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Thiazole Orange and Cy3: Improvement of Fluorescent DNA Probes with Use of Short Range Electron Transfer

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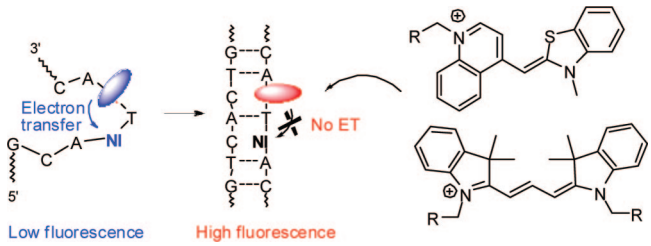

Thiazole orange was synthetically incorporated into oligonucleotides by using the corresponding phosphoramidite as the building block for automated DNA synthesis. Due to the covalent fixation of the TO dye as a DNA base surrogate, the TO-modified oligonucleotides do not exhibit a significant increase of fluorescence upon hybridization with the counterstrand. However, if 5-nitroindole (NI) is present as a second artificial DNA base (two base pairs away from the TO dye) a fluorescence increase upon DNA hybridization can be observed. That suggests that a short-range photoinduced electron transfer causes the fluorescence quenching in the single strand. The latter result represents a concept that can be transferred to the commercially available Cy3 label. It enables the Cy3 fluorophore to display the DNA hybridization by a fluorescence increase that is normally not observed with this dye.

The analytical problems in biomedicinal diagnostics, molecular genetics, and biochemistry with nucleic acids demand powerful and bright fluorescent probes and markers for DNA.[1] Organic chromophores as fluorescent probes that have been attached covalently either to the end of the oligonucleotide or to the DNA bases represent important tools, e.g., for genetic analysis on DNA microarrays,[2] for the molecular beacon technique,[3] for energy transfer systems,[4] or for the single base mismatch analysis.[5,6] On the other hand, a variety of organic fluorescent dyes have been synthetically incorporated at specific positions within the oligonucleotide sequence (e.g., cyanine dyes,[7] acridine,[8] coumarin,[9] ethidium,[10] flavin,[11] perylene bisimide,[12] pyrene,[13] and other fluorosides[14]). Among the powerful and broadly applied cyanine dyes, [7] thiazole orange (TO) represents a bright fluorescent probe that should be of potential interest for DNA analytical problems. TO was never incorporated synthetically as a base surrogate into DNA or RNA. However, TO was linked to internucleotidic[15] or terminal[16] positions of oligonucleotides and incorporated synthetically into DNA-binding peptides for cellular staining[17,18] and as a base surrogate into peptide nucleic acids.[19,20] The latter TO-modified PNAs were studied extensively by the group of Seitz et al. Remarkably, they were able to detect single nucleotide polymorphisms in a very sensitive and reliable way using TO-PNA-conjugates in a homogeneous assay.[20,21]

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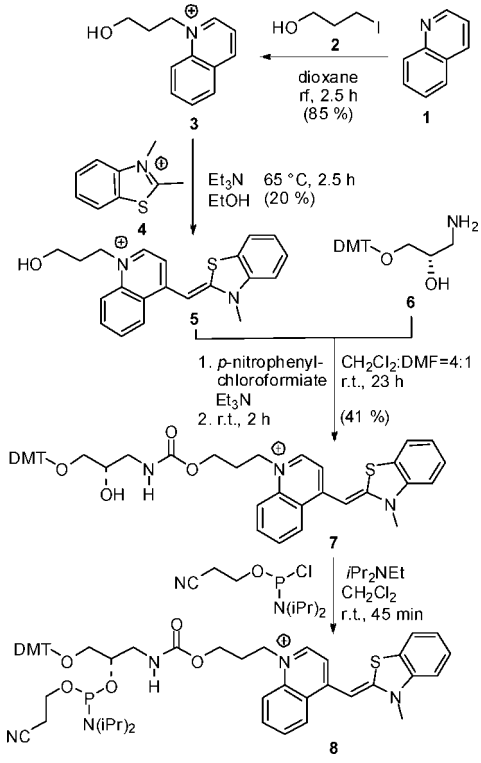
SCHEME 1. Synthesis of DNA Building Block 8

