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# Tautomeric Equilibria of 5-Fluorouracil Anionic Species in Water

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It has long been postulated that rare tautomeric or ionized forms of nucleic acid bases may play a role in mispair formation. Therefore, ab initio quantum chemical investigations on the tautomeric equilibrium in 5-fluorouracil (5FU) and its anions (deprotonated from N1, AN1, and from N3, AN3) and their tautomeric forms in water were performed. The effect of the water as solvent was introduced using solute–solvent clusters (four water molecules). The influence of the water molecules on the tautomeric reactions between different forms was considered by multiple proton transfer mechanisms. We show that when a water dimer is located in the reaction site between the two pairs of N–H and C=O groups, the assistive effect of the water molecules is strengthened. All calculations of the solute–water complexes were carried out at an MP2 level of theory and supplemented with correction for higher order correlation terms at CCSD(T) level, using the 6-31+G(d,p) basis set. The ab initio calculated frequencies and Raman intensities of 5FU and its anions AN1, AN3, and dianion are in good agreement with the experimental Raman frequencies in aqueous solution at different pH. In order to establish the pH-induced structural transformation in the molecule of 5FU, further <sup>1</sup>H, <sup>19</sup>F, and <sup>13</sup>C NMR spectra in water solution for pH = 6.9–13.8 were acquired and the chemical shift alterations were determined as a function of pH. On the basis of NMR spectroscopic data obtained for 5FU in aqueous solution at alkaline pH, we suggest the existence of a mixture of the anionic tautomeric forms predicted by our theoretical calculations.

## 1. Introduction

The 5-substituted pyrimidines comprise a biologically important class of base analogues. The replacement in uracil of the hydrogen in position 5 with fluorine, to give 5-fluorouracil (5FU), an antineoplastic agent, introduces an additional heteroatom, which is a potential H-bond acceptor, and significantly alters the electronic properties of the molecule.<sup>1–6</sup> In aqueous solution, this can induce significant changes in its physical and biological properties.

It has long been postulated that the presence of rare tautomeric forms might be involved in base mispair formation during polymerase-mediated DNA replication, resulting in genetic mutations. According to Watson and Crick,<sup>7</sup> “Spontaneous mutation may be due to... less likely tautomeric forms”. This idea has been advanced by Topal and Fresco<sup>8</sup> and it has also been estimated that these unpreferred tautomeric forms might be present, under physiological conditions, at a low frequency of 10<sup>–6</sup> to 10<sup>–5</sup>. Alternatively, hydrogen-bonding interactions and base pair formation could be perturbed by base ionization.<sup>9</sup> In particular, ionization of the pyrimidine moiety in nuclear acids could change the coding properties of the base during polymerase-mediated replication, resulting in a base substitution mutation.<sup>10</sup> The ionization constants (pK<sub>a</sub> values) of the normal DNA bases are roughly 2–3 pH units away from physiological pH, predicting that the ionized forms might exist several orders of magnitude more frequently than the rare tautomeric forms.

The 5FU behavior in water solution at different pH values gives important information about the role of pH in controlling

the uptake of the anticancer drug. This could be used to improve cancer-targeting and selective activity of the antineoplastic agent and in the design of pH-dependent drug release systems.<sup>11–13</sup> Additionally, the tautomeric conversions of 5FU and its anions could play a key role in the reactivity of the nucleophilic centers and in the design of novel polymer based drug delivery systems.<sup>14,15</sup> Recently, the inclusion of 5FU as an initiator of the polymerization process for 5FU-loaded poly(butylcyanoacrylate) nanoparticle formation was reported.

The main goal of this work is to investigate tautomeric equilibrium in 5FU anions (AN1 and AN3) and their tautomeric forms in water and compare it to the tautomeric conversion in the neutral molecule. The influence of the water molecules on the tautomeric reactions between different forms was considered by a multiple proton transfer mechanism.

Therefore, to identify and investigate for the first time the 5FU species present at different pH values in water solution, ab initio quantum chemical calculations and NMR spectroscopy were employed. Recently, due to their high sensitivity, normal Raman and SERS spectroscopy were used to investigate the conversion between two tautomeric forms related by a keto-enolic equilibrium (diketo and dienolic) and two anions of the diketo form, N1 and N3, respectively.<sup>16,17</sup> To correctly discriminate this species in water solution at different pH values, ab initio quantum chemical calculations of vibrational wavenumbers were performed.

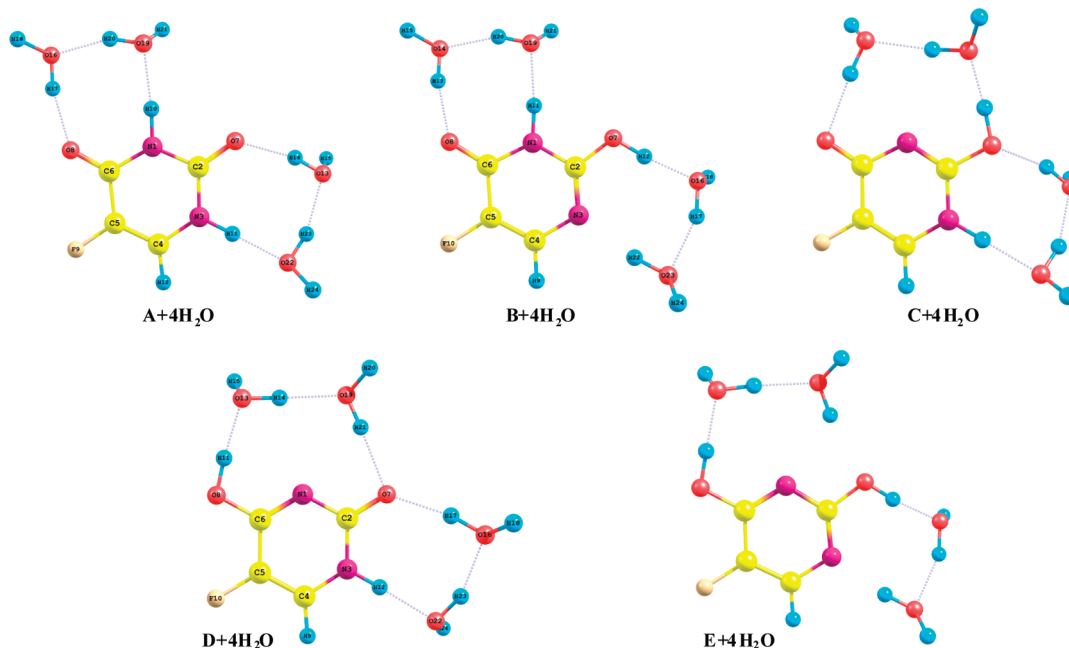
## 2. Theoretical Calculations

Ab initio quantum chemical calculations were performed for 5-fluorouracil and its anionic species in gas phase and in a four-water cluster. The calculations were carried out using the GAMESS (US) quantum chemistry package.<sup>18</sup> Full geometry

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**Figure 1.** MP2/6-31+G(d,p) optimized geometries of four-hydrated tautomers of 5-fluorouracil.

optimization of the 5FU, its anions, dianion, and their four-water complexes was performed at a MP2/6-31+G(d,p) level of theory without symmetry constraints by the gradient procedure. A gradient convergence threshold of  $1 \times 10^{-5}$  hartree·Bohr<sup>-1</sup> was used. The local minima and transition states were verified by establishing that the Hessians have zero and one negative eigenvalues, respectively. The MP2/6-31+G(d,p) calculations were then continued to obtain the Raman scattering activity of each band.

