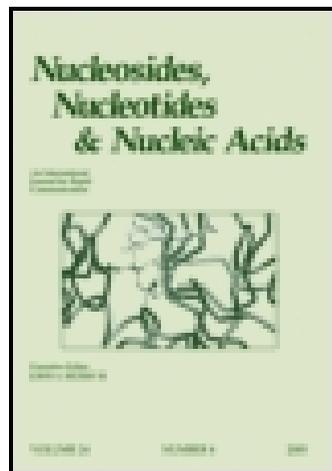


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NMR STUDIES SHOW MONOMERIC 5-FLUOROURIDINE FORMS BASE PAIRS OF INCREASED STABILITY COMPARED WITH URIDINE IN NON-AQUEOUS SOLVENTS

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Abstract - The binding constants and geometries for nucleoside base pairs involving 5-fluorouridine (FUr) and adenosine were determined by ¹H, ¹³C, and ¹⁹F NMR to better understand how FUr may perturb RNA structure. 5FU:A base pairs are more stable than U:A base pairs.

5-Fluorouracil (5-FU) is in widespread use for the treatment of colorectal and other cancers in humans¹. 5-FU is converted to 5-FdUMP intracellularly and this metabolite acts as a potent inhibitor of thymidylate synthase. As envisioned by Heidelberger², in the presence of 5-FU cancer cells cannot undergo DNA replication due to a lack of thymidine. Recently, it has been shown that 5-FU is also metabolized to 5-FUTP and incorporated into RNA³. Patient response to 5-FU treatment correlates well with its incorporation into RNA⁴. 5-Fluorodeoxyuridine has been synthetically incorporated into oligonucleotides and these have been studied by NMR spectroscopy and by other methods^{5,6}. In all oligonucleotides into which it has been incorporated, 5-fluorodeoxyuridine has been shown to destabilize the helix⁷. The structural consequences of 5-fluorouridine in RNA are currently under investigation.

NMR techniques have been widely used to study the structure and stability of nucleic acid base pairs. Katz and Penman⁸ were the first to show large downfield shifts for the resonances of the imino and amino hydrogens in guanosine and cytidine upon formation of base pairs in dimethyl sulfoxide. Newmark and Cantor⁹ used ¹H chemical shifts to obtain equilibrium constants and heats of formation for the same system. Iwahashi and Kyogoku demonstrated that ¹³C resonances are sensitive monitors of hydrogen bond formation¹⁰. Petersen and Led showed that heat of formation for guanosine-cytidine Watson Crick base pairs could be determined by using ¹³C NMR spectroscopy¹¹. Studies of the interactions among nucleoside monomers have been and continue to be informative about the structures and stabilities of RNA and DNA.

The effect of fluorouracil incorporation on DNA structure has been attributed to the ionization of the base. Values of between 7.58 and 8.00 have been reported in the literature for the pK_A of 5-fluorouridine indicating that as much as 40% of 5-fluorouridine molecules may be ionized at physiological pH. The monoanion of 5-fluorouracil has been studied by UV spectroscopy and in contrast to the other 5-halogenouracils the charge resides predominately on N3¹². Kremer et al.⁵ found the pK_A of 5-fluorodeoxyuridine in oligonucleotides to be dependent on the length and sequence of the oligonucleotide. The ionization of 5-fluorouridine and subsequent changes in base pair stability and stacking interactions undoubtedly play a major role in determining the effects that 5-fluorouracil incorporation has on RNA and DNA structures. 5-fluorouridine nonetheless exists mainly unionized under physiological conditions and in large RNA complexes may not be appreciably ionized. Little is known about the structure and

stability of base pairs involving neutral 5-fluorouridine compared to uridine. We have conducted a study on the structure and stability of A:5FU base pairs in order to better understand the affinity that neutral 5-FU has for its potential base pairing partners in duplex RNA.

Experimental Section

Materials. Uridine, 5-fluorouridine and 2',3'-isopropylidene-5'-O-acetyladenosine (**3**) were purchased from Sigma. Deuteriochloroform was purchased from Cambridge Isotopes. All other compounds were purchased from the Aldrich chemical company.

