Reaction of 5-Trifluoromethyl-2′-deoxyuridine and 1-Methyl-5-trifluoromethyluracil with Methoxyamine: Model Studies for the Interaction between Thymidylate Synthetase and 5-Trifluoromethyl-2′-deoxyuridine 5′-Phosphate

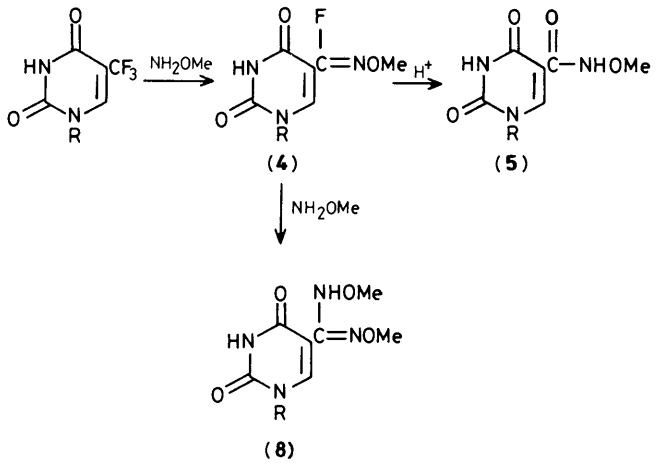
Yusuke Wataya * **and Yumi Sonobe**
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan
Mitsuaki Maeda, Ziro Yamaizumi, and Misako Aida
National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan
Daniel V. Santi
Departments of Biochemistry and Biophysics, and Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, U.S.A.

The reaction of 5-trifluoromethyl-2′-deoxyuridine (CF_3dUR) and 5-trifluoromethyl-1-methyluracil (1-MCF_3U) with methoxyamine in aqueous solution was studied to provide insight into the mechanism of inhibition of thymidylate synthetase by 5-trifluoromethyl-2′-deoxyuridine 5′-phosphate. 5-(Methoxyiminofluoromethyl)-2′-deoxyuridine (**4b**) and 5-(methoxyiminomethoxyaminomethyl)-2′-deoxyuridine (**8b**) were the products of the reactions of CF_3dUR with methoxyamine. No other major product was detected by radiochromatography of [6-^{3}H]CF_3dUR after treatment with methoxyamine. The decrease in CF_3dUR on treatment with methoxyamine followed pseudo-first-order kinetics, and (**4b**) and (**8b**) were formed sequentially, in that order. At pH 7.5 and 37 °C in 0.5M methoxyamine, the pseudo-first-order rate constant, k_{obs}, for the decrease in CF_3dUR (0.4 mM) was 0.355 h^{-1} and that for the decrease in (**4b**) (0.4 mM) was 0.06 h^{-1}. The k_{obs} for the decrease in CF_3dUR at 37 °C had an optimum at pH 7.5; it was 0.355 h^{-1} with 0.5M methoxyamine. Increasing the concentration of methoxyamine at pH 7.5 resulted in greater increases in the rate of CF_3dUR degradation than expected for a first-order reaction. This suggests that more than one molecule of methoxyamine participates in the degradation of CF_3dUR. 1-MCF_3U Reacted with methoxyamine to give 5-(methoxyiminofluoromethyl)-1-methyluracil (**4a**) and 5-(methoxyiminomethoxyaminomethyl)-1-methyluracil (**8a**) as products. Under acidic conditions, (**4a**) was converted into 5-(*N*-methoxycarbamoyl)-1-methyluracil. The formation of (**4a**) and (**4b**) as products of these reactions provides evidence for the formation of a highly reactive intermediate with an exocyclic difluoromethylene group at the 5 position which subsequently reacts with methoxyamine. An analogous mechanism was proposed for the inhibition of thymidylate synthetase by 5-trifluoromethyl-2′-deoxyuridine 5′-phosphate in which a nucleophilic group of the active site of the enzyme participates in the activation of the trifluoromethyl group.

5-Trifluoromethyl-2′-deoxyuridine 5′-phosphate (CF_3dUMP) is a strong inhibitor of thymidylate synthetase. In 1965 Reyes and Heidelberger reported that CF_3dUMP causes irreversible inhibition of thymidylate synthetase from Ehrlich ascites cells.[1] Based on the observation that 5-trifluoromethyluracil acylates amines in aqueous solution to give uracil-5-carboxyamides,[2] it was suggested that the irreversible inactivation of thymidylate synthetase may result from similar acylation of an amino group at the active site of the enzyme.[1]

A question arises as to the reason why the trifluoromethyl group at the 5-position of uracil derivatives should be at all susceptible to these reactions. The carbon–fluorine bond is quite stable and an outstanding characteristic of most trifluoromethyl groups is their resistance to chemical degradation. In contrast, 5-trifluoromethyluracil (CF_3U) is rapidly converted into 5-carboxyuracil in basic media,[2] and nucleosides of CF_3U are also converted into the corresponding nucleosides of 5-carboxyuracil.[3–6] *In vivo*, the metabolism of CF_3U and 5-trifluoromethyl-2′-deoxyuridine (CF_3dUR) gives 5-carboxyluracil.[7] To explain this unusual reactivity of the trifluoromethyl group, Santi and Sakai proposed a mechanism in which a nucleophilic group of the enzyme adds to the 6-position of CF_3dUMP, promoting the explusion of the fluorine in the form of fluoride ion and resulting in the formation of a reactive exocyclic difluoromethylene intermediate; this reactive intermediate is then trapped by a nucleophilic group of the enzyme to give an acylated enzyme.[6]

