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Novel asymmetric Cy5 dyes: Synthesis, photostabilities and high sensitivity in protein fluorescence labeling

Li Wang[a], Jiangli Fan[a], Xiaoqiang Qiao[b], Xiaojun Peng[a,*], Bin Dai[a], Bingshuai Wang[a], Shiguo Sun[a], Lihua Zhang[b], Yukui Zhang[b]

[a] State Key Laboratory of Fine Chemicals, Dalian University of Technology, 158 Zhongshan Road, Dalian 116012, China
[b] CAS Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic Research & Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

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ABSTRACT

Several novel water-soluble asymmetric pentamethine cyanine dyes were synthesized. The maximum absorption and emission wavelengths of the dyes in different solvents were in the range from 647 to 665 nm and exhibited negative solvatochromism with increasing solvent polarity. The fluorescence quantum yields of the dyes were about 0.1 in water, and were obviously higher than those of hydrophobic cyanine dyes. Dyes with *N*-benzyl groups and *N*-sulfo-groups displayed greater photostability than dyes with *N*-carboxypentyl groups in water. The limit of detection of dye **5a** for BSA was 1.2×10^{-8} mol L^{-1} by high performance liquid chromatography with fluorescent director about 100-fold lower than that by UV detection (1.0×10^{-6} mol L^{-1}). Therefore, Dye **5a** could be used to improve photostability and detection sensitivity in protein analysis.

1. Introduction

In the post-genome sequencing era, there has been more and more attention paid to protein analysis. Until now, fluorescence detection has proved to be one of the most sensitive methods for protein analysis [1,2]. Fluorescence detection combined with electrophoresis [3,4] and high performance liquid chromatography (HPLC) techniques enable qualitative and quantitative analysis of proteins. However, most proteins with important biological functions, such as the drug targets and biomarkers, are of an extremely low concentration [5]. Therefore, the study of more suitable fluorophores for the improvement on detection sensitivity for proteins is an imperative task.

Cyanine dyes play an indispensable role in biomedical applications [6,7], particularly in fluorescence detection of antibodies and DNA [8], the imaging of biological targets in vivo [9], and fluorescent labeling compounds for proteins [10,11]. This is due to their excellent spectral properties, including large molar extinction coefficients and broad wavelength tunabilities.

In order to effectively reduce the background signal arising from auto-fluorescence of the biological matrix and light scattering, longwavelength 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 the photostability [12].

In our previous work, we firstly employed rigid *N*-*p*-carboxybenzyl (*N*-*p*-$CH_2C_6H_4COOH$) as substituents in 3*H*-indolenine trimethine cyanine dyes (Cy3) [13], and 3*H*-indolenine heptamethine cyanine dyes (Cy7) [14], that not only improved the photostability, but also increased the yields of intermediates and products. The properties of pentamethine cyanine dyes (Cy5) of 3*H*-indolenines, however, remain unreported. The bio-labeling of the dyes to proteins via covalent conjugations include the activation of *N*-*p*-carboxybenzyl in the dyes and the reaction of the activated dyes to reactive groups (such as $-NH_2$) on proteins in aqueous buffer solutions under mild conditions. Sulfo-groups are important for this kind of application because they prevent protein precipitation during the derivatization procedures [15,16].

In this paper, we describe the synthesis of the water-soluble Cy5 dyes, the relationship between the molecular structure and photostability and labeling performance on BAS. The structures of water-soluble Cy5 dyes were shown in Fig. 1. Compared with well-

* Corresponding author. Tel.: +86 411 39893899; fax: +86 411 39893800.
E-mail address: pengxj@dlut.edu.cn (X. Peng).

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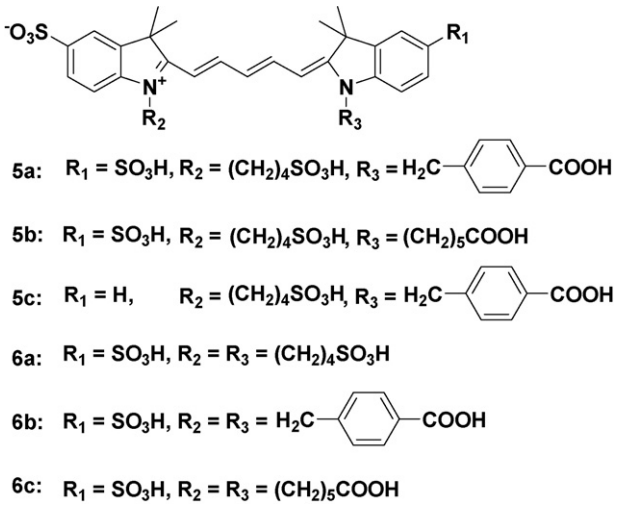

