Stereochemistry of the Metabolism of the DNA Base Thymine and the Drug 5-Fluorouracil

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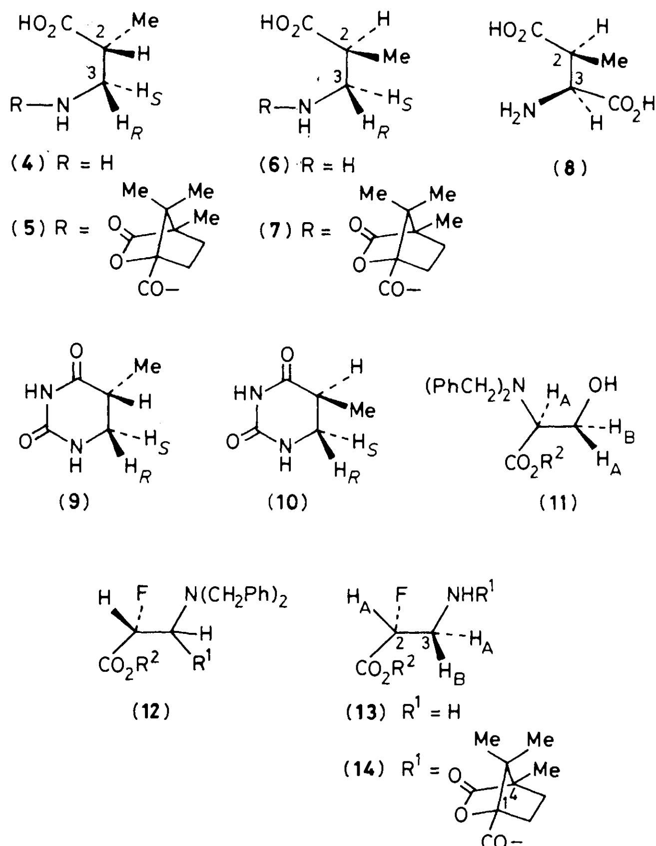
The enzyme dihydrothymine dehydrogenase reduces *both* the DNA base thymine and the anti-cancer drug 5-fluorouracil by overall *trans* addition of hydrogen, at the *si*-face at C-5 and the *si*-face at C-6.

The DNA base thymine (**1**, R=Me) is synthesised from the RNA base uracil (**1**, R=H) in a process which involves a target enzyme for the anti-cancer drug 5-fluorouracil (**1**, R=F).[1] It is interesting, therefore, that the enzyme system which normally catabolises thymine can also degrade 5-fluorouracil.[2] In view of our interest[3] in comparing the stereochemistry of binding of drugs and natural substrates to enzymes, we have investigated the stereochemistry of the catabolism of thymine and 5-fluorouracil using the mixed enzyme system we have isolated from bovine liver.[4] The catabolic process is summarised in Scheme 1. The rate limiting FAD-containing enzyme, dihydrothymine dehydrogenase (EC 1.3.1.2), catalyses the reduction of the pyrimidine (**1**) to a dihydropyrimidine (**2**). Subsequent hydrolysis then yields a β-amino-acid (**3**).

Because (2*R*)-3-amino-2-methylpropanoic acid (**4**) is excreted in human urine,[5] it is likely that the metabolic product (**3**, R=Me) has this configuration. The finding of the enantiomeric amino-acid (**6**) in some peptides[6] and the fact that the (2*S*)-isomer (**6**) is itself metabolised, whilst the (2*R*)-isomer (**4**) is not,[7] however, make it imperative that the absolute configuration at C-2 of the metabolic product (**3**, R=Me) be confirmed. (2*S*,3*S*)-3-Methylaspartic acid (**8**) was therefore prepared from mesaconic acid using the enzyme β-methylaspartase from *Clostridium tetanomorphum*.[8] The absolute stereochemistry of this amino-acid is known[9] and we were able to decarboxylate it to the amino-acid (**6**) by pyrolysis in a melt with *p*-methoxyacetophenone followed by hydrolysis in 3 M aqueous hydrochloric acid. Camphanic acid amides (**7**),† [(**7**) + (**5**)], and (**5**) were prepared from the amino-acid (**6**), commercial (2*RS*)-3-amino-2-methylpropanoic acid, and a compound isolated from an incubation of thymine with our bovine liver enzyme system, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. The 360 MHz ^{1}H n.m.r. spectra of these three compounds in C^2HCl_3 or in 10%



Scheme 1

† These compounds had the expected analytical and spectroscopic properties.

