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Synthesis of Dihydroquinoline-Based Derivatives of Fluorescent Rhodamine Dyes for Nucleic Acid Analysis by a Real-Time Polymerase Chain Reaction

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Abstract—An optimized synthesis scheme for the dihydroquinoline-based derivatives of xanthene fluorescent dye has been developed. For the first time, their use as effective fluorophores in hybridization probes for real-time PCR has been demonstrated with the detection on an individual spectral channel.

Keywords: fluorescent rhodamine dyes, real-time polymerase chain reaction, nucleic acids, quinolones
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INTRODUCTION

Rhodamine fluorescent dyes are widely used for visualization of biological macromolecules due to their good photochemical and photophysical spectral characteristics, i.e., high extinction coefficient and quantum yield [1]. Compared with fluorescent dyes of the fluorescein series, rhodamine dyes are more photostable, their fluorescence spectrum does not depend on the pH values in the range from 4 to 10 [2]. These dyes are widely used not only in biotechnology for the introduction into proteins and nucleic acids but also in medicine for diagnostic imaging of living cells or living organisms in preclinical studies [3]. In recent years, a wide variety of rhodamine dyes have been marketed for conjugation with biomolecules [2]. Nevertheless, the synthesis of efficient dyes that fluoresce in the spectral range of 610–665 nm remains relevant because the most common dyes used for the introduction into nucleic acids, proteins, and other biological macromolecules fluoresce either in the shorter wavelength range of the spectrum (5-carboxy rhodamine 6G (5-R6G, λ_{em} = 562 nm), tetramethylcarboxy rhodamine (5-TAMRA, λ_{em} = 583 nm), carboxy-X-rhodamine (6-ROX, λ_{em} = 610 nm), or in the near IR region (3,3,3',3'-tetramethyl-indocarbocyanine (Cy5, λ_{em} = 669 nm). Earlier, Liu Jixiang et al. [4] synthesized fluorescent dyes that contained the 2,2-dimethyl-1,2-dihydroquinoline fragment, which was annelated with thiophene, benzothiophene, or naphthalene.

The goal of this work was to develop the method for the synthesis of rhodamine dyes that contain a similar structural annealed dihydroquinoline fragment with improved spectral characteristics and to study their properties in hybridization probes for real-time PCR.

RESULTS AND DISCUSSION

The synthesis of compound (**V**) (Scheme 1) was chosen as the target structure. The scheme of the synthesis of compounds **I**–**IV** has been described for the first time.

Abbreviations: BHQ2, 4'-(4-nitrophenylazo)-2',5'-dimethoxy-4''-(*N*,*N*-diethanolamino)azobenzene, fluorescence extinguisher; RT PCR, real-time polymerase chain reaction; HPLC, high-performance liquid chromatography; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quadruplet; qt, quintet; m, multiplet; b, broad signal; *J*, spin–spin interaction constant, Hz; Tos, tosylate; *p*-TsOH, *p*-toluenesulfonic acid; DSC, disuccinimidyl carbonate; DMAP, 4-dimethylaminopyridine; TBTA, tris(benztriazolylmethyl)amine; TCEP, tris(2-carboxyethyl)phosphine; TSTU, *N*,*N*,*N*,*N*',*N*'-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; *p*-TsOEt, ethyl tosylate.

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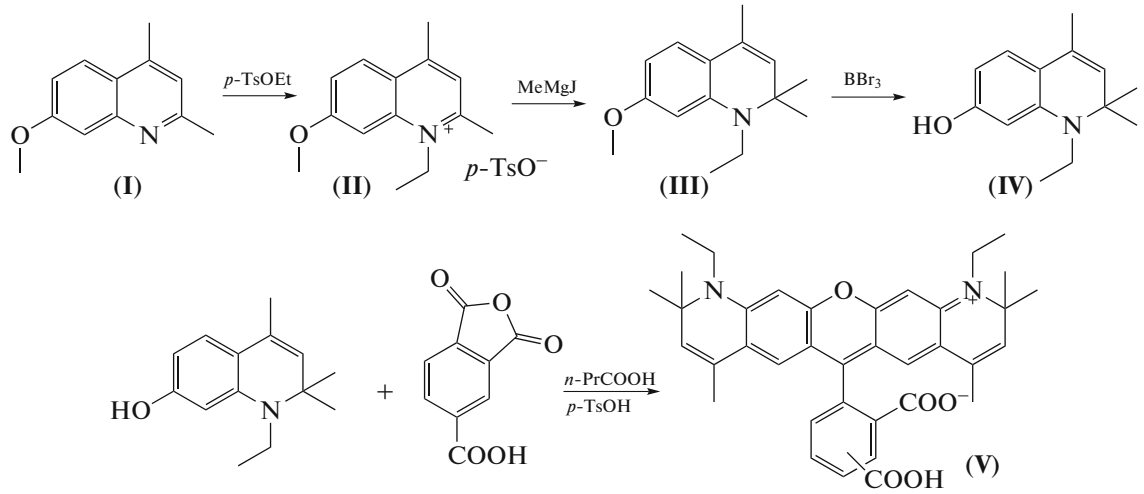
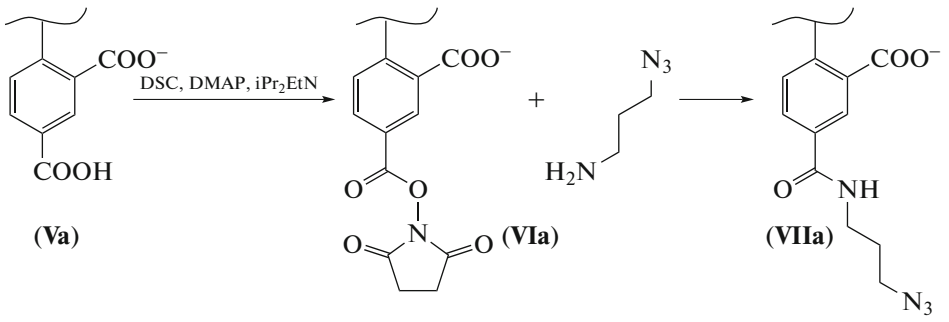


Scheme 1. Synthesis of target dye, (5(6)-carboxy-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2*H*-pyrano[3,2-g:5,6-g']dihydroquinolin-6-yl)benzoate, (5/6-Sy630).

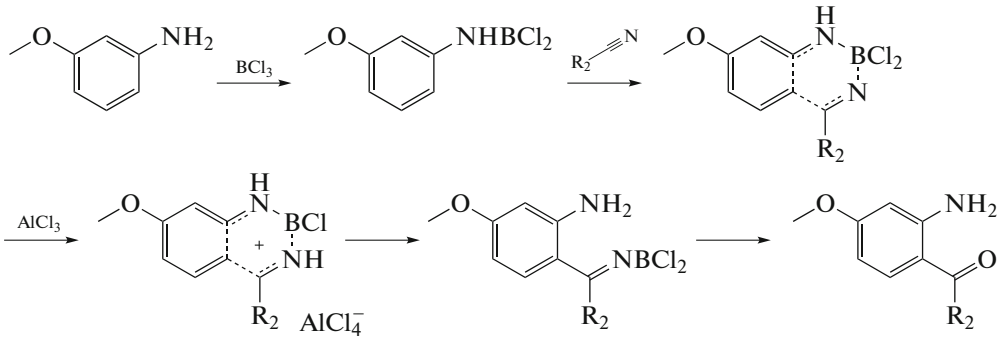
When boiling substituted quinolines with ethyl tosylate in chlorobenzene, the corresponding quaternary ammonium salts were obtained, which gave 1-ethyl-1,2-dihydroquinoline derivatives after the treatment with an excess of the Grignard reagent. The methoxy group was removed by the treatment with boron tribromide at room temperature. Since 7-hydroxy-1,2-dihydroquinoline derivatives are sensitive to the action of mineral acids and Lewis acids, the dye core was synthesized by their boiling with trimellitic anhydride in butyric acid that contained catalytic amounts of *p*-toluene sulfonic acid. The resulting mixture of isomers was separated by chromatography.

