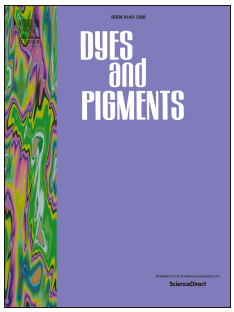
# Accepted Manuscript

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PII: S0143-7208(16)30080-8

DOI: 10.1016/j.dyepig.2016.03.021

Reference: DYPI 5146

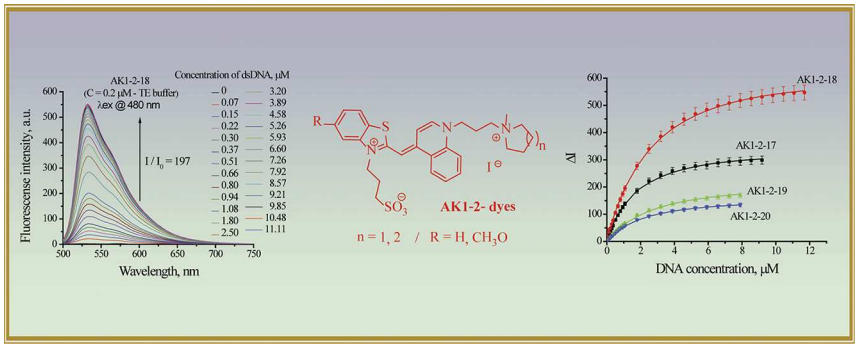
To appear in: *Dyes and Pigments*

Received Date: 28 January 2016

Revised Date: 11 March 2016

Accepted Date: 13 March 2016

Please cite this article as: Kurutos A, Ryzhova O, Trusova V, Tarabara U, Gorbenko G, Gadjev N, Deligeorgiev T, Novel asymmetric monomethine cyanine dyes derived from sulfobetaine benzothiazolium moiety as potential fluorescent dyes for non-covalent labeling of DNA, *Dyes and Pigments* (2016), doi: 10.1016/j.dyepig.2016.03.021.



# Novel asymmetric monomethine cyanine dyes derived from sulfobetaine benzothiazolium moiety as potential fluorescent dyes for non-covalent labeling of DNA

Atanas Kurutos[a*], Olga Ryzhova[b], Valeriya Trusova[b#], Ulyana Tarabara[b], Galyna Gorbenko[b], Nikolai Gadjev[a], Todor Deligeorgiev[a]

[a]*Sofia University "St. Kliment Ohridski", Faculty of Chemistry and Pharmacy, 1, blv. J. Bourchier, 1164 Sofia, Bulgaria*

[a]*Department of Nuclear and Medical Physics, V.N. Karazin Kharkiv National University, 4 Svobody Sq., Kharkiv 61022, Ukraine*

Addresses for correspondence:

[*]E-mail: ohtak@chem.uni-sofia.bg; tel: (+3592) 8161269; fax: (+3592) 9625438

[#]E-mail: valtrusova@yahoo.com; tel: (+38057) 3438244; fax: (+38057) 7544746

***Abstract:*** Four novel monomethine cyanine dyes derived from a condensation reaction between N-quaternary 2-thiomethylbenzothiazolic, and 4-methylquinolinuim choromophores have been synthesized and characterized by $^{1}$H-NMR, APT carbon NMR, ESI mass spectrometry, IR, absorption and fluorescence spectroscopy. The main synthetic approach for the target cyanines involves variations on both the substituent on the aromatic ring of the benzothiazolium derivative, as well as the size of the component yielding the additional positive charge linked to the quinolinium moiety. The interaction between novel fluorophores and dsDNA has been studied. The dyes were found to have negligible fluorescence in the buffer solution, but exhibited a significant emission increase upon binding to dsDNA. The binding parameters of cyanine dyes have been determined by fluorescence titration using the McGhee & von Hippel site-excluded model. The results obtained are consistent with an intercalative binding mode between cyanines and DNA.

**Keywords:** monomethine cyanine dyes; fluorescence; DNA markers; binding parameters

## 1. Introduction

The design of extrinsic fluorescent probes and labels for quantitative sensing, rapid visualization and characterization of nucleic acids has been attracting considerable attention in recent years and dictating by the very weak intrinsic emission of DNA and RNA. A wide range of fluorophores, including Hoechst 33258 [1], styrylcyanine dye DSMI [2], acridine orange [3], DAPI (4',6-diamidino-2-phenylindole) [4], ethidium bromide [5] have been employed for DNA identification. However, the presented above dyes are generally highly emissive in solution thereby creating a strong background signal. Moreover, the applicability of some commercially available dyes, like ethidium bromide, which is considered as being mutagenic, is complicated by some environmental concerns [6,7]. Therefore, significant research efforts are being directed at the developing and investigating new fluorescent compounds. To date the majority of fluorescent agents commonly used for DNA recognition belongs to the cyanine group. Due to their excellent staining properties, cyanine dyes are generally used for sizing and purification of DNA fragments [8, 9], fluorescent microscopy applications [10], DNA damage detection [11], microarray-based expression analysis [12], DNA sequencing [13,14], DNA intercalation bioanalytical assays as well as for staining of nucleic acids in electrophoresis [8,15-18]. The applicability of this class of fluorophores is based on the fact that these dyes display high affinity for nucleic acids double strands and the huge enhancement in emission upon binding to DNA. The nature of sharp increase in fluorescence is supposed to originate from the loss of mobility around the methine bridge between the two heterocyclic moieties as a result of the cyanine - DNA interaction [19].

