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Mechanism of the Isotopic Exchange Reaction of the 5-H Hydrogen of Uracil Derivatives in Water and Nonprotic Solvents

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The mechanism of the isotopic exchange reaction of the 5-H hydrogen of uracil and its methyl derivatives in water and organic solvents has been studied. The key intermediate of the reaction is a C-5 tautomer of uracil in which the carbon atom at the 5-position has two hydrogen atoms, its hybridization is changed from sp^2 to sp^3, and the aromaticity of the pyrimidine ring is lost. We have used ^1H NMR spectroscopy to follow the kinetics of the hydrogen/deuterium exchange reaction. In aqueous media a general base catalysis was observed and for exchange in organic solvents we have proposed a reaction mechanism that involves the participation of solvent molecules. The reaction rates determined by NMR can be rationalized by density functional computations. We have shown that the hydrogen-to-deuterium exchange reaction is much faster in some suitable nucleophilic solvents than in water. These findings could be used for the tritium labeling of pyrimidine nucleic acid bases.

Introduction

Nucleic acid bases (NAB) as the basic elements of DNA and RNA are responsible for storing and translating of genetic information in living cells. Different tautomers of NAB are obtained most frequently when an acidic hydrogen atom changes its position in the base.[1] Such tautomers of nucleic acids may be involved in various processes, including point mutations,[2–4] the stabilization of certain anomalous DNA structures,[5] and interactions with metal ions.[6–8] Therefore a large number of theoretical and experimental studies have been devoted to the tautomerism of the bases.[9–15]

As uracil (**1**) is one of the most common bases, its forms have been thoroughly investigated in recent years.[9,11,12,16–20] Six tautomers (one diketo, four hydroxy keto, and one dihydroxy) have traditionally been considered in which the two protons move between the four heteroatoms. When the rotational isomerism of the *exo*-OH group is taken into account, 13 tautomers/rotamers are possible. The following notation of uracil tautomers is employed throughout the work and indicate the atoms to which the active hydrogen atoms are attached. The diketo form is referred to as 1,3-U and the 2-hydroxy-4-oxo forms are labeled 1,2-U and 2,3-U (see Figure 1). NMR, UV, IR, Raman, and microwave spectroscopic experiments indicate that the 2,4-dioxo form (1,3-U, canonical) of uracil is the most stable tautomer in the gas and solid phases as well as in solution.[21] According to the calorimetric measurements, the 2-oxo-4-hydroxy tautomer of uracil is less stable than the 2,4-dioxo form by 19 ± 6 kcal/mol.[22] The experimental evidence of small amounts of noncanonical uracil tautomers in the gas phase[23,24] was not confirmed in later studies.[20,25]

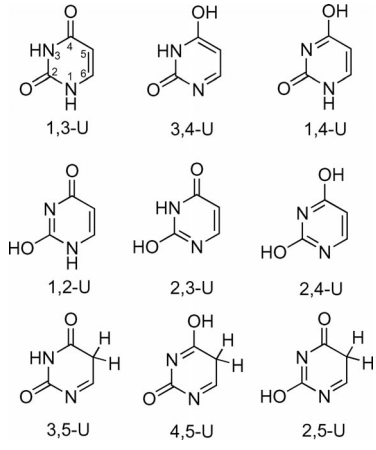

Figure 1. Tautomers of uracil.

Theoretical results have indicated that the 2,4-dioxo form is the most stable tautomer with the two hydroxy-oxo and dihydroxy tautomers being less stable by around 10 kcal/mol.[26] Microhydration and bulk solvent studies have

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shown that the water environment stabilizes the enol forms more than the diketo form when compared with, for example, the gas phase. In general, the population of the enol forms is very low.[20]

Recently a new type of uracil tautomer with a proton transferred to the C-5 carbon atom (changing its hybridization from sp^2 to sp^3, tautomers 2,5-U, 3,5-U, and 4,5-U) was proposed.[27,28] Its relative stability in the gas phase was calculated to be only slightly lower than that of the dihydroxy form.[27]

The isotopic exchange reaction of the hydrogen at the 5-position of uracil is a well-known reaction,[29–35] but several different mechanisms have been proposed for this reaction. It has previously been shown that in solution protonated pyrimidine derivatives can exist in an equilibrium of *N*- and C-5-protonated forms[36] and that C-5 protonation (deuteriation) can be used to prepare 5-H isotopically labeled pyrimidines.[28] However, the pK_a values of uracil are –2.4 and 9.4,[37] which indicates that the concentration of ionic forms in a neutral solution is very low. Another mechanism that has been proposed previously for 5-H isotopic exchange[29] involves the reversible addition of water (D_2O) to the 5,6-double bond of uracil. Another possible mechanism is the addition/elimination of a catalyst molecule to the 5,6-double bond. This type of mechanism was proposed for cysteine-catalyzed uracil deuteriation.[33] This reaction presumably involves an initial attack by the SH at the 6-position of the pyrimidine ring.