Synthesis of Protected Nucleosides. 2',3',5'-Tri-O-acetyl uridine (**1**) and 2',3',5'-Tri-O-acetyl 5-fluorouridine (**2**) were prepared from uridine and 5-fluorouridine respectively, by reaction with acetic anhydride in anhydrous pyridine for 4 h at room temperature. The desired product was purified by column chromatography on silica gel using a 90:10 chloroform:methanol mobile phase. The product was analyzed by ^1H NMR and EI Mass Spectrometry and found to analyze correctly.

Preparation of NMR Samples. Stock solutions of compounds (**1**), (**2**), and (**3**) were prepared from the dried nucleosides in deuteriochloroform solution. Stock solutions of 10 mM were prepared for the ^1H NMR studies and 0.28 M stock solutions for the ^{13}C NMR studies. NMR samples were prepared by mixing appropriate volumes of the stock solution. For the ^1H NOE study the sample was degassed by five freeze-pump-thaw cycles and placed under Argon.

Acquisition of NMR Data. ^1H NOE difference and ^{13}C NMR data were collected on a Varian UNITY 500 NMR spectrometer. For NOE experiments the imino hydrogen was irradiated for 1.5 s at a power level sufficient to just saturate the resonance. Pre-irradiation was applied on resonance and 10,000 Hz off-resonance in alternate scans. A 90° pulse was applied, 16k data points were collected and the FIDs for the control and NOE experiments were subtracted and Fourier transformed following 1 Hz line broadening. Low-power broadband proton decoupling for ^{13}C spectra was accomplished by using WALTZ-16 modulation. For ^{13}C spectra a 60° pulse was applied and a 25 kHz spectral window was scanned, and 64k points were sampled for a digital resolution of 0.76 Hz. Probe temperature was calculated to $\pm 1^\circ\text{C}$. Samples were allowed to equilibrate for 15 min before acquisition of data, and probe

temperature was controlled to ± 0.1 °C. Imino hydrogen studies were conducted on a Varian XL-300 NMR spectrometer. The experiments consisted of a 90° pulse width followed by acquisition of 16k data points over a 3600 Hz spectral window. The FIDs were weighted with 1 Hz of line broadening and Fourier transformed. ^{19}F NMR studies were conducted on a Varian XL-300 NMR spectrometer. A 90° pulse width was used and 16k points were collected over a 12 kHz spectral window. Recycle delays of 4.6s were used.

Statistical Methods of Analysis.

Estimates of dimeric chemical shifts and association constants were obtained through the application of a non-linear least squares fitting approach. When derivatives of the expression for the observed chemical shift with respect to the parameters of interest were available the Marquardt method, a compromise between Gauss and Newton and steepest descent, was used. In some instances the multivariable secant method (method of false position) was used to obtain estimates. In all cases, estimates were obtained using SAS PROC NLIN, version 6¹³.

Results

5-Fluorouridine Self-Association. The self-association constants for 2',3',5'-tri-O-acetyluridine (**1**) and 2',3',5'-tri-O-acetyl 5-fluorouridine (**2**) were determined from the concentration dependence of the ^1H chemical shift for the uridine imino hydrogen. The samples were dissolved in deuteriochloroform solution, a nonpolar aprotic solvent with a low dielectric constant ($\epsilon^{213\text{K}} = 6.8$, $\epsilon^{243\text{K}} = 4.8$) used by several groups to successfully study base pairing at the nucleoside level¹⁴. Several studies of uridine and related derivatives indicate that the nucleoside forms dimeric self-association complexes in chloroform. Uridine dimers stabilized by two hydrogen bonds are more stable than those linked by a single hydrogen bond. Three different dimer geometries involving two hydrogen bonds are possible (Fig. 1). Dimers or higher order polymers involving a single hydrogen bond do not constitute a significant portion of the equilibrium mixture. The formation of self-association dimers is easily detected by ^1H NMR.