Cyanine dyes are photosensitive compounds possessing two quaternized, nitrogen-containing, heterocyclic structures, which are linked through a polymethine bridge [20]. This dual structure of cyanine molecules gives the impetus for three predominant non-covalent

binding modes: i) intercalation between adjacent base pairs, ii) minor groove binding, and iii) electrostatic interaction of highly positively charged molecules with nucleotide phosphate backbone.The direct mode of the cyanine-DNA binding substantially vary with the minor change of the heterocyclic moieties in cyanine dye structure, multiplicity of the methine bridge, as well as with charge and bulkiness of attached substituents [21,22]. The presence of methine bridge, possessing conformational flexibility, allows the cyanine molecule to adjust to the DNA groove. It has been shown, that the majority of groove binders associate with DNA helix through forming hydrogen bonds to the base pairs, stabilizing in such a manner structure of the dye-DNA complex structure [23]. Besides, cyanine dyes devoid of hydrogen bond donors have been found bind to the minor groove also as dimers [24]. However, the numerous studies indicate that monomethine cyanine dyes, being a planar aromatic ring system predominantly belong to the group of intercalators [17, 25]. This type of binding results from the incorporation of a dye planar aromatic moiety between the DNA base pairs, followed by unwinding and lengthening of the DNA helix.

During the last decades we have investigated novel derivatives of monomethine cyanine dyes based mainly on thiazole orange and oxazole yellow chromophores for non-covalent labeling of DNA [17,21]. In a continuation of our interest in DNA reporter molecules, herein we directed our efforts towards the synthesis and evaluating the DNA-binding ability of four novel monomethine cyanine dyes differing by their structure and spectroscopic behaviour. Our goals was twofold: i) to synthesize monomethine cyanines varying the substituent on the aromatic ring of the benzothiazolium derivative, as well as the size of the component yielding the additional positive charge linked to the quinolinium moiety; ii) using fluorescence spectroscopy technique to assess the sensitivity of novel monomethine dyes to dsDNA. We have shown that novel fluorophores associate strongly with dsDNA presumably by intercalation binding mode

## 2. Materials and methods

### *2.1.Materials*

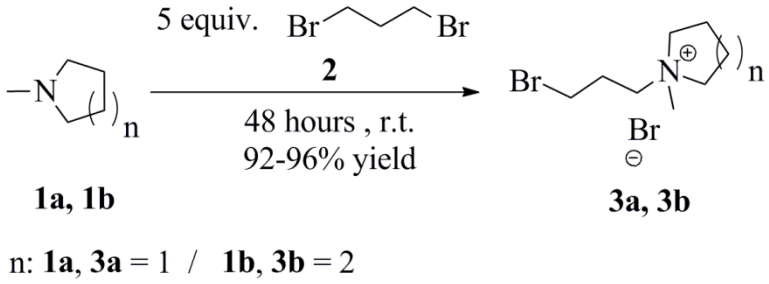
All starting materials and solvents required for the synthesis of the cyanine dyes were purchased from Sigma-Aldrich, Organica Feinchemie GmbH Wolfen, Fluka, Alfa-Aesar, TCI Europe, Deutero GmbH. Compounds 6a and 6b were commercially purchased from Organica Feinchemie GmbH Wolfen, and used without any further purification. The solvents used for the spectrophotometric and spectrofluorimetric analyses were purchased form Macron Fine Chemicals TM. Salmon testes DNA was obtained from Sigma-Aldrich. Tris-HCl and EDTA were obtained from Sigma (St.Louis, MO, USA). All other starting materials and solvents were commercial products of analytical grade and were used without further purification.

### *2.2. Analysis methods and equipment*

The title monomethinecyanine dyes were purified by recrystalisation from methanol and their chemical structures were evaluated by $^{1}$H-NMR and APT-NMR spectra recorded on a Brucker Avance III HD 500 MHz, Bruker Avance 300 and Bruker Avance 600 MHz in DMSO-$d_6$ at 25 $^{o}$C. The chemical shifts were reported in ppm in δ-values with respect to tetramethylsilane (TMS), or the deuterated solvent peak as an internal reference. Coupling constants J are expressed in Hz. IR spectra were recorder on Specord 85ER in nujol. ESI mass spectra were obtained on TSQ Quantum Access Max Masspectrometer – Triple Quadrupol in methanol/water (80:20 % v/v). The melting points were determined on a Kofler bench and are uncorrected. Absorption spectra were recorded on a Cecil Aurius CE 3021 UV-Vis spectrophotometer at room temperature. Fluorescence measurements were performed in 10 mM Tris-HCl, 0.5 mM EDTA buffer, pH 7.4 at room temperature using 10-mm path-length quartz cuvettes in a Perkin Elmer LS45 fluorescence spectrometer.

*2.3. Preparation of intermediates 3a, 3b*

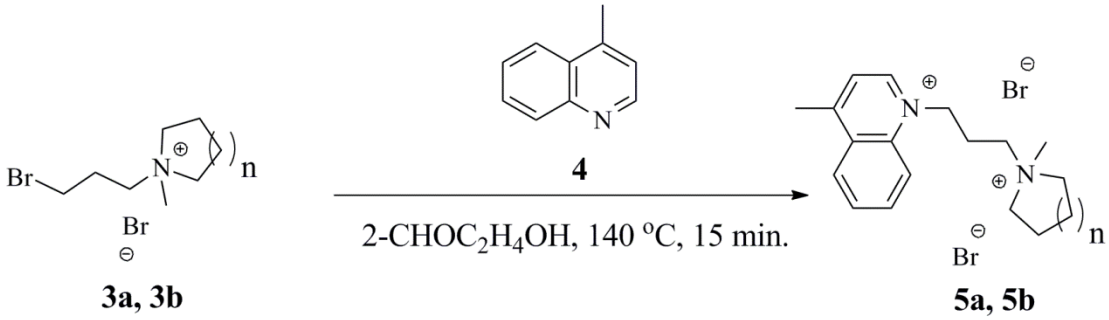
A large excess of 1,3-dibromopropane **2** (15.29 mL, 0.15 mol) was added dropwise to N-methyl pyrrolidine **1a** (3.12 mL, 0.03 mol) or N-methyl piperidine **1b** (3.65 mL, 0.03 mol) and the reaction vessel was stored in a dark place [26,27] for 48 hours (Scheme 1). The precipitated products **3a**, **3b** were collected by filtration and stored in a desiccator. Their structures were evaluated by $^{1}$H-NMR and $^{13}$C-NMR spectra.




