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The synthesis and photostability of novel squarylium indocyanine dyes

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ABSTRACT

Novel, water-soluble, squarylium indocyanine dyes with various *N*-substituents on 3*H*-indolenine were synthesized. The maxima of absorption and emission wavelengths of the dyes in different solvents were in the range 628–670 nm and exhibited negative solvatochromism with increasing solvent polarity in protic solvents. Dyes with *N*-benzyl rings (such as *N*-benzyl, *N*-carboxylbenzyl and *N*-fluorobenzyl) displayed greater photostability than dyes containing *N*-alkyl groups (such as *N*-ethyl and *N*-carboxylpentanyl) in aqueous solution. The electron-withdrawing group (such as carboxyl and fluoro group) on the *N*-benzyl group of cyanine dyes improved photostability compared to dyes that contained an electron-donating group (such as methyl). The fading constant *k* of the *N*-carboxylbenzyl dye was considerably lower than that of the *N*-carboxylpentanyl dye.

1. Introduction

Cyanine dyes play an indispensable role in biomedical applications [1,2], especially in fluorescence detection of antibodies and DNA [3], as well as the imaging of biological targets *in vivo* [4], owing to their excellent spectral properties, including large molar extinction coefficients and broad wavelength tunablility.

In order to effectively reduce the background signal arising from autofluorescence of the biological matrix and light scattering, long-wavelength cyanine dyes (>600 nm or within the near infra red region) have been developed. Increasing the length of the conjugated chain of the cyanine dyes is the main approach to impart the desired red shift; however, this reduces photostability [5].

Squrylium indocaynine dyes, possessing effective absorption >600 nm [6], narrow excitation and emission peaks [7] as well as moderate photostability [8–10], may offer potential as long-wavelength fluorescence probes and labels in biological assay [11,12]. However, the relationship between the molecular structure and photostability of this kind of dye has not been published.

The introduction of benzyl groups into terminal 3*H*-indolenine rings was shown to improve the photostability of polymethine cyanine dyes [13] and, also, that electron-donating groups on the *N*-atom of 3*H*-indolenine rings are favorable in securing greater resistance to photobleaching than are electron-withdrawing groups [14]. In this paper, the influence of the substitution on the photostability of qurylium indocaynine dyes is reported. A series of novel squarylium indocyanine dyes with good water-solubility and different photostability were synthesized. Differently from the polymethine dyes, the squrylium dyes have much better photostability when substituted by electron-withdrawing groups.

2. Experimental

2.1. Apparatus and materials

The progress of the chemical reaction and purity of product were monitored by TLC and ^{1}H NMR. ^{1}H NMR spectra were measured on a Bruke Avance-400 spectrometer in DMSO-d_6 using TMS as an internal standard. The absorption and emission spectra were determined using a Lambda 35 UV–vis spectrometer and LS 55 Luminescence Spectrometer, respectively. The purification of the dyes was performed by conventional column chromatography on reversed-phase C18 absorbent (Sinochrom C18, 40–75 μm, 100 Å, 280 m^2/g, Dalian Elite Company, China). Water–methanol mixtures were used for elution. All chemicals were of analytical grade or the best grade commercially available and used without further purification. All aqueous solutions were prepared in redistilled deionized water. All spectral measurements were carried out at room temperature unless otherwise mentioned.

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The fluorescence quantum yields (Φ_x) were calculated relative to Rhodamine B in ethanol ($\Phi_s = 0.56$) [15] using the formula [16]:

$$\Phi_x = \Phi_s(F_x/F_s)(\lambda_x/\lambda_s)(\varepsilon_s/\varepsilon_x)(n_x/n_s)^2$$

where F is the integrated area under the fluorescence emission profile, λ is the wavelength of the excitation light, ε is the molar absorptivity of the dye at the excitation wavelength, n is the refractive index of the solvent, and the subscript s and x are designated for those parameters associated with the standard and the sample respectively. Since the fluorescence quantum yield of the secondary standard used in this investigation can be considered provisional, the quantum yields reported here are intended primarily to show trends and should not be considered as absolute values.

