Prototropic Equilibria in 4-Thiouracil: A Combined Spectroscopic and ab Initio SCF-MO Investigation

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Prototropic equilibria in 4-thiouracil has been investigated by high-level ab initio calculations. Six of the most stable tautomers have been studied at various levels of theory including the MP4(SDQ)/6-311G(2d,2p)//MP2/6-31G(d,p) level. At all levels of theory, the 2-keto-4-thio form is shown to be the most stable one while the 4-thio-2-enolic form is predicted to be the second most stable tautomer. Aqueous solvation is shown only to destabilize the minor tautomers with an energy difference between the two most stable tautomers estimated to be 12.02 kcal/mol, while the corresponding difference in the gas phase is 10.4 kcal/mol. At MP4/6-311G(2d,2p)//MP2/6-31G(d,p) in the gas phase, the following order of stability is observed; 4TU1 > 4TU2 > 4TU3 > 4TU4 > 4TU5 > 4TU6, while in an aqueous phase a stability order of 4TU1 > 4TU2 > 4TU4 > 4TU3 > 4TU6 > 4TU5 is predicted. We corroborate our theoretical findings with spectroscopic studies of UV absorption and the luminescence spectra. Although the UV absorption spectrum of the neutral keto-thionic forms of 4-thiouracil in an aqueous medium is essentially a single-component one indicating that 4-thiouracil exists as a single tautomer in aqueous medium, the luminescence spectrum of these compounds in an ethanol solution was found to be a two-component one suggesting that ethanol could play a key role in stabilizing the minor tautomeric species in excited states. Using combined experimental and theoretical results, we conclude that in an aqueous medium, 4-thiouracil exists in the 2-keto-4-thio form and excludes the possible existence of minor tautomers whereas in ethanol it could exist in two tautomeric forms.

Introduction

There has been continued interest in the structural properties of modified nucleic acid bases since most of them have been widely implicated for a variety of biological activities.[1−3] For example, incorporation of heavier atoms into DNA bases leads to a therapeutically important class of nucleic acid components.[4,5] One important class of such derivatives originates from the substitution of the thio group in place of the exocyclic oxo group in both purines and pyrimidines. Some important derivatives of the purine class are 6-thioguanine[6] and 6-selenoguanine.[7] Similarly 2-thiouracil, 4-thiouracil, 2,4-dithiouracil, and thiocytosine constitute another important class of pyrimidine derivatives. Owing to their large biochemical interest,[8,9] it is not surprising that they have been the subject of extensive experimental[10−12] and theoretical studies.[13−16]

The substitution of oxygen with the bulkier sulfur significantly alters the structural patterns of nucleic acids. Such geometrical and energetic changes might be of some concern for nucleic acid conformations and hydrogen-bonding potential of thio-analogues. One can expect that such modifications would alter the recognition patterns exerted by enzymes involved in the nucleic acid metabolism. A detailed understanding of the structural and geometrical changes caused by sulfur substitution would enable us to understand the reasons behind their differential role in biological activity. For example, 2-thiouracil acts as a carcinogen, neoplastigen, tumorigen, and teratogenic agent, and its alkyl derivatives such as 6-propyl-2-thiouracil have been shown to possess antithyroid activity.[8,9] 4-Thiouracil constitutes an important derivative of the thiated pyrimidines. It is known to possess cytostatic properties and is used as the cross-linking agent in RNA transcriptional regulation.[17] It exists in prokaryotic tRNAs.[12,18,19] In addition, 4-thiouracil analoguss of dUMP are good substrates of thymidilate synthase.[20] Despite its biological importance, accurate studies of the structure and energies of 4-thiouracil tautomers are still lacking. Recent ab initio studies of tautomerism of 4-thiouracil employing smaller 3-21G basis sets with single-point calculations at the 6-31G-(d,p) level indicate that the keto-thionic form predominates over other tautomeric forms in the gas phase.[21] However, studies pertaining to the relative population of 4-thiouracil tautomers in an aqueous medium are not known. It has been obvious for a long time that solvation greatly modulates the tautomeric equilibria especially in heterocyclic systems.[22] The reported IR spectra of 4TU in an argon matrix demonstrate that 4TU under experimental conditions occurs in a keto-thionic form.[11]

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The influence of solvation on the tautomeric equilibria in 4-thiouracil is not clear. The present study focuses on the tautomeric equilibria of the 4-thiouracils both in vacuo and in aqua. The theoretical calculations will be corroborated with experimental spectroscopic investigations using UV absorption and luminescence methods.

