# Investigation of a Chemical Model for the Methylation of Deoxyuridine Monophosphate by Thymidylate Synthetase

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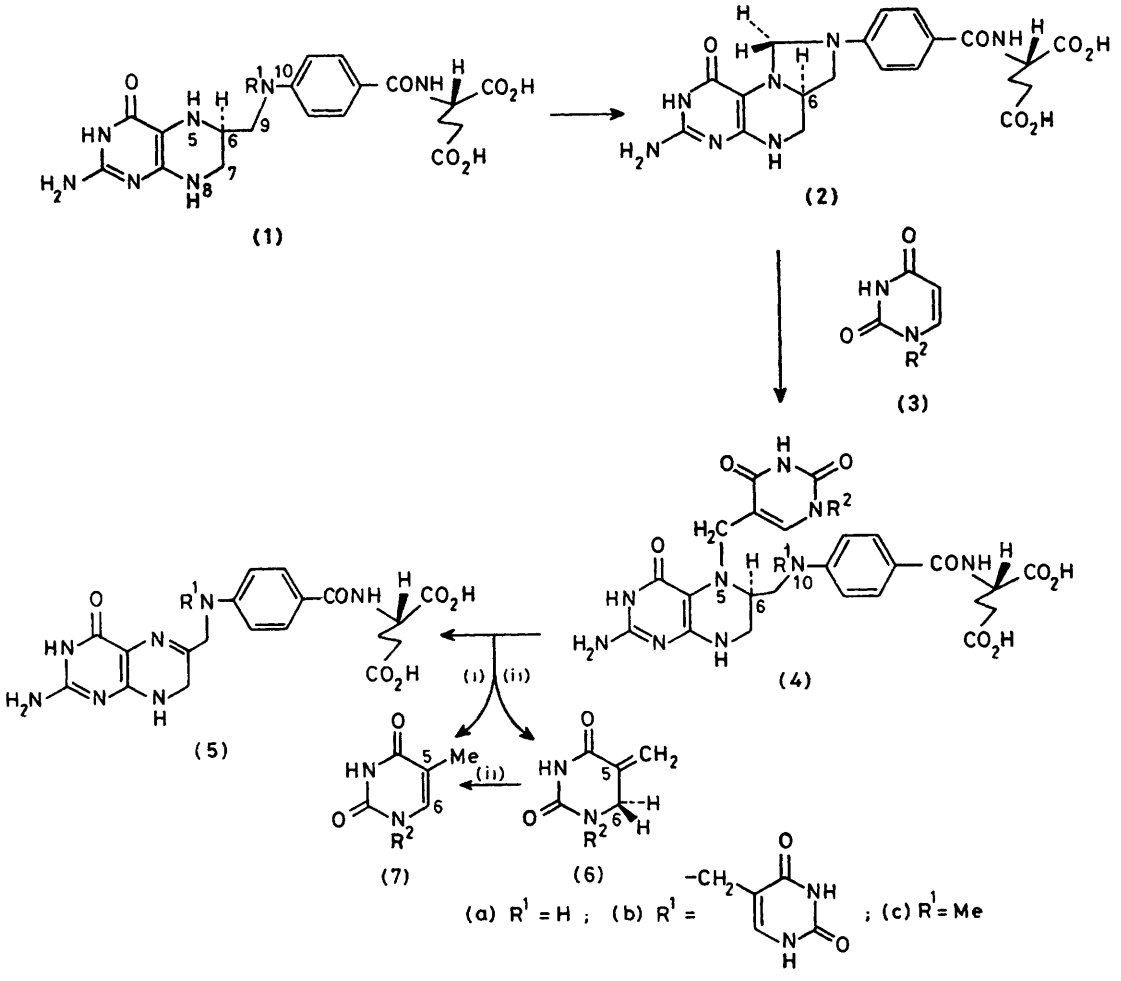
*Summary* The compound (**4a**, $R^2 = H$), long regarded as a putative intermediate in a chemical model for thymine biosynthesis, has been reformulated as the *bis*-adduct (**4b**; $R^2 = H$), a chemical model for the action of thymidylate synthetase has been achieved and the result is discussed

THYMIDYLATE SYNTHETASE, the enzyme responsible for methylation of the RNA base uracil to the DNA base thymine, is an important target enzyme in cancer chemotherapy [1] This methylation is mediated by the coenzyme 5,6,7,8-tetrahydrofolic acid (**1**, $R^1 = H$) and the one-carbon transfer involves 5,10-methylene-5,6,7,8-tetrahydrofolic acid (**2**) rather than 5-methyl-5,6,7,8-tetrahydrofolic acid which

is the normal substrate for one-carbon transfers at this oxidation level.[2] The reduction concomitant with the transfer is achieved by 'oxidation' of the cofactor to 7,8-dihydrofolic acid (**5**; $R^1$ = H), and it is known that the hydrogen at C-6 of 5,6,7,8-tetrahydrofolate (**1**; $R^1$ = H) is transferred to the methyl group of thymine (**7**; $R^2$ = H) in this process.[3]

shift might then complete the sequence [Scheme (ii)]. We now report work on the thermal conversion of a compound of type (**4**) into thymine which would be a chemical model for this proposed biochemical process.

