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Tuning excited state intramolecular proton transfer in 3-hydroxyflavone derivative by reaction of its isothiocyanate group with an amine

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Abstract

In the present work, we developed an isothiocyanate derivative of 3-hydroxyflavone, 7-isothiocyanato-4′-diethylamino-3-hydroxyflavone. This dye exhibits dual fluorescence due to an excited state intramolecular proton transfer (ESIPT). On reaction with an amine, this dye shows a dramatic change in its dual emission as well as shifts in the absorption and emission maxima. The observed phenomenon is due to the conversion of the electron acceptor isothiocyanate group into an electron donor thiourea based group. This increase in the donor properties at 7-position of the 3-hydroxyflavone probably shifts the reversible ESIPT reaction towards the ESIPT product. Moreover, we show that the conjugate of the reactive dye with an amine exhibits a two-band emission sensitive to the environment, which makes it an attractive solvatochromic label of amino groups of biomolecules.

Keywords: ESIPT; Proton transfer; 3-Hydroxyflavone; Dual emission; Fluorescent dye; Environment-sensitive; Fluorescent label

1. Introduction

3-Hydroxyflavones (3HF) are dual-band fluorescent dyes due to an excited state intramolecular proton transfer (ESIPT) reaction generating two emissive excited state species [1]. The dual emission of 3HF and especially 4′-(dialkylamino)-substituted derivatives is highly sensitive to the properties of the environment, such as polarity, H-bond donor and acceptor ability [2–6]. This unique property has already found variety of applications for studying polymers [7], host–guest complexes [8,9], reverse micelles [10,11], model lipid membranes [12–16], biomembranes [17–19] and proteins [20–23]. Both spectroscopic and solvatochromic properties of these dyes can be finely tuned by chemical substituents [4,6,24], allowing optimization of these fluorophores for a particular application. In this respect, the most interesting substitutions are in position 4′- and 7- of 3HF. These two positions correspond to the opposite sides of the 3HF fluorophore, and therefore substitutions in these positions with electron donor groups result in opposite effects [6]. Thus, as it was previously shown, introduction of a 4′-dialkylamino group in 3HF results in a dramatic decrease of the ESIPT rates and thus of the tautomer emission [2–6,25–28]. Meantime, further introduction of a dialkylamino group in 7-position results in the recovery of a fast ESIPT reaction and a high intensity of the tautomer in different media [24]. Similar, but weaker effects were observed when a weaker electron donor, such as methoxy was introduced in 7-position [6]. In contrast, introduction of an electron acceptor group in 7-position resulted in the opposite effect—a strong decrease in the tautomer emission [29]. Thus, we could conclude that 7-position plays a key role in the modulation of the dual emission of 4′-dialkylamino-substituted 3HF. This unique observation could allow the development of an ESIPT based switcher, so that a slight variation of the electron donor/acceptor properties at 7-position could modify significantly the dual emission of the dye. As a functional group that can change its electron donor/acceptor properties we selected isothyocyanate. This group exhibits considerable electron acceptor properties, while on reaction with an amine it is transformed into a thiourea derivative which exhibits electron donor properties (Fig. 1, see dyes **2** and **3**). An additional attractive feature of isothiocyanates is their applicability

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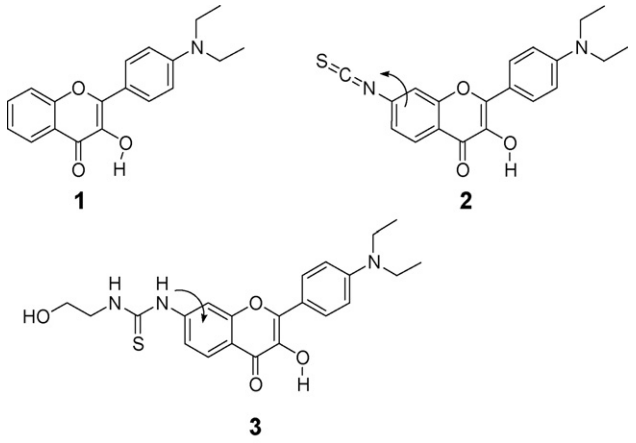


Fig. 1. 3-Hydroxyflavone dyes studied.

for fluorescence labeling of amino groups of biomolecules. In this respect, reactive isothiocyanate derivatives of 3HF could be potential fluorescent labels exhibiting a strong sensitivity to environment.

