Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology A: Chemistry 168 (2004) 53–57
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Syntheses, spectral properties and photostabilities of novel water-soluble near-infrared cyanine dyes

Fengling Song, Xiaojun Peng*, Erhu Lu, Rong Zhang, Xiuying Chen, Bo Song

State Key Laboratory of Fine Chemicals, Dalian University of Technology, 158 Zhongshan Rd., Dalian 116012, PR China

Received 16 January 2004; received in revised form 15 April 2004; accepted 6 May 2004
Available online 2 July 2004

Abstract

To develop long wavelength cyanine dyes with good water solubility and high photostability for biological application, a series of heptamethine 5-sulfo-3*H*-indocyanine dyes were synthesized and characterized. The absorption and fluorescence spectra of the dyes in various solvents are presented, the maximum absorption and emission wavelengths ranging from 680 to 830 nm. The photostabilities are influenced by the different substitutes of the dyes: *N*-benzyl on the nitrogen atom at the 3*H*-indole ring provides better photostability than *N*-ethyl, the substitution of chloro group of chlorocyclohexenyl bridge in heptamethine chain with electron-donor group (such as 4-methoxylanilino) could greatly improve the photostability. In addition, the photofading is more rapid in water than in alcohol and the mechanisms are discussed.

Keywords: Cyanine dyes; Near-infrared dyes; Photostability; Biological assay; Fluorescent dyes

1. Introduction

In the past decades many fluorescence dyes have been developed to label biomolecules. Among them, flouresceins, rhodamines, trimethine cyanines (Cy3), pentamethine cyanines (Cy5) and dipyrrometheneboron difluorides (BODIPY) are available commercially. The absorption and emission wavelengths of these dyes are in UV–visible region where biological matrix exhibits high absorption and auto-fluorescence background. It is a fatal disadvantage in trace bioanalysis. Near-infrared (NIR) fluorescent assays, which have higher sensitivity because of minimal auto-fluorescence and reduced light scattering (which is inverse fourth power dependence on the wavelength) in this spectrum, have been attractive methods in bioanalysis recently [1].

A major bottleneck in the complete utilization of NIR fluorescence for many applications is the limited number of fluorophores with high fluorescence efficiency and good stability [2]. Up to now, few NIR fluorescent dyes for biological application have been available commercially. Heptamethine cyanine dyes (Cy7) are a class of NIR fluorophores that have been used for protein labeling [3], DNA sequencing, hydrophobicity determination, proton and metal ion detection [4]. But the photostability of Cy7 dyes is much poorer than that of Cy3 and Cy5 dyes. Another disadvantage is its poor water solubility which is crucial for the fluorophore to avoid dye aggregation and nonspecific binding to irrelevant components when it is applied to biological analysis as probe in aqueous environment [5].

Recently, we found that Cy3 dyes containing *N*-carboxybenzyl group have better photostabilities [6]. In this paper, we report the syntheses, spectral properties and photostabilities of several novel water-soluble Cy7 dyes with *N*-benzyl group and substituents at cyclohexenyl bridge in heptamethine chain.

2. Experimental

Mass spectral determinations were made on HP1100 API-ES mass spectrometry. NMR spectra were recorded on a Varian 400 MHz NMR spectrometer. Fluorescence measurements were performed on a PTI-C-700 Felix and Time-Master system. Visible spectra were measured on a HP-8453 spectrophotometer. The purification of the dyes was performed by recrystallization or conventional column chromatography on C18-RP absorbent (Sinochrom C18, 40–75 μm, 100 Å, 280 m^2/g, Dalian Elite Company, China). Water–methanol mixtures were used for elution. Deionized

* Corresponding author. Tel.: +86 411 8899 3899; fax: +86 411 8899 3885.
E-mail address: pengxj@dlut.edu.cn (X. Peng).

1010-6030/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2004.05.012

water was redistilled before use. Other chemicals were of analytical grade.

