Structure of 3-Acetyl-5-fluorouracil (5-FU): Implication for Its Rearrangements During Hydrolysis and upon Heating

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Single-crystal X-ray diffraction data show that the 3-acetyl group in 1,3-diacetyl-5-FU (FU = fluorouracil) is perpendicular to the plane of the 5-FU ring, while the 1-acetyl group is coplanar with the ring. Analyses of ^{1}H NMR and IR spectra provide evidence that the 1- and 3-acyl groups are in different electronic environments, which is consistent with the X-ray diffraction structure. 3-Acetyl-5-FU is thermally unstable, giving mainly 1-acetyl-5-FU (80%) and 5-FU (20%) upon heating. The hydrolysis of 3-acyl derivatives of 5-FU showed a biexponential relationship between ln concentration and time which had not been previously observed. The behavior of 3-acetyl-5-FU during hydrolysis can be explained by postulating its initial rapid equilibrium with an intermediate, 2-acetyl-5-FU, which subsequently hydrolyzes to 5-FU or rearranges to 1-acetyl-5-FU, which hydrolyzes to 5-FU. The 2-acetyl intermediate was trapped by its reaction with formaldehyde. The formaldehyde adducts of the symmetrical 2-acetyl intermediate rearranged to yield equal amounts of 1- and 3-acetyloxymethyl-5-FU.

KEY WORDS: 5-fluorouracil (5-FU); 3-acyl-5-FU; 1,3-diacyl-5-FU; thermal rearrangement; hydrolyses; 1- and 3-acetyloxymethyl-5-FU; X-ray crystal structure.

INTRODUCTION

5-Fluorouracil (5-FU) is one of the most widely used systemic antitumor agents presently available (1). It is also useful in the topical treatment of basal cell carcinomas and actinic keratoses (2). However, the effective oral and topical use of 5-FU is limited by its highly variable bioavailability in the former case and its low lipophilicity in the latter case. Attempts to overcome the low lipophilicity of 5-FU have focused on the development of various transient chemical modifications of 5-FU known as prodrugs (3). The numerous different types of more lipophilic prodrugs of 5-FU that have been reported generally can be classified as either alkyl or acyl derivatives (4–9). Buur and Bundgaard have systematically evaluated the physiochemical properties of the various possible *N*-acyl types of derivatives. These include series of *N*-alkylcarbonyl- (4), *N*-alkyloxycarbonyl- (5,8), and *N*-alkylaminocarbonyl-5-FU (7) derivatives. Since acylation can occur at two different sites in 5-FU, 1- or 3-acyl and 1,3-diacyl derivatives, where possible, in each series were synthesized, characterized, and compared.

One unexpected feature of the results reported by Buur and Bundgaard was the finding that the 3-acyl derivatives were generally more hydrolytically stable than the 1-acyl derivatives. For instance, 1-acetyl-5-FU was reported to exhibit a $t_{1/2}$ of 6.9 min at 37°C and pH 7.4 (extrapolated to zero buffer concentration), whereas 3-acetyl-5-FU was reported to exhibit a $t_{1/2}$ of 43 min under the same conditions (4). Similarly, 1-ethyloxycarbonyl-5-FU exhibited a $t_{1/2}$ of 550 min at 37°C and pH 7.4 (8), whereas 3-ethyloxycarbonyl-5-FU exhibited no detectable hydrolysis after treatment with 0.5 *M* NaOH at 37°C for 4 hr under conditions where 1% 5-FU could have been detected (5). This difference in stabilities was attributed to the assumed difference in the leaving group ability of the respective 5-FU anions (4) based on the reported values for the pK_a of N^1-H (pK_a = 8.0) and N^3-H (pK_a = 13) in 5-FU (10). However, this conclusion is not supported by the fact that the reported pK_a of the N^3-H group in the 1-alkyloxycarbonyl series was about 6.8 (8), whereas that of the N^1-H group in the 3-alkyloxycarbonyl series was about 8.6 (5). The same relative result was observed for the two corresponding series of alkyl derivatives: the 1- and 3-acyloxymethyl-5-FU series. For example, the N^3-H group in 1-ethyloxycarbonyloxymethyl-5-FU exhibited a pK_a of 7.3 (9), whereas the N^1-H group for the corresponding 3-derivative exhibited a pK_a of 7.9 (9). Similarly, the N^3-H group in 1-acetyloxymethyl-5-FU exhibited a pK_a of 7.3 (6), whereas the N^1-H group in 3-acetyloxymethyl-5-FU exhibited a pK_a of 8.0 (6). Thus, for both *N*-acyl and *N*-acyloxymethyl derivatives of 5-FU, the N^3-anion is more stable than the N^1-anion and should be the better leaving group in their respective hydrolyses, and not vice versa.

In order to determine the basis for the above dichotomy, the physicochemical properties of selected members of one of the types of *N*-acyl derivatives have been reexamined. In this paper spectroscopic and thermal and kinetic stability data for selected 3-acyl (1 and 2)- and 1,3-diacyl-5-FU derivatives are reported as well as single-crystal X-ray diffraction data for the 1-acetyl (3) and 1,3-diacetyl (4) derivatives of 5-FU. Kinetic data are presented for the hydrolyses of the 3-acetyl derivative in the presence and absence of formaldehyde used as a trapping agent.