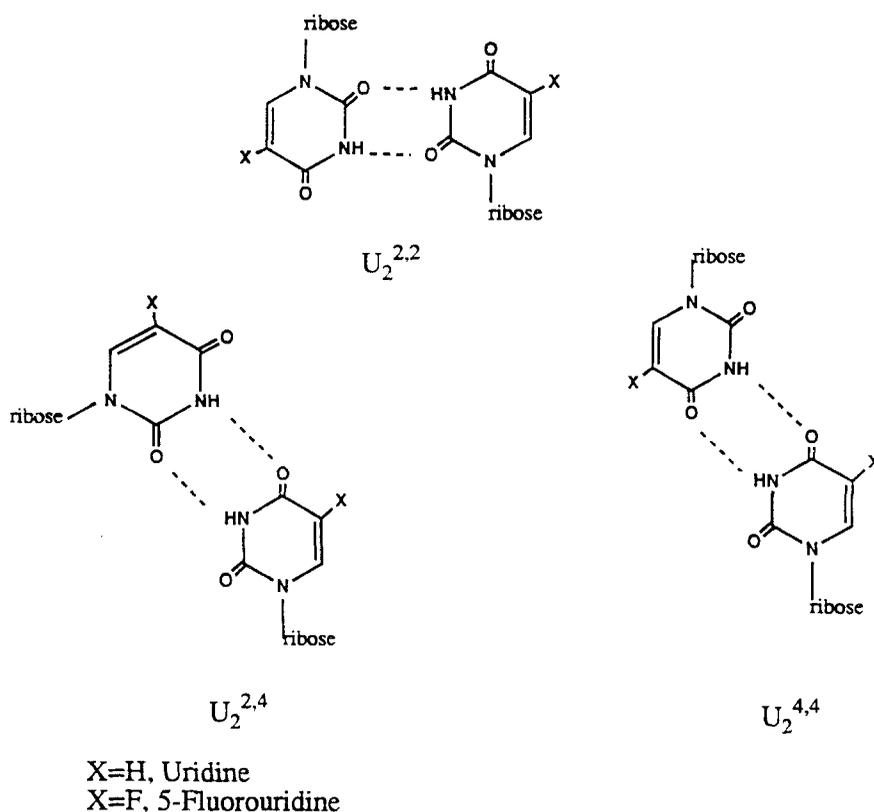


FIGURE 1 - The three geometries of self-association for uridine derivative (1) and for 5-fluorouridine derivative (2).

In order to determine self-association constants for (1) and (2) samples from .01 to 10 mM were prepared for each and the ^1H chemical shifts of the imino hydrogen were measured. These experiments were performed at -20, 0, and +20 °C. The imino hydrogen of both (1) and (2) monotonically shifts downfield as the concentration of (1) or (2) is increased and also shifts downfield at lower temperatures. Because the observed chemical shift is a weighted-average of the true monomeric shift and a shift due to the imino hydrogen resonance in each dimeric species, these trends are consistent with an increase in the equilibrium concentration of self-associated dimers.

As previously shown by Iwahashi and Kyogoku¹⁰, the equilibrium expression for self-association for uridine (U) can be written as

$$U + U = xU_2^{2,2} + yU_2^{2,4} + zU_2^{4,4} \quad (1)$$

where

$$x + y + z = 1 \quad (2)$$

Direct evidence for the proportion of each dimeric species is not available from monitoring the imino hydrogen chemical shift for (1) or (2) in deuteriochloroform since equilibrium is rapid on the time scale required for NMR measurement. An apparent association constant dependent only on the fraction of the total nucleoside concentration U_0 engaged in dimer formation (f_d) and the fraction of pure monomer (f_m), must be considered.

$$K_{app} = f_d/2f_m^2U_0 \quad (3)$$

where

$$f_m + f_d = 1 \quad (4)$$

The observed chemical shift is a weighted average of the true monomeric and the apparent dimeric chemical shifts.