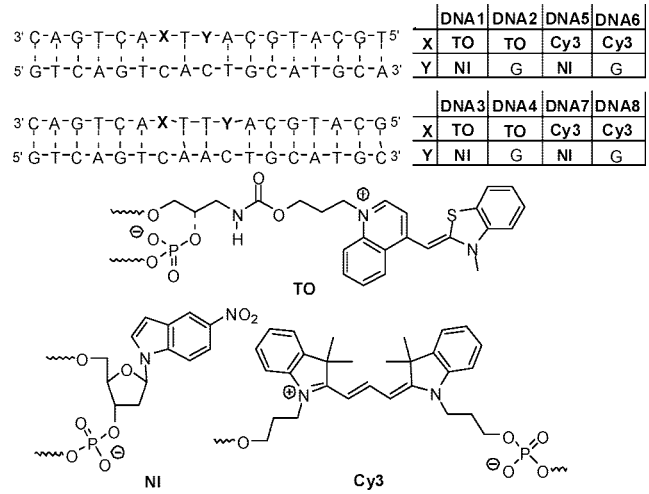


Herein, we present the synthesis and application of the TO dye as a covalently bound, internal fluorescent probe for DNA. Moreover, to be able to reference the elucidated fluorescence properties we compare TO with Cy3 as a representative fluorophore that is commercially available as a DNA building block and exhibits similar excitation and emission wavelengths.

We synthetically incorporated the TO dye into oligonucleotides using a structural approach that has been previously described for ethidium-,[10] indole-,[22] phenothiazine-,[23] and perylene bisimide-modified[12] oligonucleotides. The 2′-deoxyribofuranoside moiety of natural nucleotides was replaced by an acyclic linker system that is tethered to the nitrogen of the quinoline ring as part of the TO dye. Avoiding the acid/base labile glycosidic bond of natural nucleosides, especially in combination with a cationic dye, [24] this linker provides the chemical stability of the DNA building block that is crucial for the preparation of DNA−chromophore conjugates via automated phosphoramidite chemistry.

The synthesis of the TO-DNA building block (Scheme 1) starts with the alkylation of quinoline (**1**), using 3-iodopropane-1-ol (**2**) to give the quinoline **3**. The subsequent condensation of **3** with 1-methylbenzothiazolium iodide (**4**) yields the TO chromophore **5**. The reaction proceeds selectively to the para position of the methylated quinoline moiety as confirmed by 2D NMR measurements (HSQC, HMBC, and ROESY). Treatment with 4-nitrophenylchloroformiate furnished an active ester of compound **5** in situ that was subsequently coupled to the enantiomerically pure linker **6**[10] as a derivative of (*S*)-1-aminopropane-2,3-diol that carries already the 4,4′-dimethoxytrityl (DMT) protecting group needed for the automated DNA

SCHEME 2. Sequences of Duplexes DNA1−DNA8



phosphoramidite chemistry. The *S*-configuration of this linker has been used to mimic the stereochemical situation of the 3′-position of natural 2′-deoxyribofuranosides. The TO-chromophore is connected to the linker via a carbamate function whose N-H group is of low nucleophilicity and thus need not be protected for the phosphoramidite chemistry. The coupling of the phosphiteamide group to the free hydroxy group of **7** was performed by using standard procedures in CH_2Cl_2 yielding the phosphoramidite **8**.

By using **8** as a DNA building block, TO-modified oligonucleotides were prepared with use of standard automated DNA synthesis. In analogy to charge transfer experiments with pyrene-modified G[25] and ethidium,[26] we placed cytosine as the counterbase for TO. For the sequence design, it is important to note that the emission should compete with a fluorescence quenching caused by a photoinducable electron transfer. We recently introduced the "DETEQ" concept (detection by electron transfer-controlled emission quenching) for DNA analytics, which is based on the defined quenching of fluorescence by electron transfer processes.[27] Using combinations of pyrene-modified guanosine[25] or ethidium[26] as the fluorescent charge donors and indole or 7-deazaguanine as quenchers and charge acceptors, we were able to improve the fluorescent discrimination of DNA duplexes vs single strands and to detect single base variations. Similar to ethidium, the TO chromophore exhibits as an oxidation potential ($TO^+/TO^{\bullet\,2+}$) of 1.4 V (vs NHE).[28] Together with $E_{00} = 2.4$ V for TO, the excitation potential is not sufficient for the photoreduction of any of the DNA bases and thus prohibits electron hopping through the DNA. Together with 5-nitroindole (NI) as the charge acceptor that has a reduction potential of −0.3 V ($NI/NI^{\bullet-}$)[29] the electron transfer should follow a strongly distance-dependent superexchange mechanism.[30] Hence, in the duplexes **DNA1** and **DNA3** we placed the TO chromophore with a distance of one or two intervening T-A base pairs away from the NI moiety. The

