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Mendeleev Commun., 2017, **27**, 360–362

Mendeleev Communications

Synthesis and characterization of novel zwitterionic heptamethine indocyanine fluorophores

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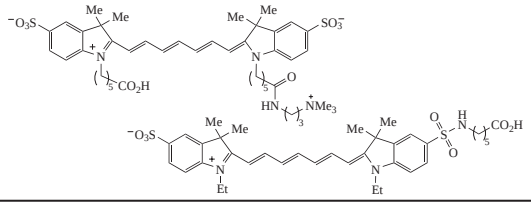
DOI: 10.1016/j.mencom.2017.07.013

Two novel electroneutral indotricarbocyanine dyes with two sulfo groups have been synthesized. They possess higher photo and thermal stability as compared to known analogue and were conjugated with aminoallyl-dUTP for enzymatic labeling of DNA by polymerase chain reaction.



Cyanine dyes are widely applied in cancer imaging, nucleic acid detection, photographic processes, nonlinear optics.[1–5] Most commonly used dyes fluoresce in the visible region of the spectrum where the background fluorescence of the matrix can cause considerable interference.[6,7] In the near-IR region of 750–1000 nm also known as 'biological window', the background can potentially be much lower than in the visible region.[8–10] Among organic dyes, indotricarbocyanine dyes with an absorption and fluorescence maximum in near-IR region are of great interest due to their relative stability, high molar extinction coefficient, and high fluorescent intensity.[11]

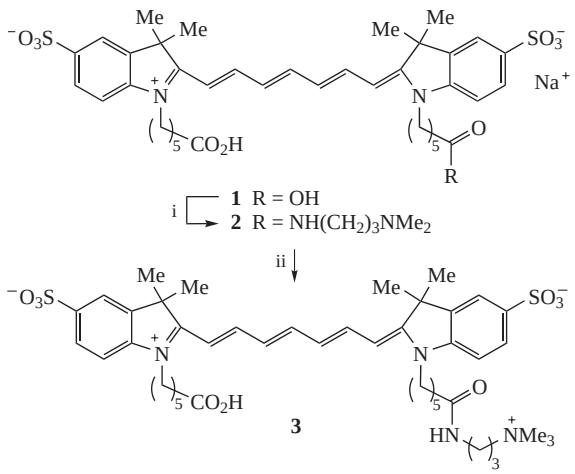
In some cases, the electric charge of fluorophore is extremely important and electroneutral dyes are required.[12] Previously, we described the relationship between molecular structure of the heptamethine 3*H*-indocyanine dyes and their spectral properties, photo and thermal stability.[13] Our studies reveal that the monosulfonated heptamethine dye shows reduced photo- and thermal stability compared with the well-known disulfonated analogue (Cy7). Here we report a synthesis of an electroneutral indotricarbocyanine dyes with enhanced photo- and thermal stability compared to dye containing one sulfo group.

The symmetrical nature of the heptamethine indocyanine dyes is an important part of their design.[14] For this reason the symmetrical dye with two sulfo and two carboxylic groups has been chosen as initial compound for subsequent modification. The neutral charge of fluorophore was achieved by covalent attachment of a ligand with electro positively charged quaternary ammonium group.[15,16]

Previously, we reported the synthesis of indotricarbocyanine dyes lacking substitution in the polyene chain.[13] The modified dye **2** was synthesized by coupling the fluorescent dye **1** with *N*,*N*-dimethylaminopropylamine in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxysuccinimide (NHS) in DMF (Scheme 1).† Quaternary ammonium salt **3** was prepared by alkylation with iodomethane and methyl *p*-toluenesulfonate as quaternizing agents in DMF. Methyl ester of acid **3** was formed as a by-product when iodomethane was used under any reaction conditions, so further acidic hydrolysis was required to remove ester methyl group. However, in acidic solutions the dye chromophore degraded. We have found that quaternization using methyl *p*-toluenesulfonate can be carried out at room temperature and only chromatographically detectable traces of unreacted dye **2** can be observed. Increase in the temperature results in the formation methyl ester of acid **3**. The amount of alkylating agent is of special importance, 3 mol per 1 mol of the starting dye being required.