Fig. 1. Structures of water-soluble Cy5 dyes.

known symmetric cyanine dye **6c**, the asymmetric dyes (**5a** and **5b**) contain only one active group on their molecules, and should benefit from quantitative protein labeling and purifying. All results demonstrated that asymmetric dye **5a** possesses better photostability, which can be a good fluorescent labeling reagent for protein labeling.

2. Experiments

2.1. Instruments and materials

Mass spectral determinations were taken on HP1100 API-ES mass spectrometer. NMR spectra were recorded on a Varian 400 MHz NMR spectrometer (USA). Chemical shifts are expressed in parts per million from D_2O (δ_H = 4.79) [17]. Fluorescence measurements were performed on a PTI-C-700 Felix and Time-Master system. Visible spectra were measured on a HP-8453 spectrophotometer. HPLC experiments were performed on the Waters 2695-2996-2475. Purification of dyes was performed by conventional column chromatography with C18-RP absorbent (Sinochrom C18, 40–75 mesh, 10 nm, 280 $m^2\ g^{-1}$, Dalian Elite Company, China). Deionized water was redistilled before use, and acetonitrile was of chromatographic grade. Other chemicals used for the experiments were of analytical grade.

2.2. Synthesis

The synthetic routes of Cy5 dyes were shown in Fig. 2. 2,3,3-Trimethyl-3*H*-indolenine and 2,3,3-trimethylindolenine-5-sulfonate (**2**) were obtained as starting materials by conventional Fisher 3*H*-indole synthesis [18].

Intermediates of 3*H*-indolium quaternary salt **3** were synthesized from the quaternization of **2** with 1,4-butane sultone, 6-bromohexanoic acid or *p*-(chloromethyl) benzoic acid, respectively, and were used in further experiments without additional purification. The yields of intermediates **3a**, **3b**, **3c** and **3d** were 78%, 58%, 39% and 80%, respectively. Symmetric Cy5 dyes, as reference dyes, were synthesized according to the previous procedure [16].

Dye **6a**: Yield 64%. ^{1}H NMR (400 MHz, D_2O): δ 1.48–1.69 (m, 8H, $4CH_2$), 1.78 (s, 12H, $C(CH_3)_2$), 2.79 (m, 4H, $CH_2SO_3^-$), 3.94 (m, 4H, N–CH_2), 6.12 (m, 2H, CH=CH), 6.39 (d, 1H, J = 13.6 Hz, CH=CH), 7.19 (m, 2H, Ar–H), 7.59 (m, 2H, Ar–H), 7.64 (s, 2H, Ar–H), 7.84 (t, 2H, J = 13.2 Hz, CH=CH). API-ES-MS, m/z: 261.2 $[M-3H]^{3-}$.

Dye **6b**: Yield 69%. ^{1}H NMR (400 MHz, D_2O): δ 1.54 (s, 12H, $C(CH_3)_2$), 5.19 (s, 4H, N–CH_2–Ar), 6.02 (d, 2H, J = 13.6 Hz, CH=CH), 6.19 (m, 1H, CH=CH), 7.04–7.76 (m, 14H, Ar–H), 7.82 (t, 2H, J = 13.6 Hz, CH=CH). API-ES-MS, m/z: 390.6 $[M-2H]^{2-}$.

Dye **6c**: Yield 60%. ^{1}H NMR (400 MHz, D_2O): δ 1.28–1.66 (m, 12H, $6CH_2$), 1.74 (s, 12H, $C(CH_3)_2$), 2.79 (m, 4H, CH_2COOH), 3.93 (m, 4H, N–CH_2), 6.02 (m, 2H, CH=CH), 6.44 (d, 1H, J = 13.2 Hz, CH=CH), 7.49 (m, 2H, Ar–H), 7.64 (m, 2H, Ar–H), 7.76 (s, 2H, Ar–H), 7.94 (t, 2H, J = 13.2 Hz, CH=CH). API-ES-MS, m/z: 740.6 $[M-2H]^{2-}$.

