

Research on Syntheses and Properties of Water-soluble Symmetrical Squarylium Double-cyanine Dyes

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Abstract: In this paper, 2,3,3-trimethyl-3H-indolin was synthesized by phenylhydrazine and 3-methyl-2-butanone as raw materials in the acetic acid reacted with different nitrogenalkylating reagents. Semi mono-cyanine dye and semi di-cyanine dye were prepared with the help of squaric acid diethyl ester. The spectral properties of the target products were checked. The results show that compared with symmetrical squarylium mono-indocyanine dyes with the same substituent, the photo-stability of the bi-cyanine dyes were much better. When dissolved in water, the dye showed shorter maximum absorption wavelength, shorter maximum emission wavelength and shorter Stokes shift and apparent aggregation. In protic solvents, dye spectra showed a blue shift effect with an increase of solution polarity, while in aprotic solvents, dye spectrum showed a red shift with solution polarity increased. Among different solvents, the photostability of the dye in methanol is strongest.

Keywords: Cyanine dye, fluorescent labeling

1. INTRODUCTION

Cyanine dyes refer to a system featured with push-pull electronic connection, conjugating methyl dyne chain with N, O, S and other heteroatoms at both ends of the chromosphere, which are connected by conjugating methyl dyne chain links between each. This special kind of structure makes the dyes be able to emit fluorescence [1]. Cyanine dye as a kind of fluorescent dyes, has been playing an important role in many areas since it was discovered by Williams in 1856 [2]. It is a kind of special fluorescent dye, which is different from the traditional printing dyes, as it is sensitive to illumination and not stable under light. Also, it cannot be strongly attached to the fabric, as a consequence, not suitable for applying it in fabric dyeing like traditional dyes. But after many years, it is found that the cyanine dye itself has special spectral properties that traditional dyes do not have, such as superior molar extinction coefficient, broad absorption wavelength range, etc. So it has been developed rapidly in various aspects in recent years.

2. CHARACTERISTICS OF CYANINE DYES

2.1. Structure and Classification of Cyanine Dyes

Cyanine dyes can be divided into the following categories according to impurity atoms in the chromosphere, one is cationic cyanine dye of nitrogen, impurity atoms are tin cyanine dye of oxygen atom, one heteroatom is oxygen atom and the other is neutral cyanine another is the conjugating zwitterion methyl dyne chain introduced with other groups.

Regarding biomarkers, the disadvantages of the traditional isotope labeling method gradually emerge. Radioactive nuclide is usually used as tracer atom in isotope tracking, which will inevitably do harm to the marked creature. However cyanine dyes as a new kind of fluorescent dyes molecular probes occupy important position among the modern analysis methods. Fluorescence analysis is gradually replacing the traditional isotope labeling method in the field of biological detection.

The acid dyes have many advantages, such as perfect light stability and thermal stability, and they doesn't decompose at 400°C. If indole ring structure is replaced with pyridine ring of indole acid dyes, the maximum absorption wavelength will go redshift, at the range of 750-1100 nm [3]. Without water soluble groups in acid dyes structure, various aggregation states occur in water, which affect its spectral performance. Indole acid dyes are also fluorescent dyes, whose structure is formed by nucleophilic replacement reaction between indole ring and square acid.

2.2. Performance of Cyanine Dyes

The general performance of cyanine dyes is very satisfactory, with good molar extinction coefficient, and long absorption wavelength [4]. In recent years, among many series of fluorescent dyes developed by molecular probe companies, cyanine dyes play the main role. Cyanine dyes dose has a very important research prospect and development potential among the fluorescent dyes used for molecular probes. However, in terms of spectral properties and the stability, cyanine dyes have the space to be improved, as well as the gathering situation of cyanine dyes in solvent.

