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A novel monomeric asymmetric tricationic monomethine cyanine dye – Thiazole Orange (TO) analog: synthesis, photophysical and dsDNA binding properties

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A new monomeric tricationic cyanine dye **TO-3c-7Cl** – analog of Thiazole Orange (**TO**), has been synthesized using an environmentally benign and simple method and its photophysical properties have been investigated and compared to those of the well-known nucleic acid stain **TO**. Dye **TO-3c-7Cl** has negligible intrinsic fluorescence in Tris-EDTA (TE) buffer, but in the presence of dsDNA its fluorescence intensity increases significantly. DFT and TDDFT calculations are used to compare and contrast the theoretically predicted structure and properties of the new tricationic cyanine dye and **TO**.

Keywords: Thiazole Orange, cyanine dyes, dsDNA, fluorescence.

INTRODUCTION

Cyanine dyes are object of unceasing attention because of their effective and fruitful applications in different scientific areas – laser optics, molecular biology, medicine [1–4].

In recent years, the use of asymmetric cyanine dyes as fluorescent labels or sensors for bioimaging and detection has been of utmost importance. These applications are related to the spectral properties of the dyes and their ability to form fluorescent complexes with variety of biological macromolecules. Such dyes have no or have very weak intrinsic fluorescence, but upon binding to a bio-object the fluorescence intensity can increase dramatically [5]. A wide range of novel molecules with similar characteristics based mainly on **TO** and Oxazole Yellow (**YO**) have been designed, synthesized and commercialized [6, 7].

Continuing our research [8–10] into development of new fluorescent probes for nucleic acids detection, here we report the synthesis of a novel asymmetric monomeric monomethyne cyanine dye **TO-3c-7Cl** – analog of the commercial dsDNA fluorescence binder Thiazole Orange. The preparation of **TO-3c-7Cl** was achieved by using an easy handling, efficient and environmentally benign synthetic procedure. The interactions of the new **TO** derivative with dsDNA have been investigated by absorption and fluorescence spectroscopy. The newly synthesized dye shows quite low fluorescence in TE buffer in the absence of dsDNA, but it becomes strongly fluorescent after binding to the biomolecule. The influence of the substituents attached to the chromophore was investigated by UV-VIS, fluorescence spectroscopy, DFT and TDDFT calculations.

EXPERIMENTAL

All solvents used in the present work were HPLC grade and commercially available. The starting materials **1** and **4** are commercially available and they were used as supplied. Melting point of **TO-3c-7Cl** was determined on a Büchi MP B-545 apparatus and is uncorrected. NMR spectra (^{1}H-, ^{13}C-NMR) were obtained on a Bruker Avance II+ NMR spectrometer operating at 600 MHz for ^{1}H- and 125 MHz for ^{13}C-NMR in DMSO-d_6 as solvent. The chemical shifts are given in ppm (δ) using tetramethylsilane (TMS) as an internal standard. UV-VIS spectra were measured on a Unicam 530 UV-VIS spectro-

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photometer and the fluorescence spectra were obtained on a Varian Cary Eclipse fluorescence spectrophotometer. The intermediate **2** was synthesized by method described in the literature [11].

Synthesis of 3-(4-(4-(dimethylamino)pyridin-1-ium-1-yl)butyl)-2-methylbenzo[d]thiazol-3-ium dibromide (3)

A mixture of 2-methylbenzo[d]thiazole (**1**) 0.76 g (5.1 mmol) and 1-(4-bromobutyl)-4-(dimethylamino)pyridin-1-ium bromide (**2**) 1.71 g (5.1 mmol) was heated at 145 °C for 1.5 h in the absence of a solvent. Then methanol (10 ml) was added and after cooling down of the reaction mixture to room temperature, the product was precipitated with the addition of diethyl ether (25 ml). The precipitate was filtered off and dried at a desiccator. The product **3** was used in the next synthetic route without any additional purification, because of its high hygroscopicity. Yield: 2.79 g (94%).

Synthesis of 4,7-dichloro-1-(4-(4-(dimethylamino) pyridin-1-ium-1-yl)butyl)quinolin-1-ium dibromide (5)

A mixture of 4,7-dichloroquinoline (**4**) 2.00 g (10 mmol) and 1-(4-bromobutyl)-4-(dimethylamino) pyridin-1-ium bromide (**2**) 3.41 g (10 mmol) was heated under argon in 50 ml round bottom flask equipped with a condenser at 145 °C for 10 min in the absence of a solvent. After cooling down of the reaction mixture to room temperature, the product was precipitated with the consecutively addition of methanol (10 ml), acetone (10 ml) and diethyl ether (15 ml). The precipitate was filtered off and dried at a desiccator. The product was used in the next stage of the reaction scheme without any additional purification, because of its high hygroscopicity and low stability. Yield 5.20 g (97%).

