Dyes and Pigments 97 (2013) 353–360

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Near-infrared heptamethine cyanine dyes. Synthesis, spectroscopic characterization, thermal properties and photostability

Valeriy E. Shershov [1], Maksim A. Spitsyn [1], Viktoriya E. Kuznetsova [1], Edward N. Timofeev [1], Olga A. Ivashkina [1], Ivan S. Abramov [1], Tatyana V. Nasedkina [1], Alexander S. Zasedatelev [1], Alexander V. Chudinov*

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russian Federation

ARTICLE INFO

Article history:
Received 23 August 2012
Received in revised form
28 December 2012
Accepted 31 December 2012
Available online 16 January 2013

Keywords:
Heptamethine dye
Near-infrared dyes
Molar extinction coefficients
Relative quantum yields
Photostability
Thermal stability

ABSTRACT

Near-infrared heptamethine cyanine dyes were synthesized without substitution to the polyene chain but with various substituents on the indoleninium fragments. To develop indotricarbocyanine dyes with improved photochemical characteristics, we studied the relationship between the structures of these compounds, their spectral properties, their photostability, and thermal stability.

1. Introduction

Enhanced research interest in cyanine dyes primarily arises from their applications in biochemistry, molecular biology, biomedicine, structural biology and other research fields [1–5]. Cyanine dyes are frequently used as reporter groups for labeling proteins, DNA sequencing, oligonucleotides, and antibodies [6–10]. Heptamethine cyanine dyes, as molecular labels, are valuable tools in optical imaging because of their low background and the autofluorescence of biological samples in the near-infrared (NIR) region [11–13]. These characteristics provide an opportunity to increase the signal-to-noise ratio and increase analytical sensitivity. However, there are a few disadvantages to using the NIR fluorescence of cyanine dyes. Reduced photostability and thermal stability of the heptamethine dyes in aqueous environments have been described in the literature [14,15] and are attributed to the flexibility of the bridge between indolenine units and rotation around the unsaturated bonds. Another disadvantage is poor water solubility, which is often responsible for dye aggregation and nonspecific binding [16,17].

The purpose of this study was the synthesis of water-soluble polymethine cyanine dyes with various substituents at the indoleninium residues and an investigation of their physicochemical properties. We report a stabilizing effect of *N*-substitution of 3*H*-indolenine that decreases photobleaching and thermal degradation and demonstrates the relationship between substituted trimethine dye structures and their photophysical properties.

2. Experimental

2.1. Materials

All reagents were purchased from Sigma–Aldrich and used without further purification. Solvents were of analytical grade or, in the case of DMF, were distilled over phthalic anhydride.

2.2. Measurements

2.2.1. Equipment

^{1}H NMR spectra were recorded with a Bruker AMX-400 spectrometer (400 MHz) (Bruker, USA) in DMSO-d_6 solutions. Chemical shifts δ are given in ppm. Coupling constants (J) are given in Hz. Mass spectral analysis was carried out with an MALDI-TOF (Matrix Assisted

* Corresponding author. Fax: +7 499 135 1405.
E-mail address: chud@eimb.ru (A.V. Chudinov).
[1] Fax: +7 499 135 1405.

0143-7208/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dyepig.2012.12.031

Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) mass spectrometer KOMPACT MALDI 4 (Kratos Analytical, Manchester, U.K.). Elemental analysis was performed on a Thermo Finnigan Flash EA 1112 Element Analyzer (Thermo Finnigan-CE Instruments, Italy). Mass spectra were obtained in the linear mode and positive ions were registered using gentisic acid (2,5-dihydroxybenzoic acid) as a matrix. Fluorescence spectra were recorded on a Cary Eclipse spectrofluorimeter (Agilent Technologies, USA). Absorption spectra were measured using a Jasco V-550 spectrophotometer (JASCO International Co., Japan). Melting points were determined on a Kofler block and are uncorrected. pH values were determined using a Thermo Orion 330 pH-meter (Thermo Scientific, USA).

Dye synthesis was performed by heating in an AccuBlock Digital Dry Bath thermoblock (Labnet, Switzerland). Analytical thin layer chromatography was performed using C18 reversed-phase plates (C18-RP) (Merck). Purification of dyes was performed using a reversed-phase C18-RP column (Analtech, Newark, DE).

2.2.2. Fluorescence quantum yields

The fluorescence quantum yields of the dyes were determined using a reference standard (Cy7, GE Healthcare QY = 0.28 in phosphate-buffered saline (PBS − 0.01 M potassium phosphate buffer, 0.9% NaCl, pH 7.4) at 25 °C [18]). The relative fluorescence quantum yield of the unknown sample was determined according to equation [19]:

$$Q_x = (D_s/D_x)(F_x/F_s)(n_x/n_s)^2 Q_s,$$

where Q is the fluorescence quantum yield, D is the optical density, F is the area under the curve of the fluorescence spectrum, and n is the refractive index of the solvent. The subscripts s and x refer to the reference fluorophore of known quantum yield and the unknown sample, respectively.

2.2.3. Molar extinction coefficients

Molar extinction coefficients were determined from absorbance values using precisely weighed dry samples. About 2 mg of a dye was dissolved in 100 mL of PBS or methanol. The absorbance was measured for the six various dilutions.

2.2.4. Photostability

Photobleaching experiments were carried out in glass vials (3 mL volume) containing a 10^{-6} M solution of probes in Milli-Q water enclosed in a light-tight box where sample solutions were exposed to a 60-W light bulb placed 30 cm from the samples. UV–vis spectra were recorded at 30 min intervals for 6 h. The data were normalized to the highest value obtained for each dye.

2.2.5. Thermal stability

The thermal stability of the cyanine dyes was tested by heating 10^{-6} M (2 mL volume) solutions in an AccuBlock Digital Dry Bath thermoblock (Labnet, Switzerland). The characterization of dyes in aqueous solution was performed using buffer solutions (PBS; 10× Taq Buffer − 500 mM KCl, 100 mM Tris HCl (pH 9.0 at 25 °C), 15 mM $MgCl_2$, and 1% Triton X-100, pH 8.6) and Milli-Q water. Buffers were prepared using Milli-Q water.

2.3. Preparation of intermediate compounds

Potassium salt of 1-(δ-sulfonatobutyl)-2,3,3-trimethylindolenium-5-sulfonate **7a** [20], 1-(5-carboxypentyl)-2,3,3-trimethylindoleninium-5-sulfonate **7b** [21], 1-benzyl-2,3,3-trimethylindolenium-5-sulfonate **7c** [22,23], 1-ethyl-2,3,3-trimethylindolenium-5-sulfonate **7d** [20], 1-(5-carboxypentyl)-2,3,3-trimethylindoleninium bromide **7e** [21], and glutaconaldehyde dianil hydrochloride **8** [24] were synthesized according to the literature.

2.3.1. Disodium diphenylamine-4,4′-disulfonate (**2**)

A three-neck flask equipped with a reflux condenser, mechanical stirrer, thermometer and dropping funnel was charged with diphenylamine **1** (20.0 g, 0.12 mol), and warmed to 80 °C for 20 min. A conc. H_2SO_4 (28.5 mL, 0.53 mol) was added dropwise under vigorous stirring while the temperature of the reaction mixture was gradually raised to 145–150 °C. The mixture was stirred at this temperature for an additional 3.5 h. Water (200 mL) was added to the hot reaction mixture, and the product was extracted with a mixture of Bu^iOH (250 mL) and tributylamine (57.2 mL, 0.24 mol). The resulting solution was washed with water (50 mL) and brine (100 mL), and concentrated *in vacuo* at 50 °C to approximately 120–130 mL. A solution of NaOH (9.6 g, 0.24 mol) in water (9.6 mL) was added dropwise to the residue. The reaction mixture was maintained at 5 °C for ~24 h. The formed crystals were filtered, washed with acetone (50 mL), and dried in a vacuum desiccator over P_2O_5 and KOH. The yield of compound **2** was 22.5 g (52%), mp >250 °C. UV–vis (PBS) λ_{max}: 311 nm, 193 nm. ^{1}H NMR (DMSO-d_6) δ: 8.48 (s, 1H, N*H*), 7.49 (d, 4H, Ar*H*, *J* 8.5 Hz), 7.01 (d, 4H, Ar*H*, *J* 8.5 Hz).

