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LETTERS TO THE EDITOR

New Indodicarbocyanine Dyes for the Biological Microchip Technology

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Abstract—New indodicarbocyanine dyes with the carboxybutyl group in position-3 of the indolenine fragment bearing methyl and sulfonic groups in positions 5 and 7 of the cycle were synthesized in order to find the most effective fluorescent labels for the biological microchip technology. The position of absorption and fluorescence maxima, the total charge of the dye molecule, and water solubility depend on the location and the total amount of methyl and sulfonic groups. The spectral characteristics of the dyes synthesized were determined. The relative fluorescence efficiencies of the dyes at equal concentrations were measured at excitation wavelengths of 635 and 655 nm and emission wavelengths of 670 and 690 nm, respectively.

Key words: biological microchips, fluorescence, hybridization analysis, indodicarbocyanine dyes

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Fluorescent labeling has become one of the basic techniques in various methods of the genetic analysis [1]. Fluorescent dyes should meet special requirements. Fluorescent labels, providing high sensitivity and accuracy of the instrumental data registration, should not interfere with biochemical process of the test. When using labels in near IR range, the self fluorescence of biological samples is low, thus increasing the signal/background ratio of the label and, therefore, raising the sensitivity of experimental data registration. Labeling with cyanine dyes is often used in the technology of biological microchips [2–4]. In this case, the test results are registered on a biochip fluorescent analyzer supplied with industrial semiconductor laser light sources with a wavelength of 635 (20 mW) and 655 nm (40 mW), providing registration of fluorescence at 670 and 690 nm, respectively.

Cyanine dyes with the reactive carboxyl group in positions 1 and 5 of the indolenine cycle had previously been reported [5–7]. The goal 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.

We have suggested and carried out the synthesis of a new series of indocarbocyanine dyes (**Va**)–(**Vh**) (scheme). Compounds (**Va**)–(**Vh**) are characterized by a number of distinctive features including water solubility. The indolenine fragment of the dye molecule contains methyl groups in positions 5 and 7. This results in the bathochromic shift of absorption and fluorescence maxima. The reactive carboxybutyl group is in position 3 of the indolenine fragment (scheme). Such structure provides a certain spatial orientation the fluorofore relative to the oligonucleotide and decreases steric hindrances. The location and the total amount of sulfonic groups make it possible to vary the total charge of the dye and its water solubility.

Indodicarbocyanines of various structures are obtained by the condensation the corresponding indoleninium bases (**Ia**)–(**Ih**) and malonic dialdehyde dianil hydrochloride (**II**) in two steps (scheme) [5–7]. Indoleninium salt (**IV**) containing the carboxybutyl group was prepared from *p*-tolylhydrazine chloride and 6-methyl-7-oxooctanoic acid by the Fisher cyclization and subsequent quaternization with 1,4-butanesulfone [7].

The first step of indocarbocyanine was carried out by heating in acetic anhydride or in acetic anhydride–acetic acid. Diisopropylethylamine or anhydrous potassium acetate was used as the condensing agent at the second step [7].

The methods for the synthesis of different dyes vary in the order of reagent mixing and the ratio of solvents. All indodicarbocyanines synthesized were isolated by reversed phase HPLC with a gradient of 0 $\longrightarrow$ 50% acetonitrile in 0.05 M triethylammonium acetate buffer solution. The yields of indocarbocyanine derivatives varied from 5 to 50% depending on the compound structure. The structure of the intermediates and the tar-

