TILDEN LECTURE. Studies on Thymidylate Synthase and Dihydrofolate Reductase – Two Enzymes Involved in the Synthesis of Thymidine

Douglas W. Young

School of Chemistry and Molecular Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ, U.K.

1 Introduction

Compounds which interfere with the biosynthesis of the nucleoside thymidine are among the most effective clinical anti-cancer drugs. The nucleic acids, DNA and RNA, use two purine bases and two pyrimidine bases in the three letter codons which ensure integrity of the genetic message on cell division and which translate to the twenty letter 'message' found in proteins. Three of these bases are common to both DNA and RNA but the pyrimidine base thymine (2, $R^1 = H$) is unique to DNA, its equivalent in RNA being the base uracil (1, $R^1 = H$). Thymidine 5'-phosphate, (2, R^1 = 2-deoxyribose-5-phosphate), must be available before cell division will occur and so inhibition of the biosynthesis of thymidine will prevent cell division. Since uncontrolled cell division is a feature of many forms of cancer, we can see how studies of the biosynthesis of the base thymidine are important in the development of new anti-cancer drugs.

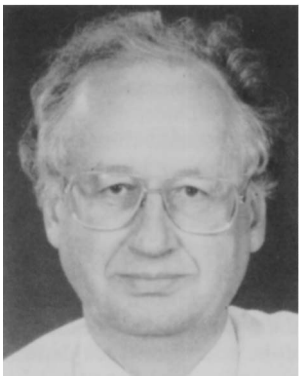
The final process in the biosynthesis of thymidine monophosphate, (2, R^1 = 2-deoxyribose-5-phosphate), is methylation of deoxyuridine monophosphate, (1, R^1 = 2-deoxyribose-5-phosphate), shown in Scheme 1 and, like many biological reactions involving the transfer of a one-carbon unit, this involves the coenzyme 5,6,7,8-tetrahydrofolic acid (5).[1] This coenzyme is a 1,2-diamine and the nitrogen atoms N-5 and N-10 are able to form the aminal (6) as in Scheme 2. This compound is a one-carbon adduct of the coenzyme and it is able to transfer this carbon to a substrate in an enzyme-catalysed reaction. Thus the amino acid glycine (9) will react with the aminal (6) in the presence of the enzyme serine hydroxymethyltransferase (EC 2.1.2.1) and pyridoxal phosphate to yield the amino acid serine (10) as in Scheme 3. This biological aldol condensation may be regarded as a one-carbon transfer at the formaldehyde oxidation level.

The aminal (6) may be oxidized by a dehydrogenase to 5,10-methenyl-5,6,7,8-tetrahydrofolic acid (7) which effects one-carbon transfers at the formic acid oxidation level such as the formylation of the imidazole (11) which is involved in synthesis of the purine (12). Enzymatic reduction of aminal (6) to 5-methyl-5,6,7,8-tetrahydrofolic acid (8) allows one-carbon transfers at the methanol oxidation level to take place. Thus methylation of the amino acid homocysteine (13) to methionine (14) involves the coenzyme 'adduct' (8).

In all of the one-carbon transfers in Scheme 3, the coenzyme (5) is regenerated and, since biosynthesis of thymidine (2) is a methylation reaction, we might expect 5-methyl-5,6,7,8-tetrahydrofolic acid (8) to be involved in the process. Nature, however, effects this methylation in a more complex manner as shown in Scheme 4. Here the aminal (6a) is used as the one-carbon source and, since it is at 'wrong' oxidation level for the methylation reaction (1) → (2a), it is oxidized in the process. The hydrogen H_C in the aminal is transferred to become the third hydrogen of the methyl group and the coenzyme is oxidized to 7,8-dihydrofolic acid (15) in the process. The overall reaction is catalysed by the enzyme thymidylate synthase (EC 2.1.1.45) and, since the coenzyme (5) is consumed in the process, a mechanism for its regeneration is required. This is achieved by the enzyme dihydrofolate reductase (EC 1.5.1.3) which reduces dihydrofolate (15) to the coenzyme (5) which is in turn converted into the aminal (6a) by the retro-aldol reaction of serine (10a) using serine hydroxymethyltransferase. The methylation of the base uracil (1) to the base thymine (2a) therefore involves a cyclic process, and two of the enzymes in it are targets for clinically used anti-cancer drugs. Thus 5-fluorouracil (16) is an anti-cancer drug since it is converted into a suicide substrate for thymidylate synthase, and the anti-cancer drug methotrexate (23) acts by inhibiting the enzyme dihydrofolate reductase.

The unusual nature of the one-carbon transfer involved in the methylation of uracil (1) attracted our attention some years ago and we have worked on the enzymes involved in the process since then. Because of this interest, we also became interested in the catabolism of the two bases uracil (1, $R^1 = H$) and thymine (2, $R^1 = H$). These are degraded in nature to β-alanine (3) and 3-amino-2-methylpropanoic acid (4), respectively, by reduction and hydrolysis. We have shown[2,3] that, in each case, the reduction involves *anti*-addition of hydrogen at the *si*-face of C-5 and the *si*-face of C-6 of the pyrimidine as indicated in Scheme 1. We have also shown[3] that the anti-cancer drug 5-fluorouracil (16, $R^1 = H$) is catabolized with the same stereospecificity.

Douglas Young is Professor of Chemistry at the University of Sussex. He completed his Ph.D. with Professor A. I. Scott and post-doctoral studies with Professor R. B. Woodward. His research covers stereochemistry and mechanism of enzyme-catalysed reactions; heterocyclic synthesis and photochemistry. In addition to the work reported here, his group were first to discover the stereochemistry of β-lactam ring-closure in penicillin biosynthesis, and have investigated the stereochemistry of a variety of other biological processes. They have developed novel syntheses of heterocyclic systems, new β-lactam systems and glutamate antagonists. Professor Young is Chairman of the Bio-organic Group of the Royal Society of Chemistry and a member of Perkin Council.

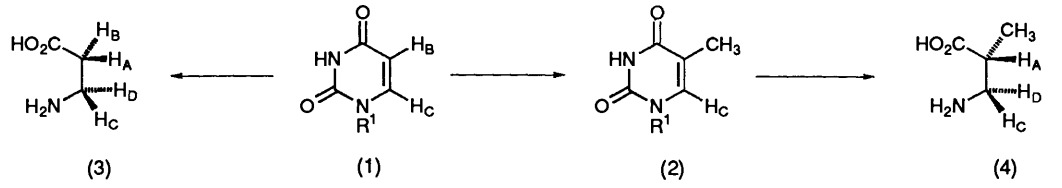


2 Studies on the Enzyme Thymidylate Synthase

2.1 A Model for the Methylation of Thymine

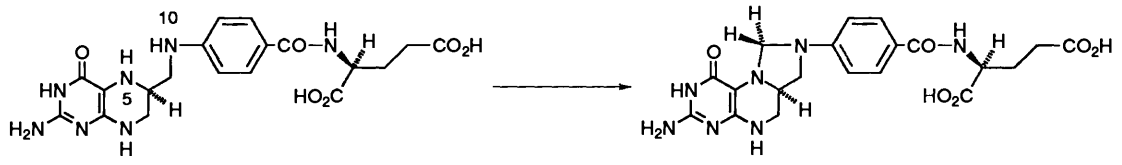
The question of a valid chemical model for the methylation of deoxyuridinemonophosphate (1, R^1 = 2-deoxyribose-5-phosphate) has excited much speculation and Friedkin revised an earlier[4] model by suggesting[5] that 2'-deoxyuridine-5'-phosphate (1, R^1 = 2-deoxyribose-5-phosphate) might act as an enamine with electrophile (6b)⇌(6c) to give the intermediate (19) as in Scheme 5. This would then rearrange in some way, with transfer of the hydrogen H_C from C-6 of the pteridine to the methyl group of the thymine moiety.

Evidence in favour of the intermediacy of the Friedkin intermediate includes the discovery of a ternary complex (18) when 5-fluorodeoxyuridinemonophosphate (16, R^1 = 2-deoxyuridine-5-phosphate) was used as a substrate for the reaction.[6,7] Here the intermediate (17) cannot rearrange to the enamine (19) when X = F. It is extremely nucleophilic and reacts with the thiol of an active-site cysteine residue.