We have made certain observations regarding the inactivation of thymidylate synthetase from *Lactobacillus casei* by CF_3dUMP. In pre-incubation experiments, CF_3dUMP inactivated the enzyme when cofactor, (±)-L-5,10-methylenetetrahydrofolic acid (CH_2-FAH_4), was absent in the enzymic reaction mixture.[8] Concomitant with the inactivation, release of fluoride ion was observed. We also described experiments that demonstrate that CF_3dUMP is an alternate substrate for the thymidylate synthetase in the presence of thiol,[8] although structures of the reaction products have not been identified. To assist the elucidation of the mechanism of the interaction between CF_3dUMP and thymidylate synthetase, the chemical model study described in this paper was performed. This study deals with the reaction of CF_3dUR and 1-methyl-5-trifluoromethyluracil (1-MCF_3U) with methoxyamine, reactions which occur under near-physiological conditions giving 5-(methoxyiminofluoromethyl)-1-methyluracil (**4a**), 5-(methoxyiminofluoromethyl)-2′-deoxyuridine (**4b**), 5-(methoxyiminomethoxyaminomethyl)-1-methyluracil (**8a**), and 5-(methoxyiminomethoxyaminomethyl)-2′-deoxyuridine (**8b**) as products (Scheme 1). The results are further evidence that nucleophilic attack at the 6-position of the heterocycle leads to the generation of a reactive difluoromethylene at the 5-position.



(a) R = Me ; (b) 2-deoxy-β-D-ribofuranosyl

Scheme 1.

Results and Discussion

When CF_3dUR was treated with methoxyamine in an aqueous solution at pH 7.5 and 37 °C, it reacted rapidly. The progress of the reaction was detected by changes in the u.v. spectrum. The spectrum obtained after 15 h of incubation was very different from that of the original compound. At time zero, the $\lambda_{max.}$ was 261 nm, which corresponded to that of CF_3dUR. During incubation the u.v. spectrum changed gradually to give, after 15 h, two $\lambda_{max.}$ values at 279 and 253 nm. Figure 1 shows the high-performance liquid chromatography (h.p.l.c.) profiles taken during the course of reaction of CF_3dUR (0.4 mM) with 0.75M methoxyamine at pH 7.5 and 37 °C. The reaction mixture gave two major products with the retention times of 11.6 and 33.6 min. These products were identified as (**4b**) and (**8b**) by n.m.r. and mass spectral analysis (see Experimental section).

In order to investigate whether non-u.v.-absorbing nucleoside materials were present in the reaction mixture, [6-^{3}H]CF_3dUR was treated with 0.5M methoxyamine at pH 7.3 and 37 °C and the reaction mixture was subjected to h.p.l.c. followed by counting of radioactivity. When the 40 h reaction mixture was analysed, radioactivity was found only in the three peaks corresponding to CF_3dUR, (**4b**) and (**8b**).

Compound (**4b**) was unstable in acidic aqueous media. The u.v. spectral changes occurring upon treatment of (**4b**) (0.05 mM) with 1M HCl at 30 °C were followed in a time-course study. At time zero, the $\lambda_{max.}$ values were 284 and 233 nm. After 6.2 h of incubation, the wavelengths for the maxima had changed to 277 and 221 nm. Three isosbestic points were observed, at 283, 254, and 228 nm and the pseudo-first-order rate constant, k_{obs}, was 0.849 h^{-1} for the degradation of (**4b**). The reaction product formed from (**4b**) in the acid was deduced to be 5-(*N*-methoxycarbamoyl)-2′-deoxyuridine (**5b**) on the basis of the properties described in the following section. Compound (**8b**) was stable in 0.1M HCl, neutral media (pH 7.0), and 0.1M NaOH at 30 °C for 10 h.

Treatment of 1-MCF_3U with methoxyamine gave two crystalline products identified as (**4a**) and (**8a**) by u.v., n.m.r., and mass spectral analysis. When (**4a**) was treated with 0.1M HCl at 30 °C, there was a shift in the u.v. maxima from 233 and 287 nm to 223 and 285 nm. During the course of the reaction, isosbestic points at 229, 257, and 287 nm were observed, indicating the absence of accumulated intermediates. The pseudo-first-order rate constant for the loss of (**4a**) was 0.848 h^{-1}. N.m.r., u.v., and mass spectral data indicated that the product of the acid-catalyzed reactions of (**4a**) was the corresponding 1-methyl-5-(*N*-methoxycarbamoyl)uracil (**5a**); compounds (**8a**) and (**5a**) were stable in 0.1M HCl, 0.1M NaOH, and neutral media (pH 7.0) at 30 °C for 10 h.