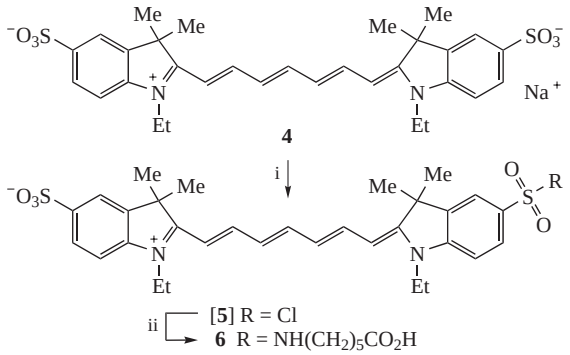
Another way to synthesize electroneutral heptamethine dye with symmetrical structure is the introduction of sulfonamide functional group directly to indolenine nuclei at the C^5 position.[17] Novel dye is much better soluble in water than the similar one with one sulfo group. Good water solubility is 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.

We developed a protocol for the synthesis of cyanine dye with sulfonamide linker arm by direct modification of disulfo cyanine dye obtained according to our previously published protocol.[13]



Scheme 1 *Reagents and conditions*: i, NHS, HBTU, DMF, 20 °C, 2 h; then $H_2N(CH_2)_3NMe_2$, $NaHCO_3/Na_2CO_3$, 20 °C, 24 h; ii, TsOMe, DMF, 20 °C, 3 h.

† For experimental details, synthetic procedures and characteristics of compounds, see Online Supplementary Materials.



Scheme 2 *Reagents and conditions*: i, PCl_5, $(MeO)_3P{=}O$, 20 °C, 5.5 h; ii, $H_2N(CH_2)_5CO_2H$, 1 M $NaHCO_3$, 0 °C, 5 h, then 20 °C, 12 h.

The most common method for sulfonamide synthesis involves the preparation of the corresponding sulfonyl chloride **5** by the treatment of sulfonic acid salt **4** with PCl_5 in trimethyl phosphate as a solvent (Scheme 2). The desired sulfonyl chloride was formed at room temperature for 5.5 h. The heating significantly reduced the yield of the monosulfonyl chloride, wherein bis-product was obtained. The other reaction conditions, such as acetonitrile as solvent and phosphoryl chloride as chlorinating agent, caused destruction of the cyanine chromophore.

Sulfonyl chloride **5** was difficult to purify, and therefore it was used crude in the reaction with 6-aminocaproic acid to afford sulfonamide dye **6**. The latter step was exothermic, so it was necessary to start the reaction on cooling. All synthesized compounds **2**, **3**, **6** were isolated using reversed-phase chromatography, their structure and purity were confirmed by ^{1}H NMR spectroscopy and mass spectrometry.

To characterize dyes **3** and **6**, spectroscopic studies were performed. Table 1 outlines their absorption (λ_{max}), emission (λ_{max}), Stokes shift, and relative fluorescence quantum yields. The correct comparison of the cyanine dye properties can be only made within a set of structures with similar core structure. So, the changes in the photophysical properties of the dyes **3** and **6** modified by quaternary ammonium group and sulfonamide linker arm, respectively, were compared to characteristics of the corresponding well-known monosulfonated heptamethine dye **7** and disulfonated analogue **8** (commercially available Cy7).

Regardless of the functional groups attached to the indolenine nuclei, the absorption, emission and the Stokes shifts show no significant variations for compounds **3**, **6**–**8**. The modification

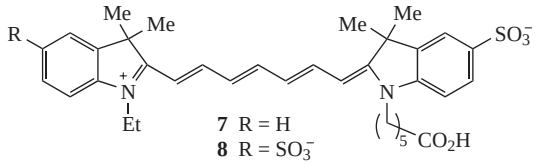


Table 1 Spectroscopic characteristics of indotricarbocyanine dyes in PBS/MeOH.[a]

Dye	λ_{max}^{abs}/nm	λ_{max}^{em}/nm	Stokes shift/nm	$\varepsilon/10^{-5}$ dm^3 mol^{-1} cm^{-1}	Φ (%)
3	750	769	19	2.45±0.03	32
	754	776	22	2.59±0.03	54
6	748	769	21	2.23±0.02	30
	748	770	22	2.47±0.03	55
7	745	769	24	0.76±0.02	19
	751	775	24	0.96±0.02	38
8	747	769	22	2.39±0.02	28
	749	773	24	2.53±0.02	52