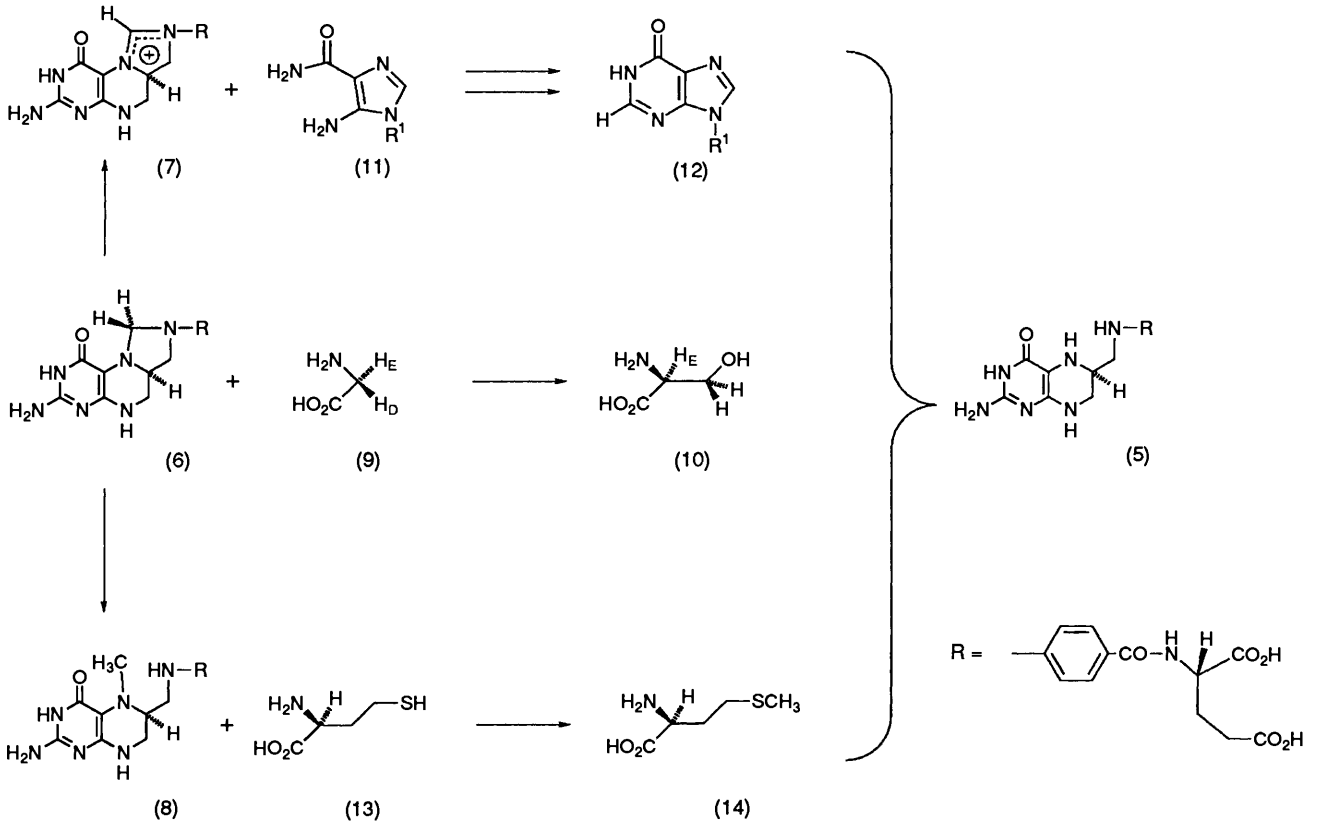
(3) (1) (2) (4)

Scheme 1



(5) (6)

Scheme 2



(7) (11) (12)

(6) (9) (10) (5)

(8) (13) (14)

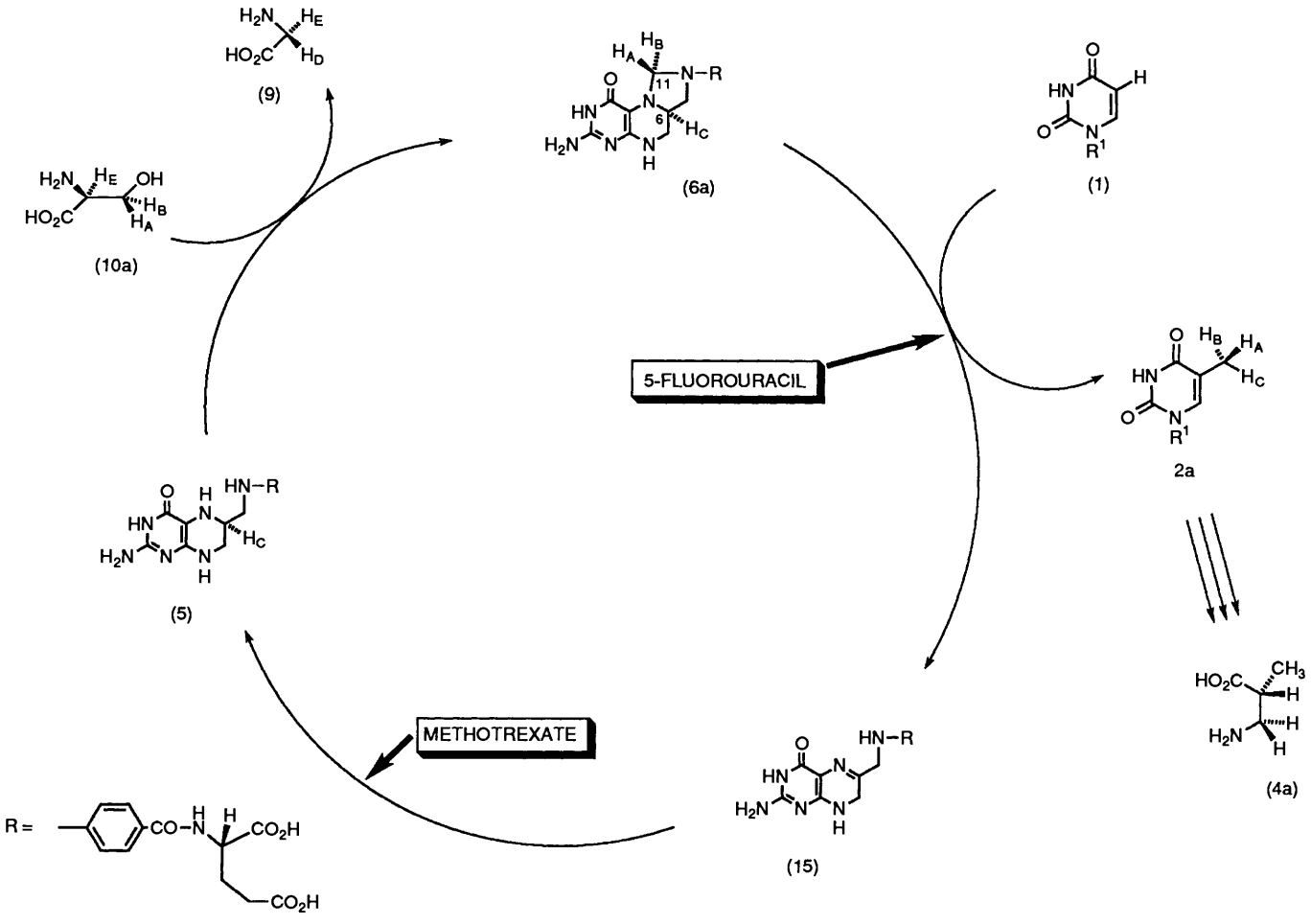
Scheme 3

An attractive explanation for the conversion of the intermediate (19) into thymidine (2b) and 7,8-dihydrofolic acid (15) seemed to us to be the retro-ene process shown in (19) → (20) of Scheme 5. This concerted reaction would lead to 7,8-dihydrofolate (15) and the quinone-methide type intermediate (20) in which the hydrogen H_C had been transferred to C-6 of the pyrimidine. A stereospecific enzyme-catalysed prototropic shift involving a single active-site base would then transfer H_C entirely to the methyl group as is found[8,9] in nature and shown in (2b).