N-Hydroxysuccinimide esters (**VI**) were synthesized by the reaction of the corresponding carboxyl derivatives with disuccinimidyl carbonate in dry methylene chloride with the addition of 1.5 equiv of the Hunig's base and 0.5 equiv of 4-dimethylaminopyridine. Their propyl amide derivatives were also synthesized by the interaction of activated esters (**VI**) with 3-aminopropylazide as shown in Scheme 2.



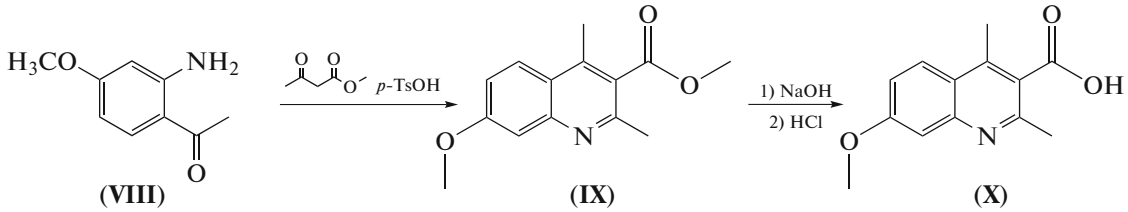
Scheme 2. Synthesis of *N*-hydroxysuccinimide ester and propylazide of the 5-carboxy-Sy630 dye.

2-Aminophenyl ketones, such as 2-aminobenzophenone and 2-aminoacetophenone and their substituted derivatives, are convenient starting compounds for the synthesis of quinolines [5]. 4-Methoxy-2-aminoacetophenone was obtained by the method of Tsutomu Sugasawa et al. [6] by the interaction of *m*-anisidine with acetonitrile in the presence of boron trichloride and aluminum chloride and subsequent hydrolysis of the resulting 2-aminophenylketimine, as shown in Scheme 3. The synthesis of quinolines based on the condensation of α-substituted aniline with ketones was first described by Friedlander in 1882 [7]. Later, numerous variations of this method were developed using different catalysts [8–20].



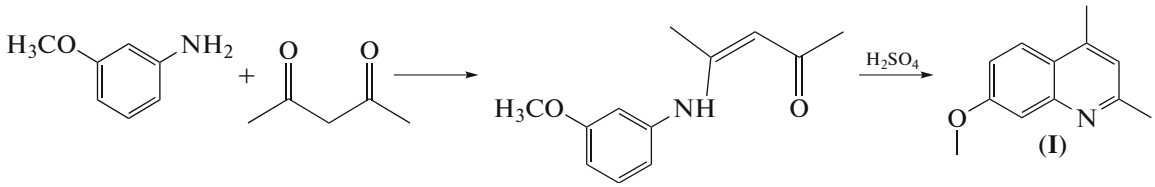
Scheme 3. Synthesis of 4-methoxy-2-aminoacetophenone.

The idea of obtaining 2,4-dimethyl-7-methoxyquinoline by the same method through the reaction of 2-amino-4-methoxyacetophenone with acetone seemed promising. However, according to [21], the interaction of 3,4-dichloro-2-aminoacetophenone with acetone occurs only when heated in a sealed vessel at 185°C for 6 h. We synthesized 2,4-dimethyl-7-methoxyquinoline-3-carboxylic acid according to Scheme 4.



Scheme 4. Synthesis of 2,4-dimethyl-7-methoxyquinoline-3-carboxylic acid.

However, this acid is very resistant to decarboxylation, and effective removal of the carboxyl group is possible only by the decomposition of its silver salt. A more rational synthesis of 2,4-dimethyl-7-methoxyquinoline is the heating of the condensation product of *m*-anisidine with acetylacetone in concentrated sulfuric acid (Scheme 5). Various chlorine derivatives of quinoline were obtained in this way [21].



Scheme 5. Synthesis of 2,4-dimethyl-7-methoxyquinoline.

The spectral characteristics of isomers 5 and 6 of carboxy-Sy630, **Va** and **Vb**, and their derivatives **VIa**, **VIb**, **VIIa**, and **VIIb** are presented in Table 1. The values of quantum yields and molar absorption coefficients were obtained as in [22].

The synthesized *N*-hydroxysuccinimide derivatives of isomers 5 and 6 of the Su630 dye (**VIa** and **VIb**) were attached to the 5' ends of oligonucleotides by condensation with the 5'-terminal amino group (Aminolink-C6) of T_{20} oligothymidylates. The corresponding azide

Table 1. Spectral characteristics of isomers of fluorescent dyes (**Va** and **Vb**) and their derivatives (**VIa**, **VIb**, **VIIa**, and **VIIb**) in an ethanol–water (1 : 1) system

Name	Designation	$\lambda_{ex}/\lambda_{em}$, nm	Quantum yield, %	ε, $M^{-1}\,cm^{-1}$
5-Carboxy-Sy630	5-Sy630 (**Va**)	589/615	70 ± 8	101900 ± 1100
6-Carboxy-Sy630	6-Sy630 (**Vb**)	586/612	88 ± 12	90300 ± 4600
5-Azidopropyl-Sy630	5-Sy630-N_3 (**VIIa**)	592/619	66 ± 8	71300 ± 2000
6-Azidopropyl-Sy630	6-Sy630-N_3 (**VIIb**)	592/617	97 ± 3	107800 ± 3900

Table 2. Results of MALDI-TOF analysis of modified oligonucleotides

Oligonucleotide, 5' → 3'	Designation	Molecular weight	*m/z* $[M + H]^+$
5-Sy630-Aminolink-C6-t-ttt-ttt-ttt-ttt-ttt-ttt-t	5-Sy630-Aminolink-C6-T20	6775.9	6780.1
6-Sy630-Aminolink-C6-t-ttt-ttt-ttt-ttt-ttt-ttt-t	6-Sy630-Aminolink-C6-T20	6775.9	6781.3
5-Sy630-Alkyn-t-ttt-ttt-ttt-ttt-ttt-ttt-t	5-Sy630-Alkyn-T20	6930.0	6942.6
6-Sy630-Alkyn-t-ttt-ttt-ttt-ttt-ttt-ttt-t	6-Sy630-Alkyn-T20	6930.0	6927.7
5-Sy630-Aminolink-C6-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	5-Sy630-Aminolink-C6-Fc	8382.0	8371.6
6-Sy630-Aminolink-C6-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	6-Sy630-Aminolink-C6-Fc	8382.0	8372.1
5-Sy630-Alkyn-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	5-Sy630-Alkyn-Fc	8536.1	8521.2
6-Sy630-Alkyn-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	6-Sy630-Alkyn-Fc	8536.1	8517.1
6-ROX-ag-cgg-ctc-cta-ctt-ctg-cag-ggg-BHQ2	FC_Pr_up_ROX	8310.8	8308.8
Cy5-ag-cgg-ctc-cta-ctt-ctg-cag-ggg-BHQ2	FC_Pr_up_Cy5	8300.6	8303.8
cac-ata-ttt-aca-gaa-tgg-caa-agg	Fc-up	7393.8	7395.1
ctg-aag-aca-cat-ttt-tac-tcc-caa	Fc-low	7255.7	7256.5

