_{14}N_6O_4$ requires 354.10765, deviation 4.2 ppm; δ_H(400 MHz; CF_3CO_2D) 2.28 (3 H, s, $COCH_3$), 5.35 (2 H, s, 2 × 9-H), 7.93 and 8.44 (2 H, d, *J* 8.6, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.6, Ar*H*[2′ and 6′], AA′BB′) and 9.24 (1 H, s, 7-H); *m/z* (EI) 354 (1%, M^+), 220 (27), 204 (30), 177 (58), 163 (30), 150 (34), 137 (100), 120 (94) and 108 (36).

***N*(2)-Acetyl-*N*(10)-trifluoroacetylpteroic acid (*p*-[*N*-(2-acetamido-4-hydroxypteridin-6-ylmethyl)-*N*-trifluoroacetylamino]-benzoic acid) 14.**[17] The *N*(2)-acetylpteroic acid **13** (275 mg, 0.78 mmol) was dissolved in a mixture of trifluoroacetic acid (8 cm^3) and trifluoroacetic anhydride (16 cm^3) and heated under reflux for 90 min. The reaction mixture, cooled to ambient temperature, was concentrated *in vacuo* to give a light brown oil which was triturated with warm water (30 cm^3), washed well with water and ether and dried to give *N*(2)-acetyl-*N*(10)-trifluoroacetylpteroic acid **14** as a pale grey crystalline solid (263 mg, 75%). δ_H(400 MHz; d^6-DMSO); forms *a*:*b*:*c* (approximate ratio by integration, 50:25:25), form *a*, 2.19 (3 H, s, $COCH_3$), 5.21 (2 H, s, 2 × 9-H), 7.66 and 7.96 (2 H, d, *J* 8.4, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.4 Ar*H*[2′ and 6′], AA′BB′) and 8.86 (1 H, s, 7-H); form *b*, 2.19 (3 H, s, $COCH_3$), 5.10 (2 H, s, 2 × 9-H), 7.31 and 7.69 (2 H, d, *J* 8.4, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.4 Ar*H*[2′ and 6′], AA′BB′) and 8.75 (1 H, s, 7-H); form *c*, 2.19 (3 H, s, $COCH_3$), 5.34 (2 H, s, 2 × 9-H), 7.41 (4 H, s, Ar*H*[2′, 3′, 5′ and 6′]) and 8.97 (1 H, s, 7-H); δ_F(376 MHz, d^6-DMSO), 97.7 (s, CF_3) and 97.77 (s, CF_3); *m/z* (LSIMS) 451 (10%, MH^+), 473 (3, M + 23[Na]), 307 (17), 289 (10), 176 (17), 154 (100) and 138 (75).

Folic acid and its precursors by the [A + (B + C)] route

***N*(2)-Acetylfolic acid 15.**[22] Glacial acetic acid (3 cm^3) was added to a finely powdered mixture of *p*-aminobenzoyl-L-glutamic acid **12** (110 mg, 0.41 mmol) and 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** (104 mg, 0.34 mmol) and the resulting paste was stirred for 15 min at ambient temperature. As far as possible the rest of the experiment was conducted in the dark. Dimethylamine–borane complex (42 mg, 0.71 mmol) in glacial acetic acid (1 cm^3) was added to the paste and the mixture was stirred for a further 20 min. The reaction mixture was heated for 10 min at 60 °C (it became homo-

geneous after heating for 5 min) and slowly cooled in the dark to 25 °C and left at 4 °C overnight. The reaction was monitored by TLC (ethyl acetate). The precipitated yellow solid was collected by centrifugation and washed with water (3 × 2 cm^3) and acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give *N(2)-acetylfolic acid* **15**. The product was recrystallised from hot water as a bright yellow powder (70 mg, 43%). This compound shows moderate instability under the conditions of recrystallisation. Accordingly the ^{1}H NMR spectrum shows minority signals attributed to *p*-aminobenzoyl-L-glutamic acid **12** (*ca.* 0.09 equiv.), which have been subtracted from the data presented below. Found *m/z* (LSIMS): MH^+, 484.15937, $C_{21}H_{22}N_7O_7$ requires *MH*, 484.15819, deviation 2.6 ppm; δ_H(400 MHz; CF_3CO_2D) 2.39–2.43 (1 H, c m, β-H_a), 2.56 (3 H, s, $COCH_3$), 2.59–2.62 (1H, c m, β-H_b), 2.83 (2 H, t, *J* 7.0, 2 × γ-H), 5.07–5.10 (1 H, m, α-H), 5.32 (2 H, s, 2 × 9-H), 7.92 and 8.15 (2 H, d, *J* 8.7, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.7 Ar*H*[2′ and 6′], AA′BB′) and 9.17 (1 H, s, 7-H); *m/z* (FAB, mNBA) 484 (50%, MH^+), 506 (24, M + 23[Na]), 337 (100, M − $C_5H_8NO_4$ [*i.e.*, M − glutamate moiety]), 273 (41), 217 (36), 199 (17) and 176 (57, $C_6H_{10}NO_5$ [*i.e.*, glutamate + CO + 2H]).

Folic acid 16.[22] The *N*(2)-acetylfolic acid **15** (65.4 mg, 0.13 mmol) was refluxed in aqueous sodium hydroxide solution (0.1 M, 80 cm^3) for 90 min in the dark under nitrogen. On completion, decolourising charcoal was added and the hot mixture was stirred for a further 10 min. The solution was filtered, the pH adjusted to 3.5 using 2 M hydrochloric acid and allowed to cool slowly to room temperature. The resulting suspension was left overnight at 5 °C. The precipitate was recovered by centrifugation, washed with water (3 × 2 cm^3), acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give folic acid **16**. The product was recrystallised from hot water as a bright yellow powder (39.2 mg, 66%); $[a]_D^{23}$ +18.0 (*c* 1 in 0.1 M NaOH) [lit.,[15] $[a]_D^{22}$ +16.0 (*c* 0.5 in 0.1 M NaOH), lit.,[33] $[a]_D^{20}$ +16 (*c* 0.76 in 0.2 M NaOH)]. Found: δ_H(400 MHz; CF_3CO_2D) 2.40–2.47 (1 H, c m, β-H_a), 2.62–2.69 (1H, c m, β-H_b), 2.85 (2 H, t, *J* 6.9, 2 × γ-H), 5.10–5.14 (1 H, m, α-H), 5.29 (2 H, s, 2 × 9-H), 7.97 and 8.16 (2 H, d, *J* 8.0, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.0 Ar*H*[2′ and 6′], AA′BB′) and 9.03 (1 H, s, 7-H); *m/z* (FAB, mNBA) 442 (18%, MH^+), 465 (19, MH + 23[Na]), 295 (49, M − $C_5H_8NO_4$[glutamate moiety]) and 176 (100, $C_6H_{10}NO_5$ [*i.e.*, glutamate + CO + 2H]); *m/z* (ES) 881 (*ca.* 2%, $[2M - H]^-$), 660.8 (*ca.* 3, $[3M - 2H]^{2-}$), 615.1 (10, $[3M - 2H - HCO_2H]^{2-}$), 440.1 (57, $[M - H]^-$), 219.7 (100, $[M - 2H]^{2-}$).