2.2.1. Spectral Performance

The structure of the dyes contains conjugated methyl dyne chain, forming a large conjugating system. The energy

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of electrons in the cyanine dye molecules increases after absorbing light energy, lead to the transition of electrons from the ground state to the excited state. When electrons fell back from the excited state to ground state, the energy is released in the form of light, and the output of light is called fluorescence. The absorption wavelength and emission wavelength of cyanine dye are among 550 ~ 1200 nm, this optical property avoids background interference fluorescence arising from the glass instrument itself. The study found that with more cyanine dyes molecular conjugating methyl dyne chain, the maximum absorption wavelength of cyanine dye molecules went redshift, generally moved about 100 nm. Moreover, the introduction of different substituent will have influence on hair base group of absorption wavelength and emission wavelength of the dyes. With the change of the solvent polarity, the ultraviolet absorption spectrum location of the dyes will reveal if it is the positive phase solvation effect or negative solvation effect.

The spectral properties of the dyes were tested in five different solvents, including UV absorption spectroscopy, fluorescence emission spectroscopy and optical stability. The results of tests show that the dye exhibited maximum absorption in the range of 602 ~ 652 nm, and maximum emission between 605 and 674 nm. Their molar absorption coefficients are about 105 mol⁻¹ cm⁻¹. Land Stokes shifts are in the range of the 3~22 nm. Compared with other organic solvents, when it was dissolved in water, the dye showed the shortest maximum absorption wavelength, as well as shortest maximum emission wavelength and shortest Stokes shift, and obvious aggregation.

2.3. Stability

When making fluorescent tags, higher stability is required for the dyes, poor stability on light, heat, oxygen are main problems. It leads to a limitation of broadening applications of cyanine dyes, so finding out the feasible ways to improve the stability of cyanine dyes is important. Both the concentration of heterocyclic mother nucleus and oxygen in chromosphere will affect the stability of the dye. Research suggested that the main factors lead to light oxidation and dye fade are REDOX active substances such as singlet oxygen, oxygen compounds and peroxide.

Compared with the corresponding mono-cyanine dyes, the double-cyanine dyes have larger Stokes shifts, a lower fluorescence quantum yield and better stability. Other advantages are the variation of the spectrum of the dye in different solvents and the more consistent stability.

2.4. The Application of Cyanine Dyes

Since the middle period of last century, the outstanding performance of cyanine dyes has drawn the attention of scientists all over the world. Industry will be more and more demanding for cyanine dyes with different structures, for different applications such as ion recognition, solar cells, biological analysis, biological fluorescence labeling technique and medical field, etc. [5].

2.4.1. Solar Cells

Solar energy, as a green energy source, is becoming more and more attractive. Dye sensitized battery is one type of

solar cells, which are composed with the dye sensitizer, REDOX electrolyte, Pt electrode *etc.* Dye sensitizer is the core part of the dye sensitized cells [6].

2.4.2. Application in Photodynamic Therapy

Photodynamic therapy is a new method of the treatment for cancer. Dyes are used in light power treatment.

Photodynamic therapy has many advantages over surgery, chemotherapy and radiotherapy treatment for cancer. Firstly, as the process is a special light sensitive materials accumulated in the targeted tumor, it will not damage the normal tissue cells. Secondly, only local anesthetic is needed. Last but not least, the side effect is small, which does not cause additional damage to the body. Because of its high selectivity and specificity, photodynamic therapy damage tumor tissue without harming the body's normal cells [7-9].

3. EXPERIMENT PART

3.1. Experiment Reagent and Instrument

Laboratory reagents mainly include: phenyl hydrazine, 3-methyl-2-butanone, sodium carbonate, sodium nitrite, dilute hydrochloric acid and n-butyl alcohol, toluene, pyridine, isopropyl alcohol, potassium hydroxide, anhydrous diethyl ether, glacial acetic acid, methanol, ethanol, acid of chlorine methyl benzoic acid, iodine ethane, benzyl bromide, ethyl acetate and dichloromethane. The purity levels are all A.R., manufacturers and other reagents are left out.

