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Synthesis, properties and bioconjugation of water-soluble asymmetric indodicarbocyanine dyes

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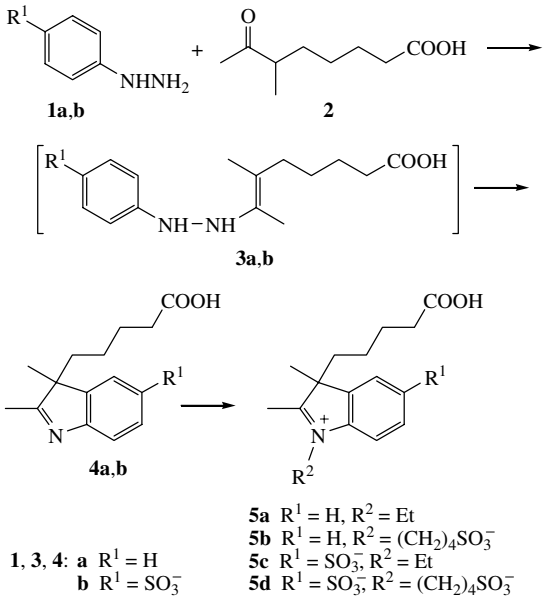
New indodicarbocyanine dyes have been synthesised and converted into succinimidyl active esters for the fluorescent labeling of proteins and oligonucleotides.

Indocyanine dyes have been used as fluorescent labeling reagents in the analysis of DNA sequence in biological microchip technology, flow cytometry, immunoassay and clinical diagnostics.[1] In the technology of 3D hydrogel biological microchips, the test results are recorded on a biochip fluorescent analyzer supplied with industrial semiconductor laser light sources with a wavelength of 635 (20 mW) or 655 nm (40 mW), providing the registration of fluorescence at 670 and 690 nm, respectively.[1(g)–(k)] When using labels in the near-IR range, the self-fluorescence of biological samples is low, thus increasing the signal/background ratio of the label and, therefore, raising the detection sensitivity. In addition, fluorescent labels, providing high sensitivity and accuracy of the instrumental data record, should not interfere with biochemical process of the test. Good water solubility of the fluorophore is crucial because it is the main factor in decreasing the extent of aggregation, and has effect on light absorption and emission properties of conjugates.[2(a)–(c)]

The purpose of this study was the synthesis of fluorescent dyes with absorption and the fluorescence maxima being the most appropriate and providing the maximal sensitivity of registration. The covalent labeling of biomolecules by carbocyanine dyes with a reactive group at the 3-position results in dye conjugates that are substantially more fluorescent on proteins and oligonucleotides, than conjugates labeled with structurally similar carbocyanine dyes bound through the nitrogen atom at the 1-position.[2(d)–(f)] In addition, these dyes have more intense fluorescence emission, greater photostability and higher absorbance at the wavelength of peak absorbance than structurally similar dyes.



Scheme 1

3-(4-Carboxybutyl)-2,3-dimethylindolenine **4a** and its analogue **4b** are expected to possess exceptional advantages as precursors of indodicarbocyanine dyes designed for biological research. Here, we describe the synthesis of indolenines **4a**,**b**, their conversion to derivatives **5a**,**b** and the preparation of dyes **9a–j**.

