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Putative phototautomerization of 4-thiouridine in the S_2 excited state revealed by fluorescence study using picosecond laser spectroscopy

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Abstract

The stationary and dynamic properties of $S_2 \rightarrow S_0$ fluorescence of 2′,3′,5′-tri-*O*-acetyl-4-thiouridine (TU) in acetonitrile solution at room temperature have been studied. In the emission spectra of the sample, measured upon excitation with picosecond laser pulses, changes in fluorescence intensity and emission band shape have been observed with increasing time of excitation. The fluorescence intensity increases and the band maximum moves to the shorter wavelength until the steady-state condition is achieved. These changes are fully reversible indicating that they are not related to the formation of stable photoproducts. No such changes have been observed in the fluorescence spectrum recorded upon excitation of the same sample of TU with continuous light from xenon lamp. Under the latter conditions the fluorescence is very weak ($\phi_F = 1 \times 10^{-4}$) and the band exhibit a maximum at $\lambda^F_{max} = 420$ nm. The decay kinetics was measured using a two-stage method in order to estimate the number of emitting species and determine the parameters characterizing their emission. Analysis of the experimental fluorescence decays at $\lambda^F = 400$, 430 and 450 nm measured upon various time intervals (<20 min) of the sample excitation with laser pulses has shown that the best fit of experimental to theoretical curve is obtained assuming the presence of three emitting species with lifetimes of $\tau_1 = 5 \pm 1$ ps, $\tau_2 = 80 \pm 20$ ps and $\tau_3 = 600 \pm 100$ ps. In contrast to the lifetimes the values of fractional coefficients depend on λ^F and the time of measurement. The results of the fluorescence study of TU with picosecond laser spectroscopy are discussed in terms of phototautomerization of TU (2-keto-4-thione) in the S_2 state leading to its two possible tautomers: 4-thione, 2-hydroxy form in the S_2 state and 4-thiol, 2-keto form in the S_1 state. The models of the tautomers of TU were synthesized and their absorption and emission features were determined.

Keywords: Fluorescence; 4-Thiouridine; Phototautomerization

1. Introduction

4-Thiouridine is a rare, modified nucleoside found in certain t-RNAs. Due to its spectral and photochemical properties it has been frequently used as a probe in studies of interactions in biological systems using the photocrosslinking method [1–3]. In order to explain the molecular mechanism of the photoreactions, this molecule, its aglycone and other derivatives containing 4-thiouracil chromophore have been subjected to spectral and photophysical studies in aqueous solution and in several aprotic, polar and nonpolar organic solvents [4–11]. The stationary and dynamic properties of these thiocarbonyl compounds in their lowest excited triplet state (T_1) at room temperature have been already described. The experimental data have been obtained by the steady-state and time-resolved phosphorescence spectroscopy [5–11] and nanosecond laser flash photolysis [4]. It has been found that the T_1 state in 4-thiouridine and in 1,3-dimethyl-4-thiouracil (DMTU) is populated with high quantum yield ($\phi_T = 0.9 \pm 0.1$) upon excitation of these compounds into very intense $S_0 \rightarrow S_2$ absorption band ($\lambda_{max} \approx 330$ nm) [1,4]. In the absence of oxygen or other addends, the major process of excited T_1 state deactivation is concentration quenching (self-quenching). The values of self-quenching rate constants (k_{sq}) determined for 4-thiouridine, 2′,3′,5′-tri-*O*-acetyl-4-thiouridine (TU, Scheme 1) and DMTU are high, approaching the diffusion controlled ones. The intrinsic lifetime of the T_1 state of DMTU in

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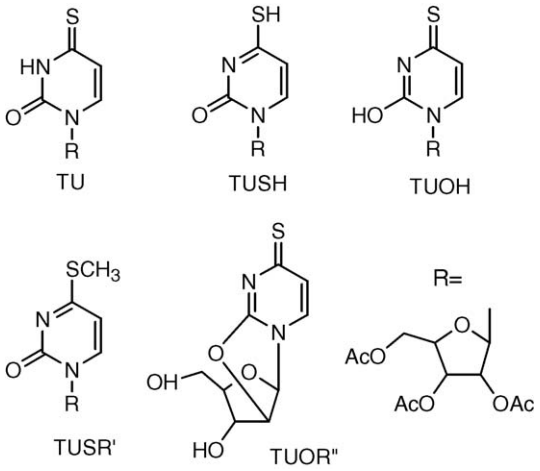

Scheme 1.

chemically inert solvent—perfluoro-1,3-dimethylcyclohexane (PFDMCH) was determined to be $\tau_T^0 = 5.3$ ms and it is significantly longer than that in any other solvent studied (e.g.: 3.6 μs in CCl_4 and 0.2 μs in H_2O). This means that quenching due to solute–solvent interactions is a second important channel of the deactivation of T_1 state of 4-thiouracil derivatives [10].