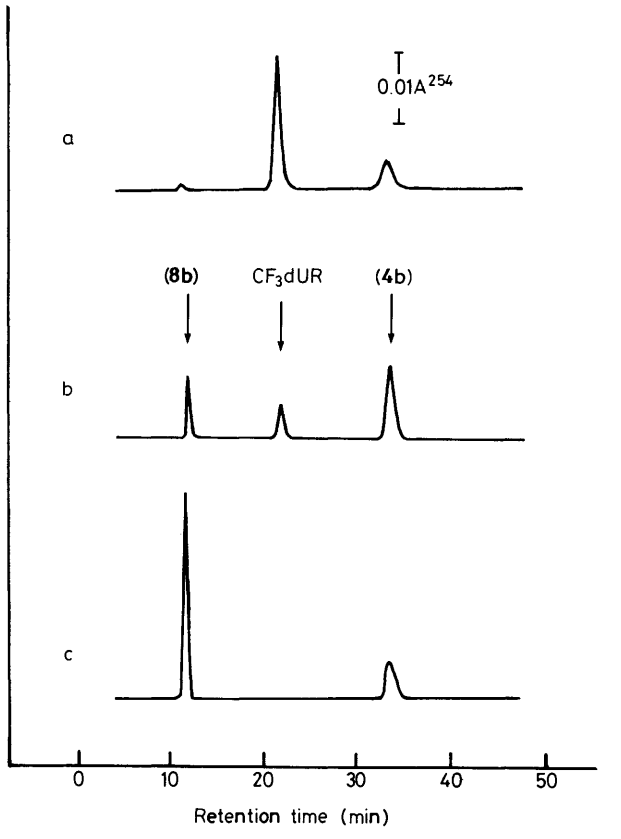


Figure 1. H.p.l.c. analysis of the reaction of CF_3dUR with methoxyamine. The reaction mixture containing CF_3dUR (0.4 mM) and methoxyamine (0.75M) was incubated at pH 7.5 and 37 °C. Aliquots of 20 μl were taken and injected onto a μBondapak C_{18} (3.9 × 300 mm) column at 0.8 h (a), 3.8 h (b), and 13.2 h (c) of the reaction

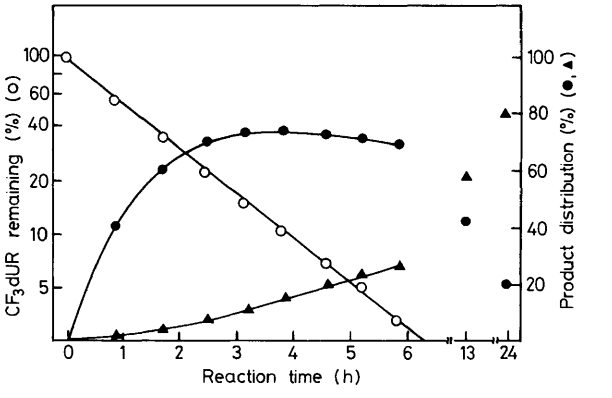


Figure 2. Course of the reaction of CF_3dUR (0.4 mM) with 0.75M methoxyamine at pH 7.5 and 37 °C. ○, ●, and ▲ represent the mole percent of CF_3dUR, (**4b**), and (**8b**), respectively, at each reaction time

Kinetic studies of the reaction of CF_3dUR with methoxyamine were carried out. H.p.l.c. separation and the use of molar absorption coefficients at pH 6.0 of CF_3dUR, (**4b**) and (**8b**) allowed the amounts of CF_3dUR, (**4b**), and (**8b**) in the reaction mixture to be measured. At all the pH values examined, CF_3dUR decreased with pseudo-first-order kinetics (see Figure 3). A typical course is shown in Figure 2; k_{obs} in this reaction was

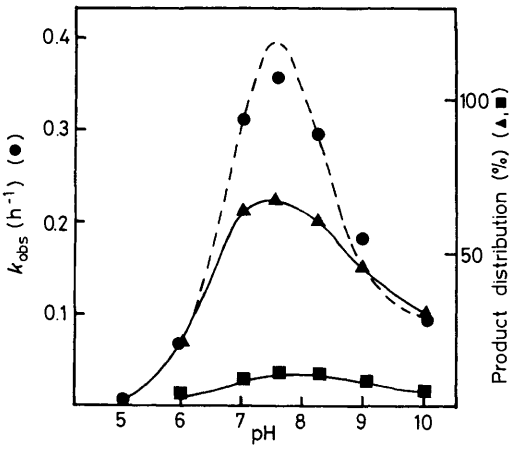


Figure 3. Dependence on pH of reaction velocity and product distribution. The reactions were done with 0.4 mM CF_3dUR and 0.5M methoxyamine at 37 °C. The calculation of pseudo-first-order rate constants (●) for the degradation of CF_3dUR and the quantification of the products were done by use of h.p.l.c. as described in the text. The percent yields are values found for the 4 h reactions: (▲) (**4b**); (■) (**8b**). Dashed line: calculated from equation (2)

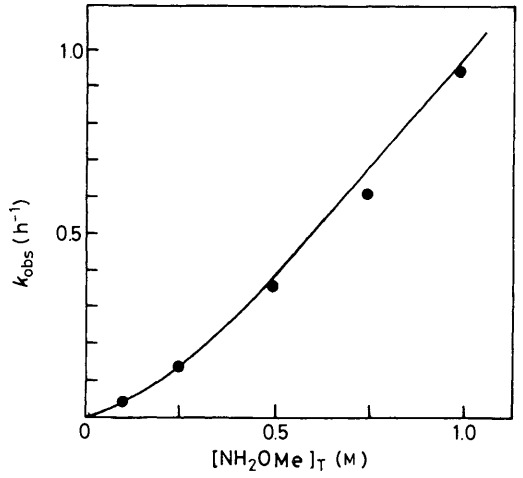
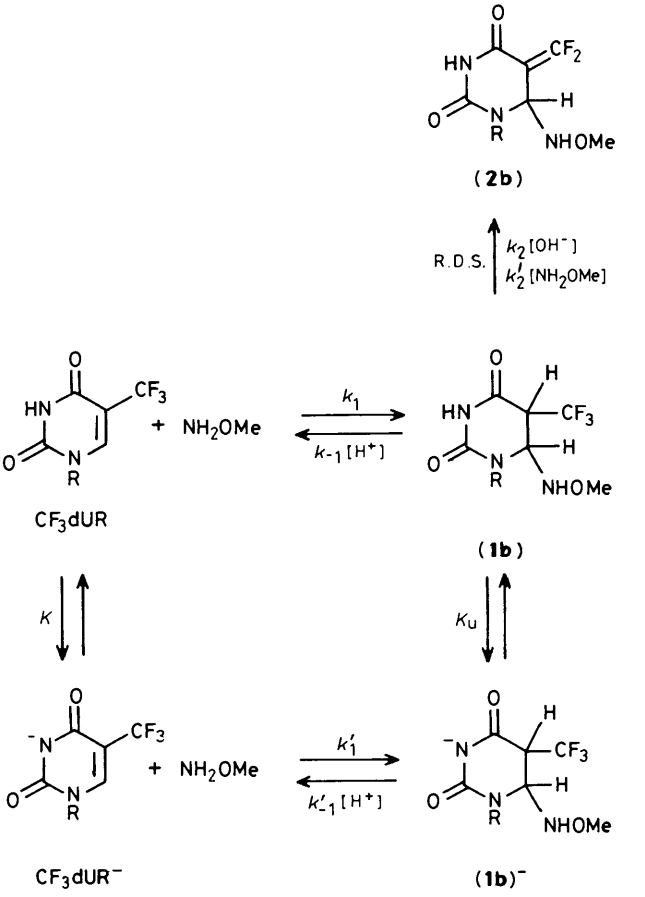