Folic acid and its precursors by the [(A + B) + C] route

Folic acid 16.[12,16,17,30] *N*(2)-Acetyl-*N*(10)-trifluoroacetylpteroic acid **14** (45 mg, 0.1 mmol) was suspended in anhydrous DMF (2 cm^3) containing triethylamine (0.075 cm^3, 0.54 mmol) and cooled to 5 °C. As far as possible the experiment was conducted in the dark. Freshly distilled isobutyl chloroformate (0.02 cm^3, 0.15 mmol) was added and the mixture was stirred for 15 min at 5 °C. Dimethyl glutamate hydrochloride **10** (45 mg, 0.2 mmol) was added in one portion. The resulting suspension was allowed to warm to room temperature slowly and was stirred for 72 h under an inert atmosphere. The reaction mixture was evaporated on a rotary evaporator and the residue was washed by trituration with water, aqueous ammonium hydrogen carbonate and ether and dried *in vacuo* to give crude dimethyl *N*(2)-acetyl-*N*(10)-trifluoroacetylfolate (32 mg, 53%). The protected folate was suspended in aqueous sodium hydroxide solution (0.1 M, 30 cm^3) and heated under reflux for 90 min in the dark in an inert atmosphere. On completion, decolourising charcoal was added and the hot mixture stirred for a further 10 min. The solution was filtered, the pH adjusted to 3.5 using 2 M hydrochloric acid and allowed to cool slowly to room temperature. The resulting suspension was left overnight at 5 °C. The yellow precipitate was recovered by centrifugation, washed with water (3 × 2 cm^3) and acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give folic acid **16**. The resulting solid was further purified by ion exchange chromatography on A-25 DEAE fractogel, eluting with a gradient of ammonium hydrogen carbonate (0.2 to 2.0 M). The relevant fractions were pooled and lyophilised to yield folic acid which was recrystallised from hot water as a bright yellow powder (15 mg, 34%); $[a]_D^{23}$ +17.5 (*c* 1 in 0.1 M NaOH); $[a]_D^{23}$ for material prepared by [**A** + (**B** + **C**)] route +18.0 (*c* 1 in 0.1 M NaOH) [lit.,[15] $[a]_D^{22}$ +16.0 (*c* 0.5 in 0.1 M NaOH), lit.,[33] $[a]_D^{20}$ +16 (*c* 0.76 in 0.2 M NaOH)]. Found: δ_H(400 MHz; CF_3CO_2D); 2.38–2.48 (1 H, c m, β-H_a), 2.59–2.69 (1H, c m, β-H_b), 2.85 (2 H, t, *J* 6.8, 2 × γ-H), 5.09–5.13 (1 H, m, α-H), 5.29 (2 H, s, 2 × 9-H), 7.95 and 8.16 (2 H, d, *J* 7.6, Ar*H*[3′ and 5′] and 2 H, d, *J* 7.6, Ar*H*[2′ and 6′], AA′BB′) and 9.04 (1 H, s, 7-H); *m/z* (ES) 903 (sodium adduct), 881 (*ca.* 5%, $[2M - H]^-$), 660.8 (*ca.* 8, $[3M - 2H]^{2-}$), 440.4 (85, $[M - H]^-$), 219.7 (100, $[M - 2H]^{2-}$) and 126.9 (59).

[$^{13}C_6$]-Labelled compounds

All labelled materials were prepared by the procedures described above for the corresponding unlabelled materials. The general section contains details of the source of the labelled starting materials and their isotopic purity.

[$^{13}C_6$]-Labelled *p*-aminobenzoic acid precursors

[1,2,3,4,5,6-$^{13}C_6$]-*p*-Iodoaniline 18*. [$^{13}C_6$]Aniline **17*** (2 g, 20 mmol) was converted to [$^{13}C_6$]-*p*-iodoaniline **18*** using the procedure described for the conversion of aniline **5** to *p*-iodoaniline **6**. *[1,2,3,4,5,6-$^{13}C_6$]-p-Iodoaniline* **18*** (2.57 g, 57%) was obtained as colourless needles from light petroleum, mp 62–63 °C (mp for unlabelled *p*-iodoaniline **6** 61.0–61.5 °C). Found *m/z* (EI): M^+, 224.97409. Calc. for $^{13}C_6H_6IN$: 224.97463, deviation 2.4 ppm; δ_H(400 MHz; $CDCl_3$) 3.4–3.9 (2 H, br, NH_2), 6.24–6.31 and 6.63–6.70 (2 H, d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 156, 2Ar*H*[2 and 6]) and 7.17–7.24 and 7.57–7.65 (2 H, d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 162, 2Ar*H*[3 and 5]);‡‡‡‡ δ_C(100 MHz; $CDCl_3$) 79.8 (t of d of t, $^1J_{C3\text{-}4}$ [$^1J_{C4\text{-}5}$] 62.0, $^3J_{C1\text{-}4}$ 9.7, $^2J_{C2\text{-}4}$ [$^2J_{C2\text{-}6}$] 2.3, C-4), 117.2 (t of c m, C 2 and 6),§§§§ 137.9 (t of c m, C 3 and 5)§§§§ and 146.0 (t of d, $^1J_{C1\text{-}2}$ [$^1J_{C1\text{-}6}$] 62.3, $^3J_{C1\text{-}4}$ 9.7, C-1); *m/z* (EI) 225 (100%, M^+), 127 (11), 112 (15), 98 (46, M − I) and 84 (8).

[1,2,3,4,5,6-$^{13}C_6$]-*p*-Iodotrifluoroacetanilide 19*. [$^{13}C_6$]-*p*-Iodoaniline **18*** (2.57 g, 11 mmol) was converted to [$^{13}C_6$]-*p*-iodotrifluoroacetanilide **19*** using the procedure described for the conversion of *p*-iodoaniline **6** to *p*-iodotrifluoroacetanilide **7**. *[1,2,3,4,5,6-$^{13}C_6$]-p-Iodotrifluoroacetanilide* **19*** (2.94 g, 80%) was obtained as colourless needles from diethyl ether–light petroleum, mp 148–149 °C (mp for unlabelled *p*-iodotrifluoroacetanilide **7** 147.2–147.5 °C); Found *m/z* (EI): M^+, 320.95664. Calc. for $^{12}C_2{}^{13}C_6H_5F_3INO$: 320.95693, deviation 0.8 ppm; δ_H(400 MHz; $CDCl_3$) 7.10–7.18 and 7.47–7.58 (2 H, overlapping d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 156, 2Ar*H*[2 and 6]), 7.47–7.58 and 7.87–7.95 (2 H, overlapping d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 152, 2Ar*H*[3 and 5]) and 7.78–7.93 (1 H, br, NH);‡‡‡‡ δ_C(100 MHz; $CDCl_3$) 90.3 (c t, C-4), 122.2 (c t, C 2 and 6), 134.5 (c t, C-1) and 138.4 (c t, C 3 and 5);§§§§,¶¶¶¶ δ_F(376 MHz; $CDCl_3$); 88.2 (d, $^4J_{C1\text{-}F}$ 1.1, CF_3); *m/z* (EI) 321 (100%, M^+), 252 (17, M − CF_3), 224 (33, M − C_2F_3O), 209 (8, M − C_2HF_3NO), 195 (3), 127 (8, I), 108 (7), 97 (44, C_2F_3O), 82 (22) and 69 (67).