We have previously reported that DMTU and TU behave as typical aromatic thiones, since in addition to phosphorescence they emit at room temperature a very weak fluorescence from the second excited singlet state (S_2) [10,11]. The $S_2 \rightarrow S_0$ fluorescence properties of DMTU in PFDMCH, CCl_4 and aqueous solutions were described [10]. Using spectrofluorimetry and picosecond laser spectroscopy the fluorescence quantum yield (ϕ_F) and fluorescence decay kinetics were determined. The values of ϕ_F were low ranging from $\phi_F = (1.5 \pm 0.7) \times 10^{-4}$ in PFDMCH to $\phi_F = (0.4 \pm 0.2) \times 10^{-4}$ in aqueous solution. In organic solvents the character of the $S_2 \rightarrow S_0$ fluorescence decays is monoexponential with the lifetimes 7.5 ps in PFDMCH and 2.5 ps in CCl_4, obtained from analysis of experimental decay curves. It has been established that the S_2 state of DMTU decays mainly through intra and intermolecular radiationless processes with the quantum yield $\phi_{NR} > 0.99$. Among these processes a reversible, intramolecular H abstraction by the excited thiocarbonyl group from N-CH_3 group in ortho position is the fastest ($k_H = 1.2 \times 10^{11}\ s^{-1}$) decay channel of the excited S_2 state of DMTU. Internal conversion is by an order of magnitude slower ($k_{s_2s_1} = 1.5 \times 10^{10}\ s^{-1}$). The mechanism and dynamics of S_2 deactivation also depend on the solvent properties. Intermolecular quenching by solvent ($k_S = 2.5 \times 10^{11}\ s^{-1}$) is responsible for the three times shorter lifetime of S_2 state of DMTU in CCl_4 as compared to that in PFDMCH solution [10].

In this paper we describe the properties of TU $S_2 \rightarrow S_0$ fluorescence revealed by the picosecond laser spectroscopy study. For the sake of comparison, the TU $S_2 \rightarrow S_0$ fluorescence study was also performed under steady-light excitation of TU. All the studies were performed at room temperature in the air-saturated acetonitrile solution. Although water is the most important biological environment of TU molecule, acetonitrile was the solvent of choice in our study, because it is polar but in contrast to water it is not involved in the specific interactions (disregarding the possibility of formation of weak hydrogen bonds; $\alpha = 0.19$, $\beta = 0.31$ [12]) which facilitates interpretation of the results.

2. Experimental

2.1. Materials

2′,3′,5′-Tri-*O*-acetyl-4-thiouridine (TU) [11], 2′,3′,5′-tri-*O*-acetyl-4-methylthiouridine (TUSR′) [13] and 2,2′-anhydro-1-(β-D-arabino-furanosyl)-4-thiouracil (TUOR″) [14] were synthesized as described in literature. The compounds were purified before measurements using the previously described HPLC system (Waters 600 E) [10,11]. The purification was performed on a RP_{18} Waters X-Terra 5 μm (7.8 × 15 mm) column eluted with 42.5% aq.CH_3CN (TU); 27.5% aq.$CH_3CN \rightarrow$ (20 min) 80% aq.CH_3CN (TUSR′), 12.5% aq.$CH_3CN \rightarrow$ (15 min) 80% aq.CH_3CN (TUOR″). The fluorescence detector was set at the same wavelengths as those used for fluorescence measurements ($\lambda_{exc} = 360$ and 365 nm, $\lambda_{em} = 410$ and 450 nm). Pure samples were collected, evaporated and dried under reduced pressure over P_2O_5. Acetonitrile was HPLC grade and the solvent was not additionally purified.

2.2. Methods

The UV absorption spectra were recorded on a Varian Cary 300 Bio or JASCO V-550 spectrophotometers. The stationary emission spectra were measured at room temperature on a previously described [15] upgraded Perkin-Elmer MPF-3 spectrofluorimeter ($\lambda_{exc} = 360$ nm) and on Edinburgh Instrument FL 900 spectrofluorimeter with the Ti: sapphire laser ($\Delta t \sim 1.5$ ps) as an excitation source ($\lambda_{exc} = 365$ nm). The air-saturated solutions of the compounds at a concentration ~0.1 mM were used. The contribution of the emission caused by solvent impurities to the total emission of a sample was small but nevertheless, the impurity emission was removed according to the published procedure [16]. The emission quantum yields were determined using quinine sulphate in 0.05 M H_2SO_4 as the reference ($\phi_F = 0.52$) [17]. Fluorescence decays ($\lambda_{exc} = 365$ nm) were measured using a time-correlated single photon counting (TCSPC) system (repetition rate: 4 MHz, count rate: 20,000 cps and time resolution: 0.61 ps per channel [16]) and picosecond lifetimes were determined as described previously [15,18,19]. CH_3CN used as a solvent was checked under the conditions of the fluorescence decay measurements and it was found to exhibit emission small enough to disregard its influence on the lifetimes.