**Scheme 1.** Preparation of intermediates 3a and 3b.

*2.4.Preparation of N-quaternary 4-methyl quinolinium derivatives 5a, 5b*

4-methylquinoline **4** (1.45 mL, 11 mmol) was mixed with the corresponding mono N-quaternary salt **3a** or **3b**, and the reaction mixture was heated to boiling point for 15 minutes with stirring in 2-methoxyethanol (10 ml) (Scheme 2). The products were isolated as semi-solids, and used without further purification in the next step yielding the target monomethine cyanine dyes.

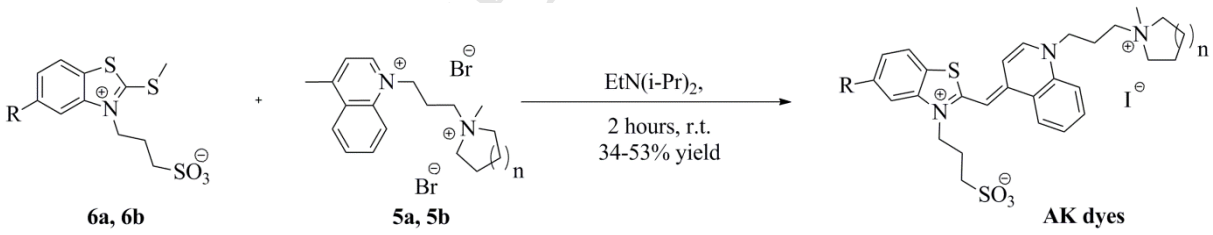



n: **3a**, **5a** = 1 / **3b**, **5b** = 2

**Scheme 2.** Preparation of intermediates 5a and 5b.

*2.5.Synthesis of the cyanine dyes*

In a reaction vessel equipped with magnetic stirrer, equimolar amounts of intermediate **5a** or **5b** (3 mmol) and **6a** or **6b** (3 mmol) respectively were dissolved in 10 ml methanol. N-Ethyldiisopropylamine (0.52 mL, 3 mmol) was added dropwise, and the reaction mixture was vigorously stirred at room temperature for 2 hours (Scheme 3), followed by addition of diethyl ether (20 ml) in order to precipitate the dyes.




n: **3a**, **5a** = 1 / **3b**, **5b** = 2
R: **6a**, **AK1-2-17**, **AK1-2-18** = H / **6b**, **AK1-2-19**, **AK1-2-20** = $CH_3O$

**Scheme 3.** Synthetic approach to asymmetric monomethine cyanine dyes.

The reactions were held in a well ventilated hood, due to the evolution of methyl mercaptan. Upon completion of the reaction, the product was dissolved in methanol and transferred to a beaker containing 200ml of an aqueous solution of potassium iodide. The

precipitated dye was suction filtered and air dried. The corresponding yields and melting point temperatures of the dyes are given in Table 1**.**

**Table 1.** Chemical structures of the monomethine cyanine dyes

| Dye | R | n | Yield (%) | M.p. ($^{o}$C) |
|---|---|---|---|---|
| AK1-2-17 | H | 1 | 34 | 270-272 |
| AK1-2-18 | H | 1 | 46 | 261-263 |
| AK1-2-19 | $CH_3O$ | 2 | 44 | 263-266 |
| AK1-2-20 | $CH_3O$ | 2 | 53 | 241-245 |

1-(3-bromopropyl)-1-methylpyrrolidin-1-ium bromide (3a); (white solid), yield = 92 %, m.p. = 173-175 $^{o}$C, lit. m.p. = 177-179 $^{o}$C [28]; $^{1}$H-NMR (DMSO-$d_6$, 500 MHz) δ/ppm: 2.06-2.12 (4H, m, 2 x $CH_2$), 2.28-2.34 (2H, m, $CH_2$), 3.03 (3H, s, $CH_3$-N), 3.45-3.49 (4H, m, 2 x $CH_2$), 3.53-3.59 (4H, m, 2 x $CH_2$), 3.60 (2H, t, J 6.5, $CH_2$-N); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz) δ/ppm: 21.5; 26.9; 31.2; 48.1; 62.1; 64.1;

1-(3-bromopropyl)-1-methylpiperidin-1-ium bromide (3b); (white solid), yield = 96 %, m.p. = 196-199 $^{o}$C, lit. m.p. = 190-193 $^{o}$C [28]; $^{1}$H-NMR (DMSO-$d_6$, 500 MHz) δ/ppm: 1.52-1.57 (4H, m, 2 x $CH_2$), 1.77-1.80 (4H, m, 2 x $CH_2$), 2.24-2.30 (2H, m, $CH_2$), 3.05 (3H, s, $CH_3$-N), 3.36-3.39 (4H, m, 2 x $CH_2$), 3.46-3.49 (4H, m, 2 x $CH_2$), 3.61 (2H, t, J 6.4, $CH_2$-N); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz) δ/ppm: 19.8; 21.0; 25.1; 31.2; 47.8; 60.6; 61.2;

4-methyl-1-(3-(1-methylpyrrolidin-1-ium-1-yl)propyl)quinolin-1-ium hexafluorphosphate (5a); (pale brown solid), obtained as dibromide, but anion was exchanged in order to obtain solid product. Yield = 73 %, m.p. = 219-226 $^{o}$C; $^{1}$H-NMR (DMSO-$d_6$, 500 MHz) δ/ppm: 2.08-2.12 (6H, m, 3 x $CH_2$), 2.42-2.48 (2H, m, $CH_2$), 2.97-3.03 (8H, m, 2 x $CH_3$ + $CH_2$), 5.03 (2H, t, J 7.4, $CH_2$), 8.07-8.11 (2H, m, 2 x ArH), 8.28-8.31 (1H, m, ArH), 8.57 (1H, d, J 8.3,

