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Synthesis and properties of novel asymmetric monomethine cyanine dyes as non-covalent labels for nucleic acids

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Abstract

Sixteen, novel monomethine cyanine dyes with benzothiazolo-[3.2-*a*]-pyrimidinium, 4-methyl-(5*H*)-pyrido-[1,2-*a*]-pyrimidinium, 1-cyano-4-methyl-(3*H*)-benzothiazolo-[3,2-*a*]-pyridinium and 4-methyl-8-methoxy-(8*H*)-benzothiazolo-[3,2-*a*]-pyrimidinium end groups have been synthesized. Their chemical structures and purity were confirmed by ^{1}H NMR and elemental analysis. The longest wavelength absorption maxima of the studied dyes are in the region 450–500 nm. Most of the dyes have very high molar absorptivities, usually over 100 000 l mol^{-1} cm^{-1}. The investigated dyes have low fluorescence in their free form but some of them become strongly fluorescent after binding to DNA.

Keywords: Cationic monomethine cyanine dyes; Nucleic acids

1. Introduction

Recent years have witnessed a growing interest in research into the bioapplication of fluorescent dyes. The current authors have investigated [1–4] novel representatives of monomethine cyanine dyes as non-covalently binding nucleic acid fluorogenic probes. In our previous work [1–4], we studied monomethine cyanine dyes based mainly on the Thiazole Orange (TO) and Oxazole Yellow (YO) chromophores. This paper concerns the synthesis of novel momomethine cyanines (with different chromophores other than TO and YO) and their properties as non-covalent nucleic acid probes.

2. Results and discussion

The intermediates **1a–1d**, **2** and **3** were prepared by known methods involving the condensation of acetylacetone with 2-amino-6-substituted-benzothiazoles [5,6] (for intermediates **1a–1d**), 2-aminopyridine [7] (for **2**) and 2-cyanomethylbenzothiazole [8] (for **3**) (Schemes 1 and 2).

Hartmann and Zhou [9] reported that mono-condensation dyes could be obtained using compounds **1a–1d** and that the 2-methyl group is the most reactive one. In a similar manner, we synthesized new dyes **5a–5h** by the condensation of intermediates **1a–1d** with compounds **4a** or **4b** (Scheme 3).

In the ^{1}H NMR spectra of dyes **5a–5h**, the singlets for CH_3 protons (in the range 2.03–3.80 ppm), NCH_3 protons (around 3.59–3.83 ppm) and CH proton (5.40–6.35 ppm) are quite characteristic for the dye structures (Table 1).

When an excess of the reagents **4a**, **4b** was used in the condensation reaction with **1a**, the bis-condensation products **6a** and **6b** were obtained (Scheme 4). Their structures were

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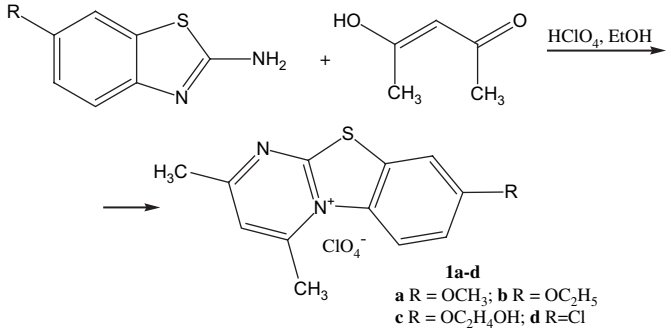


Scheme 1.

confirmed by ^{1}H NMR. The characteristic signals for CH_3 groups are at 2.90 ppm (for dye **6a**) and 3.49 ppm (for dye **6b**). The singlets for NCH_3 protons appeared at around 3.57–3.77 ppm and the singlets for CH protons are in the range 5.46–6.27 ppm (Table 1).