Table 3. Spectral characteristics of modified oligonucleotides

Oligonucleotide, 5' → 3'	Designation	$\lambda_{ex}/\lambda_{em}$, nm
5-Sy630-Aminolink-C6-t-ttt-ttt-ttt-ttt-ttt-ttt-t	5-Sy630-Aminolink-C6-T20	602/628
5-Sy630-Alkyn-t-ttt-ttt-ttt-ttt-ttt-ttt-t	5-Sy630-Alkyn-T20	602/629
6-Sy630- Aminolink-C6-t-ttt-ttt-ttt-ttt-ttt-ttt-t	6-Sy630-Aminolink-C6-T20	602/625
6-Sy630-Alkyn-t-ttt-ttt-ttt-ttt-ttt-ttt-t	6-Sy630-Alkyn-T20	601/624
5-Sy630-Aminolink-C6-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	5-Sy630-Aminolink-C6-Fc	604/629
6-Sy630-Aminolink-C6-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	6-Sy630-Aminolink-C6-Fc	603/626
5-Sy630-Alkyn-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	5-Sy630-Alkyn-Fc	602/626
6-Sy630-Alkyn-a-gcg-gct-cct-act-tct-gca-ggg-g-BHQ2	6-Sy630-Alkyn-Fc	603/626
6-ROX-ag-cgg-ctc-cta-ctt-ctg-cag-ggg-BHQ2	FC_Pr_up_ROX	588/613
Cy5-ag-cgg-ctc-cta-ctt-ctg-cag-ggg-BHQ2	FC_Pr_up_Cy5	648/668

derivatives (**VIIa** and **VIIb**) were attached to the 5' ends of the T_{20} oligothymidylates by condensation with the 5'-terminal alkyl group (Alkyne). The results of the MALDI-TOF analysis and spectral characteristics of synthesized oligonucleotide derivatives are shown in Tables 2 and 3, respectively.

The maximum emission wavelengths of carboxyl isomers 5 and 6 of the Sy630 dye differ by 4–5 nm (Table 3), which is also typical for the 5 and 6 carboxyl isomers of other xanthene dyes (FAM, R6G, TAMRA, ROX). However, this difference disappears when the 5 or 6 carboxyl isomer of the Sy630 dye is introduced into hybridization probes due, apparently, to the influence of a heterogeneous sequence compared to oligothymidylates. The maxima of the absorption and fluorescence spectra of the Sy630 dye derivatives in oligonucleotides have the intermediate values compared to those of the 6-ROX and C5 dyes (Figs. 1, 2). To detect the new Sy630 fluorescent dye in an individual spectral channel, a pair of the 580 nm (30 nm)/630 nm (30 nm) light filters was installed in an ANC-M PCR-RV device [23], where 30 nm is the value of the full bandwidth at half the amplitude. The 6-ROX dye in the hybridization probes has the characteristic excitation and emission wavelengths of 587–588 nm and 613–614 nm, respectively (Table 3). To reduce the fluorescence crosstalk of the Sy630 dye in the same spectral channel, we used a nonstandard pair of light filters with excitation/emission characteristics of 550 nm (25 nm)/600 nm (30 nm). A standard pair of 630 nm (30 nm)/685 nm (25 nm) light filters were used to detect the Cy5 dye fluorescence signal.

The results of RT PCR with simultaneous detection of 6-ROX, 5-Sy630-NHS (**VIa**), 6-Sy 630-NHS (**VIb**), and Cy5 dyes in the hybridization probes in the individual spectral channels are presented in Fig. 3.

Figure 4 presents the results of RT PCR with simultaneous detection of the 6-ROX, 5-Sy630-N3 (**VIIa**), 6-Sy630-N3 (**VIIb**), and Cy5 dyes in the hybridization probes in the individual spectral channels.

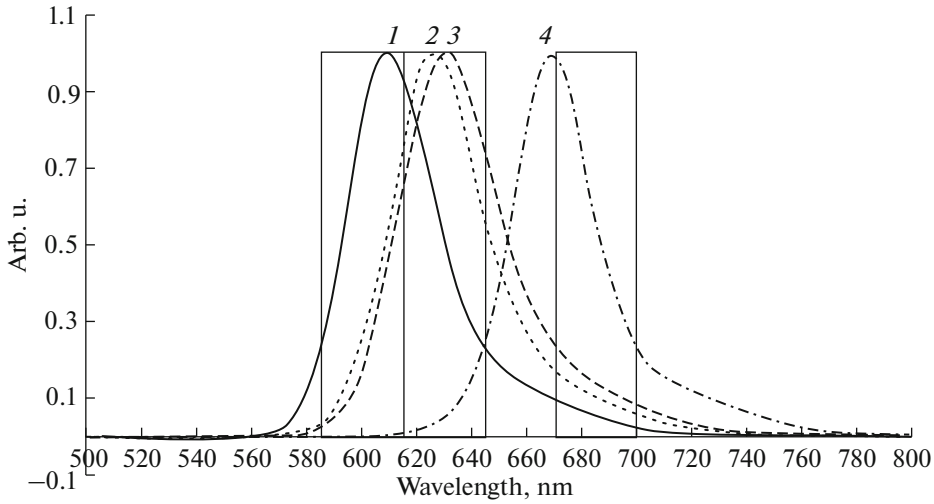


Fig. 1. Normalized excitation spectra of oligothymidylates that contained 6-ROX (*1*), 6-Sy630 (*2*), 5-Sy630 (*3*) and Cy5 (*4*) dyes at the 5' end. Rectangles indicate the spectral transmission ranges of interference light filters used for RT PCR.

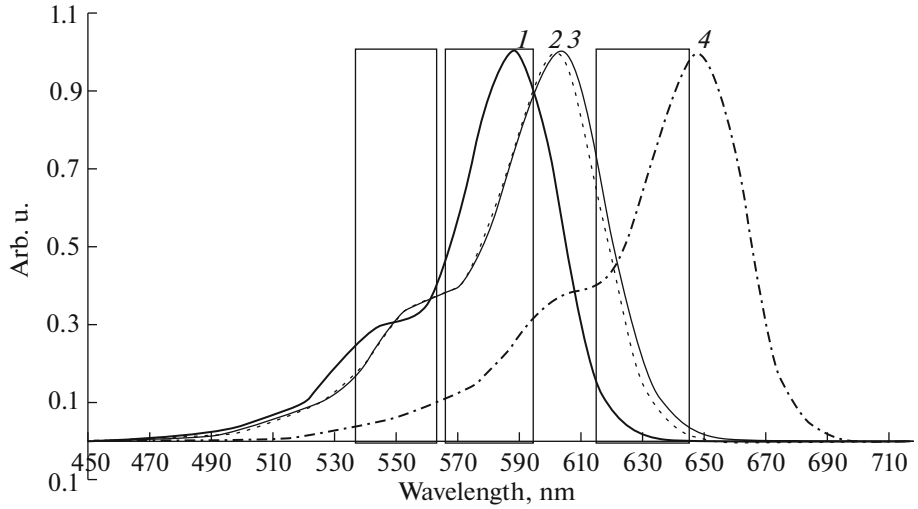


Fig. 2. Normalized emission spectra of oligothymidylates that contained 6-ROX (*1*), 6-Sy630 (*2*), 5-Sy630 (*3*) and Cy5 (*4*) dyes at the 5' end. Rectangles indicate the spectral transmission ranges of interference light filters used for RT PCR.