2.2. *Synthesis*

The synthetic routes of the squarylium cyanine dyes are shown in Fig. 1.

2,3,3-Trimethylindoleninium-5-sulfonate (**1**) was synthesized using the method described previously [17].

2.2.1. *N-benzyl-2,3,3-trimethylindoleninium-5-sulfonate (**2d**)*

The potassium salt of 2,3,3-trimethylindoleninium-5-sulfonate (**1**) (5.5 g, 0.020 mol) and 1-(bromomethyl) benzene (3.7 g, 0.032 mol) were dissolved in 35 mL toluene. The mixture was stirred and refluxed under a nitrogen atmosphere for 5 h and the ensuing mixture was cooled, the solvent was decanted and the solid (**2d**) was filtered off and washed with toluene and finally dried under high vacuum. The intermediate was used in the following steps without further purification. The other *N*-alkyl-2,3,3-trimethylindoleninium-5-sulfonates were synthesized using the similar methods. The solvents in synthesizing **3a**, **3b** are o-dichlorobenzene, in **3c** is iodoethane. The time of synthesizing **3a**, **3b** is 11–12 h, of **3c**, **3e**, **3f** is 24–26 h. The yields of **3a**, **3b**, **3c**, **3d**, **3e** and **3f** were 70%, 81%, 94%, 65%, 60% and 69% respectively.

2.2.2. *Squarylium indocyanine dye (**3d**)*

N-benzyl-2,3,3-trimethylindoleninium-5-sulfonate (**2d**) (810 mg, 1.8 mmol) in 5 mL pyridine was added to a solution of 114 mg (1 mmol) of squaric acid in 10 mL *n*-butanol and toluene (1:1) and mixed thoroughly. The mixture was refluxed in nitrogen atmosphere for 6 h. Then the mixture was cooled, the solvent was removed under high vacuum and the residue was obtained. Isopropanol was added in flask and blue solid was precipitated and filtered. The crude product was dissolved in 10 mL water and chromatographied on a reversed-phase C18 column (30 g) using a 10–20% gradient of methanol in water as eluant. Blue solution was collected and distilled under high vacuum to remove off solvent, then blue powder was obtained. Dyes **3a**–**f** were all blue powder. Melting points of them were all over 300 °C.

*Compound **3a***. Yield: 31%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70 (s, 12H, $C(CH_3)_2$), 5.46 (s, 4H, CH_2), 5.73 (s, 2H, CH), 7.22–7.25 (d, 2H, $J = 8.0$ Hz, CH), 7.29–7.31 (d, 4H, $J = 8.0$ Hz, CH), 7.55–7.57 (d, 2H, $J = 7.5$ Hz, CH), 7.72 (s, 2H, CH), 7.90–7.93 (d, 4H, $J = 8.1$ Hz, CH). ESI-MS: $[M-2H+K]^-$ ($m/z = 861.1$), $[M-H]^-$ ($m/z = 823.1$), $[M-2H]^{2-}$ ($m/z = 411.1$).

*Compound **3b***. Yield: 15%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.34–1.41 (m, 4H, CH_2), 1.54–1.62 (m, 4H, CH_2), 1.68 (s, 12H, $C(CH_3)_2$), 1.70–1.75 (m, 4H, CH_2), 2.28–2.31 (t, 4H, CH_2), 4.05 (m, 4H, CH_2), 5.82 (s, 2H, CH), 7.26–7.28 (d, 2H, $J = 8.4$ Hz, CH), 7.58–7.60 (d, 2H, $J = 8.8$ Hz, CH), 7.65 (s, 2H, CH). ESI-MS: $[M-H]^-$ ($m/z = 783.2$), $[M-3H+K]^{2-}$ ($m/z = 410.2$), $[M-2H]^{2-}$ ($m/z = 391.1$).