[a] ε is the molar extinction coefficient; Φ is the quantum yield; 10 mM potassium phosphate buffer solution (PBS), 0.9% NaCl, pH 7.4. Reference standard Cy7 (dye **8**) (GE Healthcare), Φ = 28% in PBS at 25 °C.[13,18]

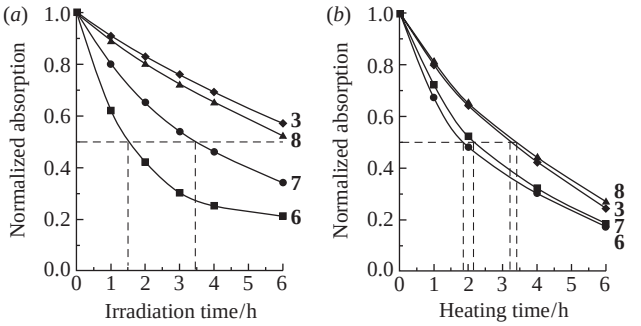


Figure 1 Comparison of (*a*) photofading and (*b*) thermal stability of the cyanine dyes **3**, **6**–**8**.

of symmetrical cyanine dyes **1** and **4** with an electro positively charged quaternary ammonium group and sulfonamide linker arm, respectively, led to increase in quantum yield and molar extinction coefficient compared to those for dyes **7** and **8**.

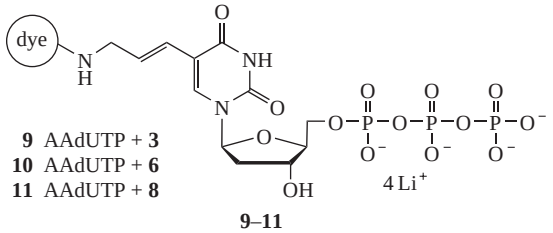
Important requirements for cyanine dyes as fluorescent labels in bioanalysis are their photo and thermal stability with high fluorescence yield.[19,20] Figure 1(*a*) shows photofading behavior of dyes **3** and **6** upon irradiation of the dye solutions in Milli-Q water with a 60-W light bulb compared to that of dyes **7** and **8**. After 6 h of irradiation, dyes **3** and **8** exhibited 50–55% photofading, while dyes **6** and **7** faded 20 and 30%, respectively. The photostabilities of commercial dye **8** with two sulfo groups and modified disulfonated dye **3** with quaternary ammonium group were close. The photostabilities of the dyes can be placed in the order: **3** > **8** > **7** > **6**. The lower photostability of non-symmetric dye **6** was due to only one sulfo group on its indolenine nuclei, while strong electron-withdrawing sulfo groups can reduce the electron density in the heptamethine chain.[21]

Information on the thermal stability of fluorescent probes is valuable for DNA analysis using polymerase chain reaction (PCR) amplification. The polymerase chain reaction involves a series of 20–40 repeated cycles of heating and cooling between 62 and 94 °C.[22] Therefore, the stability was determined by heating 10^{-6} M dye solutions in Milli-Q water at 95 °C for 6 h.[13] UV-VIS thermal analysis was used to examine the chromophore system stability depending on the features of the substituents in the dye molecule.

Figure 1(*b*) displays the thermal decomposition curves of dyes **3**, **6**–**8**. The thermal analysis demonstrated that their thermal stability depended on the fluorophore symmetry and on photostability. Symmetrical location of sulfo groups improves thermal stability from 2 h (one sulfo group, dyes **6**, **7**) to 3.5 h (two sulfo groups, dyes **3**, **8**). The incorporation of quaternary ammonium group at the N-position of a indolenine nucleus brings little effect on the thermal stability.