Figure 4. Pseudo-first-order rate constants of the reaction between CF_3dUR and methoxyamine as a function of methoxyamine concentration. The reactions were run with 0.4 mM CF_3dUR at pH 7.5 and 37 °C (●). Solid line: calculated from equation (2)

0.590 h^{-1}. Compound (**4b**) was the major product in the early stage, but after 24 h compound (**8b**) was predominant. The amount of (**4b**) was maximum at 3.8 h, when it corresponded to 73.3% of the total nucleoside.

It is clear that compound (**4b**) is an intermediate for the production of (**8b**). When the isolated (**4b**) (0.4 mM) was treated with 0.5M methoxyamine at pH 7.5 and 37 °C, the decrease in (**4b**) was associated with formation of the corresponding amount of (**8b**). The apparent first-order rate constant was 0.06 h^{-1}, about one-sixth that (0.335 h^{-1}) for the degradation of CF_3dUR under the same conditions. The effect of pH on the rate constants of the decrease in CF_3dUR is shown in Figure 3. The decrease in CF_3dUR was most rapid at pH 7.5; the rate decreased sharply at higher or lower pH. The accumulation of (**4b**) was optimum at pH 7.5, but the formation of (**8b**) was most favourable at around pH 8. The pK_a values of CF_3dUR and methoxyamine were 7.85 and 4.70, respectively. With the assumption that these two molecules react in their uncharged forms, one can expect that the optimum pH of the reaction fits into the equation, pH optimum = (pK_a CF_3dUR + pK_a NH_2OCH_3)/2. However, the observed optimum did not fit this simple equation.

The apparent rate constants for the decrease in CF_3dUR in the reactions at pH 7.5 at 37 °C were calculated as a function of the methoxyamine concentration. As Figure 4 shows, an increase in the concentration of methoxyamine resulted in a greater increase in the reaction rate than expected for a first-order reaction. This suggests that more than one molecule of methoxyamine participate in the reaction. We calculated the apparent second-order rate constant, k_{app}, from the data shown in Figure 4 by dividing each observed rate constant by the concentration of NH_2OMe: $k_{app} = k_{obs}/[NH_2OMe]_T$. The increase in k_{app} with increasing NH_2OMe concentration in the CF_3dUR degradation represents catalysis of the reaction by a second molecule of NH_2OMe. If k_{app} is plotted against the total concentration of NH_2OMe,[9] the ordinate intercept of the plot shows the rate constant k_{1app} (0.46M^{-1} h^{-1}), and the slope is the rate constant k_{2app} (0.45M^{-2} h^{-1}), according to the rate law of the following equation: $k_{obs} = k_{1app}[NH_2OMe]_T + k_{2app}[NH_2OMe]_T^2$. This equation fits the observed data shown in Figure 4. From the above observations, we propose that the mechanism of the reaction between CF_3dUR and methoxyamine is the multi-step reaction illustrated in Scheme 2. The



Scheme 2. R = 2-deoxy-β-D-ribofuranosyl R.D.S. = rate determining step

kinetic data can then be described by equation (1), which can be transformed to equation (2) when the reaction is pseudo-first-order with respect to CF_3dUR.

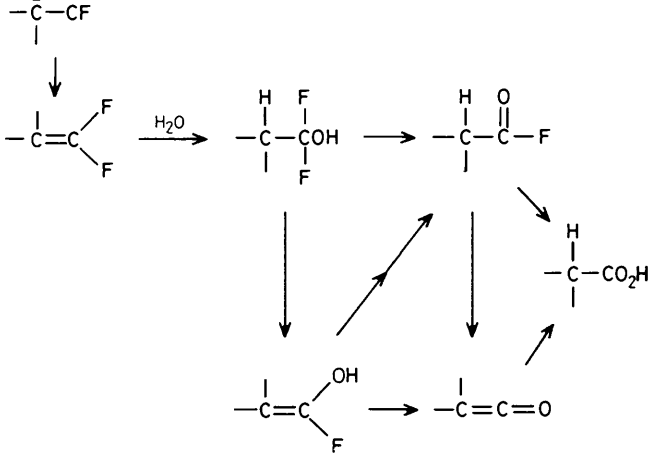
Here K and K_{ma} are dissociation constants for the NH at the 3-position of CF_3dUR (pK_a = 7.85) and NH_2OMe (pK_a =

$$v = k_1[CF_3dUR][NH_2OMe) - k_{-1}[(\mathbf{1b})][H^+] - k_2[(\mathbf{1b})][OH^-] - k'_2[(\mathbf{1b})][NH_2OMe] + k'_1[CF_3dUR^-][NH_2OMe] - k'_{-1}'[(\mathbf{1b})^-][H^+] \quad (1)$$

$$k_{obs} = \frac{\left(\frac{[H^+]}{K + [H^+]}k_1 + \frac{K}{K + [H^+]}k'_1\right)\left(\frac{K_{ma}}{K_{ma} + [H^+]}\right)k_2K_w[NH_2OMe]_T}{k_{-1}[H^+]^2 + k_2K_w + k'_2\frac{K_{ma}}{K_{ma} + [H^+]}[NH_2OMe]_T[H^+] + k'_{-1}K_u[H^+]} + \frac{\left(\frac{[H^+]}{K + [H^+]}k_1 + \frac{K}{K + [H^+]}K'_1\right)\left(\frac{K_{ma}}{K_{ma} + [H^+]}\right)^2 k'_2[H^+][NH_2OMe]_T^2}{k_{-1}[H^+]^2 + k_2K_w + k'_2\frac{K_{ma}}{K_{ma} + [H^+]}[NH_2OMe]_T[H^+] + k'_{-1}K_u[H^+]} \quad (2)$$