2.1. Synthesis

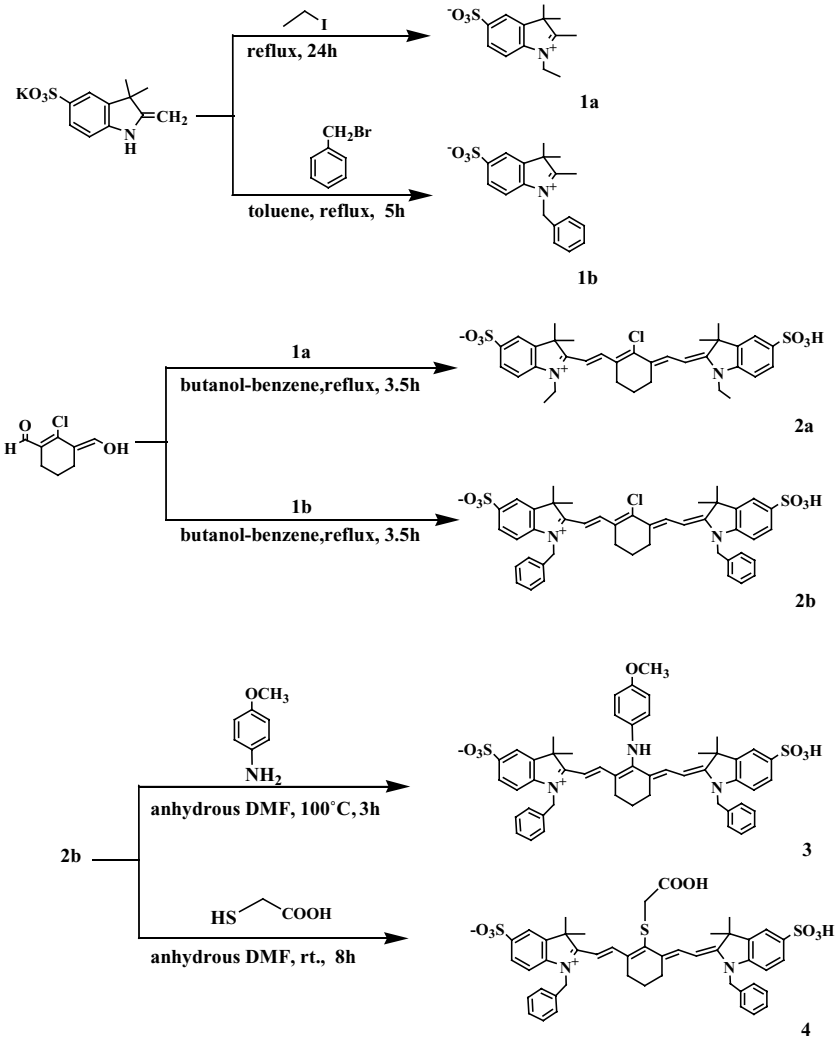
The synthesis routes are shown in Scheme 1. 2-Chloro-1-formyl-3-hydroxymethylene cyclohexene [7], 2,3,3-trimethylindolenium-5-sulfonate and *N*-ethyl-2,3,3-trimethylindolenium-5-sulfonate (**1a**) [8] were prepared according to the literature procedure.

2.1.1. *N*-Benzyl-2,3,3-trimethylindolenium-5-sulfonate (*1b*)

A mixture of 6.24 g (2.25 mmol) of potassium salt of 2,3,3-trimethylindolenium sulfonate and 4.03 g (2.35 mmol) of benzyl bromide was suspended in 20 ml toluene. The mixture was stirred and refluxed for 5 h, and then cooled to room temperature. The obtained red solid was precipitated, filtered, washed with cold acetone and dried under vacuum. The product (3.97 g, yield 96%) is pure enough for next step.

