Determination of the Tautomeric Equlibrium of Ψ-Uridine in the Basic Solution

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The pD-dependent ^{13}C shift and $^{3}J_{CH}$ of Ψ-uridine have shown that the pK_a of its N1 and N3 are 9.6 and 9.3, respectively. For the monoanionic Ψ-uridine, the N1-anion population (64%) is favored over the N3-anion (36%), suggesting a larger preference of the population for N1−Hg^{2+} complex for Ψ-uridine over its N3−Hg^{2+} complex in the neutral pH, which has also been proven by the similarity of the pD-dependent titration curve of Ψ-uridine and its N3-methyl-Ψ-uridine. Finally, ab initio molecular orbital calculations with a 6-31G** basis set (Gaussian 94) have been used to explain why the N1-anion tautomer of Ψ-uridine is thermodynamically more stable than the N3-anion tautomer of Ψ-uridine, which cannot be corroborated by the results of the calculations at 6-31+G** and 6-31++G** levels. The fact that N1 of Ψ-uridine (pK_a 9.3) is more acidic than N3 (pK_a 9.6) implies that the N1−H will compete favorably for any potential hydrogen bonding with a complementary nucleotide over the N3−H.

Introduction

Pseudouridine (Ψ-uridine) is a *C*-nucleoside occurring ubiquitously as a minor component in various tRNAs.[1] The special enzymological properties, the mode of biosynthesis, and the structural and the biological role of Ψ-uridine (5-β-D-ribofuranosyluracil) are rather unusual[2] for a nucleoside. It is distinguished by a carbon−carbon bond from C5 of uracil to C1′ of the β-D-pentoribofuranose moiety. Certain tRNAs deficient in Ψ-uridine are incapable of participating in protein synthesis.[1] A notable feature of Ψ-uridine is that six different tautomeric forms are theoretically possible because of the lactam ⇄ lactol tautomerism, all of which except for the "diketo form" (i.e. **1**) have been ruled out by Chambers et al.[3] on the basis of UV and IR. This conclusion can also be supported through the present work on the basis of ^{13}C NMR chemical shifts found for the urethane carbonyl groups at δ 153.2 ppm (for C2) and 165.7 ppm (for C4) for Ψ-uridine (**1**), which are also consistent with the ^{13}C chemical shifts of the carbonyl carbons in the analogous 1,3-dimethyluracil:[4] δ(C2) = 151.8 ppm and δ(C4) = 163.2 ppm. The presence of the two dissociable urethane protons at N1 and N3 however makes it difficult to ascertain the site of complexation for metal ions such as Hg^{2+}, which is known to bind to certain pyrimidine-2,4-dione[5a,b] residues in a polynucleotide chain and disrupt the tetiary interaction. We report here a study of the tautomeric mixture of the two monoanions **2** and **3**, as models to study the tautomeric preference in Ψ-uridine (**1**) when it is complexed with a heavy metal ion[5b] in neutral pH, which cannot be studied as such because of their very poor stability and solubility in the aqueous solution for the NMR measurement. In addition, we establish some unique structural features of Ψ-uridine (**1**)[6] by its comparison with its N1-methylated **4**[7] and N3-methylated **5**[8] analogues.

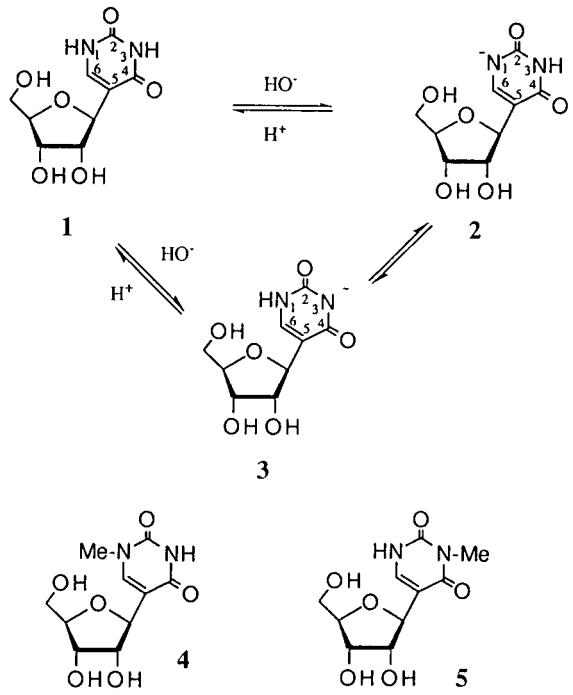

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Table 1. Experimental $\delta(^{13}C)$ and $^3J_{CH}$ Values for 1, 4, and 5 Gave the Corrected Values of 2 and 3 After Taking into Account the Substitution Corrections (Δ_1 and Δ_3) Owing to the Replacement of a Methyl for a Hydrogen, Thereby Giving an Estimate of the Percentage of the N1-Anion 2

chemical shift and coupling constant[a]	Ψ-uridine (**1**)		1-methyl-Ψ-uridine (**4**)		3-methyl-Ψ-uridine (**5**)						
	neutral	basic	neutral	basic	neutral	basic	Δ_1[b]	Δ_3	N1-anion tautomer **2**[e]	N3-anion tautomer **3**[f]	% N1-anion **2**
$\delta(C_4)$	165.7	170.8	165.4	175.5	165.1	167.0	−0.3[b]	−0.6[b]	167.6	175.8	61.0
$\delta(C_5)$	110.9	108.5	111.0	111.0	110.4	106.8	0.1[b]	−0.5[b]	107.3	110.9	66.6
$\delta(C_6)$	141.9	152.6	146.8	145.7	139.6	156.3	4.9[b]	−2.3[b]	158.6	140.8	66.3
$^3J_{C2,H6}$	9.4	12.2	8.3	7.3	9.4	14.5	−1.1[b]	0.0[c]	14.5	8.3	62.3
							−1.0[d]	0.0[c,d]	14.5	8.4	63.0

[a] δ in ppm and coupling constants in hertz. $\delta(C_2)$ cannot be used because of a too small difference in chemical shifts between N1-anion **2** and N3-anion **3** (ref 7a). [b] Δ_1 and Δ_3 are correction terms which have been elucidated using $\Delta_1 = P'_{N1-Me} - P'_{NH}$ and $\Delta_3 = P'_{N3-Me} - P'_{NH}$ (see the main text). [c] No correction term is used because there is no methyl group involved in the coupling pathway (see ref 7a). [d] The sum of the $^3J_{C2,H6}$ and $^3J_{C6,H1'}$ of the methylated analogues **4** and **5** in neutral pH and compared with the sum of the $^3J_{C2,H6}$ and $^3J_{C6,H1'}$ of Ψ-uridine in neutral pH according to ref 7b to obtain the correction of the coupling constant. [e] The chemical shift and the coupling constants for N1-anion are obtained after subtracting the correction factor (Δ_1) from the chemical shift or the coupling constant of **5** in basic solution. [f] The chemical shift and the coupling constants for N3-anion are obtained after subtracting the correction factor (Δ_3) from the chemical shift or the coupling constant of **4** in basic solution.