*Compound **3c***. Yield: 39%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.24–1.26 (t, 6H, CH_3), 1.70 (s, 12H, $C(CH_3)_2$), 4.12 (m, 4H, CH_2), 5.77 (s, 2H, CH), 7.23–7.25 (d, 2H, $J = 7.2$ Hz, CH), 7.49–7.51 (d, 2H, $J = 8.0$ Hz, CH), 7.67 (s, 2H, CH). ESI-MS: $[M-2H+K]^-$ ($m/z = 649.1$), $[M-H]^-$ ($m/z = 611.2$), $[M-2H]^{2-}$ ($m/z = 305.1$).

*Compound **3d***. Yield: 31%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.71 (s, 12H, $C(CH_3)_2$), 5.39 (s, 4H, CH_2), 5.83 (s, 2H, CH), 7.20–7.22 (d, 4H, $J = 7.6$ Hz, CH), 7.24–7.26 (d, 2H, $J = 8.8$ Hz, CH), 7.35–7.39 (t, 4H, CH), 7.28–7.31 (t, 2H, CH), 7.58–7.60 (d, 2H, $J = 8.4$ Hz, CH), 7.72 (s, 2H, CH). ESI-MS: $[M-2H+K]^-$ ($m/z = 773.1$), $[M-H]^-$ ($m/z = 735.2$), $[M-2H]^{2-}$ ($m/z = 367.1$).

*Compound **3e***. Yield: 33%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70 (s, 12H, $C(CH_3)_2$), 2.26 (s, 6H, CH_3), 5.28 (s, 4H, CH_2), 5.80 (s, 2H, CH), 7.09–7.11 (d, 4H, $J = 8.0$ Hz, CH), 7.15–7.17 (d, 2H, $J = 7.6$ Hz, CH), 7.23–7.25 (d, 4H, $J = 8.4$ Hz, CH), 7.56–7.58 (d, 2H, $J = 8.0$ Hz, CH), 7.70 (s, 2H, CH). ESI-MS: $[M-2H+K]^-$ ($m/z = 801.2$), $[M-H]^-$ ($m/z = 763.3$), $[M-2H]^{2-}$ ($m/z = 321.1$).

*Compound **3f***. Yield: 30%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70 (s, 12H, $C(CH_3)_2$), 5.38 (s, 4H, CH_2), 5.80 (s, 2H, CH), 7.18–7.20 (d, 2H, $J = 8.0$ Hz, CH), 7.22–7.24 (d, 4H, $J = 8.0$ Hz, CH), 7.24–7.28 (t, 4H, CH), 7.57–7.59 (d, 2H, $J = 8.4$ Hz, CH), 7.72 (s, 2H, CH). ESI-MS: $[M-2H+K]^-$ ($m/z = 809.2$), $[M-H]^-$ ($m/z = 771.3$), $[M-2H]^{2-}$ ($m/z = 385.1$).

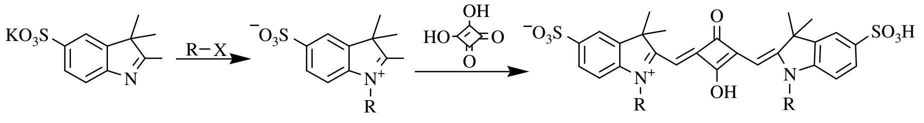
2.3. *Absorption, fluorescence and photostability measurements*

The 1×10^{-4} M dye stock solutions were prepared by dissolving of the dye in methanol and kept at 4 °C. These dyes were diluted to 2×10^{-6} M in different solvents. The samples were irradiated under a 500 W iodine–tungsten lamp at distance of 30 cm away. The photostabilites were in terms of remaining absorption calculated from the absorption intensities with absorption maximum before and after irradiated.

3. Results and discussion

3.1. *Absorption and fluorescence emission properties*

Table 1 shows the absorption and fluorescence emission spectra of the dyes **3a**–**f** in different solvents. The absorption maximum and emission wavelengths of dyes in different solvents are in the range



1 **2a~f** **3a~f**

a : R=p-$CH_2C_6H_4COOH$; b : R=$(CH_2)_5COOH$; c : R=C_2H_5;
d : R=$CH_2C_6H_5$; e : R=p-$CH_2C_6H_4CH_3$; f : R=p-$CH_2C_6H_4F$

Fig. 1. Synthetic routes of the squarylium cyanine dyes.

