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A Blue-to-Red Energy-Transfer Thymidine Analogue That Functions in DNA

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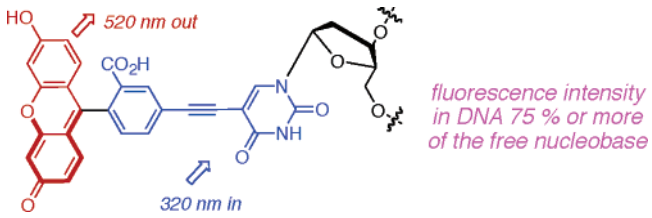
ABSTRACT



The thymidine analogue 1 is a blue-to-red energy transfer cassette designed to absorb UV light in the 300 nm region and emit it as fluorescence at around 520 nm. When incorporated into DNA, the fluorescence intensity of this modified nucleobase is not significantly reduced by the surrounding structure in the oligonucleotides.

Natural DNA nucleobases have negligible fluorescence, even as isolated nucleosides.[1] Consequently, some nucleobase analogues are interesting for their relatively enhanced fluorescence. However, fluorescent nucleobase analogues that absorb at short wavelengths (ca. 300 nm) exhibit dramatically reduced fluorescence *in DNA*.[2] This is unfortunate because there is a need for analogues that can be incorporated into DNA and fluoresce at relatively long wavelengths when excited in the UV region at around 300 nm.[3] This Letter features investigations of the thymidine analogue **1** that has potential in this regard.

These studies are an extension of our previous work on donor−acceptor cassettes for biotechnology.[4] The unifying theme is molecules with a donor part that absorbs light at relatively short wavelengths, and an acceptor part that accepts this energy and fluoresces at a much longer wavelength.[5] The key structural feature of these molecules is that the donor and acceptor parts would be electronically conjugated were it not for a twist in the molecule that keeps them from becoming planar. This barrier to planarity means that the molecules do not behave as a single, flat dye system. The fact that the molecules would otherwise be conjugated means they can transmit energy *through bonds* as well as through space, i.e., they are conceptually distinct from donor−acceptor cassettes based solely on Förster energy transfer.[6]

The distinctive feature of nucleoside **1** is that it is potentially a through-bond energy transfer system where *a*

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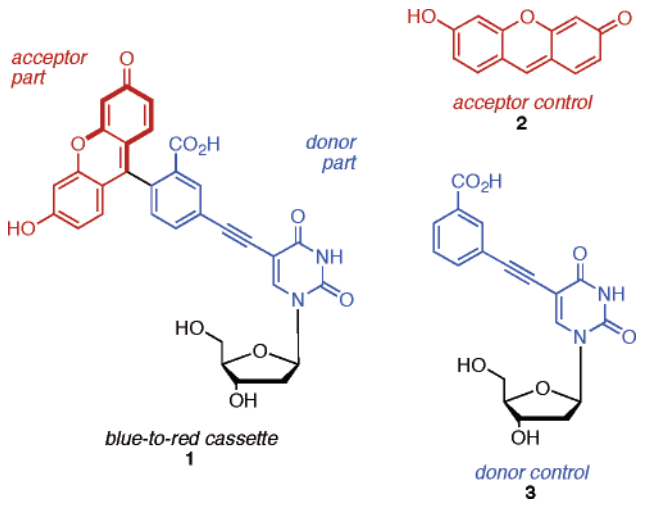

Figure 1. Featured cassette **1** and molecules resembling the donor and acceptor parts of this system.

modified thymidine forms part of the donor. Figure 2a shows UV absorption spectra for this molecule, the hydroxyxanthone **2**[7] resembling the acceptor part, and a nucleoside **3** that was prepared as a control for the donor part. The acceptor fragment **2** has a small absorption in the wavelength range around 300 nm. At 320 nm, λ_{max} for **3**, the absorption of **2** is around 5% of that of the donor control **3**. The absorption spectra of nucleoside **1** is essentially the sum of the donor−acceptor parts **2** and **3** proving that this molecule behaves as two separate conjugated systems, as required for an energy transfer cassette.

Figure 2b shows the fluorescence of equimolar **1**−**3** when excited at 320 nm. The donor control molecule **3** has almost no fluorescence at 520 nm, a wavelength close to the $\lambda_{max\ emiss}$ for **1** and **2**. Similarly, when the acceptor part is excited under the same conditions, it has negligible fluorescence. However, cassette **1** is 2 orders of magnitude more fluorescent than **3** under these conditions. Thus, while the acceptor control **2** absorbs few photons at the 320 nm and gives a correspondingly weak fluorescence, nucleoside **1** acts as an energy transfer cassette. Further, the fluorescence of the donor part is almost completely quenched in cassette **1**, implying close to 100% energy transfer. This assertion is supported by the observation of comparably high quantum yields for cassette **1** irradiated at wavelengths corresponding to absorption predominantly by the donor and by the acceptor (300 and 490 nm, respectively; Table 1).[8]