Experimental Section

The experiments were carried out on 4TU, deoxythiouridine (4dTUR), and 8-methyl-4-thiouracil (4MeTU), as supplied by the "Serva" corporation. The aqueous solutions were prepared by using deionized water. Ethanol was purified and redistilled but still contained ~7% of water. The solutions were acidified up to pH = 6.0 by adding HCl. The concentration of 4TU was in the range of $10^{-4}-10^{-5}$ M. UV absorption spectra were measured using the Hitachi spectrophotometer.

The luminescence spectra which have been obtained at 4.2 K were taken with the luminescence setup described previously.[23] The kinetic investigations were carried out using the device as described before.[24] When estimating the differential spectra of 4TU, we speculated that the luminescence intensity of the second component at α_{obs} = 540 nm was close to 0 because the kinetics of phosphorescence decay in this region are exponential and responsible for the luminescence of a single form.

Methods

The ab initio LCAO-MO[25] method was used in the present study of the tautomers of 4-thiouracils. Full-geometry optimizations were performed without imposing any symmetry constraints at the HF/6-31G(d,p) and MP2/6-31G(d,p) levels of theory, and all optimized geometries at HF/6-31G(d,p) were found to be true minima by analysis of the respective harmonic vibrational frequencies obtained from diagonalization of the force constant matrices with the corresponding Hessian eigenvalues being positive. To get more accurate energies, basis sets with inclusion of diffuse functions and electron-correlation effects were used and single-point calculations at the MP4/6-311G(2d,2p) and MP2/6-311++G(d,p) levels were performed at the HF/6-31G(d,p) and MP2/6-31G(d,p) reference geometries. The ZPE corrections were made as the sum of zero-point energies for all normal modes of vibrations scaled by a recommended factor of 0.9. The gas-phase free energies were calculated by using the standard formula $\Delta G = \Delta H - T\Delta S$; the thermodynamic quantities were evaluated at 298.5 K.

All ab initio calculations are carried out using the GAUSSIAN92 and GAUSSIAN94 packages,[26] while the semiempirical calculations in an aqueous phase were done using the AMSOL 4.5.1[27] suite of programs. The semiempirical AM1-SM2[28] method has been used in this study to evaluate the free energies of hydration of various tautomers. The AM1-SM2 method was shown to be reliable in predicting the hydration free energies.[29,30] Besides this method, we have also used the ab initio self-consistent isodensity polarizable continuum model as developed by Tomasi et al.[31] and have incorporated it into GAUSSIAN94 as an SCI-PCM model to evaluate the free energies of hydration. We used a relative permittivity constant of 78.4 to model an aqueous medium and a default 0.001*e* isodensity surface. The free energy of tautomerization in an aqueous solution was subsequently determined according to eq 1:

$$\Delta G^{aq}_{A\rightarrow B} = \Delta G^{gas}_{A\rightarrow B} + \Delta G^{hyd}_{B} - \Delta G^{hyd}_{A} = \Delta G^{gas}_{A\rightarrow B} + \Delta\Delta G^{hyd}_{A\rightarrow B} \quad \text{... (1)}$$

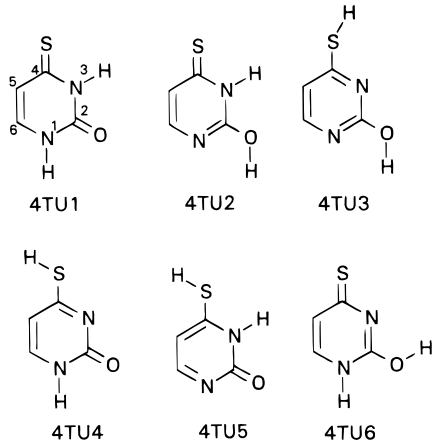

Figure 1. Schematic representation of various 4-thiouracil tautomers studied.