Experimental instruments include: electric drum wind drying oven, vacuum oven, glass instrument air drying machine, ultrasonic cleaning machine, low and constant temperature reaction bath, electronic balance, magnetic heating stirrer, circulating vacuum water pump, rotary evaporator, double beam UV-visible light spectrophotometer, nuclear magnetic resonance instrument, iodine tungsten lamp, fluorescence spectrometer, *etc.* Manufacturers and other instruments are left out [10].

3.2. The Synthesis of Intermediates

3.2.1. Synthesis of 2, 3, 3-Trimethyl-3H-Indole

Adding 2.366 g(21mmol) phenyl hydrazine into a 100 mL bottle containing 20 mL acetic acid glacial, and then adding 1.799 g (21 mmol) 3-methyl-2-butanone under the constant stirring, heating until the reaction system reached the temperature of 90 °C, kept it for 9h under nitrogen protection, and then cooled it to room temperature. After that adding fresh potash saturated solution with constant stirring, PH value was adjusted to 7, to separate the organic phase, anhydrous magnesium sulfate drying was conducted overnight, then vacuum distillation was operated to collect fraction of 120~ 140 °C (0.08~0.09 MPa) boiling range, 2.489g golden oily liquid was obtained, the yield is 74.5% [11, 12].

3.2.2. Synthesis of Carboxyl Benzyl Indole Quaternary Ammonium Salt (2a)

2,3,3-trimethyl-3h-indole and 25 mL o-dichlorobenzene were added in a 100 mL bottle stand-up 4.003 g (25 mmol), then 4.538 g (25 mmol) chlorine methyl benzoic acid was

added with constant stirring, heated to 110 °C under the condition of nitrogen protection, heating was stopped after 12h reaction, after cooling to room temperature, 2.003g light pink powder is obtained by the help of suction filter (filter cake leached with acetone), the yield is 48.7%.

3.2.3. *N*-ethyl Indole Quaternary Ammonium Salt (2c)

6.837 g (43 mmol) 2, 3, 3-trimethyl-3H-indole was put into a 100 mL bottle, soon after added 20 mL acetonitrile, then adding 6.766 g (43 mmol) iodine ethane slowly, heated to reflux under the condition of nitrogen protection, making it last for 24 hours, then cooled it to room temperature, filter cake is dealt with acetone elution, 7.378 g light pink powder was obtained, the yield is 54.5%.

3.2.4. Synthesis of *N*-Benzyl Chloride Indole Quaternary Ammonium Salt (2f)

Adding 3.214 g (20mmol) 2,3,3- trimethyl-3H-indole, 6.400 g (40mmol) benzyl chloride and 30 ml chloride anhydrous ethanol into a 50 ml bottle by turns, heating to reflux under the condition of nitrogen protection. Reaction system was cooled to room temperature after heating for 24 hours. Decompression was conducted to evaporate the solvent, and then setting it in icy water bath, precipitating. After washing filter cake with diethyl ether made it go through suction filter, 0.425 g pink solid was obtained after drying, the yield is 24.5%.

3.2.5. Synthesis of Indole Double Quaternary Ammonium Salt (Bis-3)

Dissolving 2.411 g (14mmol) 2,3,3- trimethyl-3H-indole in 25 ml toluene, then adding them into 50 ml bottle stand, exciting, dropping 14.725 (68mmol) 1, 4-dibromobutane with constant pressure funnel under constant mixture, heating to reflux. The appearance looked brown red. After 30h, cooled it down to room temperature. Ether was added to precipitate solid content. Purple solid was observed after filtration, ethyl acetate and acetone were used for recrystallization. 12.830g light purple powder was finally obtained, with a yield of 54.6%.