Synthesis of (E)-7-chloro-1-(4-(4-(dimethylamino) pyridin-1-ium-1-yl)butyl)-4-((3-(4-(4-(dimethylamino)pyridin-1-ium-1-yl)butyl)benzo[d] thiazol-2(3H)-ylidene)methyl)quinolin-1-ium triiodide (TO-3c-7Cl)

3-(4-(4-(Dimethylamino)pyridin-1-ium-1-yl) butyl)-2-methylbenzo[*d*]thiazol-3-ium bromide (**3**) (1 mmol) and 4,7-dichloro-1-(4-(4-(dimethylamino) pyridin-1-ium-1-yl)butyl)quinolin-1-ium bromide (**5**) (1 mmol) were mixed and finely ground in a mortar. The mixture was transferred to a 50 ml flask equipped with an electromagnetic stirrer and a reflux condenser and then methanol (10 ml) was added. The mixture was heated at 50 °C for 10 min and *N*-ethyldiisopropylamine (DIPEA) (2.2 mmol) was added dropwise. The reaction mixture was stirred vigorously for 2 h without heating and diethyl ether (25 ml) was added. The resulting precipitate was filtered off and the residue was dissolved in methanol (20 ml). Saturated aqueous KI (10 ml) was added to the methanol solution and the resulting precipitate was filtered off and air-dried. The yield of the crude product was over 80%. **TO-3c-7Cl** was purified by multiple recrystallizations from methanol. **TO-3c-7Cl:** Yield 25%, Mp: 260–263°C. Mw = 1047.05. ^{1}H-NMR, δ: 1.75–1.78 (m, CH_2, 4H), 1.80–1.87 (m, CH_2, 4H), 3.17 (s, NCH_3, 12H), 4.02–4.04 (m, N^+CH_2, 4H), 4.22 (t, ^3J = 6. 9 Hz, N^+CH_2, 2H), 4.59 (t, ^3J = 6.8 Hz, N^+CH_2, 2H), 6.89 (s, CH, 1H), 7.02 (d, ^3J = 7.5 Hz, 2H), 7.28 (d, ^3J = 7.2 Hz, 2H), 7.44 (dd, ^3J = 7.6 Hz, 2H), 7.62 (dd, ^3J = 8.0 Hz, 2H), 7.74 (d, ^3J = 7.6 Hz, 2H), 7.82 (d, ^3J = 8.4 Hz, 1H), 8.07 (d, ^3J = 7.8 Hz, 1H), 8.17 (s, 1H), 8.27–8.29 (m, 2H), 8.55 (d, ^3J = 7.4 Hz, 1H), 8.82 (d, ^3J = 9.3 Hz, 1H).

^{13}C, DEPT-135 NMR, δ: 25.66 (CH_2), 27.76 (CH_2), 34.70 (CH_3), 40.24 (CH_3), 53.76 (CH_2), 56.48 (CH_2), 89.22 (CH), 108.14 (CH), 108.18 (CH), 108.25 (CH), 113. 77 (CH), 113.84 (CH), 114.28 (CH), 117.72 (CH), 123.47 (CH), 125.31 (CH), 127.31 (CH), 128.58 (CH), 128.79 (CH), 142.32 (CH), 145.07 (CH). Elemental analysis for N (%): Calculated 8.03, Found 8.31.

COMPUTATIONAL

The B3LYP functional [12, 13] was used in the geometry optimization of the cationic fragments of **TO** and **TO-3c-7Cl** with/without I^- counterions. The calculations were performed with the diffuse function-augmented 6-31G(d,p) [14–16] basis set (6-31+G(d,p)) for systems without counterions and with mixed basis set for those with counterions (6-31+G(d,p) basis set for the lighter atoms (C, N, S, Cl and H) and with SDD [17] pseudopotential for the I). The selected combination method/basis set has been proven [9] to reproduce reliably the geometry of 3-(4-((3-methyl-1,3-benzothiazol-2(3H)-ylidene)methyl)-quinolinium-1-yl) propanoate tetrahydrate (CSD ENTRY OVUJUL/CCDC number 739300) [18]. C_1 symmetry was assumed for all systems and default convergence criteria were used; local minima were verified by establishing that the Hessians had zero negative eigenvalues. The structures with counterions were optimized in methanol by the method developed for implicit treatment of solute-solvent interactions of electronic properties in solution – integral equation formalism polarizable continuum model (IEFPCM) [19]. TDPBE0/6–311+G(2d,p) calculations were

performed to compute the 20 lowest excited states of each structure. Solvent effects were included in TDDFT calculations, also by the IEFPCM. All calculations were performed using Gaussian 09 [20]. The PyMOL molecular graphics system was used to generate the molecular graphics images [21].