MATERIALS AND METHODS

Kinetic Studies

The degradation of 3-acetyl-5-FU (1) and 3-propionyl-5-FU (2) were studied in phosphate buffer (pH 7.1, 0.05 *M*, I = 0.12 *M*) at 32°C. The initial substrate concentration was 1.8×10^{-4} *M*. Hydrolyses of 1 and 2 were followed by ultraviolet (UV) spectrophotometry (Cary 210; λ_{anal} = 300 nm, 1-cm pathlength). Reaction was initiated by adding 60 μL of a stock solution of substrate in acetonitrile to 3.0 mL of

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prewarmed buffer in a thermostated cuvette. Results were obtained as (absorbance at time = t) − (absorbance at time = infinity), which were then converted to substrate concentrations at t (A_t).

In each case a plot of $\ln A_t$ versus time showed a terminal linear phase for the plot, from which k_{term} was calculated. The molar concentration of 3-acetyl-5-FU (A) was then corrected for equilibrium with an intermediate (B) using Eq. (1) (11) (see Scheme I, below).

$$\ln A_{corr} = \ln A_t + k_{term}t \quad (1)$$

Typical plots of $\ln A_t$ and the corresponding $\ln A_{corr}$ versus time are shown in Fig. 4. The average of the last six data points for $\ln A_{corr}$ versus time gave A_{eq}, and K_{eq} was then calculated from $K_{eq} = B_{eq}/A_{eq}$, where $B_{eq} = A_o - A_{eq}$. At equilibrium it follows that (12)

$$\log (A_o - A_{eq})/(A_t - A_{eq}) = (k_f + k_r)\, t/2.303 \quad (2)$$

The slope of the initial data points for the left-hand side of Eq. (2) versus time forced through zero gave $(k_f + k_r)/2.303$. Since $K_{eq} = k_f/k_r$ and $k_r = 2.303$ (slope)/$(K_{eq} + 1)$, then $k_f = K_{eq}k_r$ (12). In this way the various K_{eq}, k_{term}, k_f, and k_r given in Table I were calculated.

Hydrolysis of 1 was also followed by high-performance liquid chromatography (Beckman 110A pump; Rheodyne 7125 20-μL loop injector; Lichrosorb 250 × 4.6-mm, 10-μm RP-8 column; mobile phase, 10% methanol, 90% acetate buffer, pH 5.0; flow rate, 1.0 mL/min; Beckman Model 153 fixed-wavelength detector, λ_{anal} = 254 nm; Hewlett-Packard 3392A integrator) in the presence or absence of HCHO at varying concentrations. The following retention times were found: 5-FU, 4.5 min; 1, 9.1 min; 1-acetyloxymethyl-5-FU, 11.4 min; 1-acetyl-5-FU, 13.1 min; and 3-acetyloxymethyl-5-FU, 15.7 min. The reaction was initiated at time 0 by diluting 0.4 mL of a stock solution of 1 in acetonitrile with 25 mL of the prewarmed buffer (containing HCHO if required). The solution was stored at 32°C and aliquots were chromatographed periodically. Values for pH were checked after runs and were found to be unchanged. All experiments were performed at least in duplicate. Calculations for rate and equilibrium constant estimations were performed with Excel Version 3.0 on a Macintosh SE microcomputer.

X-Ray Crystallography

The single-crystal X-ray analyses were determined from intensity data measured on an Enraf-Nonius CAD4 diffractometer (graphite-monochromated CuK_α radiation, $\omega - 2\theta$ scans). The structures were solved by a multiple-solution procedure (13) and were refined by full-matrix least squares. In the final refinement, the nonhydrogen atoms were refined anisotropically. The hydrogen atoms were included in the structure-factor calculations, but their parameters were not refined. Tables of final atomic parameters, anisotropic thermal parameters, bond distances, and bond angles have been deposited with the Crystallographic Data Centre, Cambridge, CB2 1EW England.

For 1-acetyl-5-FU the unit cell contained two independent molecules, i.e., not related by symmetry, but the essential features of their structures were identical. The size of the crystal used for data collection was approximately 0.04 × 0.07 × 0.55 mm. The crystal system was monclinic; space group $P2_1/c$; a = 6.308(2) Å, b = 16.824(1) Å, c = 13.239(1) Å, β = 93.42(2)°, Z = 8, d_{calc} = 1.630 g cm^{-3}; $\mu(CuK_\alpha)$ = 12.7 cm^{-1}. The data were not corrected for absorption. Of the 2073 independent reflections for $\theta < 60°$, 1207 were considered observed [$I > 3.0\ \sigma\ (I)$]. Eight reflections, which were strongly affected by extinction, were excluded from the final refinement and the difference map. The final discrepancy indices were R = 0.042 and wR = 0.048 for 1199 observed reflections. The final difference map had no peaks greater than ±0.2 eÅ^{-3}.

For 1,3-diacetyl-5-FU the size of the crystal was approximately 0.07 × 0.09 × 0.50 mm. The crystal system was tetragonal; space group $P4_1$; $a = b$ = 8.141 (2) Å, c = 13.776 (13) Å, Z = 4, d_{calc} = 1.558 g cm^{-3}; $\mu\,(CuK_\alpha)$ = 11.8 cm^{-1}. The data were not corrected for absorption. Of the 982 independent reflections for $\theta < 75°$, 815 were considered observed [$I > 3.0\ \sigma\ (I)$]. Five reflections, which were strongly affected by extinction, were excluded from the final refinement and the difference map. The final discrepancy indices were R = 0.042 and wR = 0.051 for the 810 observed reflections. The final difference map had no peaks greater than ±0.2 eÅ^{-3}.