[1,2,3,4,5,6-$^{13}C_6$]-*p*-Trifluoroacetamidobenzoic acid 20*. [$^{13}C_6$]-*p*-Iodotrifluoroacetanilide **19*** (2.90 g, 9.0 mmol) was

‡‡‡‡ Further coupling complex due to second order effects in 10-spin system, AA′BB′CC′DD′XY.

§§§§ Spectral signals complex due to second order effects in 6-spin system, AA′BB′XY.

¶¶¶¶ In the ^{13}C NMR spectrum, only the signals due to enriched carbon atoms are visible, the signals of the natural abundance carbon atoms are not resolved.

converted to [$^{13}C_6$]-*p*-trifluoroacetamidobenzoic acid **20*** using the procedure described for the conversion of *p*-iodotrifluoroacetanilide **7** to *p*-trifluoroacetamidobenzoic acid **8**. *[1,2,3,4,5,6-$^{13}C_6$]-p-Trifluoroacetamidobenzoic acid* **20*** was obtained as a white powder (0.95 g, 44%), mp 297–298 °C (decomp.) [mp for unlabelled *p*-trifluoroacetamidobenzoic acid **8** 297–298 °C (decomp.)]. Found *m/z* (EI): M^+, 239.05024. Calc. for $^{12}C_3{}^{13}C_6H_6F_3NO_3$: 239.05011, deviation 0.5 ppm; δ_H(400 MHz; d^6-DMSO) 7.54–7.62 and 7.96–8.04 (2 H, d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 168, 2Ar*H*[3 and 5]), 7.72–7.80 and 8.12–8.20 (2 H, d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 160, 2Ar*H*[2 and 6]);‡‡‡‡ δ_C(100 MHz; d^6-DMSO) 128.1 (t d, $^1J_{C1\text{-}2}$ [$^1J_{C1\text{-}6}$] 58.0, $^3J_{C1\text{-}4}$ 9.0, C-1), 120.9 (t of c m, C 3 and 5),§§§§ 130.8 (t of c m, C 2 and 6)§§§§ and 140.8 (t of d, $^1J_{C3\text{-}4}$ [$^1J_{C4\text{-}5}$] 63.0, $^3J_{C1\text{-}4}$ 9.0, C-4);¶¶¶¶ δ_F(376 MHz; d^6-DMSO) 90.0 (s, CF_3); *m/z* (EI) 239 (100%, M^+), 222 (18, M − OH), 194 (2, M − CO_2H), 170 (62, M − CF_3), 152 (12), 142 (41, M − CF_3CO), 127 (54, M − CF_3CONH), 97 (20, CF_3CO) and 69 (64, CF_3).††††

[1,2,3,4,5,6-$^{13}C_6$]-*p*-Aminobenzoic acid 21*. [$^{13}C_6$]-*p*-Trifluoroacetamidobenzoic acid **20*** (0.57 g, 2.38 mmol) was detrifluoroacetylated using hydrolysis with aqueous sodium hydroxide (0.1 M, 40 cm^3) as described for the conversion of *p*-trifluoroacetamidobenzoic acid **8** to *p*-aminobenzoic acid **9**. *[1,2,3,4,5,6-$^{13}C_6$]-p-Aminobenzoic acid* **21*** (0.20 g, 59%) was obtained as colourless needles from hot water, mp 187–188 °C (mp for unlabelled *p*-aminobenzoic acid 187–188 °C); δ_H(400 MHz; $CDCl_3$); 6.36–6.42 and 6.78–6.84 (2 H, d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 168, 2Ar*H*[3 and 5]), 7.59–7.65 and 7.99–8.05 (2 H, d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 160, 2Ar*H*[2 and 6]);‡‡‡‡ *m/z* (EI) 143 (100%, M^+), 126 (94, M − OH) and 98 (44, M − CO_2H).

[$^{13}C_6$]-Labelled *p*-aminobenzoyl-L-glutamate precursors

Dimethyl *p*-trifluoroacetamido{[1,2,3,4,5,6-$^{13}C_6$]benzoyl}-L-glutamate 22*. [$^{13}C_6$]-*p-Trifluoroacetamidobenzoic acid* **20*** (0.65 g, 2.72 mmol) was coupled with dimethyl L-glutamate hydrochloride **10** (0.63 g, 2.97 mmol) to yield dimethyl [$^{13}C_6$]-*p*-trifluoroacetamidobenzoyl-L-glutamate **22*** using DCC (0.61 g, 2.96 mmol), HOBT (0.73 g, 5.44 mmol) and diisopropylethylamine (0.51 cm^3, 2.93 mmol) by the procedure described for the preparation of dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11**. *Dimethyl p-trifluoroacetamido{[1,2,3,4,5,6-$^{13}C_6$]-benzoyl}-L-glutamate* **22*** (0.79 g, 73%) was obtained as a pale yellow powder, mp 135–136 °C (decomp.) [mp for unlabelled dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11** 135–136 °C (decomp.)]; $[\alpha]_D^{23}$ −15 (*c* 2.0 MeOH). Found *m/z* (EI): M^+, 396.12505. Calc. for $^{12}C_{10}{}^{13}C_6H_{17}F_3N_2O_6$: 396.12400, deviation 2.6 ppm; δ_H(400 MHz; CD_3OD) 2.08–2.15 (1 H, c m, β-H_a), 2.27–2.34 (1H, c m, β-H_b), 2.50 (2 H, t, *J* 7.2, 2 × γ-H), 3.66 (3 H, s, γ-CO_2CH_3), 3.75 (3 H, s, α-CO_2CH_3), 4.63–4.67 (1 H, m, α-H), 7.55–7.61 and 7.96–8.02 (2 H, d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 167, 2Ar*H*[3 and 5]), 7.65–7.74 and 8.05–8.13 (2 H, d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 167, 2Ar*H*[2 and 6]);‡‡‡‡ δ_C(100 MHz; CD_3OD) 26.0 (β-CH_2), 29.9 (γ-CH_2), 50.8 (γ-CO_2CH_3), 51.4 (α-CO_2CH_3), 52.3 (α-*C*H), 120.1 (t of c m, C 3 and 5),§§§§ 128.1 (t of c m, C 2 and 6),§§§§ 130.1 (t of d, $^1J_{C1\text{-}2}$ [$^1J_{C1\text{-}6}$] 57, C-1) and 139.7 (t of d, $^1J_{C3\text{-}4}$ [$^1J_{C4\text{-}5}$] 67, C-4);||||||| δ_F(376 MHz, CD_3OD) 86.79 (s, CF_3); *m/z* (EI), 396 (5%, M^+), 364 (6, M − CH_3OH), 337 (12, M − CO_2CH_3), 222 (100, M − $C_7H_{12}NO_4$ [*i.e.* M − dimethyl glutamate moiety]) and 174 (16, $C_7H_{12}NO_4$ [dimethyl glutamate moiety]).