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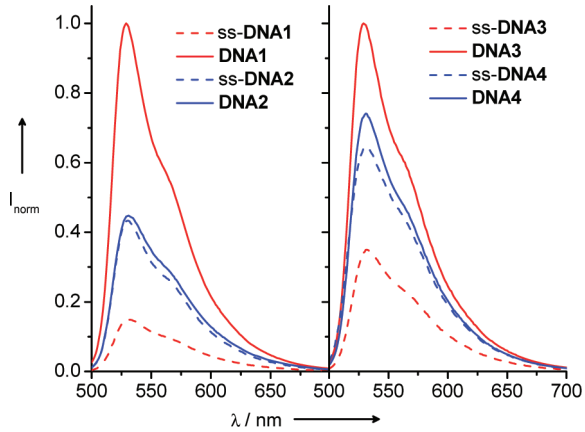

FIGURE 1. Fluorescence spectra of **DNA1**−**DNA4** (2.5 μM), 10 mM Na-P_i-buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 490 nm.

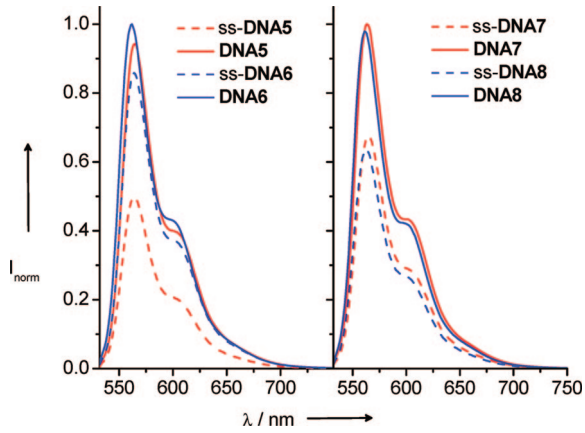

FIGURE 2. Fluorescence spectra of **DNA5**−**DNA8** (2.5 μM), 10 mM Na-P_i-buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 530 nm.

duplexes **DNA2** and **DNA4** lack the charge acceptor and serve as reference duplexes without electron transfer. In the duplex set **DNA5**−**DNA8**, the Cy3 dye replaces the TO chromophore (Scheme 2).

Comparing the NI-modified duplexes with the reference duplexes two major observations could be made: (i) the relative fluorescence intensities of the TO-modified duplexes **DNA1** and **DNA3** are increased in comparison with those of the reference duplexes **DNA2** and **DNA4** (Figure 1) and (ii) the relative fluorescence intensities of the Cy3-modified duplexes **DNA5** and **DNA7** are nearly the same (within experimental error) as those of **DNA6** and **DNA8** (Figure 2). The absence of fluorescence quenching in all four cases does not reveal that TO or Cy3 is able to photoinduce an electron transfer to NI through the duplex. It is known from the studies with the ethidium−NI DNA system that intercalation of both charge donor and acceptor in the base stack is crucial to provide the electronic coupling for an efficient charge transfer.[30] Thus, it can be concluded that at least the Cy3 chromophore is not sufficiently intercalated in the corresponding modified duplexes since it tends to bind in the minor groove. The fluorescence increase of the TO-modified **DNA1** and **DNA3** in comparison with **DNA2** and **DNA4**, respectively, occurs concomitantly with the decreased stability of the duplexes (see the melting temperatures T_m in Table 1). That indicates that the gained conformational flexibility of **DNA1** and **DNA3** allows the TO chromophore to intercalate better in the duplex yielding an intensified emission.

TABLE 1. Melting Temperatures (T_m), Quantum Yields (Φ), Extinction Coefficients (ϵ), Brightness ($B = \Phi\epsilon$), and I_{ds}/I_{ss} of Fluorescence Intensities of DNA1−DNA8

duplex	T_m (°C)	Φ	ϵ (M^{-1} cm^{-1})	B	I_{ds}/I_{ss}[c]
DNA1	61.0	0.23[a]	35800[a]	8300	6.6
DNA2	66.2	0.10[a]	35200[a]	3600	1.0
DNA3	57.0	0.15[a]	40900[a]	6100	2.9
DNA4	65.3	0.10[a]	39900[a]	4100	1.2
DNA5	50.5	0.28[b]	64300[b]	18000	1.9
DNA6	57.5	0.25[b]	86400[b]	21600	1.0
DNA7	46.7	0.30[b]	59500[b]	17900	1.6
DNA8	55.0	0.29[b]	57600[b]	16700	1.7

[a] At 490 nm. [b] At 518 nm. [c] I = integrated fluorescence intensities.