Figures 3 and 4 demonstrate that the systems of primers and probes labeled with derivatives of the new Sy630 fluorescent dye provide the high (above 97%) real-time PCR efficiency in a wide range of the DNA concentrations. The calculated R^2 values are close to 1 and the values of RT PCR efficiency are close to 100% for probes that contain the dyes, which were introduced through either *N*-hydroxysuccinimide esters or azidopropyl derivatives. These results indicate that both types of derivatives of the new dye can be used for the introduction into hybridization probes for real-time PCR. It should be noted that the azidopropyl derivatives are preferable candidates for the routine work to introduce fluorescent tags in hybridization probes for RT PCR due to their higher stability compared to *N*-hydroxysuccinimide esters. Moreover, it is best to use the azidopropyl derivative of isomer 6 because it has higher values of the quantum yield and molar absorption coefficient (Table 1), the product of which is proportional to the luminosity of the fluorophore. The chosen combination of interference light filters for three adjacent spectral channels provides effective detection of the target DNA in each spectral channel for the RT PCR device, although this combination is not optimal. To ensure the best excitation and collection of the fluorescence signal, it is necessary to use the interference light filters with the maximum transmittance shifted by 20–30 nm to the red region of

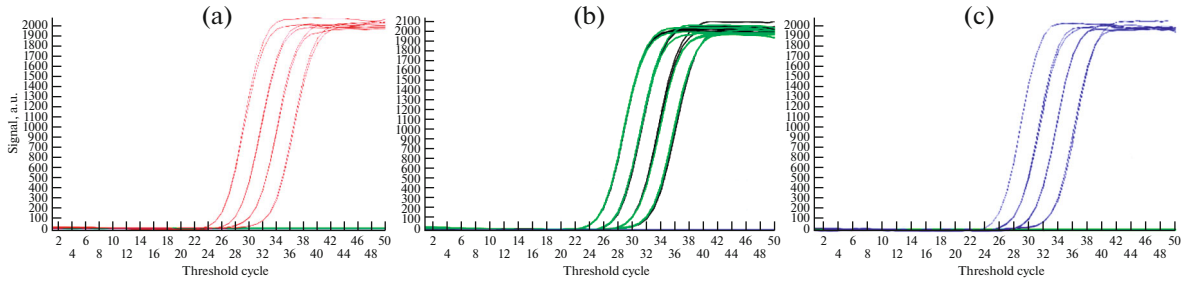


Fig. 3. Real-time PCR data (normalized to zero by the maximum) of a series of four 10-fold dilutions of human DNA at concentrations of 5, 1, 0.2, 0.04 ng/μL. Experiments were carried out in two repeats using the following hybridization probes: (a) FC_Pr_up_ROX, (R^2 = 1.0, E = 97%); (b) 5-Sy630-Aminolink-C6-Fc, (R^2 = 1.0, E = 98%) and 6-Sy630-Aminolink-C6-Fc (R^2 = 0.999, E = 100%); (c) FC_Pr_up_Cy5 (R^2 = 0.997, E = 98%).

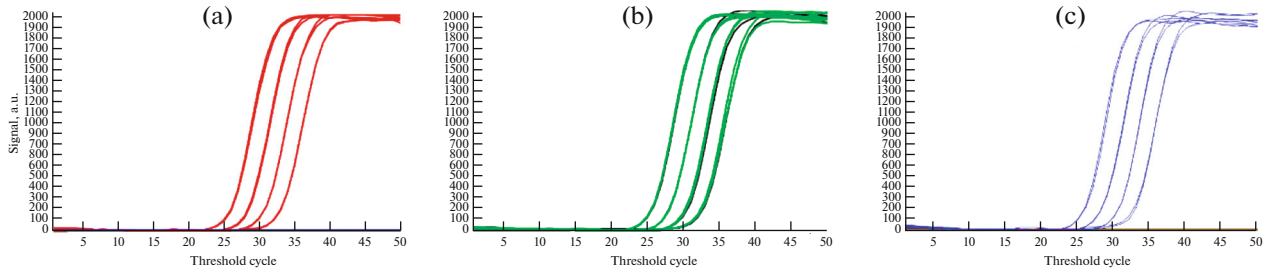


Fig. 4. Real-time PCR data (normalized to zero by the maximum) of a series of four 10-fold dilutions of human DNA at concentrations of 5, 1, 0.2, 0.04 ng/μL. Experiments were carried out in two repeats using the following hybridization probes: (a) FC_Pr_up_ROX, (R^2 = 1.0, E = 97%); (b) 5-Sy630-Alkyn-Fc, (R^2 = 0.998, E = 100%) and 6-Sy630-Alkyn-Fc (R^2 = 1.0, E = 97%); (c) FC_Pr_up_Cy5 (R^2 = 0.999, E = 101%).

the spectral range to detect the fluorescence of the Sy630 dye.

Thus, we demonstrated for the first time the possibility of real-time PCR with hybridization probes that contained the new fluorescent dye derivatives based on dihydroquinoline, i.e., (5(6)-carboxy-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2*H*-pyrano[3,2-g:5,6-g']dihydroquinolin-6-yl) benzoate-5/6-carboxy-Sy630. It was shown that these probes can be used in multiplex RT PCR with the detection in the individual spectral channel of the device for RT PCR. The use of the proposed dyes makes it possible to increase the number of simultaneously detected DNA and/or RNA targets in one test tube compared to the standard set of fluorescent dyes and corresponding combinations of interference light filters widely used today in devices for multiplex RT PCR.

EXPERIMENTAL

We used acetonitrile (pure for analysis) and benzene (chemically pure) refluxed over P_2O_5, chloroform (chemically pure). *tert*-Methylbutanol (pure for analysis) and methylene chloride (chemically pure) were kept over CaH_2; diisopropylethylamine 99% (Fisher Scientific) was refluxed over KOH; acetylacetone (pure for analysis, Cherkassk chemical plant) was refluxed in a vacuum. The following reactants were used without additional purification: ethyl *p*-toluene sulfonate (98%), *m*-anisidine (99%), cyclopentanone (99+%), 1 M boron trichloride in methylene chloride, and boron tribromide (99%) (Acros Organics); dry tetrahydrofuran (max 0.0075% water) (Panreac); granulated magnesium (12–50 mesh, 99.8%), methyl acetoacetate (99%, Alfa Aesar); 4-dimethylaminopyridine (99+%, Fluka); phosphorus(V) oxide (pure), anhydrous sodium sulfate (chemically pure), *p*-toluene sulfonic acid monohydrate (pure), ethyl acetate (chemically pure), hexane (chemically pure), diethyl ether (pure for analysis) (ChimMed); Hydrochloric acid (chemically pure), sodium hydroxide (pure for analysis), butyric acid (imp.), sulfuric acid (chemically pure), potassium bichromate (chemically pure), methyl iodide (Vecton); chlorobenzene (pure for analysis) (Ecos-1); ammonium chloride (chemically pure) (Reachim); trimellitic anhydride (97%), *N*,*N*'-disuccinimidyl carbonate (95+%), sodium azide (99.5+%), 3-bromopropylamine hydrobromide (98%), dimethylsulfoxide USP, tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (97%), cuprum (II) sulfate pentahydrate (98%), tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich); methanol (99.9%, LabScan); *N*,*N*,*N*',*N*'-tetramethyl-*O*-(N-succinim-

idyl) uranium tetrafluoroborate (Hangzhou Dayangchem). The other reagents of domestic production were chemically pure.