2.3.2. Disodium N,N′-diphenylhydrazine-4,4′-disulfonate (**3**)

A solution of $NaNO_2$ (0.41 g, 5.9 mmol) in water (0.5 mL) was added dropwise to a solution of disodium diphenylamine-4,4′-sulfonate **2** (2.0 g, 5.4 mmol) in water (5 mL). The mixture was cooled to 0 °C, and 6 M HCl (aq) (0.4 mL) was added with vigorous stirring. After additional stirring for 2 h at 20 °C, the reaction mixture was neutralized with a 5% solution of Na_2CO_3, and Pr^iOH (5 mL) and acetone (5 mL) were added. The formed crystals were filtered, washed with cold EtOH (10 mL), and dried in a vacuum desiccator over P_2O_5. The yield of disodium *N*-nitrozo-diphenylamine-4,4′-disulfonate was 2.1 g (95%), mp >250 °C. UV–vis (PBS) λ_{max}: 302 nm, 244 nm, 202 nm.

Zinc dust (1 g) was added in small portions to a stirred mixture of the foregoing disodium *N*-nitrozo-diphenylamine-4,4′-disulfonate (0.5 g, 1.2 mmol), water (1 mL) and acetic acid (0.45 mL) at 0 °C over 1 h. The reaction mixture was stirred at 20 °C for an additional 4 h. The insoluble materials were filtered, and washed with acetic acid (10 mL). The resulting solution was concentrated *in vacuo* at 50 °C, and acetone (10 mL) was added. The formed crystals were filtered, and dried in a vacuum desiccator over P_2O_5 to give 0.4 g (80%) hydrazine **3**, mp >250 °C. UV–vis (PBS) λ_{max}: 311 nm, 253 nm, 197 nm. ^{1}H NMR (DMSO-d_6) δ: 7.54 (d, 4H, Ar*H*, *J* 8.5 Hz), 6.9 (d, 4H, Ar*H*, *J* 8.5 Hz).

2.3.3. Sodium salt of 2,3,3-trimethyl-1-(4-sulfonatophenyl)-indoleninium-5-sulfonate (**5**)

A mixture containing disodium *N*,*N*′-diphenylhydrazine-4,4′-disulfonate **3** (0.7 g, 1.8 mmol), methyl isopropyl ketone **4** (0.5 mL, 4.6 mmol), acetic acid (3 mL) and a saturated solution of $ZnCl_2$ in ethanol (3 mL) was heated to 80 °C for 6 h. The solution was concentrated *in vacuo*, and acetone (15 mL) was added to the residue. The crystals formed were filtered, and dried in a vacuum desiccator over P_2O_5 to yield 0.36 g (50%) indolenine **5**, mp >250 °C. UV–vis (PBS) λ_{max}: 306 nm, 259 nm, 192 nm. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{17}H_{16}NO_6S_2^-$ 394.44; found 395.2 $[M]^+$. ^{1}H NMR (DMSO-d_6) δ: 7.05–8.03 (m, 7H, Ar*H*), 3.58 [s, 3H, CH_3(2)], 1.79 [s, 6H, 2CH_3(3)].

2.3.4. Diaminoalkane dihydrochloride (**12a–c**)

A diaminoalkane (11 mmol) was dissolved in conc. HCl (1.85 mL), and acetone (15 mL) was added to the solution. The white crystals formed were filtered, and dried in a vacuum desiccator over P_2O_5 to give dihydrochlorides **12a–c** in quantitative yield.

2.4. General procedure for the synthesis of cyanine dyes (**10a**–**d**) and (**11a**–**c**)

A solution of indolenine **5**, **7a**–**c** (for compounds **10a**–**d**) or indolenine **7a**, **d** (for compounds **11a**–**c**) (0.2 mmol) and glutaconaldehyde dianil monohydrochloride **8** (0.25 mmol) in a mixture of acetic anhydride (2 mL) and acetic acid (0.7 mL) was heated at 118 °C for 3 h. The indolenine **5**, **7a**–**c** (for compounds **10a**–**d**) or indolenine **7b**, **e** (for compounds **11a**–**c**) (0.3 mmol), anhydrous potassium acetate (0.2 g, 2 mmol), acetic anhydride (2 mL) and acetic acid (1 mL) were added to the reaction mixture, and heated at 80 °C for an additional 5 h. The solvent was evaporated, and the cyanine dyes were purified using reversed-phase (C18) chromatography. The cyanine dye was dissolved in 0.1 M triethylammonium acetate buffer (TEAA), and loaded onto the column. Elution of the dye was carried out using a linear gradient from 0.1 M TEAA to 50% acetonitrile in 0.1 M TEAA. The pure dye obtained as a triethylammonium salt was converted into its sodium salt in the following fashion: the solution of dye in Milli-Q water was loaded onto the C18-RP column, washed with 0.1 M NaCl and Milli-Q water, and eluted with an acetonitrile–Milli-Q water mixture.

2.4.1. Trisodium salt of 5,5′-disulfo-1,1′-di(4-sulfonatobutyl)-3,3,3′,3′-tetramethylindotricarbocyanine (**10a**)

Yield 93 mg (53%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{35}H_{41}N_2O_{12}S_4^{3-}$ 809.97; found 810.5 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.86 (t, 2H, β,β′-CH, *J* 12.0 Hz), 7.72 (m, 1H, δ-C*H*), 7.23–7.63 (m, 6H, Ar*H*), 6.55 (t, 2H, γ,γ′-C*H*, *J* 12.0 Hz), 6.34 (m, 2H, α,α′-C*H*), 4.12 (m, 4H, C*H*$_2$CH$_2$CH$_2$CH$_2$SO$_3$), 2.56 (m, 4H, CH$_2$CH$_2$CH$_2$C*H*$_2$SO$_3$), 1.73 (m, 8H, CH$_2$C*H*$_2$C*H*$_2$CH$_2$SO$_3$), 1.61 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)]. Anal. Calcd. for $C_{35}H_{41}N_2Na_3O_{12}S_4$ (%): C, 47.83; H, 4.70; N, 3.19. Found: C, 47.49; H, 4.89; N, 3.28.

2.4.2. Sodium salt of 5,5′-disulfo-1,1′-di(5-carboxypentyl)-3,3,3′,3′-tetramethylindotricarbocyanine (**10b**)

Yield 68 mg (43%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{39}H_{47}N_2O_{10}S_2^-$ 767.93; found 767.4 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.86 (t, 2H, β,β′-CH, *J* 12.0 Hz), 7.71 (m, 1H, δ-CH), 7.17–7.52 (m, 6H, Ar*H*), 6.54 (t, 2H, γ,γ′-CH, *J* 12.0 Hz), 6.32 (m, 2H, α,α′-C*H*), 4.1 (m, 4H, C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$COOH), 1.63 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 2.13, 1.74, 1.52, 1.36 (4m, 16H, CH$_2$C*H*$_2$C*H*$_2$ C*H*$_2$C*H*$_2$COOH). Anal. Calcd. for $C_{39}H_{47}N_2NaO_{10}S_2$ (%): C, 59.22; H, 5.99; N, 3.54. Found: C, 59.48; H, 5.74; N, 3.42.