RESULTS AND DISCUSSION

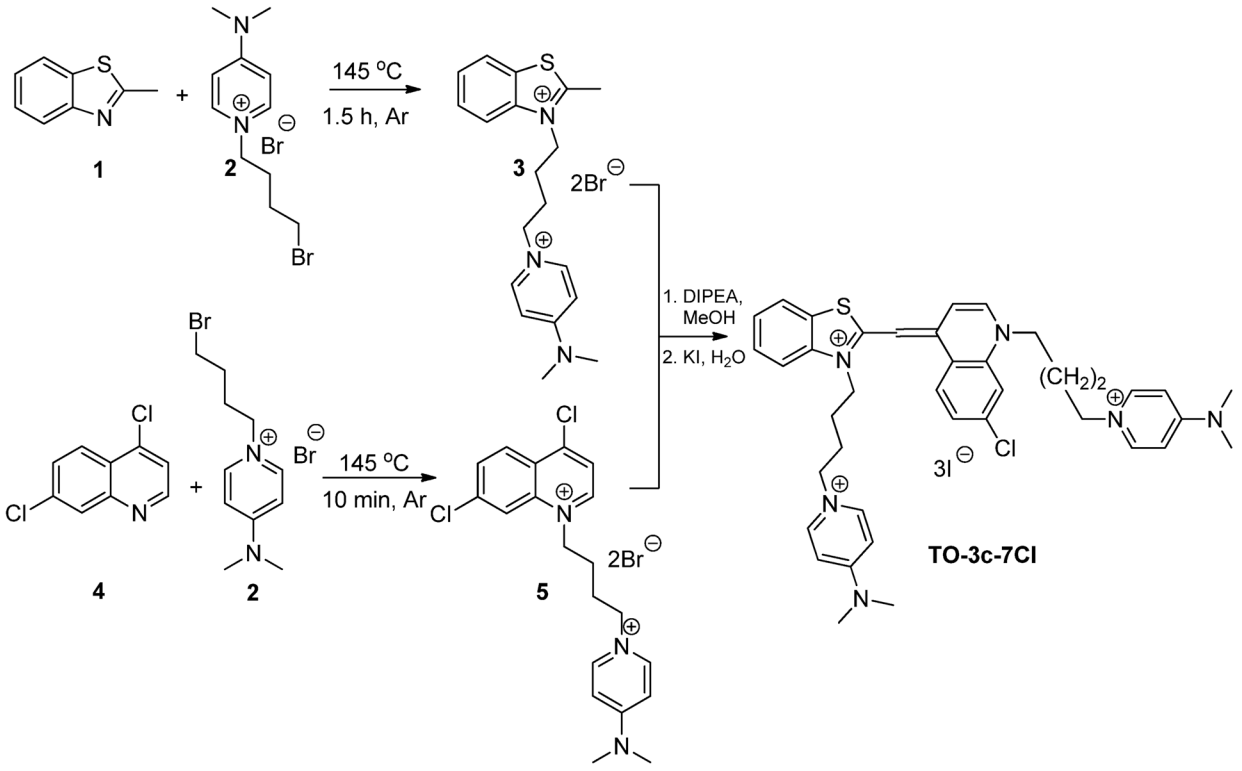
In general, monomethine cyanine dyes such as **TO** and its derivatives can be synthesized under condensation of 2-methylthio salt of alkylated benzothizole with another alkylated heterocycle with an activated methyl group (Brooker's method) [1, 22–25], but this method suffers from some disadvantages [26]. In order to avoid the problems concomitant the synthetic strategy usually applied, dye **TO-3c-7Cl** (Scheme 1) was synthesized by simple, efficient and environmentally benign procedure that has been previously used by our group [9, 10, 27, 28]. This approach involves condensation of 2-methylbenzothiazolium salt **3** and 4,7-dichloroquinolinium salt **5** in the presence of the sterically hindered *Hünig's* base (*N*-ethyldiisopropylamine, DIPEA) (Scheme 1). The reactions of quaternization of the starting 2-methylbenzo[d]thiazole (**1**) and 4,7-dichloroquinoline (**4**) with 1-(4-bromobutyl)-4-(dimethylamino)pyridin-1-ium bromide (**2**) were carried out in the absence of a solvent for a short reaction time[11]. The quaternary salts **3** and **5** were isolated in high yields and in a purity enough for the next synthetic transformation.

