# LUMINESCENCE SPECTRAL PROPERTIES OF NEW BENZOTHIAZOLE POLYMETHINE DYE

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*The absorption and fluorescence spectra of a new styryl derivative of thioflavin T 2-{(1E,3E)-4-[4-(dimethylamino)-2,6-dimethylphenyl]buta-1,3-dien-1-yl}-3-ethyl-1,3-benzothiazol-3-ium tosylate (Th-C23) in solvents with different polarity and viscosity and also incorporated in the structure of amyloid fibrils and bovine serum albumin were investigated. A characteristic feature of the dye is an extremely low quantum yield of fluorescence in low-viscosity solvents ($10^{-4}$ in water) which, however, increases significantly in viscous solutions and when it is incorporated in the structure of proteins or amyloid fibrils. In the latter case the quantum yield increases by $8 \cdot 10^3$ times. On the basis of the experimental studies and quantum chemical calculations it was shown that Th-C23 exhibits the properties of a molecular rotor. The increase of the fluorescence quantum yield in viscous solutions and in the biopolymers results from limitation of the torsional rotation of the molecular fragments, leading to fluorescence quenching. The long-wavelength location of the absorption spectrum and the fluorescence spectrum of the new dye in the red region of the spectrum (520 and 600 nm) makes it possible to use it as a fluorescent marker that is sensitive to the viscosity (hardness) of the microenvironment not only in vitro but also in vivo.*

*Keywords*: styryl derivatives of thioflavin T, fluorescent molecular rotors, amyloid fibrils.

**Introduction.** Fluorescent dyes, which are capable of being included in the structure of biopolymers without the formation of a chemical bond and therefore have little effect on the properties of objects, are widely used in biophysical research. Fluorescent molecular rotors (FMR), which are characterized by strong dependence of the quantum yield and fluorescence decay time on the viscosity of the microenvironment, are also used. Fluorescent molecular rotors have proven highly useful for detection and investigation of amyloid fibrils (AF) [1–4], DNA [5, 6], and protein aggregation processes [7, 8]. Thiazole-free derivatives of thioflavin T (ThT) with absorption and fluorescence in the red region of the spectrum have recently been synthesized [9, 10], which has made it possible to use them for both *in vitro* and *in vivo* investigations.

For quantitative analysis with fluorescent markers an important factor is the ratio of the intensity (quantum yield) of fluorescence for the incorporated dye and the free (uncombined) dye. For ThT, the quantum yield of which in aqueous solution is extremely low (0.0003 [11, 12]) this ratio is $>10^3$. For thioflavin derivatives with a larger system of $\pi$ conjugation the ratio of the fluorescence intensity of the inserted and free dye is $\leq$200–300 on account of the higher quantum yield of fluorescence of the derivatives in solution in the unbonded state. The synthesis and study of FMR derivatives with low quantum yield in aqueous solutions are relevant.

In this work the absorption and fluorescence spectra of a new benzothiazole polymethine dye 2-{(1*E*,3*E*)-4-[4-(dimethylamino)-2,6-dimethylphenyl]buta-1,3-dien-1-yl}-3-ethyl-1,3-benzothiazol-3-ium tosylate (Th-C23) in solvents with various polarities and viscosities and also its incorporation in the structure of AF and bovine serum albumin (BSA) were investigated. Th-C23 exhibits the characteristics of a molecular rotor, and the ratio of the fluorescence intensities of the incorporated and free dyes is $\sim 10^4$.

**Experimental**. The absorption spectra of solutions of the dye were obtained on a Specord 200 PC spectrophotometer (Carl Zeiss, Germany), and the fluorescence spectra were obtained on an SM2203 spectrofluorimeter (Solar, Belarus).