2.4.3. Sodium salt of 5,5′-disulfo-1,1′-dibenzyl-3,3,3′,3′-tetramethylindotricarbocyanine (**10c**)

Yield 41 mg (36%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{41}H_{39}N_2O_6S_2^-$ 719.89; found 720.8 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.83 (m, 2H, β,β′-C*H*), 7.71 (m, 1H, δ-C*H*), 7.83. 7.68, 7.08 (3m, 6H, Ar*H*), 7.83, 7.21, (2m, 10H, CH$_2$*Ph*), 6.13 (m, 2H, γ,γ′-CH), 6.08 (d, 2H, α,α′-CH, *J* 13.5 Hz), 5.46 (m, 4H, C*H*$_2$Ph), 1.64 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)]. Anal. Calcd. for $C_{41}H_{39}N_2NaO_6S_2$ (%): C, 66.29; H, 5.29; N, 3.77. Found: C, 66.64; H, 5.42; N, 3.68.

2.4.4. Trisodium salt of 5,5′-disulfo-1,1′-di(4-sulfonatophenyl)-3,3,3′,3′-tetramethylindotricarbocyanine (**10d**)

Yield 53 mg (29%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{39}H_{33}N_2O_{12}S_4^{3-}$ 849.95; found 850.3 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.85 (m, 2H, β,β′-C*H*), 7.68 (m, 1H, δ-C*H*), 7.8, 7.56, 7.18, 7.03 (3m, 14H, N*Ph*, ArH), 6.49 (m, 2H, γ,γ′-CH), 6.31 (d, 2H, α,α′-CH, *J* 13.5 Hz), 1.62 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)]. Anal. Calcd. for $C_{39}H_{33}N_2Na_3O_{12}S_4$ (%): C, 50.97; H, 3.62; N, 3.05. Found: C, 50.65; H, 3.38; N, 3.13.

2.4.5. 1-(5-carboxypentyl)-1′-ethyl-3,3,3′,3′-tetramethyl-5′-sulfoindotricarbocyanine (**11a**)

Yield 35 mg (29%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{35}H_{42}N_2O_5S$ 602.78; found 602.3 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.86 (m, 2H, β,β′-C*H*), 7.73 (m, 1H, δ-C*H*), 7.72, 7.56, 7.31 (3m, 7H, Ar*H*), 6.55 (m, 2H, γ,γ′-CH), 6.33 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.14 (m, 4H, C*H*$_2$CH$_3$, C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$COOH), 1.57 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 2.14, 1.71, 1.52, 1.34 (4m, 8H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$COOH), 1.25 (t, 3H, CH$_2$C*H*$_3$, *J* 7.0 Hz). Anal. Calcd. for $C_{35}H_{42}N_2O_5S$ (%): C, 69.74; H, 7.02; N, 4.65. Found: C, 69.51; H, 7.26; N, 4.71.

2.4.6. Sodium salt of 1-(5-carboxypentyl)-1′-ethyl-3,3,3′,3′-tetramethyl-5,5′-disulfoindotricarbocyanine (**11b**)

Yield 30 mg (21%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{35}H_{41}N_2O_8S_2^-$ 681.84; found 681.4 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.88 (m, 2H, β,β′-C*H*), 7.74 (m, 1H, δ-C*H*), 7.74, 7.63, 7.28 (3m, 6H, Ar*H*), 6.57 (m, 2H, γ,γ′-CH), 6.35 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.11 (m, 4H, C*H*$_2$CH$_3$, C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$COOH), 1.63 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 2.16, 1.72, 1.54, 1.38 (4m, 8H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$COOH), 1.27 (t, 3H, CH$_2$C*H*$_3$, *J* 7.0 Hz). Anal. Calcd. for $C_{35}H_{41}N_2NaO_8S_2$ (%): C, 59.64; H, 5.86; N, 3.97. Found: C, 59.93; H, 5.98; N, 3.91.

2.4.7. Disodium salt of 1-(5-carboxypenthyl)-3,3,3′,3′-tetramethyl-1′-(4-sulfonatobuthyl)-5,5′-disulfoindotricarbocyanine (**11c**)

Yield 27 mg (16%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{37}H_{44}N_2O_{11}S_3^{2-}$ 788.95; found 788.4 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.85 (m, 2H, β,β′-C*H*), 7.78 (m, 1H, δ-C*H*), 7.18–7.76 (m, 6H, Ar*H*), 6.55 (m, 2H, γ,γ′-CH), 6.33 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.09 (m, 4H, C*H*$_2$CH$_2$CH$_2$CH$_2$SO$_3$, C*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$COOH), 2.51, 1.76 (2m, 6H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$SO$_3$), 1.65 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 2.14, 1.7, 1.51, 1.36 (4m, 8H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$COOH). Anal. Calcd. for $C_{37}H_{44}N_2Na_2O_{11}S_3$ (%): C, 53.23; H, 5.31; N, 3.36. Found: C, 53.36; H, 5.08; N, 3.28.

2.5. General procedure for the synthesis of cyanine dyes (**13a**–**c**)

A mixture of dye **10b** (0.036 mmol), diaminoalkane dihydrochloride **12a**–**c** (0.18 mmol) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (1.8 mmol) was dissolved in anhydrous DMF (6 mL) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) (0.45 mL), and stirred at room temperature for 3 h. The mixture was diluted with 0.1 M TEAA (15 mL), and purified as described for dyes **10a**–**d**.

2.5.1. Sodium salt of 1,1′-(7,10-diaza-6,11-diketohexadecane-1,16-diyl)-3,3,3′,3′-tetramethyl-5,5′-disulfoindotricarbocyanine (**13a**)

Yield 25 mg (61%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{41}H_{51}N_4O_8S_2^-$ 792.00; found 793.1 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.85 (m, 2H, β,β′-C*H*), 7.71 (m, 1H, δ-C*H*), 7.19–7.57 (m, 6H, Ar*H*), 6.53 (m, 2H, γ,γ′-C*H*), 6.33 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.09 [m, 4H, C*H*$_2$(1), C*H*$_2$(16)], 1.6 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 3.42, 2.12, 1.71, 1.57, 1.41 [5m, 20H, C*H*$_2$(2), C*H*$_2$(3), C*H*$_2$(4), C*H*$_2$(5), C*H*$_2$(8), C*H*$_2$(9), C*H*$_2$(12), C*H*$_2$(13), C*H*$_2$(14), C*H*$_2$(15)]. Anal. Calcd. for $C_{41}H_{51}N_4NaO_8S_2$ (%): C, 60.42; H, 6.31; N, 6.87. Found: C, 60.21; H, 6.56; N, 6.75.