$$\delta_{obs} = f_m\delta_m + f_d\delta_d(app) \quad (5)$$

Solving for f_m or f_d gives

$$f_m = (\delta_d(app) - \delta_{obs}) / (\delta_d(app) - \delta_m) \quad (6)$$

$$f_d = (\delta_{obs} - \delta_m) / (\delta_d(app) - \delta_m) \quad (7)$$

Chen and Shirts¹⁵ developed a method for the simultaneous determination of δ_m , $\delta_d(app)$ and K_{app} for a self-associating system undergoing rapid interconversion. Briefly, the method involves formulating two independent expressions that relate the apparent association constant K_{app} , the total nucleoside concentration U_0 , the observed chemical shift values in the dilution study δ_{obs} , and the limiting monomeric (δ_m) and apparent dimeric ($\delta_d(app)$) chemical shift values. A linear regression analysis of the observed chemical shift δ_{obs} versus f_d from such a derivation has small errors for accurate values of K_{app} with an intercept of δ_m . Similarly, linear regression of δ_{obs} versus a parameter that depends on the value obtained for δ_m has small residual errors for

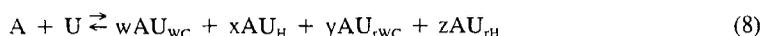
TABLE 1 - Limiting Chemical Shifts and Association Constants for Uridine Derivative (1) and 5-Fluorouridine Derivative (2)

Compound (1)	δ_m (ppm)	δ_d (ppm)	K(L/mol) ^a
20°	7.84 (7.81, 7.86)	11.4 (10.1, 12.8)	13 (04, 22)
0°	7.87 (7.85, 7.90)	11.2 (10.7, 11.7)	30 (20, 41)
-20°	8.09 (8.01, 8.17)	12.6 (11.1, 14.2)	28 (07, 50)
Compound (2)			
20°	7.93 (7.92, 7.95)	10.8 (10.3, 11.3)	21 (13, 29)
0°	7.93 (7.83, 8.02)	11.2 (10.3, 12.1)	37 (08, 66)
-20°	8.05 (8.01, 8.09)	11.1 (10.9, 11.3)	73 (55, 91)

^a 95% confidence intervals.

accurate values of δ_m . The two expressions are evaluated successively and the errors are minimized using the SAS NLIN program. The values, together with the 95% confidence intervals are summarized in Table 1 for uridine derivative (1), and 5-fluorouridine derivative (2) at -20, 0, and 20°C. The values are similar to those reported by others for the self-association of similar compounds in chloroform solution¹⁰.

Stability of 5FU:A Base Pairs. The uridine derivative (1) and the 5-fluorouridine derivative (2) each form dimeric complexes with the adenosine derivative (3) in deuteriochloroform solution. There are four base pairing arrangements possible each involving two hydrogen bonds and these are drawn in chart II. The equilibrium for formation of AU base pairs is



where

$$w + x + y + z = 1 \quad (9)$$

The ¹H chemical shift of the imino hydrogen cannot be used to distinguish base pair geometry due to the rate of interconversion of the four complexes and only an apparent equilibrium can be considered.

$$K_{app} = [AU]/[A][U] \quad (10)$$

where [AU] represents the total concentration of all four base paired species.

The observed chemical shift for the imino hydrogen of U is thus a weighted average of its chemical shift in the monomer, δ_U , in the apparent UU dimer, δ_{UU} , and in the apparent AU dimer, δ_{AU} .

$$\delta_{\text{obs}} = \delta_U([\text{U}]/[\text{U}_0]) + \delta_{UU}(2[\text{UU}]/[\text{U}_0]) + \delta_{AU}([\text{AU}]/[\text{U}_0]) \quad (11)$$

Equation (11) may be written in terms of the fractions of monomeric, self-associated, and base paired species as

$$\delta_{\text{obs}} = \delta_U f_U + \delta_{UU} f_{UU} + \delta_{AU} f_{AU} \quad (12)$$