In this paper we present the results of our mechanistic studies on the isotopic exchange reaction. We monitored the exchange by NMR spectroscopy in various buffers and solvents containing D_2O. Our hypothesis is that the rare tautomers of uracil (2,5-U, 3,5-U, or 4,5-U) are the key intermediates of the exchange reaction. The keto–enol interconversion of carbonyl compounds is one of the most investigated organic reactions in aqueous solution. Water can act as a bifunctional catalyst, that is, it can accept a proton from the donor site of the solute molecule and transfer a different proton to the acceptor site on the solute.[19,38–46] On the other hand, the available data on the kinetics of the keto–enol tautomerism of carbonyl compounds in aprotic solvents are scarce.[47]

Results and Discussion

The Isotopic Exchange of the 5-H of Uracil in Water

The hydrogen-to-deuterium exchange reaction of the 5-H of uracil was studied at 100 °C in a sealed NMR tube and ^1H NMR spectra were recorded repeatedly at defined intervals of time (see Figure S2 in the Supporting Information). The intensity of the signal from 5-H was compared with the 6-H signal. The intensity of the 5-H signal continuously decreased as a result of the exchange of the proton with deuterium, as confirmed by APT ^{13}C NMR spectra in which the signal of C-5 appeared at δ = 100 ppm as a singlet pointing downwards (a CH group). Within a few hours or days of the sample's preparation, the intensity of this signal decreased and a new signal appeared in the same position. This new signal pointed upwards (a carbon without any attached proton) and was split into a triplet with equal line intensity as a result of coupling with deuterium. This deuteriation indicated by NMR was also confirmed by MS.

The 5-H exchange reaction is apparently a first-order reaction and the rate constants were obtained from plots of the logarithm of the concentrations of the starting compound (with ^1H at the 5-position) plotted as a function of time. The data fitted first-order kinetics very well with the correlation coefficient usually higher than 0.99.

We measured the exchange rates in D_2O at different pD, in acetate buffer, and in phosphate buffers of different concentrations. The results are summarized in Table 1. It can be seen that the reaction rate is not dependent on pH in the neutral region but is dependent upon the buffer concentration. These features are typical of a general base-catalyzed reaction. At constant pH, the reaction rate increases linearly with total buffer concentration. The Brønsted plot of the logarithm of the reaction rate against the pK_a of the studied acids showed a linear relationship with a slope equal to β = 0.4, which is close to the β value observed for the keto–enol tautomerization of 2-acetylcyclohexanone.[48]

Table 1. The experimental exchange rates of 5-H in uracil in D_2O and in acetate and phosphate buffers.

	Buffer concentration [mM]	pD	k [10^{-5} s^{-1}]
D_2O	–	1.62	0.87
D_2O	–	2.10	0.25
D_2O	–	3.60	0.083
D_2O	–	4.64	0.089
D_2O	–	5.09	0.089
D_2O	–	6.60	0.092
D_2O	–	8.02	0.15
D_2O	–	9.05	0.17
D_2O	–	9.92	0.53
Phosphate	25	5.46	0.15
Phosphate	50	5.43	0.31
Phosphate	100	5.38	0.44
Phosphate	25	8.76	1.58
Phosphate	50	8.70	3.06
Phosphate	100	8.77	4.72
Acetate	100	6.60	0.87

Isotopic Exchange of the 5-H of Uracil in Organic Solvents

The hydrogen-to-deuterium exchange reaction of the 5-H of uracil was also studied in a series of protic and nonprotic solvents. Usually 100 equiv. of D_2O were added to the reaction mixture as a source of deuterium, which enabled the rapid exchange of hydrogen and deuterium of the labile protons (those attached to the oxygen or nitrogen atoms). The experimental error in the determination of the reaction rate was estimated to be less than 2% in deuteriated solvents and 5% in nondeuteriated solvents (intense signals of the solvent appeared in the spectra in these solvents and the baselines were slightly distorted). The rate constants are summarized in Table 2.

Table 2. Experimental exchange rates of 5-H in uracil in different solvents,[a] as determined by NMR, the half-lives of the reaction, and the experimental and calculated (B3LYP/cc-pVTZ, for selected solvents only) free energy barriers of the reaction. The transition states were optimized in vacuo ($\Delta G^{\#}_{calcd.,vacuum}$) and then single-point calculations with an implicit solvent model were performed ($\Delta G^{\#}_{calcd.,PCM}$).