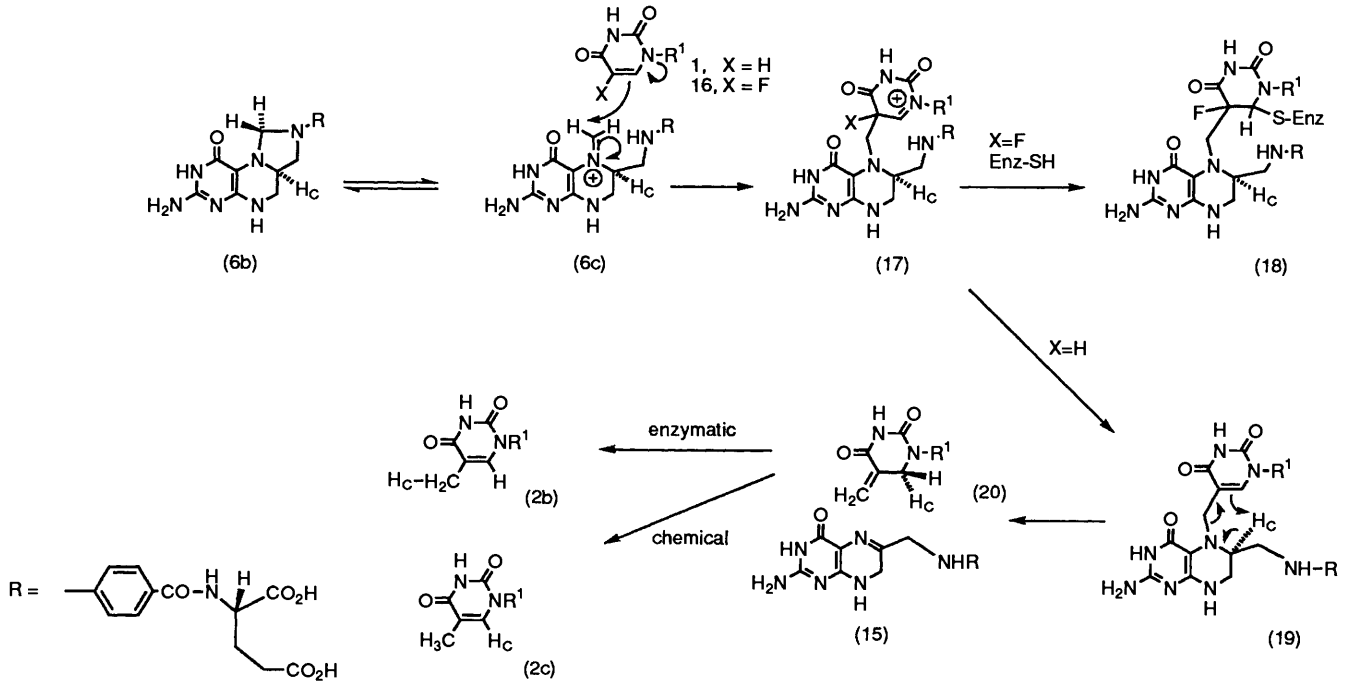
As a means of testing this hypothesis, we decided to synthesize the compound (19, R^1 = H) in which H_C was deuteriated and then attempt its chemical conversion into thymine by pyrolysis. If the retro-ene process were to give (20, R^1 = H, H_C = ^{2}H) then there would be no reason for the purely chemical reaction to be stereospecific. Indeed an isotope effect would mitigate against loss of deuterium and any thymine formed might be expected to be deuteriated at C-6 as shown in (2c).

Synthesis of the desired compound (19, R^1 = H) had been reported by Huennekens[10] to be achieved by simple alkylation of 5,6,7,8-tetrahydrofolic acid (5) with 5-chloromethyluracil (21) as shown in Scheme 6. When we repeated this reaction, however, we found that bis-alkylation to the product (22) had fortuitously the same calculated combustion data as the mono-adduct (19, R^1 = H), and our product had the reported[10] ultraviolet spectrum. The ^{1}H-NMR and mass spectral data, however, indicated that it was, in fact, the bis-adduct (22) which was obtained in this reaction.[11]

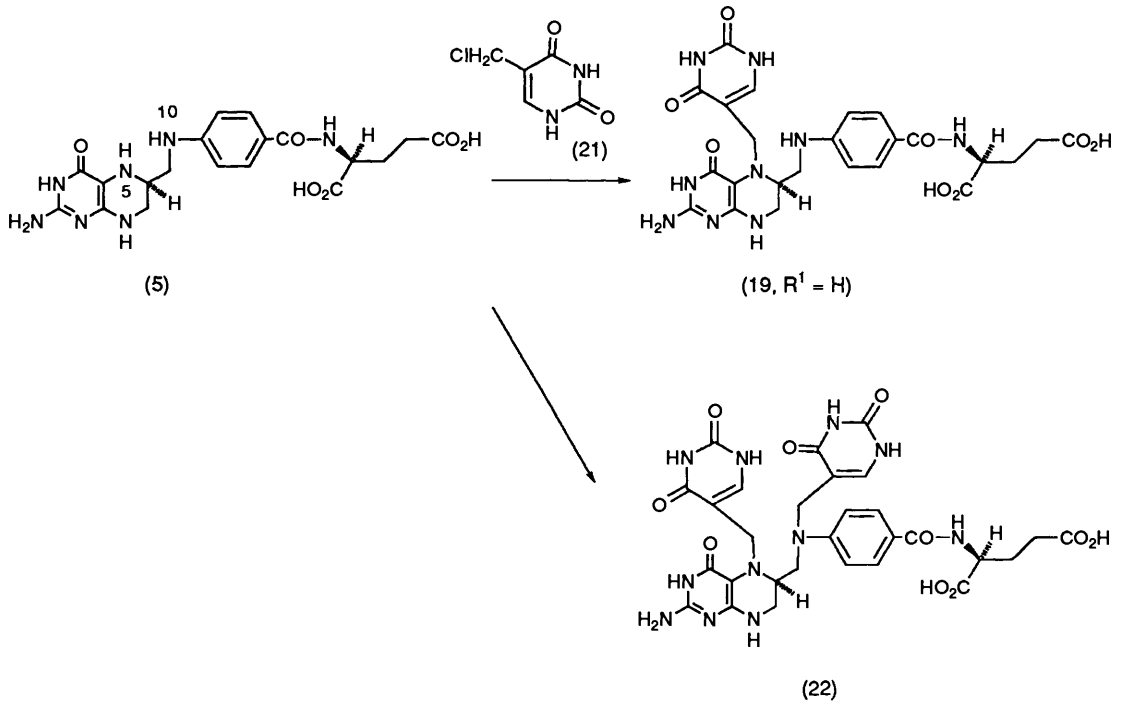
In order to obtain a suitable model we therefore needed to protect N-10 of 5,6,7,8-tetrahydrofolic acid against alkylation. We were able to achieve this[11] as outlined in Scheme 7 by hydrolysing the anti-cancer drug methotrexate (23) to the cor-



Scheme 4

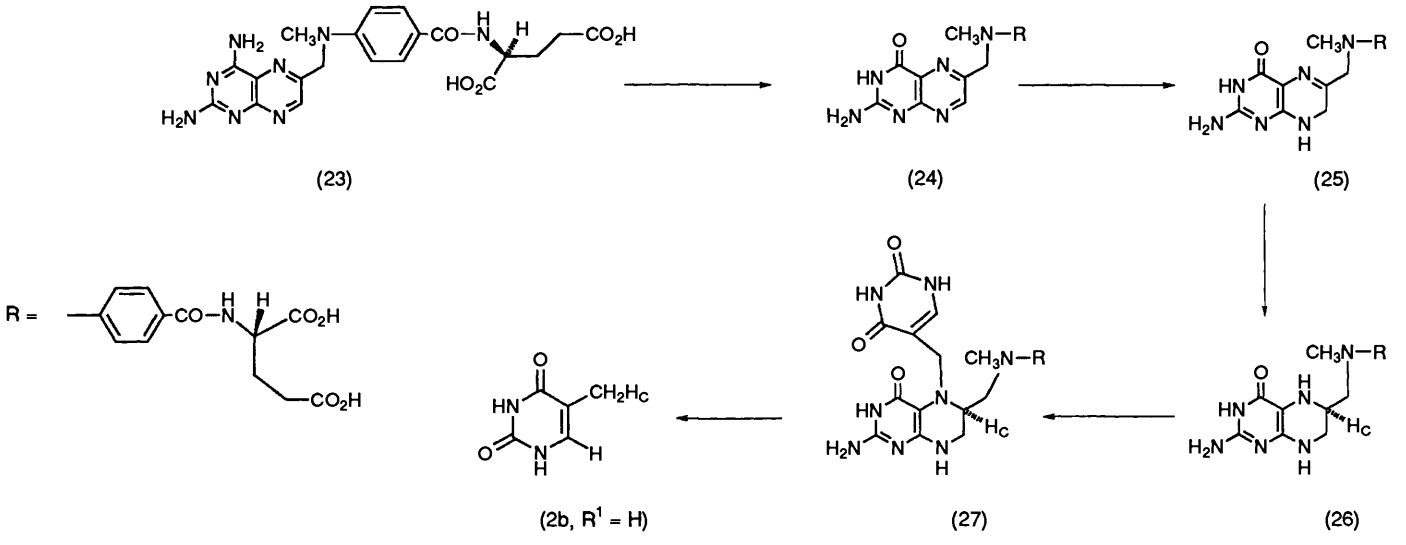


Scheme 5



(5) (21) (19, R^1 = H) (22)

Scheme 6



(23) (24) (25) (26) (27) (2b, R^1 = H)