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The obtained spectra were processed with PeakFit 4.12 and Origin 6.0 software. The quantum-chemical calculations for theTh-C23 molecule in the gas phase were done with the Firefly 8.2.0 software package [13] in terms of density functional theory (DFT) [14] with the three-parameter Becke–Lee–Yang–Parr hybrid functional (B3LYP) [15] and 6-31G($d$,$p$) basis set [16]. The geometry was optimized for the compound in the ground ($S_0$) and excited ($S_1$) singlet electronic states. The calculations for the excited state were realized in terms of density functional theory for the nonstationary systems (TDDFT) with the B3LYP functional and the 6-31G($d$,$p$) basis set. The solutions were prepared with solvents from Sigma-Aldrich (USA) without further purification. The AF was prepared from BSA (Sigma, USA) by the standard procedure [17].

**Results and Discussion**. As chromophoric group the molecule of the dye contains an aminobenzene ring and a benzothiazole ring connected by a –$(CH=CH)_2$– polymethine bridge.
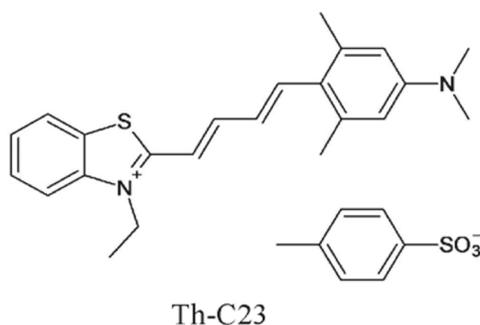


Th-C23

At neutral pH in aqueous solution the Th-C23 molecule is a cation, while the counterion is represented by the anion of $p$-toluenesulfonic acid. On account of the enlarged π-conjugation system the absorption and fluorescence spectra of the Th-C23 are shifted into the red region compared with the spectra of thioflavin T. The results of quantum chemical calculations show that the equilibrium electronic singlet ground state ($S_0$) is characterized by a planar conformation of the Th-C23 molecule (Fig. 1). The minimum energy of the excited electronic singlet state ($S_1$) corresponds to a twisted conformation of the molecule in which the aromatic rings are in an orthogonal arrangement. Here the dipole electric moment in the $S_1$ state is ∼50% greater than in $S_0$.

The angle φ between the planes in which the benzothiazole and benzene rings lie was used as parameter that defines the mutual arrangement of the aromatic fragments. The dynamics of the conformational change of the Th-C23 molecule was revealed by calculation of its energy in relation to the angle φ for the ground and excited states. The angle φ was varied in the range of 0–90$^\mathrm{o}$ with steps of 10$^\mathrm{o}$. The $E_0(\varphi, \omega_0)$ dependence (Fig. 2, curve 1) was obtained after optimization of the geometry and calculation of the energy of the $S_0$ state with fixed angles φ. The remaining parameters $\omega_0$ of the geometry of the $S_0$ state were not determined. Also, the geometry was optimized and the energy of the $S_1$ state was calculated for each fixed angle φ, and the $E_1(\varphi, \omega_1)$ dependence (curve 2) was obtained. The $E_0(\varphi, \omega_0)$ dependence shows that in the $S_0$ state the energy of the Th-C23 molecule increases by $\Delta E_0 \approx 5000\ \mathrm{cm}^{-1}$ with increase of the angle φ and reaches a maximum at $\varphi = 90^\mathrm{o}C$. In the $S_1$ state, on the contrary, the energy decreases by $\Delta E_1 \approx 5600\ \mathrm{cm}^{-1}$. This indicates that during photoexcitation the molecules pass into a nonequilibrium state. Vibrational relaxation of the nonequilibrium state leads to decrease of energy, which is accompanied by rotation of the benzene fragment. The torsional relaxation process leads the molecule into a state characterized by a twisted conformation (a TICT state). Torsional relaxation in the TICT state is accompanied by decrease of the oscillator force $f$ of the $S_1 \rightarrow S_0$ transition, which is equal to zero at $\varphi = 90^\mathrm{o}$ (curve 3), and the TICT state is consequently nonfluorescent.