TLC was carried out on Kieselgel 60 plates (Merck, Germany) in systems listed below.

Oligonucleotides were synthesized and purified as described in [22]. The sequences of primers and probes Fc-up, Fc-low, 5-Sy630-Aminolink-C6-Fc, 6-Sy630-Aminolink-C6-Fc, 5-Sy630-Alkyn-Fc, 6-Sy630-Alkyn-Fc, FC_Pr_up_ROX, and FC_Pr_up_Cy5 were matched to the Fc fragment of the human immunoglobulin G gene (FCGR3B, Sequence ID: NM_001271036.1).

We used a magnetic stirrer with heating (Heildolph MR 3001 K, Germany), a rotary evaporator (Buchi Rotavapor R200, Switzerland), a DNA synthesizer (ASM-2000, Biosset, Russia), a preparatory chromatography (Reveleris Prep Grace, United States), a thermostat (Cyclotemp-901, Russia), a shaker (Eppendorf Mixer 5432, Germany), a vacuum evaporator (Univapo 150 ECH, Germany), and a microcentrifuge-shaker (Cyclotemp-901, Russia). The absorption spectra of the dyes and their conjugates with oligonucleotides were recorded on a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, United States). The emission and fluorescence spectra were recorded on a PerkinElmer LS55 spectrofluorometer (PerkinElmer, United States) at the scanning rate of 500 nm/min and the gap widths of 15 nm and 10 nm when measuring excitation and emission, respectively. The NMR spectra were recorded on a DRX500 spectrometer (Bruker Daltonics, Germany) in the Zelinsky Institute of Organic Chemistry of the Russian Academy of Sciences (Moscow). The MALDI-TOF mass spectra were recorded on a Microflex LRF spectrometer (Bruker Daltonics, Germany) in the Center for Collective Use of Scientific Equipment Biotechnology of the All-Russian Research Institute of Agricultural Biotechnology. Real-time PCR was performed on an ANK-48 device (Institute of Analytical Instrumentation of the Russian Academy of Sciences) according to the following scheme: 95°C for 5 min, 49 cycles: 60°C for 40 s, 95°C for 15 s. The results were processed using the ANK-Shell 1.0.5.100 program.

2,4-Dimethyl-7-methoxyquinoline (I). Acetylacetone (45 mL, 43.875 g, 0.438 mol, 1.1 equiv) and *m*-anisidine (45 mL, 49.06 g, 0.398 mol, 1 equiv) were refluxed for 2.5 h at 140°C. The reaction mixture was evaporated in a vacuum at 70°C. The resulting oil was poured into concentrated sulfuric acid (250 mL) under stirring and cooling in an ice bath, followed by the heating in a boiling water bath for 1.5 h. The mixture was poured into water (1 L) and cooled in a water bath, followed by the addition of the potassium dichromate solution (135 g in 1 L of water). The mixture was cooled in an ice bath. The precipitated quinolone bichromate was filtered, thoroughly washed with water, and dissolved in 12% sodium hydroxide solution (900 mL). After the complete dissolution of the precipitate, the product was extracted with diethyl ether (4 × 200 mL), dried over anhydrous sodium sulfate, and evaporated in a vacuum. The yield of 2,4-dimethyl-7-methoxyquinoline (colorless oil, which slowly crystallizes in the form of large prisms when storing in the refrigerator at 4°C) was 38.0 g (51%). R_f 0.85 (methanol–chloroform, 1 : 3); Mass spectrum, *m/z* found: 188.165, calc.: 187.1. ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 7.73 (1H, d, J = 9.1, C_5H), 7.30 (1H, d, J = 2.4, C_8H), 7.06 (1H, dd, J = 9.1, 2.4, C_6H), 6.90 (1H, s, C_3H), 3.88 (3H, s, OCH_3), 2.61 (3H, s, C_2–CH_3), 2.53 (3H, s, C_4–CH_3). ^{13}C NMR in $CDCl_3$ (126 MHz) δ (ppm): 159.86 (C_2), 158.41 (C_7), 148.97 (C_{8a}), 143.50 (C_4), 124.16 (C_5), 120.95 (C_{4a}), 120.20 (C_3), 117.56 (C_6), 106.81 (C_8), 54.87 (OCH_3), 24.60 (C_2–CH_3), 17.95 (C_4–CH_3).

1-Ethyl 2,4-dimethyl-7-methoxyquinolinium tosylate (IIa). 2,4-dimethyl-7-methoxyquinoline (38.0 g, 0.203 mol) and ethyl tosylate (38 mL, 44.7 g, 0.233 mol, 1.1 equiv) were refluxed in chlorobenzene (115 mL) for 20 h. After cooling the reaction mixture, the precipitated crystals were filtered, washed with petroleum ether, and air-dried. The filtrate was evaporated in the vacuum of an oil pump. The resulting oil was crystallized when standing. The yield of 1-ethyl 2,4-dimethyl-7-methoxyquinolinium tosylate was 72.236 g (92%). Cream crystals, R_f 0.27 (methanol–chloroform, 1 : 3). Mass spectrum, *m/z* found: 215.969, calc.: 216.3 ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 8.08 (1H, d, J = 9.2, C_5H), 7.64 (2H, d, J 8.0, Tos(2' + 6')), 7.58 (1H, d, J = 1.9, C_8H), 7.52 (1H, s, C_3H), 7.40 (1H, dd, J = 9.2, 2.1, C_6H), 6.98 (2H, d, J = 7.9, Tos(3' + 5')), 5.09 (2H, q, J = 7.2, CH_2CH_3), 4.10 (3H, s, OCH_3), 3.04 (3H, s, C_2–CH_3), 2.81(3H, s, C_4–CH_3), 2.25 (3H, s, Tos(CH_3), 1.58 (3H, t, J = 7.3, CH_2CH_3).