In the present work, we synthesized and studied a 3HF derivative substituted with isothiocyanate at its 7-position. Our results show that, a reaction of this compound with an amine results in a dramatic change in the intensity ratio of the two emission bands of the dye, indicating a modulation of ESIPT by this reaction. In addition, the resulting conjugate shows significant solvent sensitivity, which makes the reactive isothiocyanate derivative a prospective environment-sensitive label of biomolecules.

2. Materials and methods

All the solvents were of spectroscopic grade. Absorption and fluorescence spectra were recorded on a Cary 400 spectrophotometer (Varian) and FluoroMax 3.0 spectrofluorometer (Jobin Yvon, Horiba), respectively. The excitation wavelength for the fluorescence measurements was 410 nm, unless indicated. The concentration of the dyes in the solutions for fluorescence spectroscopy corresponded to an absorbance close to 0.1.

4′-(Diethylamino)-3-hydroxyflavone (**1**) was synthesized and purified as described elsewhere [3]. 7-Isothiocyanato-4′-(diethylamino)-3-hydroxyflavone (**2**) was synthesized in four steps. First, 4′-acetamido-2′-hydroxyacetophenone (prepared from *N*-(3-hydroxyphenyl)-acetamide) with aluminium chloride in carbon disulfide) and 4-(diethylamino)benzaldehyde were condensed into the corresponding chalcone in dry DMF in the presence of sodium methoxide (rt, 24 h). The reaction mixture was diluted with several volumes of ethanol and treated with 10 mole excess of hydrogen peroxide and 12 mole excess of sodium methoxide. Refluxing the mixture for 5 min afforded 7-acetamido-4′-(diethylamino)-3-hydroxychromone. The latter was hydrolyzed into 7-amino-4′-(diethylamino)-3-hydroxychromone in 10% HCl (100 °C, 2 h) and then converted into 7-isothiocyanato-4′-diethylamino-3-hydroxyflavone (**2**) according to the following procedure. To 10 mg (1 equiv.) of **2** in 1 ml of dichloromethane was added 10 mg (3 equiv.) of sodium carbonate in 0.5 ml of water followed by addition of 10 μl (2 equiv.) of thiophosgene (caution: highly toxic!). After stirring of the mixture for 2 h the product **2** was extracted with dichloromethane and purified by silica gel column chromatography (ethyl acetate/dichloromethane, 10/1, v/v). Yield 20 %. ^{1}H NMR (300 MHz, $CDCl_3$): 1.25 (6H, t, *J* 7.1 Hz), 3.47 (4H, q, *J* 7.1 Hz), 6.78 (2H, d, *J* 8.4 Hz), 7.23 (1H, d, *J* 8.3 Hz), 7.39 (1H, s), 8.14 (2H, d, *J* 8.4 Hz), 8.21 (1H, d, *J* 8.3 Hz); ESI *m*/*z* (M^+ + 1) 367.1.

A conjugate of **2** with ethanolamine (**3**) (1-(4′-diethylamino-3-hydroxyflavone-7-yl]-3-(2-hydroxy-ethyl)-thiourea) was prepared by the following procedure. To a stirred solution of ethanolamine in THF (1 mL) under argon at room temperature, a solution of isothiocyanate **2** in THF (0.5 mL) was added drop wise during 5 min. The reaction mixture was stirred for 30 min and then evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate). Yield 82%. ^{1}H NMR (300 MHz, acetone-d_6): 1.22 (6H, t, *J* 7.1 Hz), 3.50 (6H, m), 3.78 (2H, t, *J* 7.6 Hz), 6.88 (2H, d, *J* 8.1 Hz), 7.5 (1H, d, *J* 8.4 Hz), 8.04 (1H, d, *J* 8.4 Hz), 8.5 (1H, bs) 8.19 (2H, d, *J* 8.1 Hz), 8.47 (1H, s), 9.8 (1H, bs); ESI *m*/*z* (M^+ + 1) 428.1.