{*p*-Amino[1,2,3,4,5,6-$^{13}C_6$]benzoyl}-L-glutamic acid 23*. Dimethyl [$^{13}C_6$]-*p*-trifluoroacetamidobenzoyl-L-glutamate **22*** (0.79 g, 1.99 mmol) was deprotected by hydrolysis using aqueous sodium hydroxide to give [$^{13}C_6$]-*p*-aminobenzoyl-L-glutamic acid **23*** by the procedure described for the preparation of *p*-aminobenzoyl-L-glutamic acid **12**. *{p-Amino[1,2,3,4,5,6-$^{13}C_6$]-benzoyl}-L-glutamic acid* **23*** (0.32 g, 59%) was obtained as a white solid, mp 172–173 °C (decomp.) [mp for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** 173–174 °C (decomp.)], $[\alpha]_D^{23}$ −14 (*c* 2.0 in 0.1 M HCl) [$[\alpha]_D^{23}$ for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** −15 (*c* 2.0 in 0.1 M HCl)]. Found *m/z* (EI): M^+, 272.11087. Calc. for $^{12}C_6{}^{13}C_6H_{14}N_2O_5$: 272.11040, deviation 1.7 ppm; δ_H(400 MHz; CD_3OD) 2.05–2.14 (1 H, c m, β-H_a), 2.25–2.33 (1H, c m, β-H_b), 2.48 (2 H, t, *J* 8.0, 2 × γ-H), 4.58–4.62 (1 H, m, α-H), 6.47–6.52 and 6.85–6.92 (2 H, d of c m, $^1J_{C3\text{-}H}$ [$^1J_{C5\text{-}H}$] 158, 2Ar*H*[3 and 5]), 7.41–7.48 and 7.81–7.88 (2 H, d of c m, $^1J_{C2\text{-}H}$ [$^1J_{C6\text{-}H}$] 158, 2Ar*H*[2 and 6]);‡‡‡‡ δ_C(100 MHz; CD_3OD) 26.3 (β-CH_2), 30.1 (γ-CH_2), 52.2 (α-*C*H), 113.5 (t of c m, C 3 and 5),§§§§ 128.8 (t of c m, C 2 and 6),§§§§ 121.6 (t d, $^1J_{C1\text{-}2}$ [$^1J_{C1\text{-}6}$] 68, C-1), 151.3 (t d, $^1J_{C3\text{-}4}$ [$^1J_{C4\text{-}5}$] 66, C-4), 174.0 (γ-CO_2H) and 175.3 (α-CO_2H);||||||||| *m/z* (EI) 272 (1%, M^+), 254 (34, M − H_2O), 228 (6, M − CO_2), 224 (8), 208 (17), 194 (16), 143 (38) and 126 (100, M − $C_5H_8NO_4$ [*i.e.* M − glutamate moiety]); *m/z* (ES) 853 (potassium adduct), 815 (*ca.* 5%, $[3M - H]^-$), 543 (*ca.* 12%, $[2M - H]^-$), 271.1 (100%, $[M - H]^-$). Isotopic composition of labelled *p*-aminobenzoyl-L-glutamic acid **23***: [$^{13}C_6$] 96.0% and [$^{13}C_5$] 3.8.

[$^{13}C_6$]-Labelled pteroic acid derivatives

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-*N*(2)-Acetylpteroic acid 24*. A finely powdered mixture of [$^{13}C_6$]-*p*-aminobenzoic acid **21*** (100 mg, 0.70 mmol) and 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** (213 mg, 0.70 mmol), suspended in glacial acetic acid (3.5 cm^3), was subjected to reductive amination using dimethylamine–borane complex (70 mg, 1.20 mmol) in glacial acetic acid (1.5 cm^3), as described for the reaction of unlabelled *p*-aminobenzoic acid **9** with 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4**. *[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-N(2)-Acetylpteroic acid* **24*** was obtained as a bright yellow powder (237 mg, 94%) from water. Found: δ_H(400 MHz; d^6-DMSO) 2.19 (3 H, s, $COCH_3$), 4.59 (2 H, t, $^3J_{C4'\text{-}H}$ 4.2, 2 × 9-H), 6.42–6.47 and 6.81–6.87 (2 H, d of c m, $^1J_{C3'\text{-}H}$ [$^1J_{C5'\text{-}H}$] 159, 2Ar*H*[3′ and 5′]),‡‡‡‡ 7.16–7.21 (1 H, br c, 10-N*H*), 7.42–7.48 and 7.81–7.88 (2 H, d of c m, $^1J_{C2'\text{-}H}$ [$^1J_{C6'\text{-}H}$] 159, 2Ar*H*[2′ and 6′]),‡‡‡‡ and 8.86 (1 H, s, 7-H); δ_C(100 MHz; d^6-DMSO) 24.4 (COCH_3), 46.4 (9-CH_2), 111.8 (t of c m, C 3′ and 5′),§§§§ 118.3 (t of d, $^1J_{C1'\text{-}2'}$ [$^1J_{C1'\text{-}6'}$] 58, C 1′), 131.5 (t of c m, C 2′ and 6′),§§§§ 149.7 (7 *C*H), 151.7 (t d, $^1J_{C3'\text{-}4'}$ [$^1J_{C4'\text{-}5'}$] 60, C 4′), 155.1 (C), 159.7 (C), 174.6 (*C*OCH_3);||||||||| *m/z* (EI) 360 (M^+).

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-*N*(2)-Acetyl-*N*(10)-trifluoroacetylpteroic acid 25*. [$^{13}C_6$]-*N*(2)-Acetyl pteroic acid **24*** (240 mg, 0.67 mmol) was trifluoroacetylated by refluxing with a mixture of trifluoroacetic acid (7 cm^3) and trifluoroacetic anhydride (15 cm^3) using the conditions described for the conversion of unlabelled *N*(2)-acetyl pteroic acid **13** to *N*(2)-acetyl-*N*(10)-trifluoroacetylpteroic acid **14**. *[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-N(2)-Acetyl-N(10)-trifluoroacetylpteroic acid* **25*** was obtained as a pale grey crystalline solid (207 mg, 68%). Found *m/z* (LSIMS, mNBA): MH^+, 457.11791. Calc. for $^{12}C_{12}{}^{13}C_6H_{14}F_3N_6O_5$: 457.11791, deviation 0 ppm; δ_H (400 MHz; d^6-DMSO); forms *a*:*b*:*c* (approximate ratio by integration, 50:25:25), form *a* 2.20 (3 H, s, $COCH_3$), 5.21 (2 H, s, 2 × 9-H), 7.45–7.55 and 7.84–7.92 (2 H, d of c m, Ar*H*[3′ and 5′]) and 7.74–7.80 and 8.10–8.18 (2 H, d of c m, Ar*H*[2′ and 6′])‡‡‡‡ and 8.87 (1 H, s, 7-H); form *b*, 2.20 (3 H, s, $COCH_3$), 5.11 (2 H, s, 2 × 9-H), 7.07–7.15 and 7.45–7.55 (2 H, d of c m, Ar*H*[3′ and 5′]) and 7.45–7.55 and 7.84–7.92 (2 H, d of c m, Ar*H*[2′ and 6′])‡‡‡‡ and 8.77 (1 H, s, 7-H); form *c*, 2.20 (3 H, s, $COCH_3$), 5.35 (2 H, s, 2 × 9-H), 7.18–7.26 and 7.60–7.66 (4 H, d of c m, Ar*H*[2′, 3′, 5′ and 6′])‡‡‡‡ and 8.98 (1 H, s, 7-H); δ_C(100 MHz;