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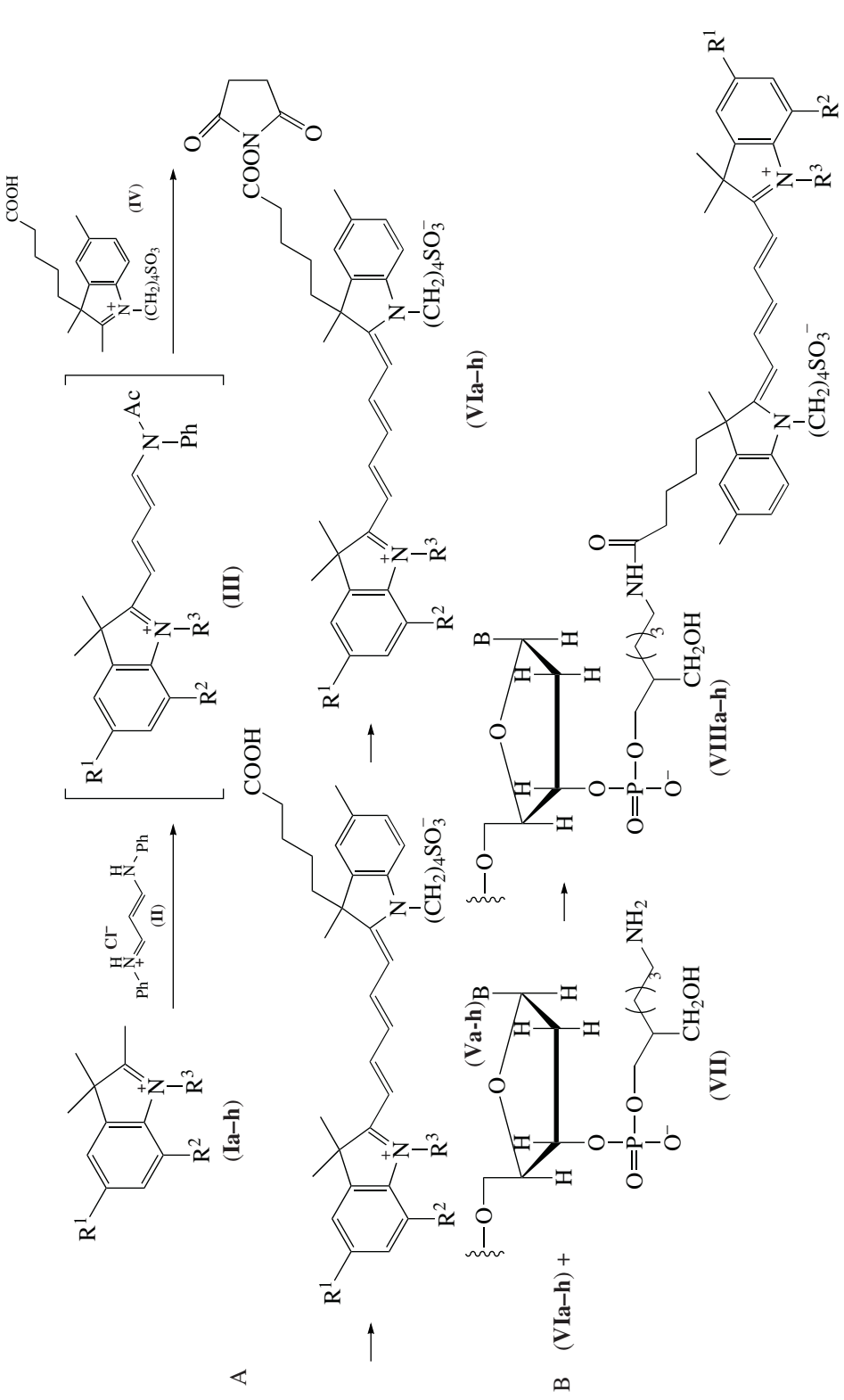

Compound	a	b	c	d	e	f	g	h
R^1	H	H	CH_3	CH_3	SO_3^-	SO_3^-	CH_3	CH_3
R^2	H	H	H	H	H	H	CH_3	CH_3
R^3	C_2H_5	$(CH_2)_4SO_3^-$	C_2H_5	$(CH_2)_4SO_3^-$	C_2H_5	$(CH_2)_4SO_3^-$	C_2H_5	$(CH_2)_4SO_3^-$
Yields of (**V**), %	18	29	27	23	18	50	17	5

The scheme of synthesis of asymmetric indocarbocyanine dyes.