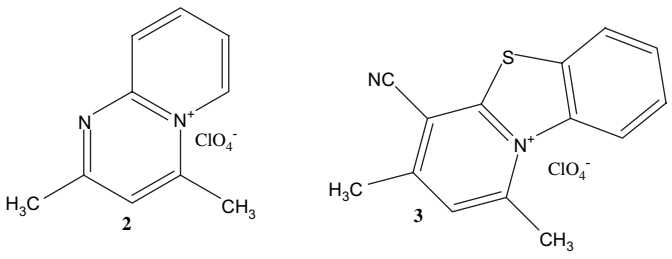
Dyes **8a** and **8b** were prepared by condensation of dye **2** in DMF and in the presence of triethylamine with 2-methylthio-4-methyloxazolo[4,5-*b*]pyridinium methosulphate **7** or 2-methylthio-3-methylbenzothiazolium methosulphate **4a** (Scheme 5, Tables 1 and 2). In the ^{1}H NMR spectra of these dyes the signals for CH_3 protons appeared at 2.58–2.63 ppm. The singlets for NCH_3 protons are in the range 3.75–4.27 ppm and those for CH protons appeared at around 6.22–6.28 ppm.

The synthesis of dyes **9a–9c** was performed by the reaction of 1-cyano-2,4-dimethylbenzothiazolo-[3,2-*a*]-pyrimidine-5-ium perchlorate **3** with dyes **7** or **4a** or **4b** (Scheme 6). The target compounds (**9a–9c**) were isolated with excellent yields (Table 2). Their structures were confirmed by the characteristic signals for CH_3 groups of protons at 2.99–3.22 ppm, for NCH_3 protons at 3.74–3.90 ppm and for CH protons in the range 5.51–6.1 ppm (Table 1).

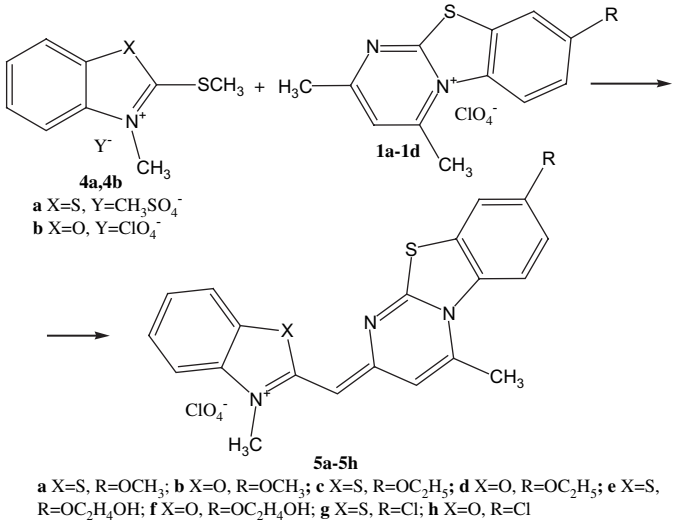
Finally dye **10** was synthesized by the reaction of intermediate **7** with compound **1a** in acetic anhydride in the presence of two equivalents of triethylamine (Scheme 7).

All dyes (**5a–5h**, **6a**, **6b**, **8a**, **8b**, **9a–9c** and **10**) of this series are new and their chemical structures and purity were confirmed by ^{1}H NMR (Table 1) and elemental analysis (Table 2). In the ^{1}H NMR spectra of dye **10**, the characteristic signals are as follows: for CH_3 protons at 3.29 ppm, for NCH_3 protons at 4.21 ppm and finally for CH at 5.47 ppm.

The longest wavelength absorption maxima of the studied asymmetric monomethine cyanine dyes in TE buffer (10 mM Tris–HCl, pH 7.0, and 1 mM EDTA) at room



Scheme 2.



Scheme 3.

temperature are in the region 450–500 nm (Table 3). The corresponding molar absorptivities range between 70 000 and 200 000 l mol^{-1} cm^{-1}. Most of the dyes exhibit very high molar absorptivities, usually over 100 000 l mol^{-1} cm^{-1}. Both the intensity and the position of the longest wavelength absorption maxima of the investigated dyes remain unchanged after binding to nucleic acids.

The investigated dyes exhibit low fluorescence in their free form but some of them become strongly fluorescent after binding to DNA (Table 3). The fluorescence maxima of the complexes are in the range 500 and 550 nm. It was found that the complexes of compound **5f** as well as of compounds **8a** and **8b** with dsDNA and ssDNA do not fluoresce. In some cases the fluorescence intensity of the dye–DNA complexes is 100- to 300-fold higher than those of the free dyes. The same holds especially for some representatives of the studied compounds **5a**, **5e**, **5g**, and **6a**. A coincidence of the fluorescence maxima of the complexes with either dsDNA or ssDNA has been observed. As a rule, the fluorescence intensity after binding to dsDNA is higher compared to that in the presence of ssDNA. The detection minimum using dye **5a** was 100 ng dsDNA in aqueous solution.