Scheme 7

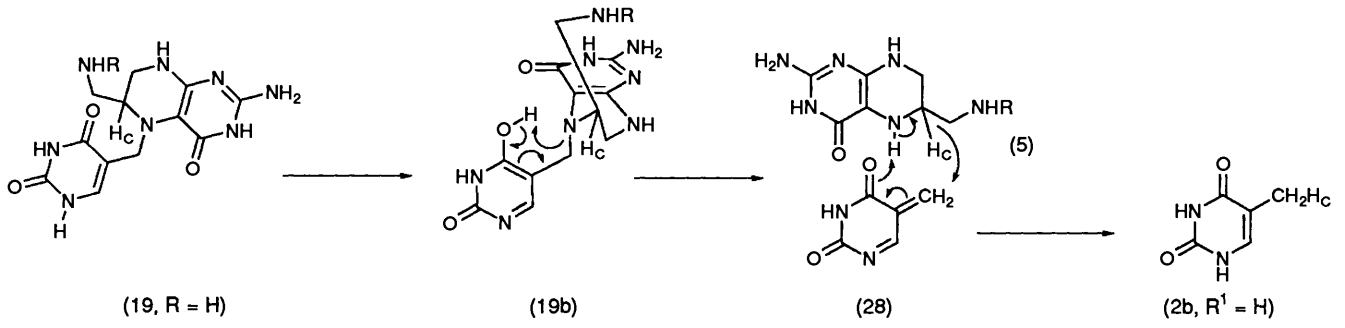
responding amide (24). This could be reduced to *N*-10-methyl-7,8-dihydrofolic acid (25) using aqueous dithionite. Reduction with either $NaBH_4$ or NaB^2H_4 then gave the unlabelled *N*-10-methyl-coenzyme (26) or its deuteriated analogue (26, $H_C = {}^2H$). These were separately alkylated to the protected putative intermediates (27) and (27,$H_C = {}^2H$) using 6-chloromethyluracil (21).[11]

Pyrolysis of the model compound (27) in the solid state gave thymine (2b, R^1 = H), identical in all respects with an authentic sample. When the deuteriated compound (27, $H_C = {}^2H$) was used, a sample of thymine was obtained in 47% yield. This had a ^2H-NMR spectrum indicating that all of the deuterium was in the methyl group, and a mass spectrum which indicated 25% monodeuteriation. The ^2H: ^1H ratio was extremely high, considering the ratio of protium to deuterium in the starting compound (27,$H_C = {}^2H$) and the fact that an isotope effect would favour transfer of protium rather than deuterium. It was evident from the fact that (2b, R^1 = H, $H_C = {}^2H$) was obtained rather than (2c,R^1 = H, $H_C = {}^2H$), that the retro-ene mechanism suggested in Scheme 5 could not account for the results.

The results of the model reaction parallel closely those found in the overall enzymatic reaction and so, if the intermediate (19) is involved in the process, the chemical results may be relevant to the enzymatic mechanism. One possible explanation of the results is suggested in Scheme 8 where the concerted process shown in (19b) may account for cleavage of the carbon–nitrogen bond. The hydrogen H_C may be sufficiently close in the crystal (or at the active site) to allow the redox process shown in (28) to occur and the labelled hydrogen would end up in the methyl group of the resultant thymine (2b,R^1 = H,$H_C = {}^2H$).

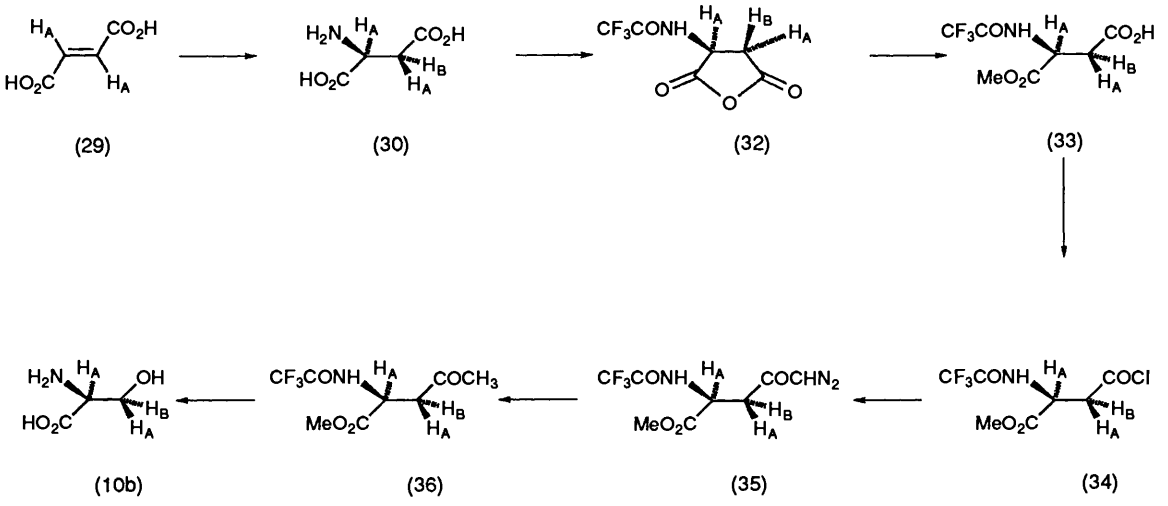
2.2 Studies on the Enzyme-catalysed Reaction

In order to understand the nature of the reaction catalysed by thymidylate synthase further, we determined to investigate the overall stereochemistry of the process. This would involve



(19, R = H) (19b) (28) (2b, R^1 = H)

Scheme 8



(29) (30) (32) (33) (34) (35) (36) (10b)

Scheme 9

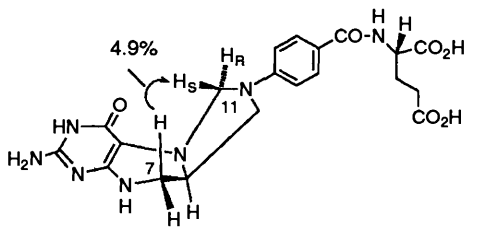
synthesis of the intermediate (6a) stereospecifically labelled at C-11 with deuterium and at C-6 with tritium (see Scheme 4). Use of this in the enzymic reaction and degradation of the resultant thymidine (2a) to acetic acid would allow us to assess the stereochemistry of the chiral methyl group and hence the overall stereochemistry of the reaction. However, when we began this work the absolute stereochemistry of the coenzyme 5,6,7,8-tetrahydrofolic acid (5) at C-6 had not been determined and so we first investigated this. This work is reported in Section 3.1 of this review.

As a first step to investigating the stereochemistry of the methylation reaction, we determined to prepare samples of L-serine, stereospecifically labelled at C-3 with deuterium and to use the enzymes serine hydroxymethyltransferase and thymidylate synthase in tandem to follow the stereochemistry from (10a) through (6a) to (2a) in the cycle represented in Scheme 4.

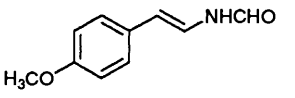
This required synthesis of samples of L-serine (10a) stereospecifically labelled at C-3. We had previously prepared samples of L-cystine stereospecifically labelled[12] at C-3 and had used these to study the stereochemistry of the cyclization which gives rise to the β-lactam ring in penicillins[12] and cephalosporins.[13] This work involved total synthesis and was unduly laborious and so, for other labelled amino acids, we devised a chemico-enzymatic method which we were able to use to prepare samples of L-glutamic acid stereospecifically labelled at C-3 with deuterium.[14] This method was adapted to the synthesis of the labelled samples of serine,[15] as shown in Scheme 9.