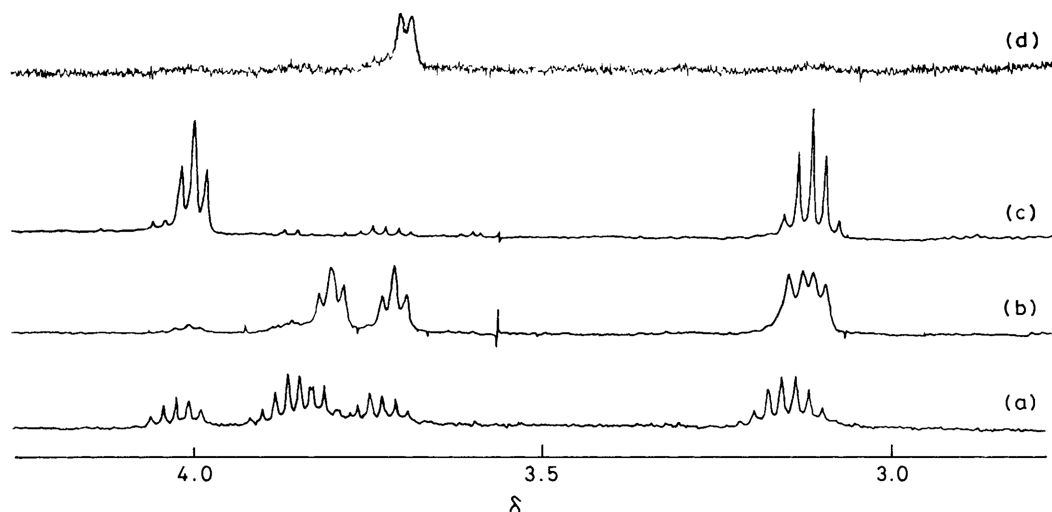


Figure 1. 360 MHz ^{1}H N.m.r. spectra in $C_5{}^2H_5N$ of (a) (2*RS*)-3-camphanoylamino-2-methylpropanoic acid; (b) (2*RS*,3*S*)-[3-$^2H_1$]-3-camphanoylamino-2-methylpropanoic acid (synthetic);‡ (c) (2*R*,3*R*)-[3-$^2H_1$]-3-camphanoylamino-2-methylpropanoic acid; (d) (2*R*,3*S*)-[2,3-$^2H_2$]-3-camphanoylamino-2-methylpropanoic acid. The samples in (c) and (d) were derived ultimately from the enzymic experiments.

NaO^2H–^2H_2O showed clearly that the C-3 protons had different chemical shifts in the (2*R*)- and (2*S*)-isomers and that, although decarboxylation of (2*S*,3*S*)-3-methylaspartic acid (**8**) had been accompanied by a small amount of racemisation at C-2, the product from thymine catabolism was clearly epimeric with the (2*S*)-isomer (**6**). Thus enzymic reduction of thymine had led to (5*R*)-dihydrothymine (**9**).

To assess the stereochemistry at C-6 in the catabolism of thymine, it was necessary to have a sample of (2*R*)- or (2*RS*)-3-amino-2-methylpropanoic acid which was stereospecifically labelled with deuterium at C-3. Witkop[10] has shown that catalytic hydrogenation of thymidine, followed by hydrolysis, gives specifically (5*S*)-dihydrothymine (**10**). Since hydrogenation should occur with *cis* addition of hydrogen, we repeated this sequence using $^2H_2$ and ^2H_2O in the reduction step. The product evidently contained deuterium at C-5, C-6, and in the methyl group. Hydrolysis of the product in hydrochloric acid, known[11] to result in racemisation at C-2, gave (2*RS*,3*S*)-[3-$^2H_1$]-3-amino-2-methylpropanoic acid.‡ The ^{1}H n.m.r. spectrum of the camphanic acid amide of this compound in $C_5{}^2H_5N$ (Figure 1b) confirmed the stereospecificity of the (3*S*)-label in both the (2*S*)- and (2*R*)-isomers and provided an assay for the absolute stereochemistry at C-3 in labelled samples of (**4**).

[6-^{2}H]Thymine[12] and thymine were now incubated in H_2O and ^2H_2O respectively with a mixture of the bovine liver enzymes,[4] NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. The samples of (2*R*)-3-amino-2-methylpropanoic acid (**4**) isolated from these experiments were converted into the amides (**5**), the ^{1}H n.m.r. spectra of which are shown in Figures 1c and 1d respectively. These clearly show that enzymic reduction of thymine has occurred from the *si*-face at C-6.

One stereoisomer of the product of catabolism of the drug 5-fluorouracil, 3-amino-2-fluoropropanoic acid, has been prepared by the reaction of *N*,*N*-dibenzyl-L-serine benzyl ester (**11**, R^2=CH_2Ph) with (diethylamino)sulphur trifluoride (DAST) followed by hydrogenolysis.[13] When we prepared the corresponding methyl ester (**11**, R^2=Me)† and treated it with DAST, we obtained a product (**12**, R^1=H, R^2=Me) which on hydrogenation and hydrolysis gave 3-amino-2-fluoropropanoic acid (**13**, R^2=H), $[\alpha]_D$ + 28.4° (H_2O). This was identical to a sample

‡ There was appreciable deuteriation in the methyl group of this sample.