with

$$f_U + f_{UU} + f_{AU} = 1 \quad (13)$$

We have developed an analytical expression that relates the apparent dimeric association constant K_{AU} to a number of parameters that may be independently calculated (equations 14-28, appendix 1). In particular, K_{AU} depends on the results obtained for the self-association of U^{9,10,14,15}. The values of K_{AU} and δ_{AU} are highly correlated and only an accurate independent determination of δ_{AU} will provide a correct value for K_{AU} . In order to determine an accurate value of δ_{AU} the ¹H chemical shift of the uridine imino hydrogen was measured in 10 mM solution of uridine derivative (1) mixed with adenosine derivative (3). The experiments were performed at -20, 0, and 20 °C in deuteriochloroform solution. In these experiments the ratio of (1) to (3) was varied from 2 to 100% but the total nucleoside concentration was invariant at 10 mM. Similar experiments were conducted with 5-fluorouridine derivative (2) mixed with adenosine derivative (3). The imino hydrogen chemical shifts of (1) and (2) are displaced monotonically downfield with increasing mole percent of (3). The chemical shift of the imino hydrogen of (1) and (2) are similar in the absence of (3) suggesting similar values for the monomeric species (as was shown by the self-association data; vide supra). The chemical shift of the 5-fluorouridine derivative (2) is more sensitive to changes in the concentration of (3). The

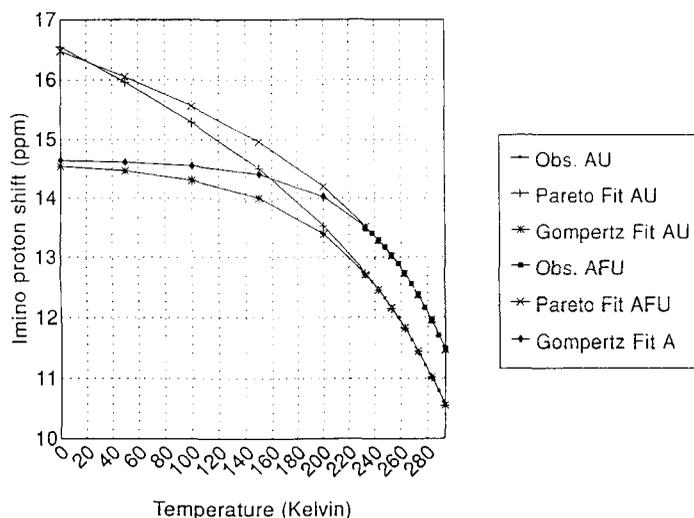


FIGURE 3 - The chemical shift of the imino hydrogen for uridine derivative (1) and for 5-fluorouridine derivative (2) as a function of temperature in the presence of a 50-fold excess of adenosine derivative (3). Experimental data points are indicated for temperatures greater than 233 K. Mathematical extrapolations using Gompertz and Generalized Pareto functions are indicated for lower temperatures.

hydrogen of (1) and (2) with a 50-fold excess of (3) is shown in Fig. 3 as a function of temperature. The imino hydrogen of both (1) and (2) is shifted downfield monotonically as the temperature is lowered. The slope of the curve for either (1):(3) or (2):(3) is not constant becoming shallower at lower temperatures. Extrapolation to 0 K should provide an estimate of δ_{AU} and δ_{AFU} . Two approaches were used. The first used a generalized Pareto function

$$Y = A (1-bx)^c \quad (29)$$

and the second used a Gompertz function

$$Y = A \exp(-b(e^{cx}-1)) \quad (30)$$

where x is temperature (K), Y is the chemical shift of the imino hydrogen and A is the chemical shift value at 0 K, an estimate of δ_{AU} . Both models were fit using non-linear least squares regression of the data using the program SAS NLIN. The resulting values are shown in Table

Table 2. Estimates of Dimeric Chemical Shifts for A:U and A:5FU Base Pairs

Method	Nucleosides	Dimer Chemical Shift ^a (ppm)
Generalized Pareto	(1):(3) U:A	16.48 ± 0.35
Gompertz	(1):(3) U:A	14.64 ± 0.12
Generalized Pareto	(2):(3) FU:A	16.55 ± 0.47
Gompertz	(2):(3) FU:A	14.54 ± 0.33

^a 95% confidence intervals.

Both models provide excellent fits to the observed data, but suggest different extrapolations. Interestingly, both models predict almost identical values for δ_{AU} as δ_{A5FU} . This is consistent with what has been observed in oligonucleotides containing 5-fluorodeoxyuridine where the imino hydrogen from the 5-FU base resonates within 0.7 ppm of the other uridine imino hydrogens⁵. The fit obtained by using the Gompertz function, $\delta_{AU} = 14.54$ ppm, is similar to values found for AU base pairs for DNA in aqueous solution.