The enzyme aspartase is commercially available and was known to add the elements of ammonia with *anti*-stereochemistry across the double bond of fumaric acid (29) to give aspartic acid. Thus by using (a) fumaric acid (29) and $N^2H_4O^2H$ and (b) [2,3-$^2H_2$]fumaric acid (29, $H_A = {}^2H$) and NH_4OH we were able to prepare large quantities of (2*S*, 3*R*)-[3-$^2H_1$]- and (2*S*, 3*S*)-[2,3-$^2H_2$]aspartic acids, (30, $H_B = {}^2H$) and (30, $H_A = {}^2H$), respectively. Conversion of these into the trifluoroacetyl anhydrides (32) and ring-opening with methanol gave primarily the α-esters (33) which were purified as the acid chlorides (34). These were converted into the diazoketones (35) which could be reduced to the methyl ketones (36) without loss of label. Although Baeyer–Villiger rearrangement was not as regiospecific as we had hoped, it proceeded with retention of stereochemistry at the migrating primary chiral centre so that hydrolysis of the products gave (2*S*,3*R*)-[3-$^2H_1$]- and (2*S*, 3*S*)-[2,3-$^2H_2$]serines(10b, $H_B = {}^2H$) and (10b, $H_A = {}^2H$), respectively.[15] In a recent synthesis of labelled D-amino acids, we have found that the quality and yield of the product from the first step in this synthesis can be improved by using immobilized cells of *Escherichia coli*.[16]

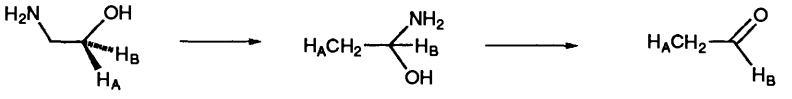
At this point in our studies, Benkovic and Floss[17] showed that the overall stereochemical outcome in the conversion of serine (10a) into thymine (2a) was as shown in Scheme 4. Benkovic[18] also prepared samples of 5,10-methylene-5,6,7,8-tetrahydrofolic acid (6a) stereospecifically deuteriated at C-11 and used the NOE between H-7*R* and H-11*S* shown in (6d) to define the absolute stereochemistry of the label. This allowed him to determine the stereospecificity of the two individual reactions (10a) → (6a) and (6a) → (2a) in Scheme 4, the stereochemistry being as indicated in the Scheme. Although our original goal had been so elegantly achieved, we were able to use our stereospecifically labelled samples of L-serine, with the biosynthesis of tuberin (37) as a 'black box', to verify that the dehydrogenase which converts (6) into (7) does so by removal of the 11-*pro*-*R*-hydrogen.[19] We have also converted our samples of labelled L-serine into stereospecifically labelled samples of ethanolamine (38) and have shown that the stereochemistry of the reaction catalysed by the enzyme ethanolamine ammonia-lyase (EC 4.3.1.7) and mediated by coenzyme B_{12} is as shown in Scheme 10.[20]



(6d)

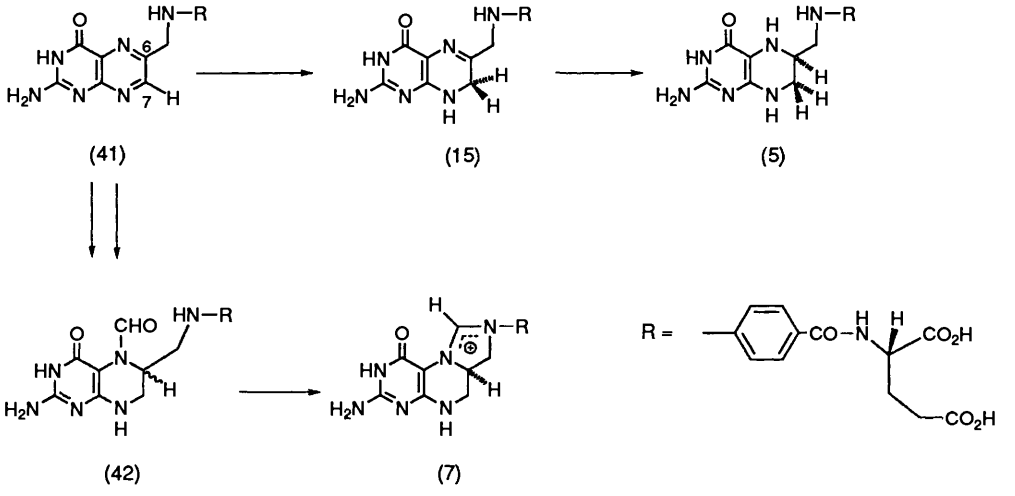


(37)



(38) (39) (40)

Scheme 10



Scheme 11

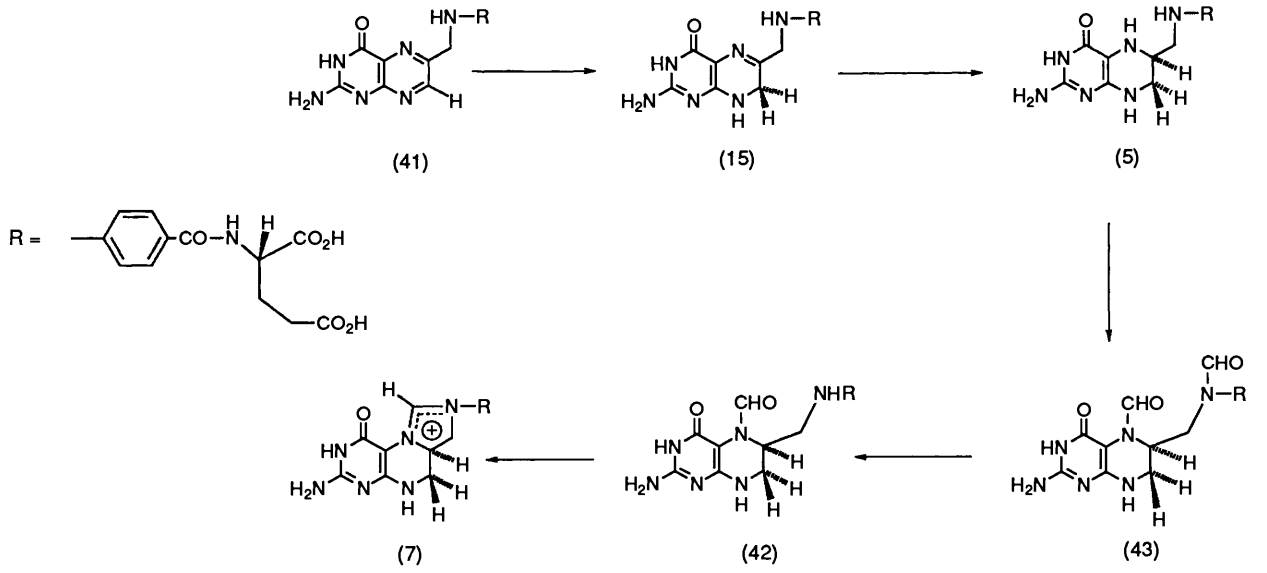
3 Studies on the Enzyme Dihydrofolate Reductase

3.1 Stereochemistry of Reduction of the Substrate

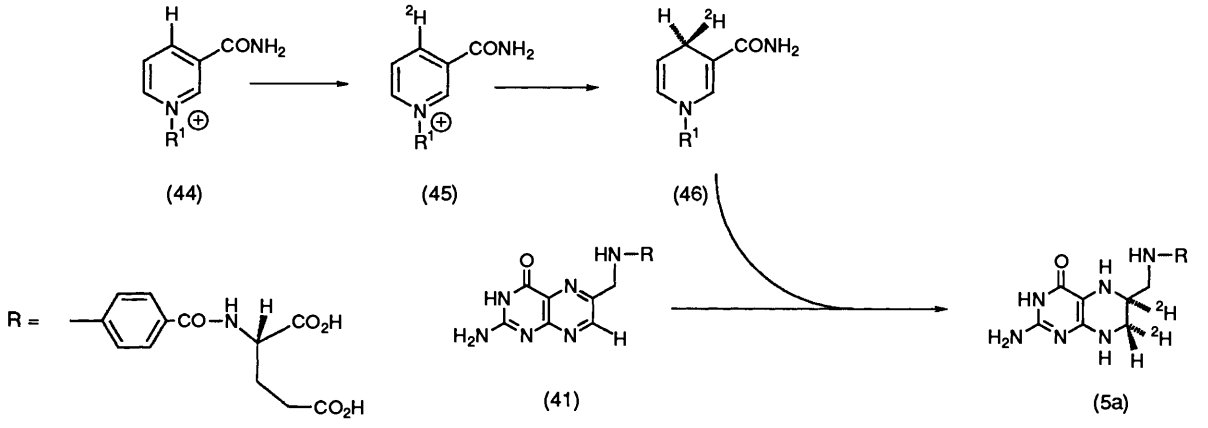
Our initial interest in this enzyme was sparked by our realization that, when we wished to assess the overall stereochemistry of the reaction catalysed by the enzyme thymidylate synthase, the absolute stereochemistry at C-6 of the coenzyme (5) was unknown. This centre is introduced during the reduction of 7,8-dihydrofolate (15) by dihydrofolate reductase, and indeed the enzyme will also reduce the vitamin folic acid (41) to the coenzyme (5) as in Scheme 11. We therefore determined to assess the stereochemistry of the reduction at both C-6 and C-7 of folic acid (41), originally intending to achieve this using comparison with unlabelled and stereospecifically labelled samples prepared by total synthesis. We had gone some way towards this goal[21] when two events changed our direction. The first was the opportunity to collaborate with Dr. J. Feeney and his colleagues and the second was a report by Fontecilla-Camps *et al.*[22] who had separated the diastereoisomers of the 'cancer rescue' agent folinic acid (42) and converted them separately into the bromide hydrobromides (7) as in Scheme 11. The *X*-ray crystal structures of these allowed their absolute stereochemistry to be assigned.