Spectroscopy

The ^{1}H NMR spectra were run on a Varian EM390 90-MHz spectrometer and the IR spectra were run on a Perkin-Elmer 1420 ratio recording spectrometer.

Thermal Rearrangement

Melting points were determined in a Mel-Temp capillary melting point apparatus and are uncorrected. Differential

Table I. Rate and Equilibrium Constants for the Reversible Hydrolysis of 3-Acyl-5-fluorouracil Derivatives (Phosphate Buffer, 0.05 M, pH 7.1, I = 0.12 M, 32°C) in the Presence or Absence of Formaldehyde (Mean ± Range Except Where Noted)

Compound	[HCHO]	$10^3 \cdot k_{term}$ (min^{-1})	$10^3 \cdot k_f$ (min^{-1})	$10^3 \cdot k_r$ (min^{-1})	K_{eq}
1. 3-Acetyl (UV)	0.00	3.25 (±0.25)	5.01 (±0.06)	3.9 (±0.3)	1.3 (±0.2)
2. 3-Acetyl (HPLC)	0.00	—[a]	5.45 (±0.25)	3.6 (±0.3)	1.50 (±0.05)
3. 3-Propionyl (UV)	0.00	2.29 (±0.05)	5.77 (±0.01)	13.1 (±0.5)	0.440 (±0.018)
4. 3-Acetyl (HPLC)	0.0036		7.19 (±0.14)[b]		
5. 3-Acetyl (HPLC)	0.036		7.00 (±0.28)[b]		

[a] k_{term} taken from UV data.
[b] Monophasic degradation. Errors given are 3 × SD.

scanning calorimetry was done with a Perkin-Elmer DSC-7 and thermogravimetric analyses were performed under nitrogen at atmospheric pressure with a Perkin-Elmer TGA-7. TLC were run on Brinkman Polygram Sil G/UV 254 plates.

RESULTS AND DISCUSSION

Synthesis

The various *N*-acyl derivatives of 5-FU were synthesized by modifications of literature procedures (14) and have been reported previously (15).

Single-Crystal X-Ray Diffraction

The single-crystal X-ray diffraction data (see Fig. 1) of 1-acetyl (3)- and 1,3-diacetyl-5-FU (4) are the most important pieces of information available for explaining the dichotomy between the relative rates of hydrolysis of the N^1-acyl and N^3-acyl derivatives, on one hand, and the apparent pK_a values of the N^1-H and N^3-H groups, on the other hand. The salient feature of the X-ray diffraction structures of 1,3-diacetyl-5-FU and 1-acetyl-5-FU is that the 3-acetyl group is perpendicular to the plane of the 5-FU ring (the torsional angle $C^4-N^3-C^{13}=O^{13}$ is 90.6°), whereas the 1-acetyl group in both molecules is essentially coplanar with the 5-FU ring with the $C^{11}=O^{11}$ carbonyl group orientated toward the C^6-H group (the torsional angle $C^6-N^1-C^{11}=O^{11}$ is −8.8°) (Fig. 1). It was not possible to obtain well-formed crystals of 3-acetyl-5-FU. In addition, 3-acetyl-5-FU was thermally unstable even at room temperature in a desiccator, where it decomposed completely in a matter of 3–4 months to give primarily 1-acetyl-5-FU and 5-FU (see below). However, since the ^1H NMR and IR spectra of 1,3-diacetyl-5-FU (see below) are essentially a composite of the spectra of 1- and 3-acetyl-5-FU, there is no reason not to believe that the acetyl group in 3-acetyl-5-FU is also perpendicular to the plane of the 5-FU ring.

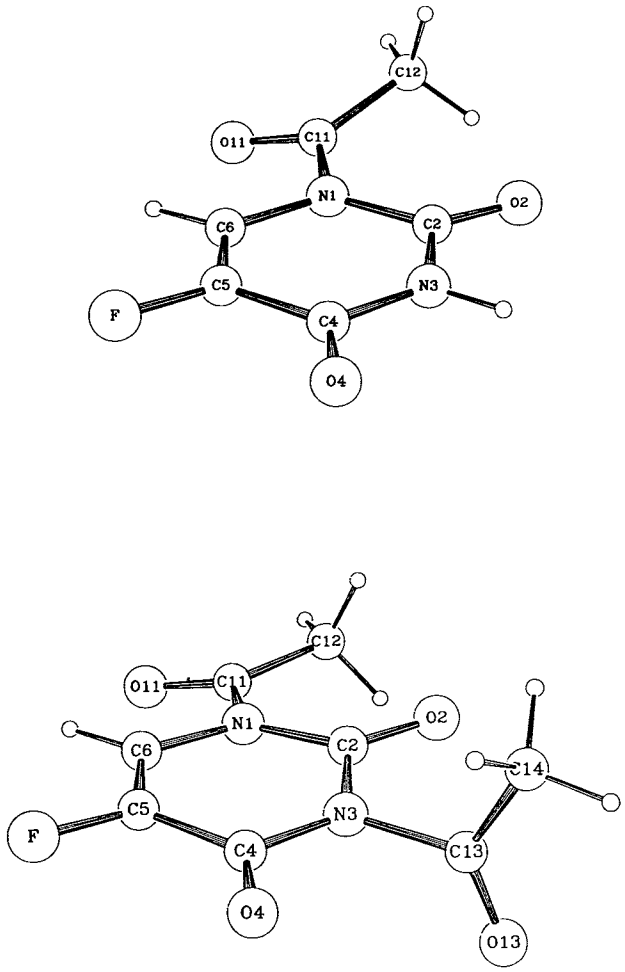

Fig. 1. Single-crystal X-ray diffraction structures of 1-acetyl-5-FU and 1,3-diacetyl-5-FU.