Starting from the transition state, the reaction path was generated as the steepest descent path in mass-scaled coordinates. This path, called intrinsic reaction coordinate (IRC), was generated using the Gonzalez–Schlegel algorithm<sup>19</sup> by employing a step size of 0.05 Bohr (1 Bohr corresponds to 0.53 Å). The distance of a point on the potential energy surface to the transition state is given by a “−” sign if on the reagent side and a “+” sign on the product side. On both branches of the reaction coordinate, the path was stopped when 40 steps were done.

Additional single-point calculations at CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p)<sup>20–22</sup> level were performed.

The values of Gibbs free energies ( $\Delta G$ ) and activation barriers ( $\Delta G^\ddagger$ ) were calculated for a temperature of 298.15 K by the formulas  $\Delta G = \Delta H - T\Delta S$  and  $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ , respectively. The classical rate constant at 298.15 K was calculated using the Eyring equation,  $k = (k_B T/h) \cdot e^{-\Delta G^\ddagger/RT}$ , where  $k_B$  and  $h$  are the Boltzmann and Planck constants, respectively. The values of the populations ( $p_i$ ) were calculated by the standard formula  $p_i = e^{-\Delta G_i/RT} / \sum_j e^{-\Delta G_j/RT}$ .

The NMR chemical shieldings were calculated at B3LYP/6-31++G(3df,2p) level using the GIAO (gauge-including atomic orbitals) approach<sup>23,24</sup> and MP2/6-31+G(d,p) optimized geometry. In order to compare with experiment, the calculated absolute shieldings were transformed to chemical shifts using the following reference compounds: tetramethylsilane (TMS) for carbon and hydrogen atoms, and KF for fluorine atoms:  $\delta = \delta_{\text{calcd}}(\text{reference}) - \delta_{\text{calcd}}$ . Both  $\delta_{\text{calcd}}(\text{reference})$  and  $\delta_{\text{calcd}}$  were evaluated with the same method and basis set. The NMR calculations were carried out using Gaussian 03.<sup>25</sup>

Natural bond orbital (NBO) calculations were performed with the NBO code<sup>26</sup> included in Gaussian03 at MP2/6-31+G(d,p) level. The energy and character of the selected unoccupied and occupied MO's of 5FU and its anion complexes also correspond to MP2/6-31+G(d,p).

### 3. Experimental Methods

All NMR experiments were recorded on a Bruker Avance III 400 spectrometer, operating at 400.15 MHz for protons, 376.52 MHz for fluorine, and 100.62 MHz for carbon, equipped with pulse gradient units, capable of producing magnetic field pulsed gradients in the  $z$ -direction of 50 G/cm. The NMR spectra were acquired using standard Bruker pulse sequences, at a temperature of 298 K in H<sub>2</sub>O:D<sub>2</sub>O (9:1) solution. Chemical shifts were referenced to internal sodium trimethylsilyl-[2,2,3,3-<sup>d</sup><sub>4</sub>]-propionate (TSP) for <sup>1</sup>H and <sup>13</sup>C, and to internal KF for <sup>19</sup>F. The NMR spectra were recorded at different pH (pH = 6.9–13.8). The pH values were adjusted using 0.1 M NaOD and measured on pH Meter Basic 20+, (CRISON). <sup>1</sup>H NMR experiments with water suppression using excitation sculpting with gradients,<sup>27</sup> acquisition time 5.45 s, relaxation delay 2 s, 16 transients of a spectral width of 3500 Hz were collected into 32 K time domain points. The following acquisition parameters were used for the full range broad-band proton decoupled <sup>13</sup>C NMR spectra: a pulse width of 8  $\mu$ s, an acquisition time of 1.41 s, a relaxation delay of 2 s, a spectral width of 17 000 Hz, 64 K time domain points and 2 K transients were collected. The <sup>19</sup>F NMR spectra were recorded with inverse gated proton decoupling, a pulse width of 12  $\mu$ s, an acquisition time of 1.75 s, a relaxation delay of 1 s, a spectral width of 37 000 Hz. A total of 128 K time domain points and 16 transients were collected.

### 4. Results and Discussion

**4.1. Tautomeric Equilibrium of 5-Fluorouracil at Neutral pH.** Recently, we studied the tautomeric conversion of 5-fluorouracil in water<sup>28</sup> through a double proton transfer mechanism. The fluorescence spectroscopic study and quantum chemical calculations of 5FU in aqueous medium confirm the possible presence of rare hydroxy tautomeric forms. We have shown<sup>29</sup>

**TABLE 1: Relative Free Energies,  $\Delta G_{298}$  (kcal mol<sup>-1</sup>), of the Tautomers of 5FU (Figure 1) and Its Monoanions Calculated at MP2/6-31+G(d,p) and CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) Levels**

tautomer	MP2/6-31+G(d,p)	CCSD(T)/6-31+G(d,p)// MP2/6-31+G(d,p)	
	$\Delta G_{298}$	$\Delta G_{298}$	population (%)
5FU			
A+4H <sub>2</sub> O	0.00	0.00	99.99
B+4H <sub>2</sub> O	7.24	7.34	$4.2 \times 10^{-4}$
C+4H <sub>2</sub> O	11.61	11.87	$1.0 \times 10^{-7}$
D+4H <sub>2</sub> O	7.48	7.51	$3.1 \times 10^{-4}$
E+4H <sub>2</sub> O	8.66	9.03	$2.4 \times 10^{-5}$
anions			
AN3	0.00	0.00	
CN3	8.90	9.12	
DN3	17.41	17.60	
AN1	11.91	10.41	
BN1	10.22	11.69	
AN1 + 4H <sub>2</sub> O	5.34	5.31	
AN3 + 4H <sub>2</sub> O	0.00	0.00	99.99
CN3 + 4H <sub>2</sub> O	6.50	6.87	$9.2 \times 10^{-4}$
DN3 + 4H <sub>2</sub> O	7.84	8.16	$1.0 \times 10^{-4}$

that the participation of two water molecules in a proton transfer reaction should be more probable than the participation of only one water molecule. Ren et al.<sup>30</sup> have shown that the tautomerization between 2-imidazolone.(H<sub>2</sub>O)<sub>n</sub> and 2-hydroxyimidazolole.(H<sub>2</sub>O)<sub>n</sub> clusters proceeds by a concerted double to quadruple proton transfer mechanism. The triple proton transfer process through an eight-membered ring formed by imidazolone and two water molecules has been found to be most efficient.

Here we consider the solvent effect on the tautomeric conversion of 5-fluorouracil through a supermolecule model. In this model, the two pairs of water molecules forming a cluster are situated between the two pairs of N–H and C=O groups (Figure 1).

The values of the free energies ( $\Delta G_{298}$ ) for four-hydrated complexes of dioxo A and hydroxy tautomers B–E of 5-fluorouracil obtained at MP2/6-31+G(d,p) and CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) levels are given in Table 1. According to our calculations, taking into account electron correlation at MP2 and CCSD(T) level, the most stable is the complex of the 2,4-dioxo form A followed by the hydrated hydroxy forms B and D. This is consistent with our previous

results<sup>28</sup> for trihydrated 5FU. The calculated at CCSD(T) level relative free energy difference between the A+4H<sub>2</sub>O and D+4H<sub>2</sub>O complexes (7.51 kcal mol<sup>-1</sup>) is slightly larger than that between the A+4H<sub>2</sub>O and B+4H<sub>2</sub>O ones (7.34 kcal mol<sup>-1</sup>). Due to these differences (Table 1) the populations of the species B and D are  $4.2 \times 10^{-4}$  % and  $3.1 \times 10^{-4}$  %, respectively, and these species should coexist with tautomer A in water solution at neutral pH.