The latter is explained by the fact that the $S_1 \rightarrow S_0$ transition is forbidden since the wave functions of the ground and excited states for the TICT conformation of the Th-C23 molecule do not overlap spatially. This is seen from the localization of the calculated frontier molecular orbitals (MO) represented by the square of the wave function of the electronic states (Fig. 3). The lowest unoccupied MO (LUMO) is localized predominantly on the benzene fragment whereas the highest occupied MO (HOMO) is localized on the benzothiazole fragment and on the polymethine bridge. Thus, according to the quantum-chemical calculations, torsional relaxation of the Th-C23 molecule leads it to the nonfluorescent TICT state, and the process can be regarded as competing with the radiative transition from the excited state with planar conformation to the ground state.

To determine the role of torsion relaxation in the fluorescence quenching of Th-C23 we studied the dependence of the fluorescence quantum yield of a 98% glycerol solution of the dye on temperature. The fluorescence spectra of Th-C23
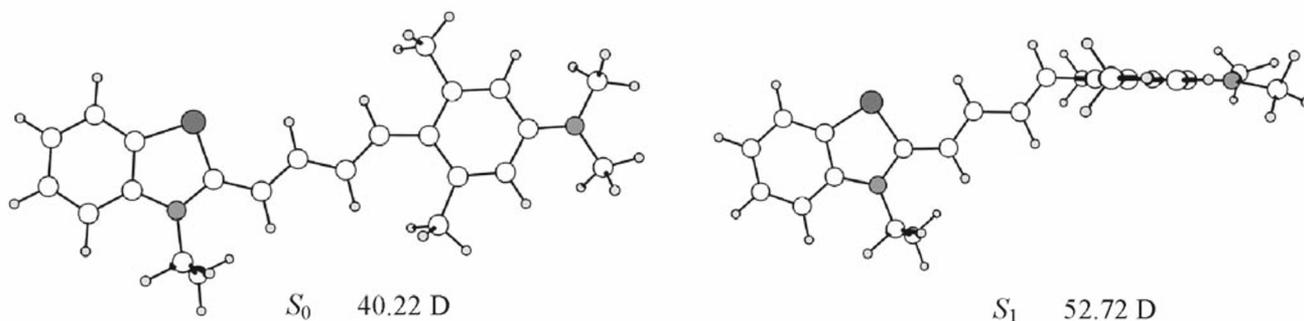
Fig. 1. Conformations of Th-C23 molecule and its electric moments in the ground ($S_0$) and excited ($S_1$) singlet electronic states (frontal view).
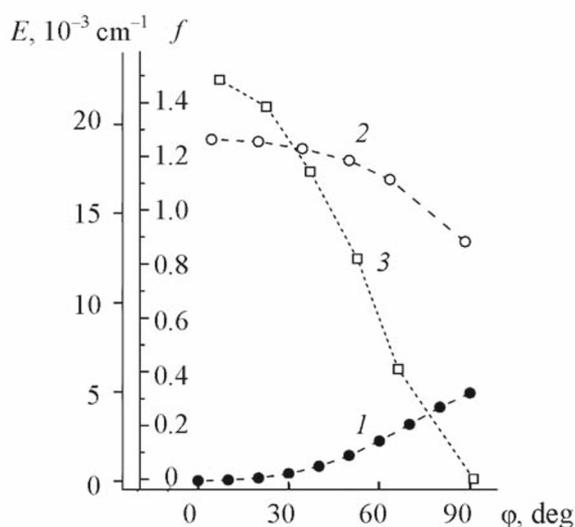


Fig. 2. The $E_0(\varphi, \omega_0)$ (1) and $E_1(\varphi, \omega_1)$ (2) dependences and also the oscillator forces $f$ of the $S_1 \rightarrow S_0$ transition (3) of the Th-C23 molecule on the angle $\varphi$.

in glycerol with variation of the temperature from 20 to 50°C are presented in Fig. 3. It is seen that change of the viscosity from 939 to 104 mPa·s leads to decrease of the fluorescence intensity at the maximum by 6.5 times.