The most reasonable explanation for the fact that the 3-acetyl group in 5-FU is perpendicular to the plane of the 5-FU ring is an electronic repulsion effect. The two unshared pairs of electrons on each of the carbonyl oxygens ($C^2=O^2$ and $C^4=O^4$) in 5-FU are coplanar with the plane of the 5-FU ring and one of the unshared pair of electrons on each carbonyl oxygen would occupy the same space as (a) one of the unshared pair of electrons on the 3-acetyl carbonyl oxygen ($C^{13}=O^{13}$) or (b) one of the C^{14} hydrogens, if the 3-acetyl group were coplanar. On the other hand, the electronic repulsion effect for the 1-acetyl derivative can be minimized by orientating the acetyl $C^{11}=O^{11}$ group away from the one adjacent ring carbonyl group ($C^2=O^2$). An interaction between the unshared pair of electrons on O^2 and the C^{12} hydrogens is still likely. However, as shown in Fig. 1, those hydrogens in the crystals of 1-acetyl- or 1,3-diacetyl-5-FU can be rotated so as to be on either side of the unshared pair of electrons on the O^2 oxygen. Thus, the 1-acetyl group can still be reasonably coplanar with the 5-FU ring and its carbonyl group conjugated with the ring as well.

The different orientation of 1- and 3-acetyl groups to the plane of the 5-FU ring should have an effect on the accessibility of their partially positive carbonyl carbons (C^{11} and C^{13}) to nucleophiles such as hydroxide and hence on their rates of hydrolysis. This assumes that the positive charges on the two carbonyl carbons are approximately the same, which seems reasonable, since the N^1-C^{11} and N^3-C^{13} bond lengths are similar—1.459 and 1.487 Å, respectively—in 1,3-diacetyl-5-FU, and the $C^{11}=O^{11}$ and $C^{13}=O^{13}$ bond lengths are identical—1.195 and 1.193 Å, respectively. The C^{13} is much less accessible because of the electronic repulsion effect of the unshared pair of electrons on the flanking $C^2=O^2$ and $C^4=O^4$ groups on electron-dense nucleophiles which have to approach at an angle of about 109° from above (or below) the plane of the $C^{13}=O^{13}$ group (i.e., slightly behind C^{13}) (16). On the other hand, C^{11} in the 1-acetyl group is more easily accessible to nucleophilic attack based on the same considerations. The result is that the $t_{1/2}$ for 3-acetyl-5-FU is about 210 min (k_{term}, Table I), while the $t_{1/2}$ for 1-acetyl-5-FU is about 4.8 min (15). Thus, regardless of the relative leaving group abilities of the N^1- or N^3-anions, the rates of hydrolyses will be proportional to the accessibility of the respective acetyl groups by nucleophiles such as hydroxide ion and hence to differences in their conformations. This phenomenon appears to be an example of stereoelectronic control (16).

^{1}H NMR Spectra

The ^{1}H NMR spectra show that the absorptions due to $-CH_2-C=O$ or $CH_3-C=O$ in the 1-acyl series of derivatives appear downfield or are deshielded compared to the same CH_2 or CH_3 absorptions in the 3-acyl series. For example, the absorption due to CH_3 in 3-acetyl-5-FU appears at δ 2.58, whereas that in 1-acetyl-5-FU appears at δ 2.73. Similarly, the absorption due to CH_2 in 3-propionyl-5-FU appears at δ 2.86, whereas that in 1-propionyl-5-FU appears at δ 3.14. The relative positions of these absorptions are consistent throughout the remaining members of both series of derivatives. Also, the corresponding absorptions due to $-CH_2-C=O$ or $CH_3-C=O$ in the 1,3-diacyl-5-FU series appear at approximately the same position as those in the 1- and 3-acyl series. Thus, the absorptions due to CH_3 in 1,3-diacetyl-5-FU appear at δ 2.72 and δ 2.58, respectively, and those due to CH_2 in 1,3-dipropionyl-5-FU appear at δ 3.11 and δ 2.85. The nearly identical positions of the $-CH_2-C=O$ and $CH_3-C=O$ absorptions in the 1- and 3-acyl derivatives compared to those of the 1,3-diacyl derivatives support the conclusion that, in solution, the acetyl group in 3-acetyl 5-FU is in the same electronic environment as it is in 1,3-diacetyl-5-FU.

The positions of the $-CH_2-C=O$ and $CH_3-C=O$ absorptions are consistent with the fact that the 3-acyl group is attached to a group capable of stabilizing a negative charge. The N^3-anion of 5-FU is highly stabilized by resonance. Thus, the positions of the $-CH_2-C=O$ and $CH_3-C=O$ absorptions in the 3-acyl derivatives are comparable to those observed for acid chlorides (δ 2.88 for propionyl chloride and 2.67 for acetyl chloride) (17). On the other hand, the $-CH_2-C=O$ and $CH_3-C=O$ absorptions for the 1-acyl derivatives are shifted even farther downfield compared to acid chlorides because of their deshielding by the $C^2=O^2$ group (see Fig. 1) (18). The deshielding effect of a carbonyl group is known to cause as much as a 0.5-ppm shift downfield so the observed shift of about 0.2 ppm is not unusual.