	k [10^{-5} s^{-1}]	$t_{½}$ [h]	$\Delta G^{\#}_{exp.}$ [kcal/mol[b]]	$\Delta G^{\#}_{calcd.,vacuum}$ [kcal/mol]	$\Delta G^{\#}_{calcd.,PCM}$ [kcal/mol]
[D_6]Acetone	0.014	1386	33.2		
[D_3]Acetonitrile	insoluble[c]	–	–		
[D_8]THF	0.019	990	33.0		
[D_4]Methanol	0.022	866	32.9		
[D_4]Acetic acid	0.058	330	32.2		
D_2O	0.092	210	31.8	47.6	48.4
[D_7]DMF	0.23	86	31.2		
[D_6]DMSO	0.13	147	31.6	41.0	37.8
HMPA	0.12	157	31.6		
2,6-Lutidine	1.33	14	29.8		
Pyridine	2.14	9.0	29.5	38.2	29.4
4-Picoline	2.70	7.1	29.3		
2-Picoline	2.77	7.0	29.3		
4-Methoxypyridine	12.2	1.6	28.2	36.7	29.0
4-Cyanopyridine	0.17	114	31.4		

[a] 0.5 mL of the solvent with 30 μL of D_2O. [b] The $\Delta G^{\#}$ values were calculated from doubled rate constants (see the text). [c] In a 1:1 mixture of acetonitrile/D_2O the reaction rate was 0.019×10^{-5} s^{-1}.

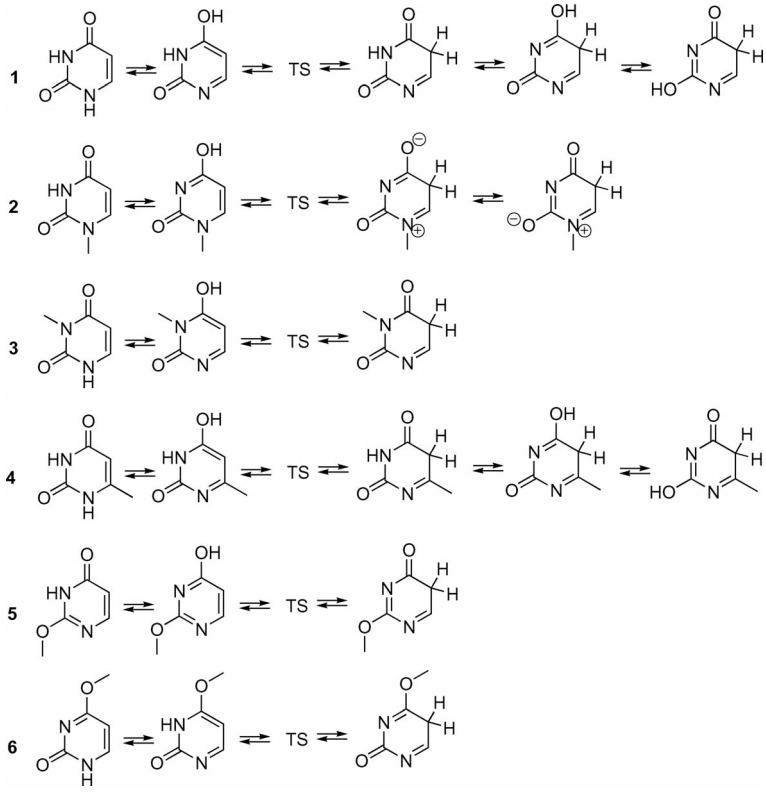
The reaction rates depend strongly on the solvent used. The highest reaction rate was observed in 4-methoxypyridine, the reaction being three orders of magnitude faster than in acetone and two orders of magnitude faster than in water. The reaction in acetone, acetonitrile, THF, methanol, and acetic acid was slower than in water. In DMF, DMSO, HMPA, and derivatives of pyridine, the reaction was faster than in water. Such a strong solvent effect cannot be explained by the different polarities of the solvents because the reaction rate does not correlate with the dielectric constants (for example, pyridine and THF have similar dielectric constants yet the reaction was 100 times faster in pyridine than in THF).

We also performed the isotopic exchange experiment in extremely dry DMSO (no traces of H_2O were observable in the ^1H NMR spectrum, see Figure S1 in the Supporting Information) in which dry uracil—with labile N1–H and N3–H protons, exchanged with deuterium—was dissolved, the exchange rate (0.0071 h^{-1}) being even higher than in DMSO with 100 equiv. of D_2O (0.0047 h^{-1}) and in pure water (0.0033 h^{-1}). It has previously been observed that increasing the DMSO concentration increased the rate of keto–enol tautomerization of 2-acetylcyclohexanone and 2-acetyl-1-tetralone.[49] From this experiment it follows that the exchange reaction can also proceed in nonprotic solvents and that no source of protons in the reaction media is required. The source of the deuterium atom that appeared at the 5-H position is the uracil molecule itself.