Since Gupta and Huennekens[6] had reported a synthesis of (**4a**; $R^2$ = H) by alkylation of 5,6,7,8-tetrahydrofolic acid with two moles of 5-chloromethyluracil, we reduced 7,8-




SCHEME

One suggestion as to how this sequence might be carried out chemically is outlined in the Scheme. A uracil derivative (**3**) might undergo Mannich reaction with the cofactor (**2**) to yield the product (**4**). This intermediate might then rearrange to the thymine derivative (**7**) [Scheme (i)] and 7,8-dihydrofolate (**5**). The intermediacy of compounds of type (**4**) was first suggested by Friedkin[4] and a nominal 1,3-hydride shift from C-6 of (**4**) to the methyl of thymine would be required to complete the process. Since a *concerted* 1,3-hydride shift is not allowed,[5] it is appealing to consider the possibility that the intermediate (**4**) might undergo an allowed concerted *retro*-ene reaction to yield 7,8-dihydrofolate (**5**) and the intermediate (**6**) in which the hydrogen at C-6 in (**4**) would be delivered to C-6 of the pyrimidine (**6**) [Scheme (ii)]. A stereospecific enzyme-catalysed proton

dihydrofolic acid (**5**)[7] with $NaBH_4$, took the pH to 0—1 with conc. HCl and alkylated the product at pH 7·5 with two moles of 5-chloromethyluracil. The product had u.v. spectra at acid, neutral, and basic pH which were identical in every way with those reported for (**4a**; $R^2$ = H).[6] The compound ran as a single quenching spot on paper chromatography and analysed as $C_{24}H_{27}N_9O_8 \cdot 4H_2O$. Coincidentally the analytical figures also fitted a *bis*-adduct, $C_{29}H_{31}N_{11}O_{10} \cdot 4H_2O$. The $^1$H-n.m.r. spectrum[8a] of the compound in 1 M NaOD–$D_2O$ showed, in addition to the typical tetrahydrofolate resonances, a one-proton singlet at $\delta$ 7·05, a one-proton doublet ($J$ 1·8 Hz) at $\delta$ 7·79, a two-proton AB system centred at $\delta$ *ca.* 3·6 and a further two protons overlapping with the α-CH of the glutamate residue at $\delta$ *ca.* 4·2. It would appear, therefore, that the product was the *bis*-adduct

(**4b**, $R^2$ = H) and this was confirmed by a field desorption mass spectrum[8b] which showed a major ion at *m/e* 694 ($C_{29}H_{31}N_{11}O_{10}$ requires 693 64)

In view of the similarity of synthesis and the identity of the spectra of our product with those of the compound previously assigned structure (**4a**, $R^2$ = H),[6] we repeated the further alkylation of this compound [6] This reaction had afforded a product claimed[6] to be (**4b**, $R^2$ = H) The $^1$H-n m r spectrum[8a] of the crude product indicated that peralkylation had occurred

Although we had obtained the *bis*-adduct (**4b**, $R^2$ = H), we resolved to investigate its pyrolysis $^1$H-N m r spectra indicated that, at most, a negligible amount of thymine was present in the compound, but when it was heated to 255 °C *in vacuo*, thymine sublimed in 39% yield The [6-$^2H_1$]-analogue of (**4b**, $R^2$ = H) was prepared by substituting $NaB^2H_4$ for $NaBH_4$ in the synthesis and on pyrolysis this gave a 46% yield of thymine which was shown to be 29 1% monodeuteriated by mass spectroscopy $^2$H-N m r spectroscopy[8c] indicated that the isotope was located entirely on the methyl group of thymine ($\delta$ −2 93 from $^2H_2O$)

These results, while interesting, were confused by the presence of the second thymine residue at N-10 It was necessary therefore to synthesise a compound which contained only the N-5 thymine unit To this end methotrexate was hydrolysed to 10-methylfolic acid with 1 M NaOH [9] The protected compound was now reduced with dithionite to 7,8-dihydro-10-methylfolic acid (**5**, $R^1$ = Me) † Further reduction with $NaBH_4$ yielded (**1**, $R^1$ = Me) and this was alkylated *in situ* to yield the product (**4c**, $R^2$ = H) ‡ The [6-$^2H_1$]-analogue‡ of (**4c**, $R^2$ = H) was prepared by substituting $NaB^2H_4$ for $NaBH_4$ in the synthesis and both compounds were shown to contain, at most, a negligible amount of thymine by $^1$H-n m r spectroscopy [8a] Pyrolysis of both compounds gave thymine The [6-$^2H_1$]-analogue gave a 47% yield of thymine which was shown to be 25 3% monodeuteriated by mass spectroscopy $^2$H-N m r spectroscopy[8c] again showed that all of the label was in the methyl group of thymine

It is evident that the *retro*-ene mechanism for the biochemical reaction is ruled out by the isotopic experiments, since C-6 of thymine would be labelled in this pathway The labelling pattern is in fact the same as is found in nature and a radical cage mechanism would seem to account best for the high discrimination for the hydrogen at C-6 of (**4**) over all of the other hydrogens present A nominal 1,3-hydride shift of this type has been observed in the pyrolysis of dihydroquinoline adducts[10] and a radical cage mechanism may be implied here also An Arrhenius calculation suggests $t_{\frac{1}{2}}$ for radical formation to be *ca* 2 s at 250 °C

We thank the S R C for financial support and for a studentship (to P A C )

(*Received*, 28*th March* 1980, *Com* 332 )

† A satisfactory $^1$H-n m r spectrum was obtained for this compound

‡ This compound had satisfactory analytical and spectroscopic properties

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