3. Results and discussion

The absorption and emission spectra of TU in CH_3CN at room temperature are presented in Fig. 1 and the relevant data are compiled in Table 1. Within 270–450 nm the absorption spectrum exhibits an intense band with a maximum at $\lambda_{max}^A = 328$ nm, related to $S_0 \rightarrow S_2$ ($\pi \rightarrow \pi^*$) electronic transition [1,4].

The weakly intense, longer wavelength absorption band corresponding to the $S_0 \rightarrow S_1$ ($n \rightarrow \pi^*$) transition well separated

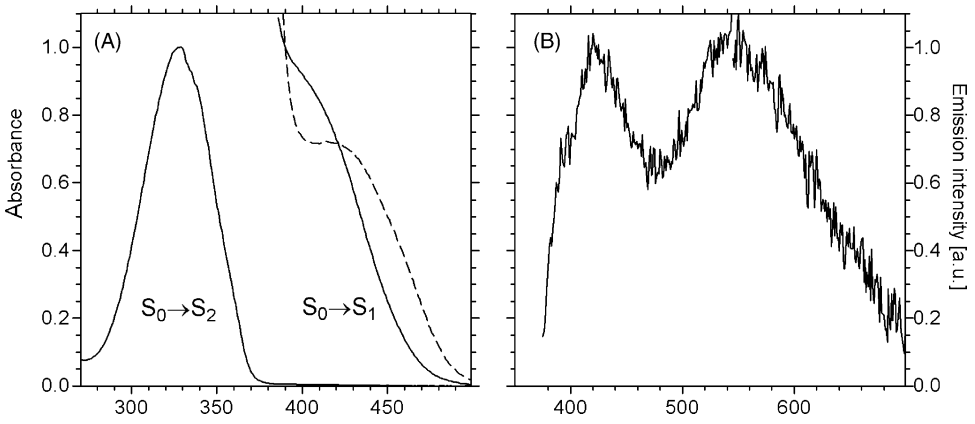

Fig. 1. (A) The normalized $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_1$ absorption bands of TU in CH_3CN solution (solid line) and the normalized $S_0 \rightarrow S_1$ absorption band of TU in CCl_4 solution (broken line) from ref. [11]; the spectra were measured at $c = 0.08$ mM ($S_0 \rightarrow S_2$) and $c = 2$ mM) ($S_0 \rightarrow S_1$). (B) The normalized emission spectrum of TU in the air saturated CH_3CN solution at room temperature measured on an MPF-3 spectrofluorimeter; $\lambda_{exc} = 360$ nm (see Section 2).

in the spectra of TU measured in the CCl_4 solution [11] is less resolved in CH_3CN solution from the $S_0 \rightarrow S_2$ band due to the solvent induced hypsochromic shift. The emission spectrum presented in Fig. 1 was recorded under excitation of a sample solution with a continuous $\lambda_{exc} = 360$ nm light from xenon lamp using an MPF-3 spectrofluorimeter. The intensity of the long-wavelength emission band ($\lambda_{max} = 550$ nm) remarkably increases (~13 times) upon deaeration of the solution. This property and the spectral range of the emission band identical to that reported for phosphorescence of TU in CCl_4 solution [11] suggest that this band corresponds to the radiative $T_1 \rightarrow S_0$ transition. The band with a maximum at 420 nm and the quantum yield $\phi_F = 1 \times 10^{-4}$ was assigned to $S_2 \rightarrow S_0$ fluorescence. The shape and intensity of the bands in the emission spectrum presented in Fig. 1 do not depend on the time required for its measurement and these features remained unchanged on multiple repetition of the spectrum recording (see below).

Kinetics of the TU fluorescence decay was measured by TCSPC. The sample was excited at $\lambda_{exc} = 365$ nm to the S_2 state. The fluorescence intensity (I_F) was monitored at three wavelengths $\lambda^F = 400$, 430 and 450 nm. TCSPC measurement of $I_F(t)$ for the sample studied was performed for up to 10,000 counts at the maximum emission intensity [15,19]. Despite TU being a weakly emitting compound (Table 1) only 2–3 min were required to measure I_F (t). On measurements of TU fluorescence decay a very distinct increase in the I_F was observed with increasing time of irradiation, prior to the experiment. The intensity enhancement was dependent on the monitored emission wavelength and was the greatest in the short-wavelength emission range, especially at $\lambda^F = 400$ nm. The same tendency was found in the emission spectra (see Fig. 2) measured on a spectrofluorimeter FL 900 in the laser excitation conditions (λ_{exc}, I_0) the same as used in the fluorescence decay kinetics measurements.