The newly synthesized cyanine dye **TO-3c-7Cl** was characterized by NMR-, UV-VIS-spectroscopy and by elemental analysis.

The photophysical properties of the new tricationic dye **TO-3c-7Cl** were evaluated and the data were compared to those of its analog **TO**. The absorption maxima of the studied dyes are at 502 and 513 nm for **TO** and **TO-3c-7Cl**, respectively. The higher value of the molar absorptivity corresponds to **TO-3c-7Cl** – 97000 $L \cdot mol^{-1} \cdot cm^{-1}$, while **TO** in our conditions is characterized by a molar absorptivity of 86100 $L \cdot mol^{-1} \cdot cm^{-1}$ (Table 1).

The newly synthesized dye **TO-3c-7Cl** in TE buffer in the absence of dsDNA absorbs at 513 nm and this peak is bathochromically shifted to 521 nm in the same buffer in the presence of dsDNA (Table 1). As we mentioned in our previous work [10], this effect could be attributed to an intercalation of the dye into dsDNA, as assumed in other investigations into cyanine dyes [9, 29].

In TE buffer at 538 nm **TO-3c-7Cl** shows fluorescence with low intensity (4.3 au at 1.10^{-7} M and almost zero (below 0.4) at 1.10^{-8} M, Table 1, Fig. 1),



Scheme 1. Synthesis of tricationic monomeric monomethyne cyanine dye **TO-3c-7Cl**.

Table 1. Comparison of the photophysical properties of **TO-3c-7Cl** and TO in the absence and in the presence of dsDNA

	Absorption			Fluorescence				
Dye	Abs[a]	Abs[b]	Abs[c]	λ_{max} [d]	I_{fl0} [d]	λ_{max} [e]	I_{fl} [e]	Ratio I_{fl}/ I_{fl0}
TO-3c-7Cl	513 (97 000)	513	521	538	0.43*	538	875	>2030
TO	502 (86 100)	502	510	549	0.55	529	252	458

[a] λ_{max} (nm) and molar absorptivity ε (L.mol^{-1}.cm^{-1}) of free dyes in methanol;
[b] λ_{max} (nm) of free dye in TE buffer;
[c] λ_{max} (nm) of dye-dsDNA complex in TE buffer;
[d] λ_{max} (nm) and I_{fl0} of free dye in TE buffer;
[e] λ_{max} (nm) and I_{fl} of complex dye-dsDNA in TE buffer.
*Due to the extremely high fluorescence intensity at 1.10^{-7} M the spectra of the complex dye **TO-3c-7Cl**/dsDNA the spectra was measured at 1.10^{-8} M concentration. The intrinsic fluorescence of the free dye at this concentration is near zero and hardly distinguishable from the instrumental noise.

but excitation at 538 nm in the presence of dsDNA led to a dramatic increase in the fluorescence intensity over 2030-fold (Table 1, Fig. 1). The significant increase of the fluorescence can be attributed to the presence of three positive charges in the dye molecule increasing the binding affinity to dsDNA.

B3LYP/6-31+G(d,p) optimized structures of the cationic fragments of **TO** and **TO-3c-7Cl** are presented in Figure 2 with the respective atom color scheme. A simple structural superimposition of the dyes reveals structural similarity between them with almost identical "TO core" fragment.

TDPBE0 (time-dependent density functional theory calculations using Perdew–Burke–Ernzerhof exchange-correlation functional) calculations with the 6–311+G(2d,p) basis set for all atoms except I and with the Stuttgart-Dresden SDD effective core

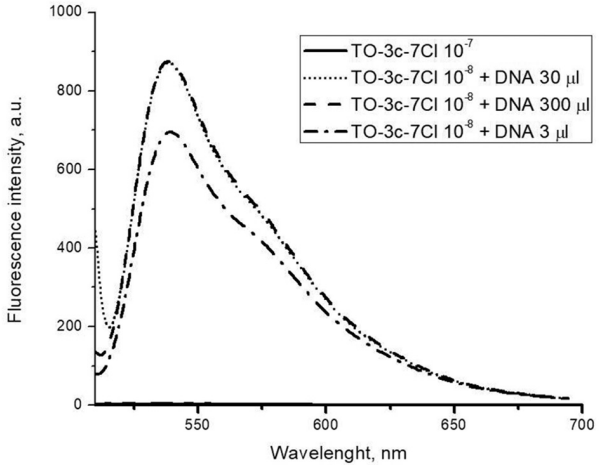


Fig. 1. Fluorescence spectra of free dye **TO-3c-7Cl** in TE buffer and in the presence of dsDNA.