TABLE 1: Relative Energies of Various Tautomers of 4-Thiouracil with Respect to 4TU1 (All Values in kcal/mol)

method	4TU1	4TU2	4TU3	4TU4	4TU5	4TU6
HF/6-31G(d,p)//HF/6-31G(d,p)	0.00	9.82	12.83	17.79	21.19	20.55
MP2/6-31G(d,p)//MP2/6-31G(d,p)	0.00	10.26	11.89	16.59	19.89	19.89
MP4(SDQ)/6-311G(2d,2p)//MP2/6-31G(d,p)	0.00	10.39	12.72	15.27	19.11	29.73
MP2/6-311++G(2d,2p)//MP2/6-31G(d,p)	0.00	9.13	9.88	14.19	17.85	28.35

The relative free energies of hydration ($\Delta\Delta G_{A\rightarrow B}$) are computed at the AM1-SM2 and HF/SCRF level from the absolute free energies of hydration (ΔG^{hyd}_{A} and ΔG^{hyd}_{B}).

The excited-state energies of the 4-thiouracil tautomeric forms were calculated by the CNDO/S methods as described previously.[32] The resonance integral of the sulfur atom was taken to be equal to 14 eV, and the one-center integral of the interelectronic repulsion was taken to be equal to 7 eV.[33,34]

Results and Discussion

Six of the most stable tautomers of 4-thiouracil are considered in the present study, out of which three are in the thionic form and the rest are in the thiolic form. Figure 1 illustrates the various tautomers studied. In all tautomeric forms, the labile hydrogen atoms that are connected to either the N1 or N3 atoms migrate to either the C2-oxo group or the C4-thio group. The calculated relative energies of tautomers at various levels of theory are tabulated in Table 1. From the data, it is clear that the 2-keto, 4-thio form of thiouracil turns out to be the most stable one while the 2-hydroxy, 4-thio form 4TU2 emerges as the next most stable form (Figure 1). The energy difference between the two most stable tautomers 4TU1 and 4TU2 is 9.13 kcal/mol in the gas phase at the MP2/6-311++G(2d,2p)//MP2/6-31G(d,p) level and amounts to 10.4 at the MP4(SDQ)//6-311G(2d,2p)//MP2/6-31G(d,p) approximation. The third most stable tautomer is the 4-thiol-2-enolic form (4TU3); the energy difference between 2 and 3 is, however, less than 1 kcal/mol. Overall, the following stability order may be observed in the gas phase for 4-thiouracils: 4TU1 > 4TU2 > 4TU3 > 4TU4 > 4TU5 > 4TU6.

It is important to be able to understand the geometric and energetic changes caused by substitution of sulfur in place of oxygen. Previous studies of uracil at the MP2/631G(d,p)//MP2/6-31G(d,p) and MP4(SDTQ)/6-31G(d,p)//HF/6-31G(d,p) levels suggest that the energy difference between the two most stable tautomers (2,4-diketo and 2-enol-4-keto forms) of uracil is 11.07