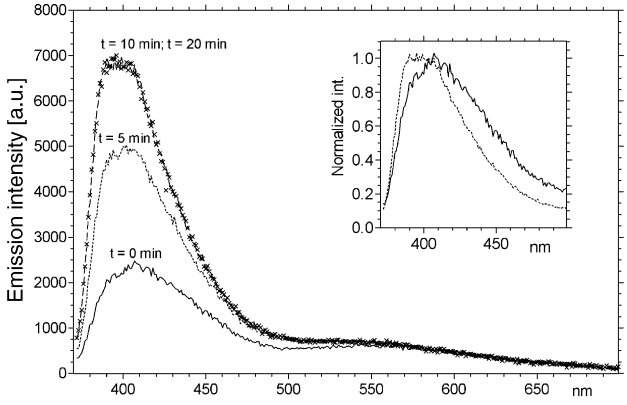

Fig. 2. The emission spectra of TU ($c = 0.08$ mM) in the air saturated CH_3CN solution at room temperature measured on an FS 900 spectrofluorimeter; $\lambda_{exc} = 365$ nm (see Section 2). The spectra were recorded without pre-irradiation ($t = 0$ min) and after pre-irradiation of a sample for $t = 5$ min, $t = 10$ min and $t = 20$ min. Inset: The normalized emission spectra recorded for samples $t = 0$ (solid line) and $t = 10$ min (dotted line).

As follows from a comparison of the spectra recorded immediately after starting the measurements (Fig. 2, $t = 0$ min) and after a short time of the sample pre-irradiation (Fig. 2, $t = 5$ min,

Table 1
Absorption (A) and emission (F, Ph) maxima (λ_{max}), molar absorption coefficients (ε) and emission quantum yields[a] (ϕ_F, ϕ_{Ph}) of TU and model compounds in air saturated CH_3CN solution at room temperature

Compound	λ^A_{max}(nm) ε(M^{-1} cm^{-1})	λ^F_{max} (nm)	λ^{Ph}_{max} [nm]	$\phi_F \times 10^{4}$[b]	$\phi_{Ph} \times 10^{4}$[c]
TU	328 (20,600)	420	550	1.0	2.0
TUSR′	296 (13,200)	380	460	0.6	2.6
TUOR″	332 (20,000)	435	595	1.4	1.6

[a] Estimated error ± 50%.
[b] Quantum yield of fluorescence measured in the spectral region: 375–475 nm, 320–400 nm and 375–510 nm for TU, TUSR′ and TUOR″, respectively.
[c] Quantum yield of phosphorescence measured in the spectral region: 475–700 nm, 400–650 nm and 510–700 nm for TU, TUSR′ and TUOR″, respectively.

10 min), besides a distinct increase in the intensity, the shape of the short-wavelength emission band changes (Fig. 2, inset), and the emission maximum λ^{F}_{max} shifts to the blue by ≈10 nm. Further increase in the pre-irradiation time did not lead to other spectral changes, which indicates that the steady-state condition was achieved after 10 min (Fig. 2). It should be emphasised that the changes observed in the TU fluorescence spectra under laser excitation (Fig. 2) are fully reversible. This was proved experimentally by the observation that the initial emission spectrum of TU (Fig. 2, $t = 0$ min) was fully recovered when taken for the same sample immediately after stopping its laser irradiation lasting for 20 min (Fig. 2, $t = 20$ min). Moreover, the UV absorption spectrum of TU after laser excitation for a time period $t = 20$ min was identical to that recorded before the measurement, which means that we do not observe the formation of stable photoproducts.

In order to estimate the number of emitting species and determine the possibly accurate values of the parameters of their emission decay, a special two-stage method of $I_F(t)$ measurements was applied. The first stage was the measurement of the emission decay kinetics at $\lambda_{em} = 400$ nm. At this wavelength at which the emission intensity (I_F) was the greatest, the time needed to get the desired number of counts was the shortest. Therefore, it can be assumed that the time of measurement has relatively the lowest effect on the emission decay parameters obtained from this curve. At the second stage the same TU sample in CH_3CN was pre-irradiated with laser pulses for 20 min to achieve the steady-state condition and from that moment the fluorescence decay was measured. In the steady-state condition the concentration of the emitting species remains constant as long as laser irradiation occurs. An additional measurement of $I_F(t)$ was made at $\lambda_{em} = 400$ nm for the sample of TU in CH_3CN pre-irradiated for $t = 5$ min. The experimental fluorescence decays at $\lambda_{em} = 400$ nm for $t = 0$, 5 and 20 min are presented in Fig. 3, and the lifetimes and fractional coefficients obtained from the curves are given in Table 2.