We therefore prepared 7,8-dihydrofolic acid (15) by dithionite reduction of folic acid (41) and reduced this, as in Scheme 12, to the biologically active coenzyme (5) using dihydrofolate reductase.[23] Clean monoformylaton of the biologically produced compound proved difficult but we were able to bis-formylate using formic acetic anhydride. The product (43) could be specifically deformylated at N-10 using alkali to yield a sample of folinic acid (42) which was compared with the samples whose stereochemistry had been assessed by *X*-ray structure analysis of the cyclization product (7). This showed that the stereochemistry at C-6 of the active coenzyme could formally be defined as (*S*).[23]

To complete our study of the stereochemistry of reduction of folic acid (41) by the enzyme dihydrofolate reductase we needed to discover the stereochemistry of reduction at C-7 and also the stereochemical origin of the hydrogen at C-4 of NADPH (46) which was transferred to C-6 and C-7. This was achieved by the particularly economical experiment shown in Scheme 13. (4*R*)-[4-$^2H_1$]NADPH (46) was first synthesized by reduction of [4-^{2}H]NAD$^+$ (45) (itself prepared using the method of San Pietro[24]) using the *proS*-specific enzyme glucose-6-phosphate dehydrogenase and glucose-6-phosphate.[25] This was then used in an 'NMR tube experiment'. Figure 1A shows the ^{1}H-NMR spectrum of the coenzyme (5) obtained using unlabelled NADPH to reduce folic acid (41). The two protons H-7 of the coenzyme (5) are part of an ABX system and, by careful comparison with model compounds,[23] we were able to assign the proton with the larger vicinal coupling to H-7*R*, *anti* to H-6 and that with the smaller vicinal coupling to H-7*S*, *syn* to H-6. Figure 1B shows the spectrum when (4*R*)-[4-^{2}-H_1]NADPH was



Scheme 12



Scheme 13

used in the experiment. Only H-7*R* remains, indicating that H-6 and H-7*S* are deuteriated and that reduction has occurred at both centres using the 4-*pro-R* hydrogen of NADPH.[23]

An *X*-ray structure of a ternary complex of the anti-cancer drug methotrexate, the coenzyme NADPH, and dihydrofolate reductase has shown,[26] that, whilst H-4*R* of NADPH is close to C-6 and C-7 of the pteridine ring of methotrexate (23), it is the opposite face of the pteridine system which is close to this reducing hydrogen from that face of the pteridine ring which is actually reduced when folic acid (41) is the substrate. Put in terms of the Fischer 'lock/key' analogy for enzymes, one key is entering the lock 'upside down' compared to the other. Figure 2 is an attempt to illustrate this in cartoon form.

3.2 Stereochemical Aspects of the Protein

After these initial studies on substrate binding, Feeney and his colleagues went on to show[27] that dihydrofolate reductase exists in three conformations whose proportions are pH-dependent and that *both* folate (41) and methotrexate (23) bind *in the same way* in two of these conformations. Only folic acid (41) binds in the third conformation where the opposite face of the pteridine ring is exposed to the coenzyme NADPH from that exposed in the other two conformations. It is this third conformation which is catalytically competent.

Feeney *et al.* also showed in this work that methyl protons from Leu-19 and Leu-27 of the enzyme came within nOe distance of H-7 of bound methotrexate.[27] At this time the prochiral methyl groups of leucine and valine residues in pro-

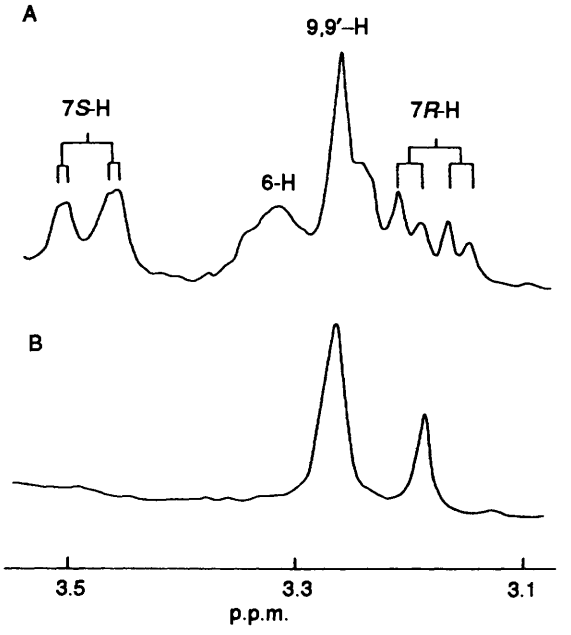


Figure 1 Part of the 270 MHz ^{1}H-NMR spectrum in ^2H_2O of 5,6,7,8-tetrahydrofolic acid (5) prepared by reduction using dihydrofolate reductase and (A) NADPH; (B) (4*R*)-[4-$^2H_1$]NADPH. The chemical shift scale is referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

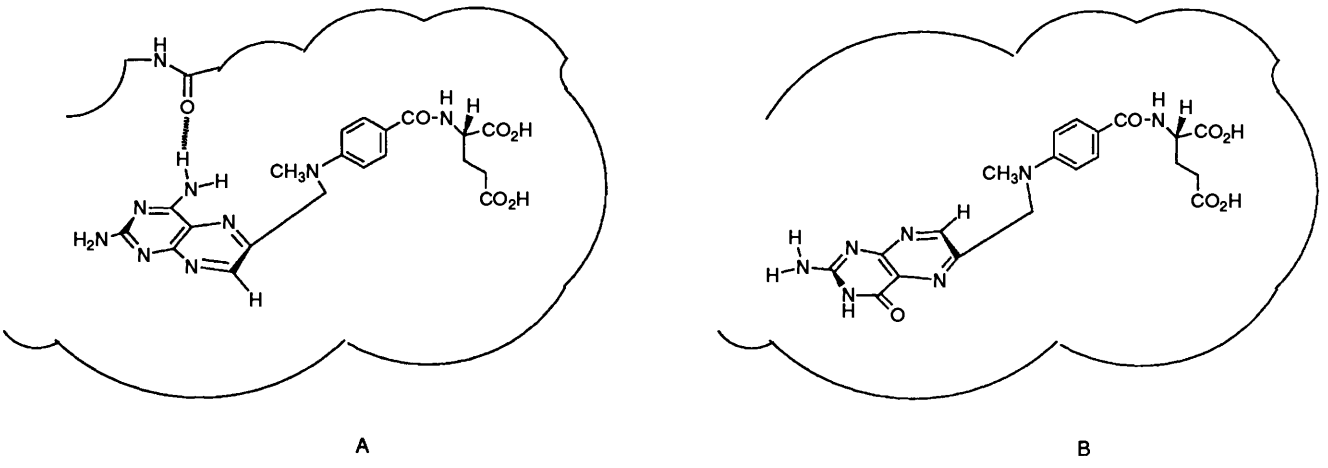
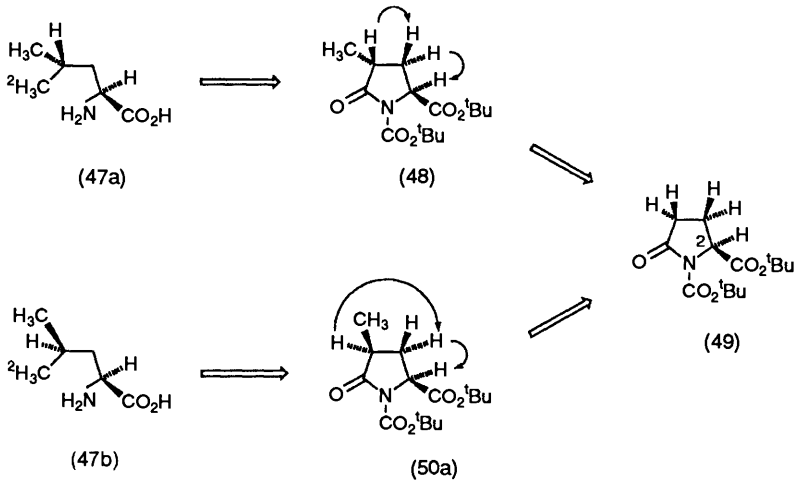


Figure 2 Representation of the binding of (A) methotrexate (23) and (B) folic acid (41) at the active site of dihydrofolate reductase.