The fluorescence quantum yield depends on the ratio of the rate constants of the radiative transition $k_r$ and all the other processes leading to nonradiative deactivation of the excited state:

$$q = k_r/(k_r + \Sigma\, k_{nonr}),\qquad(1)$$

where $\Sigma\, k_{nonr}$ is the sum of the rate constants of the nonradiative deactivation processes of the excited state. For many aromatic dyes that are not molecular rotors intercombination conversion to the triplet state is regarded as the main channel for deactivation of the excited state. However, we did not observe any noticeable phosphorescence from the dye, including at low temperatures (77 K). On the basis of the quantum-chemical calculations we suppose that torsional relaxation is the main fluorescence-quenching process. In such a case $\Sigma\, k_{nonr} \approx k_\varphi$ ($k$ is the internal conversion rate constant brought about by rotation of the aromatic fragments of the molecule in relation to each other in the excited state). According to the Debye–Stokes–Einstein equation the torsional relaxation time is directly proportional to $\eta/T$:
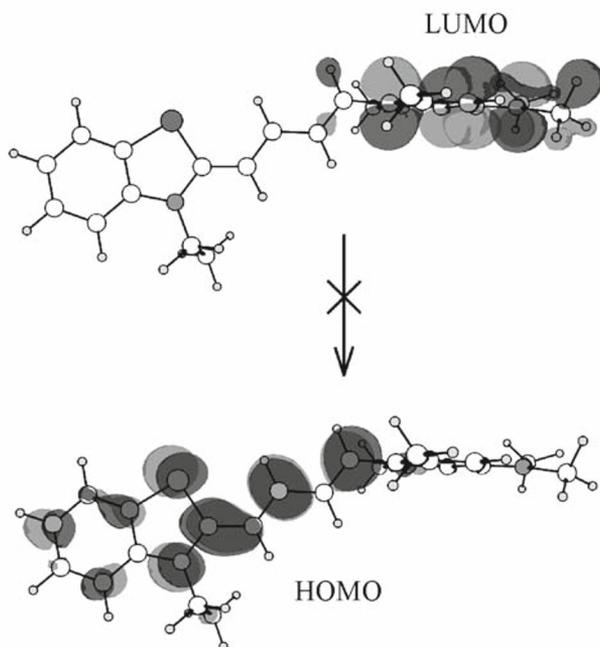
$$\tau = \frac{4\pi a^3}{3k_B}\, \eta/T\,,$$

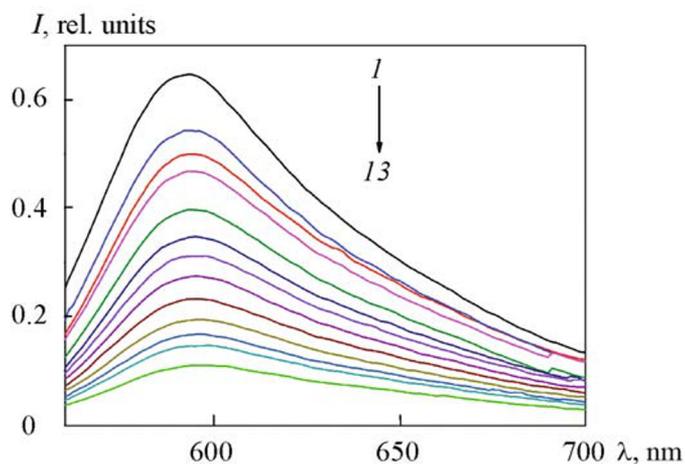Fig. 3. The frontier molecular orbitals for the TICT conformation of the Th-C23 molecule.



Fig. 4. The fluorescence spectra of the Th-C23 dye in 98% glycerol solution at temperatures 20, 21, 22.5, 25, 27.5, 30, 32.5, 35, 37.5, 40, 42.5, 45, and 50°C (1–13); $\lambda_{exc} = 520$ nm.

where $a$ is the hydrodynamic radius of the fragment of the molecule; $k_B$ is the Boltzmann constant; $\eta$ is the viscosity; $T$ is the temperature of the solution. Since $k_\varphi \sim 1/\tau_\varphi$, then $k_\varphi \sim T/\eta$.