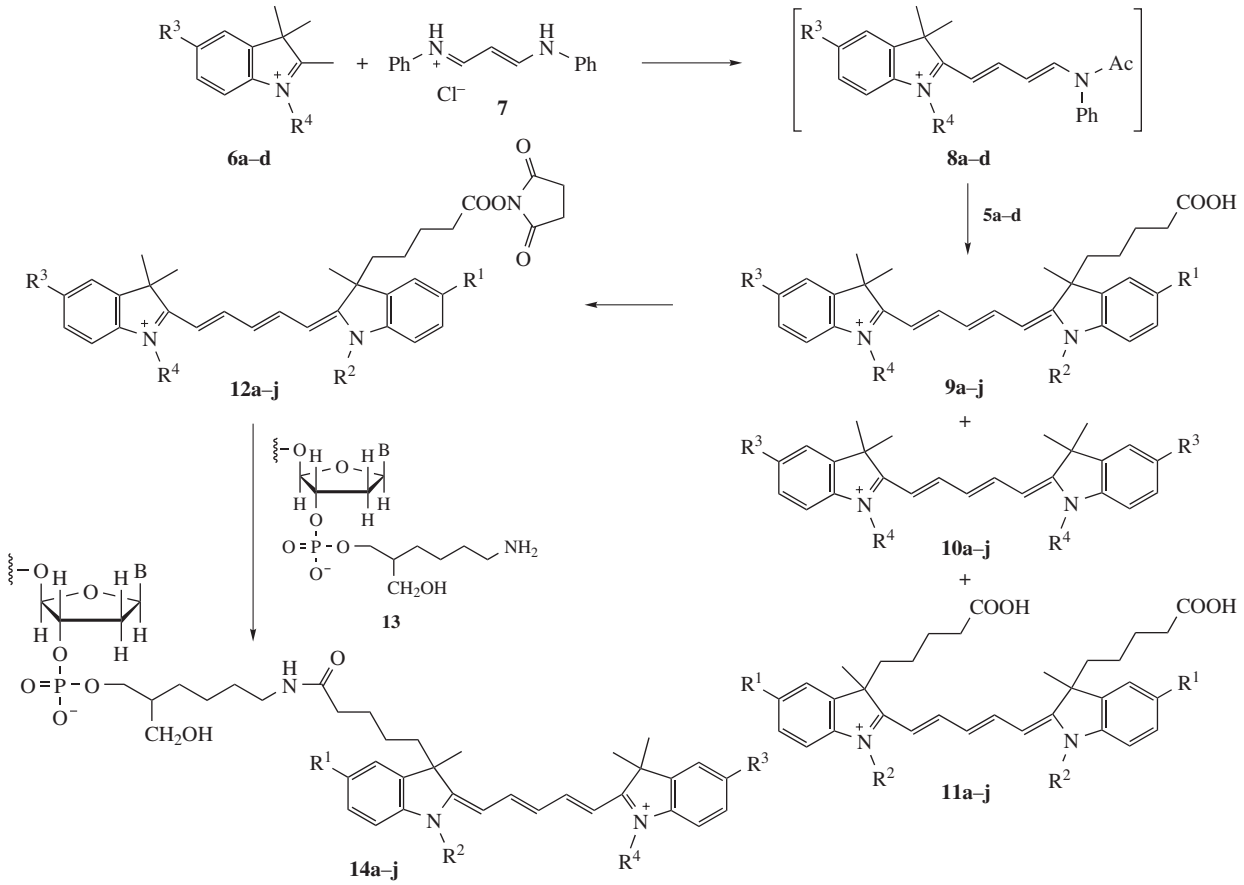
The Fischer cyclisation of phenylhydrazine derivatives is a useful synthetic route to the indolenine nucleus (Scheme 1).[3] Carboxyindolenines **4a**,**b** were prepared in good yields by heating 6-methyl-7-oxooctanoic acid **2** and substituted hydrazines **1a**,**b** in acetic acid. Indolenine **4b** thus obtained is converted into a potassium salt before quaternization.[2(c)]

The reactions of compounds **4a**,**b** with ethyl iodide and 1,4-butansultone result in substituted indoleninium salts **5a**,**b** (Scheme 1).[2(c)] The reaction products were isolated as solids by precipitation with diethyl ether to give pure substances in 95–99% yields.

The sulfoindocyanine dyes designed in this work are shown in Scheme 2. Carbocyanine dyes are characterised by two heteroaromatic residues connected by a conjugation bridge of polyethylene units. The length of the conjugating bridge affects the absorbance and fluorescence maxima.[2(c)] The spectral characteristics, reactivity, charges and solubility of cyanine dyes can be readily controlled by placing appropriate substituents R^1, R^2, R^3 and R^4.[4] Thus, dyes with enhanced or decreased chemical or photostability can be synthesised.