We suggest the mechanism for isotopic exchange depicted in Scheme 1 and Figure 2. The exchange reaction involves a very rare uracil tautomer with an sp^3-hybridized carbon atom at the 5-position. First, the canonical diketo form of uracil is changed to a keto–enol form (3,4-U), which is a fast stepwise process.[19,47,50] The 3,4-U form is then transformed into the rare 3,5-U tautomer. The organic solvents could act as catalysts in the formation of this rare tautomer. In the transition state a solvent molecule could form a complex with the uracil molecule and the free electron pair on the heteroatoms of the solvent lowers the barrier for the shift of the proton from oxygen to carbon C-5. The influence of the electronic structure of the solvent molecules is best demonstrated by the series of pyridine derivatives. Electron-donating substituents (CH_3, OCH_3) at the 4-position of the pyridine led to an increase (with respect to pyridine) in the reaction rates, whereas the electron-withdrawing carbonitrile substituent decreased the exchange reaction rate (see Table 2). In 2-picoline (2-methylpyridine), the reaction rate is very similar to that in 4-picoline. In 2,6-dimethylpyridine, the reaction was two-fold slower. This observation can be explained by the steric hindrance of the methyl groups in the transition-state complex.

The reaction rate for the isotopic exchange depends on the nucleophilicity of the solvent rather than its basicity. For example, 2,6-lutidine (2,6-dimethylpyridine) is a stronger base than 2-picoline, but the reaction is faster in 2-picoline, which has a less hindered nitrogen atom. A solvent molecule is supposedly directly involved in the transition state of the reaction. Nucleophilic attack of a solvent molecule on the hydrogen atom of the 4-hydroxy group with subsequent transfer of the hydrogen to the carbon at the 5-position is thus the most likely reaction mechanism, the solvent acting as a nucleophilic catalyst.

Another mechanism that has been proposed previously[29] involves the reversible addition of water (D_2O) to the 5,6-double bond of uracil. However, this mechanism is not consistent with our experimental findings because the exchange also proceeds in nonprotic solvents. Another possible mechanism is the addition/elimination of a solvent molecule to the 5,6-double bond. This type of mechanism was proposed for the cysteine-catalyzed deuteriation of uracil.[33] However, this mechanism cannot be considered in our case because the deuteriation of 1-methyluracil was much slower (see below) than the deuteriation of uracil. On the other hand, the deuteriation of uracil derivatives with a



Scheme 1. The tautomeric equilibrium of the uracil derivatives involved in the 5-H isotopic exchange.

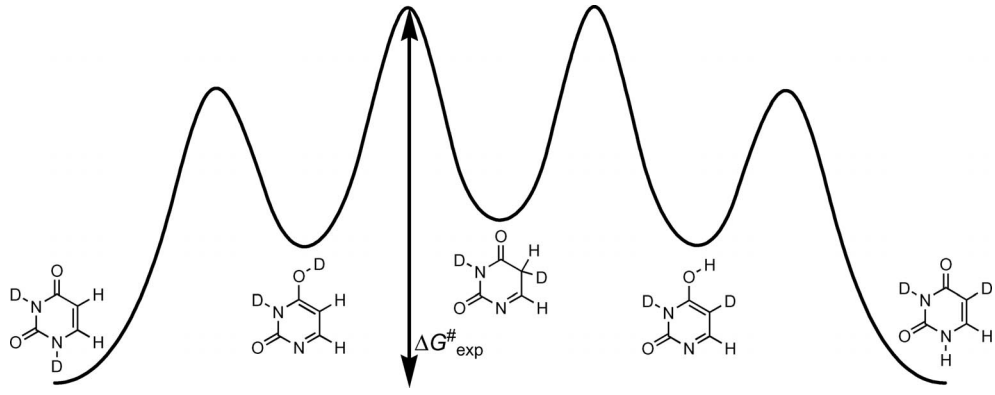


Figure 2. Mechanism for the isotopic exchange of hydrogen at the 5-position of uracil.

substituent at the 1-position is readily achieved by cysteine catalysis. In addition, the isotopic exchange reaction of 6-methyluracil is faster than for uracil (see below), which would not be possible if addition to the 5,6-double bond was a rate-limiting step of the reaction.

The intermediate C-5 tautomer has an equal likelihood of reverting to its original 5-H form or switching to the 5-D form (Figure 2) because the energy barrier is the same for both reactions (the deuterium isotopic effect has no or very little effect).[28] Thus, we can conclude that the rate constant for the canonical/C-5 tautomer interconversion is twice that for the C-5 proton/deuterium exchange. The experimental $\Delta G^{\#}$ values were obtained from the Eyring equation $\Delta G^{\#} = RT[23.76 - \ln(k/T)]$ using a rate constant k twice that of the observed rate constant for 5-H exchange.

Isotopic Exchange of Uracil Derivatives

To obtain further insight into the mechanism of isotopic exchange we measured the reaction rates of the methylated uracil derivatives 1-methyluracil (**2**), 3-methyluracil (**3**), 6-methyluracil (**4**), 2-methoxypyrimidin-4-one (**5**), and 4-methoxypyrimidin-2-one (**6**), cytosine (**7**), and isocytosine (**8**; see Figure 3). The reaction rates in D_2O and [D_5]pyridine are summarized in Table 3.