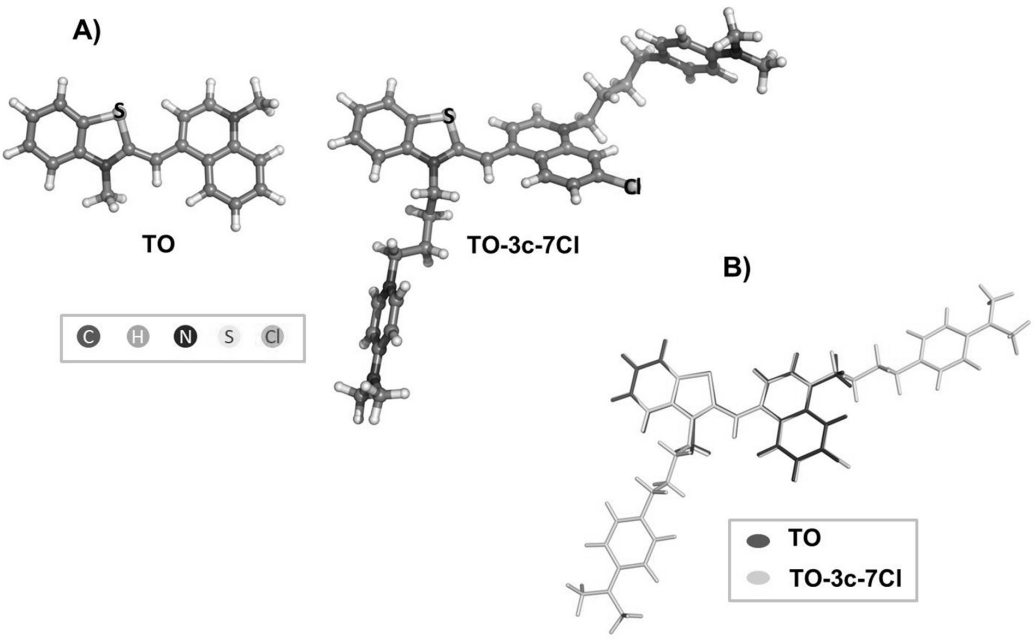


Fig. 2. A) B3LYP optimized structures of the cations of **TO** and **TO-3c-7Cl**; B) superimposed structures of **TO** and **TO-3c-7Cl** cations.

potential (ECP) basis set for I in the range 400–500 nm predict an intensive band for **TO** (at 452 nm) and an intensive band for its tricationic analogue (at 456 nm). The calculated optical parameters such as the absorption maximum (λ_{max}), oscillator strength (f) and frontier orbital energy levels are provided in Table 2.

The $S_0 \rightarrow S_1$ excitation process in the studied dyes can be assigned mainly to HOMO (highest occupied molecular orbital) → LUMO (lowest unoccupied molecular orbital) transition. An examination of the frontier molecular orbitals of the compounds under investigation can be useful. The qualitative frontier molecular orbital representations for **TO** and **TO-3c-7Cl** are shown in Figure 3.

The oscillator strengths calculated for HOMO → LUMO transition are 0.777 and 1.035 for **TO** and **TO-3c-7Cl**, respectively. The HOMO–LUMO gaps are similar: 3.27 eV and 3.25 eV for the compounds (**TO** and **TO-3c-7Cl**, respectively) in methanol. In **TO-3c-7Cl** the HOMO was found to be populated over the "TO core" fragment. There is no difference between the delocalization of the LUMO in both compounds (Figure 3).