3. Experimental part

Melting points were determined on a Kofler apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Bruker 500 MHz instrument in DMSO-d_6. Absorption spectra were scanned on a Unicam 530 UV–vis spectrophotometer (1×10^{-5} mol/l in MeOH) and the corrected fluorescence spectra (excitation at 460 nm) were obtained on a Perkin–Elmer MPF44 spectrofluorimeter. The emission spectra were corrected using a standard tungsten lamp, while the excitation spectra were corrected with Rhodamine B.

Stock solutions were prepared by dissolving 1 mM of each dye in 1 ml DMSO and subsequent dilution with TE buffer (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA) to a final concentration of 1×10^{-7} M. The fish sperm dsDNA was

Table 1
Chemical structures and ^{1}H NMR spectra of dyes **5a–5h**, **6a**, **6b**, **8a**, **8b**, **9a–9c** and **10**

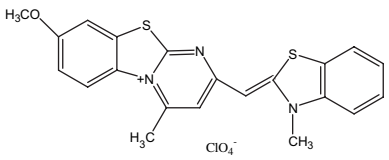
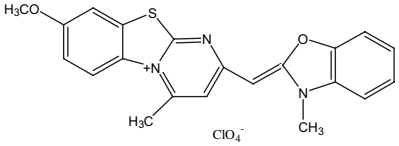
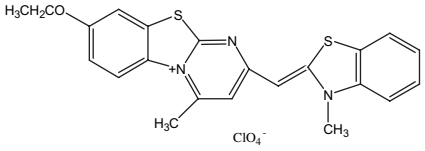
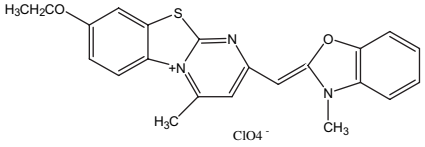
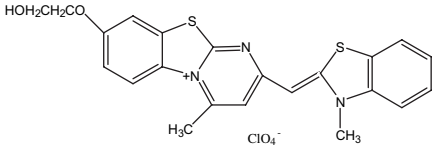
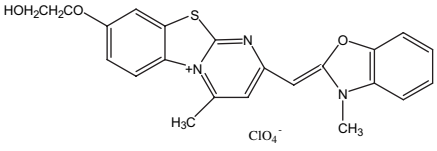
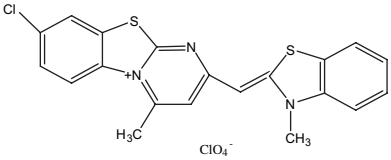
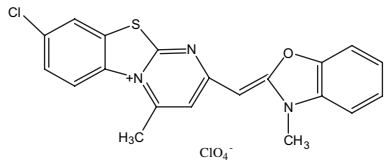
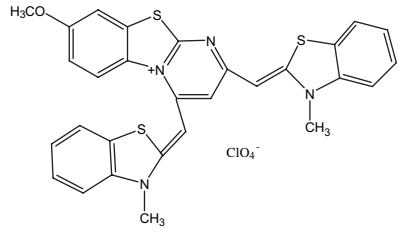
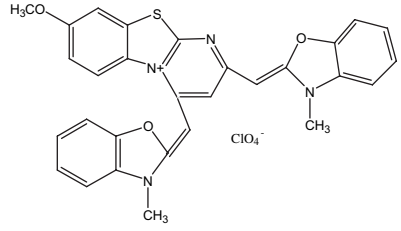
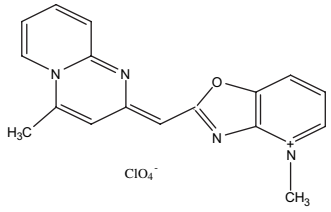
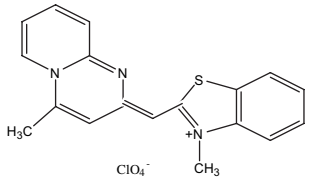
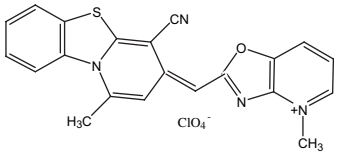
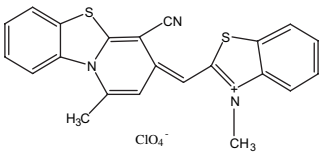
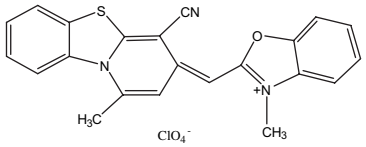
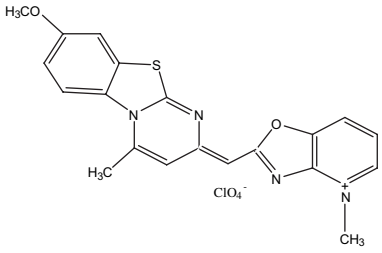
Dye no	Chemical structure/name	^{1}H NMR (DMSO-d_6, δ (ppm))