2,2,4-Trimethyl-1-ethyl-7-methoxy-1,2-dihydroquinoline (IIIa). The Grignard reagent, which was obtained from magnesium (2.43 g, 100 mmol, 4 equiv) and methyl iodide (6.23 mL, 100 mmol, 14.194 g) in *tert*-butyl methyl ether (100 mL), was added dropwise in a dark to the solution of 1-ethyl-2,4-dimethyl-7-methoxyquinolinim tosylate (9.438 g, 24.36 mmol) in absolute tetrahydrofuran (100 mL) in the argon atmosphere. The reaction mixture was stirred for two days, followed by the addition of a saturated solution of ammonium chloride (150 mL) under cooling in an ice bath. The precipitate was filtered and washed with a small amount of water and *tert*-butyl methyl ether. The filtrate was separated in a separatory funnel. The water phase was extracted with diethyl ether (2 × 100 mL). The combined organic phase was dried over anhydrous sodium sulfate and evaporated in a vacuum. The resulting product was purified by flash chromatography on silica gel using a hexane-chloroform mixture

(9 : 1) as an eluent. The yield of 2,2,4-trimethyl-1-ethyl-7-methoxy-1,2-dihydroquinoline was 49%. Mass spectrum, *m/z* found: 231.168, calc: 231.2. ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm) : 6.97 (1H, d, J = 8.3, C_5H), 6.14 (1H, dd, J = 8.3, 2.3, C_6H), 6.08 (1H, d, J 2.3, C_8H), 5.10 (1H, s, C_3H), 3.79 (3H, s, OCH_3), 3.32 (2H, q, J = 7.0, N–CH_2CH_3), 1.97 (3H, s, C_4–CH_3), 1.32 (6H, s, C_2–$(CH_3)_2$), 1.22 (3H, t, J = 7.0, N–CH_2CH_3). ^{13}C NMR in $CDCl_3$ (126 MHz) δ (ppm): 160.69 (C_7), 145.11 (C_{8a}), 127.48 (C_4), 127.12 (C_3), 124.59 (C_5), 116.73 (C_{4a}), 98.91 (C_6), 97.65 (C_8), 57.02 (C_2), 55.19 (OCH_3), 38.32 (N–CH_2CH_3), 28.80 (C_2–$(CH_3)_2$), 18.90 (C_4–CH_3), 14.39 (N–CH_2CH_3).

2,2,4-Trimethyl-1-ethyl-7-hydroxy-1,2-dihydroquinoline (IVa). 2,2,4-Trimethyl-1-ethyl-7-hydroxy-1,2-dihydroquinoline (2.655 g, 11.5 mmol) was dissolved in dry chloroform (20 mL), followed by the dropwise addition of bromine trichloride (2.22 mL, 5.762 g, 23 mmol, 2 equiv) in dry chloroform (10 mL) in the dark in an ice bath in the argon atmosphere. The reaction mixture was kept for 1 h at –3°C and then for 40 min at room temperature, followed by the addition of 12% sodium hydroxide (22.5 mL) under ice-cooling. The lower layer was separated on a separatory funnel. The water phase was acidified and extracted with chloroform. The combined extracts were dried under anhydrous sodium sulfate and passed through a silica gel layer using chloroform as eluent. The eluate was evaporated in a vacuum. The yield of 2,2,4-trimethyl-1-ethyl-7-hydroxy-1,2-dihydroquinoline in the form of oil darkening in the air was 1.106 g (44%). Mass spectrum, *m/z* found: 217.230, calc.: 217.1. ^{1}H NMR in $CDCl_3$ (50 MHz) δ (ppm): 6.87 (1H, d, C_5H), 6.03 (2H, m, C_6H + C_8H), 5.05 (1H, s, C_3H), 3.27 (2H, q, NCH_2), 1.92 (3H, s, C_4–CH_3), 1.29 (6H, s, C_2–$CH_3)_2$), 1.18 (3H, t, CH_2CH_3).

5(6)-Carboxy-Sy630 4(5)-carboxy-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2*H*-pirano[3,2-g:5,6-g']diquinolin-1-ium-6-yl)benzoate (Va) and (Vb). Toluene sulfonic acid hydrate (60 mg, 0.32 mmol) and trimellitic anhydride (1.603 g, 8.35 mmol, 1 equiv) were added to the solution of 2,2,4-trimethyl-1-ethyl-7-hydroxy-1,2-dihydroquinoline (3.63 g, 16.7 mmol, 2 equiv) in butyric acid (130 mL). The reaction mixture was refluxed in an argon atmosphere for 24 h, followed by evaporation in a vacuum at 80°C. The residue was filled with hexane, and hexane was removed the next day. The product was initially purified by chromatography on neutral aluminum oxide using isopropanol containing water (0–15%) and trimethylamine (5%) as an eluent. Fractions that contained isomer 5 were evaporated, and the residue was dissolved in dry methylene chloride (5 mL), followed by the dropwise addition to absolute diethyl ether (100 mL) under stirring on a magnetic stirrer. The precipitate was filtered, washed with ether, and dried in a vacuum. The yield of pure 5-carboxy isomer was 0.265 g. The other product-containing fractions were additionally purified by silica gel chromatography using elution with a gradient of methanol in chloroform containing 1% trimethylamine (0–20%). 6-Carboxy isomer (**Vb**) and 5-carboxy isomer (**Va**) were eluted by 10% and 15–20% of methanol, respectively. 6-Carboxy-Sy630 isomer was obtained in a yield of 0.796 g (16%) in the form of purple foam. R_f 0.24 (methanol-chloroform, 1 : 4). Mass spectrum, *m/z:* found: 591.315, calc.: 590.3; quantum yield, 0.77 (in an ethanol–water system, 1 : 1); molar absorption coefficient at λ_{max}, 9×10^4 M^{-1} cm^{-1}; maximum absorption wavelength, 584 nm (in an ethanol-water system, 1 : 1); maximum fluorescence wavelength, 609 nm (in the same system). ^{1}H NMR spectrum of triethylammonium salt in $CDCl_3$ (500 MHz) δ (ppm): 8.23 (1H, d, J = 7.9), 7.98 (1H, d, J = 8.1), 7.80 (1H, s), 6.37 (2H, s), 6.24 (2H, s), 5.09 (2H, s), 3.36 (4H, q, J = 6.9, 2*NCH_2CH_3), 3.01 (6H, q, J = 7.3, $N(CH_2CH_3)_3$), 1.63 (6H, s, C_4–CH_3 + C_8CH_3), 1.31 (12H, s, 2*C_2–CH_3 + 2*C_{11}–CH_3), 1.24 (15H, m, 2*NCH_2CH_3 + $N(CH_2CH_3)_3$).

5-Sy630-NHS 5-Carboxy-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2*H*-pyrano-[3,2-g:5,6-g']diquinolin-1-ium-6-yl)benzoate N-hydroxysuccinimide ester (VIa). 5-Carboxy-Sy630 (95 mg, 0.161 mmol) was dissolved in dry methylene chloride (9.5 mL), followed by the addition of diisopropylethylamine (42 μL, 31 mg, 0.242 mmol, 1.5 equiv), disuccinimidyl carbonate (50 mg, 0.193 mmol, 1.2 equiv), and 4-dimethylaminopyridine (10 mg, 0.08 mmol, 0.5 equiv). The mixture was stirred at room temperature for 2 h, followed by the addition of disuccinimidyl carbonate (25 mg) and stirring for another 1 h. The reaction mixture was poured into 0.6 M hydrochloric acid (10 mL), and the bottom layer was separated in a separating funnel. The organic phase was dried over anhydrous sodium sulfate and evaporated in a vacuum. The residual compound was dissolved in dry methylene chloride (2 mL) and added dropwise into absolute *tert*-butyl methyl ether under stirring. The precipitate was filtered, washed with *tert*-butyl methyl ether, and dried in a vacuum. The yield of compound (**VIa**) in the form of purple powder was 43 g (39%). R_f 0.75 (methanol–chloroform, 1 : 3). Mass spectrum, *m/z:* found: 688.1, calc.: 687.3. Maximum absorption wavelength, 592 nm (in an ethanol–water system, 1 : 1); maximum fluorescence wavelength, 641 nm (in the same system). ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 9.08 (1H, s), 8.41 (1H, d, J = 7.7), 7.40 (1H, d, J = 7.7), 6.61 (2H, s), 6.58 (2H, s), 5.44 (2H, s), 3.62 (4H, d, J = 6.5), 2.98 (4H, s), 1.76 (6H, s), 1.48 (12H, s), 1.38 (6H, unresolved triplet).