Table 1
Spectral data of the cyanines in different solvents.

Dye	Solvent	Absorption λ_{ab} (nm)	Emission λ_{em} (nm)	Stokes shift (nm)	ε (10^5 mol^{-1} cm^{-1} L)	Φ
3a	Water	631	646	15	2.42	0.021
	Methanol	638	655	17	2.11	0.170
	Ethanol	641	657	16	2.28	0.101
	DMF	654	668	14	2.22	0.185
	DMSO	656	670	14	2.39	0.157
3b	Water	632	643	11	0.75	0.023
	Methanol	638	652	14	1.17	0.184
	Ethanol	641	656	15	1.23	0.111
	DMF	653	667	14	1.11	0.147
	DMSO	656	668	12	1.16	0.153
3c	Water	628	639	11	1.92	0.008
	Methanol	634	648	14	2.54	0.047
	Ethanol	638	650	12	2.56	0.048
	DMF	649	662	13	2.25	0.088
	DMSO	652	666	14	2.31	0.089
3d	Water	631	646	15	2.44	0.017
	Methanol	638	652	14	2.72	0.065
	Ethanol	641	654	13	2.33	0.136
	DMF	653	667	14	2.62	0.117
	DMSO	656	672	16	2.67	0.114
3e	Water	631	646	15	2.11	0.011
	Methanol	638	653	15	2.57	0.068
	Ethanol	642	655	13	2.03	0.144
	DMF	653	667	14	2.33	0.136
	DMSO	656	669	13	2.38	0.119
3f	Water	631	647	16	0.67	0.014
	Methanol	637	651	14	0.73	0.117
	Ethanol	640	653	13	0.76	0.164
	DMF	652	662	10	0.81	0.221
	DMSO	655	665	10	0.69	0.151

from 628 to 670 nm. The dyes display a strong absorption in red visible region with a high molar absorption coefficients ε up to 10^5 mol^{-1} cm^{-1} L in solutions. Typical absorption and emission spectra are shown in Fig. 2.

As seen from Fig. 2, the peak shapes of both absorption and fluorescence emission are narrow, Stokes shift is small ($\approx$15 nm). As seen from Table 1, these dyes exhibited absorption maxima in the range from 628 to 632 and fluorescence emission maxima from 639 to 647 nm in aqueous media, whereas in other various solvents, they exhibited a red shift of about 6–25 nm. In protic solvents, the spectra of all these dyes exhibited negative solvatochromism which was a blue shift of the absorption and emission maximum with increasing solvent polarity. In aprotic polar solvents, the spectra of all these dyes exhibited positive solvatochromism which was a red shift of the absorption and emission maximum with increasing solvent polarity. The red shifts in aprotic polar solvents were not obviously compare to blue shifts in aprotic polar solvents. The effect of the protic solvent polarity on λ_{max} can be illustrated by interactions between the dye molecules and the solvents as the interactions make ground state of dye more stable by forming hydrogen bond. Compared with normal cyanine dyes, the ground states of ionic squarylium dyes have a much more polar character than that of the excited states, so increase of solvent polarity is more beneficial to lower the energy of the ground states than that of the excited states of the dye molecules [18]. The squarylium dyes were easy to be ionized and created oxygen anion which was stabilized by hydrogen bond.