Analysis of the experimental fluorescence decay curve measured in possibly the shortest time (Fig. 3, $t = 0$) has shown that the best fit of the theoretical to the experimental curve is obtained assuming the presence of three emitting species with the lifetimes of $\tau_1 = 4$ ps, $\tau_2 = 60$ ps and $\tau_3 = 590$ ps (Table 2, entry 1). Taking into account the error in the lifetimes determined (see below), the values of the lifetimes τ_1, τ_2, τ_3 determined from

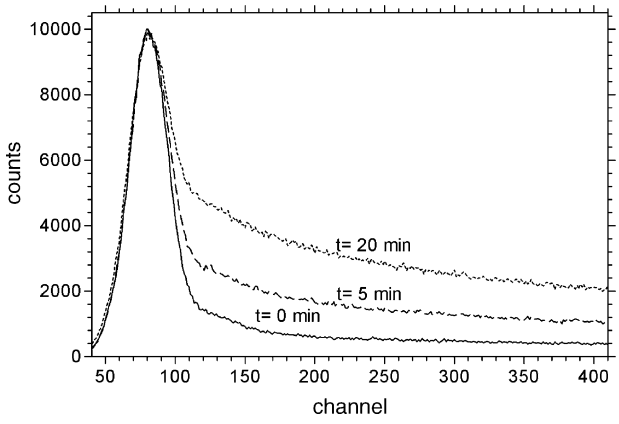

Fig. 3. The experimental decay traces of $\lambda^{F} = 400$ nm emission measured upon excitation of TU ($c = 0.08$ mM) in the air saturated CH_3CN solution, at room temperature; $\lambda_{exc} = 365$ nm. The decay curves were measured without pre-irradiation ($t = 0$ min) and after pre-irradiation of the sample for $t = 5$ min and $t = 20$ min.

the fluorescence decay curve, measured for the TU sample pre-irradiated for $t = 20$ min (Table 2, entry 2), and obtained from the kinetic curve for $t = 0$ min are the same within error range. This means that they do not depend on the pre-irradiation time. On the other hand, the fractional coefficients being a direct measure of the contribution of the three individuals in the experimentally observed total emission, do depend significantly on the time of the sample irradiation. Extension of this time causes a decrease in f_1, an increase in f_2, and an even more pronounced increase in f_3. The results obtained from the emission decay curve for the sample pre-irradiated for $t = 5$ min (Table 2, entry 3) confirm the above conclusions. Fluorescence decay was also measured at $\lambda^{F} = 430$ nm and $\lambda^{F} = 450$ nm for TU samples pre-irradiated for 5 min. The lifetimes and fractional coefficients obtained from these curves are given in Table 2 (entry 5, 7).

As follows from analysis of all lifetime values determined and presented in Table 2, they do not depend on the time of the sample irradiation or on λ^{F} and are $\tau_1 = 5 \pm 1$ ps, $\tau_2 = 80 \pm 20$ ps, $\tau_3 = 600 \pm 100$ ps (the maximal errors are given). This result indicates the presence of the same three emitting species, irrespective of the wavelength λ^{F} and the irradiation time. For a few reasons the lifetimes τ_2 and τ_3 are charged with an error significantly greater than the normal lifetime error expected from the TCSPC measurements of $I_F(t)$ in a typical system in which no light induced reversible processes occur. The main reason is

Table 2
Lifetimes (τ_i), fractional coefficients (f_i) and the parameter (χ^2) of the fit of the experimental decay curves of TU fluorescence ($\lambda_{exc} = 365$ nm)

Entry	t[b] (min)	λ^{F} (nm)	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)	τ_R[a] (ps)	f_1	f_2	f_3	χ^2
1	0	400	4	60	590	14.0	0.65	0.07	0.28	1.00
2	20	400	5	100	660	14.5	0.16	0.13	0.71	1.10
3	5	400	4	80	610	14.4	0.33	0.11	0.56	1.05
4	5	400	5	–	500	14.4	0.21	–	0.79	10.8
5	5	430	5	70	590	14.5	0.37	0.08	0.55	1.03
6	5	430	6	–	470	14.5	0.40	–	0.60	2.95
7	5	450	5	70	630	14.5	0.46	0.08	0.46	0.98
8	5	450	5	–	490	14.4	0.51	–	0.49	2.23

[a] Fluorescence lifetime of xanthione (reference) in CH_3CN solution, $\tau_R = 14.3$ ps [19].
[b] Time of the sample irradiation prior to the measurement of $I_F(t)$.