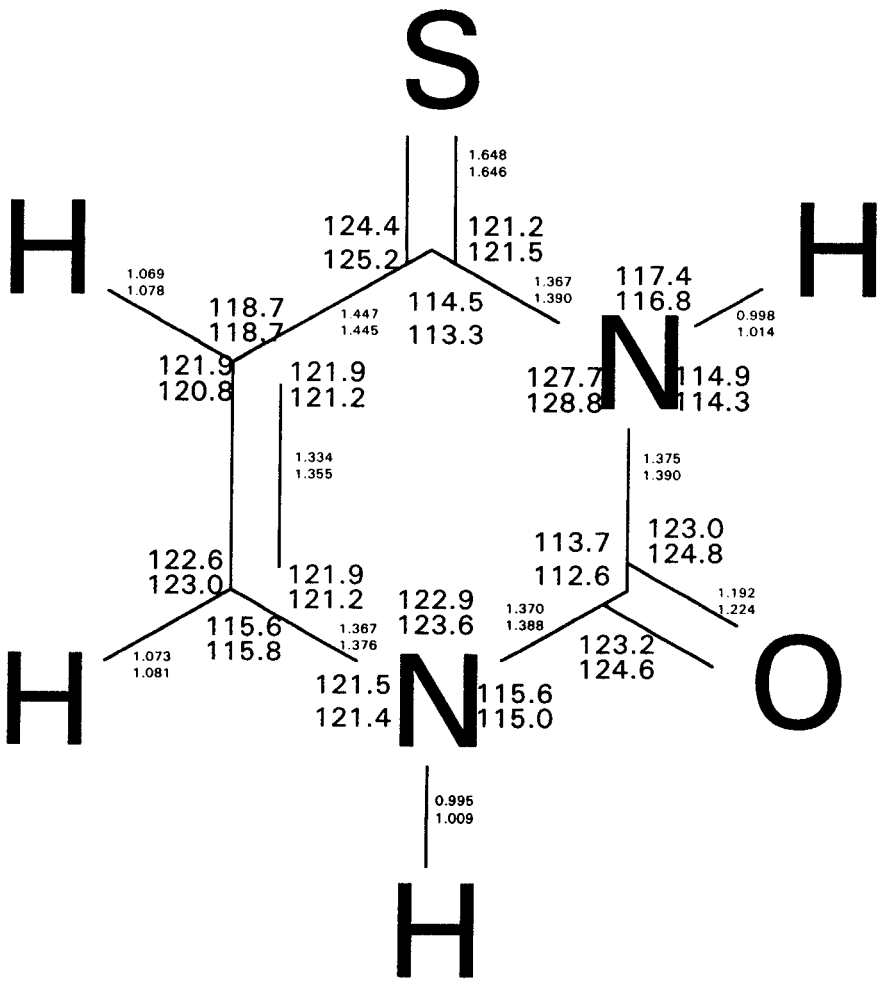

Figure 2. Bond angles and lengths for 4TU1, calculated at the HF/6-31G** and MP2/6-31G** levels.

and 11.50 kcal/mol, respectively,[35] whereas the same energy difference between the thione and thiolic forms of 4-thiouracil at the MP2/6-31G** level is 10.26 kcal/mol. Thus, substitution of oxygen by sulfur at the C4 position diminishes the energy difference between uracil and 4-thiouracil to 0.8 kcal/mol at the MP2/6-31G(d,p)//MP2/6-31G(d,p) level. Figures 2 and 3 show the geometrical parameters of two of the most stable tautomers of 4-thiouracil computed at HF/6-31G(d,p) and MP2/6-31G(d,p). The bond distances of the pyrimidine ring computed at the MP2/6-31G(d,p) level show different trends for the 2-keto-4-thiouracil tautomer (4TU1, Figure 2) and the corresponding 2-enolic tautomer (4TU2, Figure 3). While, in case of 4TU1, the MP2 bond distances of the ring atoms are, to a large extent, longer than the corresponding bond distances computed using the HF/6-31G(d,p) basis set, the enolic tautomer 4TU2 shows an opposite trend making the HF/6-31G(d,p) bond distances longer than that of the MP2 geometries. This tendency in thiopyrimidines is somewhat different from those in thiopurines where the MP2 bond lengths are shown to be consistently longer than those calculated at the HF/6-31G(d,p) level. Very recent studies of thioguanine indicate that there is a general expansion of the ring geometry at the MP2/6-31G(d,p) level when compared with the HF/6-31G(d,p) level geometries for both 6-thione and 6-thiolic tautomers.[36]

A number of studies of the influence of aqueous solvation on the geometries and energies of several heterocyclic systems, nucleic acid bases, and their derivatives indicate that solvation plays a major role in altering the tautomeric equilibrium.[22,37,38] For instance, while in the gas phase guanine exists predominantly in the N7-protonated form, upon aqueous solvation, the tautomeric preference shifts toward the N9-protonated form.[39] A similar effect is seen in the case of 6-thioguanine.[36] In order to understand the influence of aqueous solvation on the tautomeric energies of the thiouracil system, we have estimated the free energies of hydration by the polarized continuum models SCI-PCM and AM1-SM2. The computed results are tabulated in Table 2 along with computed gas-phase dipole moments. As far as the trends are concerned, it is encouraging to observe that both methods predict a qualitatively similar trend. The 2-enol, 4-thio form (4TU6) is predicted to be the most hydrated species by both methods, which is in line the with the larger dipole moment of 4TU6 (8.04 D at MP2/6-31G(d,p)). The thiol−enol form, 4TU3, is estimated to be the least hydrated form with the corresponding values of 7.86 and 5.86 kcal/mol by the SCI-PCM and AM1-SM2 models. Despite the methodological differences between the two solvation models, one can observe good qualitative similarity between the two models. These trends of free energies of hydration are very much in line with the similar trends in dipole moments. The dipole moments predicted by the MP2/631G(d,p) method are in general smaller than the corresponding HF/6-31G(d,p) values. The 2-enolic form (4TU6) of 4-thiouracil is predicted to have the highest dipole moment (6.81 D at the MP2/6-31G(d,p) level), while the 4-thiol, 2-enolic form (4TU3) possess a dipole moment of 1.79 at the same level.