ArH), 8.63 (1H, d, J 8.9, ArH), 9.35 ( 1H, d, J 5.7, ArH); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz) δ/ppm: 20.2; 21.5; 21.6; 24.4; 26.8; 48.3; 54.3; 60.2; 64.4; 119.6; 123.2; 127.7; 129.4; 130.2; 135.8; 137.3; 149.1; 159.6; $^{19}$F-NMR (DMSO-$d_6$, 470 MHz) δ/ppm: -69.4; -70.9;

4-methyl-1-(3-(1-methylpiperidin-1-ium-1-yl)propyl)quinolin-1-ium hexafluorphosphate (5b); (pale brown semi-solid) product was found to be highly hygroscopic. It's structure is evaluated on dyes AK1-2-18 and AK1-2-20.

(E)-3-(2-((1-(3-(1-methylpyrrolidin-1-ium-1-yl)propyl)quinolin-4(1H)-ylidene)methyl) benzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate iodide (AK1-2-17); **(**red crystals), yield = 14 %, M.p. = 270-272 $^{o}$C; IR (nujol, cm$^{-1}$): 570, 740, 760, 880, 1040, 1150, 1200, 1290, 1320, 1380, 1460, 1500, 1600, 1620; $^{1}$H NMR (DMSO-$d_6$, 600 MHz) δ/ppm: 2.09-2.19 (6H, m, 3 x $CH_2$), 2.31-2.37 (2H, m, $CH_2$), 2.72 (2H, t, J 6.1, $CH_2$), 3.00 (3H, s, $CH_3$-N), 3.47-3.54 (6H, m, 3 x $CH_2$), 4.61 (2H, t, J 7.4, $CH_2$), 4.83 (2H, t, J 7.6, $CH_2$), 7.18 (1H, s, CH), 7.36 (1H, d, J 7.2, ArH), 7.40-7.43 (1H, m, ArH), 7.62 (1H, ddd, J 1.2, 7.3, 8.4, ArH), 7.68 (1H, ddd, J 0.7, 7.1, 8.0, ArH), 7.89 (1H, d, J 8.4, ArH), 7.97 (1H, ddd, J 1.0, 7.2, 8.5, ArH), 8.01 (1H, dd, J 1.0, 8.0, ArH), 8.13 (1H, d, J 8.7, ArH), 8.58 (1H, d, J 7.2, ArH), 9.07 (1H, dd, J 1.0, 8.9, ArH); APT-NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 21.1; 23.2; 23.4; 44.9; 47.5; 47.7; 50.9; 59.8; 63.7; 88.4; 107.9; 113.0; 117.4; 122.8; 124.0; 124.3; 124.5; 126.7; 127.0; 128.2; 133.2; 136.8; 139.8; 144.0; 149.0; 159.6; ESI: m/z: Found [M+] 524.46 $C_{28}H_{34}N_3O_3S_2$+ requires [M+] 524.20; UV/VIS (methanol): $\lambda_{max}$ = 507 nm, ε= 84 000 l.mol$^{-1}$.cm$^{-1}$, $\lambda_{fl}$ = 567 nm**;**

(E)-3-(2-((1-(3-(1-methylpiperidin-1-ium-1-yl)propyl)quinolin-4(1H)-ylidene)methyl) benzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate iodide (AK1-2-18); (red crystals), yield = 46 %, M.p. = 261-263 $^{o}$C; IR (nujol, cm$^{-1}$): 550, 740, 880, 1040, 1150, 1200, 1300, 1330, 1380, 1460, 1510, 1600; $^{1}$H NMR (DMSO-$d_6$, 600 MHz) δ/ppm: 1.56 (2H, quint, J 5.7, $CH_2$), 1.81 (4H, q, J 6.2, 2 x $CH_2$), 2.12-2.18 (2H, m, $CH_2$), 2.27-2.33 (2H, m, $CH_2$), 2.71 (2H, t, J 6.1,

$CH_2$), 3.00 (3H, s, $CH_3$-N), 3.33 (4H, q, J 5.9, 2 x $CH_2$), 3.51-3.55 (2H, m, $CH_2$), 4.62 (2H, t, J 7.4, $CH_2$), 4.83 ( 2H, t, J 7.6, $CH_2$), 7.20 (1H, s, CH), 7.38 (1H, d, J 7.3, ArH),7.43 (1H, m, ArH), 7.63 (1H, ddd, J 1.2, 7.4, ArH), 7.70 (1H, ddd, J 0.8, 7.0, 8.1, ArH), 7.89 (1H, d, J 8.4, ArH), 7.99 (1H, ddd, J 1.1, 7.0, 8.3, ArH), 8.02 (1H, dd, J 1.0, 7.9, ArH), 8.14 (1H, d, J 8.7, ArH), 8.59 (1H, d, J 7.3, ArH), 9.10 (1H, dd, J 1.2, 9.0, ArH); APT-NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 19.2; 20.5; 21.6; 23.2; 44.9; 47.5; 50.8; 60.3; 88.4; 107.9; 113.0; 117.4; 122.8; 124.0; 124.3; 124.5; 126.7; 126.9; 128.2; 133.2; 136.8; 139.8; 144.0; 148.9; 159.6; ESI: m/z: Found [M+] 538.55 $C_{29}H_{36}N_3O_3S_2$+ requires [M+] 538.22; UV/VIS (methanol): $\lambda_{max}$ = 507 nm, ε= 78 000 l.mol$^{-1}$.cm$^{-1}$, $\lambda_{fl}$ = 568 nm;