Since the rate constant of the radiative transition does not depend on the viscosity and temperature it follows from Eq. (1) that $(1/q - 1) \sim T/\eta$. The linear dependence of $(1/q - 1)$ on $T/\eta$ (Fig. 5a) confirms that in accordance with the mechanism of fluorescence quenching of the dye the change of the quantum yield in the investigated temperature range is determined unambiguously by the $T/\eta$ ratio.

As known, the viscosity of the solvent depends on the temperature according to the Arrhenius principle [18]:
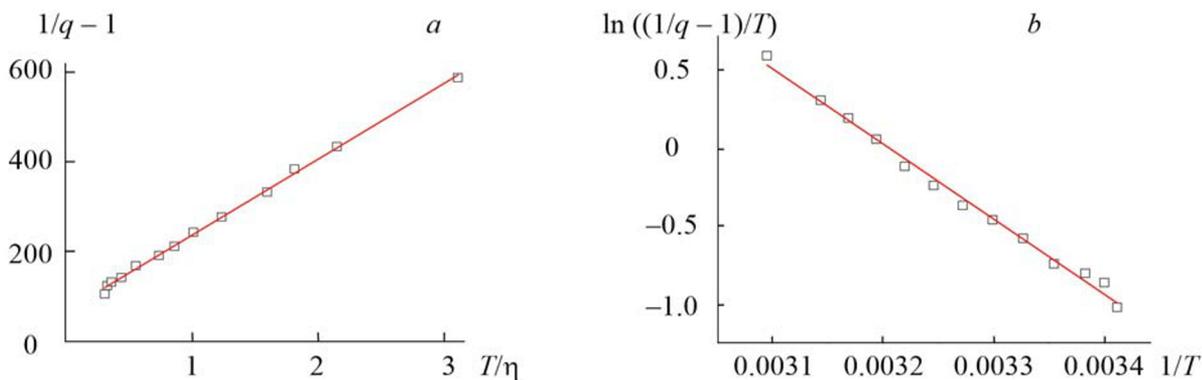
Fig. 5. Dependence of $(1/q - 1)$ on $T/\eta$ (a) and of $\ln\left((1/q - 1)/T\right)$ on $1/T$ (b).

$$\eta^{-1} = \eta_0^{-1} \exp\left(-\frac{E_a}{k_B T}\right),$$

where $\Delta E_a$ is the activation energy of viscous flow of the solvent.

Using the dependence of the quantum yield on $T/\eta$ it is possible to write $1/q - 1 \sim T \exp\left(-\Delta E_a/k_B T\right)$ or $\ln\left((1/q - 1)/T\right) \sim \left(-\Delta E_a/k_B T\right)$. From Fig. 5b it is seen that the dependence of $\ln\left((1/q - 1)/T\right)$ on $1/T$ is linear, and from the slope the activation energy of the quenching process was determined as $4.0 \cdot 10^3$ cm$^{-1}$, which is close to the activation energy of viscous flow of glycerol ($4.7 \cdot 10^3$ cm$^{-1}$). This confirms that the torsion relaxation of the fragments of the Th-C23 molecule and, hence, the change in the fluorescence quantum yield are determined not by the activation energy of rotation of the fragments but by the viscosity of the solvent.

The obtained results show that Th-C23 is a fluorescent molecular rotor. In low-viscosity solutions the main fluorescence-quenching process is torsional relaxation. The significant increase of the quantum yield of fluorescence of the dye in viscous solutions is due to decrease in the rate of torsional rotation of the fragments of the molecule, leading to quenching of the fluorescence.