5a	 2-[(3-Methyl-(3*H*)-benzothiazol-2-ylidene)-methyl]-4-methyl-8-methoxybenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	2.93 s (3H, OCH_3), 3.80 s (3H, CH_3), 3.83 s (3H, NCH_3), 6.35 s (1H, CH), 7.06–8.16 m (8H, Ar)
5b	 2-[(3-Methyl-(3*H*)-benzooxazol-2-ylidene)-methyl]-4-methyl-8-methoxybenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	3.07 s (3H, OCH_3), 3.66 s (3H, CH_3), 3.81 s (3H, NCH_3), 5.58 s (1H, CH), 7.12–8.19 m (8H, Ar)
5c	 2-[(3-Methyl-(3*H*)-benzothiazol-2-ylidene)-methyl]-4-methyl-8-ethoxybenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	1.34 t (3H, OCH_2CH_3), 2.95 s (3H, CH_3), 3.83 s (3H, NCH_3), 4.02–4.05 m (2H, OCH_2), 6.34 s (1H, CH), 7.10–8.20 m (8H, Ar)
5d	 2-[(3-Methyl-(3*H*)-benzooxazol-2-ylidene)-methyl]-4-methyl-8-ethoxybenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	1.27 t (3H, OCH_2CH_3), 3.03 s (3H, CH_3), 3.59 s (3H, NCH_3), 3.80–3.92 m (2H, OCH_2), 5.40 s (1H, CH), 6.94–8.06 m (8H, Ar)
5e	 2-[(3-Methyl-(3*H*)-benzothiazol-2-ylidene)-methyl]-4-methyl-8-(2-hydroxyethoxy)-benzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	2.78 s (3H, CH_3), 3.59 br s (3H, NCH_3), 3.93 br s (2H, OCH_2), 4.26 br s (2H, OCH_2), 5.99 s (1H, CH), 6.80–7.90 m (8H, Ar)
5f	 2-[(3-Methyl-(3*H*)-benzooxazol-2-ylidene)-methyl]-4-methyl-8-(2-hydroxyethoxy)-benzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	2.03 s (3H, CH_3), 3.13 br s (2H, CH_2), 3.68 s (3H, NCH_3), 4.36 br s (1H, OH), 4.36 t (2H, $HOCH_2$), 5.72 s (1H, CH), 6.97–8.21 m (8H, Ar)
5g	 2-[(3-Methyl-(3*H*)-benzothiazol-2-ylidene)-methyl]-4-methyl-8-chlorobenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	2.89 s (3H, CH_3), 3.82 s (3H, NCH_3), 6.98 s (1H, CH), 7.25 s (1H, CH), 7.27–8.21 m (7H, Ar)

Table 1 (*continued*)