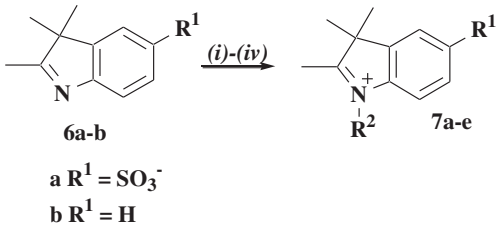
2.5.2. Sodium salt of 1,1′-(7,12-diaza-6,13-diketooktadecane-1,18-diyl)-3,3,3′,3′-tetramethyl-5,5′-disulfoindotricarbocyanine (**13b**)

Yield 18 mg (53%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{43}H_{55}N_4O_8S_2^-$ 820.05; found 821.2 $[M]^+$. ^{1}H NMR (DMSO-d_6) *δ*: 7.82 (m, 2H, β,β′-C*H*), 7.73 (m, 1H, δ-C*H*), 7.2–7.6 (m, 6H, Ar*H*), 6.55 (m, 2H, γ,γ′-C*H*), 6.32 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.13 [m, 4H, C*H*$_2$(1), C*H*$_2$(18)], 1.62 [s, 12H, C*H*$_3$(3,3), C*H*$_3$(3,3′)], 3.46, 2.19, 1.75, 1.58, 1.36 [5m, 24H, C*H*$_2$(2), C*H*$_2$(3), C*H*$_2$(4), C*H*$_2$(5), C*H*$_2$(8), C*H*$_2$(9), C*H*$_2$(10), C*H*$_2$(11), C*H*$_2$(14), C*H*$_2$(15), C*H*$_2$(16), C*H*$_2$(17)]. Anal. Calcd.

for $C_{43}H_{55}N_4NaO_8S_2$ (%): C, 61.26; H, 6.58; N, 6.65. Found: C, 61.59; H, 6.81; N, 6.73.

*2.5.3. Sodium salt of 1,1′-(7,14-diaza-6,15-diketoicosane-1,18-diyl)-3,3,3′,3′-tetramethyl-5,5′-disulfoindotricarbocyanine (**13c**)*

Yield 26 mg (59%), dark-green powder. MS (MALDI-TOF) *m*/*z*: calcd. for $C_{45}H_{59}N_4O_8S_2^-$ 848.10; found 849.7 $[M]^+$. ^{1}H NMR (DMSO-d_6) δ: 7.85 (m, 2H, β,β′-C*H*), 7.71 (m, 1H, δ-C*H*), 7.16–7.68 (m, 6H, Ar*H*), 6.54 (m, 2H, γ,γ′-C*H*), 6.35 (d, 2H, α,α′-CH, *J* 13.5 Hz), 4.14 [m, 4H, CH_2(1), CH_2(20)], 1.64 [s, 12H, CH_3(3,3), CH_3(3,3′)], 3.39, 2.15, 1.72, 1.52, 1.34 [5m, 28H, CH_2(2), CH_2(3), CH_2(4), CH_2(5), CH_2(8), CH_2(9), CH_2(10), CH_2(11), CH_2(12), CH_2(13), CH_2(16), CH_2(17), CH_2(18), CH_2(19)]. Anal. Calcd. for $C_{45}H_{59}N_4NaO_8S_2$ (%): C, 62.05; H, 6.83; N, 6.43. Found: C, 62.38; H, 6.69; N, 6.34.



Scheme 2. Synthesis of *N*-substituted 2,3,3-trimethylindolenine bases **7a**–**e**. Reagents and conditions: *(i)* 1,4-butane sultone, C_3H_7CN, N_2, 115 °C, 24 h, 92% (**7a** – $R^1 = SO_3^-$, $R^2 = (CH_2)_4SO_3^-$); *(ii)* $Br(CH_2)_5COOH$, $C_6H_4Cl_2$, N_2, 145 °C, 30 h, 75% (**7b** – $R^1 = SO_3^-$, $R^2 = (CH_2)_5COOH$), 83% (**7e** – R^1 = H, $R^2 = (CH_2)_5COOH$); *(iii)* C_6H_5Br, C_3H_7CN, N_2, 115 °C, 25 h, 96% (**7c** – $R^1 = SO_3^-$, R^2 = Bn); *(iv)* ethyl *p*-toluenesulfonate, $(C_2H_5)_3N$, C_3H_7CN, N_2, 115 °C, 24 h, 82% (**7d** – $R^1 = SO_3^-$, $R^2 = C_2H_5$).

3. Result and discussion

3.1. Indotricarbocyanine dye synthesis

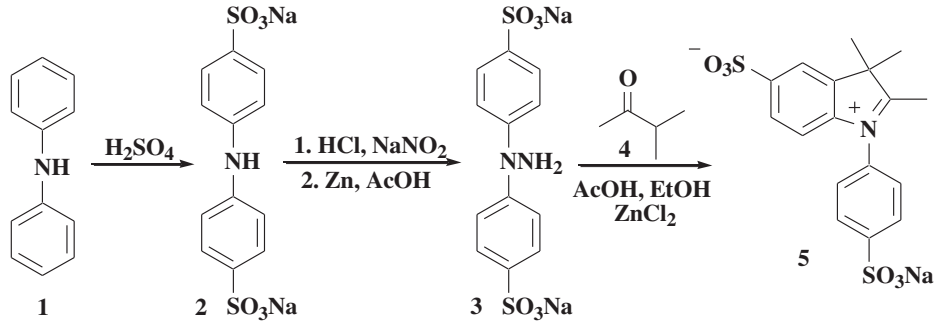
The synthesis of cyanine dyes began with the preparation of the indolinium salts and glutaconaldehyde dianil monohydrochloride **8** synthesized according to literature procedures [17,25–27]. Fischer cyclization of phenyl hydrazine derivatives provides a convenient synthetic route to the indolenine nucleus [28–30]. The biphenylamine **1** was selectively sulfonated at 145 °C using concentrated H_2SO_4 to give the sulfo derivative **2** in a 52% yield (Scheme 1) [31–35]. A substituted hydrazine **3** was formed by diazotization of disulfobiphenylamine **2** with $NaNO_2$ [36], with subsequent reduction of the nitroso derivative using zinc in acetic acid [37,38]. The product was condensed with methyl isopropyl ketone **4** following Fisher indole synthesis. Alkylation of indolenines **6a**–**b** with ethyl *p*-toluenesulfonate [20], 6-bromohexanoic acid [21], 1,4-butane sultone [20], or benzyl bromide [22,23] yielded substituted indolinium salts **7a**–**e** (Scheme 2). The reaction products were isolated as solids by precipitation with ethyl ether or acetone to give pure substances.

According to the literature [39], symmetric cyanine dyes were synthesized using a one-step condensation reaction of the appropriate indoleninium salt containing an activated methyl group in the 2-position and glutaconaldehyde dianil **8**. The reaction is usually catalyzed by sodium acetate [18,39] or triethylamine [40], using either a mixture of acetic acid and acetic anhydride [39] or ethanol [40] as the solvent. In our efforts to synthesize symmetric cyanine dyes **10a**–**d** using the one-step condensation, the desired products were obtained in 3–6% yields. Therefore, we carried out a two-step condensation under a variety of conditions (Scheme 3). The synthesis of the symmetric cyanine dyes **10a**–**d** was performed in different solvents, including Ac_2O, DMF, DMF/Ac_2O, AcOH/Ac_2O, EtOH/Ac_2O, and MeOH/Ac_2O in the presence of either DIPEA or anhydrous potassium acetate as a condensation agent. The best results were obtained using an AcOH/Ac_2O mixture in the presence of anhydrous potassium acetate, heating to 118 °C for 3 h in the first step and to 80 °C for 5 h in the second step. Also, the dyes **10a**–**d** were synthesized using a Py/Ac_2O mixture without condensation agent. It should be noted, however, that the latter conditions caused byproducts formation which complicated isolation of dyes **10a**–**d** by reversed-phase chromatography. The yields of target compounds **10a**–**d** obtained by either method did not exceed 53%.