Finally, we evaluated the free energies of tautomerization by combining the gas-phase free energies with the hydration free energies according to eq 1, and the results are tabulated in Table

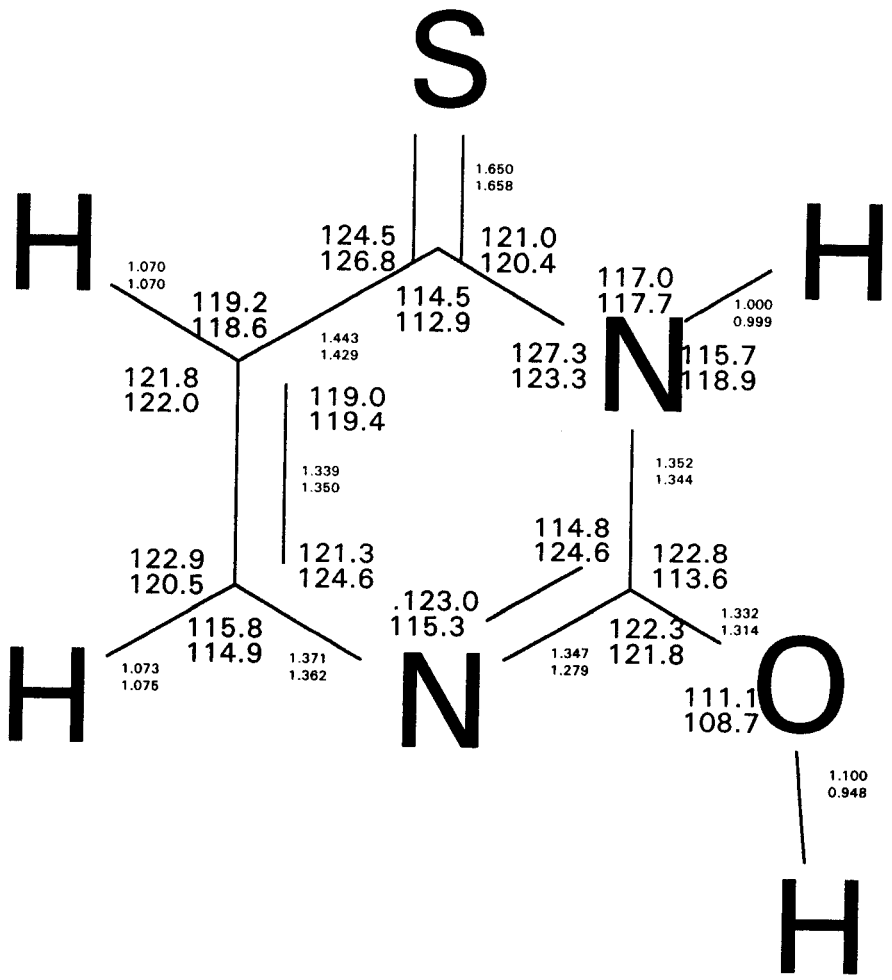

Figure 3. Bond angles and lengths for 4TU2, calculated at the HF/6-31G** and MP2/6-31G** levels.