Discussion

(A) Estimation of the Dynamic Tautomeric Population of Monoanionic Pseudouridine. It has been concluded by UV titration studies[3] that the N1-anion tautomer of Ψ-uridine **2** is greatly favored over the N3-anion tautomer **3** in a ratio of ~4:1 at 298 K. We herein show that this ratio is 6.4:3.6 by the use of pD-dependent ^{13}C chemical shifts[9] as well as three-bond $^{13}C,^1H$ spin−spin coupling constant analysis.[10]

The relative rate of the dynamic tautomeric proton shift is usually very fast in the NMR time scale; hence one observes only a weighted average of $\delta(^{13}C)$ or $^3J_{CH}$ from the participating tautomers. In the procedure for the determination of the tautomeric equlibrium by NMR, both the $\delta(^{13}C)$ and $^3J_{CH}$ of the pure participating tautomers are required to analyze their population in the actual equilibrium. Since the individual anions cannot be isolated because of the rapid equilibrium, we have used the N1-methyl-(**4**[7]) and N3-methyl-Ψ-uridines (**5**[8]) to estimate the $\delta(^{13}C)$ and $^3J_{CH}$ values for each of the two tautomeric monoanions **2** and **3**.

The following general equation[9,10] has been used to calculate the population of the N1-anion tautomer **2**

$$[\%\text{N1-anion}] = \frac{P_{obs} - [P_{N1-Me} - \Delta_1]}{[P_{N3-Me} - \Delta_3] - [P_{N1-Me} - \Delta_1]} \quad (1)$$

where P_{obs}, P_{N1-Me}, and P_{N3-Me} are respectively the observed $\delta(^{13}C)$ and $^3J_{CH}$ values of the uracil-5-yl carbons in basic solution for Ψ-uridine (**1**) and the *N*-methylated analogues **4** and **5** in basic solution.

Because of the use of N-substituted analogues **4** and **5** in basic solution for estimation of the chemical shifts and $^3J_{CH}$ of the two monoanionic species **2** and **3**, any change in the chemical shifts and the $^3J_{CH}$ values due to the methyl group must be taken into account by making the appropriate corrections (Δ_1 for N3 tautomer and Δ_3 for the N1 tautomer). At neutral pH, there is no tautomeric equilibrium in Ψ-uridine (**1**), and the observed change in the chemical shift and the coupling constant by replacing the proton in Ψ-uridine (**1**) with a methyl to give **4** and **5** is entirely due to the methyl substituent. Therefore Δ_1 is defined as $\Delta_1 = P'_{N1-Me} - P'_{NH}$ and $\Delta_3 = P'_{N3-Me} - P'_{NH}$, where P'_{NH}, P'_{N1-Me}, and P'_{N3-Me} are respectively the experimental NMR data for Ψ-uridine (**1**) and the N1- and N3-methylated derivatives at neutral pH. The results are presented in Table 1.

Wasylishen and Tomlinson[10a] first studied the imidazole tautomerism problem on the basis of the $^3J_{CH}$ coupling constant analysis, and later Schumacher et al.[10b] employed this on the purine tautomerism. They noticed that the value of a particular three-bond coupling constant considerably decreases in magnitude if an N-substituent is present in the coupling pathway. This is consistent with our data for **4** where the methyl group is present in the coupling pathway of C_2 with H_6 (i.e. $^3J_{C2,H6}$), and it is only this coupling constant which decreases by about 1.1 Hz by methylation on N1 in Ψ-uridine (**1**). All other coupling constants (i.e. $^3J_{C4,H6}$, $^3J_{C4,H1'}$, and $^3J_{C6,H1'}$) remain more or less unchanged. On the basis of the chemical shifts of the methylated analogues **4** and **5** and the Δ_1 and Δ_3 correction terms (eq 1), we can derive the chemical shifts and $^3J_{CH}$ values for the two monoanionic N1 and N3 tautomers of Ψ-uridine **2** and **3** (Table 1).

Clearly, these methods[10a] are only valid when the corrected NMR data (i.e. the carbon chemical shift and the coupling constant) for tautomers are sufficiently different. This is consistent with our own observation on **2** and **3** that the difference between their C_2 chemical shifts (161.2 ppm for the N1-anion **2** and 161.3 ppm for the N3-anion **3**) is only 0.1 ppm, and this is too small to be used for determination of their relative population: hence the C_2 chemical shifts were rejected for the calculation of the tautomeric population. The ^{13}C chemical shift differences in C_4 (8.2 ppm), C_5 (3.6 ppm), and C_6 (17.8 ppm) between the two tautomers **2** and **3** are, on the other hand, significant enough to be used in eq 1. The difference in the coupling constants ($^3J_{C4,H6} = 0.8$ Hz, $^3J_{C4,H1'} = 1.5$, Hz and $^3J_{C6,H1'} = 0.2$ Hz) between the two tautomers (**2** and **3**) was also considered to be too small to be of any practical use. Only the $^3J_{C2,H6}$ which is