Scheme 14

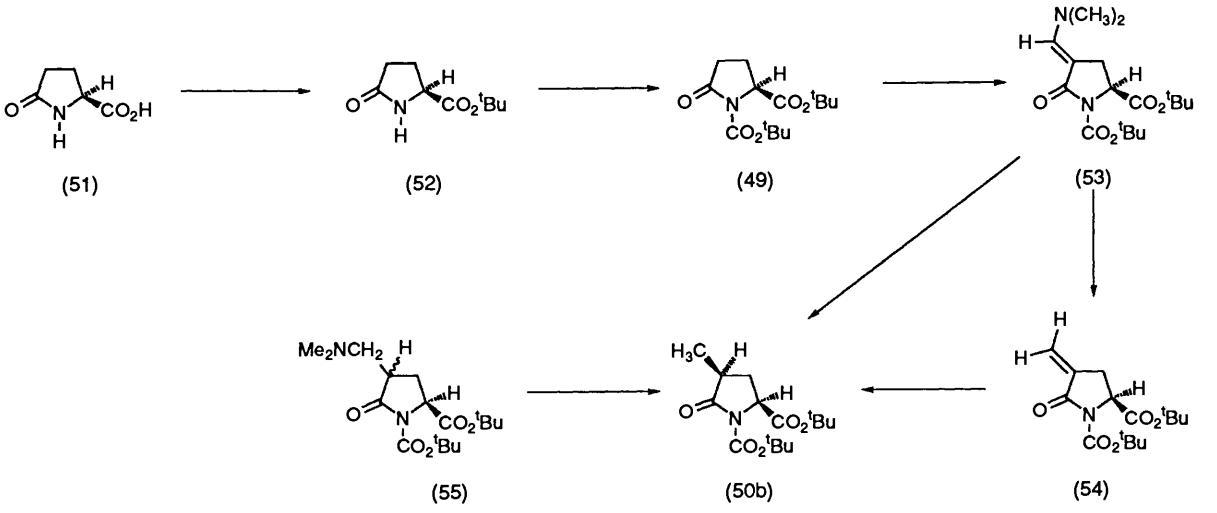
teins could not be distinguished by NMR spectroscopy, although such side chains were important in hydrophobic binding. We therefore resolved to prepare a sample of dihydrofolate reductase in which the leucine residues had their terminal methyl groups distinguishable by stereospecific isotopic labelling, and to use these to assign the prochiral methyls of these residues in the protein.

Our first task was to synthesize stereospecifically labelled samples of leucine and to this end we were able to adapt some work which we had undertaken to assess the stereochemistry of the reaction catalysed by the enzyme glutamate γ-carboxylase.[28] The plan was to use a protected pyroglutamic acid derivative (49) as a chiral template as shown in Scheme 14. Here the bulky group at C-2 would have a large 1,3-interaction at C-4 so that either *cis* or *trans* products, (50a) or (48) respectivelv, might be prepared stereospecifically. Further, the NOEs shown in (50a) and (48) could be used unambiguosly to define the stereochemistry. Ring-opening and functionalization would then give the labelled samples of leucine, (47b) or (47a) respectively.

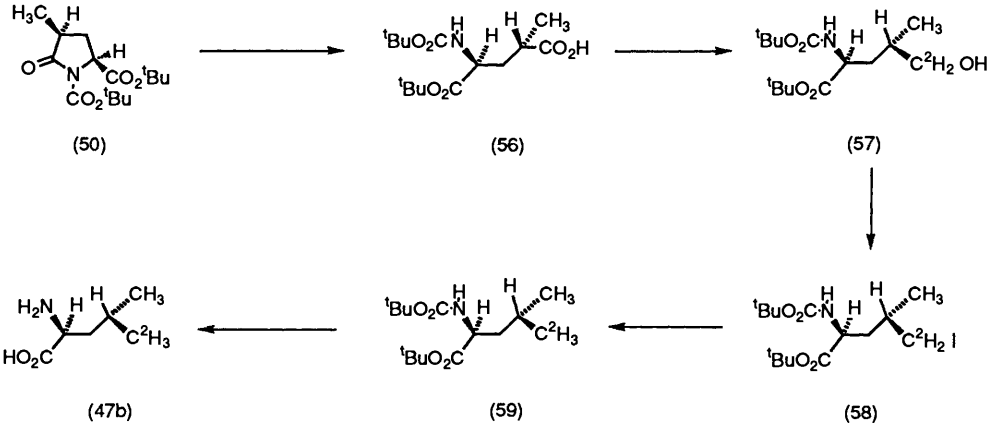
The protected pyroglutamic acid derivative (49) proved surprisingly difficult to obtain, but we were eventually able to prepare it in good yield and in large amounts as shown in Scheme 15 by esterification of pyroglutamic acid (51) using t-butyl acetate and perchloric acid followed by preparation of the urethane (49), using di-t-butyldicarbonate and DMAP in acetonitrile. The urethane (49) was converted into the enaminone (53) using Bredereck's reagent as shown in Scheme 15.[29] This enaminone has served as a useful synthetic intermediate in our studies on glutamate antagonists,[30] glutamate-γ-carboxylase,[28] and amino acid synthesis[31] and our intention was to convert it into the enone (54) so that we might investigate asymmetric induction in hydrogenation of this compound.

The enamine (53) was readily converted into the enone (54) in excellent yield using DIBAL.[29] A small amount of a mixture of the diastereoisomers of the Mannich base (55) was also obtained as a by-product. Hydrogenation of the enone (54) specifically gave the *cis*-methylated product (50b), the catalyst directing attack from the less-hindered face. The stereochemistry of the product was verified by observation of the NOEs indicated in (50a) in Scheme 14. When the diastereoisomeric mixture (55) was subjected to catalytic hydrogenation, the *cis*-product (50b) was again obtained in excellent yield, suggesting an elimination/addition mechanism with stereochemical control in the addition step. This encouraged us to investigate direct catalytic hydrogenation of the enaminone (53) which gave the *cis*-4-methyl-substituted pyroglutamate derivative (50b) in good yield.[29]

We had now succeeded in the first part of our synthetic plan, outlined in Scheme 14. All that remained was to effect ring-opening and to convert the carbonyl group of the pyroglutamate to C^2H_3. The ring-opening reaction was achieved without epimerization using aqueous LiOH in the THF, yielding the optically pure acid (56) as shown in Scheme 16. This was converted into the deuteriated alcohol (57) by formation of the mixed anhydride and reduction with NaB^2H_4. The alcohol was converted into the iodide (58) using methyltriphenoxyphosphonium iodide in HMPA, and this was reduced *in situ* at 70 °C to give the protected labelled leucine derivative (59). Deprotection was achieved by hydrolysis in 6N aqueous HCl at room temperature giving (2*S*,4*R*)-[5,5,5,-$^2H_3$]leucine hydrochloride (47b), the ^{13}C-NMR spectrum of which is shown in Figure 3, allowing



Scheme 15



Scheme 16

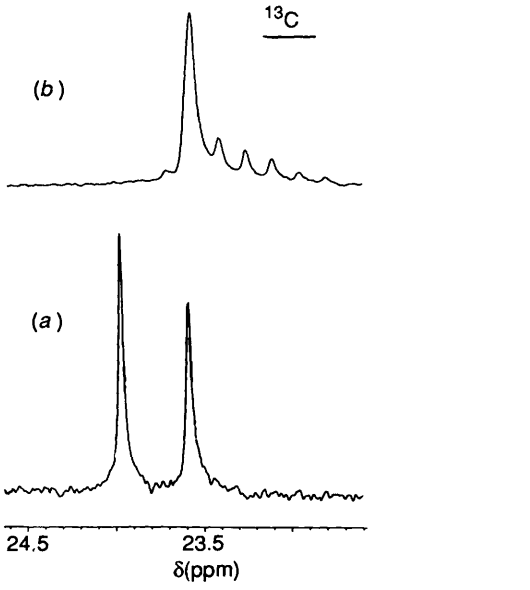


Figure 3 Part of the ^{1}H-decoupled ^{13}C-NMR spectrum in 20% ^{2}HCl/$^{2}H_2O$ at 125 8 MHz of (a) (2*S*)-leucine hydrochloride, and (b) (2*S*,4*R*)-[5,5,5-$^{2}H_3$]leucine hydrochloride (47b)