The association constants K_{AU} and K_{A5FU} may be determined by using equation 28 in conjunction with the self-association data (Table I). Either statistical fitting of the data predicts the association constants are different and that K_{A5FU} is greater than K_{AU} by about a factor of 2. The association constants calculated at 20°C are 58 L/mol for the (1)-(3) dimer and 97 L/mol for the (2)-(3) dimer. These values are similar to those obtained by other methods.⁹ This shows that under these experimental conditions A:5FU base pairs are more stable than A:U base pairs and suggests that neutral A:5FU base pairs enhance the stability of DNA and RNA duplexes relative to AU base pairs. Thus, base pairing involving neutral 5-FU may not contribute to the destabilization of nucleic acid helices. Therefore, the observed destabilization of nucleic acid double helices may result from the decreased stability of base pairs in which 5-FU exists as the monoanion and/or from unfavorable stacking interactions that result from fluorine substitution.

The enhanced stability of A:5FU base pairs relative to A:U base pairs could potentially arise from the participation of fluorine as a hydrogen bond acceptor. We, therefore, monitored the ¹⁹F chemical shifts for compound (2) as a function of the mole percent of adenosine

derivative (3). The experimental conditions were identical to the ^1H NMR experiments described above. The data are shown in Table 3. The ^{19}F resonance changes only slightly (0.2 ppm) as the ratio of (3) to (2) is increased from zero to 50. Such a small dependence of the chemical shift on base pair formation is not consistent with the hypothesized action of fluorine as a hydrogen bond acceptor. This small change in chemical shift also argues against major reorganization of the electron density about fluorine upon base pair formation. Fluorine, therefore, appears to impact base pair formation chiefly through its electron withdrawing inductive effect rendering the imino hydrogen more acidic and stabilizing negative charge on the carbonyl oxygens.

Mole Percent of Adenosine	^{19}F Chemical Shift
0	-100.75
20	-100.73
35	-100.72
50	-100.68
65	-100.66
80	-100.62
90	-100.61
95	-100.61

Table 3. ^{19}F Chemical Shifts for 5-Fluorouridine Derivative (2) Versus Mole Percent Adenosine Derivative (3) at 10mM Total Nucleoside Concentration

Base Pair Geometry by ^{13}C

NMR. The association constants and limiting chemical shifts obtained for mixtures of compounds (1) and (2) with (3) are apparent values that reflect the

formation of four different dimer geometries (Figure 2). The higher affinity of (3) for the 5-fluorouridine derivative (2) relative to the uridine derivative (1) might be due to the increased formation of one of the four geometries or all might be equally enhanced. The proximity of fluorine to C4 might lead to selective enhancement of this position through stabilization of negative charge on the hydrogen bonding acceptor carbonyl. This would result in a higher ratio of Watson-Crick and Hoogsteen base pairs relative to base pairs of reversed geometry. Iwahashi and Kyogoku¹⁰ observed that chemical shifts of C2 and C4 are selectively moved downfield in dimeric complexes of uridine and adenosine. They concluded that only the resonance for the carbonyl that acts as the hydrogen bond acceptor in base pair formation is moved downfield, and that the chemical shift moves downfield in proportion to the extent of hydrogen bond formation

TABLE 4. ^{13}C Chemical Shifts for Uridine Derivative (1) and 5-Fluorouridine Derivative (2) in A:U and A:5FU Base Pairs at 0.28 M Total Nucleoside Concentration

	Conc. of Uridine	C2 shift at 20 C	C4 shift at 20 C	C2 shift at -20 C	C4 shift at -20 C
A:U	0.25	150.39	163.25	150.45	163.68
A:U	0.20	150.56	163.46	150.67	163.91
A:U	0.15	150.79	163.78	150.89	164.20
A:U	0.10	150.92	163.97	150.97	164.34
A:U	0.05	151.03	164.16	150.99	164.41