(E)-3-(5-methoxy-2-((1-(3-(1-methylpyrrolidin-1-ium-1-yl)propyl)quinolin-4(1H) ylidene)methyl) benzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate iodide (AK1-2-19); (red crystals), yield = 44 %, M.p. = 263-266 $^{o}$C; IR (nujol, cm$^{-1}$): 550, 720, 760, 1030 , 1170 , 1220, 1300, 1330, 1380, 1460, 1500, 1600; $^{1}$H NMR (DMSO-$d_6$, 600 MHz) δ/ppm: 2.09 (4H, quint, J 3.6, 2 x $CH_2$), 2.12-2.17 (2H, m, $CH_2$), 2.30-2.38 (2H, m, $CH_2$), 2.70 (2H, t, J 6.0, $CH_2$), 2.99 (3H, s, $CH_3$-N), 3.44-3.53 (6H, m, 3 x $CH_2$), 3.92 (3H, s, $CH_3$-O), 4.61 (2H, t, J 7.3, $CH_2$), 4.85 (2H, t, J 7.9, $CH_2$), 7.05 (1H, d, J 2.3, ArH), 7.07 (1H, dd, J 2.3, 8.8, ArH), 7.20 (1H, s, CH), 7.34 ( 1H, d, J 7.2, ArH), 7.55 (1H, d, J 2.3, ArH), 7.69-7.73 (1H, m, ArH), 7.93 (1H, d, J 8.7, ArH), 8.00 (1H, ddd, J 1.1, 7.1, 8.1, ArH), 8.14 (1H, d, J 8.7, ArH),, 8.53 (1H, d, J 7.4, ArH), 9.10 (1H, dd, J 1.1, 9.0, ArH); APT-NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 21.1; 23.1; 23.4; 44.9; 47.5; 47.7; 50.8; 56.1; 59.9; 63.7; 88.5; 98.3; 107.7; 112.6; 115.3; 117.4; 123.4; 124.3; 126.7; 126.8; 133.1; 136.8; 141.3; 143.8; 148.4; 160.3; 160.6; ESI: m/z: Found [M+] 554.49 $C_{29}H_{36}N_3O_4S_2$+ requires [M+] 554.21; UV/VIS (methanol): $\lambda_{max}$ = 517 nm, ε= 77 000 l.mol$^{-1}$.cm$^{-1}$, $\lambda_{fl}$ = 567 nm**;**

(E)-3-(5-methoxy-2-((1-(3-(1-methylpiperidin-1-ium-1-yl)propyl)quinolin-4(1H)-ylidene)methyl) benzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate iodide (AK1-2-20); (red

crystals); yield = 53 %, M.p. = 241-245 $^{o}$C**;** IR (nujol, $cm^{-1}$): 550, 720, 760, 910, 1020, 1170, 1200, 1300, 1330, 1370, 1450, 1500, 1600; $^{1}$H NMR (DMSO-$d_6$, 600 MHz) δ/ppm: 1.52-1.59 (2H, m, $CH_2$), 1.81 (4H, q, J 5.9, 2 x $CH_2$), 2.12-2.17 (2H, m, $CH_2$), 2.26-2.32 (2H, m, $CH_2$), 2.70 (2H, t, J 6.0, $CH_2$), 3.00 (3H, s, $CH_3$-N), 3.32(4H, t, J 5.9, 2 x $CH_2$), 3.50-3.53 (2H, m, $CH_2$), 3.92 (3H, s, $CH_3$-O), 4.61 (2H, t, J 7.3, $CH_2$), 4.85 (2H, t, J 8.1, $CH_2$), 7.06 (1H, dd, J 2.3, 8.8, ArH), 7.20 (1H, s, CH), 7.35 (1H, d, J 7.3, ArH), 7.54 (1H, d, J 2.2, ArH), 7.68-7.72 (1H, m, ArH), 7.92 (1H, d, J 8.8, ArH), 8.00 (1H, ddd, J 0.9, 7.1, 8.2, ArH), 8.14 (1H, d, J 8.7, ArH), 8.54 (1H, d, J 7.4, ArH), 9.09 (1H, dd, J 0.9, 8.9, ArH); APT-NMR (DMSO-$d_6$, 75 MHz) δ/ppm: 19.2; 20.5; 21.6; 23.1; 44.9; 47.5; 50.7; 56.1; 60.3; 88.5; 98.3; 107.7; 112.6; 115.2; 117.3; 123.4; 124.3; 126.6; 126.8; 133.1; 136.8; 141.3; 143.8; 148.4; 160.3; 160.6; ESI: m/z: Found [M+] 568.40 $C_{30}H_{38}N_3O_4S_2$+ requires [M+] 568.23; UV/VIS (methanol): $\lambda_{max}$ = 517 nm, ε= 71 000 $l.mol^{-1}.cm^{-1}$, $\lambda_{fl}$ = 568 nm**;**

*2.6. Preparation of dye and DNA solutions*

The dye stock solutions were prepared by dissolving the dyes in DMSO to achieve final concentrations of 1 mM. To determine the photophysical characteristics of the novel monomethine cyanine dyes these stock solutions were diluted with methanol up to 5 μM, and TE buffer up to 0.10 μM. Salmon testes dsDNA solution was prepared in 10 mM Tris-HCl, 0.5 mM EDTA buffer, pH 7.4. The concentrations of cyanines and dsDNA were determined spectrophotometrically using their molar absorptivities and $\varepsilon_{260} = 6.4 \times 10^3\ M^{-1}cm^{-1}$ for DNA. The molar absorptivities of the dyes are given in Table 2.

To determine the fluorescence spectra of cyanine-dsDNA complexes appropriate amounts of the stock solution of DNA added to each dye in 10 mM Tris-HCl, 0.5 mM EDTA buffer, pH 7.4. The final dye concentration was $2\times10^{-7}\,M$.