The hydrogen-to-deuterium exchange rates for 3-methyluracil (**3**) and 2-methoxypyrimidin-4-one (**5**) in D_2O are very similar to those of uracil. In pyridine, the reaction rates of compounds **3** and **5** are slightly lower. The similar behavior of compounds **3**, **5**, and uracil is expected from the proposed reaction mechanism because the 2- and 3-positions

Table 3. Exchange rates of 5-H in uracil (**1**) and its derivatives **2**–**8** in D_2O and [D_5]pyridine[a] and the calculated (B3LYP/cc-pVTZ) free-energy barriers of the reaction.

Compound	CH_3 in position	D_2O			[D_5]Pyridine	
		k [10^{-6} s^{-1}]	$\Delta G^{\#}_{exp.}$[b] [kcal/mol]	$\Delta G^{\#}_{calcd.,vacuum}$ [kcal/mol]	k [10^{-6} s^{-1}]	$\Delta G^{\#}_{exp.}$[b] [kcal/mol]
1	–	0.92	31.8	47.6	21.4	29.5
2	1	0.044	34.1	57.1	–	–
3	3	0.97	31.8	46.9	12.8	29.9
4	6	6.2	30.4	46.3	37.5	29.1
5	2	0.78	31.9	45.8	3.7	30.8
6	4	–	–	–	0.55	32.2
7	–	0.038	34.2	–	0.0031	36.1
8	–	4.9	30.6	–	21.0	29.5

[a] 0.5 mL of [D_5]pyridine with 30 μL of D_2O. [b] The experimental $\Delta G^{\#}$ values were calculated from doubled rate constants (see text).

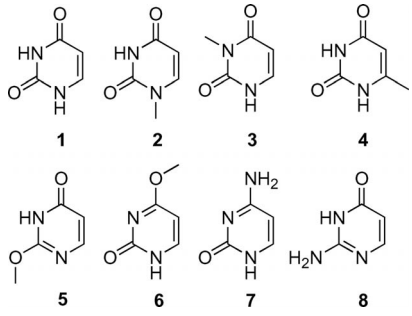


Figure 3. Pyrimidine derivatives studied in this work.

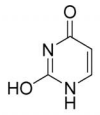
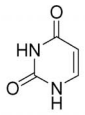
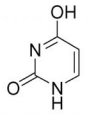
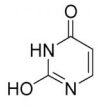
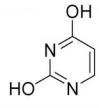
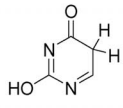
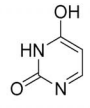
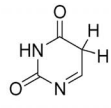
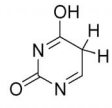
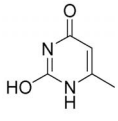
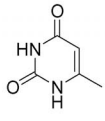
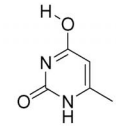
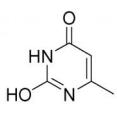
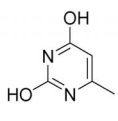
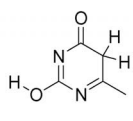
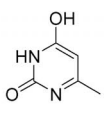
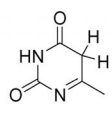
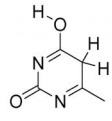
are not involved in the reaction. The reaction rates of 6-methyluracil (**4**) are slightly higher than those of uracil in both solvents. This can be explained by the stabilization of the 1,6-double bond in the key intermediate (3,5-tautomer) by the methyl group at the 6-position, which is also confirmed by theoretical calculations (see below). The exchange reaction of 1-methyluracil (**2**), on the other hand, is much slower than that of uracil in D_2O and no isotopic exchange was observed in pyridine. This finding is not surprising because, in this case, the C-5 sp^3 intermediate must have a zwitterionic structure (see Scheme 1), which is not stable (as also indicated by the calculations, see below). After heating compound **2** for a long time in D_2O, we also observed traces of a C-6 deuteriated compound; this indicates that another mechanism is probably involved in the exchange reaction of compound **2**. The exchange reaction of 4-methoxypyrimidin-2-one (**6**) is much slower than that of uracil in pyridine. In D_2O, no exchange reaction was observed at all. This is probably caused by a higher reaction barrier because for the 4-methoxy derivative it is not possible to draw a simple transition state for the hydrogen transfer from a heteroatom (4-OH) to carbon C-5 involving a solvent molecule, unlike for the other derivatives, and it is known that proton transfer from the γ position of enones or to the γ position of dienolates is slower than the corresponding reactions at the α positions. The methoxypyrimidinones **5** and **6** are unstable in solution; in water they hydrolyze to uracil and in pyridine a complex mixture of transmethylated pyrimidine derivatives appeared during heating. However, the signals from 5-H and 6-H of the parent compounds **5** and **6** were well separated from the signals of the side-products in the ^1H NMR spectra and careful integration of the signals made it possible to determine the exchange rates. The reaction mixtures of compounds **5** and **6** in pyridine were analyzed by GC–MS. The retention times and fragmentation patterns were compared with authentic samples of other methylated uracil derivatives. Uracil, 2,4-dimethoxypyrimidine, and 1,3-dimethyluracil were found in the reaction mixtures of both methoxypyrimidinones **5** and **6**, along with the parent compounds and two unknown compounds with masses corresponding to dimethylated uracils (a combination of *N*- and *O*-methylated derivatives).