	Conc. of 5-FU	C2 shift at 20 C	C4 shift at 20 C	C2 shift at -20 C	C4 shift at -20 C
A:5FU	0.25	149.18	156.89	149.26	157.11
A:5FU	0.20	149.40	157.33	149.45	157.59
A:5FU	0.15	149.66	157.79	149.64	158.00
A:5FU	0.10	149.74	157.92	149.68	158.11
A:5FU	0.05	149.81	158.03	149.68	158.17

at that site. To test whether the ratio of base pairing through the C2 and C4 carbonyls is altered in A:FU base pairs compared to A:U base pairs we measured the ^{13}C shifts for C2 and C4 of mixtures of (1) and (3) and (2) and (3) in deuteriochloroform solution. The total nucleoside concentration was constant at 0.28 M while the concentration of (1) or (2) was varied from .05 M to .28 M. A monotonic downfield shift was observed for C2 and C4 in (1) and (2). The limiting chemical shifts are summarized in Table 4.

The downfield shift for C2 is similar for mixtures of (1) and (3) and for (2) and (3). The concentration of base pairs that form by using the C2 carbonyl as the hydrogen bond acceptor is thus, similar for uridine derivative (1) and 5-fluorouridine derivative (2). The greater association constant for A:5FU base pair formation must therefore result from a greater equilibrium concentration of base pairs that form by using the C4 carbonyl. The observed downfield shifts at C4 are consistent with this analysis. Compound (2) displays nearly a 50% larger downfield shift as compound (1) in this concentration range. This is consistent with a

greater equilibrium concentration of normal Watson-Crick and Hoogsteen base pairs that use the C4 carbonyl as the hydrogen bond acceptor. The larger association constant for A:5FU base pairs therefore stems from an activation of the C4 carbonyl as a hydrogen bond acceptor with little if any effect on the hydrogen bond formation at C2.

To determine the relative ratios of Watson-Crick and Hoogsteen base pairs ^1H NOE experiments were performed on mixtures of (1) and (3) and (2) and (3) in deuteriochloroform solution. In either Watson-Crick or Hoogsteen geometry the distance from the imino hydrogen of uridine to only one of the base protons of adenosine is short enough for efficient NOEs. In Watson-Crick base pairs this is the H2 proton while in Hoogsteen base pairs this is the H8 proton. This strategy has been used to assign base pair geometry in tRNA¹⁶ and also in nucleoside base pairs¹⁴. We found that upon irradiating the imino hydrogen in mixtures of (1) and (3) or (2) and (3) NOEs are observed to both H2 and H8 of adenosine. The relative enhancements in the difference spectra are not significantly different between the two mixtures indicating that fluorine substitution has little effect on the relative stabilities of Watson-Crick and Hoogsteen base pairs.

Discussion

The present study is a detailed investigation of the structure and stability of nucleoside base pairs involving neutral 5-fluorouridine done by using NMR spectroscopy. Many previous studies have been conducted on nucleoside base pair formation in deuteriochloroform and other organic solutions^{8,9,10,14}. These studies have been helpful in understanding the stabilities and geometries for base pair formation. The present study extends these methods to examine base pairs involving 5-fluorouridine. Fluorine substitution impacts base pair stability and, for the neutral nucleoside used in the present study, results in the formation of base pairs of greater stability. A:5FU base pairs of four different geometries may be formed but only those involving the C4 carbonyl as the hydrogen bond acceptor appear to be stabilized relative to uridine.

The study of complementary base pairing involving uridine or uridine derivatives is hampered by competing equilibria between nucleoside self-association and hydrogen bonds to

adenosine derivatives. Analytical expressions have been developed by others that may be used in conjunction with statistical methods to determine limiting monomeric and dimeric chemical shifts and association constants for self-associating systems. These have been applied here for uridine derivative (**1**) and 5-fluorouridine derivative (**2**). A new series of analytical expressions (appendix 1) has been developed that relates the observed chemical shift values for a two component system in which one component undergoes self-association to the limiting monomeric and dimeric chemical shifts and the association constant for heterodimer formation. It is impossible to define a unique solution that simultaneously satisfies all chemical shift and association constant data without prior information about the true dimeric chemical shift or the association constant for heterodimer formation, because these variables are highly correlated. A reasonable estimate of the true dimeric chemical shift may be obtained by extrapolation of the observed chemical shift data at high ratios of adenosine to uridine (or 5-fluorouridine) to low temperatures where the association constants are very large. The exact results depend on the type of statistical model used to fit the data. Both the Gompertz and generalized Pareto models tightly fit the experimental data and indicate no difference in the intrinsic chemical shift of A:5FU and A:U dimers. The method developed here indicates that the chemical shift for the imino hydrogen in an A:U base pair is similar to that for an A:5FU base pair, in agreement with values reported for duplex DNA in aqueous solution⁵. The enhanced stability of A:5FU base pairs determined by using this method is consistent with the results of ¹³C NMR experiments that suggest increased formation of base pairs that use the C4 carbonyl as a hydrogen bond acceptor.