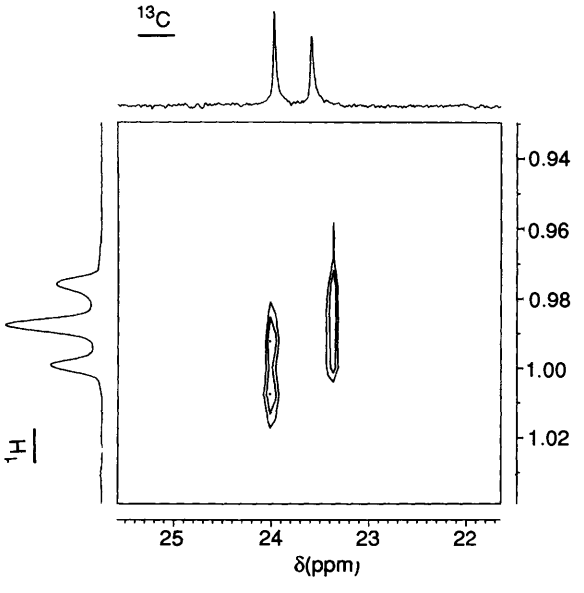


Figure 4 Two-dimensional ^{1}H–^{13}C shift correlation of leucine hydrochloride in 20% ^{2}HCl/$^{2}H_2O$

assignment of the methyl absorptions. Because of overlap in the ^{1}H-NMR spectrum, a two-dimensional ^{13}C–^{1}H shift correlation, shown in Figure 4, was required to assign the methyl proton absorptions In both ^{1}H- and ^{13}C-NMR spectra, the signals due to the 4-*pro-R* methyl group were to lower field The remainder of the ^{13}C-NMR spectrum of the labelled sample of leucine was as expected, except that there was a second resonance in the region of the absorption due to C-4. This could be explained by the presence of a small amount of (2*S*, 4*R*)-[5,5-$^{2}H_2$]leucine which would arise due to the $NaB^{2}H_3CN$ having been less than 100% isotopically pure. The β-isotope shift would

cause the C^2H_3-*C* and C^2H_2H-*C* resonances to absorb differently This interpretation was confirmed by a DEPT experiment and the ^{1}H-NMR spectrum indicated that there was *ca* 8% protium in the 4-*pro R*-methyl group

The stereospecifically labelled sample of leucine (47b) was used as the sole source of this amino acid in growing an auxotrophic strain of *Lactobacillus casei* The resultant dihydrofolate reductase was isolated and purified and a binary complex with methotrexate (23) was prepared [32] Comparison of the leucine methyl absorptions in the ^{1}H-NMR spectrum of this sample with those of an unlabelled sample, as shown in the Table, allowed twelve of the thirteen methyl resonances to be assigned [32]

Table 1 Chemical shifts (measured from dioxan and then referenced to DSS) of the leucine methyl resonances in the complex *L casei* dihydrofolate reductase–methotrexate–NADPH

	High field	Low field
Leu 4	1 17*	0 52†
Leu 12	0 93*	0 80†
Leu 19	0 66*	0 46†
Leu 23	0 65*	0 06†
Leu 27	0 64*	− 0 01†
Leu 54	0 36†	− 0 07*
Leu 62	0 66†	0 43*
Leu 94	0 78	0 78
Leu 113	0 30†	− 0 96*
Leu 114	1 07†	0 98*
Leu 118	− 0 33*	− 0 52†
Leu 131	0 44†	− 0 02*
Leu 151	1 15*	0 85†

* Absent in enzyme from (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine † Present in enzyme from (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine

This method is applicable to other proteins although, since we commenced this work, the elegant use of 'biosynthetic fractional ^{13}C labelling' has allowed the methyl resonances in valine and leucine residues in some proteins to be assigned [33]

4 Conclusions and Prospects

In this article I have concentrated mainly on our own experiments on two of the enzymes involved in the synthesis of the DNA base thymine in nature Where appropriate, I have referred briefly to other areas of study that this work has taken us into The importance of these enzymes as targets for anticancer drugs means that work will continue and our knowledge of them will continue to be enhanced The details of the chemical mechanism of the methylation of uracil will continue to present an intellectual challenge for some time to come

It is perhaps appropriate that, in this year of the centenary of Emil Fischer's lock/key analogy for enzymes,[34] I have reviewed our work on a case which required a less rigid view of this analogy Our recent work on examination of protein interactions is currently being applied to proteins other than dihydrofolate reductase, and we are looking at details of hydrophobic binding in leucine zipper proteins and ubiquitin

5 References

1 For a review on one-carbon transfer mediated by tetrahydrofolic acid see D W Young in 'Chemistry and Biology of Pteridines', ed J A Blair, Walter de Gruyter, Berlin, 1983, p 321
2 D Gani and D W Young, *J Chem Soc Chem Commun*, 1983, 576, *ibid*, *J Chem Soc Perkin Trans 1*, 1985, 1355
3 D Gani, P B Hitchcock, and D W Young, *J Chem Soc Chem Commun*, 1983, 898, *ibid*, *J Chem Soc Perkin Trans 1*, 1985, 1363
4 M Friedkin and A Kornberg in 'The Chemical Basis of Heredity', ed W D McElroy and B Glass, Johns Hopkins Press, Baltimore, 1957, p 609
5 M Friedkin in 'The Kinetics of Cellular Proliferation', ed F Stohlman, Grune and Stratton, New York and London, 1959, p 97
6 C A Lewis, P D Ellis, and R B Dunlap, *Biochemistry*, 1981, **20**, 2275
7 P C Plese and R B Dunlap *J Biol Chem*, 1977, **252**, 6139 and references cited therein
8 E J Pastore and M Friedkin, *J Biol Chem*, 1962, **237**, 3802
9 M Y Lorenson, G F Maley, and F Maley, *J Biol Chem*, 1967, **242**, 3332
10 V S Gupta and F M Huennekens, *Biochemistry*, 1967, **6**, 2168
11 P A Charlton and D W Young, *J Chem Soc Chem Commun*, 1980, 614, *ibid*, *J Chem Soc Perkin Trans 1*, 1982, 1363
12 D J Morecombe and D W Young, *J Chem Soc Chem Commun*, 1975, 198, D W Young, D J Morecombe, and P K Sen, *Eur J Biochem*, 1977, **75**, 133
13 J A Huddleston, E P Abraham, D W Young, D J Morecombe, and P K Sen, *Biochem J*, 1978, **169**, 705
14 S J Field and D W Young, *J Chem Soc Chem Commun*, 1979, 1163, *ibid*, *J Chem Soc Perkin Trans 1*, 1983, 2387
15 D Gani and D W Young, *J Chem Soc Chem Commun*, 1982, 867, *ibid*, *J Chem Soc Perkin Trans 1*, 1983, 2393
16 B S Axelsson, K J O'Toole, P A Spencer, and D W Young, *J Chem Soc Chem Commun*, 1991, 1085, *ibid*, *J Chem Soc Perkin Trans 1*, 1994, 807
17 C Tatum, J Vederas, E Schleicher, S J Benkovic, and H Floss, *J Chem Soc Chem Commun*, 1977, 218
18 L J Shieker and S J Benkovic, *J Am Chem Soc*, 1984, **106**, 1833
19 K M Cable, R B Herbert, V Bertram, and D W Young, *Tetrahedron Lett*, 1987, **28**, 4101
20 D Gani, O C Wallis, and D W Young, *Eur J Biochem*, 1983, **136**, 303
21 D W Young, *Chem Ind (London)*, 1981, 556
22 J C Fontecilla-Camps, C E Bugg, C Temple, J D Rose, J A Montgomery, and R L Kisliuk, *J Am Chem Soc*, 1979, **101**, 6114
23 P A Charlton, D W Young, B Birdsall, J Feeney, and G C K Roberts, *J Chem Soc Chem Commun*, 1979, 922, *ibid*, *J Chem Soc Perkin Trans 1*, 1985, 1349
24 A San Pietro, *J Biol Chem*, 1955, **217**, 579
25 D W Young in 'Isotopes in Organic Synthesis' ed E Buncel and C C Lee, Elsevier, Amsterdam, 1978, Vol 4, pp 184—188
26 D A Matthews, R A Alden, J T Bolin, D J Filman, S T Freer, R Hamlin, W G J Hol, R L Kisliuk, E J Pastore, L T Plante, N Xuong, and J Kraut, *J Biol Chem*, 1978, **253**, 6946
27 B Birdsall, J Feeney, S J B Tendler, S J Hammond, and G C K Roberts, *Biochemistry*, 1989, **28**, 2297
28 R A August, A N Bowler, P M Doyle, X J Durand, P Hudhomme, J A Khan, C M Moody , and D W Young in 'Molecular Recognition Chemical and Biochemical Problems II', ed S M Roberts, Special Publication No 111, The Royal Society of Chemistry, Cambridge, 1992, p 121
29 R A August, J A Khan, C M Moody, and D W Young, *Tetrahedron Lett*, 1992, **33**, 4617
30 A N Bowler, P M Doyle, and D W Young, *J Chem Soc Chem Commun*, 1991, 314
31 C M Moody and D W Young, *Tetrahedron Lett*, 1993, **34**, 4667
32 G Ostler, A Soteriou, C M Moody, J A Khan, B Birdsall, M D Carr, D W Young, and J Feeney, *FEBS Lett*, 1993, **318**, 177
33 D Neri, G Otting, and K Wuthrich, *Tetrahedron*, 1990, **46**, 3287 and references cited therein
34 E Fischer, *Ber Dtsch Chem Ges*, 1894, **27**, 2985