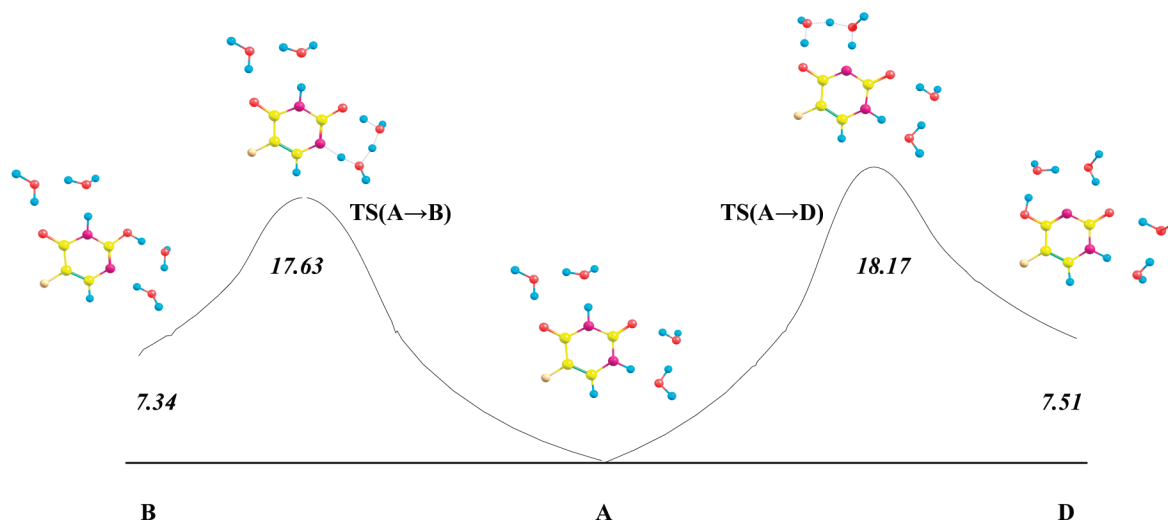
On the basis of IRC analysis of the A → B reaction (Figure 2) the following conclusions could be made: both water molecules which are situated near the N3 and O7 atoms of 5FU (Figure 1) come closer to each other and to the fluorouracil molecule. When the distances N3...O22, O7...O13, and O22...O13 become 2.5 Å, 2.55 Å and 2.4 Å, respectively, protons H11, H23, and H14 start transferring to the acceptor oxygen atoms. When the distances N3–H11, O22–H23, and H14...O13 become 1.4, 1.25, and 1.35 Å, respectively, the N3...O22, O7...O13, and O22...O13 distances again start to return to their equilibrium values. Obviously, the process is highly cooperative. A similar mechanism holds for the A → D proton transfer reaction.

For the four-hydrated complex of 5FU, the proton transfer activation energies for the two parallel reactions A → B and A → D are very close (Figure 2). The reaction barrier of A → B calculated at CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) level of theory amounts to 17.63 kcal mol<sup>-1</sup>, which is by 0.54 kcal mol<sup>-1</sup> lower than that of the A → D proton transfer reaction. Both investigated proton transfer reactions involve concerted atomic movement.

Transition state theory was applied to estimate the rate constants *k* of the concurrent processes A → B and A → D using the Eyring equation. The computed classical rate constants *k* of the proton transfer in 5FU are listed in Table 2. According to the rate constant values obtained at both levels of calculation, the forward assisted proton transfer reaction can occur (*k* = 10<sup>-1</sup>–10<sup>1</sup>). The reaction rate constant is large enough to generate a concentration of rare tautomeric forms significant enough to reproduce the frequency of point mutations.

#### 4.2. Tautomeric Equilibrium of 5-Fluorouracil Anions.

The deprotonation of the most stable 2,4-dioxo tautomeric form, A, of 5FU can occur in two possible sites by formation of two anions: AN1 and AN3. Anion AN1 can theoretically exist in



**Figure 2.** Energy profile of the tautomerization reactions D ← A → B for four-hydrated 5-fluorouracil, calculated at CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) level. The relative free energies and activation barriers are given in kcal mol<sup>-1</sup>.

**TABLE 2: Calculated Energy Barriers  $\Delta G_{298}^\ddagger$  (in kcal mol<sup>-1</sup>) for A  $\rightarrow$  B, A  $\rightarrow$  D, AN3  $\rightarrow$  CN3, and AN3  $\rightarrow$  DN3 Reactions in Four-Hydrated Complexes of 5-Fluorouracil and Its Monoanion N3<sup>a</sup>**

computational level	$\Delta G_{298}^\ddagger$	$k$	$\nu^\ddagger$
<b>A + 4H<sub>2</sub>O <math>\rightarrow</math> B + 4H<sub>2</sub>O</b>			
MP2/6-31+G(d,p)	15.29	$3.8 \times 10^1$	1183i
CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p)	17.63	$7.4 \times 10^{-1}$	
<b>A + 4H<sub>2</sub>O <math>\rightarrow</math> D + 4H<sub>2</sub>O</b>			
MP2/6-31+G(d,p)	16.00	$1.2 \times 10^1$	960i
CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p)	18.17	$3.0 \times 10^{-1}$	
<b>AN3 + 4H<sub>2</sub>O <math>\rightarrow</math> DN3 + 4H<sub>2</sub>O</b>			
MP2/6-31+G(d,p)	16.88	2.62	1078i
CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p)	19.17	$5.5 \times 10^{-2}$	
<b>AN3 + 4H<sub>2</sub>O <math>\rightarrow</math> CN3 + 4H<sub>2</sub>O</b>			
MP2/6-31+G(d,p)	14.33	$1.94 \times 10^{-2}$	979i
CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p)	16.48	5.16	

<sup>a</sup> The barriers for the forward reactions are given. Rate constants are in s<sup>-1</sup>. Imaginary frequencies  $\nu^\ddagger$  are in cm<sup>-1</sup>.

two tautomeric forms (AN1 and BN1) while AN3 in three tautomeric forms (AN3, CN3, and DN3) shown in Figure 3.

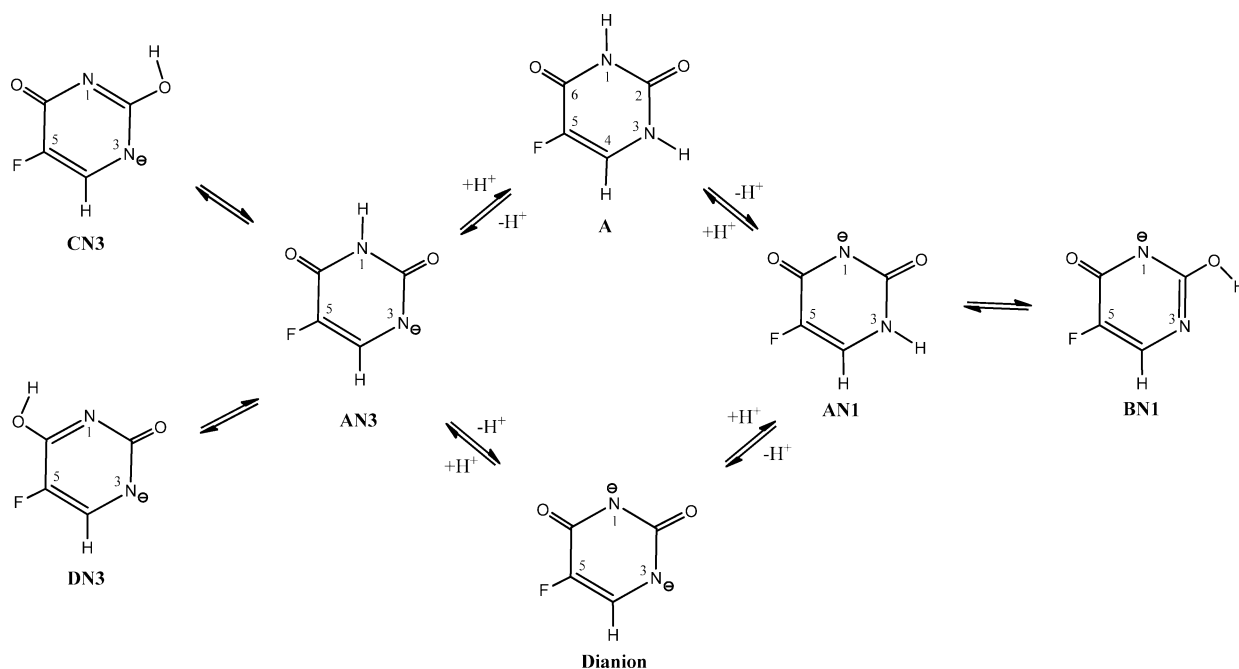
So far the equilibrium between anions AN1 and AN3 has been considered in the literature only.<sup>16,17,31,32</sup> Wierzchowski et al.<sup>31</sup> reported that the monoanions of 5-fluorouracil are in equilibrium a mixture of two tautomeric forms corresponding to dissociation of the N1 and N3 protons. The UV spectrum of the monosodium salt of 5FU in 0.001 *N* NaOH shows that: (i) in H<sub>2</sub>O, there is a mixture of AN1 (63%) and AN3 (37%) and (ii) in 67–85% aqueous dioxane, the proportion of AN1 to AN3 is 1:3.