Key issues in this project relate to the properties of the cassette when incorporated into oligonucleotides. Consequently, oligonucleotides **4**−**6** containing **1** were prepared via solid-phase syntheses (Figure 3a).[9] It was critical to determine the extent to which the organized hydrogen bonding network and base stacking effects in DNA would

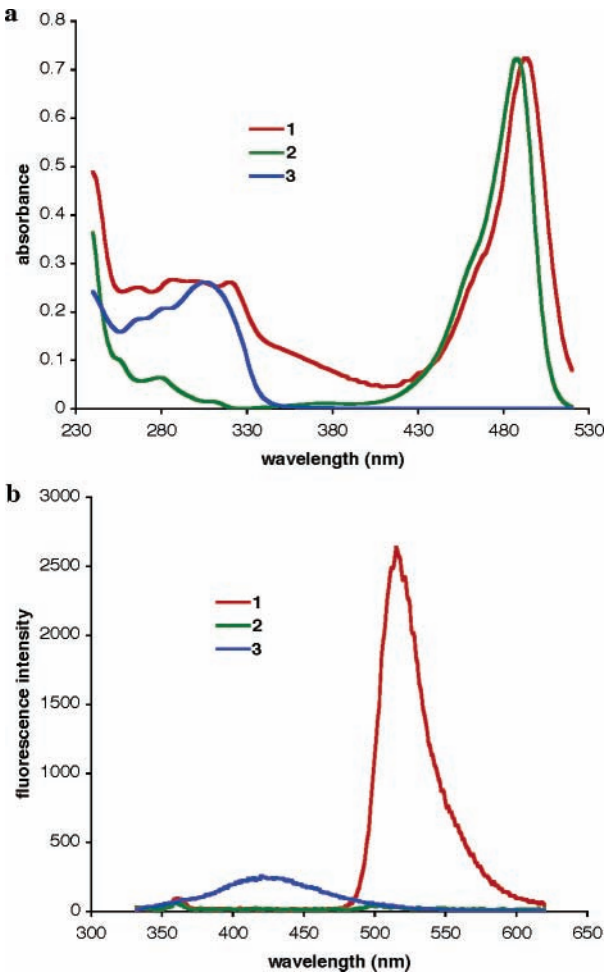

Figure 2. (a) UV absorption spectra of **1**−**3**, as equimolar solutions in Tris·HCl solution (50 mM, pH = 7.5). (b) Fluorescence spectra of **1**−**3**; equimolar solutions in Tris·HCl solution (50 mM, pH = 7.5) excited at 320 nm (uncorrected for the spectral response of the instrument).

tend to quench the fluorescence of cassette. To explore this, it was necessary to generate solutions of **1** and of the oligonucleotides that were precisely equimolar. Practically, this was a challenge because the small amounts involved precluded accurate weighing. Moreover, the extinction coefficients of **1** and the oligonucleotides containing it cannot be assumed to be the same, so determining concentrations via UV absorbance (or fluorescence) would not be simple. Consequently, the following procedure was used. Stock solutions of the oligonucleotides were prepared and diluted with buffer for the spectroscopic studies. To obtain equimolar solutions of compound **1**, the same stock solutions were mixed with phosphodiesterase and incubated. HPLC analyses were used to confirm that the oligonucleotides had been completely hydrolyzed to the constituent bases. The digested

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(8) Donor control compound **3** shows 114 nm of Stokes' shift and 200 times greater quantum yield than native thymidine.

(9) Jiao, G.-S.; Burgess, K. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2785. This paper also reports thermal denaturation data that show **1** has a slight destabilizing effect on annulation with complementary oligonucleotide sequences, though not as much as A:A mismatches.

Table 1. Selected Fluorescence Data

compound	relative fluorescence intensity[a]	quantum yield (ϕ)[b]
1	1	0.81 ± 0.04 @ 300 nm
		0.85 ± 0.02 @ 490 nm
2	na	0.91 ± 0.02 @ 490 nm
3	na	0.015 ± 0.003 @ 300 nm
ss-4	0.84[c]	nd
ds-4	0.75[c]	nd
ss-5	0.78[c]	nd
ds-5	0.71[c]	nd
ss-6	0.89[c]	nd
ds-6	0.76[c]	nd

[a] Equimolar concentrations in Tris·HCl buffer (50 mM, pH = 7.5) excited at 320 nm. [b] Determined in 10 mM Tris·HCl buffer pH = 8.0. Rhodamine 6G in the same solvent was used as a reference (ϕ = 0.95) for **1**, fluorescein (ϕ = 0.79) for **2**, coumarin 1 (ϕ = 0.73) for **3**. [c] Compared with cassette **1**; na = not applicable; nd = not determined.

mixtures were then diluted to the same concentration as the oligonucleotides.