2.1.2. Dyes *2a* and *2b*

The 8.0 mmol of **1a** or **1b** and 692 mg (4.0 mmol) of 2-chloro-1-formyl-3-hydroxymethylene cyclohexene were dissolved in 300 ml of a mixture of *n*-butanol and benzene (7:3 v/v) in a 500 ml round bottom flask equipped with a Dean-Stark trap. The mixture was heated to reflux with stirring and the water formed was collected in the trap. After 3.5 h, the mixture was cooled to room temperature and the solvents were evaporated under vacuum. The residue was dissolved in a small amount of methanol, and then the solution was added to 350 ml of ether with violent stirring. The green precipitate was collected on a sintered glass filter, washed with ether(3 × 50 ml) and dried under vacuum. The crude dye was recrystallized twice in water. Compound **2a**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 1.31 (t, 6H, CH_3), 1.67 (s, 12H, CH_3), 1.83 (m, 2H, CH_2), 2.70 (t, 4H, CH_2), 4.25 (q, 4H, CH_2), 6.31–6.35 (d, 2H, J = 13.6 Hz, CH), 7.40 (d, 2H, CH), 7.70 (d, 2H, CH), 7.81 (d, 2H, CH) 8.24–8.27 (d, 2H, J = 13.6 Hz, CH). MS, m/z: 669.2 (M^-). Compound **2b**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 1.58 (m, 2H, CH_2),



Scheme 1. Synthesis of the near-infrared dyes.

1.72 (s, 12H, CH_3), 2.09 (t, 4H, CH_2), 5.52 (s, 4H, CH_2), 6.36–6.40 (d, 2H $J = 13.6$ Hz, "CH), 7.26–7.40 (m, 12H, CH), 7.62–7.64 (d, 2H, CH), 7.85(s, 2H, CH), 8.22–8.25 (d, 2H, $J = 13.6$ Hz, CH). MS, *m/z*: 794.2 (M^-).

2.1.3. Dye **3**

The 79.5 mg (0.1 mmol) of dye **2b** and 85 mg (0.7 mmol) of 4-methoxyaniline were dissolved in 15 ml anhydrous DMF in a 50 ml round bottom flask. The mixture was stirred at 110 °C under argon for 3 h. Then it was cooled and added to 300 ml of ether with violent stirring. The obtained blue solid was filtered and dried under vacuum, then purified on C18-RP column using methanol–water mixture as eluent. ^{1}H NMR (400 MHz, DMSO-d_6), δ: 1.36 (s, 12H, CH_3), 1.68 (t, 2H, CH_2), 2.33–2.34 (t, 4H, CH_2), 3.69 (s, 3H, CH_3), 5.27 (s, 4H, CH_2), 5.86–5.89 (d, 2H $J = 13.6$ Hz, CH), 6.90–6.92 (d, 2H, CH), 7.05 (d, 2H, CH), 7.12 (d, 4H, CH), 7.19–7.30 (m, 8H, CH), 7.51–7.53 (d, 2H, CH), 7.60 (s, 2H, CH), 7.83–7.86 (d, 2H, $J = 13.6$ Hz, CH). MS, *m/z*: 880.3 (M^-).

2.1.4. Dye **4**

The 690 mg (7.5 mmol) of mercaptoacetic acid was added to 25 ml anhydrous DMF and 120 mg (1.5 mmol) of chloro dye **2b**, and then the vessel was purged with dry argon. The mixture was stirred for 8 h at room temperature in the dark. Then it was added to 350 ml of ether with violent stirring. The obtained solid was filtered and purified on C18-RP column using methanol–water mixture as eluent. ^{1}H NMR (400 MHz, DMSO-d_6), δ: 1.63 (m, 2H, CH_2), 1.71 (s, 12H, CH_3), 2.40 (t, 4H, CH_2), 5.48 (s, 4H, CH_2), 6.31–6.34 (d, $J = 13.6$ Hz, 2H, CH), 7.26–7.27 (m, 8H, CH), 7.33–7.35 (d, 4H, CH), 7.61 (d, 2H, CH), 7.80 (s, 2H, CH), 8.79–8.82 (d, 2H, $J = 13.2$ Hz, CH). MS, *m/z*: 850.3 (M^-). ^{13}C NMR (400 MHz, DMSO-d_6), δ: 20.5, 25.6, 27.6, 47.0, 49.0, 102.3, 110.5, 120.0, 126.3, 126.6, 127.8, 129.0, 133.5, 135.0, 140.3, 142.7, 144.8, 145.6, 157.3, 172.4.