To investigate the oxo-hydroxy tautomerism in anionic forms of 5-fluorouracil, we calculated the free energies of all the possible tautomers. According to our calculations at CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) level, anion AN3 is more

stable than AN1 in gas phase and in water solution by 10.41 and 5.31 kcal mol<sup>-1</sup>, respectively (Table 1 and Figure 4). In gas phase, the 2,4-dioxo tautomer of anion AN3 is most stable followed by the 2-hydroxy-4-oxo CN3 (9.12 kcal mol<sup>-1</sup>) and 2-oxo-4-hydroxy DN3 (17.60 kcal mol<sup>-1</sup>). These values are close to the results obtained at MP2 level. Upon complexation with four water molecules, the energy difference between the most stable anionic tautomer AN3 and CN3 becomes 6.87 kcal mol<sup>-1</sup>. For tautomer DN3, the change is more strongly expressed: 17.60 kcal mol<sup>-1</sup> before and 8.16 kcal mol<sup>-1</sup> after complexation.

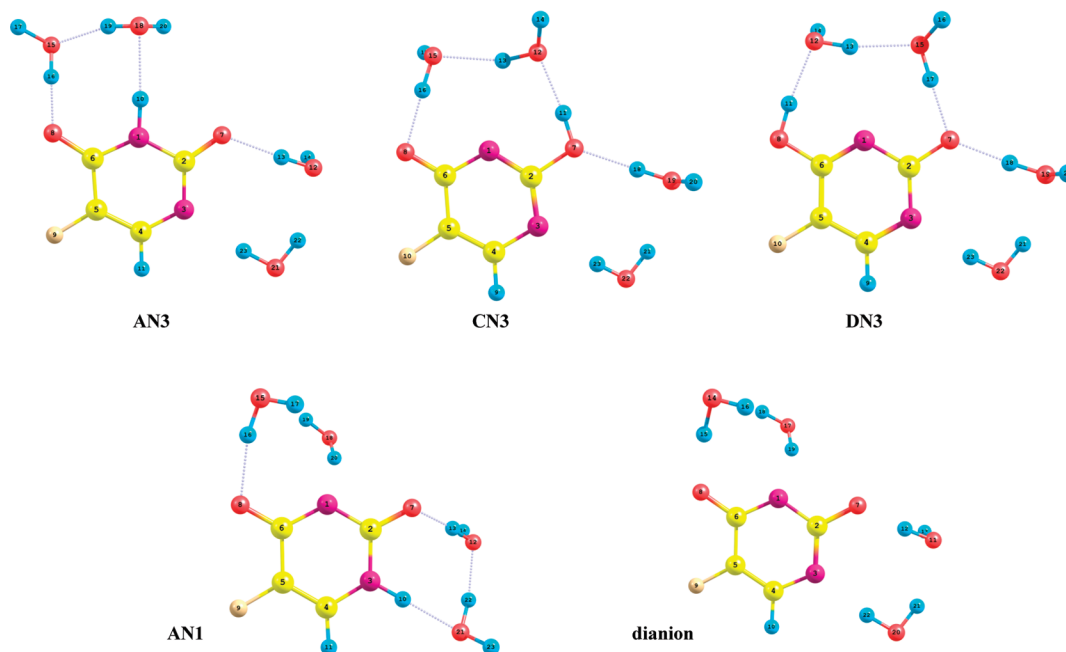
We considered the solvent effect on the tautomeric conversion of the 5FU anions, shown on Figure 3, through a multiple proton transfer mechanism. Complexes containing four water molecules around the anions and their tautomeric forms were used for simulation of the interactions in aqueous medium. The tautomers of 5FU anions considered in this study (AN3, CN3, DN3, and AN1) are shown in Figure 4, and their relative free energies in gas phase and water surrounding are given in Table 1. As was shown above, anion AN3 is more stable than AN1 in water solution by 5.31 kcal mol<sup>-1</sup>. A paltry fraction of the enol forms CN3 and DN3 cannot be ruled out, since these tautomers are 6.87 and 8.16 kcal mol<sup>-1</sup> less favored than AN3. According to the relative stabilities calculated at CCSD(T) level (Table 1) the populations of the species CN3 and DN3 are  $9.2 \times 10^{-4}$  % and  $1.0 \times 10^{-4}$  %, respectively. These values are comparable to the calculated ones for 5FU tautomers, B ( $4.2 \times 10^{-4}$ ) and D ( $3.1 \times 10^{-4}$ ). Tautomer BN1 is not considered because it could be produced from the energetically unfavorable AN1. Thus, we studied the tautomeric equilibria of monoanion AN3 only: AN3  $\rightarrow$  DN3 and AN3  $\rightarrow$  CN3.

The TS structures corresponding to the water-assisted proton transfer reactions for monoanion AN3 were found and the energy barriers of multiple proton transfer reactions were calculated. The tautomeric conversion AN3  $\rightarrow$  CN3 is characterized by a lower energy barrier ( $\Delta G_{298}^\ddagger$ ) than that for the AN3  $\rightarrow$  DN3 one by 2.55 and 2.69 kcal mol<sup>-1</sup> at MP2/6-31+G(d,p) and CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) levels of theory, respectively (Table 2 and Figure 5). Therefore, it can be seen