Dye no	Chemical structure/name	^{1}H NMR (DMSO-d_6, δ (ppm))
5h	 2-[(3-Methyl-(3*H*)-benzooxazol-2-ylidene)-methyl]-4-methyl-8-chlorobenzothiazol-[3.2-*a*]-pyrimidine-5-ium perchlorate	3.08 s (3H, CH_3), 3.71 s (3H, NCH_3), 5.67 s (1H, CH), 6.97–8.28 m (8H, Ar)
6a	 2,4-Bis-[(3-methyl-(3*H*)-benzthiazol-2-ylidene)-methyl-8-methoxybenzothiazol-[2,3-*a*]pyrimidine-5-ium perchlorate	2.90 s (3H, CH_3), 3.74 s (3H, NCH_3), 3.77 s (3H, NCH_3), 5.72 s (1H, CH), 6.27 s (1H, CH), 7.02 s (1H, CH), 7.05–8.09 m (11H, Ar)
6b	 2,4-Bis-[(3-Methyl-(3*H*)-benzooxazol-2-ylidene)-methyl-8-methoxybenzothiazol-[2,3-*a*]pyrimidine-5-ium perchlorate	3.49 s (3H, CH_3), 3.57 s (3H, NCH_3), 3.75 s (3H, NCH_3), 5.46 s (1H, CH), 5. 72 s (2H, 2 × CH), 6.98–8.34 m (11H, Ar)
8a	 2-[(4-Methyl-(5*H*)-pyrido-[1,2-*a*]-pyrimidine-2-ylidene)-methyl]-4-methyloxazolo[4,5-*b*]pyridinium perchlorate	2.63 s (3H, CH_3), 3.75 s (3H, NCH_3), 6.22 s (1H, CH), 7.20 s (1H, CH), 7.22–8.51 m (6H, Ar)
8b	 2-[(4-Methyl-(5*H*)-pyrido-[1,2-*a*]-pyrimidine-2-ylidene)-methyl]-3-methylbenzothiazolium perchlorate	2.58 s (3H, CH_3), 4.21 s (3H, NCH_3), 6.28 s (1H, CH), 7.30–9.12 m (8H, Ar)
9a	 2-[(1-Cyano-4-methyl-(3*H*)-benzothiazolo-[3,2-*a*]-pyrido-2-ylidene)-methyl]-4-methyloxazolo-[4,5-*b*]-pyridinium perchlorate	2.99 s (3H, CH_3), 3.77 s (3H, NCH_3), 5.51 s (1H, CH), 7.01–8.22 m (8H, Ar)

(*continued on next page*)

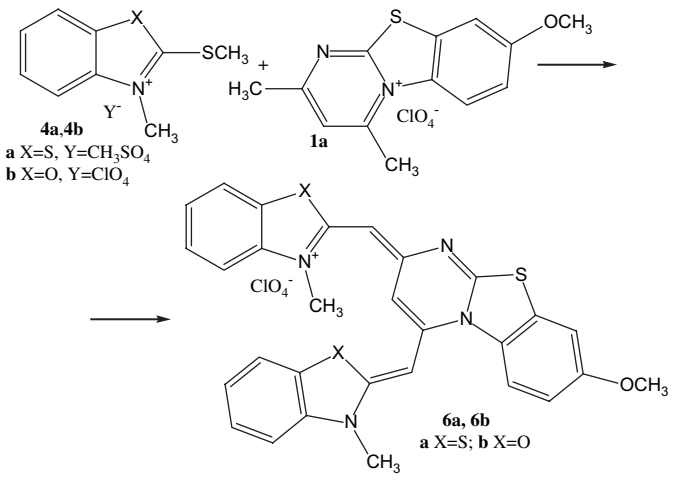
Table 1 (*continued*)

Dye no	Chemical structure/name	^{1}H NMR (DMSO-d_6, δ (ppm))
9b		3.22 s (3H, CH_3), 3.90 s (3H, NCH_3), 6.1 s (1H, CH), 7.43–8.48 m (9H, Ar)
	2-[(1-Cyano-4-methyl-(3*H*)-benzothiazolo-[3,2-*a*]-pyrido-2-ylidene)-methyl]-3-methylbenzothiazolium perchlorate	
9c		3.22 s (3H, CH_3), 3.74 s (3H, NCH_3), 5.36 s (1H, CH), 7.93 s (1H, CH), 7.40–8.48 m (8H, Ar)
	2-[(1-Cyano-4-methyl-(3*H*)-benzothiazolo-[3,2-*a*]-pyrido-2-ylidene)-methyl]-3-methylbenzooxazolium perchlorate	
10		3.12 s (3H, CH_3), 3.29 s (3H, CH_3), 4.21 s (3H, NCH_3), 5.47 s (1H, CH), 7.39–8.37 m (7H, Ar)
	2-[(4-Methyl-8-methoxy-(8*H*)-benzothiazolo-[3,2-*a*]-pyrimido-2-ylidene)-methyl]-4-methyloxazolo-[4,5-*b*]-pyridinium perchlorate	

purchased from Sigma (USA). The ssDNA was obtained after thermal denaturation of dsDNA. The intermediates **1a–1d** [5,6], **2** [7], **3** [8], **4a** and **4b** [10] were prepared according to the known procedures [5–8,10].