6-Sy630-NHS 4-Carboxy-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2*H*-pyrano-[3,2-g:5,6-g']diquinolin-1-ium-6-yl)benzoate N-hydroxysuccinimide ester (VIb). 6-Carboxy-Sy630 (227 mg, 0.38 mmol) was dissolved in dry methylene

chloride (22 mL), followed by the addition of diisopropylethylamine (100 μL, 75 mg, 0.576 mmol, 1.5 equiv), disuccinimidyl carbonate (117 mg, 0.456 mmol, 1.2 equiv), and 4-dimethylaminopyridine (23 mg, 0.19 mmol, 0.5 equiv). The mixture was stirred at room temperature for 1 h, followed by the addition of disuccinimidyl carbonate (12 mg) and stirring for another 1.5 h. The mixture was poured into saturated potassium dihydrogen phosphate (25 mL) and shaken in a separating funnel. The organic phase was separated, dried over anhydrous sodium sulfate, and evaporated in a vacuum. The product was purified by reverse-phase chromatography using a water–acetonitrile gradient. The product-containing fractions eluting with 40% acetonitrile were combined and evaporated to half the volume, extracted with methylene chloride, dried with anhydrous sodium sulfate, and evaporated in a vacuum. The yield of compound (VIb) in the form of purple foam was 67 mg (26%). R_f 0.72 (methanol–chloroform, 1 : 3). Mass spectrum, *m/z:* found: 688, calc.: 687.3. Maximum absorption wavelength, 603 nm (in water). ^{1}H NMR in $CDCl_3$ (400 MHz) δ (ppm): 8.51 (1H,d, J = 8.2), 8.44 (1H, dd, J = 8.2, 1.6), 7.99 (1H, d, J =1.5), 6.64 (2H, s), 6.60 (2H, s), 5.46 (2H, s), 3.61–3.64 (4H, m), 2.94 (4H, s), 1.77 (6H, s), 1.48 (12H, d), 1.39 (6H, t, J = 7.0).

5-Sy630-N_3 5-((3-Azidopropyl)carbamoyl)-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2H-pyrano[3,2-g:5,6-g']diquinolin-1-ium-6-yl)-benzoate (VIIa). 5-Carboxy-Sy630 (0.265 g, 0.45 mmol) was dissolved in dry methylene chloride (10 mL), followed by the addition of TSTU (0.162 g, 0.54 mmol, 1.2 equiv), diisopropylethylamine (94 μL, 0.54 mmol, 1.2 equiv, 70 mg), and DMAP (27 mg, 0.22 mmol, 0.5 equiv) under stirring. After stirring for 80 min, the mixture was poured into 1% hydrochloric acid (100 mL). The product was extracted with chloroform (2 × 50 mL), dried over aqueous sodium sulfate, and evaporated in a vacuum. The residue was dissolved in dry methylene chloride (10 mL), followed by the addition of aminopropylazide (68 μL, 0.675 mmol, 1.5 equiv) and diisopropylamine (118 μL, 87 mg, 0.675 mmol, 1.5 equiv). The mixture was stirred for 20 min and evaporated in a vacuum. The product was purified by silica gel chromatography eluting with a gradient of methanol in chloroform (0–5%) and then a gradient of methanol in chloroform (6–8%) containing 1% trimethylamine. The solution of the product was evaporated in a vacuum and dried over P_2O_5. The yield of compound (**VIIa**) in the form of purple powder was 212 mg (70%). R_f 0.54 (methanol–chloroform, 1 : 4). Mass spectrum, *m/z:* found: 673.423, calc.: 672.3; quantum yield, 0.74 (in an ethanol–water mixture 1 : 1); molar absorption coefficient at λ_{max}, 1.286×10^5 M^{-1} cm^{-1}; maximum absorption wavelength, 596 nm (in water); maximum fluorescence wavelength, 620 nm (in an ethanol–water mixture 1 : 1). ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 8.62 (1H, s, C_4'H), 8.19 (1H, d, J = 7.8, C_7'H), 7.58 (1H, t, J = 5.6, CONH), 7.20 (1H, d, J = 7.8, C_6'H), 6.72 (2H, s, $C_{12}H + C_{13}H$), 6.50 (2H, s, $C_5H + C_7H$), 5.32 (2H, s, $C_3H + C_9H$), 3.61 (2H, dd, J = 12.6, 6.4, $CONHCH_2$), 3.53 (4H, m, $N_1CH_2CH_3 + N_{11}CH_2CH_3$), 3.45 (2H, t, J = 6.7, CH_2N_3), 1.97 (2H, qt, J = 6.7, $CONHCH_2CH_2$), 1.72 (6H, s, $C_4–CH_3 + C_7–CH_3$), 1.42 (12H, d, J = 5.3, $C_2–2*CH_3 + C_{10}–2*CH_3$), 1.34 (6H, m, $N_1CH_2CH_3 + N_{11}CH_2CH_3$).

6-Sy630-N_3 4-((3-Azidopropyl)carbamoyl)-2-(1,11-diethyl-2,2,4,8,10,10-hexamethyl-10,11-dihydro-2H-pyrano[3,2-g:5,6-g']diquinolin-1-ium-6-yl)-benzoate (VIIb). 6-Sy630-NHS (66 mg, 0.096 mmol) was dissolved in dry methylene chloride (10 mL), followed by the addition of 3-aminopropylazide (19 mg, 0.192 mmol, 2 equiv). The mixture was stirred at room temperature for 4 h, diluted with methylene chloride (10 mL), and washed with 0.1% trifluoroacetic acid (2 × 10 mL), sodium bicarbonate (saturated solution–water, 1 : 1; 2 × 10 mL), and saturated solution of sodium chloride (20 mL). The product was purified by silica gel chromatography eluting with 5% ethanol in chloroform containing 1% trimethylamine. The yield of compound (**VIIb**) in the form of a blue-purple powder was 41 mg (64%). R_f 0.27 (methanol–chloroform, 1 : 10 with 5% triethylamine). Mass spectrum, *m/z:* found: 673.248, calc.: 672.3. ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 8.24 (1H, d, J = 8.1), 8.11 (1H, d, J = 8.0), 7.75 (2H, s), 6.66 (2H, s), 6.53 (2H, s), 5.35 (2H, s), 3.58 (4H, nonresolved q, J = 6.8, $2*CH_2N$), 3.50 (2H, dt, J = 15.8, 7.9, CH_2NHCO), 2.36 (2H, t, J = 6.9, CH_2N_3), 1.92 (2H, qt, J = 6.8, $–CH_2–$), 1.66 (6H, s, $2*CH_3$), 1.44 (12H, s, $4*CH_3$), 1.33 (6H, t, J = 6.9, $2*CH_2CH_3$). Based on the HMBC and HSQC spectrum, the ^{13}C NMR spectrum was correlated, and the structures of each isomer were confirmed. ^{13}C NMR in $CDCl_3$ (126 MHz) δ (ppm): 166.93 ($COO^–$), 165.61 (CONH), 157.46 (2C, F), 157.29 (2), 151.54 (2C, D), 136.27 (6), 136.10 (1), 133.02 (3), 131.29 (2C, X), 131.18 (1), 128.45 (5), 127.79 (4), 125.41 (2C, E), 123.04 (2C, A), 122.29 (2C, H), 113.63 (2C, C), 94.90 (2C, B), 59.42 (2C, $2*C(CH_3)_2$), 48.96 (a), 39.56 (2C, $2*NCH_2CH_3$), 37.27 (c), 29.22, 29.05 (4C, $4*CH_3$), 28.16 (b), 17.86 ($2*CH_3$), 12.76 ($2*CH_2CH_3$).