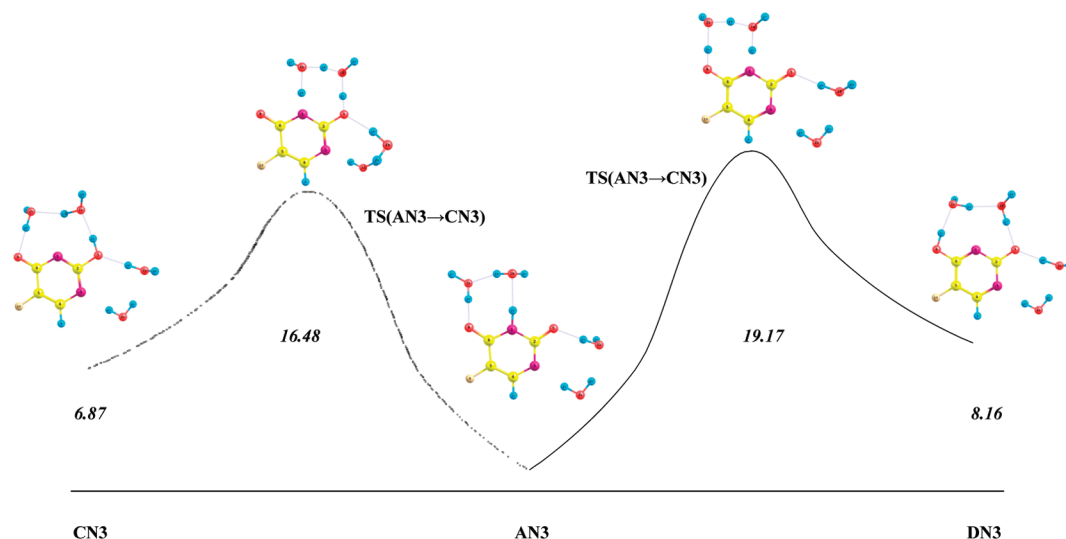


**Figure 3.** Two possible 5-fluorouracil anions (AN1 and AN3), their tautomeric forms and dianion.





**Figure 4.** MP2/6-31+G(d,p) optimized structures of the solvated tautomeric forms of 5FU anions (AN1 and AN3).



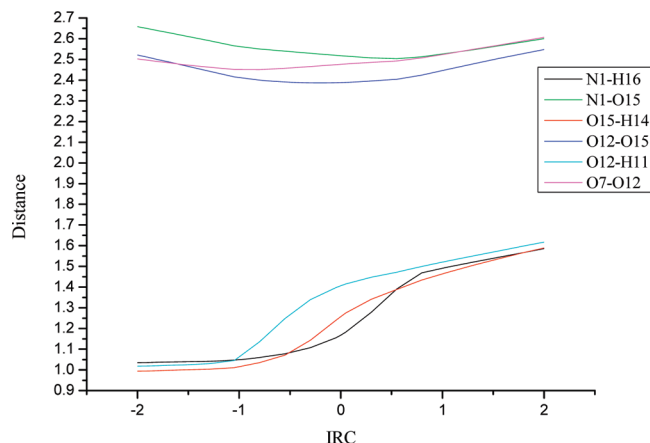
**Figure 5.** Energy profile of the tautomerization reactions of 5FU monoanion AN3 ( $\text{DN3} \leftarrow \text{AN3} \rightarrow \text{CN3}$ ), calculated at the CCSD(T)/6-31+G(d,p)//MP2/6-31+G(d,p) level. The relative free energies and activation barriers are given in  $\text{kcal mol}^{-1}$ .

that there exists the possibility for two parallel reactions  $\text{DN3} \leftarrow \text{AN3} \rightarrow \text{CN3}$  because both barriers are close in energy. The calculated rate constants of the  $\text{AN3} \rightarrow \text{CN3}$  ( $k = 5.16 \text{ s}^{-1}$ ) and  $\text{AN3} \rightarrow \text{DN3}$  ( $k = 5.5 \times 10^{-2} \text{ s}^{-1}$ ) reactions are sufficiently large to be able to generate a concentration of rare hydroxy anionic tautomeric forms enough to reproduce the frequency of point mutations.

In Figure 6 the selected distances between pairs of atoms along the classical reaction coordinate for the  $\text{AN3} \rightarrow \text{CN3}$  triple proton transfer reaction are presented. Proton H16 (involved in the  $\text{N1} \cdots \text{H16} \cdots \text{O15}$  hydrogen bond) stays at a constant distance of slightly more than 1 Å from the donor N1 atom, while proton H14 (involved in the  $\text{O12} \cdots \text{H14} \cdots \text{O15}$  hydrogen bond) stays at a constant distance of 1 Å from the donor O15 atom. At the same time the  $\text{N1} \cdots \text{O15}$  distance decreases from 2.7 Å equilibrium distance to 2.5 Å. In the case of  $\text{O12} \cdots \text{O15}$  distance the equilibrium value 2.6 Å decreases to 2.4 Å. Then there is a point on the reaction coordinate where protons H16 and H14 suddenly start the transfer to the acceptor oxygen atoms, which

can be seen from the sudden increase of the proton distance to the acceptor oxygen atoms. When the distances  $\text{N1} \cdots \text{H16}$  and  $\text{O15} \cdots \text{H14}$  become 1.45 and 1.25 Å, respectively, the  $\text{N1} \cdots \text{O15}$  and  $\text{O12} \cdots \text{O15}$  distances again start to return to their equilibrium values. At the same time the  $\text{O7} \cdots \text{O12}$  distance decreases to 2.45 Å and H11 starts to transfer to the O7 atom. When the  $\text{H11} \cdots \text{O12}$  distance becomes 1.35 Å, the  $\text{O7} \cdots \text{O12}$  distance starts to return to its equilibrium value. Obviously the proton transfer is triggered by short  $\text{N} \cdots \text{O}$  and  $\text{O} \cdots \text{O}$  distances. It can be seen from Figure 6 that when more than one proton is transferred, all  $\text{N} \cdots \text{O}$ ,  $\text{O} \cdots \text{O}$ ,  $\text{N} \cdots \text{H}$ , and  $\text{O} \cdots \text{H}$  distances vary at the same time, i.e., the process becomes highly cooperative. From a mechanistic point of view, this means that the proton transfers happen in a single step without any intermediates. However, the concerted triple proton transfer occurs asynchronously.

To understand the electronic effects induced by the deprotonation on the stability of the 5FU anions, NBO analysis using MP2/6-31+G(d,p) data in water was carried out, and correlated well with changes in bond lengths in 5FU and its anions under



**Figure 6.** Distances between two selected atoms along the intrinsic reaction coordinate (IRC) of triple proton transfer reaction  $\text{AN3} \rightarrow \text{CN3}$  in the four-hydrated anion of 5-fluorouracil (Figure 3) as calculated at MP2/6-31+G(d,p) level of theory. Distances are in Å and IRC in  $\text{amu}^{1/2}$  bohr. The numbering of the atoms is given in Figure 4.

**TABLE 3: Main NBO Second-Order Stabilisation Energies  $E^{(2)}$  ( $\text{kcal mol}^{-1}$ ) Calculated at MP2/6-31+G(d,p) Level of Theory, for Four-Hydrated Complexes of 5FU and its Anions**

delocalization	$E^{(2)}$	delocalization	$E^{(2)}$
	5FU+4H <sub>2</sub> O		AN1+4H <sub>2</sub> O AN3+4H <sub>2</sub> O
$n_{\text{O7}} \rightarrow \sigma^*_{\text{O13-H14}}$	9.47	$n_{\text{N}} \rightarrow \sigma^*_{\text{O-H}}$	8.49 14.63
$n_{\text{O8}} \rightarrow \sigma^*_{\text{O16-H17}}$	6.68	$n_{\text{O7}} \rightarrow \sigma^*_{\text{O12-H13}}$	12.90 17.33
$n_{\text{O16}} \rightarrow \sigma^*_{\text{O19-H20}}$	20.33	$n_{\text{O8}} \rightarrow \sigma^*_{\text{O15-H16}}$	13.03 17.92
$n_{\text{O13}} \rightarrow \sigma^*_{\text{O22-H23}}$	22.87	$n_{\text{O15}} \rightarrow \sigma^*_{\text{O18-H19}}$	3.62 19.01
$n_{\text{O22}} \rightarrow \sigma^*_{\text{N3-H11}}$	28.46	$n_{\text{O12}} \rightarrow \sigma^*_{\text{O21-H22}}$	25.55 9.01
$n_{\text{O19}} \rightarrow \sigma^*_{\text{N1-H10}}$	29.87	$n_{\text{O21}} \rightarrow \sigma^*_{\text{N3-H11}}$	15.84
		$n_{\text{O18}} \rightarrow \sigma^*_{\text{N1-H10}}$	17.28

hydration. In this analysis, a stabilization energy  $E^{(2)}$ <sup>33,34</sup> related to the delocalization trend of electrons from donor to acceptor orbitals, was calculated via perturbation theory. If the stabilization energy  $E^{(2)}$  between a donor bonding orbital and an acceptor orbital is large, there is a strong interaction between them. For each donor NBO(*i*) and acceptor NBO(*j*), the stabilization energy  $E$  is associated with  $i \rightarrow j$  delocalization, given by the following equation:

$$E^{(2)} = \Delta E_{ij} = q_i \frac{F^2(i,j)}{\varepsilon_j - \varepsilon_i}$$

where  $q_i$  is the  $i^{\text{th}}$  donor orbital occupancy;  $\varepsilon_j$  and  $\varepsilon_i$  are diagonal elements (orbital energies) and  $F(i,j)$  are off diagonal elements, respectively, associated with NBO Fock matrix.