Spectral characteristics of indocarbocyanine dyes*

Compound	λ_{max}^{abc}, nm	λ_{max}^{em}, nm	Δλ, nm	$\varepsilon \times 10^{-5}$, $M^{-1}cm^{-1}$	F	Relative efficiency of fluorescence		
						λ_{max}^{abc} / λ_{max}^{em}	655/690	635/670
(**Va**)	649	665	16	2.1 ± 0.03	0.10	0.492	0.104	0.313
	651	668	17	2.14 ± 0.03	0.14	0.736	0.276	0.469
(Vb)	650	666	16	2.17 ± 0.02	0.12	0.664	0.181	0.400
	651	670	19	2.33 ± 0.02	0.18	1	0.373	0.604
(Vc)	653	670	17	1.96 ± 0.02	0.09	0.459	0.185	0.263
	654	674	20	2.05 ± 0.02	0.16	0.755	0.442	0.409
(Vd)	654	671	17	1.95 ± 0.02	0.10	0.477	0.216	0.261
	655	675	20	2.08 ± 0.01	0.15	0.714	0.456	0.367
(Ve)	651	666	15	1.73 ± 0.02	0.08	0.336	0.098	0.222
	652	670	18	2.00 ± 0.01	0.12	0.558	0.239	0.349
(Vf)	651	668	17	1.71 ± 0.02	0.09	0.357	0.120	0.222
	652	672	20	2.07 ± 0.02	0.14	0.664	0.342	0.392
(Vg)	656	676	20	1.82 ± 0.03	0.07	0.328	0.164	0.172
	654	672	18	2.13 ± 0.03	0.11	0.564	0.382	0.292
(Vh)	654	671	17	1.49 ± 0.02	0.06	0.137	0.071	0.097
	655	676	21	1.56 ± 0.02	0.09	0.274	0.151	0.180

Note: * For each dye, the first line refers to the solution in PBS (10 mM potassium phosphate buffer solution, 0.9 % NaCl, pH 7.4); the second, to the solution in MeOH.

get compounds was confirmed with UV and ^{1}H NMR spectroscopy and MALDI TOF mass spectrometry.

The corresponding succinimide esters of (**VIa**)–(**VIh**) for labeling synthetic oligonucleotides (**VII**) bearing the amino linker with the C_6 spacer in aqueous solutions were synthesized (scheme).

The ultimate sensitivity of detection was determined for oligonucleotides (**VIIIa**)–(**VIIIh**) labeled with dyes (**Va**)–(**Vh**). When using the biochip fluorescent analyzer with a light source at 655 nm and registration at 690 nm, the ultimate sensitivity of detection was 3×10^{-18} mol.

Dyes (**Va**)–(**Vh**), when being used as labels for the oligonucleotide primers, were found not to inhibit the polymerase chain reaction (PCR). Labeled and the corresponding unlabeled primers participate in PCR with equal efficiency.

The molar extinction coefficient (ε) and the quantum yield of fluorescence (F) in MeOH and aqueous phosphate buffered saline (PBS, 10 mM potassium phosphate buffer, 0.9 % NaCl, pH 7.4) were found (the table). The highest values of these parameters have been found for dye (**Vb**).

The relative fluorescence efficiency was measured at the equal concentrations of dyes (**Va**)–(**Vh**) in MeOH and in PBS in absorption and fluorescence maxima at excitation wavelengths of 635 and 655 nm and registration wavelengths of 670 and 690 nm, respectively (table).

Compound (**Vd**) is the most appropriate in sensitivity for aqueous solutions in a range of 655 nm/690 nm, whereas compound (**Vb**) is the most appropriate in a range of 635 nm/670 nm.

The dyes synthesized can be recommended for the use in biological microchip technology, in scientific research and medicinal diagnostics.

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