an over a hundred times difference in the lifetimes determined (τ_1 and τ_3). In order to determine exact lifetime of a short-lived species (τ_1) we apply the highest possible time resolution (0.61 ps/channel). To get the lifetime of long-lived species (τ_3) to the highest possible accuracy, it would be necessary to perform $I_F(t)$ measurements in the time window $t = 3\ \tau_3$, that is up to 1800 ps. To get $I_F(t)^{max} = 10{,}000$ counts in these conditions would need a much longer ($\sim 5\times$) time of measurements than that in the above-described experiments. In such a long time the jitter would be too high to ensure reliable values of τ_1. Moreover, the long sample irradiation time would always lead to the steady-state condition, which would prevent the use of the special two-stage method of $I_F(t)$ measurements proposed in our paper. In view of the above, the conditions of the $I_F(t)$ measurement (time window ~650 ps) were chosen to ensure the maximum reliability and repeatability of the results, although the values of τ_3 are charged with a relatively large error. The error in determination of τ_2 is first of all dependent on a small contribution of this component in the total emission ($f_2 = 0.07$–014; Table 2). It should be emphasised that although the contribution of the component characterized by the lifetime τ_2 in the total emission is not great, still it must be taken into regard for all λ^F. The presence of three fluorescent species is indicated by a substantial worsening of the fit of all the experimental decays to the theoretical curve (χ^2, standard deviations, autocorrelation function, residuals) performed assuming the presence of only two emitting species. The selected results of fitting to a two component model are included in Table 2 (entry 4, 6, 8). A comparison of the values of f_1, f_2 and f_3 obtained from the experimental curves of the fluorescence decay recorded at $\lambda_{em} = 400$, 430 and 450 nm (Table 2, entry 3,5,7) shows that the values of fractional coefficients depend on the emission wavelength at which the monitoring is performed. Apparently, the emission maximum of the longer-lived species with lifetimes 80 ± 20 ps and 600 ± 100 ps is located at shorter wavelengths than that of the short-lived species. Reproducibility of results was confirmed by measurements taken for a few independently prepared TU solutions in CH_3CN.

We suggest that the described above, unusual fluorescence behaviour of TU in CH_3CN revealed by picosecond laser spectroscopy studies can be accounted for by assuming phototautomerism of this molecule in the S_2 excited state. We have found no other reasonable explanation of the steady-state and dynamic results of the emission study. It should be pointed out that CH_3CN does not play an important role in the process, since the identical fluorescence behaviour of TU was observed when CCl_4 was used as a solvent. The fluorescence of TU in CCl_4 solution was not studied further by picosecond laser spectroscopy, because of some permanent changes in the UV absorption spectra evidencing irreversible photochemical decay of TU and formation of stable photoproducts. On the other hand, under identical experimental conditions, the $S_2 \rightarrow S_0$ fluorescence of DMTU, which has a methyl group instead of a hydrogen at N(3) of pyrimidine ring and thus, the molecule is not capable of undergoing tautomerization, decays monoexponentially with $\tau_{S_2} = 2.5$ ps [10]. This appears to be the rationale indication for phototautomerization of TU in the S_2 state.

Phototautomerization is a known excited state process and it has been described for several heteroaromatic thiones: 2(1*H*)-pyridinethione [20], 3(2*H*)-pyridazinethione, 4(3*H*)-pyrimidine-thione [21], 2(1*H*)-quinolinethione [22] and also for two 4-thiouracil derivatives [23,24]. The thermal instability of the phototautomers of thiones in the ground state prevents their observation at room temperature, but they were unequivocally identified by IR spectroscopy in low temperatures. Irradiation ($\lambda > 335$ nm) of 2,4-dithiouracil in low-temperature argon or nitrogen matrices gives 2,4-pyrimidinedithiol (the ditiol form of 2,4-dihydroxyuracil). The experimental IR and UV spectra of the photoproduct are reported [23]. The phosphorescence studies of 4-thiouracil in ethanol glass, both steady-state at 4.2 K as well as time-dependent at 77 K revealed the presence of two emitting components, which were ascribed to a normal 4-thione, 2-keto form and its enolic tautomer, i.e.: 4-thione, 2-enol form [24].

N(1)-substituted 4-tiouracyl derivatives, including TU, may potentially exist in three tautomeric forms shown in Scheme 1: 4-thione, 2-keto (TU), 4-thiol, 2-keto (TUSH) and 4-thione, 2-hydroxy (TUOH). Theoretical [24–31] and experimental data (IR, UV, X-ray) [14,24,26,32–36] indicate that in the ground state 4-thiouracil and its N(1)-substituted derivatives exist exclusively in 4-thione, 2-keto form in vapour, inert matrix, solid and in solution. Calculations of the stability of 4-thiouracil tautomers in the gas-phase indicate that 4-thione, 2-keto form is more stable than 4-thiol; 2-keto form by 15.27 kcal/mol (14.19 kcal/mol, depending on the level of theory of ab initio method [24], 11.31 kcal/mol DFT method [27]) and by 29.73 kcal/mol (28.35 kcal/mol [24]) more stable than 4-thione; 2-enol form. Moreover, in a solution, the stability of different tautomeric forms is also affected by the energy of interactions with the solvent. The values of the ground state dipole moments computed for the gas phase indicate that 4-thione; 2-keto tautomer of 4-thiouracil ($\mu = 5.66$ or 4.60 D depending on the theory level) is a bit less polar than the two other forms: 4-thiol; 2-keto ($\mu = 7.48/6.74$ D) and 4-thione; 2-enol ($\mu = 8.17/6.81$ D) [24]. However, as follows from the calculations performed taking into regard the free energy of hydration, also in a H_2O solution the 4-thione; 2-keto tautomer is still at least by 12.08 kcal/mol more stable than 4-thiol; 2-keto tautomer [24] (13.53 kcal/mol [27]) and by 22.02 kcal/mol more stable than 4-thione; 2-enol tautomer [24]. The results of these calculations are in agreement with the experimental results. The experimental study of 4-thiouracil tautomerism performed in H_2O, DMSO, $CHCl_3$ by UV and IR spectroscopy indicate the presence of a single tautomeric form: 4-thione; 2-keto of 4-thiouracil molecule in these media [14]. Hence, we assume that 4-thiouridine exists exclusively in this form in a polar medium such as CH_3CN.