Table 3 lists the calculated second order interactions energies  $E^{(2)}$  between donor–acceptor orbitals within the four hydrated complexes of 5FU and its anions (AN1 and AN3). In all of these cases, antibonding  $\sigma^*(\text{N-H}, \text{O-H})$  participate as electron acceptor and “N” or “O” lone pair electrons as electron donors in intermolecular charge transfer interactions, hence the stabilization energy  $E^{(2)}$  ( $n_{\text{N}} \rightarrow \sigma^*_{\text{O-H}}$ ,  $n_{\text{O}} \rightarrow \sigma^*_{\text{O-H}}$  and  $n_{\text{O}} \rightarrow \sigma^*_{\text{N-H}}$ ) is found to be large. In the case of AN3, our data indicate that the lone pair of nitrogen N3 mixes more strongly with the antibonding (O21–H23) bond orbital than N1 with O18–H20 in AN1 (Figure 4 and Table 3). The higher value of this energy indicates that this interaction plays an important role in the stabilization of the AN3 water complex. It can be seen that significant hydrogen bond type interactions between LP(O) from

carbonyl groups of all complexes and  $\sigma^*(\text{O-H})$  exist. These interactions exhibit the highest  $E^{(2)}$  values for all studied series, which decrease in the order: AN3, AN1, 5FU. The interaction of type  $n_{\text{O}} \rightarrow \sigma^*_{\text{O-H}}$  ( $n_{\text{O15}} \rightarrow \sigma^*_{\text{O18-H19}}$  in anions and  $n_{\text{O16}} \rightarrow \sigma^*_{\text{O19-H20}}$  in 5FU) related to the water cluster in the region between the N1–H and O8=C groups is of high importance due to the anti arrangement of the corresponding orbitals; the same is true in the case of interaction  $n_{\text{O}} \rightarrow \sigma^*_{\text{O-H}}$  ( $n_{\text{O12}} \rightarrow \sigma^*_{\text{O21-H22}}$  in anions and  $n_{\text{O13}} \rightarrow \sigma^*_{\text{O22-H23}}$  in 5FU) in the region between C7=O and N3–H. The value of the first transference is the lowest for AN1. The strongest  $n_{\text{O12}} \rightarrow \sigma^*_{\text{O21-H22}}$  delocalization is observed for AN1. Therefore, the water clusters near the C=O8 group are connected by a strong intermolecular hydrogen bond in 5FU and AN3, while in the region between C7=O and N3–H a strong hydrogen bond is formed in 5FU and AN1. Hydration has an important effect on the  $n_{\text{O}} \rightarrow \sigma^*_{\text{N-H}}$  interaction energies as can be seen in Table 3. They are  $\sim 12.6$   $\text{kcal mol}^{-1}$  higher in 5FU than in the anions. At the same time the  $E^{(2)}$  magnitude of the transference  $n_{\text{O22}} \rightarrow \sigma^*_{\text{N3-H11}}$  is somewhat lower in AN1, than that for transference  $n_{\text{O19}} \rightarrow \sigma^*_{\text{N1-H10}}$  in AN3 ( $\sim 1.4$   $\text{kcal mol}^{-1}$ ). Consequently, the delocalization  $n_{\text{O19}} \rightarrow \sigma^*_{\text{N1-H10}}$  stabilizes the hydrogen bonding of AN3 complex in the region of the C=O8 group more expressively than the delocalization  $n_{\text{O22}} \rightarrow \sigma^*_{\text{N3-H11}}$  in the region of the C=O7 group of AN1. Generally these results show stronger intermolecular hydrogen bonds near the C=O7 group than in the C=O8 region. Higher relative conjugative stabilization in AN3, involving the lone pair on the nitrogen atom, in comparison to AN1, also accounts for the greater stability of the AN3 anion. In conclusion, our results from NBO analysis predict the AN3 water complex of 5FU as more probable than the AN1 one.

In addition, when water molecules are inserted as bridges for the protons, the van der Waals spheres of the atoms situated in the region of the N1–H and C=O8 groups (Figure 7) do not overlap, whereas the spheres in another region, between N3–H and C–O7 do, albeit slightly. Due to the larger distances between donor (N1–H) and acceptor (water oxygen O19), the hydrogen bonds in this region are weaker than these in the region between the N3–H and C–O7 groups. As is well-known, short strong H-bonds occur when the distance between the heteroatoms is less than the sum of the van der Waals radii (effective radius for closest molecular packing). This is supported by the natural charges of the oxygen and nitrogen atoms of 5FU and water molecules, obtained from NBO analysis. The negative charges of the N1, O13, and O22 atoms are larger than these of N3, O16, and O19. For this reason, the deprotonation of N3 is more probable.

To verify this hypothesis, we compared our ab initio calculated wavenumbers and Raman activities for the dioxo form of 5FU (Figure 1) and its anions AN1, AN3 and dianion (Figure 4) with recently published Raman wavenumbers in aqueous solution in different pH ( $\text{pH} = 7-14$ )<sup>17</sup> (Table 4). There is good agreement between the calculated and experimentally observed wavenumbers for 5FU at neutral pH. The typical strong carbonyl band at  $1691 \text{ cm}^{-1}$  is calculated to be at  $1705 \text{ cm}^{-1}$ . Good correspondence is found for the characteristic  $\nu(\text{C-F})$  and  $\delta(\text{C-H})$  and other low-frequency bands. The calculated wavenumbers for both deprotonated AN3 and AN1 anions are also presented in Table 4. Generally speaking, the experimental Raman wavenumbers obtained at  $\text{pH} = 10$  are in better correspondence with the calculated ones for the AN3 anion. The calculated carbonyl  $\nu(\text{C2=O})$  band for 5FU at  $1731 \text{ cm}^{-1}$  is shifted down to  $1622 \text{ cm}^{-1}$  and  $1624 \text{ cm}^{-1}$  for the AN3 and