3.2.6. Synthesis of Acid Ethyl Ester

Adding 4.032 g (35mmol) acid and 40 ml anhydrous ethanol in 100 ml bottle, keeping it reacted under circumfluence temperature for 3h and then put the liquid into distillation unit, 20 ~ 25 ml solvents were obtained. Adding 20 ml of ethanol into it and conducted reflux for 1 hour, then steaming out 20 ml solvent; repeat the process again. Pink white solid adhesive was finally obtained, which can be used directly during the next step.

3.3. Separation and Purification of Target Dye

In this paper, we used the self-filled C-18 reversed phase column for separating and purifying the complex water-soluble acid double cyanine dyes.

The separation operations are:

- (1) Dissolving double cyanine dyes for separating it into a certain proportion of mobile phase, light absent is necessary.

- (2) Carrying on gradient elution of already packed chromatographic column: the ratios of water and methanol are respectively 8:1, 6:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:6,1:8. Until the gradient of dye, mobile phase was dissolved.
- (3) Use dissolved dye to filter absorbent cotton, remove the water insoluble substance in the dye, and then added it into the processed chromatographic column for separation and purification.

During the separation, at first a small amount of mobile phase should be added for elution, watching it until the flow of new phase is out of color, and then adding a lot of mobile phase. After every phase separation, flushing it with pure methanol of relatively small polarity. Then silica gel has to be stirred with a glass rod for restoring the chromatographic column, it can be reused after soaking for at least 8h with acceptable column efficiency.

3.4. Light Stability Test of Target Dyes

Putting the prepared dye solution in four cuvettes respectively, sealed. And then put a 500W halogen lamp in the front of the cuvettes at a distance of 38cm; a glass display tank with 50.0 g/L sodium nitrite aqueous solution should be set between cuvettes and light source as a filter, also it helped to keep the stability of the dye as the cold trap can reduce the influence of high heat when the light is on. After setting up the devices, observe the dye absorbance value every 30 minutes, photo bleaching curves were written down according to the records of absorbance values. The light stability of synthetic dyes was also observed and recorded.

4. RESULTS AND DISCUSSION

4.1. Synthesis of Intermediates and Indole Acid Cyanine Dyes

The synthesis of indole acid cyanine dyes is the reaction between indole acid half-cyanine dyes and the corresponding indole quaternary ammonium salt. This experiment uses pyridine as alkaline catalyst, enhancing the nuclear capability of indole quaternary ammonium salt, which makes the cyanine dyes synthesis even more easy.

This paper adopts two methods of synthesis of three different substituents of indole acid pairs of cyanine dyes:

- (1) Taking n-butyl alcohol and toluene as solvent, pyridine as catalyst, make indole acid double half cyanine dye reaction (bis-4) with the corresponding indole quaternary ammonium salt to obtain indole acid double cyanine dyes;
- (2) First, indole acid cyanine dyes 4a, 4c, 4f were obtained through the condensation of different substituent of indole quaternary ammonium salt 2a, 2c, 2f and acid ethyl ester, respectively, then reacting them with indole double-quaternary ammonium salts (bis-3) to obtain targeted products.

During the experiments, taking *N*-benzyl chloride synthesis of cyanine dyes (5f) as an example, the effect of reaction time on the reaction was investigated, as shown in the Table 1.

Table 1. Results of bi-cyanine dye 5f.