**Table 2.** Spectral characteristics of cyanine dyes in different solutions.

| Dye | Methanol (C = 10 μM) | | | TE buffer (C = 0.2 μM) | | | $I_0$ | I | $I/I_0$ |
|---|---|---|---|---|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | $\lambda_{fl}$ (nm) | ε ($mol^{-1}$ $dm^3$ $cm^{-1}$) | $\lambda_{max}$ (nm) | $\lambda_{max}$ (nm) +dsDNA | $\lambda_{fl}$ (nm) +dsDNA | (a.u.) | (a.u.) | |
| AK1-2-17 | 507 | 554 | 83.200 | 507 | 514 | 532 | 2.26 | 301.15 | 13 |
| AK1-2-18 | 507 | 553 | 77.200 | 508 | 515 | 532 | 2.64 | 551.78 | 20 |
| AK1-2-19 | 517 | 562 | 70.700 | 518 | 515 | 549 | 1.63 | 170.41 | 10 |
| AK1-2-20 | 517 | 561 | 59.300 | 518 | 515 | 548 | 1.97 | 136.18 | 69 |

*2.7. Analysis of dye-DNA binding parameters*

Thermodynamic analysis of DNA-cyanine complexes was made in terms of the McGhee & von Hippel excluded site model allowing calculation of the binding constant and stoichiometry [29]:

$$\frac{B}{F}=KP(1-\frac{nB}{P})\left[\frac{1-(nB/P)}{1-(n-1)(nB/P)}\right]^{n-1} \quad (1)$$

where *B* and *F* are concentrations of bound and free dye, respectively, *P* is DNA concentration, *K* denominates association constant and *n* represents the site exclusion parameter (i.e. number of DNA base pairs covered by one dye molecule). The values of *K* and *n* were estimated using the nonlinear least-square fitting procedure.

## 3. Results and discussion

*3.1. Synthesis and structural analysis of cyanine dyes*

Four novel monomethine cyanine dyes obtained via a condensation reaction between N-quaternary 2-thiomethyl benzothiazolic, and lepidinium choromophores have been synthesized with medium yields. The structures of the novel analogues of the Thiazole Orange (Table 1) were evaluated by $^1$H-NMR, APT carbon NMR spectroscopy, IR and ESI

mass spectrometry (see Materials and methods). Reaction yields and melting points are summarized in Table 1.

As seen from Table 1, the cyanines under study are asymmetric monomethine dyes, containing a benzothiazole fragment. The dyes are cationic in nature due to the substituent yielding the additional positive charge linked to the quinolinium moiety. Presented in Table 2 are the basic photophysical characteristics of the examined cyanines in methanol: absorption maximum ($\lambda_{max}$); molar absorptivities ($\varepsilon$) and emission maximum ($\lambda_{em}$). Absorption and fluorescence spectra of the dyes are typical for Thiazole Orange derivatives [17,25] with absorption maximum in methanol in a range from 507 to 517 nm and emission maxima from 553 to 562 nm depending on dye structure. Adding one methoxy group to the benzothiazole moiety of AK1-2-19 and AK1-2-20, resulted in 10 nm and 6 nm shift in absorption and fluorescence maxima position, respectively.

### *3.2. Absorption and fluorescent properties of free cyanine dyes in buffer solution and when bound to dsDNA*

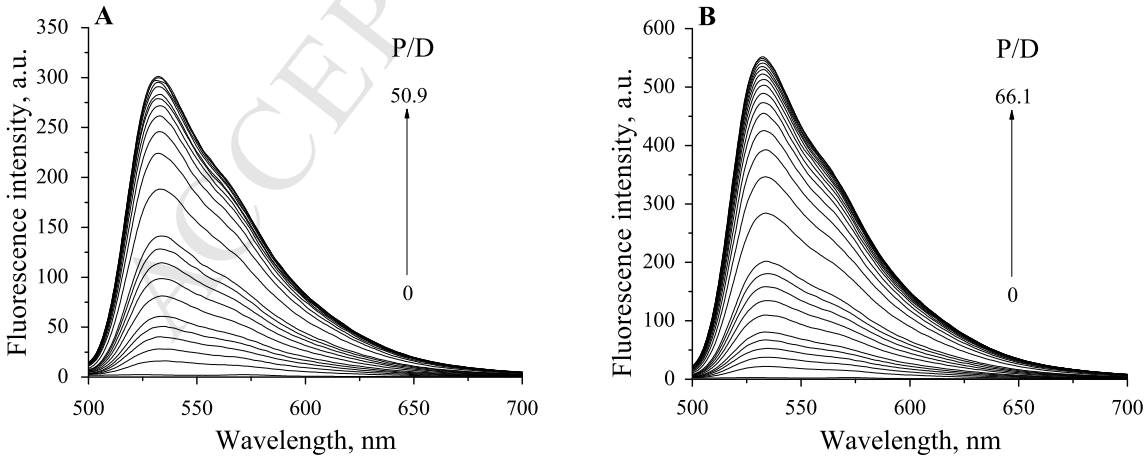
A common feature of cyanine dyes is their tendency to aggregate in water solutions [30,31]. Dimerization with substantial aggregation of cyanine dyes upon binding to DNA has also been reported in previous studies [32,33]. Since the aggregation of cyanines is concentration-dependent process [34], in order to prevent the formation of highly organized molecular assemblies of dyes, their concentrations do not exceede 0.2 μM. In a buffer solution the studied cyanines are characterized by one-peak absorption spectrum (monomeric dye form) with the absorption maximum in the range of 507-518 nm depending on the dye structure. The maxima of the fluorescence band for the free and DNA-bound dyes, along with the absolute and relative fluorescence intensities of the free dye in TE buffer ($I_0$) and dye-DNA solutions ($I$) are given in Table 2. The dyes were found to have only negligible fluorescence in TE buffer in the absence of DNA (Fig.1). It is believed that Thiazole Orange