3.1. Preparation of dyes *5a–5h*

The corresponding compounds (0.001 mol) **1a–1d** and 0.0011 mol of dyes **4a** or **4b** were finely powdered together and suspended in 20 ml acetic anhydride in a reaction vessel, equipped with electromagnetic stirrer. Double excess of triethylamine (0.002 mol) was added dropwise for about a minute and the reaction mixture was stirred at room temperature for 4 h. The formed precipitate was suction filtered, washed with diethyl ether and air-dried.



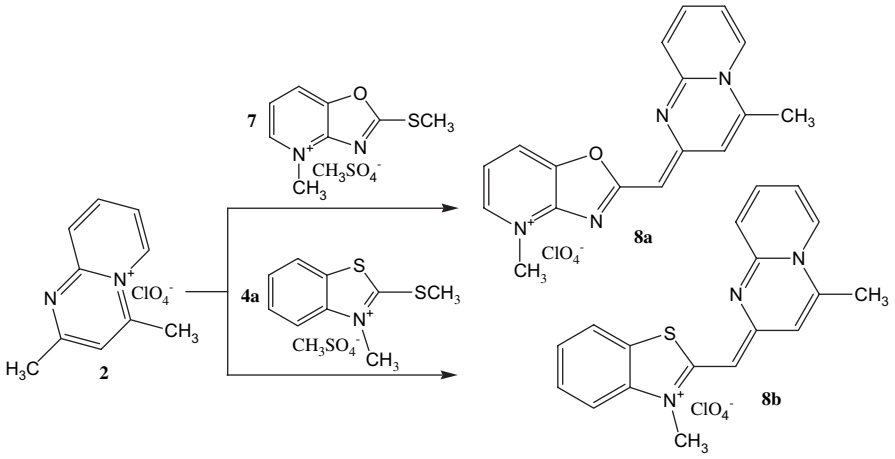
Scheme 4.

3.2. Preparation of dyes *6a and 6b*

2,4-Dimethyl-(8-methoxybenzothiazol-[3,2-*a*])pyrimidin-5-ium perchlorate **1a** (1.4 g, 0.004 mol) and 3.7 g (0.012 mol) 2-methylthio-3-methyl-benzothiazolium perchlorate **4a** or 3.35 g (0.012 mol) 2-methylthio-3-methyl-benzooxazolium methosulphate **4b** were suspended in 20 ml DMF and 2 ml (0.014 mol) triethylamine was added. The reaction mixture was refluxed for 10 min. After one night at room temperature the formed precipitate was suction filtered, washed with ethanol and air-dried.

3.3. Preparation of dyes *8a and 8b*

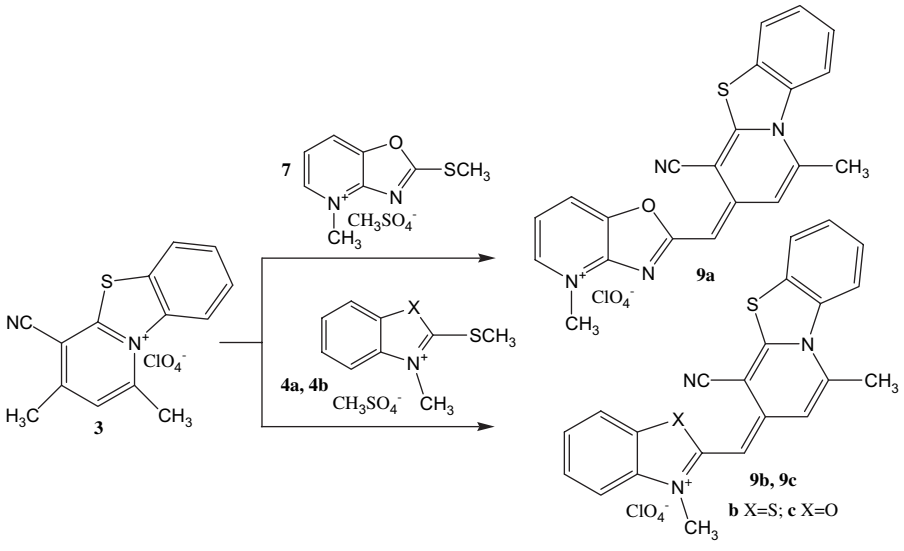
2,4-Dimethylpyridinio-[1,2-*a*]-pyrimidinium perchlorate **2** (1.0 g, 0.00386 mol), 1.13 g (0.00386 mol) 2-methylthio-4-methyl-oxzolo-[4,5-*b*]-pyridinium methosulphate **7** or 1.2 g (0.00386 mol) 2-methylthio-3-methylbenzothiazolium methosulphate **4a** was suspended in 10 ml acetic anhydride, and