||||||||| The ^{13}C NMR data is incomplete. The differential in signal intensity between enriched ^{13}C atoms and those present in natural abundance is so great that only the strongest of the natural abundance signals are just visible.

d^6-DMSO) 24.4 ($COCH_3$), 54.2 (9-CH_2), 115.8 (q, $^1J_{C\text{-}F}$ 290, CF_3), 127.1–131.4 (complex overlapping signals, $Ar^{13}C$), 136.9 (t of c m, Ar^{13}C),§§§§ 140.5 (t of c m, Ar^{13}C),§§§§ 142.8 (t of c m, Ar^{13}C),§§§§ 147.2 (t of c m, Ar^{13}C),§§§§ 150.0 (7-CH), 155.0 (C), 158.7 (q, $^2J_{C\text{-}F}$ 38, $COCF_3$), 174.5 ($COCH_3$); *m/z* (LSIMS, mNBA) 457 (15%, MH^+), 479 (5, M + 23[Na]), 439 (4, M − OH), 307 (10), 289 (8), 176 (27), 154 (100) and 136 (90).

[$^{13}C_6$]-Labelled folic acid and its precursors by the [A + (B + C)] route

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-*N*(2)-Acetylfolic acid 26*. A finely powdered mixture of [$^{13}C_6$]-*p*-aminobenzoyl-L-glutamic acid **23*** (98 mg, 0.36 mmol) and 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** (92 mg, 0.30 mmol), suspended in glacial acetic acid (1.4 cm^3), was subjected to reductive amination using dimethylamine–borane complex (36.4 mg, 0.62 mmol) in glacial acetic acid (0.8 cm^3), as described for the reaction of unlabelled *p*-aminobenzoyl-L-glutamic acid **12** with 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4**. *[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-N(2)-Acetylfolic acid* **26*** was obtained as a bright yellow powder (59 mg, 40% [repetitions of this experiment gave yields in the region 32–40%]); *m/z* (FAB, mNBA), 490 (84%, MH^+), 343 (100, M − $C_5H_8NO_4$ [*i.e.* M − glutamate moiety]), 273 (81), 217 (76) and 176 (63).

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]Folic acid 27*. [$^{13}C_6$]-*N*(2)-Acetylfolic acid **26*** (65.4 mg, 0.134 mmol) was deacetylated by hydrolysis with refluxing aqueous sodium hydroxide solution (0.1 M, 130 cm^3) for 90 min in the dark, under nitrogen, as described for the conversion of *N*(2)-acetylfolic acid **15** to folic acid **16**. Recrystallisation of the crude material (51.0 mg, 85%) from hot water gave the product, *[$^{13}C_6$]folic acid* **27***, as a bright yellow powder (39.2 mg, 66%); $[a]_D^{23}$ +18.0 (*c* 1 in 0.1 M NaOH) [$[a]_D^{23}$ for unlabelled folic acid **16** +18.0 (*c* 1 in 0.1 M NaOH)]. Found: δ_H(400 MHz; CF_3CO_2D), 2.39–2.48 (1 H, c m, β-H_a), 2.61–2.68 (1H, c m, β-H_b), 2.85 (2 H, t, *J* 7.1, 2 × γ-H), 5.10–5.14 (1 H, m, α-H), 5.29 (2 H, s, 2 × 9-H), 7.72–7.78 and 8.13–8.18 (2 H, d of c m, $^1J_{C3'\text{-}H}$ [$^1J_{C5'\text{-}H}$] 169, 2Ar*H*[3′ and 5′]), 7.92–7.98 and 8.34–8.40 (2 H, d of c m, $^1J_{C2'\text{-}H}$ [$^1J_{C6'\text{-}H}$] 166, 2Ar*H*[2′ and 6′]),‡‡‡‡ and 9.02 (1 H, s, 7-H); *m/z* (FAB, mNBA) 448 (41%, MH^+), 347 (7), 327 (17), 301 (64, M − $C_5H_8NO_4$ [glutamate moiety]), 286 (49), 273 (34), 217 (94), 195 (31), 176 (28, [M − $C_5H_8NO_4$] − [C_7H_5ON], *i.e.* M − glutamate moiety − *p*-aminobenzoate moiety) and 133 (100); *m/z* (ES), 468.1 (10%, [M + Na − 2H]$^-$), 446.1 (100, [M − H]$^-$), 271.1 (10, [$^{12}C_6{}^{13}C_6H_{14}N_2O_5$ − H]$^-$) and 222.7 (89, [M − 2H]$^{2-}$). Isotopic composition of labelled folic acid **27***: [$^{13}C_6$] 95.7%, [$^{13}C_5$] 3.9 and [$^{13}C_4$] 0.4.