Appendix

The values of δ_U , δ_{UU} , and $K_{app}(UU)$ are known from the analysis of the self-association data above. The apparent equilibrium constant $K_{app}(UU)$ can thus be written as

$$K_{app}(UU) = \frac{([UU]/[U_o])}{([U]/[U_o])([U]/[U_o])([U_o])} = \frac{1/2f_{UU}}{f_U^2[U_o]} \quad (14)$$

Thus

$$f_{UU} = 2K_{app}(UU)f_U^2[U_o] \quad (15)$$

Note

$$[U_o] = [U] + [AU] + 2[UU] \quad (16)$$

which may be written as

$$[AU] = [U_o] - [U] - 2K_{UU}[U]^2 \quad (17)$$

Equation 12 can thus be rewritten in terms of equations 15 and 17 as

$$[U_o]\delta_{obs} = \delta_U[U] + \delta_{UU}2K_{UU}[U]^2 + \delta_{AU}\{[U_o] - [U] - 2K_{UU}[U]^2\} \quad (18)$$

Equation 18 may be rearranged to give

$$[U]^2\{2K_{UU}(\delta_{UU}-\delta_{AU})\} + [U](\delta_U-\delta_{AU}) + [U_o](\delta_{AU}-\delta_{obs}) = 0 \quad (19)$$

which in turn may be written in terms of the fraction of monomeric U as

$$f_U^2[U_o]\{2K_{UU}(\delta_{UU}-\delta_{AU})\} + f_U(\delta_U-\delta_{AU}) + (\delta_{AU}-\delta_{obs}) = 0 \quad (20)$$

Equation 20 may be rewritten by using the quadratic formula to yield.

$$f_U = \frac{(\delta_{AU} - \delta_U) \pm \sqrt{(\delta_U - \delta_{AU})^2 - 8K_{UU}[U_o](\delta_{UU} - \delta_{AU})(\delta_{AU} - \delta_{obs})}}{4K_{UU}[U_o](\delta_{UU} - \delta_{AU})} \quad (21)$$

Now at constant total nucleoside concentration i.e., $[U_o] + [A_o]$ is constant,

$$K_{AU} = [AU]/[A][U] = f_{AU} = [AU]/[U_o] = K_{AU}[A]f_U \quad (22)$$

But

$$[A] = [A_o] - [AU] \quad (23)$$

Thus

$$f_{AU} = K_{AU}\{[A_o]/[U_o] - [AU]/[U_o]\}f_U[U_o] \quad (24)$$

$$f_{AU} = K_{AU}[A_o]f_U - K_{AU}f_{AU}f_U[U_o] \quad (25)$$

$$f_{AU}(1 + K_{AU}f_U[U_o]) = K_{AU}[A_o]f_U \quad (26)$$

$$f_{AU} = K_{AU}[A_o]f_U/(1 + K_{AU}f_U[U_o]) \quad (27)$$

This implies that K_{AU} can be calculated as follows:

$$K_{AU} = f_{AU}/\{([A_o]/[U_o] - f_{AU})f_U[U_o]\} \quad (28)$$

For a fixed choice of δ_{AU} one can calculate f_U from equation 21, f_{UU} from equation

15 and f_{AU} from equation 13 and knowing these defines K_{AU} exactly according to

equation 28. The values of K_{AU} and δ_{AU} are highly correlated and only an accurate determination

of δ_{AU} will provide a correct value for K_{AU} .

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