2.2. Absorption and fluorescence measurements

The NIR dyes synthesized were dissolved in methanol to prepare stock solutions (1×10^{-4} M), and kept at 4 °C. Dyes **2a**, **2b** and **4** were diluted to 1×10^{-6} M in various solvents, while dye **3** was diluted to 1×10^{-5} M. All samples were allowed to stand for more than 5 min at room temperature before detection. The fluorescence quantum yields were determined in reference to the NIR laser dye IR-125 in DMSO ($\Phi_f = 0.13$) [9].

2.3. Photofading of dyes

Dyes were dissolved in different solvents with concentration of 1×10^{-5} M. The samples were irradiated with a 500 W iodine–tungsten lamp at distance of 25 cm away. Sodium nitrite aqueous solution (50 g/L), as light filter (to cut off the light shorter than 400 nm) and heat filter, was placed between the samples and the lamp. The photostabilities were in the terms of remaining absorption (%) calculated from the change of absorption intensities at the absorption maximum before and after irradiation.

3. Results and discussion

3.1. Synthesis and design of dyes

The structure design of heptamethine indocyanine dyes has been deeply investigated by Patonay and coworkers [4,10] and Waggoner and coworkers [3,8]. It has been widely accepted that a rigid cyclohexenyl ring in the methine chain can increase the photostability and enhance the fluorescence quantum yield. It also provides the dye with a reactive site for chemical substitution at the central ring. In addition, it is suggested that the attachment of sulfonate groups to 3*H*-indo rings can remarkably improve water solubility and increase the photostability [11] of the dyes, although it makes their purification difficult. Moreover, such arylsufonate dyes have a minimal tendency to form aggregates in aqueous solution.

In this paper, we report the syntheses of four heptamethine indocyanine dyes with such cyclohexenyl ring and sulfonate groups (see Scheme 1). Dyes **2a** and **2b** were synthesized from quaternary salt **1a** and **1b** [10]. Two derivative dyes **3** and **4** were prepared from dye **2b** by nucleophilic substitution reactions according to a modified procedure from literatures [4,7,12]. A carboxyl as a reactive functional group was introduced into dye **4**, which permits covalent attachment of the dye to any biological molecule via the ester of *N*-hydroxy-succinimide.

3.2. Absorption and emission of dyes

The maximum absorption and emission wavelengths (λ_{max}) of dyes in different solvents are in the range from 680 to 830 nm. Like most other cyanine dyes, four dyes all exhibit negative solvatochromism (see Table 1).

The absorption and emission spectra of dyes **2a** and **2b** in methanol are shown in Fig. 1. It can be found that dye **2b** are similar to dye **2a** in λ_{max}, molar extinction coefficient (ε) and fluorescence quantum yield (Φ_f). The change of the substituent on the nitrogen atoms just makes the λ_{max} of dye **2b** a slight bathochromic shift (about 6 nm) compared with those of dye **2a**.

Dye **3**, derived from dye **2b** by the substitution of the central chloro group with 4-methoxyphenylamino, has weaker absorbance and emission intensity, as well as a larger Stokes shift (≈127 nm in methanol). But dye **4** with a carboxymethyl mercapto group maintains the similar spectral properties of dye **2b**. The spectra of dyes **3** and **4** in methanol were presented in Fig. 2.