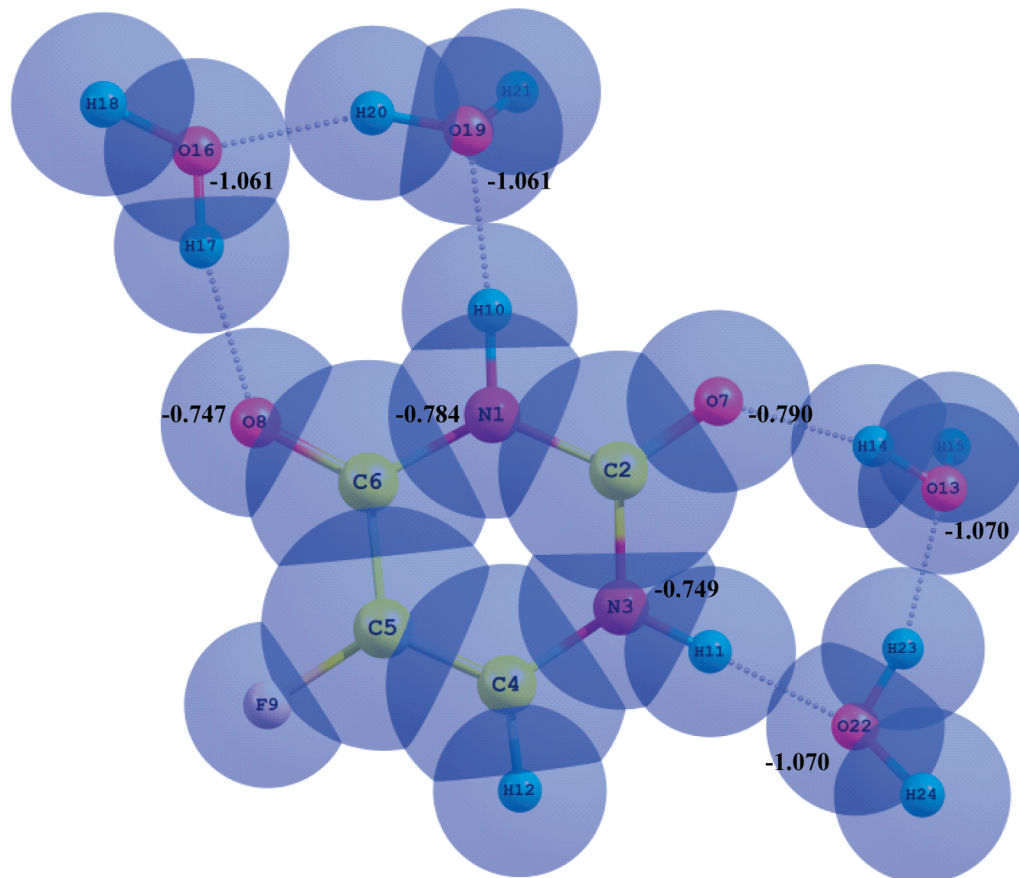


Figure 7. van der Waals radii and natural charges of the atoms in the 5FU water complex optimized at MP2/6-31+G(d,p) level.

TABLE 4: Selected Vibrational Data Calculated at MP2/6-31+G(d,p) Level for Four-Hydrated Complexes of 5FU (Dioxo Form) and Its Anions AN1 and AN3 (Figures 1 and 4)<sup>a</sup>

assignment	wavenumbers (Raman activities)						
	pH = 7	5FU	pH = 10	AN3	AN1	pH = 14	dianion
$\nu(\text{N3-H})$		3207 (324)		3325 (105)	3420 (137)		
$\nu(\text{N1-H})$		3124 (148)		3110 (120)	3170 (116)		3068 (139)
$\nu(\text{C-H})$		3184 (114)		1590 (32)	1671 (15)	1547	1597 (39)
$\nu(\text{C=C})$		1668 (8)	1531	1663 (46)	1577 (24)	1680	1539 (19)
$\nu(\text{C6=O})$	1691	1705 (191)	1679	1622 (6)	1624 (64)	1609	1445 (5)
$\nu(\text{C2=O})$		1731 (11)	1621		1467 (8)		
$\delta(\text{N3-H})$		1527 (25)		1389 (29)			
$\delta(\text{N1-H})$		1466 (16)	1354	1305 (32)	1267 (1)	1285	1285 (16)
$\delta(\text{C-H})$	1354	1341 (73)	1290	1187 (5)	1222 (5)	1210	1130 (2)
$\nu(\text{C-F})$	1262	1256 (17)	1187	976 (2)	965 (12)	1024	977 (11)
$\delta(\text{C4-N3-C2}) + \nu(\text{ring})$	-	979 (6)	1014	887 (2)	809 (1)	816	851 (1)
$\gamma(\text{C-H})$	824	838 (2)	822	786 (17)	785 (5)	783	781 (17)
$\delta(\text{ring})$	767	789 (5)	771	582 (1)	606 (1)	581	628 (2)
$\gamma(\text{ring})$	555	570 (1)	573				

<sup>a</sup> The wavenumbers, scaled by 0.963, are in  $\text{cm}^{-1}$  and Raman activities (in brackets) are in  $\text{\AA}^4 \text{amu}^{-1}$ . Experimental data at different pH are taken from ref 17.

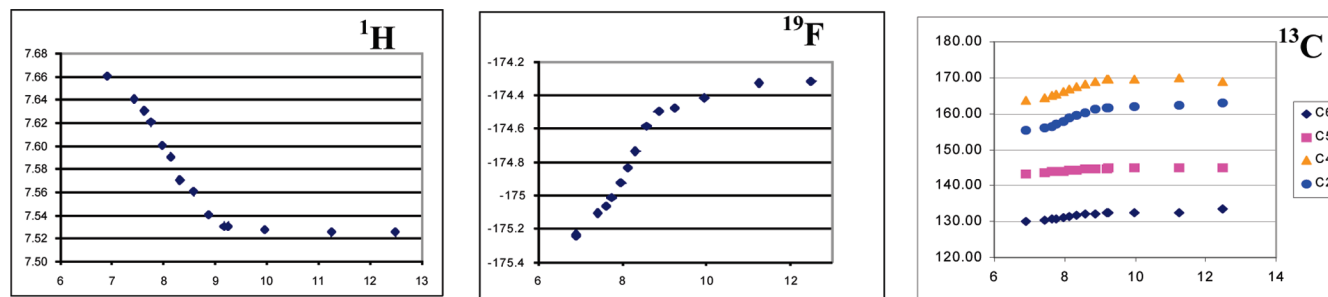
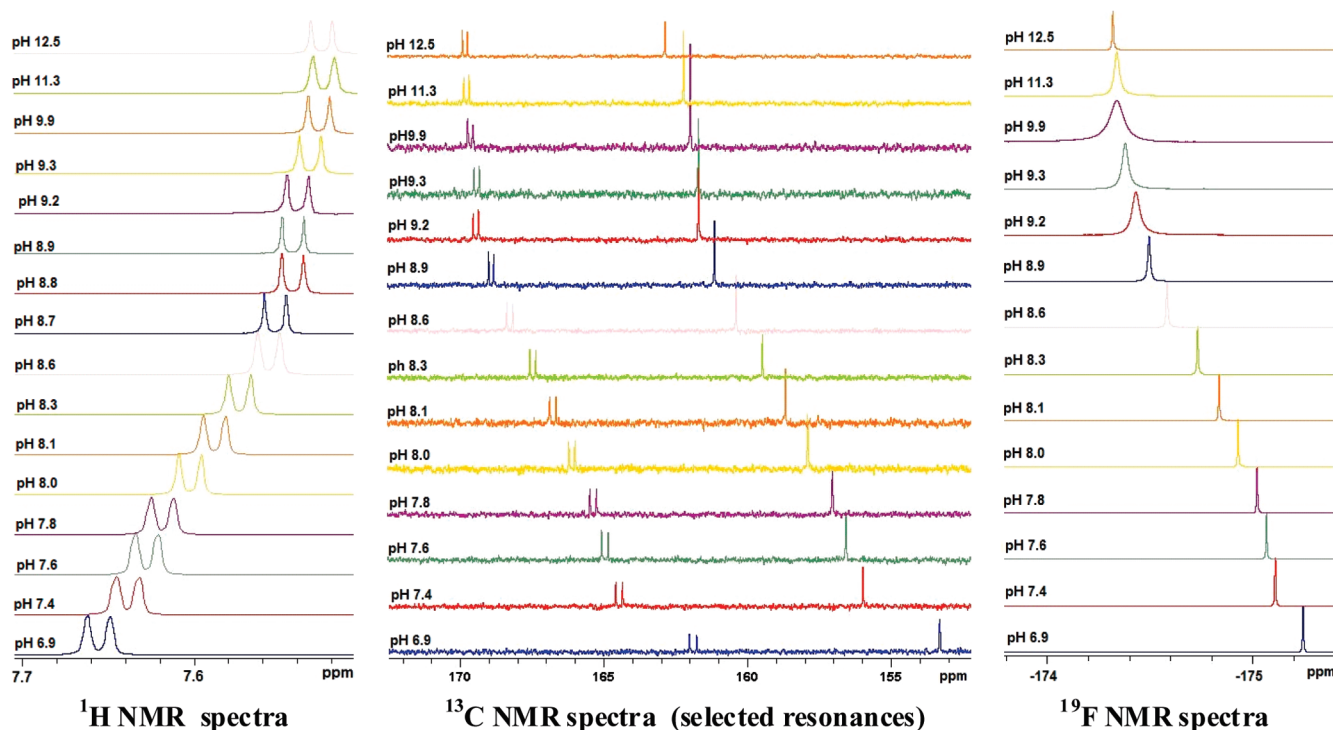
AN1 anions, respectively. More indicative is the change for the  $\nu(\text{C6=O})$  band at  $1705 \text{ cm}^{-1}$  which is shifted down to  $1663 \text{ cm}^{-1}$  for AN3 and to  $1577 \text{ cm}^{-1}$  for AN1, and their Raman activities decrease strongly. Generally, the calculated carbonyl bands at  $1663 \text{ cm}^{-1}$  and  $1622 \text{ cm}^{-1}$  are closer to the experimental values at pH = 10. Similarly, ab initio calculated in-plane deformations,  $\delta(\text{N1-H})$  and  $\delta(\text{C-H})$ , and  $\nu(\text{C-F})$  stretching mode for AN3 are also close to the experimental data. In this sense, at pH = 10 AN3 is the major form, probably. At alkaline pH = 14 the behavior is slightly different—based on the comparison of the theoretical and experimental data it could be suggested that a mixture between AN1 and a dianion of 5FU predominates.