Reaction Time	12	24	36	40	48
Yield	11.9	13.6	15.3	14.6	13.5

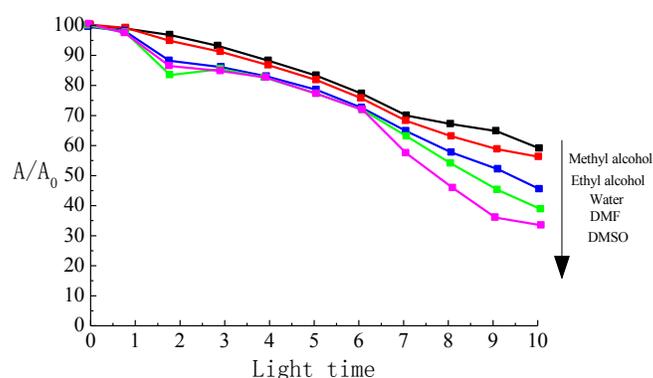
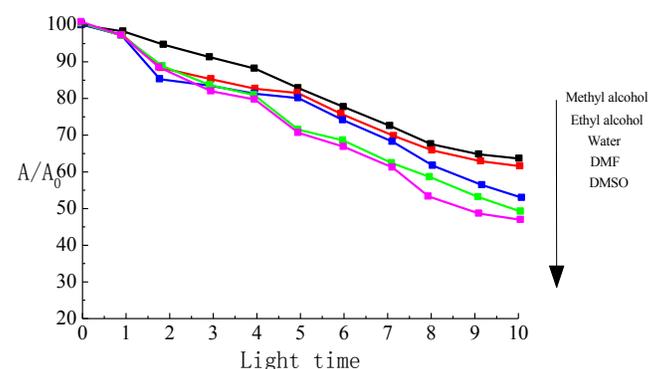
Table 1 shows that in the process of synthesis of cyanine dyes, with a relatively short time, the reaction was incomplete, product yield was low. In the meanwhile, the yield of dyes did not rise if the time of reaction is relatively long, this may be because the reaction is over, side effects, or/and chromatography of ribbon increased the difficulty of the separation and purification column chromatography.

4.2. Purification of the Indole Acid Double Dyes

In this paper, a suitable solvent could not be found for the three synthetic double-cyanine dyes; ideal pure product can't be obtained by recrystallization. And indole ring of dye molecules don't adapt to substituent polarity, which made the polarity of the dye small, so normal phase silica gel column is used to carry out the separation and purification of the products. As for N-p-carboxyl benzyl indole acid double cyanine dye (5a), using a mixture of methanol and acetone solution in the normal phase column (V methanol: V acetone = 1:5 ~ 1:2), and gradient elution for separation and purification, more than one blue ribbon appeared in the column during the separation process. Through the structure identification, it can be identified that which blue ribbon is the product. The eluent used for purifying N-ethyl indole party acid double cyanine dye (5c) is the methanol and ethyl acetate (ethyl acetate methanol: V = 1:12~1:7). Elution was conducted at the same gradient, when the eluent proportion reaches V methanol: V ethyl acetate = 1:10, picking up the first blue belt, at the time of the methanol eluent proportion reaches V methanol: V ethyl acetate = 1:7, accessing all four blue bands. According to the structure identification, the second blue ribbon ought to be the targeted product. N-p-benzyl chloride indole acid double cyanine dye (5f) eluent is the mixture solution of ethyl acetate and petroleum ether (V methanol: V ethyl acetate = 2:1).

4.3. Research on the Light Stability of the Indole Acid Double-Dyes

Test results of light stability of three synthetic pairs of cyanine dyes in different solvents are shown in Figs. (1-3). It can be seen from the figures that the dye with different solvents are shined by iodine tungsten lamp irradiation after 10h at the distance of 38 cm. N-p-carboxyl benzyl indole acid cyanine dyes (5a) faded by 30% in methanol, 32% in ethanol, 39% in water, 42% in DMF, 44% in DMSO; N-ethyl indole acid double cyanine dye (5c) faded 54% in methanol, 55% in ethanol, 69% in water fade, 72% in DMF, 78% in DMSO; N-p-benzyl chloride double cyanine dye (5f) faded 36% in methanol, 36% in ethanol, 38% in water, 44% in DMF, 47% in DMSO.

**Fig. (1).** Photo-bleaching of double-cyanine dye 5a in different solutions.**Fig. (2).** Photo-bleaching of double-cyanine dye 5c in different solutions.

SUMMARY

From Figs. (1-3), it can be seen that the light stability of three kinds of dyes in different solvents are consistent. The light stability of the same kind of dye in different solvents is methanol > ethanol > water > DMF > DMSO.