We also studied the isotopic exchange reaction of cytosine (**7**) and isocytosine (**8**). The exchange rate is 130 times higher for isocytosine than for cytosine, which is consistent with the formation of a stabilized transition state for hydrogen transfer from oxygen to carbon C-5. The exchange rate of isocytosine is four times higher in pyridine than in D_2O, but the exchange rate of cytosine is 12 times lower in pyridine than in D_2O. These findings support the hypothesis of hydrogen transfer from oxygen O-4 to carbon C-5 with the participation of a solvent molecule.

Calculations

We performed geometry optimizations of all the possible tautomers/rotamers (including C-5 tautomers) of all the studied compounds at the B3LYP/cc-pVTZ/CPCM level of theory; the resultant relative free energies (referenced to the most stable structure) are summarized in Tables 4 and 5. The relative stabilities of the uracil tautomers/rotamers are the same as found previously.[27] The 3,5-tautomer of uracil is 15.61 kcal/mol less stable than the canonical 1,3-tautomer; similar values were found also for the hydroxy keto and dihydroxy tautomers. These values are well below the barriers for 5-H isotopic exchange determined experimentally. Therefore these tautomers may be involved in the reaction. The relative stability of the C-5 tautomer of compound **3** is very similar to that of uracil, the C-5 tautomer of **4** is slightly more stable, and the C-5 tautomers of **5** and **6** are less stable than the corresponding tautomer of uracil. The relative stabilities of the C-5 tautomers of these compounds correlate very well with the barriers to isotopic exchange determined experimentally. The relative stability of

Table 4. Calculated (B3LYP/cc-pVTZ/CPCM) relative free energies (referenced to the lowest energy structure) of the tautomers/rotamers[a] of compounds **1**, **4**, **7**, and **8**.

	12	13	14	23	24	25	34	35	45
1									
	a 19.48	0.00	c 10.29	a 12.88	ac 16.83	a 32.13	c 18.73	15.61	c 31.80
	b 15.64		d 12.71	b 16.43	ad 18.72	b 31.95	d 17.06		d 34.67
					bc 17.10				
					bd 18.82				
4									
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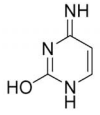
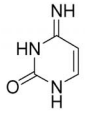
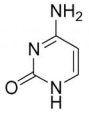
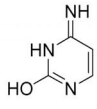
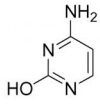
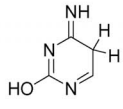
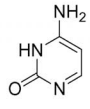
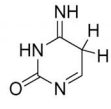
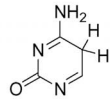
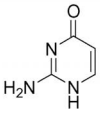
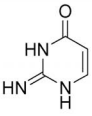
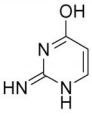
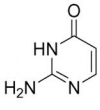
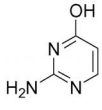
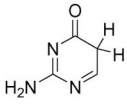
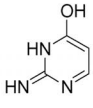
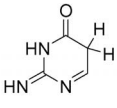
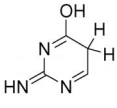
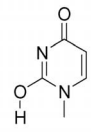
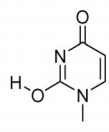
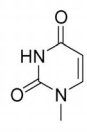
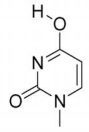
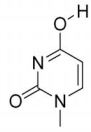
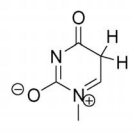
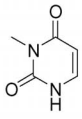
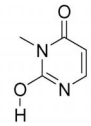
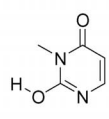
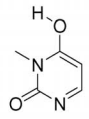
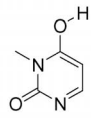
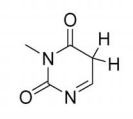
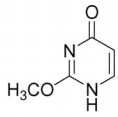
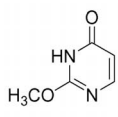
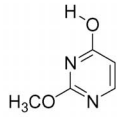
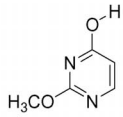
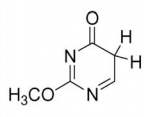
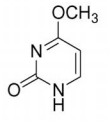
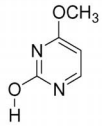
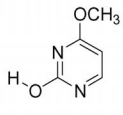
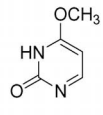
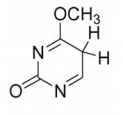
	b 16.36		d 12.99	b 17.24	ad 19.23	b 29.96	d 16.92		d 32.45
					bc 17.75				
					bd 18.82				
7									
	ac 26.47	c 7.57	0.00	ac 22.42	a 6.15	ac 36.98	3.39	c 21.96	19.44
	ad 27.75	d 6.45		ad 21.11	b 6.28	ad 38.41		d 21.24	
	bc 22.79			bc 26.22		bc 36.76			
	bd 23.97			bd 24.80		bd 38.08			
8									
	3.68	a 6.55	ac 18.92	0.00	c 4.30	15.35	ac 24.92	a 16.80	ac 31.48
		b 6.29	ad 21.37		d 6.18		ad 23.12	b 18.97	ad 34.22
			bc 16.62				bc 27.43		bc 31.56
			bd 19.00				bd 34.34		bd 34.35