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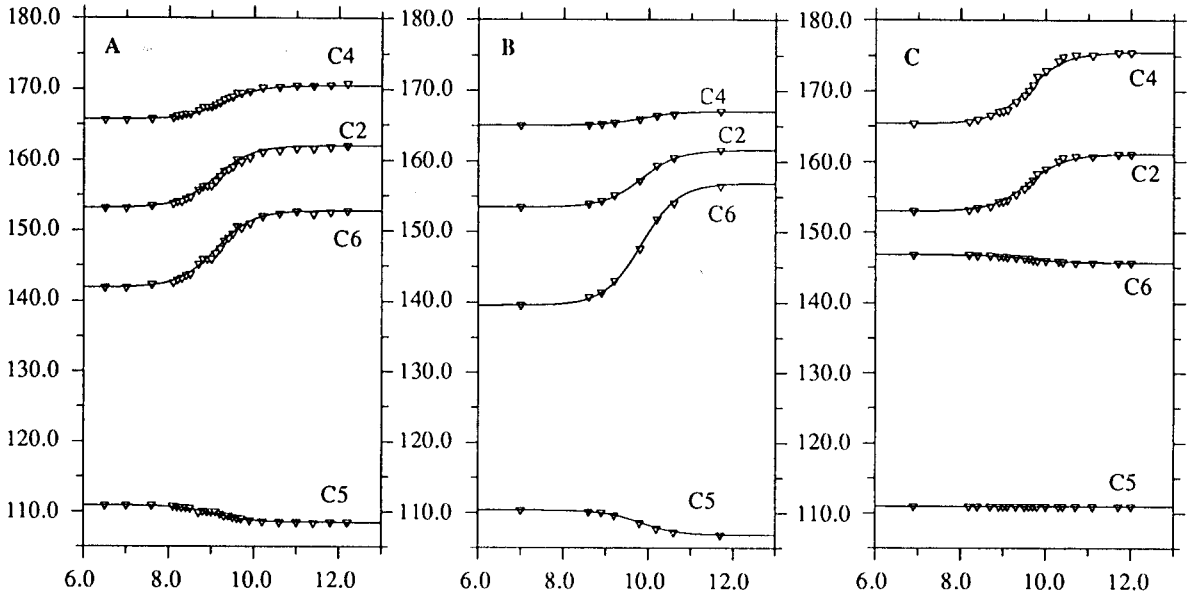

Figure 1. ^{13}C chemical shift−pH profiles of aromatic carbons of Ψ-uridine (**1**) (panel A), 1-methyl-Ψ-uridine (**4**) (panel C), and 3-methyl-Ψ-uridine (**3**) (panel B) at 298 K.

sufficiently different in both tautomers (6.1 Hz) is used to calculate the population of the N1-anion tautomer **2**. We find good agreement between the two methods (the first one is based on the difference in chemical shifts, and the other relies on the difference in coupling constants) in estimating the relative populations of the two tautomers (average %N1-anion tautomer ∼64%), which are presented in Table 1.

(B) Estimation of pK_a of N1 and N3 in Ψ-Uridine (1) from the Population of Tautomers Derived from the NMR Data. The tautomeric distribution (K_T)[3,11] in the alkaline pH is determined by the pK_as of the N1 and N3 in Ψ-uridine (**1**), which can be obtained from the NMR data by using eqs 2 and 3.

$$pK_{N3} = pK_{\Psi} + \log(1 + K_T) \quad (2)$$

$$pK_{N1} = pK_{N3} - \log(K_T) \quad (3)$$

where, pK_{N3} is the pK_a of N3−H in Ψ-uridine (**1**), pK_{N1} is the pK_a of N1−H in Ψ-uridine (**1**), and pK_Y is the average pK_a of Ψ-uridine (**1**). The ΔpK between the two tautomers is expressed as log K_T, where K_T = ([N1-anion]/[N3-anion]) = 1.8 and ΔpK = log K_T = 0.3. This means that N3 of Ψ-uridine (**1**) is a slightly stronger base than N1, and hence we have 64% of N1−H tautomer for the monoanionic species of Ψ-uridine (**1**). *This also means that the population of a potential N1−Hg^{2+} complex will be ∼64%, whereas the N3−Hg^{2+} complex will be the other ∼36%.* This new result contradicts an earlier spectrophotometric determination[3] of the tautomer population of the monoanionic species of Ψ-uridine (**1**), which has shown that K_T = ([N1-anion]/[N3-anion]) = 4.0 and ΔpK = 0.6, thereby showing an 80% population for the N1−H tautomer for the monoanionic species of Ψ-uridine (**1**).

It is noteworthy that this observed difference in the ratio of the tautomer population obtained by our NMR result (K_T = 1.8 and ΔpK = 0.3) compared with the older spectrophotometric method (K_T = 4 and ΔpK = 0.6) makes a difference in the estimation of the pK_as of N1 and N3 of Ψ-uridine (**1**), which can be seen in the following discussion.

The ^{13}C chemical shift (heteronuclear HMBC experiment[12] with gradients) titration as a function of pD at 298K for Ψ-uridine (**1**) (Figure 1A) showed sigmoidal curves, which was subsequently used for Hill plots[13] (Figure 2, Tables 5−7) to extract their pK_as. The average pK_a of Ψ-uridine (**1**) was thus found to be 9.2 ± 0.1, in comparison with the spectrophotometric measurements,[3a] which gave a pK_a 9.0. Substituting our average pK_a of Ψ-uridine (**1**) as 9.2 in eq 2 gave the pK_a of 9.6 for N3 in Ψ-uridine (**1**) (see eq 4). Similarly, applying the result of eq 4 into eq 3 gave the pK_a of 9.3 for N1 in Ψ-uridine (**1**) (see eq 5).

$$pK_{N3} = 9.2 + \log(1 + 1.8) = 9.6 \quad (4)$$

$$pK_{N1} = 9.6 - \log(1.8) = 9.3 \quad (5)$$

(C) Structure of Ψ-Uridine (1) by NMR and *ab Initio* Calculations. Similarly, the ^{13}C chemical shift (heteronuclear HMBC experiment[12] with gradients) titration as a function of pD at 298 K for 1-methyl-Ψ-uridine (**4**) (Figure 1C) and 3-methyl-Ψ-uridine (**5**) (Figure 1B) showed sigmoidal curves, which were subsequently used for Hill plots (Figures 2 and 3 and Tables 5−7) to extract their pK_as, which are found to be 9.6 and 9.8, respectively. These pK_as (9.6 and 9.8) suggest that the N3-anion would be more stable than the N1-anion for methylated Ψ-uridines. In this context, it should be emphasized that the pK_a of N1 in Ψ-uridine (**1**) derived by NMR method (see above) is 9.3, not 9.8 as found for 3-methyl-Ψ-uridine (**5**), whereas the pK_a of N3 in Ψ-uridine (**1**) is the same (i.e. 9.6) as that of 1-methyl-Ψ-uridine (**4**).

We have performed ab initio calculations of the neutral Ψ-uridine (**1**) and its N1 and N3 anions **2** and **3**,

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(13) Wyman, J.; Gill, S. J. *Binding and Linkage. Functional Chemistry of Biological Macromolecules*; University Science Books: Mill Valley, CA, 1990, p 330.