Taking into account the values of τ_i and f_i (Table 2) as well as the changes in the latter values upon laser irradiation, we identified the three fluorescent species as the TU in the S_2 excited state ($\tau_1 = 5$ ps), and the singlet excited tautomers: TUOH in the S_2 state ($\tau_2 = 80$ ps) and TUSH in the S_1 excited state ($\tau_3 = 600$ ps) (Scheme 1). We propose that the processes occurring upon excitation of TU sample by the first laser pulse can be described by Eqs. (a)–(f). The equations do not cover non-radiative deacti-

vation processes of the molecules, although, taking into regard low quantum yield of fluorescence (Table 1, Fig. 2) they are the major deactivation channel of the singlet excited states of TU and its tautomers.

$$TU(S_0) + h\nu \rightarrow TU^*(S_2) \quad (a)$$

$$TU^*(S_2) \rightarrow TUOH^*(S_2) \quad (b)$$

$$TU^*(S_2) \rightarrow TUSH^*(S_1) \quad (c)$$

$$TU^*(S_2) \rightarrow TU(S_0) + h\nu_F \quad (d)$$

$$TUOH^*(S_2) \rightarrow TUOH(S_0) + h\nu'_F \quad (e)$$

$$TUSH^*(S_1) \rightarrow TUSH(S_0) + h\nu''_F \quad (f)$$

Because of much greater energy (>10 kcal/mol) [27] the tautomers TUOH and TUSH are unstable in the ground state at room temperature and undergo back proton transfer to regenerate TU (Eqs. (g) and (h)):

$$TUOH(S_0) \rightarrow TU(S_0) \quad (g)$$

$$TUSH(S_0) \rightarrow TU(S_0) \quad (h)$$

On the laser excitation, if the lifetimes of tautomers in the ground state (S_0) are longer or comparable ($\tau_{S_0} \geq 250$ ns) to the interval between adjacent laser pulses (repetition rate: 4 MHz), the tautomers built up to higher concentration and they may undergo direct excitation. This process would be responsible for a gradual increase in the contribution of the emitting $TUOH^*(S_2)$ molecules and in particular $TUSH^*(S_1)$ molecules in the total emission, depending on the duration of the experiment, until the stationary state is achieved (Eqs. (i) and (j)).

$$TUOH(S_0) + h\nu \rightarrow TUOH^*(S_2) \quad (i)$$

$$TUSH(S_0) + h\nu \rightarrow TUSH^*(S_1) \quad (j)$$

The increase in the concentration of these excited individuals is evidenced by an increase in the values of f_2 and f_3 (Table 2) observed in the dynamic measurements and the enhancement of the total fluorescence intensity (Fig. 2).

At this stage of the study, it is not possible to prove the proposed mechanistic scheme. Neither UV absorption spectra nor fluorescence properties of TU tautomers: TUSH and TUOH have been reported. In order to get some information that would help verify the mechanism suggested, the compounds: 2′,3′,5′-tri-*O*-acetyl-4-methyl-thiouridine (TUSR′, Scheme 1) and 2,2′-anhydro-1-(β-D-arabinofuranosyl)-4-thiouracil (TUOR″, Scheme 1) were synthesized and their absorption and emission spectra in CH_3CN were measured. These compounds may serve as models of TUSH and TUOH, the tautomeric forms of TU. The UV absorption and room-temperature emission spectra of the compounds in CH_3CN solution in the presence of air are presented in Fig. 4 and the relevant parameters are compiled in Table 1.

The model compound TUOR″ containing a thiocarbonyl group, similarly as TU, retains the major spectroscopic properties of TU. Both the absorption spectra and emission spectra are very similar to those of TU. The longest wavelength emission band ($\lambda_{max} = 595$ nm), whose intensity increases in the absence of oxygen is assigned to the $T_1 \rightarrow S_0$ phosphorescence. By analogy to TU, the band with a maximum at 435 nm is assigned to $S_2 \rightarrow S_0$ emission.