3. Results and discussion

The new dye, 7-isothiocyanato-4′-diethylamino-3-hydroxyflavone (**2**, Fig. 1), has been synthesized in four steps starting from 4′-acetamido-2′-hydroxyacetophenone and 4-(diethylamino)-benzaldehyde. On the final step, 7-amino-4′-diethylamino-3-hydroxyflavone was converted into a 7-isothiocyanate derivative in the presence of thiophosgene. The absorption and fluorescence properties of the new dye were studied in several organic solvents of different polarity [30] and compared with those of its parent non-substituted analog **1**. Interestingly, dye **2** shows absorption and emission maxima significantly (ca 20 nm) red shifted with respect to **1** (Table 1). In addition, the Stokes shift of the N* band of dye **2** is significantly larger than that of dye **1** in all studied solvents (Table 1). Moreover, the ratio of the two emission bands, which characterizes the ESIPT reaction, is also strongly affected by the 7-isothiocyanate group. Indeed, the intensity ratio of the short-wavelength band of the normal excited state (N*) to the long-wavelength band of the tautomer (ESIPT product, T*), I_{N^*}/I_{T^*}, increases more than three-fold compared to dye **1** (Fig. 2, Table 1). Recently, it has been shown that an electron acceptor group (methoxycarbonylvinyl) at 7-position of 3-hydroxyflavones also provides strong red shifts in absorption and emission as well as an increase in the I_{N^*}/I_{T^*} ratio, [25] while a 7-electron donor (methoxy or dialkylamino) group provides opposite effects [3,24]. Accordingly, the observed spectroscopic effects are connected with the electron acceptor properties of 7-isothiocyanate group that increases the charge transfer character of the N* excited state of the 3HF dye. Moreover, the ESIPT of dye **1** has been shown to be reversible and occurs on a much shorter time scale than the fluorescence lifetime, so that its I_{N^*}/I_{T^*} ratio depends on the relative energies of the N* and T* states [3,5,27,28]. The increase in the charge transfer of the N* state of dye **2** is expected to favour its dielectric stabilization, and thus make this state more energetically favourable than the T* state. Therefore, the reversible

Table 1
Spectroscopic properties of studied dyes in organic solvents[a]

Solvent $E_T(30)$	Dye	λ_{max} abs (nm)	λ_{max} fl N* (nm)	Stokes shift, N* (cm^{-1})	λ_{max} fl T* (nm)	I_{N^*}/I_{T^*}	φ
THF	**1**	405	477	3730	574.5	0.240	0.05
37.4	**2**	423	532	4840	607	1.08	0.25
	3	403	486	4240	587	0.089	0.29
Ethyl	**1**	401	475	3890	570	0.253	0.05
acetate	**2**	422	529	4790	603	1.04	0.23
38.1	**3**	403	458	2980	587	0.065	0.26
Dichloromethane	**1**	411	492	4010	568	0.621	0.17
40.7	**2**	432	531	4320	598	1.67	0.40
	3	421	501	3790	590	0.773	0.54
Acetone	**1**	404	502	4830	574	0.936	0.05
42.2	**2**	423	554	5590	–	–	0.10
	3	407	482	3820	592	0.445	0.21
Acetonitrile	**1**	404	509	5110	571	1.30	0.09
45.6	**2**	424	564	5850	–	–	0.07
	3	409	509	4800	591	0.730	0.29
Ethanol	**1**	412	523	5150	–	–	0.52
53.7	**2**	433	562	5300	–	–	0.30
	3	416	525	4990	–	–	0.58

[a] $E_T(30)$: empirical polarity index of solvents from Ref. [30]. λ_{max} abs: positions of the absorption maxima, λ_{max} fl N* and λ_{max} fl T*: positions of the fluorescence maxima of the N* and T* bands. Stokes shift, N*: Stokes shift of the N* band. φ: fluorescence quantum yield determined with 1 as a reference ($\varphi = 0.52$ in ethanol, see Ref. [2]). Data on dye **1** are from Ref. [5].

ESIPT reaction should be shifted more towards the N* state in dye **2** compared to dye **1**, in line with our observations.

Due to its isothiocyanate substituent, the dye **2** reacts fast with an amine (2-ethanolamine) in organic solvents resulting in the formation of the fluorescent conjugate **3** (Fig. 1). This conjugate was isolated and its absorption and fluorescence properties were further characterized.

The conjugation with amine modified significantly the absorption and emission spectra of the dye, as it can be seen from Fig. 3 and Table 1. We observe that the absorption and emission spectra of conjugate **3** are blue shifted as compared to these of dye **2** (Fig. 3) in all the studied organic solvents (Table 1). Conjugate **3** also demonstrates smaller values of the Stokes shift of the N* band (Table 1). Moreover, the blue shifts are accompanied by a dramatic decrease in the relative intensities of the N* band (i.e. in the I_{N^*}/I_{T^*} ratio) (Fig. 3, Table 1). Noticeably, the dyes **2** and **3** show similar fluorescence quantum yields in medium polar solvents. This suggests that the observed strong decrease in the I_{N^*}/I_{T^*} ratio on transformation of **2** into **3** is connected with the shift of the ESIPT reaction towards its product T* state. The observed phenomena can be explained by the electron donor properties of the 7-thiourea group that is formed from the 7-isothiocyanate group and the amine (Scheme 1). This change from electron acceptor to electron donor at 7-position of 3HF decreases the charge transfer character of the N* state of the dye, resulting in the observed blue shifts and decrease in the Stokes shifts. Moreover, this change makes the N* state dielectrically less stabilized, so that the reversible ESIPT reaction is shifted towards the product T* state. These conclusions are in accordance with previous studies on variation of the electron donor group in 7-position of 3HF derivatives [6,24].