IR Spectra

The relative positions of the infrared absorptions due to the $C^{11}=O^{11}$ and $C^{13}=O^{13}$ groups are also consistent with the fact that the 3-acyl group is perpendicular to the plane of the 5-FU ring, whereas the 1-acyl group is coplanar and conjugated with the 5-FU ring. The carbonyl absorptions at 1805 cm^{-1} for the 3-acetyl and at 1815 cm^{-1} for the 3-propionyl groups are clearly separated from the broad absorptions due to the heterocyclic ring system which ends at about 1750 cm^{-1}. The positions of these absorptions are similar to those observed for acid chlorides (18) and are consistent with the inductive effect of the 5-FU ring being similar to that of chloride. On the other hand, the absorptions due to the 1-acyl carbonyl group are buried within the broad absorption due to the heterocyclic ring except for those due to the 1-acetyl group in 1,3-diacetyl-5-FU (1750 cm^{-1}). The conjugation of the 5-FU ring with the 1-acyl carbonyl group leads to a decrease in the polarity of the carbonyl bond and hence in the vibrational stretching energy (18). This is consistent with the observed decrease in the stretching frequency for conjugated carbonyl functional groups in other systems.

Thermal Rearrangement of 3-Acetyl-5-FU

The observed melting points of all the 3-acyl derivatives were dependent on the rate of heating. For example, differential scanning calorimetry (DSC) analysis of 3-acetyl-5-FU samples showed that an endotherm occurred with a peak at 102°C when the heating rate was 2°C/min, at 114°C when the rate was 5°C/min, and at 126°C when the rate was 20°C/min. The 3-acyl derivatives also appear to rearrange and lose mass upon heating. For example, careful heating of 3-acetyl-5-FU at approximately 5°C/min in a capillary tube resulted in melting of the sample at about 118–122°C, almost immediate resolidification and remelting at 160–170°C. When the sample was cooled and reheated, most of the sample melted at 132°C—the capillary melting point of 1-acetyl-5-FU. Thermogravimetric analysis (TGA) of samples of 3-acetyl-5-FU showed onset of mass loss at 164°C and 71.6% mass loss by 188°C, at which point an abrupt decrease in the rate of mass loss occurred.

The thermal rearrangement of 3-acetyl-5-FU was also examined on a larger scale (15–50 mg). In experiment (a), 3-acetyl-5-FU was heated in a glass container until a clear liquid was obtained. After the residue had cooled, TLC (15) and ^{1}H NMR analysis identified the products as 1-acetyl-5-FU (80%) and 5-FU (20%) with only a trace of 1,3-diacetyl-5-FU based on the positions and intensities of the C^6-H and $CH_3-C=O$ absorptions. In two other experiments, 1,3-diacetyl-5-FU alone (b) or an equimolar mixture of 1,3-diacetyl-5-FU and 5-FU (c) was heated in ethyl acetate until the ethyl acetate evaporated and the solid residues had melted. In the former experiment, 61% of 1,3-diacetyl-5-FU remained, while 39% of the sample was converted to 1-acetyl-5-FU, presumably by the loss of the 3-acetyl group as ketene (Fig. 2). In the latter experiment, 76% of 1-acetyl-5-FU, 20% of 5-FU, and 4% of 1,3-diacetyl-5-FU were obtained. The product distribution was based on the positions and intensities of the C^6-H and $CH_3-C=O$ absorptions. In all of these experiments some overall loss of acetyl groups was observed.

Thus, the thermal rearrangement can take place intermolecularly since some 1,3-diacetyl-5-FU was observed in

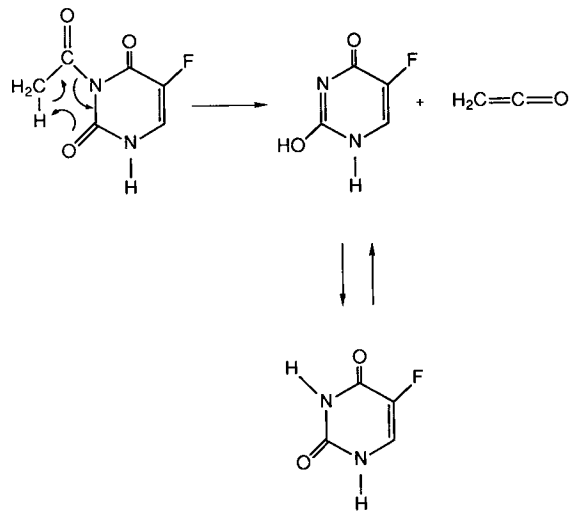

Fig. 2. Thermal decomposition of 3-acetyl-5-FU to 5-FU.

experiment (a) and greater than 50% of 1-acetyl-5-FU was observed in experiment (c). However, the experiments that were run cannot exclude the possibility of an intramolecular rearrangement taking place concurrently through a transient O^2-acyl intermediate (see below). Regardless of the mechanism, the driving force for the thermal rearrangement is probably the stabilization of the acyl group in the 1-position by conjugation of its carbonyl group with the π system of the heterocyclic ring. No 3-acetyl-5-FU was observed when 1-acetyl-5-FU was heated under similar conditions.