Table 1
Photophysical characteristics of dyes in different solvents

Dye	Solvent	Absorption, λ_{max} (nm)	Emission, λ_{max} (nm)	ε[a] ($\times 10^5$)	Φ_f[b]
2a	Water	778	800	1.6	
	Methanol	785	806	2.6	
	Ethanol	790	812	2.0	
	DMF	801	824	1.7	
	DMSO	805	820	1.1	0.082
2b	Water	783	803	2.0	
	Methanol	791	813	2.7	
	Ethanol	796	817	2.5	
	DMF	807	824	2.3	
	DMSO	811	827	1.1	0.065
3	Water	686	783	0.30	
	Methanol	689	788	0.47	
	Ethanol	694	793	0.43	
	DMF	709	802	0.23	
	DMSO	712	807	0.41	0.025
4	Water	786	808	2.0	
	Methanol	789	813	1.8	
	Ethanol	795	816	1.6	
	DMF	804	823	1.8	
	DMSO	815	827	1.9	0.096

[a] Molar extinction coefficients are in cm^{-1} M and in the maximum of the highest peak.
[b] The fluorescence quantum yields were determined in reference to IR-125 in DMSO ($\Phi_f = 0.13$).

The difference between the spectra of dyes **3** and **4** might be from the electron-donor property of 4-methoxylanilino group which perturbs the fluorophore's π-electron system.

3.3. Study of photostability

It is necessary for fluorescence dyes to have a considerable photostability when used in bioassays. Compared with Cy3 and Cy5 dyes, Cy7 dyes have a longer methine chain which is easier to be attacked by dioxygen. To resolve this problem, Patonay groups developed a robust polymethine chain with chlorocyclohexenyl bridge such as the case of dye **2a**. In our previous paper [6], we have reported that *N*-(*p*-carboxylbenzyl) group at 3*H*-indo rings (such as the case of dye **2b**) can stabilized the flourophore to some extent. Here we further investigated this problem and found that the photostabilities of the dyes is surprisingly changed as the substitution of central chloro group of dye **2b** is different or the solvents used varied.

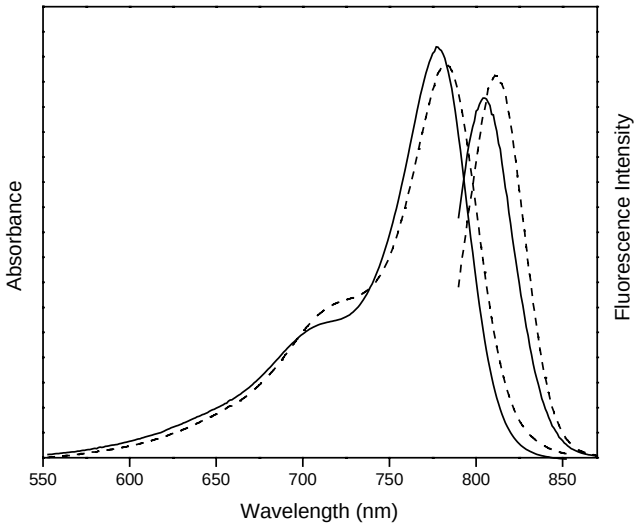


Fig. 1. The absorption and emission spectra of dyes **2a** (solid line) and **2b** (dashed line) in methanol.

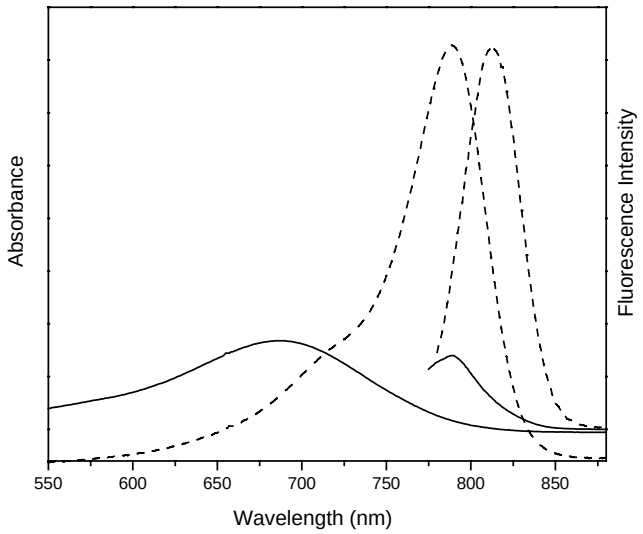


Fig. 2. The absorption and emission spectra of dyes **3** (solid line) and **4** (dashed line) in methanol.