4.70), respectively. K_u, the dissociation constant of intermediate (**1b**) was assumed to be similar to the structurally analogous compound 1-methyl-5,6-dihydrouracil ($pK_a = 12$).[10] K_w Is the ion product of water. $[H^+]$ and $[OH^-]$ Are the concentrations of protons and hydroxide ions as measured by a glass electrode, and $[NH_2OMe]_T$ is the total concentration of methoxyamine present. We were not able to calculate the rate constants k_1, k_{-1}, k_2, k'_2, k'_1, and k'_{-1} directly from these experiments. However, by computer simulation, the most significant fit for k_{obs} was obtained and is shown in Figure 3 and in Figure 4. The best-fit pH profile was obtained when the rate constants were estimated as follows: K_1, 1.5; k_{-1}, 5 000; k_2, 0.01; k'_2, 0.001; k'_1, 0.18; and k'_{-1}, 1.2. The mechanistic interpretations of these data are discussed in the next section.

The facile reaction of CF_3dUR with methoxyamine to give compounds (**4b**) and (**8b**) was not unexpected, because 5-trifluoromethyluracil is known to react with glycine giving uracil-5-carboxamide as product.[2] In addition, hydrolysis of CF_3dUR and CF_3U in alkaline solution gives 5-carboxydeoxyuridine and 5-carboxyuracil, respectively.[6] In considering the mechanism of the CF_3dUR-methoxyamine reaction, it should be noted that most of the cleavages of C–F bonds in organic compounds can be explained by mechanisms analogous to that shown in Scheme 3;[11] the ability of the compound to form the difluoro-olefinic intermediate seems to be necessary for such reactions to occur. The mechanism by which the olefinic intermediates are transformed to products is believed to involve alternating addition of nucleophile to the intermediate and elimination of fluoride ions. A mechanism for hydrolysis of the trifluoromethyl group is proposed in Scheme 3, which assumes



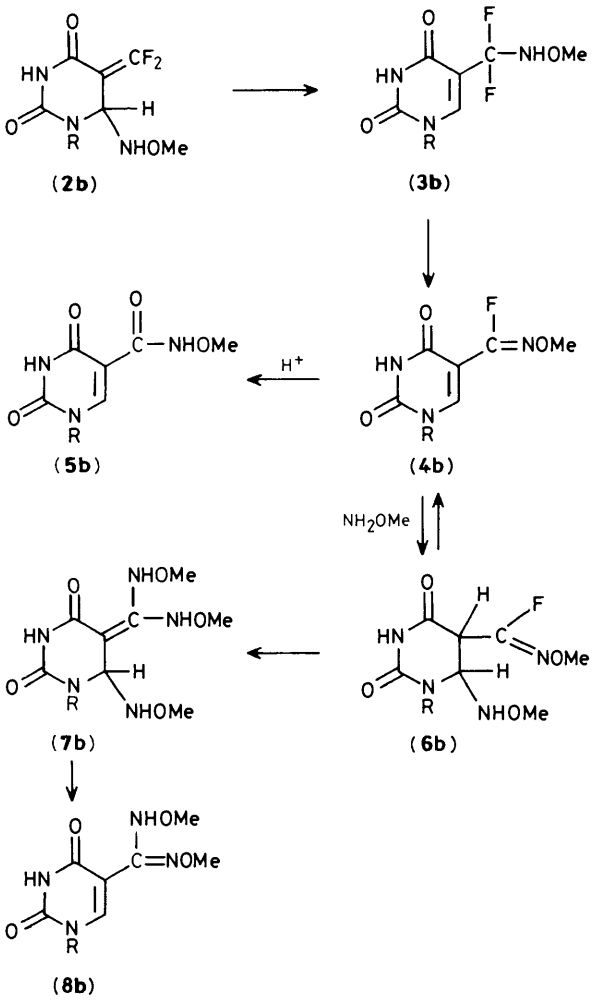
Scheme 3.

the intermediacy of acyl fluorides in the transformation of a trifluoromethyl group to a carboxylate function. The above provides insight into the possible mechanisms by which CF_3dUR reacts with methoxyamine as a nucleophile to form (**4b**) and (**8b**), and reveals evidence for the formation of reactive intermediates in the reaction.

Nucleophiles add across the 5,6-double bond of uracil to give 5,6-dihydro-6-substituted derivatives. Examples of such reactions are seen in the bisulphite addition to uracil to form 5,6-dihydrouracil-6-sulphonate,[12–14] the intramolecular nucleophilic attack of 5′-deoxy-5′-mercapto-2′,3′-isopropylideneuridine to give 6,5′-*S*-cyclo-5,6-dihydro-2′,3′-isopropylideneuridine[15] and the hydroxylamine addition to uridine to form 6-hydroxyamino-5,6-dihydrouridine.[16] These kinds of dihydrouracil derivatives all exhibit enhanced reactivity at the 5 position. The hydrogen exchange at position 5 of uridine catalyzed by bisulphite,[17] cysteine,[18,19] and cysteine methyl ester[20] are all believed to occur through such adduct formation. Further examples of the enhanced reactivity are seen in the rapid dehalogenation of 5-halogenouracil mediated by bisulphite, which also proceeds through the formation of the adduct 5,6-dihydro-5-halogenouracil-6-sulphonate,[21,22] and in the cysteine reaction with 5-bromodeoxyuridine to form deoxyuridine and *S*-5-(2′-deoxyuridyl)cysteine.[23,24] It is generally believed that methoxyamine can add at the 6-position of uracil forming 6-methoxyamino-5,6-dihydrouracil. We also observed that bisulphite readily reacts with CF_3dUR and isolable reaction products are formed (Wataya, unpublished results).