It is important to note that in case of the TO-modified duplexes without the NI modification, the relative fluorescence intensities of the single strands (ss-**DNA2**, ss-**DNA4**) are nearly the same as that of the corresponding duplexes (**DNA2**, **DNA4**). Due to the covalent fixation of the TO dye as a DNA base surrogate, the TO-modified oligonucleotides do not exhibit the TO-typical increase of fluorescence upon hybridization with the counterstrand. However, if NI is present as a second artificial DNA base (two or three base pairs away from the TO dye) a fluorescence increase upon DNA hybridization can be observed. In fact, the relative fluorescence intensities of the TO-modified single strands that contain the NI charge acceptor (ss-**DNA1**, ss-**DNA3**) are significantly reduced compared to those of the corresponding reference oligonucleotides (ss-**DNA2**, ss-**DNA4**). This result suggests that a photoinduced electron transfer could take place exclusively in the random-coil single strands causing a significant fluorescence quenching. This is a remarkable result since it clearly improves the optical discrimination of duplexes vs single strand by the fluorescent TO base surrogate (Table 1).

A similar result was obtained with the Cy3-modified duplex pair **DNA5/DNA6**. This is even more remarkable since the placement of NI as an electron acceptor at a distance of one intervening base pair enables the Cy3 fluorophore to display the DNA hybridization by a fluorescence increase (**DNA5**) compared to the duplex without NI (**DNA6**).

The photochemical data (Table 1) display the quantification of the steady-state fluorescence spectra. The quantum yields of the TO-modified duplexes lie in the range between 10% and 23% and the brightness in the range of 4000−9000 M^{-1} cm^{-1}. These values make the TO-modified oligonucleotides clearly suitable for fluorescence applications in chemical biology and fluorescent diagnostic technologies. Especially **DNA1** shows a high quantum yield (23%) that is comparable to that of the Cy3-modified **DNA5** (28%), and a significant fluorescence discrimination ratio of 6.6 between duplex and single strand due to the presence of the NI moiety. In comparison, the Cy3-modified duplexes are brighter (17000−22000 M^{-1} cm^{-1}) and exhibit higher quantum yields (25−30%). However the fluorescent duplex discrimination ratio in **DNA5** is lower (1.9, compared to **DNA1**). Please note that the quantum yields were not determined at the absorption maxima. That means that the brightness of all the duplexes is significantly higher if excited at the extinction maxima.

Taken together, there are two major results from these studies: (i) TO represents a bright fluorescent DNA base surrogate and thus a promising alternative to the commercially available cyanine dyes for applications in fluorescent DNA analytics and

cell biology. Due to the short methine bridge in TO we expect a better photostability of this dye compared to Cy3. (ii) The fluorescence increase of TO and Cy3 upon DNA hybridization can be improved by a short-range electron transfer in the single strand. The latter result could represent a concept that is potentially applicable also for other fluorescent labels, which is currently under investigation.

Experimental Section

1-(3-Hydroxypropyl)quinolinium Iodide (3). 2 (2.45 mL, 25.5 mmol) was added to a solution of **1** (1.01 mL, 8.5 mmol) in 9 mL of dioxane. The solution was stirred under reflux for 2.5 h. After cooling to rt the product was precipitated by addition of 6 mL of acetone. The residue was separated by filtration and washed with acetone yielding 2.28 g (85%) of **3** as a yellow solid compound: for ^{1}H and ^{13}C NMR, see the Supporting Information. FAB-HRMS: M^+ calcd for $C_{12}H_{14}NO$ 188.1075, found 188.1076.

1-(3-Hydroxypropyl)-4-(3-methyl-3*H*-benzothiazol-2-ylidenmethyl)quinolinium Iodide (5). 3 (2.28 g, 7.2 mmol) and **4** (2.11 g, 7.2 mmol) were dissolved in 40 mL of EtOH. Dry NEt_3 (3.01 mL) was added and the mixture was stirred for 2.5 h at 65 °C. The solution was cooled to rt and stirred for 1 h at rt. The product was precipitated by addition of 80 mL of Et_2O at −18 °C. After filtration, the product was suspended in a mixture of 30 mL of MeOH and 42 mL of Et_2O for 1 h. The product was collected by filtration and dried under vacuum yielding 0.68 g (20%) of **5** as a red solid compound: R_f 0.21 (9:1 CH_2Cl_2/MeOH); for ^{1}H and ^{13}C NMR, see the Supporting Information. FAB-HRMS: M^+calcd for $C_{21}H_{21}N_2OS^+$ 349.1375, found 349.1431.