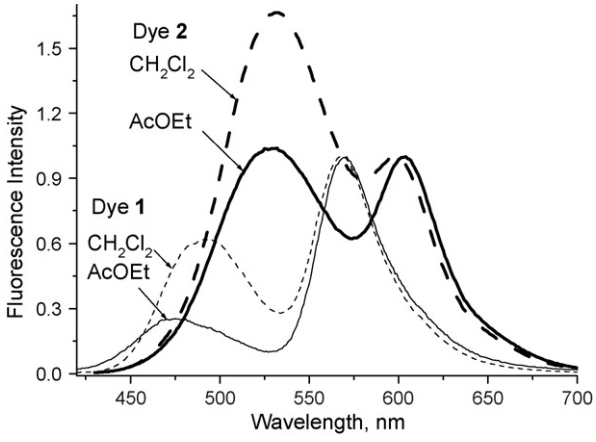


Fig. 2. Fluorescence spectra of dyes **1** and **2** in organic solvents. Excitation wavelength was 410 nm.

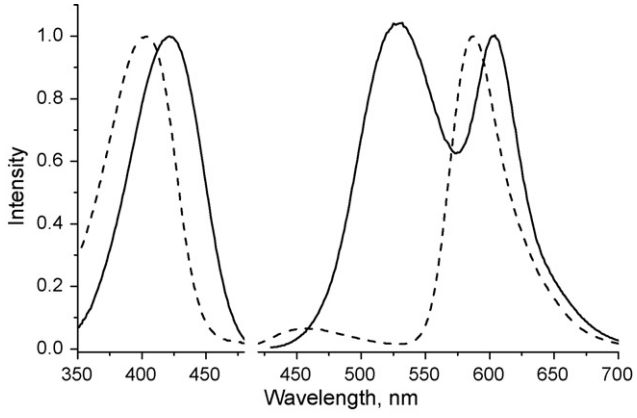
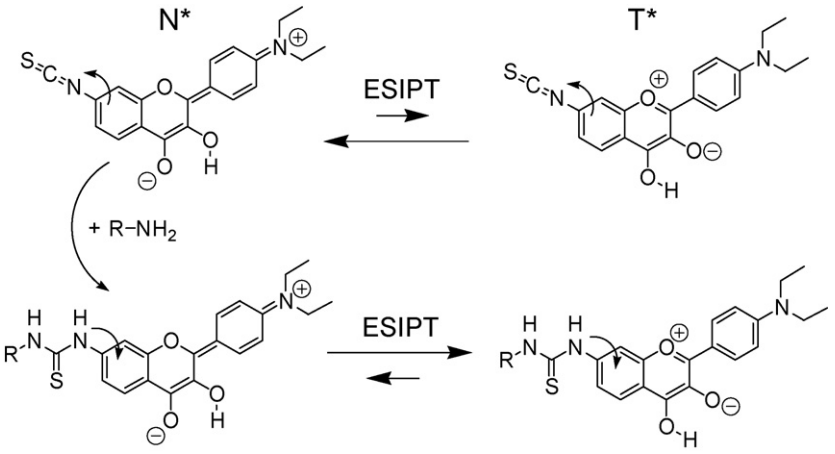


Fig. 3. Absorption (left) and fluorescence (right) spectra of label **2** (solid line) and its conjugate with ethanolamine, **3** (dashed line), in ethyl acetate.



Scheme 1. Mechanism of the effect of isothiocyanate coupling with amines on the ESIPT reaction in 3-hydroxyflavone derivative. Only excited states are shown.

Since conjugation of dye **2** with an amine results in dramatic changes of the fluorescence spectra, we recorded the fluorescence spectra of solution of **2** in THF after addition of ethanolamine as a function of time. Prior addition of ethanolamine the fluorescence spectrum of **2** shows predominant emission of the N* band. After addition of 1.2 mole equivalent of ethanolamine, the spectra progressively change with time, so that the intensity of the N* band decreases with respect to the T* band (Fig. 4). After ca 40 min no more changes are observed and the resultant spectra correspond well to that of **3** in THF (Fig. 4) suggesting that the reaction of conjugation is completed. Thus, we observe that the reaction of **2** with the amine results in a dramatic change of its dual emission. Conjugation with the amine transforms the electron acceptor isothiocyanate group into the electron donor thiourea based group, which shifts the ESIPT reaction towards the emissive T* state of the dye. The present result is the first demonstration of an ESIPT based two-colour fluorescence switching driven by a chemical reaction. This phenomenon allows easy monitoring of the process of the conjugation as well as determination of the time of its completion.