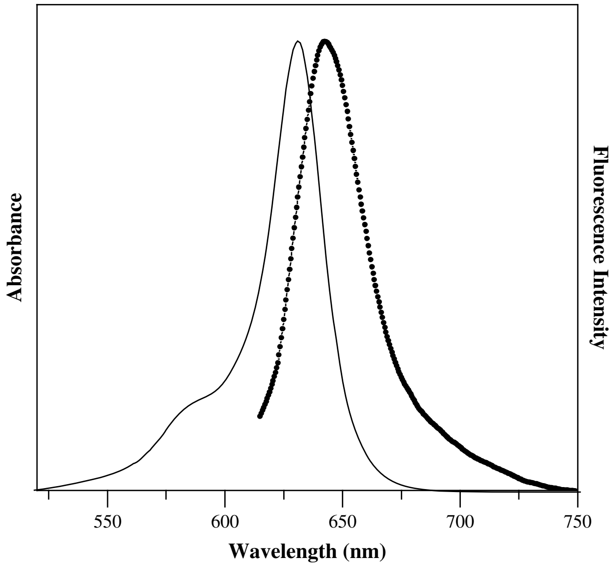


Fig. 2. Absorption and emission spectra of dye **3d** in water.

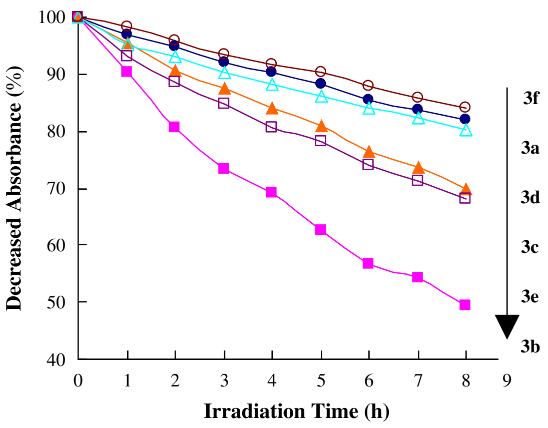


Fig. 3. Photofading behavior of dyes **3a–f** in water.

Table 2
Rate constants of photofading.

Molecule	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**
Rate constants k ($\times 10^{-4}$ mol min^{-1})	4.109	14.70	7.490	4.597	8.004	3.657

All these dyes possess reasonably good fluorescence quantum yields in organic solvents. However, they exhibited around lower quantum yields of fluorescence in aqueous media when compared to those in organic solvents. This could be attributed to the interactions of these dyes with surrounding water molecules and the interactions can result in increased non-radiative decay processes [8]. It has been proposed that the major non-radiative decay pathway for the excited states of the squaraine dyes is by the rotation of the C–C bond between the indole ring and the C_4O_2 unit which is at the center of the molecule [19].

3.2. Photostability of the dyes

The good photostability is an important requirement for the application of fluorescent dye in fluorescence microscopy. The photostabilities of the dyes with different substituents on *N*-position of 3*H*-indolenine were studied in the aqueous media to get a deeper insight into relationship of structure and photostability of the cyanine dyes.

Fig. 3 shows photofading behavior of dyes **3a**–**f** in water. After irradiation of 8 h, dye **3e** and **3c** showed 31.5% photofading, and dye **3b** lost 51%, but dye **3a**, **3d** and **3f** showed about only 16–19% decrease in maximal absorbance. The photostabilities of the dyes can be placed in an order: **3a**, **3d**, **3f** > **3c**, **3e** > **3b**. We can calculate the speed constant of the photodegradation reaction (k) according to the formula (1) [14] from the experimental data.

$$\ln\left(\frac{A_0}{A_t}\right) = kt \qquad (1)$$

A_0 is the absorbance in maximal wavelength before the irradiation, and A_t is the absorbance in maximal wavelength after the irradiation. The rate constants of photoreaction based on the experiments are shown in Table 2.

These data show that the dyes with benzyl ring in the *N*-substituents (**3a**, **3d** and **3f**) are more stable than those with *N*-alkyl substitution (**3b** and **3c**). For example, fading constant *k* of *N*-carboxylbenzyl dye (**3a**) is less than one third of the *k* of *N*-carboxylpentanyl dye (**3b**) where the *N*-carboxylpentanyl group is commercially used in cyanine dyes for bio-labeling. Furthermore, the electron-withdrawing groups on the benzyl group (**3f**) improve the photostability compared with electron-donating group (**3e**). The main reason of this might be that the electron-withdrawing group decreases the density of the electron cloud on the π-conjugated system. In the case of low electron density, the oxidation by singlet oxygen on the conjugate chain should not be easy to carry out. The electron-withdrawing group would enlarge the π-conjugated system and make the dye more stable [20].