**Effect of the Polarity of the Solvents**. In order to determine the effect of the polarity of the solvents on the spectral characteristics of Th-C23 the absorption and fluorescence spectra of the dye were studied in water, glycerol, ethanol, and chloroform with permittivity $\varepsilon = 80$, 42, 24, and 4.8 respectively. The absorption and fluorescence spectra are presented in Figs. 6 and 7. The principal spectral characteristics of the new dye are presented in Table 1. It is seen that the absorption spectrum of the dye has the shortest wavelength in aqueous solution. In low-polarity chloroform there is a significant bathochromic shift (~50 nm) and a decrease in the half-width of the spectrum. A hypsochromic shift of the absorption spectrum in polar solvents is also characteristics of thioflavin T and most of the styryl derivatives [9, 17]. This is a result of solvate and torsional relaxation and significant stabilization of the ground state of the molecule in the polar microenvironment [17, 19].

The absorption spectrum of the aqueous solution of the dye in the presence of the AF undergoes significant broadening (Fig. 6, curve 3), which is due to the appearance of a long-wave band from the absorption of the molecules of the Th-C23 dye inserted into the fibrils in monomeric form. The recorded spectrum represents the superimposition of the spectrum of the free dye and that combined with the AF. The result of breakdown of the spectrum is also shown in Fig. 6. It is seen that the dye is integrated into the low-polarity region of the fibrils and has an absorption spectrum at ~570 nm. The absorption spectrum of the dye also undergoes a significant bathochromic shift of ~30 nm in the presence of BSA. When incorporated into the low-polarity region of a protein or an AF groove the dye molecules enter a rigid low-polarity environment that limits the relaxation processes. In this case stabilization and decrease in the energy of the ground state do not occur, and the absorption spectrum of the dye is more long-waved.

The intensity and quantum yield of fluorescence of Th-C23 increase with decrease of permittivity of the solvent (Fig. 7, Table 1), i.e., do not depend only on the viscosity of the solvent. Also a small bathochromic shift (~10 nm) of the spectra is observed in polar solvents. Induced absorption measurements with femtosecond time resolution show that the rate constant of nonradiative deactivation of the molecules of ThT and its derivatives increases in polar solvents [20]. On the
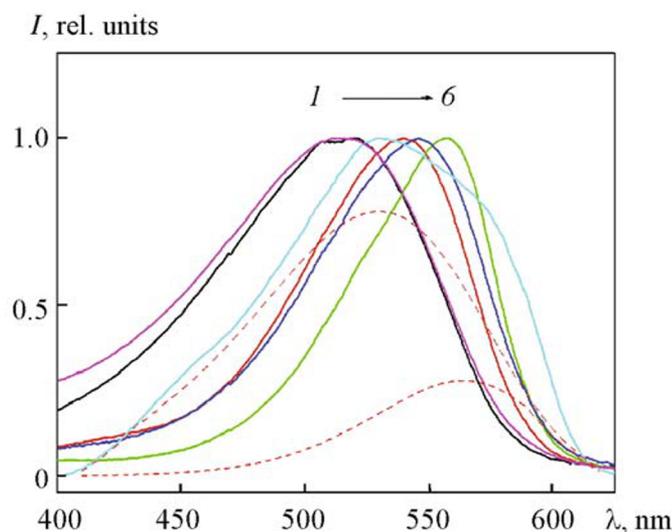
Fig. 6. Normalized absorption spectra of Th-C23 dye (2.5 μmole/L) in various solvents: water (1); glycerol (2); water + 0.1 mg/mL AF (3); ethanol (4); water + 1.0 mg/mL BSA; chloroform (6); the spectrum of the dye in the presence of AF is presented after deduction of Rayleigh scattering; the dashed curves represent the result of separating spectrum 3 into two log-normal components.