The existence of intramolecular 1,3-acyl migrations of both N to O and O to N has been well documented (19). Of the several possible mechanisms for thermal rearrangement, the synchronous mechanism depicted in Fig. 3 is preferred (20). The perpendicular orientation of the N^3-acetyl group would facilitate such a rearrangement. All steps in Fig. 3 are shown as reversible since acyl or aroyl rearrangements are potentially reversible, regardless of whether they are intramolecular or intermolecular (21–23). However, the fact that no 3-acetyl-5-FU was observed on heating 1-acetyl-5-FU suggests that the final equilibrium is greatly in favor of the latter. It is also worth noting that melting behavior similar to that seen for the 3-acyl derivatives in this study was also observed for O to N benzoyl migration in 6-phenanthridinone (23).

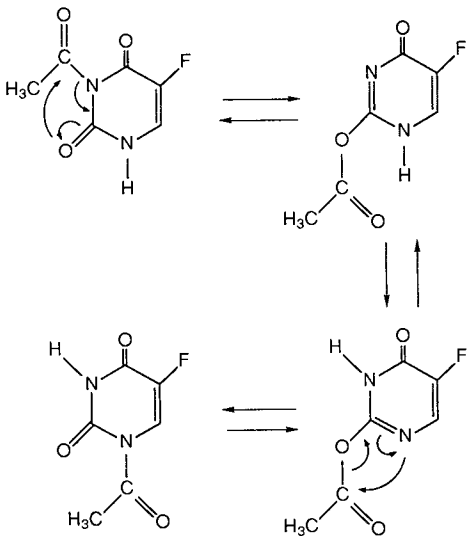

Fig. 3. Thermal intramolecular rearrangement of 3-acetyl-5-FU to 1-acetyl-5-FU.

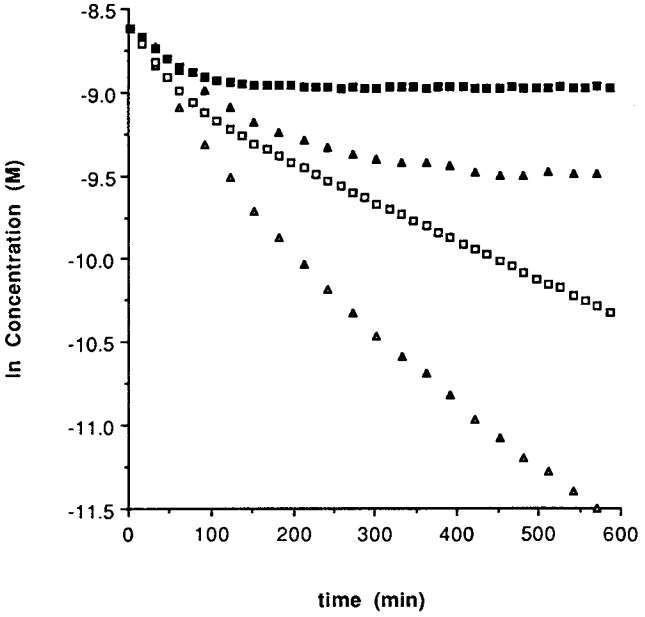

Fig. 4. Plots of ln concentration as a function of time (min) for 3-acetyl-5-FU (1; triangles) and 3-propionyl-5-FU (2; squares), either before (open symbols) or after (filled symbols) correction for the terminal phase hydrolytic step.

Hydrolysis of 3-Acyl-5-FU Derivatives

The chemical hydrolysis kinetics of 3-acyl-5-FU derivatives have previously been reported to be pseudo-first order and much slower than the kinetics for hydrolysis of the corresponding 1-acyl-5-FU derivatives (4). In the present work, disappearance of 3-acetyl-(1) and 3-propionyl-5-FU (2) in UV studies is shown in Fig. 4. Clearly, the disappearances of 1 and 2 do not follow pseudo-first order kinetics. Similar plots for the disappearance of 1 in the absence of HCHO were obtained by HPLC (Fig. 5). The UV spectrum at the end of each reaction was consistent with that of 5-FU. Reactions followed by HPLC also gave quantitative conversion of substrate to 5-FU in the absence of HCHO.

Instead of appearing to be linear, the ln concentration versus time plots in the absence of HCHO in Figs. 4 and 5 appear to be biexponential. This indicates an initial rapid equilibrium reaction to given an intermediate, followed by a terminal pseudo-first order reaction of either the substrate or the intermediate (or both) to give 5-FU. Kinetic reactions involving an initial equilibrium step have been recently dis-

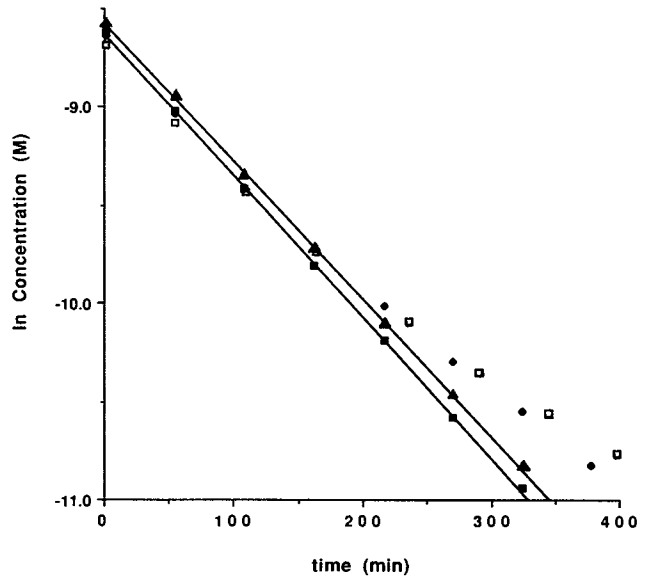

Fig. 5. Plots of ln concentration (HPLC) versus time (min) for hydrolysis of 3-acetyl-5-FU in 0.05 M phosphate buffer (pH 7.1, I = 0.12) at 32°C without formaldehyde (n = 3) (□) and with 3.6×10^{-4} M (◆), 3.6×10^{-3} M (■), and 3.6×10^{-2} M (▲) formaldehyde (n = 2).

