ELSEVIER

Dyes and Pigments 54 (2002) 107–111

DYES and PIGMENTS

www.elsevier.com/locate/dyepig

Syntheses and spectral properties of fluorescent trimethine sulfo-3*H*-indocyanine dyes

Liqiu Wang[a,b], Xiaojun Peng[a,*], Rong Zhang[a], Jingnan Cui[a], Guoquan Xu[a], Fenggang Wang[a,c]

[a]*State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116012, PR China*
[b]*Qiqihar University, Qiqihar 161006, People's Republic of China*
[c]*Lines Technologies Corporation, 6398D Angus Dr., Raleigh, NC 27612, USA*

Received 5 November 2001; received in revised form 3 December 2001; accepted 23 April 2002

Abstract

Novel fluorescent sulfo-3*H*-indocyanine dyes containing at least one *p*-carboxybenzyl group on the nitrogen atoms in 3*H*-indocyanine rings were synthesized. Their λ_{abs} are in the range of 547–551 nm and λ_{em} 562–567 nm in water. When solvents change from water to ethanol or DMF, the λ_{abs} and λ_{em} of the dyes exhibit red shifts. Because of the presence of sulfonate groups and carboxyl groups, these dyes have good aqueous solubility. The *p*-carboxybenzyl group let the dyes have better photo-stability than commercial 5-carboxylpentynyl- containing dyes. The carboxyl groups can be converted into their *N*-hydroxylsuccinimidyl esters to label proteins, oligonucleotides or other bio-chemicals.

Keywords: Sulfo-3*H*-indocyanine; Fluorescent dyes; Biological label

1. Introduction

3*H*-indocyanine dyes as fluorescent labeling reagents have been used in the analysis of DNA sequence, immunoassay and clinical diagnostics [1–3]. For these applications good water solubility for the fluorophore is crucial because aqueous solubility of the dyes is the main factor to decrease the degree of aggregation, and influences their light absorption and emission properties when conjugated to substrates [4–6]. In fluorogenic labeling a fluorescent molecule is normally coupled either covalently or non-covalently to a non-fluorescent molecule. For covalent labeling, highly reactive functional groups, such as *N*-hydroxysuccinimidyl ester (NHS) or isothiocyanate, are the most widely employed in commercial fluorescence labels. Because of the mild reaction conditions (neutral medium and room temperature) and less influence on activity of labeled bio-molecules, NHS-carboxyl group has gradually replaced isothiocyanate group in fluorescence labeling [7]. The NHS-carboxyl dyes are easily prepared from the esterification of *N*-hydroxysuccinimide with carboxyl dyes.

In Scheme 1, **1b** and **1e** containing *N*-5-carboxypentynyl synthesized by Waggoner et al.

* Corresponding author. Tel./fax: +86-411-3673467.
E-mail address: visitor@dlut.edu.cn (X. Peng).

0143-7208/02/$ - see front matter
PII: S0143-7208(02)00036-0

[7,8] are commercial fluorescent label compounds for proteins, oligonucleotides and other compounds containing amino, mercapto or hydroxyl groups and have been applied to bioanalysis. They have excellent fluorescent properties combined with good aqueous solubility and a reduced tendency to form aggregates when they are used in aqueous systems due to the presence of the negatively charged sulfonate groups [8,9]. However, photostability of these dyes has been a problem. Yao et al. reported that polymethine 3*H*-indocyanine dyes containing *N*-benzyl substituents in the heterocyclic rings were more photo-stable than *N*-*n*-alkyl dyes [10]. To improve photostability of the water-soluble cyanine dyes, we employed *p*-carboxybenzyl (*p*-$CH_2C_6H_4COOH$) as the substituents on the nitrogen atoms in 3*H*-indole rings, designed and synthesized dyes **1a** and **1d**, and investigated spectral properties of these dyes.

2. Results and discussion

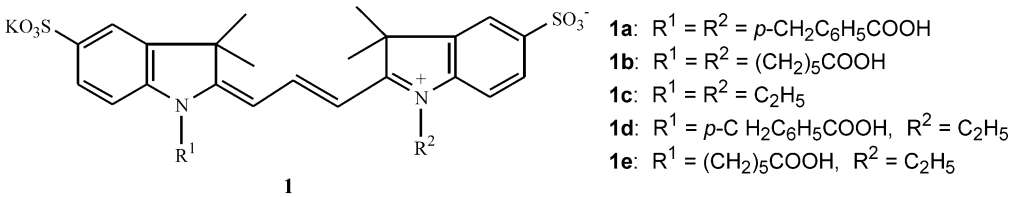
2.1. Syntheses

As shown in Scheme 2, dyes **1** were synthesized by condensation of intermediates 3 which were from the quaternization of **2** with *p*-(chloromethyl) benzoic acid, 6-bromohexanoic acid or ethyl iodide, respectively. Compound **2** was prepared by conventional Fisher indole synthesis [11]. Compared with **3b** (from 6-bromohexanoic acid), **3a** (from *p*-(chloromethyl) benzoic acid) was more convenient to prepare.