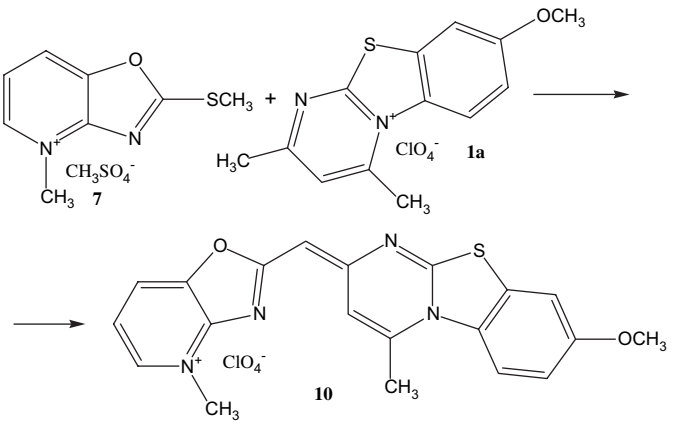
Scheme 5.

Table 2
Melting points (m.p.), yields and elemental analysis of dyes **5a–5h**, **6a**, **6b**, **8a**, **8b**, **9a–9c** and **10**

Dye no	M.p. (°C)	Yield (%)	Molecular formula (Mw)	Elemental analysis Calcd./found		
				C	H	N
5a	>300	82	$C_{21}H_{18}ClN_3O_5S_2$ (491.5)	51.3/51.0	3.7/4.1	8.6/8.7
5b	267–269	87	$C_{21}H_{18}ClN_3O_6S$ (475.5)	53.0/52.7	3.8/3.8	8.8/8.9
5c	>300	88	$C_{22}H_{20}ClN_3O_5S_2$ (546.5)	52.7/52.5	4.2/4.1	10.25/10.1
5d	>300	99	$C_{22}H_{20}ClN_3O_6S$.0.5 CH_3CN (510)	–	–	9.6/9.5
5e	282–284	72	$C_{22}H_{20}ClN_3O_6S_2$. (521.5)	50.62/50.9	3.8/4.2	8.05/8.2
5f	245–247	73	$C_{22}H_{20}ClN_3O_7S$. (505.5)	52.2/51.9	4.0/4.3	8.3/8.5
5g	>300	74	$C_{20}H_{15}Cl_2N_3O_4S_2$. (469)	48.4/48.3	3.0/2.6	8.5/8.9
5h	>300	77	$C_{20}H_{15}Cl_2N_3O_5S$.0.5 CH_3CN (500.5)	50.35/49.7	3.3/3.5	9.8/10.2
6a	282–284	55	$C_{29}H_{23}ClN_4O_5S_3.3H_2O$ (692.5)	50.25/50.2	4.2/4.05	8.1/8.45
6b	>300	43	$C_{29}H_{23}ClN_4O_7S$.0.5 CH_3CN (627)	57.4/57.0	3.8/4.3	9.2/9.8
8a	>310	44	$C_{17}H_{15}ClN_4O_5$ (390.5)	–	–	14.3/14.0
8b	308–310	74	$C_{18}H_{16}ClN_3O_4S$ (405.5)	53.3/53.6	3.95/4.1	10.4/10.7
9a	>310	81	$C_{21}H_{15}ClN_4O_5S$.0.5 (479.5)	52.55/52.6	3.3/2.8	11.7/11.4
9b	>310	88	$C_{22}H_{16}ClN_3O_4S_2$ (485.5)	–	–	8.65/8.3
9c	>310	95	$C_{22}H_{16}ClN_3O_5S$.0.5 H_2O (478.5)	55.2/55.4	3.55/3.3	8.8/8.9
10	>310	65	$C_{20}H_{17}ClN_4O_6S$ (490.2)	–	–	12.4/12.3



Scheme 6.