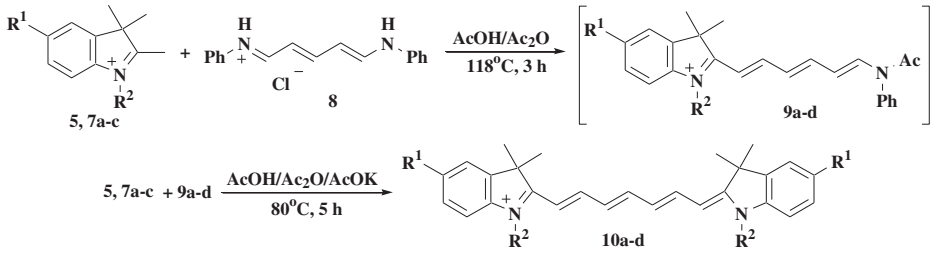
The synthesis of asymmetric cyanine dyes **11a**–**c** (Fig. 1) involved sequential condensation reactions of the appropriate indoleninium salts **7a**, **d** with glutaconaldehyde dianil **8** and acetanilide **9** with carboxyindoleninium salts **7b**, **e** in an AcOH/Ac_2O mixture in the presence of anhydrous potassium acetate. Addition of acetyl chloride to the reaction mixture at the first step substantially reduced the yield of the desired dyes **11a**–**c**.

As reviewed in the literature [15,41,42], rotation of two indolium heads of the heptamethine dye around the polyene chain results in a decrease in fluorescence, and thermo- and photo-stabilities. This so-called “loose belt effect” can be eliminated by crosslinking the two heads [41]. Non-reactive 1,1′-crosslinked intramolecular heptamethine dyes with polymethine chains **13a**–**c** were synthesized to investigate their physicochemical properties (Scheme 4). The 1,1′-crosslinked heptamethine dyes were prepared in good yields by coupling the symmetric reactive dye **10b** with diamine hydrochloride **12a**–**c** in the presence of HBTU and DIPEA in DMF at room temperature for 3 h.

All synthesized dyes were isolated using reversed-phase chromatography. The yields ranged from 16% to 61% depending on the structure of cyanine dyes. The structure and purity of compounds **10a**–**d**, **11a**–**c** and **13a**–**c** were confirmed by ^{1}H NMR spectroscopy, elemental analysis and mass spectrometry.



Scheme 1. Synthesis of sodium salt of 2,3,3-trimethyl-1-(4-sulfonatophenyl)-indoleninium-5-sulfonate **5**.



Scheme 3. Synthesis of symmetrical cyanine dyes **10a**–**d**: **9a**, **10a** – $R^1 = SO_3^-$, $R^2 = (CH_2)_4SO_3^-$; **9b**, **10b** – $R^1 = SO_3^-$, $R^2 = (CH_2)_5COOH$; **9c**, **10c** – $R^1 = SO_3^-$, $R^2 = Bn$; **9d**, **10d** – $R^1 = SO_3^-$, $R^2 = PhSO_3^-$.

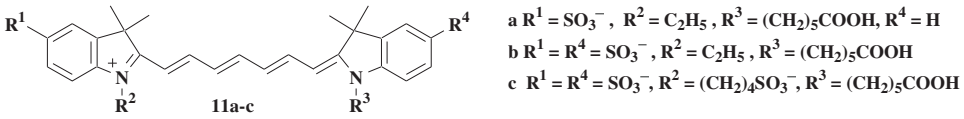
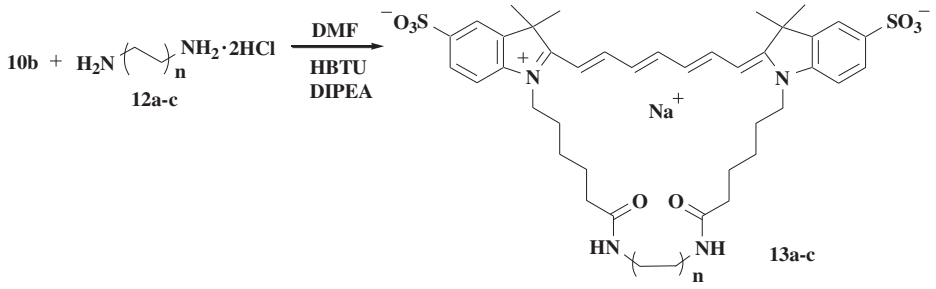


Fig. 1. Asymmetric cyanine dyes **11a**–**c**.



Scheme 4. Synthesis of intramolecularly 1,1′-crosslinked heptamethine dyes **13a**–**c**: **12a**, **13a** – $n = 1$; **12b**, **13b** – $n = 2$; **12c**, **13c** – $n = 3$.

3.2. Spectral properties of indotricarbocyanine dyes

A series of tricarbocyanine dyes containing a polyene chain and various substituents were synthesized as presented in Schemes 3 and 4. To characterize these dyes, spectroscopic studies were performed, as shown in Table 1. Table 1 outlines absorption (λ_{max}), emission (λ_{max}), Stokes shift, and relative fluorescence quantum yield of the synthesized cyanine dyes. Regardless of the functional groups attached to the indolenine nuclei, the spectroscopic profiles of the synthesized dyes **10a**–**d** and **11a**–**c** were nearly identical. The absorption, emission and the Stokes shifts show no significant change as a result of substituting alkyl (dyes **10a**, **b**, **11a**–**c**) and benzyl groups (dye **10c**) onto the nitrogen of the indolenine nuclei. The absorption and emission maxima of *N*-sulfophenyl substituted dye **10d** were red-shifted by approximately 7 nm compared to those of *N*-sulfobutyl substituted dye **10a**, whereas the Stokes shifts was nearly identical to dye **10a**, ~25 nm. The intramolecularly 1,1′-crosslinked heptamethine dyes **13a**–**c** exhibited spectral characteristics of a typical heptamethine cyanine dye **10b**, with absorption and emission maxima at ~750 and ~770 nm, respectively. These results suggest that *N*-alkyl and *N*-benzyl groups and 1,1′-crosslinking do not affect the spectral properties of the dyes.

As can be seen in Table 1, molar extinction coefficients of heptamethine cyanine dyes **10**, **11** and **13** range from 0.99 to $2.62 \cdot 10^5$ L mol^{-1} cm^{-1}. The dye **11a** with one sulfo group and the dye **10c** with *N*-benzyl groups showed low molar extinction coefficients in comparison with dye **11c** containing three sulfo groups. The molar extinction coefficient of dye **10d** with *N*-(4-sulfophenyl) groups is less in comparison with dye **10a** with *N*-(4-sulfobutyl) groups.

Quantum yield (QY) is one of the most important characteristics of NIR molecular labels. The quantum yield of fluorophores was measured at 25 °C using a reference compound with a known quantum yield [18]. Fluorescence quantum yields are higher for dyes with three (dye **11c**) and four (dye **10a**) sulfo groups (Fig. 2).

Table 1
Spectral characteristics of heptamethine cyanine dyes.

Dye no.	Solvent	λ_{max}^{abs} (nm)	λ_{max}^{em} (nm)	Stokes shift (nm)	$\varepsilon \cdot 10^{-5}$ (L mol^{-1} cm^{-1})	QY[a] (%)
10a	PBS	747	771	24	2.38 ± 0.03	29
	MeOH	752	776	24	2.41 ± 0.02	54
10b	PBS	749	771	22	2.57 ± 0.02	29
	MeOH	755	776	21	2.62 ± 0.02	55
10c	PBS	748	771	23	1.04 ± 0.02	28
	MeOH	753	773	20	1.19 ± 0.02	62
10d	PBS	754	779	25	1.93 ± 0.01	21
	MeOH	759	782	23	1.81 ± 0.02	47
11a	PBS	745	769	24	0.76 ± 0.02	19
	MeOH	751	775	24	0.96 ± 0.02	38
11b	PBS	747	769	22	2.39 ± 0.02	28
	MeOH	749	773	24	2.53 ± 0.02	52
11c	PBS	749	771	22	2.42 ± 0.03	30
	MeOH	753	774	21	2.59 ± 0.02	55
13a	PBS	749	773	24	2.36 ± 0.03	32
	MeOH	754	776	22	2.48 ± 0.02	57
13b	PBS	750	770	20	2.32 ± 0.03	33
	MeOH	755	778	23	2.51 ± 0.02	57
13c	PBS	749	768	19	2.28 ± 0.02	36
	MeOH	754	776	22	2.55 ± 0.03	58

[a] Reference standard – Cy7 (GE Healthcare), QY = 0.28 in PBS at 25 °C [18].