2.2.1. Hemicyanine intermediate *4*

The quaternary salt **3a** (375 mg, 1 mmol) and malonaldehyde dianil hydrochloride (310 mg, 1.2 mmol) were dissolved in a mixture of acetic acid (5 mL) and acetic anhydride (5 mL), and then heated to reflux. The reaction was monitored by thin-layer chromatography (TLC). Extended heating produced some symmetrical dye (<5%). After 50 min, the mixture was cooled to room temperature and diluted with diethyl ether. The supernatant fluid was removed by decantation. The brown powder thus obtained was simply separated by flash-column.

2.2.2. Asymmetrical dyes

A solution of crude hemicyanine intermediate **4** (585 mg, 1 mmol) and quaternary salt **3b**, **3c** or **3d** (1 mmol) in acetic anhydride (5 mL) was heated to 120 °C for 40 min. The reaction was monitored by TLC. The mixture was cooled to room temperature and diluted with diethyl ether. After filtration, the crude dye was chromatographied in a C18-RP column using a methanol–water mixture as the eluent.

Dye **5a**: Yield 56%. ^{1}H NMR (400 MHz, D_2O): δ 1.53 (m, 4H, $2CH_2$), 1.74 (s, 12H, $C(CH_3)_2$), 2.74 (m, 2H, $CH_2SO_3^-$), 3.92 (s, 2H, N–CH_2), 5.18 (s, 2H, N–CH_2–Ar), 6.02 (m, 2H, CH=CH), 6.26 (t, 1H, J = 13.2 Hz, CH=CH), 7.02–8.42 (m, 12H, Ar–H), 7.76 (m, 2H, CH=CH). API-ES-MS, m/z: 391.1 $[M-2H]^{2-}$.

Dye **5b**: Yield 30%. ^{1}H NMR (400 MHz, D_2O): δ 1.27–1.67 (m, 10H, $5CH_2$), 1.68 (s, 12H, $C(CH_3)_2$), 2.08 (m, 2H, CH_2COOH), 2.81 (m, 2H, $CH_2SO_3^-$), 3.94 (m, 4H, N–CH_2), 6.14 (m, 2H, CH=CH), 6.46 (t, 1H, J = 12.8 Hz, CH=CH), 7.19 (m, 2H, Ar–H), 7.62 (m, 2H, Ar–H), 7.69 (d, 2H, J = 5.2 Hz, Ar–H), 7.84 (m, 2H, CH=CH). API-ES-MS, m/z: 381.0 $[M-2H]^{2-}$.

Dye **5c**: Yield 64%. ^{1}H NMR (400 MHz, D_2O): δ 1.71 (m, 4H, $2CH_2$), 1.73 (s, 12H, $C(CH_3)_2$), 3.17 (m, 2H, $CH_2SO_3^-$), 4.15 (m, 2H, N–CH_2), 5.36 (s, 2H, N–CH_2–Ar), 6.37 (m, 2H, CH=CH), 6.42 (m, 1H, CH=CH), 7.13–7.78 (m, 11H, Ar–H), 8.34 (t, 2H, J = 12.8 Hz, CH=CH). API-ES-MS, m/z: 703.2 $[M-H]^-$.

2.2.3. Synthesis of the succinimidyl esters *7* of Cy5 dyes

The dye with carboxyl group was dissolved in dry *N*,*N*-dimethylformamide (DMF, 2 mL/100 mg of the dye). *N*,*N*′-dicyclohexyl carbodiimide (DCC, 5 eq./carboxyl group) and *N*-hydroxysuccinimide (NHS, 10 eq./carboxyl group) was added. The mixture was left at room temperature for 10 h. After diluting the mixture with dry ethyl acetate, the supernatant was centrifuged and collected. By TLC, a nearly 100% yield of the active succinimidyl esters of Cy5 dyes (Cy5-NHS) was obtained. Because active esters easily become deactivated via hydrolysis, the products were prepared on the spot and used immediately without further purification.

2.3. Determination of quantum yield

The corresponding fluorescence quantum yields were calculated relative to a standard solution of Rhodamine B in ethanol