Conventional procedures were used for the synthesis of unsymmetrical cyanine dyes **9a–j**.[1(f)] The main reaction involved the formation of substituted acetanilides **8a–d** from quaternised indolenines **6a–d** and malonaldehyde dianil hydrochloride **7**. These intermediates have poor stability. Note that compounds **8a–d** were difficult to purify; therefore, we used the reaction mixture at the subsequent stage. The completion of the reaction was monitored by absorption spectra in methanol, which showed that the absorption maximum at 270–280 nm diminished as the reaction progressed and the absorption at 440–450 nm increased correspondingly. Symmetrical dyes **10a–j** (< 5%) were always formed if heating was continued for a long time.

The subsequent condensation of intermediates **8a–d** and second activated quaternised indoleninium salts **5a–d** in acetic anhydride in the presence of diisopropylethylamine or anhydrous potassium acetate afforded a mixture of symmetrical **10a–j**, **11a–j** and unsymmetrical dyes **9a–j**. All dyes were isolated by reversed-phase chromatography. Pure dyes were obtained as sodium salts. The yields ranged from 14 to 48% depending



5, **6**, **8**: **a** R^3 = H, R^4 = Et
b R^3 = H, R^4 = $(CH_2)_4SO_3^-$
c R^3 = SO_3^-, R^4 = Et
d R^3 = SO_3^-, R^4 = $(CH_2)_4SO_3^-$

9–**12**, **14**: **a** R^1 = R^3 = H, R^2 = R^4 = $(CH_2)_4SO_3^-$
b R^1 = R^3 = H, R^2 = Et, R^4 = $(CH_2)_4SO_3^-$
c R^1 = R^3 = H, R^2 = $(CH_2)_4SO_3^-$, R^4 = Et
d R^1 = H, R^2 = R^4 = Et, R^3 = SO_3^-
e R^1 = SO_3^-, R^2 = R^4 = Et, R^3 = H
f R^1 = R^3 = SO_3^-, R^2 = R^4 = Et
g R^1 = SO_3^-, R^2 = Et, R^3 = H, R^4 = $(CH_2)_4SO_3^-$
h R^1 = H, R^2 = $(CH_2)_4SO_3^-$, R^3 = SO_3^-, R^4 = Et
i R^1 = R^3 = SO_3^-, R^2 = Et, R^4 = $(CH_2)_4SO_3^-$
j R^1 = R^3 = SO_3^-, R^2 = R^4 = $(CH_2)_4SO_3^-$

Scheme 2

Table 1 Spectral-luminescent characteristics of the dilute solutions of cyanine dyes.[a]

Dyes	Solvent	λ_{max}^{abs}/ nm	λ_{max}^{em}/ nm	$\varepsilon/\times10^5$ dm^3 mol^{-1} cm^{-1}	Q (%)	Relative efficiency of fluorescence[b]		
						$\lambda_{max}^{abs}/\lambda_{max}^{em}$	655 nm/ 690 nm	635 nm/ 670 nm
9a	PBS[c]	647	661	2.43±0.02	0.17	0.567	0.085	0.357
	MeOH	648	664	2.40±0.02	0.27	0.837	0.186	0.570
9b	PBS	646	660	2.13±0.02	0.15	0.433	0.054	0.262
	MeOH	647	662	2.20±0.02	0.26	0.730	0.134	0.493
9c	PBS	643	657	2.06±0.02	0.13	0.372	0.025	0.123
	MeOH	644	662	2.47±0.02	0.25	0.737	0.153	0.598
9d	PBS	646	663	1.80±0.03	0.14	0.353	0.049	0.207
	MeOH	647	660	1.78±0.02	0.23	0.514	0.102	0.359
9e	PBS	646	659	1.91±0.02	0.15	0.385	0.052	0.229
	MeOH	648	663	2.07±0.03	0.23	0.627	0.124	0.424
9f	PBS	650	663	2.13±0.03	0.28	0.859	0.147	0.448
	MeOH	650	667	1.99±0.01	0.32	0.798	0.204	0.484
9g	PBS	648	661	1.90±0.03	0.18	0.446	0.070	0.269
	MeOH	649	664	1.93±0.02	0.27	0.657	0.164	0.418
9h	PBS	645	660	2.17±0.02	0.14	0.411	0.073	0.272
	MeOH	646	664	2.29±0.03	0.27	0.695	0.188	0.563
9i	PBS	650	664	1.77±0.03	0.29	0.711	0.146	0.383
	MeOH	651	668	1.59±0.03	0.34	0.713	0.229	0.410
9j	PBS	650	665	2.33±0.02	0.31	1.000	0.234	0.531
	MeOH	651	669	2.21±0.02	0.32	0.873	0.340	0.472