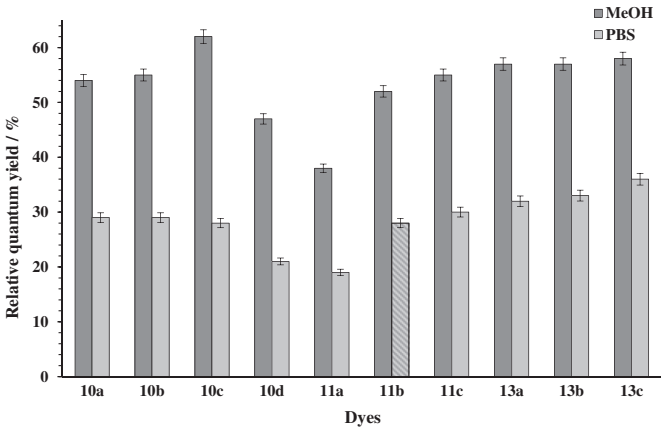


Fig. 2. Relative quantum yields of the NIR dyes.

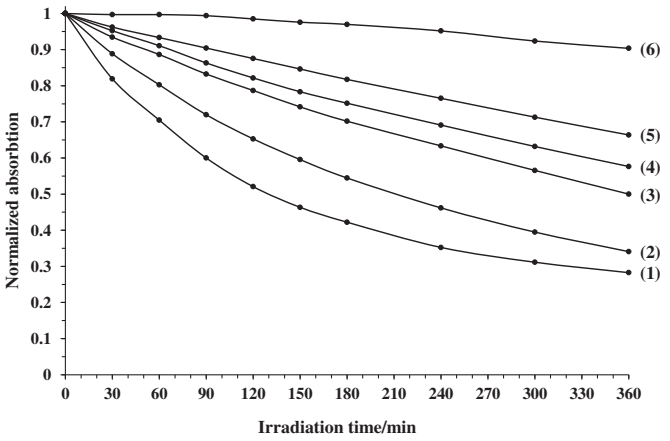


Fig. 3. Comparison of photofading of NIR dyes: (1) – **10c**; (2) – **11a**; (3) – **11b**; (4) – **10b**, **11c**; (5) – **10a**, **13c**; (6) – **10d**.

The quantum yield of dye **11a** with one sulfo group was lowest. The dyes **10a**–**c** with *N*-(5-carboxypentyl), *N*-(4-sulfobutyl), and *N*-benzyl groups showed approximately equal quantum yields, while dye **10d** with *N*-(4-sulfophenyl) groups showed a low fluorescence quantum yield compared to dye **10a**. The modification of the symmetrical cyanine dye **10b** bearing two carboxyl groups with a saturated chain bridge of varying length led to some increase in quantum yield (dyes **13a**–**c**). Solvent choice may have considerable influence on the QY of dyes. We compared the effect of PBS and MeOH on QY. In general, the quantum yields of all synthesized dyes **10a**–**d**, **11a**–**c**, and **13a**–**c** increased in methanol as compared to PBS. This effect can be accounted for by the fact that these dyes tend to aggregate considerably in aqueous environments.

3.3. Photostability

An important requirement for cyanine dyes as fluorescent labels in bioanalysis is high photostability. Photobleaching is typically observed with highly useful long-wavelength cyanine dyes due to flexibility of the polymethine chain between the indolenine units [15,22,23,43,44]. To investigate the relationship between the structure and photostability of the NIR cyanine dyes, photobleaching behavior of compounds **10a**–**d**, **11a**–**c**, and **13a**–**c** (with different substitutions at the *N*-positions of 3*H*-indolenines) was studied.

It was found that the optical properties of all probes in the dark at room temperature remained constant for over 3 weeks but observed photodegradation upon irradiation of the dye solutions with a 60-W light bulb. Fig. 3 shows photofading behavior of dyes **10a**–**d**, **11a**–**c**, and **13a**–**c** in Milli-Q water. The results show that *N*-(4-sulfophenyl) groups at 3*H*-indolenine rings (dye **10d**) stabilize the fluorophore more than *N*-(4-sulfobutyl) groups (dye **10a**). The photostability of the dyes decreases in the order **10d** > **10a** > **10b**, **11c** > **11b** > **11a** > **10c**. These data demonstrate that dyes containing four sulfo groups (dyes **10a**, **d**) are water-soluble, and have improved photostability compared to the dye (**11a**) containing one sulfo group. Compound **10c**, which was characterized by sterically hindered *N*-benzyl groups at the nitrogen atoms, exhibited the least photostability. Although the difference between dyes **10b** and **13c** is not very large, 1,1′-cross-linked heptamethine dye **13c** exhibits better photostability than symmetric dye **10b** with carboxy groups. The photodegradation data obtained for **13a** and **13b** fell between curves 2 and 3 in Fig. 3 (data not shown).

3.4. Thermal stability

Temperature is another factor that has a significant role in destabilizing the cyanine molecular structure. Resistance to heat-induced decomposition at elevated temperatures is one of the principal properties required for cyanine dyes used in bioanalysis. The polymerase chain reaction (PCR) technique most frequently used in DNA analysis is based on thermal cycling consisting of cycles of repeated heating and cooling between 62 and 94 °C [45]. The thermal stability in the dark of compound **11b**, a widely used fluorescent label, has been investigated and the results are presented in Fig. 4. Aqueous solutions of dye at a concentration of 10^{-6} M were employed for thermal stability testing. To determine the limits of thermal stability sample **11b** was heated from 10 to 95 °C using 10 °C intervals, and the samples were maintained for 2 h at each temperature point. Thermal stability was calculated from the change in absorption intensity at the absorption maximum after heating, and was then normalized to the highest value. The thermal analysis of dye **11b** demonstrated that the dye noticeably decomposes when the temperature increases from 80 °C to 95 °C.

The effect of solvent type on the thermal stability of compound **11b** was also studied. Fig. 5 shows the influence of two aqueous buffers and Milli-Q water on the thermal stability of the dye. The 10^{-6} M solutions of the dye **11b** in Milli-Q water, 0.1 M PBS (pH 7.4),

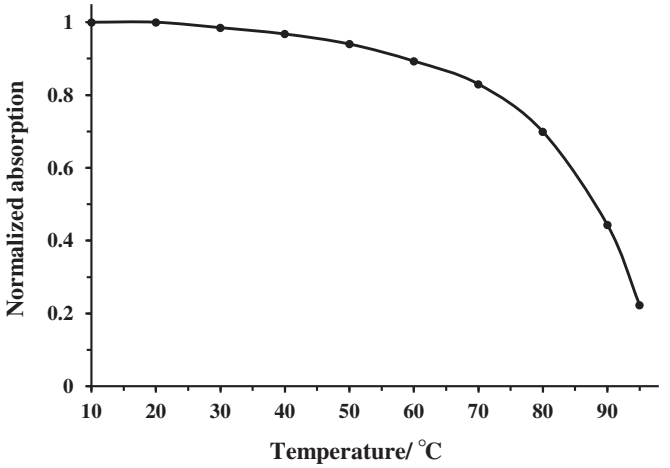


Fig. 4. The thermal stability of dye **11b** in Milli-Q water.