Fig. (4) shows light stability curves of double cyanine dyes of different substituents in methanol. It can be seen from Fig. (4) that in the same solution, the light stability sequence of three kinds of different substituent double-dyes is N-p carboxyl benzyl > (5a) N-p-benzyl chloride(5f) > N-ethyl (5c), which means that the introduction of electron-withdrawing substituent of the nitrogen atom on the indole ring can strengthen the light stability of dyes enhancement, strengthen the electron-withdrawal ability of substituent and strengthen the stability of the dyes. This is because the electron clouds of the dye molecules reduced due to the existence of electron-withdrawal substituent, which enhances the oxidation resistance and light stability of the dye molecules. The performance of Dye 5a is good with the substituent containing carboxyl, which can be combined with covalently binding protein, making it ideal for application of protein markers.

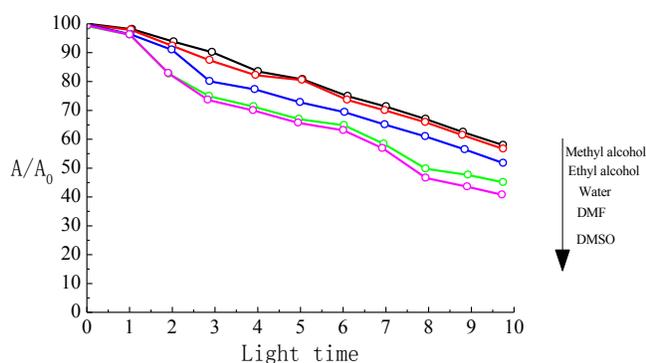


Fig. (3). Photo-bleaching of double-cyanine dye 5f in different solutions.

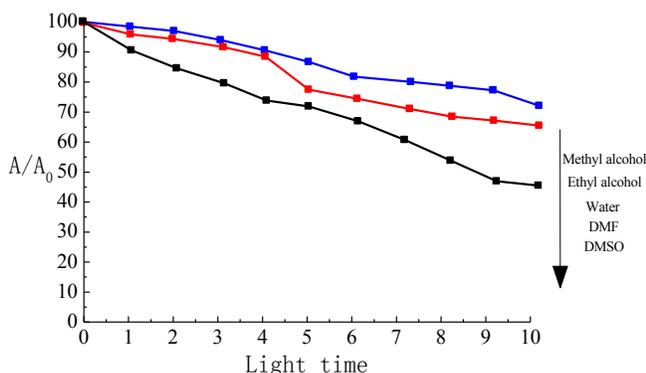


Fig. (4). Photo-bleaching of double-cyanine dye 5a, 5c, 5f in different solutions.

CONCLUSION

Compared with the light stability data of the double cyanine dyes with the corresponding single-cyanine dye, the light stability of the double cyanine is better. That is probably because in double cyanine dyes, two single cyanine dyes connected to butyl chain can be the base of each other, that help the introduction of the substituent with high space steric, which can hinder the attack of singlet oxygen or ultra-oxygen anion, so the oxidation resistance and light stability of the dye are improved.

Three kinds of double cyanine dyes in proton solvent can lead to the increase of solvent polarity, spectral blue shift, and characterize negative solvation effects. However, in aprotic solvents, they increased the polarity of solvent, with the redshift of spectrum, characterized by the positive phase solvation effects. The structural characteristic of the complex dual dyes is the introduction of chain of butyl with two single dyes connected. This characteristic makes the fluorescent output of double cyanine dyes lower compared to single cya-

nine dyes, different degrees of increase happen with the Stokes displacement, and enhancement of light stability.

Above all, compared with the single cyanine dye, double cyanine dyes have better spectral performance and strong stability, which promises a positive prospect in application.

CONFLICT OF INTEREST

The authors confirm there is no conflict of interest in this article.

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