TABLE 2: Relative Free Energies of Hydration Estimated by Various Continuum Models (in kcal/mol) and Dipole Moments of 4-Thiouracil Tautomers

method	4TU1	4TU2	4TU3	4TU4	4TU5	4TU6
SCI-PCM/6-311G(2d,2p)//MP2/6-31G(d,p)	0.00	1.63	7.56	2.86	4.68	−6.33
AM1-SM2	0.00	2.72	5.86	−2.11	−1.68	−2.98
			Dipole Moments			
HF/6-31G(d,p)	5.66	5.17	1.93	7.48	6.87	8.17
MP2/6-31G(d,p)	4.60	3.89	1.79	6.74	6.22	6.81

TABLE 3: Free Energies of Tautomerization of Various Tautomers with Respect to 4TU1 (kcal/mol)

method[a]		4TU1	4TU2	4TU3	4TU4	4TU5	4TU6
MP4(SDQ)/6-311G-(2d,2p)//MP2/6-31G(d,p)	(a)	0.00	12.02	20.28	18.13	23.79	23.40
	(b)	0.00	13.11	18.58	13.16	17.43	26.75
MP2/6-311++G-(2d,2p)//MP2/6-31G(d,p)	(a)	0.00	10.76	17.44	17.05	22.53	22.02
	(b)	0.00	11.84	15.74	12.08	16.17	25.37

[a] (a) and (b) values correspond to free-energies of tautomerization estimated using the SCI-PCM and AM1-SM2 values of free energies of hydration respectively.

3. Our best estimation of the free energy of tautomerization at MP2/6-311++G(2d,2p)//MP2/6-31G(d,p) indicates that the tautomeric transition between 4TU1 and 4TU2 involves a free energy of about 10.8 kcal/mol. One important observation is that aqueous solvation makes 4TU4 more stable than 4TU3. Similarly, 4TU6 turns out to be more stable than 4TU5. Thus in an aqueous phase, the following order of stability may be established: 4TU1 > 4TU2 > 4TU4 > 4TU3 > 4TU6 > 4TU5.

To gain further insight into the tautomerism of 4-thiouracil, we investigated the UV absorption and luminescence (phosphorescence) spectra of aqueous and ethanol solutions of 4TU, 4dTUR, and 4MeTU at pH = 6.0. Under these conditions one can observe only the phosphorescence spectra. The data on the UV absorption and luminescence spectra of 1,3-dimethyl-4-thiouracil (1,3Me4TU) in polar solutions were taken from previous studies,[40,41] and those on luminescence spectra of 1-methyl-4-thiouracil (1Me4TU) were derived from a crystal matrix of 1-methyluracil.[42] We also studied the kinetics of phosphorescence decay of the ethanol solutions of 4TU at different wavelengths of observation. As is evident from the UV absorption and luminescence spectra of 4dTUR, 1,3Me4TU, and 4MeTU (Figure 4), the UV and luminescence spectra of 4MeTU is shifted toward a high-frequency region relative to those of 4dTUR, and the luminescence spectrum of 1,3Me4TU is somewhat shifted toward a low-frequency region relative to that of 4dTUR. It is interesting to observe that although the luminescence spectra of 4TU and 1,3Me4TU differ in shape the UV absorption spectra of 4dTUR and 1,3Me4TU are almost identical in shape and position.

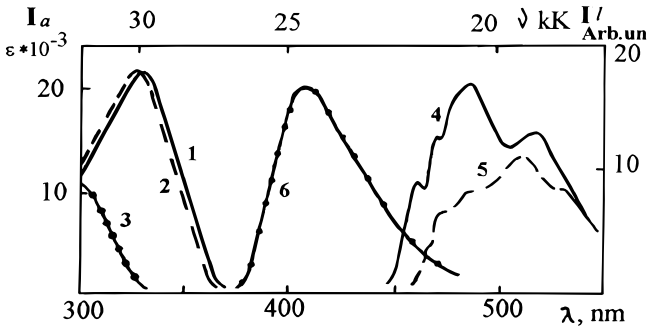

Figure 4. UV−absorption spectra (left) at 293 K in water and luminescence spectra (right) at 4.2 K in ethanol of 4dTUr (1, 4), 1,3 Me4TU (2, 5), and 4MeTU (3, 6).