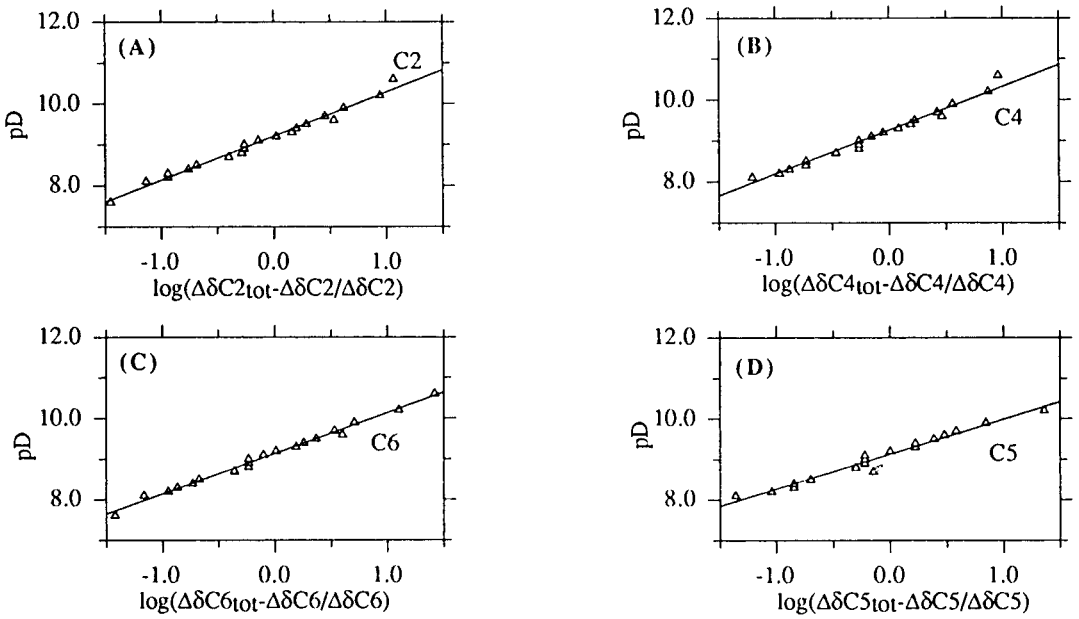

Figure 2. Hill plots for Ψ-uridine (**1**) to determine its pK_a value (see Experimental Section and Tables 5−7).

Table 2. Bond Lengths (Å) of Optimized Ψ-Uridine and Its Monoanions 1−3 Using HF/6-31G, HF/6-31+G**, and HF/6-31++G** Basis Sets and SCRF Mimicking the Aqueous Environment (ϵ = 78.3)**

	Ψ-uridine **1**			N1-anion of Ψ-uridine **2**			N3-anion of Ψ-uridine **3**		
bonds	6-31G**	6-31+G**	6-31++G**	6-31G**	6-31+G**	6-31++G**	6-31G**	6-31+G**	6-31++G**
N1−C2	1.363	1.361	1.361	1.337	1.333	1.333	1.390	1.383	1.383
N1−C6	1.378	1.378	1.378	1.343	1.348	1.348	1.370	1.372	1.372
C2−N3	1.370	1.369	1.369	1.393	1.390	1.390	1.331	1.330	1.330
C2−O2a	1.198	1.201	1.201	1.229	1.236	1.236	1.234	1.242	1.242
N3−C4	1.388	1.388	1.388	1.378	1.378	1.379	1.359	1.362	1.362
C4−O4a	1.198	1.201	1.201	1.219	1.221	1.221	1.225	1.227	1.227
C4−C5	1.461	1.461	1.461	1.433	1.433	1.433	1.483	1.482	1.482
C5−C6	1.331	1.333	1.333	1.364	1.365	1.365	1.328	1.331	1.331
C5−C7	1.505	1.506	1.506	1.506	1.507	1.507	1.505	1.508	1.508
C7−O8	1.388	1.389	1.389	1.400	1.400	1.400	1.396	1.395	1.395
O8−C9	1.401	1.403	1.403	1.393	1.396	1.396	1.398	1.403	1.403
N1−H1a	0.994	0.995	0.995				0.991	0.992	0.992
N3−H3a	0.996	0.997	0.997	0.994	0.994	0.995			
C6−H6a	1.071	1.072	1.072	1.078	1.075	1.075	1.072	1.073	1.073
C7−H7a	1.089	1.089	1.089	1.091	1.090	1.090	1.090	1.089	1.089
C7−H7b	1.089	1.089	1.089	1.091	1.090	1.090	1.090	1.089	1.089
C9−H9a	1.087	1.087	1.087	1.089	1.088	1.088	1.088	1.087	1.087
C9−H9b	1.080	1.080	1.080	1.083	1.082	1.082	1.081	1.080	1.080
C9−H9c	1.087	1.087	1.087	1.089	1.088	1.088	1.088	1.087	1.087

respectively, to understand which of these two anions is most stable in terms of their total HF energies, electronic distributions, and bond lengths.

The structures Ψ-uridine (**1**) and its N1-anion **2** and N3-anion **3** were first completely optimized using HF/6-31G** basis in a self-consistent reaction field (SCRF) with the dielectric constant of 78.3 to mimic the aqueous environment. The optimized geometries were subsequently used as input for full optimization at the HF/6-31+G** level with SCRF, where s- and p-type functions are used for the correct description of the negative charge in an anion as in **2** or **3**. This calculation was further extended by using the HF/6-31++G** basis set (with SCRF) in order to include diffusion function to hydrogens (see Experimental Section for details).

An inspection of the optimized bond lengths and bond angles in **1**, **2**, and **3** (Tables 2 and 3) at all three levels, HF/6-31G**, HF/6-31+G**, and HF/6-31++G**, shows that there are only very small differences in these three basis sets. A comparison of the ESP charges of **1**, **2**, and **3** (Figure 5) at the three levels shows that the charges for the anions change more than the charges for the neutral **1**, which is consistent with the fact that the 6-31+G** and 6-31++G** basis sets are necessary for the description of anions.

The carbonyl bond lengths are longer for C2═O than C4═O in both N1- and N3-anions in all the three basis sets suggesting the anion delocalization is relatively more centered at C2═O (Table 2). Similarly, the double-bond character of C2−N3 is more pronounced than that of N3−C4 in N3 anion, and for the N1-anion the N1−C2 is shorter than the N1−C6 suggesting that both N3- and N1-anion prefers to delocalize to the C2═O. This is also consistent with relatively higher point charges for oxygens of C2═O than C4═O (Figure 5).