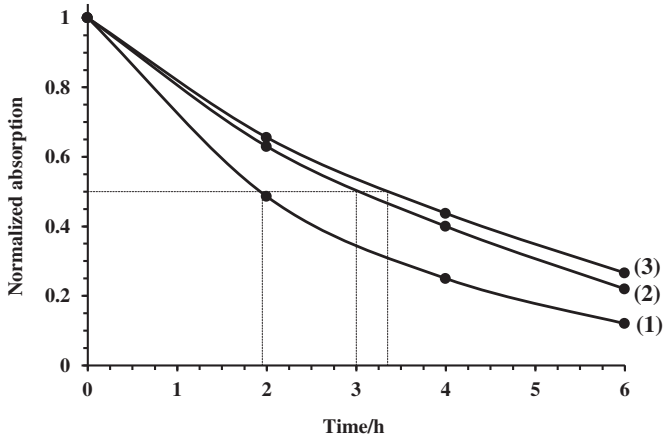


Fig. 5. Thermal stability of dye **11b** in PBS (1), Taq buffer (2), and Milli-Q water (3).

and Taq buffer (pH 8.6) were heated at 95 °C for 6 h. The UV–vis spectra were recorded at 2 h intervals. The half-life time ($t_{1/2}$) for the dye **11b** in PBS was 1.95 h; the $t_{1/2}$ values for the dye in Milli-Q water and Taq buffer were 3.4 h and 3 h, respectively.

The experimental data presented in Figs. 4 and 5, and the requirements of the PCR amplification procedure (62–94 °C) gave us the idea for a protocol to study the thermal stability of synthesized dyes **10a**–**d**, **11a**–**c**, and **13a**–**c**. The stability was determined by heating 10^{-6} M solutions in Milli-Q water at 95 °C for 6 h. Fig. 6 shows curves for the thermal decomposition of these dyes. The thermal analysis of the cyanine dyes demonstrated that the stability of the compounds depended on the nature and position of the substituents in the dye structures. From the thermal curves, it can be observed that increasing the number of sulfo groups from one (dye **11a**) to four (dye **10a**) substantially improves thermal stability. Half-life time was enhanced from 1.9 h (**11a**) to more than 6 h (**10a**). It should be noted that the tetrasulfonated dye **10d** containing two *N*-(4-sulfophenyl) groups at the nitrogen atoms in the 3*H*-indo-rings had lower thermal stability than the tetrasulfonated dye **10a** with two *N*-(4-sulfobutyl) groups. Additionally, the disulfonated dye **10c** with *N*-benzyl groups at the nitrogen atoms and dye **11c** with one sulfo group had identical half-life times (1.9 h). The symmetrical cyanine dye **10b** bearing two carboxyl groups was modified with saturated chain bridges of various lengths that led to increased thermostability (for example, dye **13c**). The thermal stability data for dyes **13a** and **13b** were arranged between thermal curves 5 and 6 in Fig. 6 (data not shown).

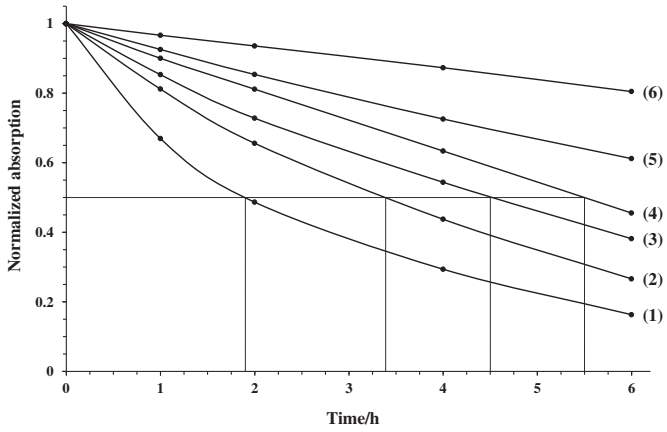


Fig. 6. Comparisons on thermal stability of the NIR dyes: (1) – **10c**, **11a**; (2) – **10d**, **11b**; (3) – **10b**; (4) – **11c**; (5) – **10a**; (6) – **13c**.

4. Conclusion

In summary, ten water-soluble near-infrared heptamethine 3*H*-indocyanine dyes were synthesized in order to investigate the relationship between molecular structure, spectral properties, photostability, and thermal stability. The 4-sulfophenyl-*N*-substituted dye **10d** shows improved photostability and reduced thermal stability compared with the well-known carboxypentyl-*N*-substituted dye **11b**. The benzyl groups at the *N*-atoms of 3*H*-indolenines of dye **10c** had a negative effect on photostability and thermal stability. The intramolecularly 1,1′-crosslinked heptamethine dye **13c** exhibited enhanced photostability and thermal stability. The results of the present study provide the rationale for the design of tricarbocyanine dyes with improved photophysical properties suitable for molecular labeling and bioimaging applications.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (Projects nos. 11-04-01998 and 11-04-01950 a) and by Contract no. 16.522.12.2011 of the Ministry of Science and Education of the Russian Federation.

References

[1] Leevy WM, Gammon ST, Johnson JR, Lampkins AJ, Jiang H, Marquez M, et al. Noninvasive optical imaging of *Staphylococcus aureus* bacterial infection in living mice using a bis-dipicolylamine-zinc(II) affinity group conjugated to a near-infrared fluorophore. Bioconjug Chem 2008;19(3):686–92.
[2] Zhang Z, Bloch S, Achilefu S. Synthesis and evaluation of novel galactose–carbocyanine fluorescent contrast agents with enhanced hydrophilicity and rigid molecular constraint. Photonics West 2004: Biomedical Optics (BiOS); 2004 Sept 3; Bellingham, WA, USA. In: Savitsky AP, Bornhop DJ, Raghavachari R, Achilefu SI, editors. Genetically engineered and optical probes for biomedical applications II; 2004. p. 262–8.
[3] Li C, Greenwood TR, Bhujwalla ZM, Glunde K. Synthesis and characterization of glucosamine-bound near-infrared probes for optical imaging. Org Lett 2006;8(17):3623–6.
[4] Wang W, Ke S, Kwon S, Yallampalli S, Cameron AG, Adams KE, et al. A new optical and nuclear dual-labeled imaging agent targeting interleukin 11 receptor alpha-chain. Bioconjug Chem 2007;18(2):397–402.
[5] Licha K, Riefkea B, Ntziachristosb V, Beckerd A, Chancee B, Semmlerf W. Hydrophilic cyanine dyes as contrast agents for near-infrared tumor imaging: synthesis, photophysical properties and spectroscopic in vivo characterization. Photochem Photobiol 2000;72(3):392–8.
[6] Zhang S, Metelev V, Tabatadze D, Zamecnik PC, Bogdanov A. Fluorescence resonance energy transfer in near-infrared fluorescent oligonucleotide probes for detecting protein–DNA interactions. Proc Natl Acad Sci U S A 2008;105(11):4156–61.
[7] Peng X, Chen H, Draney DR, Volcheck W, Schutz-Geschwender A, Olive DM. A non-fluorescent, broad range quencher dye for FRET assays. Anal Biochem 2008;388(2):220–8.
[8] Pham W, Medarova Z, Moore A. Synthesis and application of a water-soluble near-infrared dye for cancer detection using optical imaging. Bioconjug Chem 2005;16(3):735–40.
[9] Chen Y, Grushuk A, Achilefu S, Ohylchansky T, Potter W, Zhong T, et al. A novel approach to a bifunctional photosensitizer for tumor imaging and phototherapy. Bioconjug Chem 2005;16(5):1264–74.
[10] Zheng G, Li H, Yang K, Blessington D, Licha K, Lund-Katz S, et al. Tricarbocyanine cholesteryl labeled LDL: new near infrared fluorescent probes (NIRFs) for monitoring tumors and gene therapy of familial hypercholesterolemia. Bioorg Med Chem Lett 2002;12(11):1485–8.
[11] Daehne S, Resch-Genger U, Wolfberg OS. Near-infrared dyes for high technology applications. High Technology. In: NATO ASI Series, Series 3. Trest: Kluwer Academic Publishers; 1998.
[12] Patonay G, Antoine MD. Near-infrared fluorogenic labels: new approach to an old problem. Anal Chem 1991;63(6):321a–7a.
[13] Soini E, Hemmila I. Fluoroimmunoassay: present status and key problems. Clin Chem 1979;25(3):353–61.
[14] Geddes CD, Cao H, Lakowicz JR. Enhanced photostability of ICG in close proximity to gold colloids. Spectrochim Acta A: Mol Biomol Spectrosc 2003;59(11):2611–7.
[15] Sanchez-Galvez A, Hunt P, Robb MA, Olivucci M, Vreven T, Schlegel HB. Ultrafast radiationless deactivation of organic dyes: evidence for a two-state