cussed for ebifuramin and for rifampicin, both of which contain an azomethine functional group that exhibits a reversible degradation step in acidic solutions followed by subsequent pseudo-first order degradation (11,24). In those cases, the equilibrium step was for an A in equilibrium with B plus C type (Scheme Ia). In the present work, the equilibrium step appears to be that of an A in equilibrium with B type (Scheme Ib):

$$A \underset{k_r}{\overset{k_f}{\rightleftharpoons}} B + C \quad (a)$$

$$A \underset{k_r}{\overset{k_f}{\rightleftharpoons}} B \quad (b)$$

Scheme I

These reactions are characterized by a forward rate constant (k_f), a reverse rate constant (k_r), an equilibrium constant (K_{eq}), and a terminal rate constant (k_{term}) for the final pseudo-first order reaction leading to products. The use of k_{term} to correct the concentration versus time data for the effects of the final step has been described previously (11,24), while estimation of k_f, k_r, and K_{eq} from reactions where A and B do not react further is also well-known (12). The rate and equilibrium constants are reported in Table I.

The constants in Table I are the same within experimental error for both the UV and the HPLC studies of hydrolysis of **1**. Similar results were found when the initial concentration of **1** was reduced to 1.2×10^{-4} *M* (not reported in Table I). The similarity in K_{eq} values from reactions starting with different substrate concentrations suggests that the first step follows Scheme Ib, rather than Scheme Ia, for which a dependence on concentration would be expected, due to the second order reverse reaction. For **2**, the value for k_f is slightly larger than those for **1**, although not significantly, and the value for k_{term} is somewhat less. However, the reverse rate constant (k_r) is substantially greater for **2** than **1** and the disparities in k_f and k_r result in an equilibrium constant (K_{eq}) which is significantly less for **2** (i.e., favoring the reactant). This is easily seen in the plots for **1** and **2**, after correction for the terminal reaction (Fig. 4, filled symbols). The corrected plot for **1** reaches an equilibrium concentration of reactant ($7.6 \pm 0.3 \times 10^{-5}$ *M*) which is significantly less than that for **2** ($1.23 \pm 0.01 \times 10^{-4}$ *M*), and equilibration takes considerably longer for **1**.

The fact that the k_{term} for **2** is less than that for **1** is consistent with normal steric effects on the rates of hydrolysis of acyl derivatives (25). It is not clear why the k_r for **2** is so much greater than that for **1**, except that k_r contains a contribution from the intermediate B rearranging to the 1-acyl derivative, which hydrolyzes further to 5-FU. In that regard, it should be noted that the 1-propionyl derivative hydrolyzes about 50% faster than the 1-acetyl derivative under these conditions ($t_{1/2}$ = 3.1 and 4.8 min, respectively) (15), which is the opposite of normal expected steric effects on hydrolysis.

The use of HCHO as a trapping reagent in reversible reactions has been reported previously (11). The purpose of adding a trapping agent is to prevent the reverse reaction from taking place, thus converting the equilibrium step into a pseudo-first order reaction, presumably with the same rate constant as k_f. Behavior similar to that in Fig. 4 was seen for the lowest concentration of added HCHO (3.6×10^{-4} *M*; Fig. 5), while higher concentrations (3.6×10^{-3} and 3.6×10^{-2} *M*) resulted in linear plots of ln concentration as a function of time (Fig. 5), which are recorded in Table I as k_f. These rate constants are significantly larger than those for k_f in the absence of HCHO. Increased pseudo-first-order rate constants in the presence of HCHO have also been reported previously (11,15). This rate enhancement by the added HCHO could be due to either (a) general base catalysis or (b) a medium effect, such as a change in dielectric constant. The lack of HCHO concentration dependence, as indicated by the last two entries of Table I, is not surprising in view of the fact that almost 97% of the total 5-FU species in the reaction mixture are converted to hydroxymethyl adducts even at the lower of those two HCHO concentrations (26).

Table II. Reaction Products Formed During Hydrolysis of 3-Acetyl-5-FU in 0.05 *M* Phosphate Buffer (pH 7.1, I = 0.12) with Formaldehyde at 32°C.

Reactant (*M*)		Product (%)		
3-Acetyl-5-FU	Formaldehyde	5-FU	1-AOM-5-FU[a]	3-AOM-5-FU[b]
1.8×10^{-4}	3.6×10^{-4}	91	4.7	4.9
1.8×10^{-4}	3.6×10^{-3}	65	18	17
1.8×10^{-4}	3.6×10^{-2}	57	22	21

[a] 1-Acetyloxymethyl-5-FU. [b] 3-Acetyloxymethyl-5-FU.