[a] a denotes a rotamer with torsion angle N1–C2–X–H close to 0° (X is oxygen or nitrogen atom), b N1–C2–X–H ca. 180°, c N3–C4–X–H ca. 0°, d N3–C4–X–H ca. 180°.

the C-5 tautomer of compound **2** is much lower because of its zwitterionic structure; its relative energy of 31 kcal/mol is close to the experimental barrier, which suggests that this tautomer may not be involved in the exchange reaction (in agreement with our experimental observations). The relative energy of the C-5 tautomer of cytosine is 4 kcal/mol higher than that of isocytosine, which is in perfect agreement with the experimentally determined reaction barriers. The optimized geometries of the C-5 tautomers are planar or almost planar structures; the torsion angle C2–N3–C4–C5 is usually within ±2°.

We were also looking for transition-state structures in the formation of the C-5 tautomer of uracil. We found transition-state structures of the 3,4-U to 3,5-U interconversion reaction (the shift of a hydrogen atom from oxygen O-4 to carbon C-5). In a vacuum, the transition-state free-energy barrier is much higher than the experimental value (53.4 kcal/mol). The first-order saddle-point structures on the reaction coordinate of the uracil complexes with DMSO, pyridine, and 4-methoxypyridine were localized by using the QST3 method and the free-energy barriers to transition were calculated in vacuo and with a polarizable continuum model of solvation. As an example, the assumed transition state of the uracil–pyridine complex is visualized in Figure 4. As can be seen in Table 2, the free-energy barrier heights calculated in vacuo for the organic solvents are around 9 kcal/mol higher than the experimental values. We expected a lowering of the barriers if the solvent effects were treated correctly because the transition states have higher dipole moments than the starting uracil and therefore they can be stabilized by polar solvents. When single-point calculations with an implicit solvation model were

Table 5. Calculated (B3LYP/cc-pVTZ/CPCM) relative free energies (referenced to the lowest-energy structure) of the tautomers/rotamers[a] of compounds **2**, **3**, **5**, and **6**.

		1	2a	2b	3	4c	4d	5
2		-						
		-	21.57	16.77	0.00	10.65	14.12	28.81
3					-			
		0.00	12.83	16.87	-	19.34	17.12	15.70
5			-	-				
	a	7.44	-	-	0.00	4.85	6.72	20.66
	b	2.87	-	-	4.32	5.14	6.97	18.91
6						-	-	
	c	0.00	6.83	7.08	8.53	-	-	19.78
	d	3.07	7.07	6.83	6.16	-	-	24.38

[a] a denotes a rotamer with torsion angle N1–C2–X–H close to 0° (X is oxygen or nitrogen), b N1–C2–X–H ca. 180°, c N3–C4–X–H ca. 0°, and d N3–C4–X–H ca. 180°.

performed, the free-energy barriers for DMSO, pyridine, and 4-methoxypyridine were reduced significantly and were much closer to the experimental barriers.

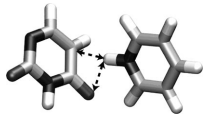


Figure 4. Transition-state structure for the hydrogen transfer from oxygen O-4 to carbon C-5 with the participation of a pyridine molecule.

The situation is different for complexes of uracil and its derivatives with water. A saddle point with a vibrational mode corresponding to the transfer of proton 1 to the water molecule and proton 2 from water back to uracil was found (see Figure 5). The calculated free-energy barrier heights are significantly higher (ca. 15 kcal/mol) than the experimental ones although the overall trend is retained (Table 3). One can also imagine a different transition state in which only one proton is transferred during the isomerization. Such a "single proton" TS (Figure 5) was found at the AM1 level of theory but was not confirmed at the B3LYP/cc-pVTZ level. The "single proton" structure was energetically rich and optimized to the "2-proton" state in all the calculations. The implicit solvent description was thus tested for the uracil–water complex with similar results. Although it was possible to converge this calculation, it did not yield a single-proton TS. These findings are in agreement with previous studies on the mechanism of the keto–enol tautomerism of nucleic acid bases,[19,44–46] pyridine derivatives,[38–41] and other compounds[43,51] in aqueous media. It was found that the tautomerization reactions proceed preferentially by a multiple proton-transfer mechanism with the assistance of two to three water molecules.