2-Amino-4-methoxyacetophenone (VIII). The solution of *m*-anisidine (2.777 mL, 24.72 mmol, 3.044 g) in dry benzene (20 mL) was added dropwise to 1 M boron trichloride solution (25 mL) under ice-cooling in an apparatus equipped with a calcium chloride tube, followed by the addition of acetonitrile (2.58 mL, 49.44 mmol, 3.03 g, 2 equiv) and aluminum chloride (3.626 g, 27.19 mmol, 1.1 equiv). The mixture was refluxed for 2 h; methylene chloride was evaporated with dephlegmator until the temperature reached

80°C; the mixture was refluxed for another 5 h, followed by the addition of acetonitrile (1.29 mL, 1 equiv); the mixture was refluxed for another 8 h, followed by the addition of 2 M hydrochloric acid (90 mL) under ice-cooling. The mixture was refluxed for 75 min. After cooling, the organic phase was separated, and the aqueous phase was alkalinized with 2 M sodium hydroxide (101 mL) to pH 3. The mixture was cooled to +3°C, and the resulting precipitate was filtered out. The product was extracted from the filtrate with methylene chloride (2 × 50 mL), dried over anhydrous sodium sulfate, and the solution was evaporated in a vacuum. The residue was combined with the precipitate and recrystallized from aqueous ethanol. The yield of 2-amino-4-methoxyacetophenone in the form of brilliant colorless prisms was 1.078 g (27%). ^{1}H NMR in $CDCl_3$ (500 MHz) δ (ppm): 7.64 (1H, d, $J = 9.0$, C_6H), 7.30 (1.5H, b, NH_2), 6.23 (1H, d, $J = 2.3$, C_3H), 6.13 (1H, dd, $J = 9.0$, 2.5, C_5H), 3.73 (3H, s, OCH_3), 2.41 (3H, s, $COCH_3$).

2,4-Dimethyl-7-methoxyquinoline-3-carboxylic acid methyl ester (IX). 2-Amino-4-methoxyacetophenone (0.772 g, 4.67 mmol), methyl acetoacetate (0.605 mL, 0.651 g, 5.6 mmol, 1.2 equiv), and *p*-toluenesulfonic acid (0.804 g, 1 equiv) were mixed and slowly heated on an oil bath to 100°C. After heating at this temperature for 30 min, the reaction mixture was cooled, followed by the addition of water (14 mL). The solution was neutralized with the addition of 12% sodium hydroxide (1.56 mL). After stirring for 5 min, the resulting precipitate was filtered out and washed three times with water. The product was purified by silica gel chromatography using a 2–50% gradient of ethyl acetate in hexane as an eluting system. The product was eluted at 25–30% of ethyl acetate. The yield of 2,4-Dimethyl-7-methoxyquinoline-3-carboxylic acid methyl ester in the form of a pale yellow mass was 0.664 g (64%). R_f 0.73 (methanol–chloroform, 1 : 9), ^{1}H NMR in $CDCl_3$ (500 MHz), δ (ppm): 7.82 (1H, d, $J = 9.2$, C_5H), 7.30 (1H, s, C_8H), 7.13 (1H, dd, $J = 9.1$, 2.5, C_6H), 3.96 (3H, s, C_7–OCH_3), 3.92 (3H, s, COO–CH_3), 2.65 (3H, s, C_2–CH_3), 2.59 (3H, s, C_4–CH_3). NMR ^{13}C in $CDCl_3$ (126 MHz), δ (ppm): 170.04 (COO), 161.36 (C_7), 155.08 (C_{8a}), 149.34 (C_2), 141.90 (C_4), 125.90 (C_3), 125.39 (C_{4a}), 120.81 (C_5), 119.38 (C_6), 107.54 (C_8), 55.69 (C_7–OCH_3), 52.54 ($COOCH_3$), 24.07 (C_2–CH_3), 15.94 (C_4–CH_3).

2,4-Dimethyl-7-methoxyquinoline-3-carboxylic acid (X). 2 M sodium hydroxide (8 mL) was added to 2,4-dimethyl-7-methoxyquinoline-3-carboxylic acid methyl ester (0.66 g, 3 mmol), and the mixture was refluxed for 15 h, followed by the addition of water (12 mL) and 6 M hydrochloric acid (2.66 mL). The mixture was kept overnight. The precipitate was filtered out, washed with water, and dried over P_2O_5. The yield of 2,4-dimethyl-7-methoxyquinoline-3-carboxylic acid in the form of pale yellow crystals was 0.436 g (70%). ^{1}H NMR in DMSO-d_6 (500 MHz), δ (ppm): 8.27 (1H, d, $J = 9.4$, C_5H), 7.62 (1H, d, $J = 2.3$, C_8H), 7.47 (1H, dd, $J = 9.3$, 2.4, C_6H), 3.96 (3H, s, OCH_3), 2.83 (3H, s, C_2–CH_3), 2.79 (3H, s, C_4–CH_3).

N-hydroxysuccinimide esters of 5- and 6-carboxy isomers of Sy630 were attached to the amino groups of 5'-amino-containing oligonucleotides according to the following general method. A five-fold excess of N-hydroxysuccinimide ester of the dye in DMSO (20 μL, 10 mg/mL) was added to 5'-amino-containing oligonucleotide (200 μL, 5 μM) in 0.1 M sodium bicarbonate (pH 8). The mixture was stirred on a shaker (1400 rpm) at 25°C for 12 h. Modified oligonucleotides were purified by electrophoresis in denaturing polyacrylamide gel followed by repurification by reverse-phase HPLC. The azide derivatives of 5- and 6-carboxy isomers of Sy630 were attached to the alkyne groups of 5'-alkyne-containing oligonucleotides according to [22].

Real-time PCR was performed according to the following cyclogram. Denaturation: 95°C for 5 min; 49 cycles: 60°C for 40 s, 95°C for 15 s. The working concentrations of primers and probes were 5 μM and 2.5 μM, respectively. The amount of SynTaq DNA polymerase containing antibodies that inhibit the activity of the enzyme was 5 U (Syntol, E-039-1000).

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with the use of humans as objects of research.

Conflict of Interests

The authors state that there is no conflict of interest.

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