derivatives undergo rapid deactivation from the singlet excited state by rotating around double bond joining benzothiazole and quinoline ring, a well-established decay mechanism for alkenes [35]. This energy wasting mechanism manifests itself in a dramatic reduction of fluorescence. The situation is changed when the dye is placed in the DNA solution with the restricted motional freedom, that results in cyanine energy dissipation predominantly throughout emission *via* fluorescence. As a consequence, in the presence of dsDNA all dyes under study show enhanced fluorescence (approximately from 69 to 197-fold increase in fluorescence intensity) therby creating prerequisites for their use as fluorescence dyes for DNA detection and characterization. Comparison of the spectral properties of free and dsDNA-bound dyes under the same experimental conditions revealed that the most pronounced fluorescence increase is observed for AK1-2-18, pointing out to significantly greater ability of this dye (in comparison with the other examined dyes) to respond to the DNA-induced changes in the dye microenvironment. Interestingly, the minor differences in cyanine structure can substantially affect the magnitude of fluorescence enhancement ($I/I_0$) in the presence of dsDNA. As seen from Table 1, cyanine dyes under investigation possess similar structure. The only difference was the presence of methoxy group in the benzothiazole ring of AK-1-2-19 and AK1-2-20 and methylpyrrolidin (AK1-2-17, AK1-2-19) or methylpiperidin (AK1-2-18, AK1-2-20) group in the quinoline fragment. As seen from Table 2, the fluorescence enhancement for the dyes containing methoxy group diminished to 1.24- and 2.85-fold for AK1-2-19 and AK1-2-20 respectively. The most probable explanation for the observed fluorescence decrease in the presence of methoxy group lies in the fact, that –O-$CH_3$ group elongates the dye molecule approximately for 3 Å. According to the Lerman's intercalation model [36], introduction of bulky substituent into the dye molecule negatively affects on fluorescence intensity of the dye-DNA complexes. The similar results were obtained by Yarmoluk and coworkers who showed that bulky substitution in the

benzothiazole residue led to a significant decrease in fluorescence enhancement [25]. Replacement of methylpiperidin group by methylpyrrolidin one in the structure of dye AK1-2-18 caused a 1.5-fold increase in $I/I_0$ ratio. However, in the case of AK1-2-20 the introduction of methylpiperidinium group brought about the opposite effect (1.5-fold decrease in $I/I_0$ ratio in comparison with methylpyrrolidin-containing dye AK1-2-19). The observed differences may come from the presence of methoxy group in AK1-2-19 and AK1-2-20, that exerts influence on electron-donor ability of heterocycle in the latter case displacing the charge from the benzothiazole residue to the quinolinium moiety and affecting in such a manner the fluorescent properties of these dyes. The validity of this assumption stems from the fact that dyes AK1-2-19 and AK1-2-20 display the most pronounced Stock's shifts.

*3.3. Cyanine binding to the dsDNA*

In order to characterize the stability of cyanine-DNA complexes and to make an assumption about the complexation mechanisms, the DNA binding parameters of the examined novel monomethine cyanine dyes were obtained by fluorescence titration of the solutions of cyanine dyes with salmon testes DNA (Fig.1).



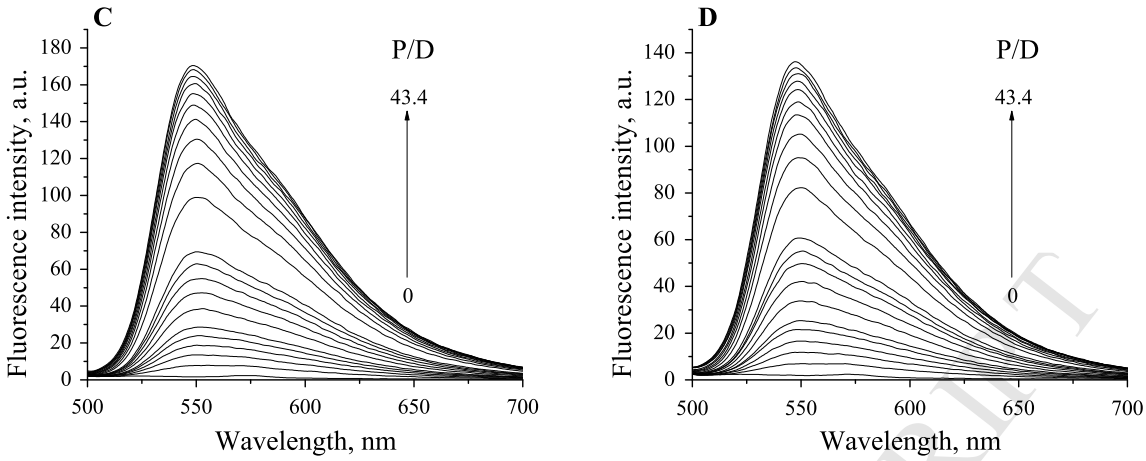



**Fig. 1.** Fluorescence spectra of AK1-2-17 (A), AK1-2-18(B), AK1-2-19(C) and AK1-2-20(D) recorded at varying concentration of salmon testes dsDNA. Dye concentration was 0.2 μM.

Fluorescence intensity was found to increase upon the dye transfer from aqueous phase to dsDNA, with emission maximum being shifted towards lower wavelengths. These findings can be explained by the reduced polarity of the dye surroundings and restricted fluorophore mobility within the DNA binding sites. The experimental dependencies of the dye fluorescence increase upon binding to DNA ($\Delta I$) as a function of DNA concentration are presented in Fig.2.