Figure 4 shows the dependence on the methoxyamine concentration of the pseudo-first-order rate constants for the reaction of methoxyamine with CF_3dUR at pH 7.5 and 37 °C. The reaction is greater than first order with respect to methoxyamine concentration. In the proposed mechanism for the reaction of CF_3dUR with methoxyamine (Scheme 2), the first step is an addition of methoxyamine at position 6 to form the intermediate (**1b**). Then, the release of hydrogen fluoride is catalyzed by hydroxide ion or methoxyamine, and it produces the exocyclic difluoromethylene intermediate (**2b**). From the data described in this paper, we suggest that this is the rate-determining step of the reaction. Addition of methoxyamine across the difluoromethylene bond followed by elimination of methoxyamine at the 6-position would yield (**3b**); with release of a fluoride ion and a proton, probably through successive addition-elimination reactions as described above, the stable intermediate (**4b**) is formed. A similar reaction cycle would convert (**4b**) into (**8b**) with intermediacy of (**6b**) and (**7b**) as depicted in Scheme 4.

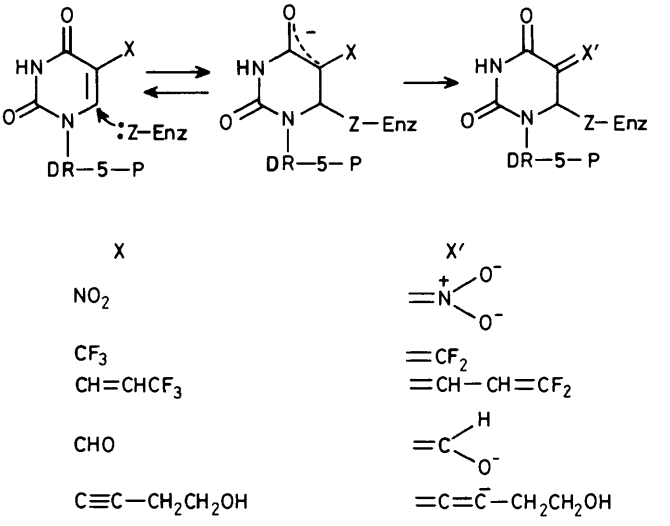
The results described here can be directly related to earlier observations on the interaction of CF_3dUMP and thymidylate synthetase.[8] CF_3dUMP can inactivate the thymidylate synthetase in the absence of the cofactor CH_2-FAH_4. The difference spectra of enzyme plus CF_3dUMP *versus* enzyme in the presence of dithiothreitol, when compared with the spectrum of CF_3dUMP, clearly demonstrated changes in the structure of the heterocycle of the inhibitor. With time, the difference spectra gradually changed. The intensity at the maximum of CF_3dUMP (261 nm) decreased, and a transient broad peak appeared which has absorbance up to 340 nm. After 1 h, the final



Scheme 4. R = 2-deoxy-β-D-ribofuranosyl

spectrum exhibited a peak at 276 nm. Two nucleotide products, A and B, of unknown structure were recovered from the reaction mixture. Product A was formed more rapidly than B. Product A was stable to aqueous base but was converted into B by acid. In turn, B was converted into 5-carboxy-dUMP by base (details will be published elsewhere). This clearly indicates that conversions take place at the 5-trifluoromethyl moiety of CF_3dUMP. The acid-lability of the product A is similar to that of products (**4a**) and (**4b**) obtained from the reaction with methoxyamine. We assumed that product A had one fluorine atom, because it was converted into the product B by treatment with acid, as observed with compounds (**4a**) and (**4b**). We would like to propose that the product B may be a thioester of dithiothreitol, because it was converted into 5-carboxy-dUMP by alkaline treatment. We suppose that a nucleophilic group of the enzyme is added to the 6-position of CF_3dUMP, promoting the explusion of fluoride ion; this would result in the formation of reactive exocyclic difluoromethylene intermediate. Subsequent addition of dithiothreitol across the difluromethylene bond, followed by elimination of the enzyme at 6-position would release the product A.

In our model in which methoxyamine was used as the nucleophile, the trifluoromethyl group was activated by addition of a nucleophile at the 6-position, and a difluoromethylene species was formed, with release of a fluoride ion. This provides support for mechanisms in which a nucleophilic group of the enzyme adds to the 6-position of CF_3dUMP, promoting the expulsion of fluoride ion, and thereby forming an inactive binary complex. In the binary complex, the difluoromethylene group may be stabilized due to the shielding by the enzyme. The inactivation of thymidylate synthetase by 5-nitro-2′-deoxyuridine 5′-phosphate (5-NO_2dUMP),[25–27] 5-formyl-2′-deoxyuridine 5′-phosphate (5-CHOdUMP),[28] *trans*-5-(3,3,3-trifluoropropenyl)-2′-deoxyuridine 5′-phosphate (5-CF_3HC=CHdUMP)[29] and 5-(4-hydroxybutyl)-2′-deoxyuridine 5′-phosphate (HOBdUMP)[30] has also been explained in terms of the formation of a covalent binary complex between the inhibitor and the enzyme. A generalized mechanism for the formation of such stable binary complexes is shown in Scheme 5.