The NMR predicted values of chemical shifts for 5FU in water solution are unsatisfactory if the tautomer A is considered as an isolated molecule.<sup>35,36</sup> To simulate the solvent influence on the chemical shifts a discrete arrangement of four water molecules around dioxo tautomer of 5FU were assumed (Figure 1). The calculated  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts were found to be close to the experimental data obtained (Table 5). Thus, the intermolecular interactions are a substantial factor determining the chemical shifts of the different nuclei. The NMR calculated chemical shifts for the carbons of the different 5FU anionic structures (Figure 4) are distinguished from those of the neutral molecule and should be indicative of the presence of anionic species in the pH range investigated.



**TABLE 5: GIAO  $^1\text{H}$  and  $^{13}\text{C}$  Chemical Shifts of 5-Fluorouracil (Four-Hydrated Neutral and Deprotonated Structures) Calculated at B3LYP/6-31++G(2df,p) Level and Experimental Data at Different pH<sup>a</sup>**

nuclei	pH = 7.43	5FU	pH = 9.96	AN3	CN3	AN1	pH = 13.8	dianion
C2	155.98	153.61	162.00	161.28	162.11	160.36	166.48	171.86
C4	130.46	127.05	132.45	144.42	138.44	121.84	137.73	138.07
C5	143.69	145.72	144.80	144.31	150.86	147.60	145.89	148.93
C6	164.40	160.20	169.67	160.46	167.04	167.91	170.04	168.24
H12	7.64	7.36	7.53	7.86	7.61	6.93	7.53	7.41

<sup>a</sup> The geometry is optimized at MP2/6-31+G(d,p) level.**Figure 8.** The graphically represented experimental  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  NMR data (ppm) of 5FU in water solution ( $\text{H}_2\text{O}:\text{D}_2\text{O} = 9:1$ ) at pH = 6.9–12.5.**Figure 9.** Selected  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  NMR spectra of 5FU in water solution ( $\text{H}_2\text{O}:\text{D}_2\text{O} = 9:1$ ) at pH = 6.9–12.5.

In order to establish the pH induced structural changes in the molecule of 5FU, further  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  NMR spectra in water solution ( $\text{H}_2\text{O}:\text{D}_2\text{O} = 9:1$ ) in the pH range of 6.9 to 13.8 were acquired and the chemical shift alterations determined as a function of pH. For optimal comparison and monitoring of pH-induced alterations on the chemical shifts, all NMR spectra were recorded at 298 K and pH of the samples was monitored using pH meter. The experimental  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  NMR data are graphically presented in Figure 8. In the pH range studied, only one set of resonance signals was observed in the  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  NMR spectra of 5FU.

A downfield chemical shift of the  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR resonances in 5FU in pH = 6.9–13.8 was observed (Figure 9). In contrast, pH dependent upfield chemical shift of H4 nuclei

was registered in the same pH range. An overall trend can be seen—a sudden leap of the chemical shifts of  $^1\text{H}$ ,  $^{19}\text{F}$ , and  $^{13}\text{C}$  resonances in the pH = 6.9–7.4 range. Then, a smooth downfield chemical shift for the  $^{19}\text{F}$  and  $^{13}\text{C}$  and an upfield chemical shift of  $^1\text{H}$  are observed until pH = 9.2. Above this pH value, only insignificant chemical shift alterations in all spectra were detected. The changes described above could be a result of the N–H chemical bond dissociation and 5FU anion formation. Moreover, the GIAO calculated carbon chemical shifts of AN3 and CN3 anions are closer to experimental data at pH = 9.96 (Table 4). The broadening of the fluorine resonance signals in the pH range between 9 and 10 suggests the existence of a mixture of different 5FU anion species.

In the  $^{13}\text{C}$  NMR spectra, the most significant pH dependent chemical shift changes are observed for the two carbonyl C2 and C6, and methine C4 carbons due to their close proximity to both NH groups. The fluorine substituted carbon (C5) is relatively unaffected (Figure 8). The equivalent pH induced chemical shifts alterations for C2 and C6 resonances could be the result of a fast proton transfer between the nitrogen and oxygen atoms of the AN3 anion leading to formation of both tautomers CN3 and DN3. This is verified by the values of the rate constants  $k$  of the tautomeric conversions  $\text{AN3} \rightarrow \text{CN3}$  ( $k = 5.16 \text{ s}^{-1}$ ) and  $\text{AN3} \rightarrow \text{DN3}$  ( $k = 5.5 \times 10^{-2} \text{ s}^{-1}$ ) (Table 2 and Figure 4).

## 5. Conclusions

According to our ab initio quantum chemical calculations, taking into account electron correlation at MP2 and CCSD(T) level using the 6-31+G(d,p) basis set, the most stable is the four-hydrated complex of the dioxo form A followed by the hydrated hydroxy forms B and D. The populations of the species B and D are  $4.2 \times 10^{-4} \%$  and  $3.1 \times 10^{-4} \%$ , respectively, and these species should be coexisting with tautomer A in water solution at neutral pH.

The deprotonation of the most stable dioxo tautomeric form, A, of 5FU can occur in two possible sites by formation of two anions: AN1 and AN3. Complexes containing four water molecules around the anions and their tautomeric forms are used for simulation of the interactions in aqueous medium. Anion AN3 is more stable than AN1 by  $5.31 \text{ kcal mol}^{-1}$  in water. Therefore, only the AN3 anion tautomeric conversion is considered. Our theoretical results for Raman wavenumbers, NMR chemical shifts and NBO analysis of neutral and AN1 and AN3 deprotonated ions of 5-FU allow us to deduce that the deprotonation at alkaline pH should occur at the N3–H bond. Therefore, the coexistence of different tautomeric forms of AN3 in water solution at pH = 7.8–10 is probable.

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