[$^{13}C_6$]-Labelled folic acid and its precursors by the [(A + B) + C] route

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]Folic acid 27*. [$^{13}C_6$]-*N*(2)-Acetyl-*N*(10)-trifluoroacetylpteroic acid **25*** (300 mg, 0.66 mmol) was suspended in anhydrous DMF (10 cm^3) containing triethylamine (0.93 cm^3, 6.6 mmol), and cooled to 5 °C. As far as possible the experiment was conducted in the dark. Freshly distilled isobutyl chloroformate (0.14 cm^3, 1.0 mmol) was added and the mixture stirred for 15 min at 5 °C. Dimethyl glutamate hydrochloride **10** (698 mg, 3.3 mmol) was added in one portion. The resulting suspension was allowed to warm to room temperature slowly and was stirred for 72 h under an inert atmosphere. The reaction mixture was evaporated on a rotary evaporator and the residue was washed by trituration with water, aqueous ammonium hydrogen carbonate and ether and dried *in vacuo* to give crude [$^{13}C_6$]dimethyl *N*(2)-acetyl-*N*(10)-trifluoroacetylfolate (213 mg, 53%). The protected folate was suspended in aqueous sodium hydroxide solution (0.2 M, 150 cm^3) and heated under reflux for 90 min in the dark in an inert atmosphere. On completion, decolourising charcoal was added and the hot mixture was stirred for a further 10 min. The solution was filtered, the pH adjusted to 3.5 using 2 M hydrochloric acid and the solution allowed to cool slowly to room temperature. The resulting suspension was left overnight at 5 °C. The precipitate was recovered by centrifugation, washed with water (3 × 2 cm^3) and acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give [$^{13}C_6$]folic acid **27***. The resulting solid was further purified by ion exchange chromatography on A-25 DEAE fractogel, eluting with a gradient of ammonium hydrogen carbonate (0.2 to 2.0 M). The relevant fractions were pooled and lyophilised to yield *[$^{13}C_6$]folic acid* **27*** as a bright yellow powder (118 mg, 40%); $[a]_D^{23}$ +17.5 (*c* 1 in 0.1 M NaOH) [$[a]_D^{23}$ for material prepared by the [**A** + (**B** + **C**)] route +18.0 (*c* 1 in 0.1 M NaOH), $[a]_D^{23}$ for unlabelled folic acid **16** +17.5 (*c* 1 in 0.1 M NaOH)]. Found: δ_H(400 MHz; CF_3CO_2D) 2.38–2.48 (1 H, br c m, β-H_a), 2.61–2.69 (1H, br c m, β-H_b), 2.82–2.87 (2 H, br t, *J* 5.4, 2 × γ-H), 5.09–5.13 (1 H, m, α-H), 5.26–5.31 (2 H, br s, 2 × 9-H), 7.72–7.80 and 8.14–8.24 (2 H, d of br c m, $^1J_{C3'\text{-}H}$ [$^1J_{C5'\text{-}H}$] 168, 2Ar*H*[3′ and 5′]), 7.92–7.99 and 8.33–8.42 (2 H, d of br c m, $^1J_{C2'\text{-}H}$ [$^1J_{C6'\text{-}H}$] 163, 2Ar*H*[2′ and 6′]),‡‡‡‡ and 9.03–9.07 (1 H, br s, 7-H); δ_C(100 MHz; CF_3CO_2D) 125.8 (t of c m, C 3′ and 5′),§§§§ 132.3 (t of c m, C 2′ and 6′),§§§§ 137.4 (t of d, C-1′) and 139.7 (t of d, C-4′);¶¶¶¶ *m/z* (ES), 468.1 (10%, [M + Na − 2H]$^-$), 446.1 (100, [M − H]$^-$), 271.1 (10, [$^{12}C_6{}^{13}C_6H_{14}N_2O_5$ − H]$^-$) and 222.7 (89, [M − 2H]$^{2-}$). Isotopic composition of labelled folic acid **27***: [$^{13}C_6$] 95.7%, [$^{13}C_5$] 3.9 and [$^{13}C_4$] 0.4.

[$^2H_4$]-Labelled compounds

All labelled materials were prepared by the procedures described for the corresponding unlabelled materials. The general section contains details of the source of the labelled starting materials and their isotopic purity.

Labelled glutamic acid precursor

[3,3,4,4-$^2H_4$]-Dimethyl L-glutamate hydrochloride 28#.[12,20] Dried [3,3,4,4-$^2H_4$]-L-glutamic acid (1.04 g, 7.1 mmol) was esterified using thionyl chloride (3.25 cm^3, 37 mmol) in anhydrous methanol (10 cm^3) as described for the formation of dimethyl L-glutamate hydrochloride **10**. *[3,3,4,4-$^2H_4$]-Dimethyl L-glutamate* **28#** (1.14 g, 75%) was obtained as fine white crystals from acetone–diethyl ether, mp 78–80 °C (mp for unlabelled dimethyl glutamate **9** 78–80 °C.); $[a]_D^{23}$ +24.9 (*c* 5.0 in water) [$[a]_D^{23}$ for unlabelled dimethyl glutamate hydrochloride **10** +25.5 (*c* 5.0 in water)]. Found: δ_H(400 MHz, CD_3OD) 3.73 (3 H, s, γ-CO_2CH_3), 3.87 (3 H, s, α-CO_2CH_3) and 4.13 (1 H, s, α-H), 4.80 (br s, 3H, NH_3^+); *m/z* (EI), 180 (MH^+).

Labelled *p*-aminobenzoyl-L-glutamate precursors

[3′,3′,4′,4′-$^2H_4$]-Dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate 29#. *p*-Trifluoroacetamidobenzoic acid **8** (150 mg, 0.64 mmol) was coupled with [$^2H_4$]dimethyl L-glutamate hydrochloride **28#** (135 mg, 0.62 mmol) using HOBT (0.167 g, 1.24 mmol), DCC (0.141 g, 0.68 mmol) and diisopropylethylamine (0.13 cm^3, 0.72 mmol) as described for the preparation of unlabelled dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11**. *[3′,3′,4′,4′-$^2H_4$]-Dimethyl p-trifluoroacetamidobenzoyl-L-glutamate* **29#** (150 mg, 61%) was obtained as a pale yellow solid, mp 134–135 °C (mp for unlabelled dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11** 135–136 °C). Found: *m/z* (EI): M^+, 394.12760. Calc. for $C_{16}{}^1H_{13}{}^2H_4F_3N_2O_6$: 394.128979, deviation 3.5 ppm; δ_H(400 MHz, CD_3OD) 3.66 (3 H, s, γ-CO_2CH_3), 3.75 (3 H, s, α-CO_2CH_3), 4.65 (1 H, s, α-H), 7.67 and 8.04 (2 H, d, Ar*H*[3 and 5] and 2 H, d, Ar*H*[2 and 6], AA′BB′); *m/z* (EI), 394 (6%, M^+), 335 (15, M − CO_2CH_3), 216 (100, M − $C_7H_8{}^2H_4NO_4$ [*i.e.* M − [$^2H_4$]dimethyl glutamate moiety]).

[3′,3′,4′,4′-$^2H_4$]-*p*-Aminobenzoyl-L-glutamic acid 30#. Dimethyl [$^2H_4$]-*p*-trifluoroacetamidobenzoyl-L-glutamate **29#** (150 mg, 0.38 mmol), was deprotected by hydrolysis using aqueous sodium hydroxide (0.1 M, 65 cm^3) to give [$^2H_4$]-*p*-aminobenzoyl-L-glutamic acid **30#** by the procedure described for the preparation of unlabelled *p*-aminobenzoyl-L-glutamic acid **12**. *[3′,3′,4′,4′-$^2H_4$]-p-Aminobenzoyl-L-glutamic acid* **30#** was obtained as a white solid (86 mg, 83%) mp 174–175 °C (decomp.) [mp for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** 173–174 °C (decomp.)], $[a]_D^{23}$ −14 (*c* 2.0 in 0.1 M HCl) [$[a]_D^{23}$ for unlabelled *p*-aminobenzoyl-L-glutamic acid −15 (*c* 2.0 in 0.1 M HCl)]. Found *m*/*z* (EI): M$^+$, 270.11549. Calc. for $C_{12}{}^1H_{10}{}^2H_4N_2O_5$: 270.11538, deviation 0.4 ppm; δ_H(400 MHz, CD_3OD) 4.60 (1 H, m, α-H), 6.68 and 7.65 (2 H, d, *J* 8.5 2Ar*H*[3 and 5] and 2 H, d, *J* 8.5 2Ar*H*[2 and 6], AA′BB′); *m*/*z* (EI), 270 (1%, M$^+$), 252 (2, M − H_2O), 226 (7, M − CO_2), 120 (100, M − $C_5H_4{}^2H_4NO_4$[*i.e.*, M − glutamate moiety]) and 92 (34); *m*/*z* (ES) 539 (*ca.* 13%, [2M − H]$^-$) and 269.2 (100%, [M − H]$^-$). Isotopic composition of labelled *p*-aminobenzoyl-L-glutamic acid **30#**: [$^2H_4$] 90.0%, [$^2H_3$] 9.0 and [$^2H_2$] 1.2.