1-(3-{3-[Bis(4-methoxyphenyl)phenylmethoxy]-2-hydroxypropylcarbamoyloxy}propyl)-4-(3-methyl-3*H*-benzothiazol-2-ylidenmethyl)quinolinuim Iodide (7). To a mixture of **5** (400 mg, 0.84 mmol) and NEt_3 (0.41 mL, 2.94 mmol) in 40 mL of DMF was added a solution of 4-nitrophenylchloroformiate (559 mg, 2.77 mmol) in 160 mL of CH_2Cl_2 and the solution was stirred for 23 h at rt. Subsequently, **6**[10] (495 mg, 1.26 mmol) was added and the mixture was stirred for 2 h at rt. The solvents were removed under vacuum and the residue was purified via silica chromatography with CH_2Cl_2/MeOH 96:4 +0.1% NEt_3 as an eluent to afford 306 mg (41%) of **7** as a red powder: R_f 0.38 (9:1 CH_2Cl_2/MeOH); for ^{1}H and ^{13}C NMR, see the Supporting Information. FAB-HRMS: M^+ calcd for $C_{46}H_{46}N_3O_6S^+$ 768.3107, found 768.3106.

1-(3-{3-[Bis(4-methoxyphenyl)phenylmethoxy]-2-[(2-cyanoethoxy)diisopropylaminophosphanyloxy]propylcarbamoyloxy}propyl)-4-(3-methyl-3*H*-benzothiazol-2-ylidenmethyl)quinolinuim Iodide (8). To a solution of **7** (306 mg, 0.34 mmol) in 10 mL of CH_2Cl_2 were added ethyldiisopropylamine (178.5 μL 1.03 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (111 μL, 0.5 mmol). The mixture was stirred for 45 min at rt and was washed with saturated aq $NaHCO_3$, dried over Na_2SO_4, and concentrated under vacuum. Due to the hydrolytic lability the resulting red solid compound was solved in MeCN and applied directly for automated DNA synthesis. Quantitative conversion was assumed based on tlc; R_f 0.50 (9:1 CH_2Cl_2/MeOH); for ^{31}P NMR, see the Supporting Information.

Oligonucleotides (DNA1−DNA8). Oligonucleotides were prepared on a synthesizer by using standard phosphoramidite chemistry. For the TO-phosphoramidite the coupling time was enhanced from 96 to 3700 s. Commercially available reagents and CPG (1 μmol) were used. After preparation the trityl-off oligonucleotide was cleaved off the resin and deprotected by treatment with concd NH_4OH at rt for 24 h. The oligonucleotide was dried and purified by HPLC on a RP-C18 column, using the following conditions: A = NH_4OAc buffer (50 mM; pH 6.5); B = MeCN. The oligonucleotides were lyophilizied and quantified by their absorbance at 260 nm on a photometer. Duplexes were formed by heating to 90 °C (15 min) followed by slow cooling. UV/vis: ϵ_{260} (M^{-1} cm^{-1})/yield (%) 161000/13.7 (ss-**DNA1**), 155500/7.1 (ss-**DNA2**), 161000/15.7 (ss-**DNA3**), 155500/6.0 (ss-**DNA4**), 145000/ 21.9 (ss-**DNA5**), 155000 /11.7 (ss-**DNA6**), 145000/18.5 (ss-**DNA7**), 155000/24.3 (ss-**DNA8**). ESI-MS: M^+ calcd for ss-**DNA1** 5418, found 1356.2 $[M/4]^{4+}$; M^+ calcd for ss-**DNA2** 5407, found 1353.3 $[M/4]^{4+}$; M^+ calcd for ss-**DNA3** 5418, found 1356.1 $[M/4]^{4+}$; M^+ calcd for ss-**DNA4** 5407, found 1353.4 $[M/4]^{4+}$; M^+ calcd for ss-**DNA5** 5398, found 1350.8 $[M/4]^{4+}$; M^+ calcd for ss-**DNA6** 5387, found 1348.1 $[M/4]^{4+}$; M^+ calcd for ss-**DNA7** 5398, found 1350.7 $[M/4]^{4+}$; M^+ calcd for ss-**DNA8** 5387, found 1346 $[M/4]^{4+}$.

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Supporting Information Available: ^{1}H NMR spectra, ^{13}C NMR spectra, and MS spectra for **3**, **5**, and **7**, and an ^{31}P NMR spectrum for **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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