two-mode pathway in polymethine cyanines. J Am Chem Soc 2000;122(12):2911–24.
[16] Encinas C, Miltsov S, Otazo E, Rivera L, Puyol M, Alonso J. Synthesis and spectroscopic characterization of heptamethine NIR dyes for their use in optochemical sensors. Dyes Pigm 2006;71(1):28–36.
[17] Patonay G, Antoine MD, Devanathan S, Strekowski L. Near-infrared probe for determination of solvent hydrophobicity. Appl Spectrosc 1991;45(3):457–61.
[18] Lin Y, Weissleder R, Tung CH. Novel near-infrared cyanine fluorochromes: synthesis, properties, and bioconjugation. Bioconjug Chem 2002;13(3):605–10.
[19] Pham W, Lai WF, Weissleder R, Tung CH. High efficiency synthesis of a bioconjugatable near-infrared fluorochrome. Bioconjug Chem 2003;14(5):1048–51.
[20] Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. Bioconjug Chem 1993;4(2):105–11.
[21] Chen X, Peng X, Cui A, Wang B, Wang L, Zhang R. Photostabilities of novel heptamethine 3*H*-indolenine cyanine dyes with different *N*-substituents. J Photochem Photobiol A: Chem 2006;181(1):79–85.
[22] Ernst LA, Gupta RK, Mujumdar RB, Waggoner AS. Cyanine dye labeling reagents for sulflhydryl groups. Cytometry 1989;10(1):3–10.
[23] Song F, Peng X, Lu E, Zhang R, Chen X, Song B. Synthesis, spectral properties and photostabilities of novel water-soluble near-infrared cyanine dyes. J Photochem Photobiol A: Chem 2004;168(1–2):53–7.
[24] Fisher NI, Hamer FM. Tricarbocyanines. J Chem Soc 1933:189–93.
[25] Li Q, Tan J, Peng BX. Synthesis and characterization of heptamethine cyanine dyes. Molecules 1997;2(6):91–8.
[26] Wang LQ, Peng XJ, Lu EH, Cui JN, Gao XQ. Novel heptamethine 3*H*-indocyanines and their spectral properties. Chin Chem Lett 2005;16(4):461–4.
[27] Chapman G, Henary M, Patonay G. The effect of varying short-chain alkyl substitution on the molar absorptivity and quantum yield of cyanine dyes. Anal Chem Insights 2011;6:29–36.
[28] Rosenstock PD. 2,3-dimethyl-3*H*-indole-3-acetic acid. J Heterocycl Chem 1966;3(4):537–9.
[29] Illy H, Funderburk L. Fisher indole synthesis. Direction of cyclisation of isopropylmethyl ketone phenylhydrazone. J Org Chem 1968;33(11):4283–5.
[30] Sajjadifar S, Vahedi H, Massoudi A, Louie O. New 3*H*-indole synthesis by Fischers method. Part 1. Molecules 2010;15(4):2491–8.
[31] Dutta PC, Mandal D. Studies in indigoid dyes. Part XIV. Thioindigoid dyes derived from diphenyl-4,4′-disulphonic acid. J Indian Chem Soc 1955;32(6):339–43.
[32] Eichenauer U, Neumann P, inventors. BASF AG, assignee. Process for the production of alkali salts of biphenyl-4,4′-disulphonic acid. European patent. EP 0325975. 1989 Aug 2.
[33] Koji N, Takeshi H, Shunsaku T, Katsuhiko T, inventors. Sugai Chemical Ind Co Ltd, assignee. Process for producing 4,4′-diphenyldisulfonic acid and its monopotassium salt. United States patent. US 4382896. 1983 May 10.
[34] Martin TI, Mychajlowskij W, inventors. Xerox Corp, assignee. Process for preparing di-tertiary aryl amines. United Kingdom patent. GB 2147897. 1985 May 22.
[35] Meister O. Correspondenzen. Chem Ber 1872;5:283–7 [in German].
[36] Fierz-David HE, Blangey L, Streiff H. Zur kenntnis der p-oxo-azo-farbstoffe. Helv Chim Acta 1946;29:1718–65 [in German].
[37] Fischer E. Ueber die hydrazinverbindungen. Liebigs Ann 1877;174:167–83 [in German].
[38] Clusius K, Vecchi M. Reaktionen mit ^{15}N. Zum crackmechanismus des *N*,*N*-diphenylhydrazins. Helv Chim Acta 1953;35:933–7 [in German].
[39] Licha K, Hessenius C, Becker A, Henklein P, Bauer M, Wisniewski S, et al. Synthesis, characterization, and biological properties of cyanine-labeled somatostatin analogues as receptor-targeted fluorescent probes. Bioconjug Chem 2001;12(1):44–50.
[40] Southwick PL, Ernst LA, Tauriello EW, Parker SR, Mujumdar RB, Mujumdar SR, et al. Cyanine dye labeling reagents – carboxymethylindocyanine succinimidyl esters. Cytometry 1990;11(3):418–30.
[41] Divu Z, Zhang J, Tang Y, Guobing X, inventors. Anaspec Inc, assignee. Reactive 1,3′-crosslinked carbocyanines and their bioconjugates. United States patent. US 2010029954. 2010 Febr 4.
[42] Singh R, Gorski G, Frenzel G, inventors. Surromed Inc, assignee. Bridged fluorescent dyes, their preparation and their use in assays. United States patent. US 6403807. 2002 June 11.
[43] Strekowski L, Lee H, Mason JC, Say M, Patonay G. Stability in solution of indolium heptamethine cyanines and related pH-sensitive systems. J Heterocycl Chem 2007;44(2):475–8.
[44] Henary M, Mojzych M. Stability and reactivity of polymethine dyes in solution. In: Strekowski L, editor. Heterocyclic polymethine dyes: synthesis, properties and applications. Topics in Heterocyclic Chemistry, vol. 14. Berlin: Springer; 2008. p. 221–38.
[45] Gra OA, Glotov AS, Nikitin EA, Glotov OS, Kuznetsova VE, Chudinov AV, et al. Polymorphisms in xenobiotic-metabolizing genes and the risk of chronic lymphocytic leukemia and Non-Hodgkin's lymphoma in adult Russian patients. Am J Hematol 2008;83(4):279–87.