Remarkable changes were noted in the total and relative HF energies at all three levels of calculations as shown in Table 4. The relative stabilization of the N1- and N3-anions with respect to the neutral Ψ-uridine (**1**) shows that the N1-anion **2** is energetically more favored than the N3-anion **3** by 2.2 kcal/mol in the 6-31G** calculations (Table 4), which is in excellent agreement

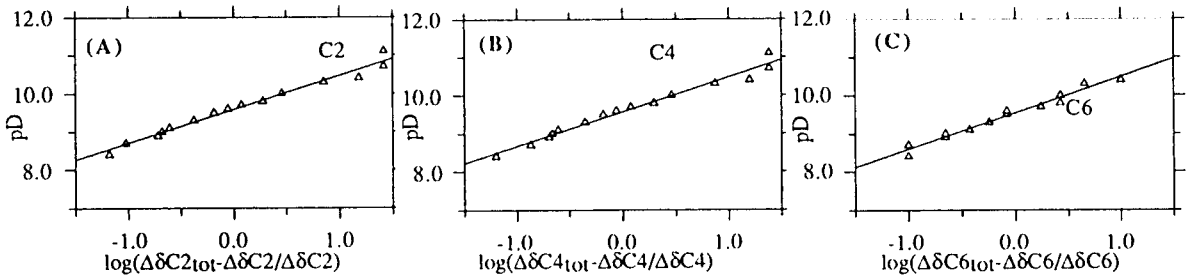

Figure 3. Hill plots for 1-methyl-Ψ-uridine (**4**) to determine its pK_a value (see Experimental Section and Tables 5−7).

Table 3. Bond Angles (deg) of Optimized Ψ-uridine and Its Monoanions 1−3 Using HF/6-31G, HF/6-31+G**, and HF/6-31++G** Basis Sets and SCRF Mimicking the Aqueous Environment (ϵ = 78.3)**

	Ψ-uridine **1**			N1-anion of Ψ-uridine **2**			N3-anion of Ψ-uridine **3**		
bonds	6-31G**	6-31+G**	6-31++G**	631G**	6-31+G**	6-31++G**	6-31G**	6-31+G**	6-31++G**
N1−C2−N3	113.46	113.66	113.66	117.38	117.65	117.65	118.52	118.69	118.69
N1−C2−O2a	123.75	123.72	123.73	125.78	125.65	125.65	116.49	116.69	116.69
N1−C6−C5	122.47	122.51	122.51	127.98	127.77	127.77	120.18	120.40	120.40
N1−C6−H6a	115.92	115.91	115.91	114.63	114.66	114.66	117.04	116.94	116.94
H1a−N1−C2	115.98	116.15	116.15				116.42	116.79	116.79
H1a−N1−C6	120.54	120.44	120.45				120.87	120.61	120.61
C2−N3−H3a	115.80	115.90	115.89	116.31	116.31	116.31			
C2−N3−C4	127.73	127.56	127.56	127.42	127.16	127.16	121.57	121.61	121.61
N3−C2−O2a	122.79	122.61	122.61	116.84	116.70	116.70	124.98	124.63	124.63
C2−N1−C6	123.48	123.40	123.40	117.20	117.27	117.27	122.71	122.60	122.60
N3−C4−O4a	120.05	119.77	119.76	119.89	119.56	119.56	121.71	121.24	121.24
N3−C4−C5	114.55	114.73	114.73	113.16	113.49	113.49	119.44	119.40	119.40
H3a−N3−C4	116.48	116.54	116.54	116.27	116.30	116.30			
C5−C4−O4a	125.40	125.52	125.52	126.95	126.95	126.95	118.85	119.36	119.36
C5−C7−O8	108.49	108.43	108.44	110.55	110.47	110.47	109.47	109.22	109.22
C5−C7−H7a	109.63	109.67	109.67	110.24	110.11	110.11	109.86	109.88	109.88
C5−C7−H7b	109.63	109.68	109.67	110.24	110.12	110.12	109.86	109.88	109.88
C6−C5−C4	118.31	118.14	118.14	116.86	116.66	116.66	117.58	117.31	117.31
C6−C5−C7	123.40	123.30	123.32	125.58	125.42	125.42	122.75	122.40	122.40
H6a−C6−C5	121.60	121.58	121.58	117.38	117.57	117.57	122.79	122.66	122.66
C7−C5−C4	118.29	118.56	118.55	117.55	117.91	117.91	119.67	120.29	120.29
C7−O8−C9	114.54	114.73	114.73	113.71	113.84	113.84	114.92	115.16	115.16
O8−C7−H7a	111.12	110.95	110.94	109.83	109.76	109.76	110.64	110.53	110.53
O8−C7−H7b	111.11	110.94	110.93	109.83	109.76	109.76	110.63	110.53	110.53
O8−C9−H9a	111.15	110.93	110.94	111.63	111.37	111.37	111.40	111.10	111.10
O8−C9−H9b	107.41	107.27	107.28	107.87	107.64	107.64	107.71	107.51	107.51
O8−C9−H9c	111.14	110.91	110.93	111.62	111.34	111.34	111.41	111.10	111.10

with the experimentally determined tautomeric ratio of 1.8:1 for **2** and **3** as found by our ^{13}C NMR study. In contrast, the total and relative HF/6-31+G** and HF/6-31++G** energies of Ψ-uridine (**1**) and its two monoanionic tautomeric forms **2** and **3** show that the N3-anion **3** is energetically favored over the N1-anion **1** by only 0.2 kcal/mol, which however is in excellent agreement with the experimentally determined pK_a values of the N1- and N3-methylated Ψ-uridine derivatives (**4** and **5**) (Table 4) but not with the NMR observation that the N1-anion is more stable than the N3-anion in Ψ-uridine (**1**). Two points emerge from this study.

(i) Similarity of the Titration Curve of Ψ-Uridine (1) and Its N3-Methyl Analogue 5. A comparison of the ^{13}C chemical shift−pH profiles for Ψ-uridine (**1**) (Figure 1A) and the methylated analogues (**4** and **5**), Figures 1C and 1B, reveals that they are for all the base carbons (C_2, C_4, C_5, and C_6) of 3-methyl-Ψ-uridine (**5**) identical in evolution with those for Ψ-uridine (**1**), while the ^{13}C chemical shift−pH profile for C_6 in 1-methyl-Ψ-uridine (**4**) is totally inconsistent with that of the titration profile of Ψ-uridine (**1**), thereby suggesting that Ψ-uridine (**1**) has a predominance of the N1-anion tautomer **2**, which is indeed the case (see our NMR results, vide supra).