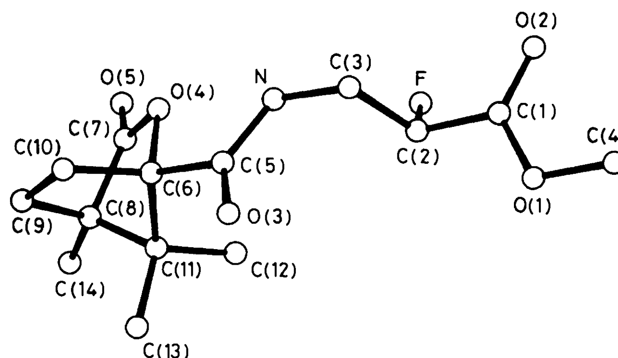


Figure 2. Molecular structure of the amide (**14**, R^2=Me).

obtained by incubating 5-fluorouracil with our bovine liver-glucose dehydrogenase system. Since the low anomalous dispersion of fluorine made the use of Bijvoet's method difficult, we converted (**13**, R^2=H) into the ester (**13**, R^2=Me)† and thence into the amide (**14**, R^2=Me).† *X*-Ray structure analysis§ of this compound showed the stereochemistry to be as in Figure 2. (−)-Camphanic acid, derived from (+)-camphor of known[14] absolute stereochemistry, has the (1*S*,4*R*) stereochemistry, so that the absolute stereochemistry of the metabolite of 5-fluorouracil must be as shown in (**13**, R^2=H). The enzymic reduction of 5-fluorouracil has therefore occurred from the *si*-face at C-5.

When threonine and allothreonine replaced L-serine in the α-fluoro-β-amino-acid synthesis,[13] the products were the *threo*- and *erythro*-isomers respectively of (**12**, R^1=Me, R^2=CH_2-Ph). This stereospecificity was accounted for[13] by a mechanism involving an aziridinium ion intermediate and our finding of

§ *Crystal data* (**14**, R^2=Me), $C_{14}H_{20}FNO_5$, M = 301.3, orthorhombic, space group $P2_12_12_1$, a = 7.375(1), b = 9.522(2), c = 21.793(2) Å, U = 1530.4 Å^3, Z = 4, D_c = 1.31 g cm^{-3}, $F(000)$ = 640. R = 0.101 based on 1132 reflections with $|F^2| > \sigma(F^2)$ collected on a diffractometer using Cu-K_α radiation, λ = 1.5418 Å, μ = 9.2 cm^{-1}. Hydrogen atoms are not included. The atomic co-ordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW. Any request should be accompanied by the full literature citation for this communication.

the absolute stereochemistry of (**13**, R^2=H) further supports this suggestion. Since we had prepared samples of L-serine stereospecifically labelled at C-3 with deuterium,[15] we were able to prepare (**11**, R^2=Me, H_B=^{2}H) and (**11**, R^2=Me, H_A=^{2}H) from them. These were converted into the camphanic acid derivatives (**14**, R^2=Me) in the same way as the unlabelled compound above. Assuming the stereochemistry of the reaction to be identical to that found with threonine and allothreonine, the sample from (**11**, R^2=Me, H_B=^{2}H) was (**14**, R^2=Me, H_B=^{2}H) and that from (**11**, R^2=Me, H_A=^{2}H) was (**14**, R^2=Me, H_A=^{2}H). The ^{1}H and ^{2}H n.m.r. spectra of these compounds in C^2HCl_3 showed the absorption for the *pro*-3*R*-hydrogen to be δ 3.7 and that for the *pro*-3*S*-H to be δ 3.96. [6-^{2}H]-5-Fluorouracil[16] and 5-fluorouracil were now incubated in H_2O and ^2H_2O respectively with the bovine liver enzymes,[4] NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. The samples of 3-amino-2-fluoropropanoic acid obtained from these incubations were converted into the amides (**14**, R^2=Me), the n.m.r. spectra of which indicated that the *pro*-3*R*-hydrogen was deuteriated in the sample derived from [6-^{2}H]-5-fluorouracil whilst the *pro*-3*S*-hydrogen was deuteriated in the sample from the ^2H_2O incubation. Enzymic reduction of 5-fluorouracil had therefore occurred from the *si*-face at C-6.

These experiments show that catabolism of *both* the DNA base thymine and the anti-cancer drug 5-fluorouracil involves *trans*-addition of hydrogen to the pyrimidines at the *si*-face at C-5 and the *si*-face at C-6. This suggests that these compounds are bound similarly at the active site of the enzyme when being reduced by the flavin coenzyme. This contrasts with the case of the substrate folic acid and the anti-cancer drug methotrexate which bind at the active site of dihydrofolate reductase.[3] The coenzyme NADPH approaches these pteridines from opposite faces and the drug methotrexate is not reduced.

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