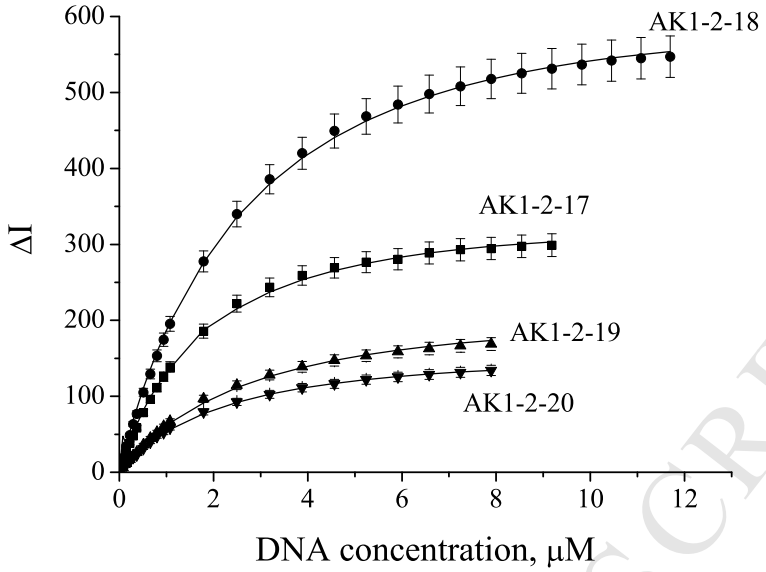



**Fig. 2.** The isotherms of binding of cyanine dyes to dsDNA.

The observed binding isotherms were analyzed in terms of the McGhee & von Hippel excluded site model (Eq.(1)). The results obtained are summarized in Table 3.

**Table 3.** Parameters of cyanine-DNA binding calculated using McGhee & von Hippel model.

| Dye | $K$, $\times 10^5$, $M^{-1}$ | $n$ | $\alpha$, $\times 10^3$, $M^{-1}$ |
|---|---|---|---|
| AK1-2-17 | 5.7 ± 1.1 | 1.9 ± 0.6 | 1.9 ± 0.6 |
| AK1-2-18 | 3.3 ± 0.8 | 1.4 ± 0.4 | 3.9 ± 1.2 |
| AK1-2-19 | 3.1 ± 0.5 | 1.7 ± 0.5 | 1.3 ± 0.4 |
| AK1-2-20 | 4.0 ± 0.4 | 1.5 ± 0.4 | 1.0 ± 0.3 |

It appeared that the site exclusion parameters and molar fluorescence have the values typical for DNA-dye complexation. The association constants were found to have the magnitude of $10^5$ $M^{-1}$, suggesting that the dyes under study form stable complexes with DNA.

The highest value of association constant was observed for dye AK1-2-17, whereas the molar fluorescence was the largest in the case of dye AK1-2-18. To explain the observed finding, it should be noted that the binding constant reflects the strength of binding, whereas the term "molar fluorescence" in generally determined by the fluorophore ability to respond to the changes in its microenvironment.

The observed value of association constant, being of the order of $10^5$ $M^{-1}$ imply that cyanine dyes under study can be placed to the category of molecules that interact with DNA by intercalation binding mode. Detailed analysis of the available literature on DNA-dye interactions indicates that association constants for intercalators that bind to DNA by hydrophobic, Van der Waals and electrostatic forces do not exceed $10^7$ $M^{-1}$ , whereas the *K* value observed for DNA complexes of groove binders, especially those stabilized by hydrogen bonds (netropsin), may even be larger than $10^8$ $M^{-1}$ [37,38]. For example, *K* values for ethidium bromide, acridine orange and thiazole orange, well-known intercalating agents, are equal to $1.5\times10^5$ $M^{-1}$, $2.69\times10^4$ $M^{-1}$, and $10^6$ $M^{-1}$, respectively [38-40]. The dye Cyan 2 has association constant even smaller, of the order $10^4$ $M^{-1}$ order [38]. The binding constants for asymmetric thiazole orange derivatives reported in [17] fall in the range $(0.66-7.58)\times10^5$ $M^{-1}$ suggesting the intercalation binding mode. Other monomethine dyes (TOTO, YO, YOYO, etc.), the planar aromatic moiety of which incorporates between the DNA base pairs, have the binding constants of the order $10^6$ -$10^7$ $M^{-1}$ [41].

The assumption about intercalating binding mode of cyanine dyes is confirmed also by the fact, that the size of the examined dyes does not exceed 1.5 nm, that is comparable with DNA space available for intercalation between the base pairs (the diameter of dsDNA is approximately equal to 2 nm). In addition, the groove binders are generally crescent-shaped structures possessing conformational flexibility, that is more usual for polymethines, while the dyes under study belong to the monomethine cyanine group. Finally, according to the

principle of the nearest neighbor exclusion, the binding of one intercalating molecule between two base pairs hinders access of the next binding site to another intercalator [11], so the highest possible dye-base pair ratio for intercalation is 1:2. For groove binding agents one dye molecule occupies at least 3-5 base pairs which is a consequence of more elongated and ribbon like fluorophore structure. As shown in Table 3, the obtained estimates of site exclusion parameters do not exceed 2, lending the additional support to the assumption on the intercalation binding mode.

## 4. Conclusions

To summarize, four novel monomethine cyanine dyes derived from a condensation reaction between N-quaternary 2-thiomethyl benzothiazolic, and 4-methyl quinolinuim choromophores have been synthesized and spectroscopically characterized. The potential application of these dyes as fluorescent probes for non-covalent labeling and characterization of dsDNA has been evaluated using the fluorescence spectroscopy technique. The examined dyes were found to have negligible fluorescence in the buffer solution, but exhibited a significant emission increase upon their binding to dsDNA. The binding studies indicate that monomethine cyanine dyes associate with salmon testes DNA presumably by intercalating mode. The huge fluorescence enhancement upon formation of the dye-DNA complexes allowed us to recommend these dyes for DNA detection studies.

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## Highlights

- Synthesis of novel asymmetric monomethine cyanine dyes and structural analysis.
- Absorption and fluorescent studies - potential markers for nucleic acid detection.
- Evaluation of binding constants to dsDNA.