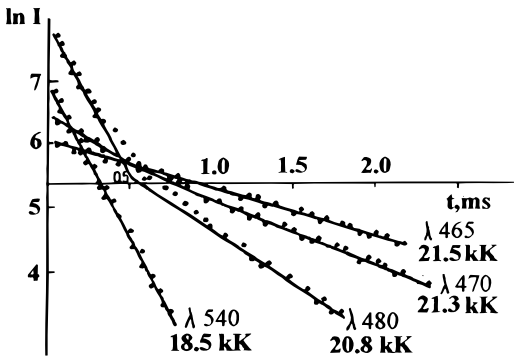

Figure 5. Kinetics of phosphorescence quenching of ethanol 4TU solutions for different observed wavelengths at 77 K.

As can be seen from Figure 5, the kinetics of phosphorescence decay of 4TU at the observed wavelengths (21.5 and 18.5 kK) is exponential while at the intermediate frequencies it is nonexponential. The data is in good correlation with those of 4TUR as previously published.[43] According to our observations, the phosphorescence polarization of 4TU varies with frequency.[23,44]

Close consideration of the data shown in Figures 4 and 5 and the previous data[23,43] reveals that the spectrum of 4TU in the ethanol solution is a two-component one. Subtraction of the luminescence spectrum of 1,3Me4TU in a similar solution[41] or that of 1Me4TU in a crystal matrix[42] from the spectrum of 4dTUR results in similar data as shown in Figure 6. As evident from the figure, the differential spectrum has a distinct vibrational structure and is shifted toward a high-frequency region relative to the spectra of 1,3Me4TU and 1Me4TU.

As the luminescence spectra shown in Figure 4 were taken at pH = 6.0, which is well below the pKa value of 4TU and 4dTUR, the spectra under consideration should be considered as those not due to ionization of 4dTUR and 4TU. The shape and position of the spectra from the 4dTUR and 4TU ionic forms are considered in detail previously.[23,44] The investigation of the concentration variations in the luminescence spectrum of 4TU in the concentration range of $10^{-5}-10^{-3}$ M suggests that the generation of a new spectral component is not associated with a possible aggregation of 4TU and 4dTUR.

Calculations of the excited state energies of the SH−, OH−, and keto-thionic forms of 4TU (Table 4) show that the UV absorption and luminescence spectra of the thiolic tautomer must be shifted to a high-frequency region while the excited-states energies of the keto and enol forms of 4TU are similar. This correlates with the spectra shown in Figure 4.

One can conclude that the second component in the luminescence spectra originates from the presence of the enolic

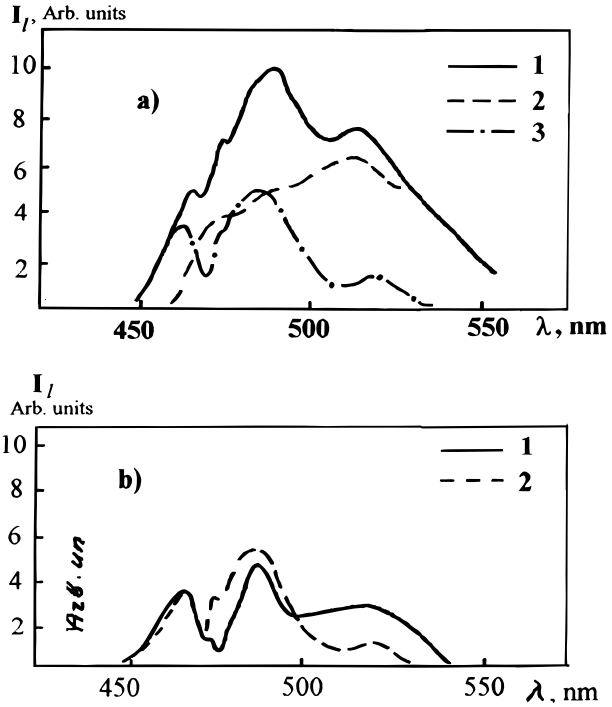

Figure 6. (a) Phosphorescence spectra of 4-dTUR (1) and 1,3-Me4TU[41] (2) and their differential spectrum (3). (b) Differential spectra of (4-dTUR-1,3 Me4TU) (1) and (4-dTUR-1-Me4TU) (2).