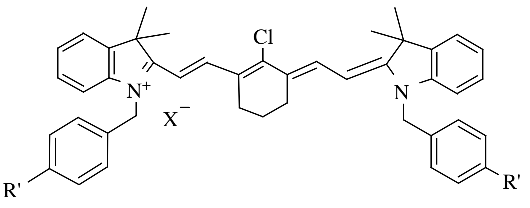


Fig. 4. The structure of dyes **4**.

Table 3
HOMO and LUMO energy levels.

Molecule	HOMO (a.u.)	LUMO (a.u.)	HOMO (eV)	LUMO (eV)
3a (CO_2H)	−0.18630	−0.10777	−5.07	−2.93
3d (H)	−0.18193	−0.10341	−4.95	−2.81
3e (CH_3)	−0.17965	−0.10112	−4.89	−2.75
3f (F)	−0.18471	−0.10610	−5.03	−2.89

We reported in our previous papers [14,21] that the dyes **4** (Fig. 4) with electron-donating groups (R′) on the nitrogen atoms perform improved photostability compared with the dyes with electron-withdrawing groups. It seems incompatible with the present cases.

We considered that the effect in the case of dyes **4** might be from photo-induced electron transfer. As the chromophore is a cation, it has low electron density. After excited by light, the excited chromophore should be quenched quickly by intramolecular electron transfer from R′ (rich electron) to the dye chromophore, and go back to ground state before the chromophore is attacked by singlet oxygen [21]. On the contrary, in the cases of present squarylium, the central hydroxyl group donates electron density to the chromophore via intramolecular charge transfer, lets the chromophore have higher electron density than that of dyes **4**, and photo-induced electron transfer from *N*-substituent to the chromorphore is a difficulty in thermodynamics.

To examine the effect of substituents linked to *N*-position on the photostability of the squarylium dyes, the ground and excited states geometries and electronic structures of compounds **3a**, **3d**, **3e** and **3f** were computed using B3LYP/6-31G* method. The results are listed in Table 3. According to the calculation, HOMO and LUMO energy levels change regularly with the substituents. As the substituent becomes more electron withdrawing, the HOMO and LUMO energy levels become lower. The lower energy levels make the dyes increase the oxidative potential, and become more resistant to photo-oxidation.

4. Conclusion

Six novel water-soluble squarylium indocyanine dyes were synthesized. The maxima of absorption and emission wavelengths of these dyes in different solvents are in the range from 628 to 670 nm. The dyes display a strong absorption in red visible region. The spectra of these dyes exhibited negative solvatochromism with increasing solvent polarity in protic solvents. All these dyes possess reasonably good fluorescence quantum yields in organic solvents compare with in aqueous medium. The photostabilities of the dyes can be placed in an order: **3a** (with carboxylbenzyl), **3d** (with benzyl), **3f** (with fluorobenzyl) > **3c** (with ethyl), **3e** (with methylbenzyl) > **3b** (with carboxylpentanyl). These data show that the dyes with benzyl ring in the substituents which is linked to *N*-position are indeed more stable. The electron-withdrawing group on the benzyl group on nitrogen atom of cyanine dyes improves photostability compared with the dyes with electron-donating group which decreases photostability obviously. The general commercial cyanine dyes for bio-labeling always contain *N*-carboxylpentanyl. The results, however, show that the dye containing *N*-carboxylbenzyl has much better photostability. It may be very useful for the consideration in the design of new bio-labeling dyes.

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The calculations of the ground state geometries and electronic structures have been performed with the Gaussian 03 package of programs by the author's friend Yun-Xiao Liang who works at Faculty of Materials Science and Chemical Engineering, Ningbo University.

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