(ii) The N1-Anion of Ψ-Uridine (1) Is Thermodynamically More Stable Than the N3-Anion. A perusal of pK_as of Ψ-uridine (**1**) show that N1 (pK_a 9.3) is more acidic than N3 (pK_a 9.6). This is consistent with the point charge of −0.566 at N1 and −0.665 at N3 for neutral **1**, and −0.999 at N1 atom in N1-anion and −1.106 at N3 atom in N3-anion derived by ab initio calculations with a 6-31G** basis set[11] (see Figures 5A−C). This shows that the charge at the N1 anion is more delocalized than in N3-anion, which is consistent with the fact that the N1- anion shows more stabilization than the N3-anion by 2.2 kJ/mol in 6-31G** calculations, which is in excellent agreement with the experimentally determined tautomeric ratio of 1.8:1 for the monoanionic Ψ-uridines **2** and **3** by our ^{13}C NMR study. The use of 6-31+G** (Figures 5D−F) and 6-31++G** (Figures 5G−I) calculations on the point charges also shows a very similar trend, but the relative stabilization energy of the N3-anion is reversed (Table 4) in that the N3-anion is slightly more stable (by 0.2 kcal/mol) than the N1-anion, which is consistent with the pK_a values of 1-methyl-Ψ-uridine (**4**) and 3-methyl-Y-uridine (**5**) but in disagreement with the pK_a values of monoanions of Ψ-uridine (**1**).

Conclusion

It is known that Ψ-uridine is present ubiquitously in active tRNA, and certain tRNAs deficient in Ψ-uridine

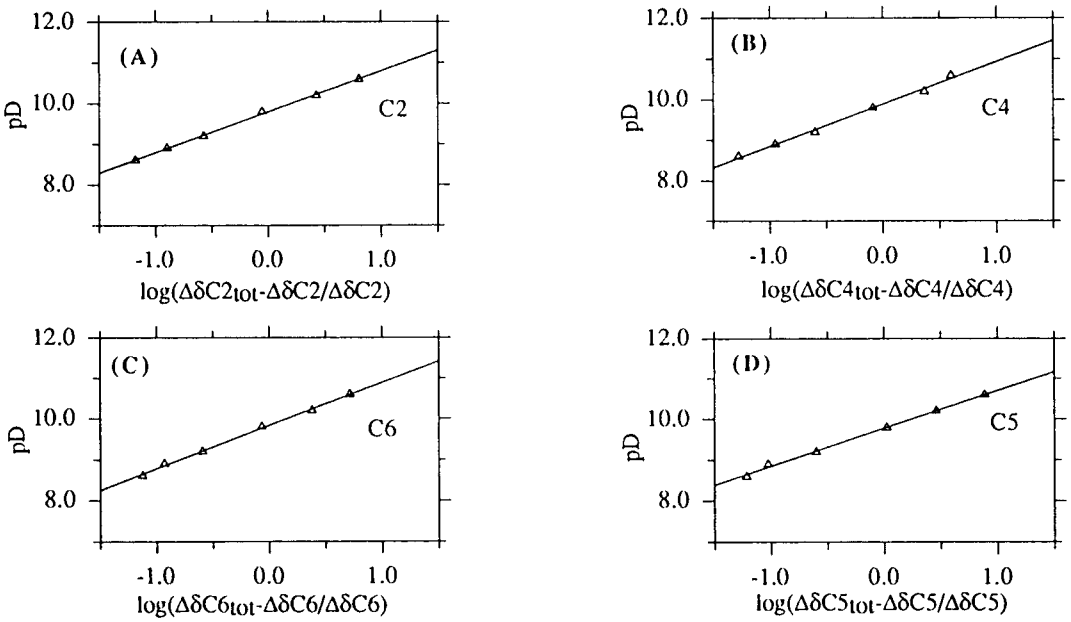

Figure 4. Hill plots for 3-methyl-Ψ-uridine (**5**) to determine its pK_a value (see Experimental Section and Tables 5−7).

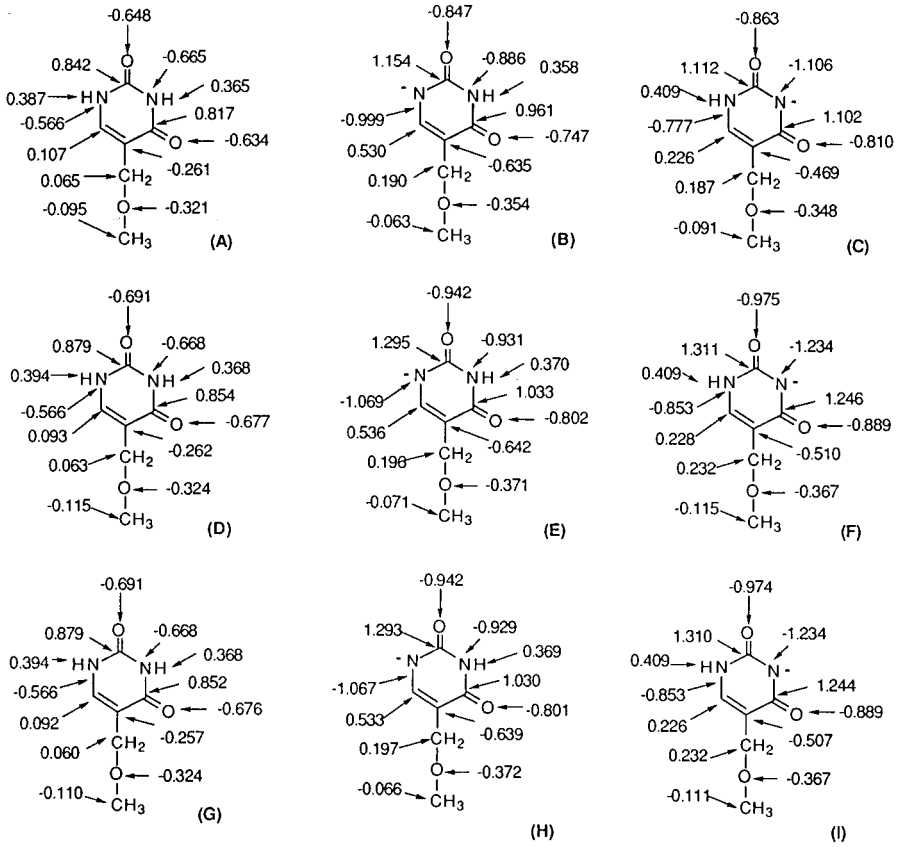

Figure 5. ESP point charges (shown by arrows) of various atoms in the neutral Ψ-uridine (**1**) [panels A, D, and G] and in their tautomeric monoanionic forms (**2**) [panels B, E, and H] and (**3**) [panels C, F, and I] at 6-31G** (panels A−C), 6-31+G** (panels D−F), and 6-31++G** (panels G−I) levels.