Scheme 5. :Z-Enz = thymidylate synthetase and DR-5-P = phospho-2-deoxyribosyl

Experimental

CF_3dUR and CF_3U were purchased from Sigma. Methoxyamine hydrochloride was purchased from Eastman Kodak. [6-^{3}H]CF_3dUR was obtained from Moravek Biochemicals. ^{1}H (300 MHz) and ^{19}F (94 MHz) N.m.r. spectra were recorded on Bruker CXP-300 and JEOL-JNM-PS-100 n.m.r. spectrometers. Chemical shift values (δ) were recorded in [$^2H_6$]dimethyl sulphoxide ([$^2H_6$]-DMSO) or D_2O from an external standard in a capillary (TMS for ^{1}H and CF_3CO_2H for ^{19}F). Mass spectra were recorded on JEOL DX-300 and Varian MAT-731 instruments. Thin layer chromatography (t.l.c.) was done with silica gel 60F_{254} (Merck). 1-MCF_3U Was prepared from CF_3U as reported elsewhere.[31] Separation and quantification of nucleosides in the reaction mixtures were achieved by high performance liquid chromatography (h.p.l.c.). In h.p.l.c., a Waters 6000A pump equipped with a 440 absorbance detector (at 254 nm) and a Hewlett Packard 3390A integrator was used. Aliquots of reaction mixtures were injected onto a 3.9 × 300 mm μBondapak C_{18} column and eluted with 40 mM ammonium phosphate (pH 6.0) containing 24% methanol at a flow rate of 1 ml min^{-1} and at the ambient temperature. The methoxyamine solution was freshly prepared before each use.

Isolation of Reaction Products (**4a**) *and* (**8a**).—1-MCF_3U (10 mg) was incubated with 1M NH_2OMe (pH 8.3; 1.5 ml) at 90 °C for 45 min. After incubation, the reaction mixture (500 μl) was injected onto a μBondapak C_{18} column (Sumitomo, 8 × 250 mm) and the column was eluted with 10% methanol–water at the flow rate of 2 ml/min. The reaction products (**8a**) and (**4a**) were eluted at the retention time of 22 and 40 min,

respectively. 1-MCF_3U Was eluted at 28 min. The fractions containing (**8a**) and (**4a**) were collected and evaporated. During the evaporation, the compounds crystallized. Two-dimensional t.l.c. of (**4a**) and (**8a**) gave single spots [1st solvent, benzene–MeOH (3:1); and 2nd $CHCl_3$-EtOH (6:1)].

In the ^1H n.m.r., signals were observed that we interpreted in terms of the structures of (**4a**) and (**8a**); in D_2O, (**4a**) at δ (p.p.m.) 3.74 (s, 3 H, N-Me), 4.15 (s, 3 H, O-Me), 7.77 (s, 1 H, H, of the 6-position) and (**8a**) at 3.67 (s, 3 H, N-Me), 3.80 (s, 3 H, O-Me), 4.00 (s, 3 H, O-Me), 7.44 (s, 1 H, H of the 6-position); in [$^2H_6$]-DMSO, (**4a**) at 3.32 (s, 3 H, N-Me), 3.85 (s, 3 H, O-Me), 8.25 (d, *ca.* 1 Hz, H of the 6-position) and (**8a**) at 3.26 (s, 3 H, N-Me), 3.45 (s, 3 H, O-Me), 3.66 (s, 3 H, O-Me), 7.88 (s, 1 H, H of the 6-position). The 94 MHz ^{19}F n.m.r. spectrum of compound (**4a**) in [$^2H_6$]-DMSO containing D_2O showed a fluorine peak, which was 13.1 p.p.m. upfield of the signal from the external reference, trifluoroacetic acid. Chemical ionization mass spectra gave signals consistent with the structures; (**4a**) at *m/z* 202 (MH^+) and 152 ($MH^+ - 50$), and (**8a**) at *m/z* 229 (MH^+), 199 ($MH^+ - 30$), and 152 ($MH^+ - 77$).

Results in the high-resolution mass spectroscopy (electron impact ionization) were as follows; (**4a**) at observed *m/z* (composition, interpretation, calculated millimass) 201.055 472 ($C_7H_8N_3O_3F$, M^+, 201.054 969) and 151.037935 ($C_6H_5N_3O_2$, $M^+ - CH_3OF$, 151.038176) and (**8a**) at observed 228.085 002 ($C_8H_{12}N_4O_4$, M^+, 228.085 860), 198.075 929 ($C_7H_{10}N_4O_3$, $M^+ - HCHO$, 198.075 290), 151.038 426 ($C_6H_5N_3O_2$, $M^+ - (HCHO + CH_3ONH_2)$, 151.038 176). The u.v. spectrum of (**4a**) at pH 1 had maxima at 232 and 284 nm and a minimum at 254 nm, and (**8a**) at pH 1 had maxima at 218 and 267 nm and a minimum at 247 nm; (**4a**) (at pH 6) 233 and 285 nm (max.) and 255 nm (min.), (**8a**) (at pH 6) 216 and 263 nm (max.) and 247 nm (min.); (**4a**) (at pH 12) at 279 nm (max.), 233 nm (sh) and 259 nm (min.), (**8a**) (at pH 12) at 265 nm (sh).