[a] ε is the molar extinction coefficient; Q is the quantum yield. [b]Relative efficiency of fluorescence at three wavelengths of excitation/registration is presented. [c]PBS is a 10 mM potassium phosphate buffer solution, 0.9% NaCl, pH 7.4.

on the structure of cyanine dyes. The structure and purity of compounds **4a**,**b**, **5a–d** and **9a–j** were confirmed by ^{1}H NMR spectroscopy, mass spectrometry (MALDI-TOF) and elemental analysis.[†,‡] The ^{1}H NMR spectra of sulfoindolenines **4b**, **5b–d** in D_2O did not contain a singlet of the 2-methyl group, but this signal appeared when spectra were recorded in [$^2H_6$]DMSO.[2(c)]

The spectral properties of fluorophores **9a–j** are presented in Table 1. Indodicarbocyanines are near-IR dyes.[4(c)] These dyes absorb at 650 nm with Stokes shifts within 15–18 nm making them suitable for excitation with inexpensive laser diodes.

[†] Purification of dyes was performed on a reverse-phase C18 column (10 mm×160 mm) (Analtech), connected with a Progr gradient pump (Mikrotechna). Cyanine dyes were dissolved in a 0.1 M triethylammonium acetate buffer (TEAA) and loaded on the column. Elution of the dyes was carried out with a linear gradient concentration running from 0.05 M TEAA to 50% acetonitrile in 0.1 M TEAA. The solutions of dyes in deionized water were loaded on the C18 column, washed with 0.1 M NaCl, deionized water and then eluted with acetonitrile–deionized water.

Molar absorption coefficients were determined from the absorbance of dried samples and their estimated molecular weights.

Quantum yields (Q) were calculated relative to a Cy5.5 (Amersham-Pharmacia) with a quantum yield of 32 (methanol) or 27% (PBS).

DMF was distilled over phthalic anhydride. 6-Methyl-7-oxooctanoic acid **2**,[6] 1-(δ-sulfonatobutyl)-2,3,3-trimethylindoleninium **6a**,[2(a)] 1-ethyl-2,3,3-trimethylindoleninium iodide **6b**,[2(c)] 1-ethyl-2,3,3-trimethylindolenium-5-sulfonate **6c**,[2(c)] potassium salt of 1-(δ-sulfonatobutyl)-2,3,3-trimethylindoleninium-5-sulfonate **6d**,[2(c)] malonaldehyde dianil hydrochloride **7**[7] were synthesised according to published procedures.

[‡] For general synthetic procedures and characteristics of compounds **4a**,**b**, **5a–d** and **9a–j** see Online Supplementary Materials.

Cyanine dyes are characterised by intense absorption and emission bands, high molar absorption coefficients (160000–250000 $dm^3\ mol^{-1}\ cm^{-1}$) and acceptable quantum yields (0.14–0.34). Note that some dyes (Table 1) have spectral properties superior over the commercial fluorescent probe Cy5.5[2(*g*)] (ε 220000 $dm^3\ mol^{-1}\ cm^{-1}$, Q 0.27 in PBS).

The direct attachment of SO_3^- groups to the aromatic system of the chromophore insignificantly changed the indodicarbocyanine dye absorption and emission wavelengths. The nature of the substituent at the heterocyclic nitrogen had also a very limited effect on the absorption maxima. Dyes **9f**,**i**,**j** had a slight bathochromic shift, as compared to other indodicarbocyanines.