Table 2. TDDFT/PBE0 excitation energies (eV), wavelengths (nm) (in parentheses), oscillator strength *f*, HOMO and LUMO energies and energy differences (HOMO-LUMO gap) (eV) in methanol for **TO** and **TO-3c-7Cl**

	TO	TO-3c-7Cl
HOMO→LUMO		
Excitation energy (wavelength)	2.74 (452)	2.72 (456)
Oscillator strength, *f*	0.777	1.035
HOMO	–5.99	–6.08
LUMO	–2.72	–2.83
HLG	3.27	3.25

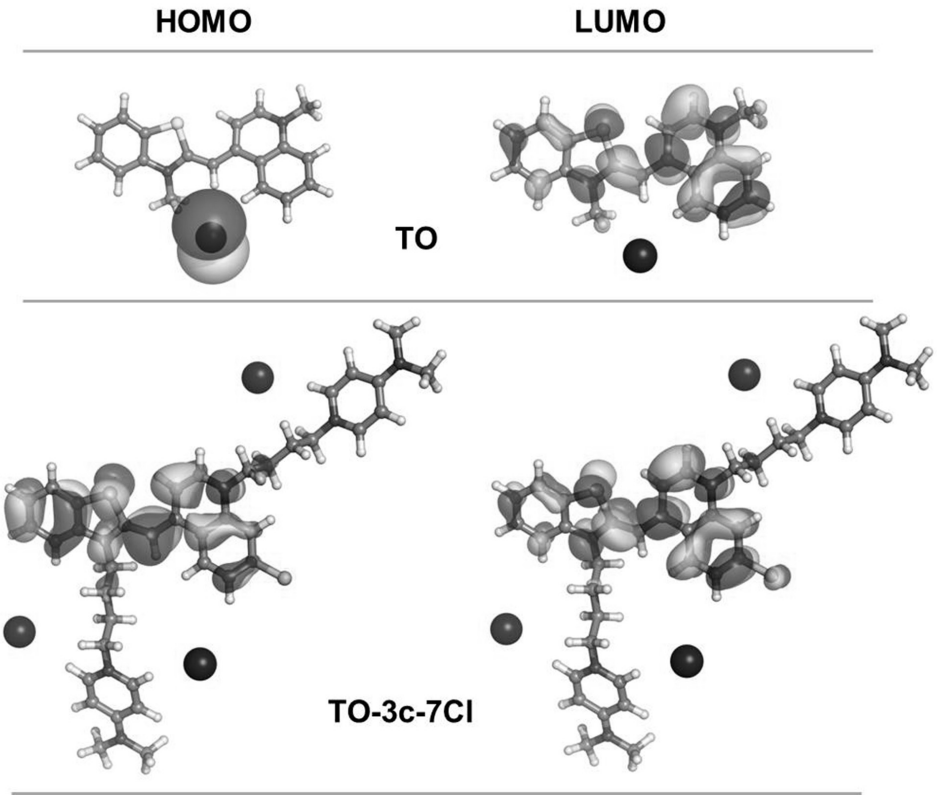


Fig. 3. Graphical representation of the frontier orbitals (isodensity plot, isovalue = 0.02 a.u.).

CONCLUSION

A novel tricationic analog of the commercial nucleic acids binder **TO** was synthesized and its photophysical properties were investigated. **TO-3c-7Cl** possesses typical features of fluorescent DNA label. In the absence of DNA, the dye has negligible fluorescence, but after binding to DNA a significant increase in the fluorescence intensity (2030-fold) was observed. Theoretically predicted (DFT and TDDFT calculated) structure and properties of the new tricationic cyanine dye and **TO** are compared and contrasted. The promising results stimulate us for further investigations in the design, synthesis and application of new polycationic halogen containing analogs of TO as nucleic acid binders.

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НОВ МОНОМЕРЕН ТРИКАТИОНЕН МОНОМЕТИНЦИАНИНОВ АНАЛОГ НА ТИАЗОЛ ОРАНЖ (ТО): СИНТЕЗ, ФОТОФИЗИЧНИ И двДНК-СВЪРЗВАЩИ СВОЙСТВА

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(Резюме)

Синтезирано е ново мономерно трикатионно цианиново багрило **TO-3c-7Cl** – аналог на Тиазол оранж (**TO**) посредством лесно приложим и щадящ околната среда метод. Изследвани са фотофизичните свойства на съединението и са сравнени с тези на известния търговски продукт **TO**. Багрилото **TO-3c-7Cl** се характеризира с много ниска собствена флуоресценция в Tris-EDTA (TE) буфер, но в присъствието на двДНК, интензитетът му на флуоресценция се увеличава значително. DFT и TDDFT-изчисления са използвани за сравнение и разграничаване на теоретично предсказаните структури и свойства на новополученото трикатионно цианиново багрило и **TO**.