Isolation of Compound (**5a**).—Compound (**4a**) was incubated with 0.1M HCl at 37 °C for 10 h, and after neutralization of the reaction mixture with conc. NH_4OH, the solution was put onto an h.p.l.c. column (8 × 250 mm μBondapak C_{18}; eluant, 9% methanol–water; flow rate, 3 ml/min). The reaction product (**5a**) was eluted at 15 min, while (**4a**) at 30 min. Compound (**5a**) was further purified by rechromatography. The ^1H n.m.r. spectrum of compound (**5a**) in [$^2H_6$]-DMSO containing D_2O showed signals at 3.33 p.p.m. (s, 3 H, N-Me), 3.82 (s, 3 H, O-Me) and 8.11 (s, 1 H, 6-H). Chemical ionization mass spectra of (**5a**) had signals at *m/z* 200 (MH^+) and 170 ($MH^+ - 30$), and fast-atom bombardment mass spectrum of (**5a**) had a signal at *m/z* 200 ($M^+ + 1$) (calc. for $C_7H_9N_3O_4$, 199). Characteristics of the u.v. spectra were as follows: at pH 1, 233 and 281 nm (max.) and 247 nm (min.); at pH 6, 223 and 281 (max.) and 247 (min.); at pH 12, 268 (max.) and 252 (min.).

Products in the Reaction of CF_3dUR *with Methoxyamine.*—CF_3dUR (44 mg) Was incubated with 1.0M NH_2OCH_3 (pH 7.1; 2 ml) at 37 °C for 24 h. After the incubation, the reaction mixture (500 μl) was subjected to h.p.l.c. as described above for the 1-MCF_3U reaction. The products (**8b**) and (**4b**) were eluted at 24 and 62 min, respectively, and CF_3dUR at 42 min. The fractions of (**4b**) and (**8b**) were collected, evaporated, and further purifed by rechromatography in the same system. The purified products gave single spots on two-dimensional t.l.c. (see above for the solvents). N.m.r. data were as follows: ^1H ([$^2H_6$]-DMSO containing 40% D_2O), (**4b**) at δ 2.26 (m, 2 H, 2′-H), 3.70 (octet, 2 H, 5′-H), 3.88 (s, 3 H, O-Me), 3.93 (m, 1 H, 4′-H), 4.33 (m, 1 H, 3′-H), 6.16 (t, 1 H, 1-H), and 8.58 (d, *ca.* 1 Hz, 1 H, 6-H); (**8b**) at 2.27 (m, 2 H, 2′-H), 3.38 (s, 3 H, O-Me), 3.62 (s, 3 H, O-Me), 3.64 (octet, 2 H, 5′-H), 3.88 (m, 1 H, 4′-H), 4.28 (m, 1 H, 3′-H) 6.08 (t, 1 H, 1′-H), and 8.01 (s, 1 H, 6-H). The 94 MHz ^{19}F n.m.r. spectrum of compound (**4b**) in [$^2H_6$]-DMSO containing D_2O had a fluorine peak 13.2 p.p.m. upfield from the external trifluoroacetic acid. Fast-atom bombardment mass spectra had signals at *m/z* 304 ($M^+ + 1$) for (**4b**) (calc. for $C_{11}H_{14}N_3O_6F$, 303) and at *m/z* 331 ($M^+ + 1$) for (**8b**) (calc. for $C_{12}H_{18}N_4O_7$, 330). Data for u.v. spectra were as follows: (**4b**) (at pH 1) at 233 and 285 nm (max.) and 256 nm (min.), (**8b**) (at pH 1) at 218 and 267 nm (max.) and 246 (min.); (**4b**) (at pH 6) at 233 and 285 nm (max.) and 255 nm (min.), (**8b**) (at pH 6) at 216 and 263 nm (max.) and 247 nm (min.); (**4b**) (at pH 12) at 279 nm (max.) and 259 (min.), (**8b**) (at pH 12) at 265 (sh).

Calculation of Molar Absorption Coefficients of Products (**4b**) *and* (**8b**).—CF_3dUR (2.7 mg) Containing [6-^3H]CF_3dUR (4 μCi) was incubated with 0.5M NH_2OMe (pH 7.3; 0.5 ml) at 37 °C for 40 h. After the incubation, the reaction mixture was subjected to h.p.l.c. as described above. The fractions for CF_3dUR, (**4b**) and (**8b**) were individually collected and evaporated. The residues were dissolved in 0.1M potassium phosphate buffer (pH 6.0). The u.v. spectra were made and the radioactivities were counted. The radioactivity per mole of each nucleoside should be the same, and the molar absorption coefficient (M^{-1} cm^{-1}) of CF_3dUR was 9 960 at 262 nm and 8 380 at 254 nm (measured in a separate experiment). So, the molar absorption coefficients of product (**8b**) at 263 and 254 nm were calculated to be 6 460 and 6 070, and those of (**4b**) at 285 and 254 nm were 8 580 and 3 970, respectively.

Velocity of Reaction between CF_3dUR *and Methoxyamine.*—The reaction mixture (10 ml) consisted of 0.4 mM CF_3dUR and 0.5M methoxyamine. Methoxyamine solutions of various pH values were prepared by the addition of 10M NaOH to a stock methoxyamine solution. The reaction mixture was incubated at 37 °C. Portions (20 μl) were withdrawn at desired intervals and directly injected onto a μBondapak C_{18} column (Waters, 3.9 × 300 mm). The eluant was 24% (v/v) methanol containing 40 mM $(NH_4)_2HPO_4$ (pH 6.0). A flow of 1.0 ml/min was maintained throughout the run, which was at the ambient temperature. Absorption was measured at 254 nm with a sensitivity of 0.2 a.u.f.s. Mole percents were calculated based on the molar absorption coefficients at pH 6.0 of CF_3dUR, (**4b**), and (**8b**). CF_3dUR was eluted at the retention time of 22.0 min, and (**4b**) and (**8b**) were eluted at 33.6 and 11.6 min, respectively. The salt concentrations of the reaction mixtures were adjusted to 2.0M by addition of KCl.

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