are incapable of participating in protein synthesis.[1c] Among all known N- and C-nucleosides, Ψ-uridine (**1**) is the only C-nucleoside which has two lactam functions that can be potentially involved in the folding of the tRNA as well as in the interaction with any ligand. The choice of which of these two lactam functions in Ψ-uridine (**1**) (i.e. N^1H or N^3H) in a Ψ-uridine containing tRNA is actually involved in the hydrogen bonding or binding to a soft or hard metal ion clearly depend upon their relative basicity. We have investigated this by studying the N1- and N3-anion population of Ψ-uridine in the alkaline medium by NMR. It has been found that N1-anion population of Ψ-uridine, formed in the alkaline medium, is favored (64%) over the corresponding N3-anion (36%),

Table 4. Total and Relative Energies of Optimized Ψ-uridine and Its Monoanions 1−3 Using HF/6-31G, HF/6-31+G**, and HF/6-31++G** Basis Sets and SCRF Mimicking the Aqueous Environment (ϵ = 78.3)**

	6-31G**		6-31+G**		6-31++G**	
compd	HF energy[a]	rel energy[b]	HF energy[a]	rel energy[b]	HF energy[a]	rel energy[b]
1	−565.40764	0[c]	−565.42336	0[c]	−565.42362	0[c]
2	−564.84457	353.3	−564.87368	344.9	−564.87389	345.0
3	−564.84109	355.5	−564.87403	344.7	−564.87424	344.8

[a] Energies in au (= 627.5095 kcal/mol). [b] Relative energies in kcal/mol. [c] The HF energy of the neutral Ψ-uridine has been taken as the reference point

Table 5. Experimental δ^{13}C and $^3J_{CH}$ Values and Δδaromatic carbon$_{(total)}$ for 1 Used To Determine the pK_a Values through Hill Plots of p*D* versus log((Δδaromatic carbon$_{(total)}$ − Δδaromatic carbon/Δδaromatic carbon) (see Figure 2)

	Ψ-uridine (**1**)					
chemical shift	neutral	basic	Δδ aromatic carbon$_{(total)}$[b]	slope	pK_a	R^a
$\delta(C_2)$	153.2	162.0	6.8	1.1 (σ = 0.2)	9.2 (σ = 0.2)	0.993
$\delta(C_4)$	165.7	170.8	5.1	1.1 (σ = 0.1)	9.3 (σ = 0.1)	0.989
$\delta(C_5)$	110.9	108.5	2.4	0.9 (σ = 0.1)	9.1 (σ = 0.1)	0.996
$\delta(C_6)$	141.9	152.6	10.7	1.0 (σ = 0.1)	9.2 (σ = 0.2)	0.984

[a] R = correlation coefficient. [b] Δδaromatic carbon$_{(total)}$ = total difference in chemical shift between the neutral and the anionic state.

Table 6. Experimental δ(^{13}C) and $^3J_{CH}$ Values and Δδaromatic Carbon$_{(total)}$ for 4 Used To Determine the pK_a Values through Hill Plots of p*D* versus log((Δδaromatic carbon$_{(total)}$ − Δgdaromatic carbon/Δδaromatic carbon) (see Figure 3)

	1-methyl-ψ-uridine **4**					
chemical shift	neutral	basic	Δδ aromatic carbon$_{(total)}$[b]	slope	pK_a	R^a
$\delta(C_2)$	153.0	161.1	7.1	0.9 (σ = 0.1)	9.6 (σ = 0.1)	0.991
$\delta(C_4)$	165.4	175.5	10.1	0.9 (σ = 0.1)	9.6 (σ = 0.1)	0.980
$\delta(C_5)$	111.0	111.0	0	0		
$\delta(C_6)$	146.8	145.7	1.1	0.9 (σ = 0.1)	9.5 (σ = 0.1)	0.985

[a] R = correlation coefficient. [b] Δδ aromatic carbon$_{(total)}$ = total difference in chemical shift between the neutral and the anionic state.

Table 7. Experimental δ(^{13}C) and $^3J_{CH}$ Values and Δδaromatic carbon$_{(total)}$ for 5 Used to Determine the pK_a Values through Hill Plots of p*D* versus log((Δδaromatic carbon$_{(total)}$ − Δδaromatic carbon/Δδaromatic carbon) (see Figure 4)

	3-methyl-Ψ-uridine **5**					
chemical shift	neutral	basic	Δδ aromatic carbon$_{(total)}$[b]	slope	pK_a	R^a
$\delta(C_2)$	153.5	161.5	8.0	1.0 (σ = 0.1)	9.8 (σ = 0.1)	0.999
$\delta(C_4)$	165.1	167.0	1.9	1.0 (σ = 0.1)	9.9 (σ = 0.1)	0.997
$\delta(C_5)$	110.4	106.8	3.6	0.9 (σ = 0.1)	9.8 (σ = 0.1)	0.999
$\delta(C_6)$	139.6	156.3	17.7	1.1 (σ = 0.1)	9.8 (σ = 0.1)	0.999

[a] R = correlation coefficient. [b] Δδ aromatic carbon$_{(total)}$ = total difference in chemical shift between the neutral and the anionic state.

which is in agreement with the ab initio calculations at 6-31G** level but inconsistent with the results obtained with 6-31+G** or 6-31++G** basis sets.

Our observation of favored N1-anion formation over the N3-anion for Ψ-uridine has two biological implications: (i) The N1−H of Ψ-uridine (**1**) will compete favorably for any potential hydrogen bonding with a complementary nucleotide over its N3−H. (ii) A larger preference of the N1 anion of Ψ-uridine over its N3-anion also suggests that Ψ-uridine in RNA will bind to a hard metal ion such as Hg^{2+} in the neutral pH preferably to the N1 giving N1−Hg^{2+} complex over its N3−Hg^{2+} complex. The work is now in progress to delineate the nature of H-bonding as well as Hg^{2+} ion binding in the oligonucleotides containing Ψ-uridine.