Scheme 7.

1.06 ml (0.0076 mol) triethylamine was added. The reaction was led as described in the first procedure.

3.4. Preparation of dyes *9a–9c*

1-Cyano-2,4-dimethylbenzothiazolium-[3,2-*a*]-pyridine-5-ium perchlorate **3** (1.16 g, 0.0034 mol), 1.0 g (0.0034 mol) 2-methylthio-4-methyloxazolo-[4,5-*b*]-pyridinium methosulphate **7** or 1.1 g (0.0034 mol) 2-methylthio-3-methylbenzothiazolium methosulphate **4a**, or 0.95 g (0.0034 mol) 2-methylthio-3-methylbenzooxazolium perchlorate **4b** were suspended in 20 ml acetic anhydride, and 1 g (0.0072 mol) triethylamine was added. The reaction was lead as described in the first procedure.

3.5. Preparation of dye *10*

2,4-Dimethyl-(8-methoxybenzooxazol-[3,2-*a*])pyrimidin-5-ium perchlorate **1a** (1.3 g, 0.0034 mol), 1.0 g (0.0034 mol) 2-methylthio-4-methyl-oxzolo-[4,5-*b*]-pyridinium methosulphate **7** were suspended in 15 ml acetic anhydride, and 1.0 g (0.0072 mol) triethylamine was added. The reaction was led as described in the first procedure.

4. Conclusions

Sixteen novel monomethine cyanine dyes with different chromophores other than TO and YO were prepared according easy and convenient synthetic procedure. The derived cyanines were isolated with good to excellent yields.

The investigated dyes have low fluorescence in their free form but some of them become strongly fluorescent after binding to DNA. In some cases the fluorescence intensity of the dye–DNA complexes is 100- to 300-fold higher than those of the free dyes.

The fluorescence intensity after binding to dsDNA is higher compared to that in the presence of ssDNA; this fact gives the possibility for the discrimination of ds from ssDNA.

Table 3
Absorption maxima, λ_{max} (nm), molar absorptivity, ε (l mol^{-1} cm^{-1}), fluorescence maxima, λ_F (nm) of the studied dyes in TE buffer (dye concentration 1×10^{-6} M) as well as of the complexes with dsDNA and ssDNA

Dye no	λ_{max}/nm (ε/l mol^{-1} cm^{-1})	Free dye (λ_F)	Dye + dsDNA[a] (λ_F)	Dye + ssDNA[b] (λ_F)	Fluorescence enhancement	
					Dye + dsDNA	Dye + ssDNA
5a	449sh, 475 (77 000, 135 000)	630	499	497	360×	180×
5b	434sh, 452 (90 000, 124 000)	545	481	480	50×	35×
5c	450, 474 (83 200, 139 000)	495	499	497	20×	10×
5d	429, 453 (102 130, 144 300)	518	480	479	40×	20×
5e	448sh, 474 (71 400, 118 700)	599	498	496	250×	160×
5f	434, 448 (101 100, 135 400)	482	–	–	–	–
5g	451, 476 (74 000, 116 000)	500	499	498	100×	50×
5h	434sh, 451 (109 200, 124 300)	490	480	480	20×	30×
6a	449sh, 475 (106 000, 186 000)	586	498	497	100×	100×
6b	414, 500 (89 500, 89 500)	620	544	538	30×	13×
8a	484 (114 300)	–	–	–	–	–
8b	460 (73 100)	511	–	–	–	–
9a	440sh, 461 (129 300)	512	515	514	5×	2×
9b	471sh, 496 (85 400, 134 000)	525	521	520	20×	20×
9c	469sh, 500 (69 000, 194 000)	495	493	492	30×	50×
10	455sh, 495 (62 500, 158 300)	519	521	520	10×	15×

(–) – No fluorescence.
[a] Fish sperm dsDNA at a concentration of 2×10^{-6} M.
[b] Fish sperm ssDNA at a concentration of 2×10^{-6} M.

Additionally it could be pointed out that dye **5a** is very sensitive; its detection minimum is 100 ng dsDNA in aqueous solution.

In conclusion, we consider that some of these dyes are completely suitable for use as non-covalent fluorescent DNA probes.

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