TABLE 1. Spectral Characteristics of Th-C23 Dye in Various Solvents

| Solvent | $\varepsilon$ | $\eta$, mPa·s | $\lambda_{abs}$, nm | $\lambda_{fl}$, nm | $q$ |
|---|---|---|---|---|---|
| Water | 80 | 1.0 | 520 | 582 | 0.0001 |
| Glycerol | 42 | 939 | 515 | 593 | 0.0090 |
| Ethanol | 24 | 1.2 | 540 | 590 | 0.0002 |
| Chloroform | 4.8 | 0.57 | 558 | 595 | 0.0004 |
| Water + BSA (1 mg/mL) | 80 | 1.0 | 546 | 588 | 0.2 |
| Water + AF (0.1 mg/mL) | 80 | 1.0 | 570 | 597 | 0.8 |

basis of the correlation between the rate constant of nonradiative deactivation and the polarity of the solvent in [20] it was concluded that as well as viscosity the dielectric properties of the solvent also affect the photophysical processes in these molecules. Our investigations make it possible to suppose that for derivatives of ThT characterized by an elongated chain of $\pi$-conjugation the effect of the polarity of the solvent on the rate of intramolecular charge transfer accompanying torsional relaxation and on the quantum yield of fluorescence can be even more substantional than for ThT.

The quantum yield of fluorescence for the dye in aqueous solution with the addition of BSA and AF is significantly higher (0.2 and 0.8). The intensity of fluorescence in the presence of BSA and AF is $4 \cdot 10^3$ and $2 \cdot 10^4$ times higher. On the basis of a model of the molecular rotor it is possible to give an explanation for the increase of quantum yield of fluorescence for the dye with the presence of the proteins or AF in the solution. Indeed, with their incorporation into the structure of the biopolymers there is a significant (in the case of BSA) or complete limit (in the case of AF) in the torsional rotation of the molecular fragments, leading to an increase of 3–4 orders of magnitude in the quantum yield.

Limitation of the torsional movement of the fragments of the molecule has little effect on the position of the fluorescence spectra. Since the fluorescence takes place from a nonequilibrium excited state, where only partial relaxation occurs, the position of the absorption spectrum depends to a larger degree on the polarity of the solvent than the position of the fluorescence spectrum.
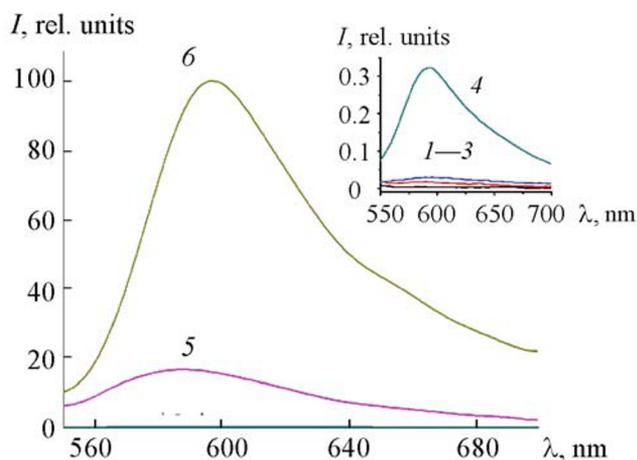
Fig. 7. Fluorescence spectra of Th-C23 (2.5 μmole/L) in various solvents: water (1), ethanol (2), chloroform (3), glycerol (4), water + 1.0 mg/mL of BSA (5), water + 0.1 mg/mL of AF (6); in the inset, spectra 1–4 enlarged scale.

**Conclusions.** On the basis of experimental data and quantum-chemical calculations it was shown that the new benzothiazole dye Th-C23 in aqueous solution has an absorption spectrum with a maximum at 520 nm and a fluorescence spectrum in the red region and belongs to the class of molecular rotors. The main process that quenches the fluorescence is torsional relaxation of the fragments of the molecule. The quantum yield of fluorescence in viscous solutions is increased as a result of the restricted torsional mobility of the molecule. With the incorporation of proteins and amyloid fibrils into the structure the torsional dynamics of the molecule is almost completely blocked, and the quantum yield here is increased by more than three orders of magnitude. The new dye can be an effective tool for the study of proteins, the detection of amyloid fibrils, and investigation of the mechanisms of their formation.

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