Conjugation of aminoallyl nucleotide (AAdUTP) with cyanine dyes **3**, **6**, and **8** to give corresponding labeled dUTPs **9**–**11** was performed using corresponding *p*-nitrophenyl esters in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.2) containing DMF.[23] The enzymatic incorporation of fluorescently labeled nucleotide into DNA was tested using PCR and hybridization analysis with 'TB-Biochip' microarray and *rpoB*-specific primer pair.[23(*b*)]

The efficiency of PCR was controlled by 5% agarose gel electrophoresis with ethidium bromide staining. The modified



9 AAdUTP + **3**
10 AAdUTP + **6**
11 AAdUTP + **8**

9–**11**

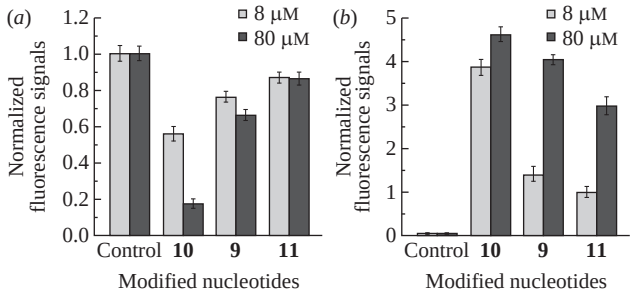


Figure 2 The fluorescence signals from the 'TB-biochip' with different concentration of the fluorescently labeled nucleotides (8 and 80 μM): (*a*) the intensity of the hybridization signals from perfect duplexes of the biochip (excitation of Cy3) normalized to native (without labeled nucleotides) PCR product to estimate the quantity of hybridized products with labeled nucleotides; (*b*) the intensity of the hybridization signals from perfect duplexes of the biochip (excitation of Cy7) normalized to 'reference' nucleotide **11** and equalized to the quantity of hybridized PCR product to estimate the efficiency of incorporation.

deoxynucleoside triphosphates exhibit inhibitory effect from minor to significant [Figure S1(*a*), see Online Supplementary Materials]. However, the yield of the full-size PCR products is sufficient for visual analysis. Inhibitory effect of nucleotide **10** becomes most noticeable with increasing the concentration up to 80 μM.

The degree of incorporation of labeled triphosphates was evaluated by the described method based on hybridization analysis.[23(*a*)] To normalize the signals from incorporated in the growing DNA chain modified triphosphates, Cy3-labeled primer was used. The number of Cy3 labels is equimolar to single-stranded PCR product used for hybridization. Parallel simultaneous use of Cy3-dye (the absorption maximum of 550 nm, the fluorescence maximum 570 nm) and Cy7-line modified nucleoside triphosphates, allowed us to independently detect the fluorescence signals at two wavelengths. Figure 2(*a*) shows the averaged (over three experiments) signals obtained from control hybridized PCR product (contains only natural nucleotides and Cy3-labeled primer) and PCR products obtained with the use of compounds **9**–**11** as substrates at their concentration in the initial PCR mixture 8 and 80 μM. The differences in the intensity of signals correlate well with the optical density observed in the electrophoresis [*cf.* Figures S1 and 2(*a*)].

To estimate the relative effectiveness of modified triphosphates, the normalization of the signals from nucleotides **9** and **10** to the signal of nucleotide **11** containing commercially available Cy7-dye was carried out. Interestingly, nucleotide **10** demonstrates the highest efficiency of incorporation into the growing DNA chain (Figure 2) and at the same time the most significant inhibition of the reaction (Figure S1), which led to a low signal level on the chip at 80 μM, and correspondingly low signal/background and 'perfect/imperfect' ratios in minor polymorphism analysis. We observed an effect of inverse relationship between the effectiveness of the incorporation of labeled nucleotides and the PCR yield. Perhaps this is due to slower incorporation of labeled nucleotides as compared with the natural counterpart.

In conclusion, we have described the synthesis and properties of novel electroneutral indotricarbocyanine dyes. Dye **3** with quaternary ammonium group shows improved spectral properties, photostability and thermal stability compared with the well-known electroneutral dye **7**. The sulfonamide group at the C^5 position of 3*H*-indolenine of dye **6** has a negative effect on its photostability and thermal stability. The inhibitory effect on the PCR and the relative efficiency of incorporation in the growing DNA chain have been evaluated, namely, the two parameters that are direct indicators of the effectiveness of fluorescently labeled triphosphates in the hybridization analysis.

This study was supported by the program 'Basic research for the development of biomedical technologies' of the Presidium of the Russian Academy of Sciences (grant no. 0103-2015-0144) and by the Russian Foundation for Basic Research (grant no. 17-04-02062).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2017.07.013.

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Received: 29th November 2016; Com. 16/5106