TABLE 4: CNDO/S Oscillator Strengths (*f*) and Excited-State Energies (in eV) of 4-Thiouracil Tautomers

	4TU1	4TU6	4TU4	4TU3	4TU2	4TU5	method
f	0.579	0.604	0.132	0.211	0.466	0.324	CNDO/S
$E(S\pi\rightarrow\pi^*)$[a]	3.90	3.78	4.86	5.01	3.82	4.57	HF
	3.87	3.75	4.69	4.91	3.78	4.42	MP2
$E(n\rightarrow\pi^*)$	2.88	2.73	4.39	4.72	3.13	4.40	HF
	2.85	2.70	4.33	4.72	3.11	4.34	MP2
$E(T\pi\rightarrow\pi^*)$	2.59	2.48	3.32	4.07	2.72	3.23	HF
	2.50	2.40	3.15	3.95	2.66	3.07	MP2

[a] Excited-state energies of single (S) and triplet (T) electronic excitations values estimated by CNDO/S method.

tautomer in a polar solvent. As is evident from Figure 4, the ratio between the areas under curves 4 and 5 is equal to 1.5. If the quantum yield of 4dTUR is taken as 0.1, contribution of a second component to the luminescence spectrum accounts for 0.03. For the ionic forms of 4TU the quantum yield was 10 times as great as that of the neutral form.[23,44] If we assume a 10-fold increase in the quantum yield for the enolic form (though it may be as much as 15−20-fold large), we may estimate the relative concentration of the enolic form. Such a rough estimation for the given experimental conditions (4dTUR in ethanol) yields an approximately 5−7% concentration of the enolic form. The long-wave band in the UV absorption spectrum of the 4TUand 4dTUR neutral forms is described by a log normal curve,[23,34,44] which is usual for the other nucleic acid components.[45] It suggests that the UV absorption spectra of 4TU and 4dTUR in water are one-component and reflect the existence of only one 4TU1 tautomer in a water solution. On the other hand, the data of Tables 1, 3, and 4 show that the relative concentration of the enolic tautomer must be less than 0.1% in a polar solvent. It suggests that the second (enolic) tautomer, which is observed in the phosphoresence spectra of 4TU or 4dTUR, is formed only upon electronic excitation (in the S or T states). Thus, the above experimental and theoretical data indicate that the UV absorption of 4TU and 4dTUR in a water solution have only one component related only to the

4TU1 (keto-thion) tautomer in solution. On the other hand, the luminescence spectrum of 4TU (4dTUR) in an ethanol solution is two-component, which could be caused by the formation of the enolic tautomer.

Conclusions

The ab initio calculations were performed on six stable tautomers of 4-thiouracil at the second and fourth-order levels of the correlated Moller−Plesset theory using large basis sets. At both MP4(SDQ)/6-311G(2d,2p) and MP2/6-311++G(2d,2p) levels using the MP2/6-31G(d,p) reference geometries, the order of stability is found to be 4TU1 > 4TU2 > 4TU3 > 4TU4 > 4TU5 > 4TU6 in the gas phase. The aqueous solvation effect was studied using the SCI-PCM model at the HF/6-311G(2d,2p) level using the MP2/6-31G(d,p) geometry and the semiempirical AM1-SM2 models. These models are shown to agree only qualitatively with each other. Aqueous solvation is shown only to destabilize the minor tautomers with an energy difference between the two most stable tautomers estimated to be about 12.02 kcal/mol, while the corresponding difference in the gas phase is 10.4 kcal/mol. Despite the conceptual difference between the two models, both continuum models predict that 4TU6 is the most hydrated species while 4TU3 is the least hydrated one. Such a trend is attested by the corresponding gas-phase dipole moment values. In an aqueous phase the following stability order is established for 4-thiouracil, 4TU1 > 4TU2 > 4TU4 > 4TU3 > 4TU6 > 4TU5.

The calculated excited-state energies of the various thione and thiolic forms of 4-thiouracil by the CNDO/S method demonstrate that the UV absorption and luminescence spectra of the thiolic tautomer should be shifted to a high-frequency region. An analysis of the UV absorption and luminescence spectra and the kinetics of phosphorescence in water and ethanol solutions suggests that 4-thiouracil exists predominantly in the keto-thionic form in an aqueous phase while in an ethanolic solution, the minor tautomeric species might be formed and stabilized.

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