Importantly, the conjugate **3** demonstrates a strong dependence of its dual emission on the solvent polarity. Indeed, an increase in the solvent polarity results in an increase in the I_{N^*}/I_{T^*} ratio, which is accompanied by a strong red shift of the N* emission band (Fig. 5). This typical behaviour of 4′-(dialkylamino)-3-hydroxyflavones [2–6] indicates that conjugate **3** preserves the strong solvatochromism of 3-hydroxyflavones. These observations make dye **2** an attractive environment sensitive label, which allows monitoring conformational changes and interactions of biomolecules [31,32]. Thus, SH/NH_2-reactive derivatives of 3HF and 2-(2′-hydroxyphenyl)benzazole have already shown potential applicability in protein research [23,33,34]. The response of the conjugate **3** to the environment is ratiometric (Fig. 5), which is being independent from the local dye concentration and some instrumental factors is highly desirable in biological measurements [35]. Moreover, taking into account that the pK_a of 3-hydroxyflavones is around 9 [36,37], we can expect that the spectroscopic properties of dye **2** conjugated to biomolecules should not show any dependence on pH in the physiological pH range. Finally, the fluorescence characteristics of the

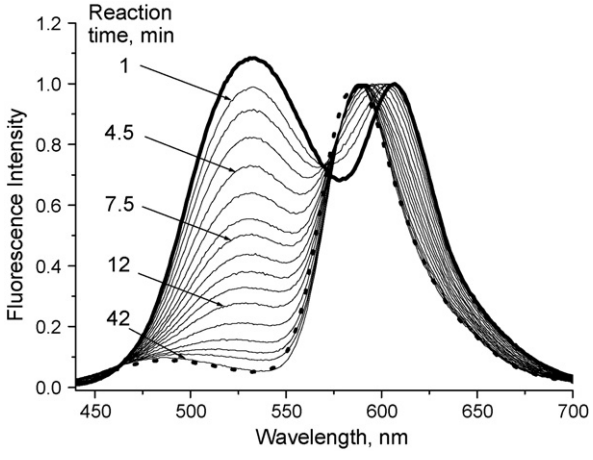


Fig. 4. Fluorescence monitoring of the reaction of dye **2** with ethanolamine. Fluorescence spectra of 1.25 μM of dye **2** in THF after addition of 1.5 μM of ethanolamine at different reaction times. The thick solid line corresponds to the spectra of the unreacted dye **2**, while the dotted line corresponds to the purified conjugate **3** in THF. All the fluorescence spectra were normalized at the T* band maximum. Excitation wavelength was 430 nm.

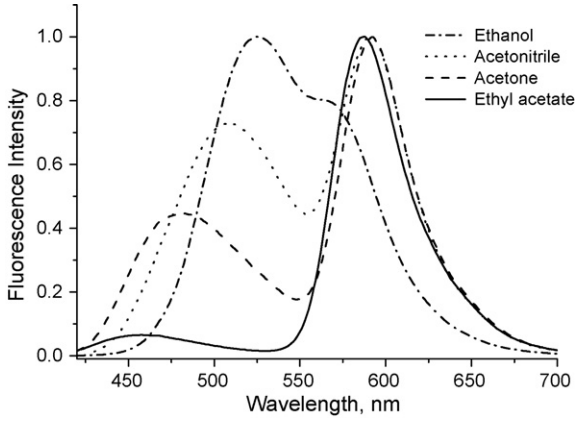


Fig. 5. Fluorescence spectra of conjugate **3** in different organic solvents. Excitation wavelength was 410 nm.

new label and its NH_2-conjugate are similar to those of the best conventional solvatochromic labels, since they absorb and emit in the visible range and demonstrate satisfactory fluorescence quantum yields (Table 1) and extinction coefficients (ca 40,000 M^{-1} cm^{-1}).

4. Conclusions

In the present work, we have synthesized an isothiocyanate derivative **2** of 3-hydroxyflavone, which shows dramatic changes in its dual emission on conjugation with an amine. This phenomenon is explained by transformation of the electron acceptor isothiocyanate group into the electron donor thiourea based group. Due to sensitivity of its dual emission to solvent polarity, dye **2** can be considered as a prospective environment-sensitive label for investigating biomolecules.

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