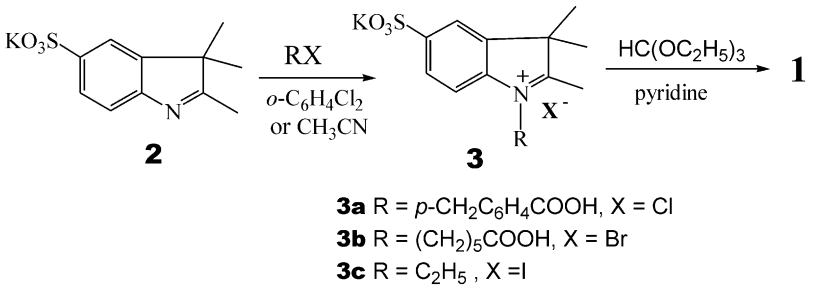
Condensation of equimolar portions of **3a** and **3c** with triethyl orthoformate in pyridine afforded a mixture of symmetrical and asymmetrical dyes: **1a**, **1c** and **1d** with molar ratio of 1:2:2. The ratio indicated that the reactivity of **3c** was relatively higher than **3a**. The unsymmetrical dye **1d** was isolated by reversed phase chromatography in 35% yield. The symmetrical dyes **1a** and **1c** were more easily obtained in 40–50% yield by the self-condensation of **3a** and **3c** with triethyl orthoformate, respectively.

2.2. Spectral properties of the sulfo-3H-indocyanine dyes

The dyes synthesized in this paper showed red fluorescent color with λ_{abs} and λ_{em} between 540 and 590 nm (Tables 1 and 2). Change of the substituent on the nitrogen atoms slightly changed the basic indocyanine absorption and fluorescence



Scheme 1.



Scheme 2. Syntheses of symmetrical and unsymmetrical indocyanines.

properties compared to the commercial dyes **1b** and **1e**. Dye **1a** had a slight red shift to **1b** and **1e**. This shift may result from electron donor property of the N-substituents. Asymmetrical **1d** had λ_{abs} and λ_{em} maxima between those of the two related symmetrical dyes **1a** and **1c**.

Their λ_{abs} and λ_{em} maxima displayed a blue shift with increasing solvent polarity. As shown in Table 2, λ_{em} exhibited a blue shift of about 15 nm for **1a**, 12 nm for **1d** and 9 nm for **1b** when the solvent changed from ethanol to water. This may be caused by interactions between the dye molecule and the solvent.

Comparing the excitation and emission spectra of **1a** and **1d** in water (Figs. 1 and 2), unsymmetrical dye **1d** had more narrow bands.

2.3. Photostability of the dyes

No noticeable change of absorption spectra of **1a** and **1d** was observed when they were stored in water solution saturated with N_2 under room-light irradiation in several weeks. However, the absorption intensity of **1b** decreased. On TLC plates, **1a** and **1d** may be kept for 1 week in air with no absorption intensity loss except that the fluorescence intensity of these dyes slightly decreased due to the fluorescence quenching by oxygen. Compared with **1b**, dyes **1a** and **1d** were with aromatic *p*-carboxybenzyl substituent on the nitrogen atoms in 3*H*-indole rings. The *p*-carboxybenzyl substitutent may let the molecule be more endurant or resistant to the attack by singlet oxygen than the 5-carboxypentynyl group in **1b**.

Table 1
Absorption λ_{max} (nm) and ε (l mol^{-1} cm^{-1}) of sulfoindocyanine dyes in various solvents

Dye no.	λ^{w}_{max}	λ^{m}_{max}	λ^{e}_{max}	λ^{d}_{max}	ϵ^{w}
1a	551.0	561.0	567.0	567.0	112745
1b	550.0	555.0	559.0	565.0	124555
1c	547.0	552.0	556.9	562.0	110076
1d	549.0	554.0	558.2	564.0	125106

w: Water, m: methanol, e: ethanol, d: DMF.