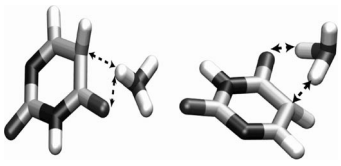


Figure 5. Transition-state structures for the hydrogen transfer from oxygen O-4 to carbon C-5 including a water molecule. The single-proton mechanism (left) and the double-proton mechanism (right).

Conclusions

We have studied the mechanism of the isotopic exchange reaction of the 5-H hydrogen of uracil derivatives. It was evidenced that the C-5 tautomers are the key intermediates of the exchange reaction. In a water environment, a general base catalysis for the isotopic exchange reaction was observed. In organic solvents, we proposed a mechanism that

involves the participation of a solvent molecule. We have shown that C-5 tautomers of uracil and its methylated derivatives are possible and should be considered in tautomeric equilibria in solution. Considering the number of uracil bases in RNA polymers, the occurrence of the C-5 tautomer could play a role in biologically important processes. We speculate that the methylation of uracil at the 5-position (thymine biosynthesis) probably decreases the formation of the C-5 tautomer, which could have played a role in the natural selection of the thymine base as a DNA building block. We calculated the relative free energy of the thymine 3,5-tautomer and it was less stable than that of uracil by 4 kcal/mol and the reaction barrier is probably much higher because of the steric hindrance of the methyl group. The mechanism of the 5-H isotopic exchange reaction with the occurrence of the C-5 tautomers of compounds **1**–**6** is depicted in Figure 2 and Scheme 1. It follows from the suggested mechanism that the isotopic exchange reaction of compound **2** is not likely because of the high energy of the C-5 tautomer intermediate. The reaction barrier for the formation of the C-5 tautomer of compound **6** is too high because solvent molecules cannot access an active hydrogen easily, which could then be transferred to the carbon at the 5-position. We have shown that the hydrogen-to-deuterium exchange reaction is much faster in suitable nucleophilic solvents than in water. These findings could be used for the tritium labeling of pyrimidine nucleic acid bases.

Experimental Section

Starting Material: Compounds **1**–**4**, **7**, and **8** were purchased commercially. Compounds **5**[52] and **6**[53] were prepared according to literature procedures. The prepared compounds and all of the reaction mixtures were analyzed using a 6890N gas chromatograph coupled to a 5975B quadrupole mass spectrometer and equipped with a Zebron ZB-5 fused silica capillary column (30 m × 0.25 mm, 0.10–3.00 μm). The carrier gas was helium at 1 mL/min. The injector was operated in split mode (100:1) at 230 °C. The temperature program was 60 °C (4 min), then 10 °C/min to 320 °C (10 min). The standard 70 eV mass spectra were recorded in the mass range of 25–800 u; a 4 min solvent delay was used. The temperatures of the transfer line, ion source, and quadrupole were 280, 230, and 150 °C, respectively.

Exchange Reactions: The pyrimidine derivatives (2 mg) were dissolved in solvent (500 μL) and D_2O (30 μL, 100 equiv.) with 1% of the sodium salt of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added. Alternatively, the samples were dissolved in buffer and the pD was then measured. The samples were sealed in NMR tubes and heated to 100 °C in an oil bath or directly in the NMR spectrometer. The ^{1}H NMR spectra of the samples were acquired periodically (with an appropriate delay between them). The NMR spectra were measured with a Bruker Avance II 500 instrument (499.84 MHz for ^{1}H). At least 10 spectra were recorded for each sample. The amount of ^{1}H at the 5-position was determined by integration of the 5-H signal relative to the integral intensity of the ^{1}H signal of 6-H. The signal of DSS was used for control integration. The rate constants were obtained from the plots of the logarithm of the 5-^{1}H concentration versus time.

Calculations: All of the structures were optimized at the DFT level of theory using the B3LYP functional[54,55] and cc-pVTZ basis set.[56–58] The solvent effects were treated implicitly by the CPCM method.[59,60]

The QST3 optimization method[61,62] was applied in the search for the transition states in vacuo with one explicit solvent molecule, that is, the structures of the reactants, products, and an estimate of the transition state were used. AM1 was used to pre-optimize the transition state. Once the transition state (a complex of a pyrimidine derivative with a solvent molecule) had been optimized in vacuo, we performed a single-point energy and frequency calculation with the CPCM method of solvation. The $\Delta G^{\#}_{calcd.}$ values are differences of the free energies of the transitions states and the sum of separated reactant free energies. The vibrational frequencies and free energies were calculated for all of the optimized structures and the stationary-point character (a minimum or a first-order saddle point) was thus confirmed. Gaussian 09[63] was used throughout this study.

Supporting Information (see also the footnote on the first page of this article): ^{1}H NMR spectrum of uracil in dry DMSO, cartesian coordinates, and electronic and free energies for all calculated compounds at the B3LYP/cc-VTZ level of theory.

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