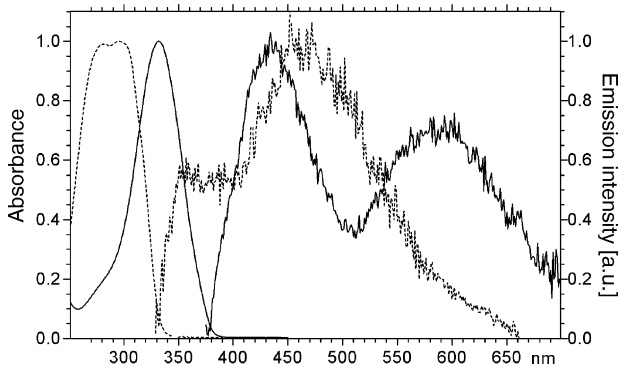

Fig. 4. Normalized absorption and emission spectra of TUSR′ (dotted line; $c = 0.4$ mM, $\lambda_{exc} = 300$ nm) and TUOR″ (solid line; $c = 0.9$ mM, $\lambda_{exc} = 360$ nm). All spectra were determined in the CH_3CN solution at room temperature, in the presence of air.

The absorption of TUSR′ is blue shifted and less intense as compared to that of TU. The emission spectrum exhibits two bands, the longest wavelength ($\lambda_{max} = 460$ nm) can be completely quenched by KI ($c = 0.1$ M) and is therefore, ascribed to phosphorescence. The short wavelength band in the emission spectrum is ascribed to $S_1 \rightarrow S_0$ fluorescence. It should be added that at room temperature the total emission of both model compounds is very weak (Table 1). Comparison of the spectra presented in Figs. 1 and 4 indicates that both model compounds do show the emission band in the spectral region similar to that of $S_2 \rightarrow S_0$ fluorescence of TU. Moreover, the absorption intensity of TUOR″ at the laser excitation wavelength ($\lambda_{exc} = 365$ nm) is comparable to that of TU. On the other hand, inspection of the absorption spectrum of TUSR′ (long-wavelength end of absorption at $\lambda \sim 350$ nm, Fig. 4) raises a question whether the 365 nm laser pulse is of sufficient energy for direct excitation of this tautomer (Eq. (j)). In answering this question it should be realized that substitution of hydrogen in TUSH and TUOH by an alkyl group in TUSR′ and TUOR″ may change the position of λ^A_{max} as well as the intensity and shape of absorption bands. Examples of such spectral changes in aromatic molecules have been reported [14,37]. However, the spectral changes induced by an alkyl substitution are not dramatic. Therefore, taking into account the absorption properties of TUSR′ (Fig. 4, Table 1) it appears unlikely that TUSH would effectively compete for the exciting photons with TU molecules and the process described by Eq. (j) can be neglected.

On the other hand, the absorption of exciting photons by $TUSH^*(T_1)$ seems more probable, provided that the triplet lifetime is long enough (>250 ns) and $TUSH^*(T_1) \rightarrow TUSH^*(T_n)$ absorption is characterized by high extinction coefficient at $\lambda_{exc} = 365$ nm. The following sequence of processes (Eq. (k)) could be proposed to account for the increase in the concentration of $TUSH^*(S_1)$ dependent on the duration of TU excitation.

$$TUSH^*(T_1) + h\nu \rightarrow TUSH^*(T_n) \rightarrow TUSH^*(S_1) \quad (k)$$

This mechanism explaining the appearance of $S_1 \rightarrow S_0$ phototautomer fluorescence as a result of absorption of the second quantum by its T_1 state has been proved for the processes of phototautomerization of other compounds, e.g. 3-hydroxyflavone derivatives [38–43].

4. Conclusions

Although the formation of tautomers upon UV irradiation of 4-thiouracil derivatives has been described, the nature of the reactive excited state has not been determined. In the photochemical study performed at low temperature, the mere presence of the "rare" tautomer in ground state, identified by IR spectroscopy, was an indication of the excited state process [23]. The presence of triplet excited (T_1) phototautomer (4-thione, 2-hydroxy form) was inferred from the phosphorescence study of 4-thiouracyl in ethanol glass. The two-exponential emission decay observed in these conditions was interpreted as an indication of the presence of the phototautomer in addition to the "normal" (4-thione, 2-keto) form in the T_1 excited states [24]. To the best of our knowledge, our results are the first experimental evidence of formation of two singlet excited phototautomers from the S_2 excited state of TU. Interpretation of the steady state (spectra) and dynamical (lifetimes) results obtained by the TCSPC two-stage method is based on the assumption of the full reversibility of the processes and their interrelations. In particular, the identification of the three singlet excited species: $TU^*(S_2)$, $TUOH^*(S_2)$ and $TUSH^*(S_1)$ is based on the values of their lifetimes and changes in the fractional coefficients taking place on increasing the time of irradiation prior to $I_F(t)$ measurement. Unfortunately, the spectral ranges of fluorescence of TU and its two tautomeric forms are so similar and their emission intensities so low that it is impossible to separate the overall fluorescence emission spectrum into the parts corresponding to individual components assignable to each species, on the basis of the time-resolved and steady-state measurements performed. In the future we are going to perform UV–vis broadband femtosecond transient absorption measurements in order to characterize non-emissive intermediates involved in the reaction pathways.

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