Direct introduction of sulfonate groups at the 5- and 5′-positions of the chromophore ring systems increase the quantum yields from 13 (compound **9c**) to 31% (compound **9j**) (PBS). The quantum yields are somewhat higher in organic solvents (methanol). For the substituents investigated here, sulfoalkyl groups (dye **9a**) increase the molar absorption coefficient to 240000.

For the covalent labeling of biomolecules with dyes, highly reactive functional groups, such as *N*-hydroxysuccinimidyl ester (NHS), are widely employed.[1(*f*),2(*c*)] The NHS esters are prepared by the esterification of *N*-hydroxysuccinimide in the presence of DCC in DMF with dyes **9a–j**. These active esters were easily separated from their carboxylic acid precursors by reversed-phase chromatography. Hydrolysis in a neutral solvent mixture (water–acetonitrile) is too slow to interfere with chromatographic results. Compounds **12a–j** were isolated in quantitative yields (90–95%).[§]

Then, the active derivatives were covalently attached to synthetic aminolinkers containing oligonucleotide **13** in acetonitrile and a carbonate buffer mixture with following incubation at room temperature (Scheme 2). Fluorescently labeled oligonucleotides **14a–j** were immobilized in the polyacrylamide gel pads of microchips.[5] The fluorescent signal of every microchip element was detected using a laboratory-built fluorescent analyzer of biochips with appropriate excitation (635 and 655 nm) and emission (670 and 690 nm) filters. The fluorescence detection limits of conjugates **14a–j** were about 10^{-18} mol dm^{-3}.

Cyanine dye **9a** was found to provide higher discrimination (ratio between signals of perfect and mismatched duplexes), as compared with the commercial fluorescent probe Cy5.5 in assays on the detection of *Mycobacterium tuberculosis* strain mutations using a TB-Biochip oligonucleotide microarray system. Note that conjugates **14a–j**, in contrast to a conjugate with Cy5.5 did not show non-specific binding with acrylamide gel pads of biochip. In PCR application, the conjugates of dyes **14a–j** were stable and did not inhibit the polymerase activity.

The relative efficiency of fluorescence in MeOH and PBS solutions at equal concentrations of dyes **9a–j** was also measured. It is equal to the ratio between input and output signals. The fluorescent signal was detected by excitation at absorption maxima of 635 and 655 nm and recorded at fluorescence maxima of 670 and 690 nm, respectively (Table 1). The bandwidth is 1 nm in all cases. The results were normalised to the highest value obtained for the dye **9j** in a PBS.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the electronic version at doi:10.1016/j.mencom.2008.05.009.

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[§] *General procedure for the synthesis of succinimidyl esters* **12a–j**. The mixture of dye **9a–j** (0.15 mmol) and *N*-hydroxysuccinimide (69 mg, 0.6 mmol) was dissolved in DMF (11 ml) and cooled to –18 °C. Dicyclohexylcarbodiimide (62 mg, 0.3 mmol) in DMF (0.5 ml) was added to the reaction mixture and the solution was stirred at room temperature for 72 h. The mixture was then diluted with water (50 ml). The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration, and the filtrate was chromatographed using a water–acetonitrile eluent. The quantitative yields (90–95%) of cyanine active esters were obtained. MS (MALDI), *m*/*z* (M^+): **12a**, 809.4; **12b**, 702.2; **12c**, 702.0; **12d**, 673.5; **12e**, 674.0; **12f**, 753.4; **12g**, 780.7; **12h**, 781.1; **12i**, 860.1; **12j**, 967.0.

General oligonucleotide labeling procedure. Synthetic oligonucleotide 5′-CTCAGTTT-NH_2-3′ **13** (200 nmol) was dissolved in a carbonate buffer (15 μl, 0.1 M, pH 9.4) and acetonitrile (15 μl) mixture and the solution was cooled to –18 °C. Active ester **12a–j** (0.6 mg) was then added and the reaction mixture was stirred at room temperature for 24 h. In some cases, conjugate **14a–j** was separated from an excess of free dye (**9a–h**) by butan-1-ol extraction. Fluorescently labeled oligonucleotides **14a–j** were purified by HPLC (C18-RP) using a 0.05 M TEAA–50% acetonitrile in 0.1 M TEAA as an eluent.