Table 2
Fluorescence spectral data (nm) of sulfoindocyanine dyes in various solvents

Dye no.	λ^{w}_{λ}	Δ^{w}_{λ}	λ^{m}_{em}	Δ^{m}_{λ}	λ^{e}_{em}	Δ^{e}_{λ}	λ^{d}_{em}	$\Delta_{\lambda^{d}}$
1a	564.8	13.4	576.3	15.3	580.0	14.0	584.8	16.1
1b	562.0	13.0	570.2	13.3	571.2	12.2	582.1	15.5
1c	560.0	13.2	567.8	12.8	569.7	13.8	580.0	15.8
1d	561.0	13.0	570.2	14.3	571.5	14.3	582.1	16.4

w: Water, m: methanol, e: ethanol, d: DMF, $\Delta\lambda$: Stokes shift.

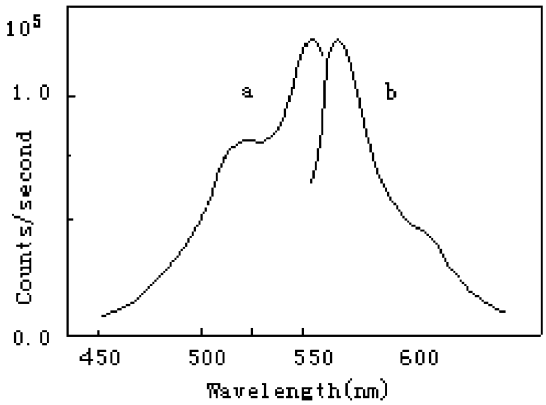


Fig. 1. Excitation (a) and emission (b) spectra of dye **1a** in water.

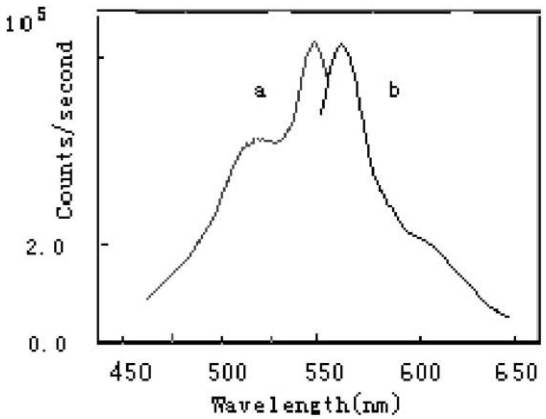


Fig. 2. Excitation (a) and emission (b) spectra of dye **1d** in water.

2.4. Solubility of the 3H-indocyanine dyes

Commercial fluorescent probes **1b** and **1e** are water-soluble [8,12] due to the presence of the sulfo-groups and carboxyl groups; **1a** and **1d** also dissolved easily in water. Even at millimolar concentrations, no new absorption band was observed in the absorption spectra of **1a** and **1d**. It implies no formation of dye dimmer or dye aggregation. This property made them very easily to be used in bio-analysis.

3. Experimental

3.1. Instruments and materials

Mass spectral determinations were made on HP1100API-ES mass spectrometry. ^{1}H NMR spectra were obtained on a VARIAN 400 MHz ^{1}H NMR spectrometer. Fluorescence measurements were performed using a PTI-C-700 Felix and Time-Master system (USA). Purification of the dyes was performed on a PU-1586 (JASCO) preparative HPLC unit equipped with a C18-RP column. Water–methanol mixtures were used for the elution. Ultraviolet-visible spectra were measured on a HP-8453 spectrophotometer. Extinction coefficients were determined from absorbance values of weighed samples of dried material and their molecular weights. 6-Bromohexanoic acid was purchased from ACROS Chemical Co.

3.2. Syntheses of 1-(P-carboxybenzyl)-2, 3, 3-trimethylindoleninium-5-sulfonate ***3a***

The potassium salt of 2, 3, 3-trimethylindoleninium-5-sulfonate **2** (11.0 g, 40 mmol) and *p*-(chloromethyl) benzoic acid (8.2 g, 48 mmol) were mixed in 1,2-dichlorobenzene (100 ml) and heated at 105–108 °C for 10 h under nitrogen. The mixture was cooled, 1,2-dichlorobenzene was decanted. Triturating the solid with 2-propanol afforded **3a**, the light purple powder (15.2 g, yield 85%) was used in further experiments without additional purification. λ_{max} (water) = 266 nm. ESI–MS: $[M–K–Cl–H]^-$ (m/z = 372.0).