Evidence that HCHO was trapping an intermediate is presented in Table II. Although a transient intermediate was not observed during the HPLC studies, two final products were identified that suggested the presence of an O^2-acylated intermediate, in addition to 5-FU. The formation of 1-acetyloxymethyl-5-FU and 3-acetyloxymethyl-5-FU in equal amounts was confirmed by comparison with authentic samples.[7] Identical retention times from HPLC were observed for the authentic samples and the reaction products from the hydrolysis studies. ^{1}H NMR spectra obtained for the products of a scaled-up hydrolysis reaction were consistent with the assigned structures, based on the chemical shifts of the C^6-H and the $N-CH_2-O-$ absorptions (28). In addition, 1-acetyloxymethyl-5-FU was isolated for the reaction mixture, and its melting point (124–125°C) was consistent with the literature value (127–128°C) (28). 1-Acetyl-5-FU was not observed as a product in any study of the hydrolysis of 3-acetyl-5-FU. Also, no acyloxymethyl-5-FU products were observed from the hydrolysis of 1-acyl-5-FU prodrugs in the presence of HCHO (15).

Possible routes for the formation of 1- and 3-acetyloxymethyl-5-FU are presented in Fig. 6. These require reversible rearrangement of 3-acetyl-5-FU to 2-acetyl-5-FU (Fig. 3), followed by reversible addition of HCHO to one or the other of the canonical forms of the 2-acetyl-5-FU anion. The resulting intermediates may then rearrange intramolec-

[7] Authentic samples were provided by Dr. Hans Bundgaard of the Royal Danish School of Pharmacy, Copenhagen, Denmark.

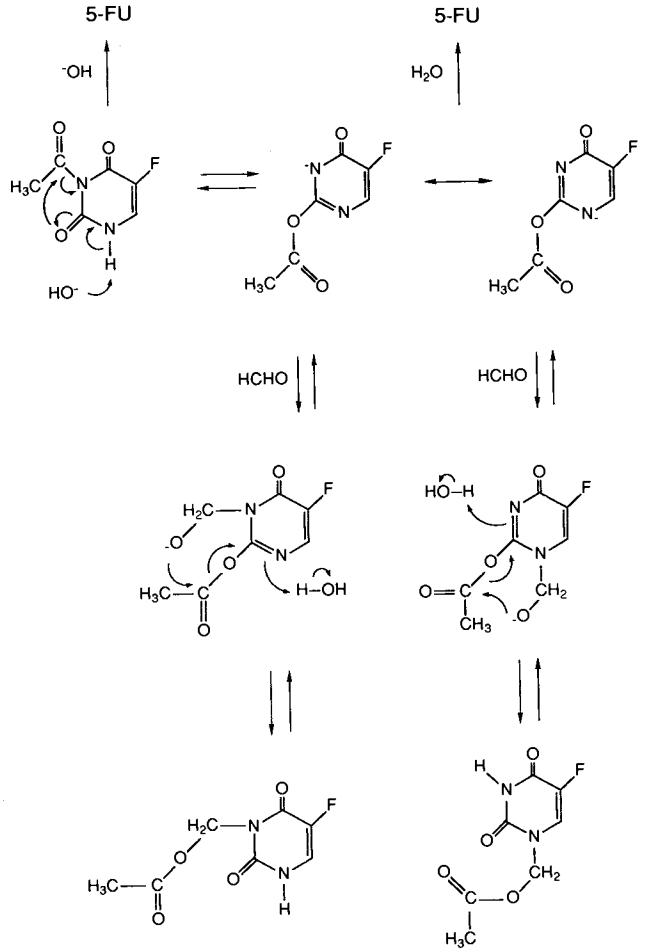

Fig. 6. Reaction of 3-acetyl-5-FU with formaldehyde to form 1-acetyloxymethyl-5-FU and 3-acetyloxymethyl-5-FU.

ularly to either 1-acetyloxymethyl-5-FU or 3-acetyloxymethyl-5-FU. A similar rearrangement was proposed in the reaction of HCHO with the free-base form of fetindomide, an amino acid prodrug (27). The equal distribution of the two acyloxymethyl derivatives observed during the hydrolyses of 1 in the presence of HCHO at all concentrations (Table II) is a persuasive argument for the intermediacy of a symmetrical 2-acyl derivative, resulting from the intramolecular rearrangement of 1, which is subsequently trapped as one of two possible hydroxymethyl adducts and rearranges to the observed products.

CONCLUSION

The X-ray diffraction, IR, and ^1H NMR spectroscopic data suggest that the 3-acyl-5-FU derivatives hydrolyze slower than the 1-acyl-5-FU derivatives because the 3-acyl group is perpendicular to and the 1-acyl group is coplanar with the plane of the 5-FU ring. Thus, the perpendicular 3-acyl carbonyl group is shielded from nucleophilic attack by the flanking 5-FU carbonyl groups, and this accounts for its slower hydrolysis in spite of the fact that the more stable N^3-anion is a better leaving group than the N^1-anion. Although it is tempting to suggest a mandatory 2-acyl intermediate during the thermal rearrangement of the 3-acyl derivatives to 1-acyl derivatives, an intermolecular rearrangement seems to be the major pathway. On the other hand, the hydrolysis of 3-acyl derivative proceeds to the extent of at least 50% by way of the 2-acyl intermediate based on trapping experiments using formaldehyde. In those trapping experiments almost 50% of the reaction products were acyloxymethyl-5-FU derivatives, and the fact that the ratio between 1- and 3-substitution was 1:1 strongly supports the existence of the symmetrical 2-acyl intermediate.

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