[3′,3′,4′,4′-$^2H_4$]-*p*-Aminobenzoyl-L-glutamic acid 30#. *p*-Trifluoroacetamidobenzoic acid **8** (150 mg, 0.64 mmol) was coupled with [$^2H_4$]dimethyl L-glutamate hydrochloride **28#** (135 mg, 0.62 mmol) using HOBT, DCC and diisopropylethylamine in tetrahydrofuran as described above. The resulting pale yellow solid was immediately deprotected with aqueous sodium hydroxide as described above to give *[3′,3′,4′,4′-$^2H_4$]-p-aminobenzoyl-L-glutamate* **30#** (93 mg, 0.32 mmol, 55%) as an off white solid, mp 173–174 °C [mp for unlabelled *p*-aminobenzoyl-L-glutamic acid **11** 173–174 °C (decomp.)], $[a]_D^{23}$ −15 (*c* 2.0 in 0.1 M HCl) [$[a]_D^{23}$ for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** −15 (*c* 2.0 in 0.1 M HCl)]; δ_H(400 MHz; CD_3OD) 4.60 (1 H, m, α-H), 6.68 and 7.65 (2 H, d, *J* 8.5 2Ar*H*[3 and 5] and 2 H, d, *J* 8.5 2Ar*H*[2 and 6], AA′BB′); *m*/*z* (EI), 270 (1%, M$^+$).

[$^2H_4$]-Labelled folic acid by the [A + (B + C)] route

[3″,3″,4″,4″-$^2H_4$]Folic acid 32#. A finely powdered mixture of [$^2H_4$]-*p*-aminobenzoyl-L-glutamic acid **30#** (20 mg, 0.074 mmol) and 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4** (22 mg, 0.072 mmol) suspended in glacial acetic acid (2.0 cm^3), was subjected to reductive amination using dimethylamine–borane complex (10 mg, 0.17 mmol) in glacial acetic acid (1.0 cm^3), as described for the reaction of unlabelled *p*-aminobenzoyl-L-glutamic acid **12** with 2-acetylamino-4-hydroxy-6-formylpteridine DMF solvate **4**. *[$^2H_4$]-N(2)-Acetylfolic acid* **31#** was obtained as a bright yellow powder (13 mg, 37%) which was immediately subjected to hydrolysis with aqueous sodium hydroxide solution (0.1 M, 25 cm^3) as described for the hydrolysis of *N*(2)-acetylfolic acid **15**. The [$^2H_4$]folic acid obtained was purified by ion exchange chromatography on A-25 DEAE fractogel, eluting with a gradient of ammonium hydrogen carbonate (0.2 to 2.0 M). The relevant fractions were pooled and lyophilised to yield *[3″,3″,4″,4″-$^2H_4$]-folic acid* **32#** as a bright yellow powder (10 mg, overall 31%), $[a]_D^{23}$ +17.0 (*c* 1 in 0.1 M NaOH) [$[a]_D^{23}$ for unlabelled folic acid **13** +18.0 (*c* 1 in 0.1 M NaOH)]; δ_H(400 MHz; CF_3CO_2D) 5.11 (1 H, s, α-H [Glu]), 5.28 (2 H, s, 2 × 9-H), 7.96 and 8.16 (2 H, d, *J* 8.9, Ar*H*[3′ and 5′] and 2 H, d, *J* 8.9 Ar*H*[2′ and 6′], AA′BB′) and 9.03 (1 H, s, 7-H); *m*/*z* (ES), 889.0 (*ca.* 10% [2M − H]$^-$), 666.4 (*ca.* 15, [3M − 2H]$^{2-}$), 444.5 (100, [M − H]$^-$), 269.6 (24, [$C_{12}{}^1H_9{}^2H_4N_2O_5$]$^-$), 265.4 (55), 221.7 (52, [M − 2H]$^{2-}$) and 206.3 (15). Isotopic composition of labelled folic acid **32#**: [$^2H_4$] 93.7%, [$^2H_3$] 5.4 and [$^2H_2$] 0.9.