Experimental Section

(A) ^{13}C NMR Spectroscopy. Ψ-Uridine (**1**),[6] N1-methyl-Ψ-uridine (**4**),[7] and N3-methyl-Ψ-uridine (**5**)[8] were synthesized using the literature procedure. The NMR spectra were recorded at 500 MHz (Bruker DRX 500) in D_2O solution [δ_{CH_3CN} = 0.3 ppm as internal reference] at 298 K. The p*D* of the solution for recording NMR spectra was changed simply either by addition of D_2SO_4 in D_2O or by NaOD at room temperature, and the p*D*s were callibrated with reference samples at pH 4 and 7. The ^{13}C chemical shifts [8.0 mM for **1**, **4**, and **5**] were measured by heteronuclear multibond correlation (HMBC) experiments (ref 12) at 125.76 MHz with *Z*-gradients with the delay of a multibond ^{13}C filter of 60 ms, relaxation delay of 4s, and datapoints of 512 in F1 and 2K in F2 dimension, F1 dimension was subsequently zero-filled to 2K. The $^3J_{CH}$ coupling constants [16.0 mM for **1**, **4**, and **5**] were extracted from ^{1}H-coupled ^{13}C NMR spectra which were measured with gated decoupling with full NOE, which after zero filling gave a digital resolution of 0.1 Hz. In the case of the *N*-methyl derivatives **4** and **5** selective methyl ^{1}H decoupling had to be used to resolve the splittings of interest. The δ(^{13}C) for **1** (neutral, deprotonated shifts are shown in *italics*): 153.2, *162.0* (C2) ($^3J_{C2,H6}$ = 9.4, *12.2*); 141.9, *152.6* (C6) ($^3J_{C6,H1'}$ = 4.9, *5.0*); 110.9, *108.5* (C5); 165.7, *170.8* (C4) ($^3J_{C4,H6}$ = 9.4, *8.4*; $^3J_{C4,H1'}$ = 4.1, *4.7*). The δ(^{13}C) for **4** (neutral, deprotonated shifts are shown in italics): 153.0, *161.1* (C2) ($^3J_{C2,H6}$ = 8.3, *7.3)*; 146.8 *145.7* (C6) ($^3J_{C6,H1'}$ = 5.0, *4.9*); 111.0, *111.0* (C5); 165.4, *175.5* (C4) ($^3J_{C4,H6}$ = 9.5, *7.9;* $^3J_{C4,H1'}$ = 4.1, *3.9*). The δ(^{13}C) for **5** (neutral, deprotonated shifts are shown in italics): 153.5, *161.5* (C2) ($^3J_{C2,H6}$ = 9.4, *14.5)*; 139.6, *156.3* (C6) ($^3J_{C6,H1'}$ = 5.0, *5.1*); 110.4, *106.8* (C5); 165.1, *167.0* (C4) ($^3J_{C4,H6}$ = 9.4, *8.6*; $^3J_{C4,H1'}$ = 4.0, *5.3*).

The HMBC experiments usually took ∼12 h in basic solutions, and under this period we did not observe any isomerization to the α-anomer or to an anomeric mixture of hexopyanosyl derivatives.

(B) Determination of the pK_a Value of the Aglycon from the Hill Plot. The plot of chemical shifts as a function of p*D* displays a sigmoidal dependence characteristic of a

typical titration curve for **1**, **4**, and **5** (Figure 1). The curves through the experimental points were fitted with the use of nonlinear least-squares fitting procedure to the Henderson−Hasselbach equation: $pD = pK_a + \log(1 - \alpha/\alpha)$, where a representing the fraction of protonated species (ref 13) was calculated from the change of chemical shift relative to the reference neutral state at a given p*D* divided by the total change in their respective values between the neutral and the protonated or the deprotonated state. This equation can then be rewrittten in to the Hill equation as $pD = pK_a + \log((\Delta\delta\text{aromatic carbon}_{(total)} - \Delta\delta\text{aromatic carbon}/\Delta\delta\text{aromatic carbon})$. The pK_a values for **1**, **4**, and **5** were thus determined through Hill plots of p*D* versus the $\log((\Delta\delta\text{aromatic carbon}_{(total)} - \Delta\delta\text{aromatic carbon}/\Delta\delta\text{aromatic carbon})$ (Figires 2, 3, and 4). Linear regression gives straight lines with Pearson correlation coefficients (*R*) above 0.98 and slopes close to 1, which is a characteristic indication for the protonation involving a single protonation site, and pK_a values were obtained at the intercepts (see Tables 5−7 for the details of the results including the chemical shifts of the neutral and deprotonated species, $\Delta\delta\text{aromatic carbon}_{(total)}$, slope, pK_a, and the correlation coefficients).

(C) Ab Initio Calculations. Ab initio calculations were performed using the GAUSSIAN 94 program[14] with Silicon Graphics Indigo R4000 computers.

The structures Ψ-uridine **1** and its N1-anion **2** and N3-anion **3** were first completely optimized using a 3-21G basis set.[14a] The HF/3-21G optimized geometries were used as input for full optimization of the HF/6-31G** level. The HF/6-31G** is a basis set formed by adding polarization functions to a 6-31G basis (d-type basis functions added to the heavy atoms and p-type functions added to hydrogens[14b]). The HF/6-31G** optimized geometries were subsequently used as input for full optimization of the HF/6-31+G**level which is an improved basis set for the correct description of anions.[14] The HF/6-31+G** is formed by adding diffusion functions (one diffuse s-and p-type Gaussian are added on the heavy atoms) to the 6-31G** basis set. Because of the presence of exchangeable protons in Ψ-uridine, the HF/6-31+G** optimized geometries were further improved by adding also some diffusion function (one s-type Gaussian) to hydrogen giving the optimized structures of **1**, **2**, and **3** at the HF/6-31++G**. All these optimizations were done in a self-consistent reaction field (SCRF) with the dielectric constant of 78.3 to mimic the aqueous environment. The radius of the spherical cavity (a_0) was obtained from the molecular volume to which 0.5 Å was added to account for the nearest approach of the solvent molecules according to the suggestions of Wiberg et al.[15] Net atomic point charges were calculated using a Besler−Merz−Kollman method[16] which performs a least-squares fit of the qauntum mechanically calculated electrostatic potential (ESP) to that of the charge model.

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