3.3. Synthesis of ***1a***

3a (6.3 g, 14 mmol) in pyridine (25 ml) was heated to refluxing. Triethyl orthoformate (4 ml, 25 mmol) was added slowly, then the solution was heated for an additional 2.5 h. The mixture was cooled and diluted with several volumes of diethyl ether. A product separated in the form of a red powder from which the supernatant fluid was removed by decantation. It was dissolved in methanol and reprecipitated with addition of 2-propanol. The product was collected on filter paper and dried. Crude dye was dissolved in water and chromatographed on a silica gel column with *n*-butyl alcohol–pyridine–water (2: 1: 0.5–1) as eluent. 4.5g of **1a** was obtained (40% yield). ^{1}H NMR (D_2O) δ8.34 (s, 1H, β proton of the bridge), 7.93 (s, 2H, 4, 4′- H), 7.79–7.77 (d, 2H, J = 8.0 Hz, 6, 6′- H), 7.69–7.67 (d, 4H, J = 7.2 Hz, γ_1, γ_1′- H), 7.26–7.24 (d, 2H, J = 8.0 Hz, 7, 7′- H), 7.11–7.09 (d, 4H, J = 7.2 Hz, β_1, β_1′- H), 6.18–6.15 (d, 2H, J = 13.5 Hz, α,α′protons of the bridge), 5.30 (s, 4H, α_1, α_1′- H), 1.62 (s, 12H, $C(CH_3)_2$). ESI–MS: $[M–K]^-$ (m/z = 755.7), $[M–K–H]^{2-}$ (m/z = 377.0), $[M–K–2H]^{3-}$ (m/z = 251.4).

3.4. Synthesis of ***1d***

The mixture of triethyl orthoformate (3 ml, 18 mmol), **3a** (2.9 g, 7 mmol) and **3c** (2.0 g, 7 mmol) in pyridine was stirred at room temperature for 5 min and was then heated at reflux for 75 min under nitrogen. The mixture was cooled to room temperature and diluted with diethyl ether. The dark purple solid was dissolved in water and chromatographed on a reversed-phase C18 column (methanol–water 1:3–4). Dye **1d** was obtained as a dark red solid (1.7 g, 35% yield). ^{1}H NMR (D_2O) δ 8.45 (s, 1H, β proton of the bridge), 7.93 (s, 2H, 4, 4′- H), 7.89–7.87 (d, 2H,J = 8.4 Hz, γ_1 - H), 7.80–7.78 (d, 2H, J = 8.4 Hz, 6, 6′- H), 7.37–7.35 (d, 2H, J = 8.4 Hz, β_1 - H), 7.30–7.28 (d, 2H, J = 8.4 Hz, 7, 7′- H), 6.34–6.26 (q, 2H, α, α'protons of the bridge), 5.43 (s, 2H, α_1-H, N–CH_2), 4.10 (q, 2H, α_2-H, N–CH_2), 1.91 (s, 12H, $C(CH_3)_2$), 1.31 (m, 3H, β_2-H,–CH_3). ESI–MS: $[M–H]^-$ (m/z = 687.0), $[M–K]^-$ (m/z = 649.0), $[M–K–H]^{2-}$ (m/z = 324.0).

4. Conclusion

Two new 3*H*-indocyanine fluorescent labeling dyes containing sulfo-groups and at least one carboxylbenzyl group were easily synthesized. These dyes not only were water soluble and had spectral properties similar to the commercial dyes **1b** and **1e**, but also were more photo-stable due to the presence of the larger substituent on the nitrogen atoms. The carboxyl group of the dyes may esterified with *N*-hydroxysuccinimide to be reactive to bio-chemicals.

Acknowledgements

The authors would like to thank National Natural Science Foundation of China and The Ministry of Education of China for providing financial support for this project.

References

[1] Dorshow RB, Achilefu S, Bugaj J, Rajagopalan R. US Patent 6 264 919; 2001.
[2] Zhu Z, Chao J, Yu H, Waggoner AS. Nucleic Acids Research 1994;22:3418–22.
[3] Narasimhachari N, Gabor P. J Org Chem 1995;60:2391–5.
[4] Reddington MV. J Chem Soc Perkin Trans 1998;1:143–7.
[5] Waggoner AS. US Patent 5 627 027; 1997.
[6] Schobel U, Egelhaaf HJ, Brecht A, Oelkrug D, Gauglitz G. Bioconjugate Chem 1999;10:1107–14.
[7] Mujumbdar SR, Mujumbdar RB, Grant CM, Waggoner AS. Bioconjugate Chem 1996;7:356.
[8] Mujumdar RB, Earnt LA, Mujumdar SR, Lewis CJ, Waggoner AS. Bioconjugate Chem 1993;4:105.
[9] Yu H, Chao J, Patek D, Mujumdar R, Mujumdar S, Waggoner AS. Nucleic Acids Research 1994;22:3226–32.
[10] Wang W, Yao ZG. Photographic Science and Photochemistry (Chinese) 1997;15:321–5.
[11] Ikeda T. Egashira. Yokkyo Koho JP 04 273 856; 1992.
[12] Waggoner AS. US Patent 5 569 766; 1996.