[$^2H_4$]-Labelled folic acid by the [(A + B) + C] route

[3″,3″,4″,4″-$^2H_4$]Folic acid 32#.[12] *N*(2)-Acetyl-*N*(10)-trifluoroacetylpteroic acid **14** (100 mg, 0.22 mmol) was suspended in anhydrous DMF (4 cm^3) containing triethylamine (0.19 cm^3, 1.35 mmol), and cooled to 5 °C. As far as possible the experiment was conducted in the dark. Freshly distilled isobutyl chloroformate (0.05 cm^3, 0.36 mmol) was added and the mixture was stirred for 15 min at 5 °C. [$^2H_4$]Dimethyl glutamate hydrochloride **28#** (130 mg, 0.61 mmol) was added in one portion. The resulting suspension was allowed to warm to room temperature slowly and was stirred for 72 h under an inert atmosphere. The reaction mixture was evaporated on a rotary evaporator and the residue was washed by trituration with water, aqueous ammonium hydrogen carbonate and ether and dried *in vacuo* to give crude [$^2H_4$]dimethyl *N*(2)-acetyl-*N*(10)-trifluoroacetylfolate (32 mg, 53%). The protected [$^2H_4$]folate was suspended in aqueous sodium hydroxide solution (0.2 M, 150 cm^3) and heated under reflux for 90 min in the dark in an inert atmosphere. On completion, decolourising charcoal was added and the hot mixture was stirred for a further 10 min. The solution was filtered, the pH adjusted to 3.5 using 2 M hydrochloric acid and the solution allowed to cool slowly to room temperature. The resulting suspension was left overnight at 5 °C. The precipitate was recovered by centrifugation, washed with water (3 × 2 cm^3) and acetone (2 × 2 cm^3) and dried *in vacuo* in the dark to give [$^2H_4$]folic acid. The resulting solid was further purified by ion exchange chromatography on A-25 DEAE fractogel, eluting with a gradient of ammonium hydrogen carbonate (0.2 to 2.0 M). The relevant fractions were pooled and lyophilised to yield [$^2H_4$]folic acid contaminated with pteroic acid (10%) (118 mg, 0.26 mmol, 40%). Pure [$^2H_4$]folic acid was obtained free of contaminating pteroic acid by semi-preparative high performance liquid chromatography using a reversed phase ion pairing technique (see general Experimental section for details). The relevant pooled fractions were concentrated *in vacuo* below 45 °C and lyophilised. The pale yellow solid was dissolved in the minimum volume of water and the pH adjusted to 3.5 using 2 M hydrochloric acid and the resulting suspension left overnight at 5 °C. The precipitate was recovered by centrifugation, washed well with water (3 × 2 cm^3) to ensure complete removal of the ion-pairing reagent and dried *in vacuo* in the dark to give *[3″,3″,4″,4″-$^2H_4$]folic acid* **32#** as a bright yellow powder; $[a]_D^{23}$ +17.5 (*c* 1 in 0.1 M NaOH) [$[a]_D^{23}$ for material prepared by the [A + (B + C)] route +17.0 (*c* 1 in 0.1 M NaOH), $[a]_D^{23}$ for unlabelled folic acid +18.0 (*c* 1 in 0.1 M NaOH)]; δ_H(400 MHz; CF_3CO_2D) 5.09 (1 H, s, α-H [Glu]), 5.27 (2 H, s, 2 × 9-H), 7.93 and 8.15 (2 H, d, *J* 8.7 2Ar*H*[3′ and 5′] and 2 H, d, *J* 8.7 2Ar*H*[2′ and 6′], AA′BB′), and 9.01 (1 H, s, 7-H); *m*/*z* (ES), 889.0 (*ca.* 10% [2M − H]$^-$), 666.4 (*ca.* 15, [3M − 2H]$^{2-}$), 444.5 (100, [M − H]$^-$), 269.6 (24, [$C_{12}{}^1H_9{}^2H_4N_2O_5$]$^-$), 265.4 (55), 221.7(5, [M − 2H]$^{2-}$) and 206.3 (15). Isotopic composition of labelled folic acid **32#**: [$^2H_4$] 93.7%, [$^2H_3$] 5.4 and [$^2H_2$] 0.9.

[$^{13}C_6$,$^2H_4$]-Labelled *p*-aminobenzoyl-L-glutamate precursors

[1,2,3,4,5,6-$^{13}C_6$,3′,3′,4′,4′-$^2H_4$]Dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate 33*#. [$^{13}C_6$]-*p*-Trifluoroacetamidobenzoic acid **20*** (150 mg, 0.63 mmol) was coupled with [$^2H_4$]dimethyl L-glutamate hydrochloride **28#** (135 mg, 0.63 mmol) to yield [$^{13}C_6$,$^2H_4$]dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **33*#** using DCC (141 mg, 0.68 mmol), HOBT (167 mg, 1.24 mmol) and diisopropylethylamine (0.13 cm^3, 0.72 mmol) in THF (10 cm^3) by the procedure described for the preparation of dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11**. *Dimethyl [$^{13}C_6$,$^2H_4$]-p-trifluoroacetamidobenzoyl-L-glutamate* **33*#** was obtained as a pale yellow powder (180 mg, 72%), mp 134–135 °C (decomp.) [mp for unlabelled dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11** 135–136 °C (decomp.)]; $[a]_D^{23}$ −15 (*c* 2.0 MeOH) [$[a]_D^{23}$ for unlabelled dimethyl *p*-trifluoroacetamidobenzoyl-L-glutamate **11** −15 (*c* 2.0 MeOH)]. Found *m*/*z* (EI): M$^+$, 400.14881. Calc. for $^{12}C_{10}{}^{13}C_6{}^1H_{13}{}^2H_4F_3N_2O_6$: 400.14911, deviation 0.7 ppm; δ_H(400 MHz; CD_3OD) 3.66 (3 H, s, γ-CO_2CH_3), 3.75 (3 H, s,

α-CO_2CH_3), 4.65 (1 H, m, α-H), 7.55–7.61 and 7.96–8.02 (2 H, d of c m, 2Ar*H*[2 and 6]), 7.74–7.65 and 8.05–8.13 (2 H, d of c m, 2Ar*H*[3 and 5]);‡‡‡‡ *m*/*z* (EI), 400 (1%, M^+), 368 (1, M − CH_3OH), 341 (7, M − CO_2CH_3), 222 (100, M − $C_7H_8{}^2H_4NO_4$[*i.e.* M − dimethyl glutamate moiety]), 205 (34) and 178 (9, $C_7H_8{}^2H_4NO_4$ [dimethyl glutamate moiety]).

[1,2,3,4,5,6-$^{13}C_6$,3′,3′,4′,4′-$^2H_4$]-*p*-Aminobenzoyl-L-glutamate acid 34*#. Dimethyl [$^{13}C_6$,$^2H_4$]-*p*-trifluoroacetamidobenzoyl-L-glutamate **33*#** (178 mg, 0.45 mmol) was deprotected by hydrolysis using aqueous sodium hydroxide (0.1 M, 65 cm^3) to give [$^{13}C_6$,$^2H_4$]-*p*-aminobenzoyl-L-glutamic acid **34*#** by the procedure described for the preparation of unlabelled *p*-aminobenzoyl-L-glutamic acid **12**. *[$^{13}C_6$,$^2H_4$]-p-Aminobenzoyl-L-glutamic acid* **34*#** (76 mg, 0.28 mmol, 62%) was obtained as a white solid, mp 172–173 °C (decomp.) [mp for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** 173–174 °C (decomp.)], $[\alpha]_D^{23}$ −14 (*c* 2.0 in 0.1 M HCl) [$[\alpha]_D^{23}$ for unlabelled *p*-aminobenzoyl-L-glutamic acid **12** −15 (*c* 2.0 in 0.1 M HCl)]. Found *m*/*z* (EI): M^+, 276.13555. Calc. for $^{12}C_6{}^{13}C_6{}^1H_{10}{}^2H_4N_2O_5$: 276.13551, deviation 0.2 ppm; δ_H(400 MHz; CD_3OD) 4.60 (1 H, s, α-H), 6.46–6.51 and 6.84–6.91 (2 H, c m of d, 2Ar*H*[2 and 6]), 7.41–7.48 and 7.81–7.88 (2 H, c m of d, 2Ar*H*[3 and 5]);‡‡‡‡ *m*/*z* (EI), 276 (0.6%, M^+), 258 (3, M − H_2O), 232 (4, M − CO_2), 143 (21) and 126 (100, M − $C_5H_4{}^2H_4NO_4$ [*i.e.* M − glutamate moiety]); *m*/*z* (ES), 551 (*ca*. 10% [2M − H]$^-$) and 275.1 (100%, [M − H]$^-$). Isotopic composition of labelled *p*-aminobenzoyl-L-glutamic acid **34*#**: [$^{13}C_6$][$^2H_4$] 82.3%, 9 atoms labelled 15.7, 8 atoms labelled 1.9 and 7 atoms labelled 0.14.

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