Fig. 3 shows photofading behavior of dyes **2a**, **2b**, **3** and **4** in methanol. It can be seen that the photostabilities of the dyes can be placed in an order: **3** > **2b** > **2a** > **4**. Although difference between dye **2a** and dye **2b** is not very large, dye **2b** with benzyl groups on the nitrogen atoms does exhibit better photostability than dye **2a** with ethyl groups. This

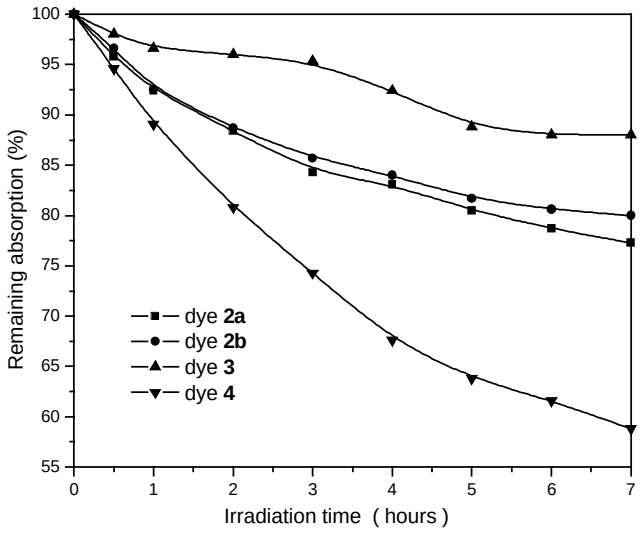


Fig. 3. Photofading behavior of dyes **2a**, **2b**, **3** and **4** in methanol.

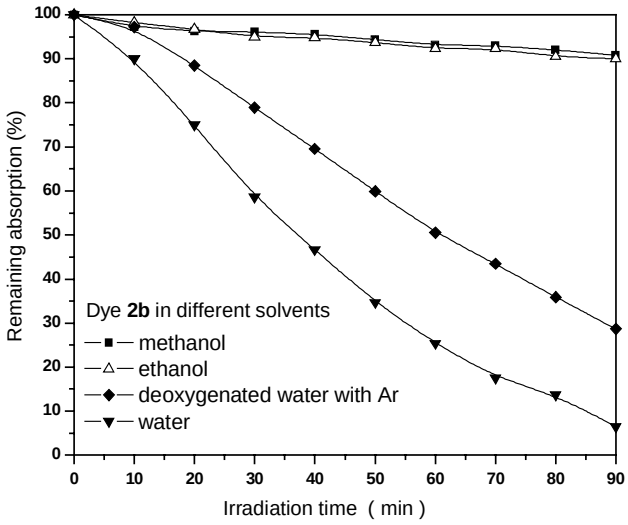


Fig. 4. Photofading behavior of dye **2b** in methanol, ethanol, water and deoxygenated water with Ar.

might result from the steric hindrance and rigidity of the benzyl groups that retard the photooxidation on polymethine chain [6].

An inconsistent effect is illustrated in Fig. 3 where dye **3** and dye **4**, both from dye **2b** via substitution on central chlorine atom of the polymethine chain, behave different photostabilities. Dye **3** has much better photostability than dye **2b**, while dye **4** has the poorest photostability. The inconsistent effect might be from electron-donor property of the substituent groups. Methoxylanilino group in dye **3** as an electron-rich group might let the excited dye molecule be quenched quickly by intra-molecular electron transfer and go back to ground state, and thus protect the dye molecule from photobleaching. But carboxymethylmercapto group in dye **4** has no such effect. It is consistent with the fluorescence quantum yields of dye **3** (0.025) and dye **4** (0.096) (see Table 1).

The effect of solvent is shown in Fig. 4. Under the same irradiation condition the absorption intensity of dye **2b** remained about 90% in methanol or ethanol after irradiation for 90 min, while less than 10% in water. After deoxygenation with argon bubbling for 30 min, the stability of dye **2b** was improved remarkably. So the presence of oxygen is a fatal factor for the photofading of the dyes. Additionally, it was found that pH had little effect on photostability when pH varied from 2.5 to 9.9 in aqueous buffers.

As is well known, there are two possible mechanisms of the interaction of an excited dye molecule and dioxygen, which lead to the formation of singlet oxygen ($^1O_2$) and superoxide anion (O_2^-), respectively [13]. It has been proved that both singlet oxygen ($^1O_2$) and superoxide (O_2^-) contribute to the photofading of polymethine cyanine dyes [14]. In water, superoxide anion (O_2^-) is unstable and can produce H_2O_2 and O_2 [13] which might accelerate the photooxidation of dyes:

$$O_2^- + H^+ \rightarrow HO_2^\bullet$$

$$H_2O + HO_2^\bullet + O_2^- \rightarrow H_2O_2 + O_2 + OH^-$$

$$H_2O_2 + O_2^- \rightarrow OH^\bullet + OH^- + O_2$$

4. Conclusion

Four water-soluble near-infrared heptamethine 3*H*-indocyanine dyes have been synthesized. The relationship between the photostability and molecular structure has been investigated. The results show that there are two other ways to improve their photostabilities besides the change of traditional linear heptamethine chain into cyclohexenyl ring: firstly, sterically hindered *N*-benzyl groups displace *N*-alkyl groups on the nitrogen atoms; secondly, the central chlorine atom at cyclohexenylene-chain is substituted by electron-donor group such as methoxyphenylamino.

Acknowledgements

This work was supported by “973 program” of the Ministry of Science and Technology of China and National Natural Science Foundation of China (project 20128005 and 20376010).

References

[1] A. Zaheer, R.E. Lenkinskl, A. Mahmood, A.G. Jones, L.C. Cantley, J.V. Frangioni, Nat. Biotechnol. 19 (2001) 1148.
[2] S.A. Soper, Q.L. Mattingly, J. Am. Chem. Soc. 116 (1994) 3744.
[3] Y.H. Lin, R. Weissleder, C.H. Tung, Bioconjugate Chem. 13 (2002) 605.
[4] L. Tarazi, H. Choi, J.C. Mason, J. Sowell, L. Strekowski, G. Patonay, Microchem. J. 72 (2002) 55.
[5] R.B. Mujumdar, L.A. Ernst, S.R. Mujumdar, C.J. Lewis, A.S. Waggoner, Bioconjugate Chem. 7 (1996) 356.
[6] L.Q. Wang, X.J. Peng, Dyes and Pigments 54 (2002) 107.
[7] D.L. Gallaher, Jr. Johnson, M.E. Johnson, Analyst 124 (1999) 1541.
[8] S.R. Mujumdar, R.B. Mujumdar, C.M. Grant, A.S. Waggoner, Bioconjugate Chem. 4 (1993) 105.
[9] R.C. Benson, H. Kues, J. Chem. Eng. Data 22 (1997) 379.
[10] N. Narayanan, G. Patonay, J. Org. Chem. 60 (1995) 2391.
[11] F. Farooqul, M.A. Michael, M.P. Reddy, US Patent 6 335 450 (2002).
[12] C.M. Yang, O. Shimelis, X.J. Zhou, G.D. Li, C. Bayle, M. Nertz, H. Lee, L. Strekowski, G. Patonay, F. Couderc, R.W. Giese, J. Chromatogr. A 979 (2002) 307.
[13] C.P. Chen, B.M. Zhou, D.H. Lu, G.Z. Xu, J. Photochem. Photobiol. A: Chem. 89 (1995) 25.
[14] J. Li, P. Chen, J. Zhao, D.S. Zheng, T. Okazaki, M. Hayami, Photogr. Sci. Photochem. 15 (1997) 343.