Figure 3b shows typical data obtained by comparing the fluorescence spectra of an oligonucleotide containing **1** (in this case **4**), the corresponding double-strand (ds) DNA

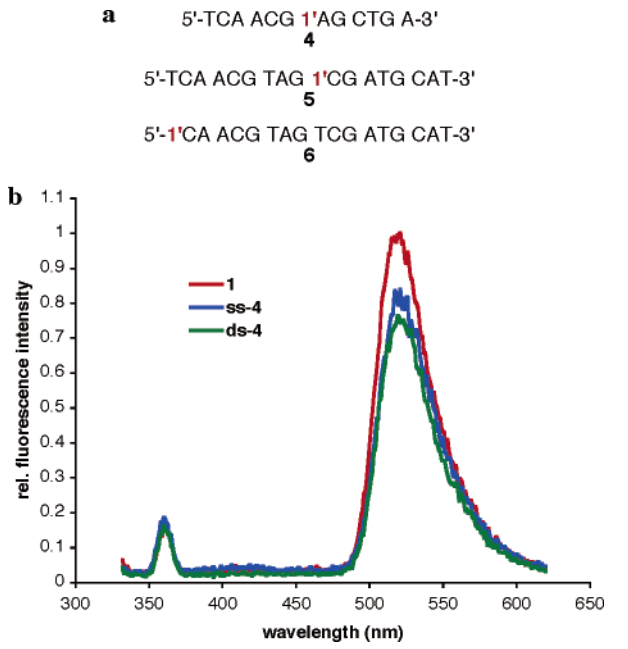
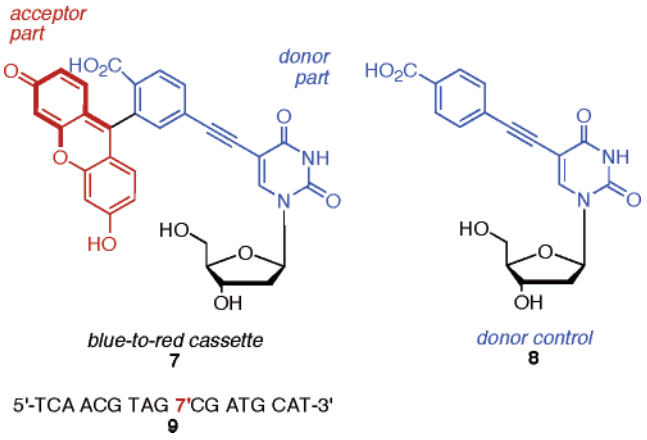


Figure 3. (a) Oligonucleotides **4**−**6** where **1′** is the phosphodiester of **1**, and (b) fluorescence emission spectra of **1**, **4**, and ds DNA wherein **1′** is paired with A in equimolar Tris·HCl solution (50 mM, pH = 7.5) excited at 320 nm.

wherein **4** is paired with A, and an equimolar solution of **1** formed via the phosphodiesterase procedure. These data show that the fluorescence intensity of **1** in single-strand or ds DNA is at least 75% of that in the parent nucleoside. Data for similar experiments with **5** and **6** are shown in Table 1.

Compounds **7** and **8**, i.e., isomers of nucleoside **1** and the control compound **3**, were also prepared and studied in parallel. The spectroscopic data obtained from these nucleosides and oligonucleotide **9** formed from them shows no significant difference from that described above (see Supporting Information).



Compounds **A**[10] and **B**[11] are typical of numerous studies by others in which fluorescent labels have been attached to nucleoside bases. The compounds described here are distinct from those in two respects. Nucleoside **A** has a fluorescent group conjugated to a modified thymidine, but the molecule can freely adopt planar conformations; hence, it does not behave as a cassette. Instead, it represents a unified dye system that absorbs at appreciably longer wavelengths than **1** but emits at much shorter wavelengths. The linker connecting the fluorescein and the nucleobase in molecule **B** precludes *through-bond* energy transfer between the label and the base. The flexible linker in **B** also opens the possibility that the fluorescein part would interact with, or even intercalate into, the DNA strand. Indeed, the fluorescence of this nucleotide is dramatically reduced in oligonucleotides. Conversely, the fluorescein is held away from the DNA strand in oligonucleotides formed from **1,** and we have demonstrated that its fluorescence intensity is similar to that in the nucleoside.

Overall, our interpretation of the data shown in this paper is as follows. Nucleobase **1** has a donor fragment that resembles an alkyne-modified T-residue. This part of the molecule dominates the UV absorption of **1** in the 320 nm region, i.e., near, but not completely overlapping with, the intrinsic absorption of DNA bases (ca. 260 nm). Introduction of the twisted linker-hydroxyxanthone system provided an acceptor part and a viable pathway for through-bond energy transfer. This was kinetically competitive with nonradiative decay processes that ensued when the donor part was

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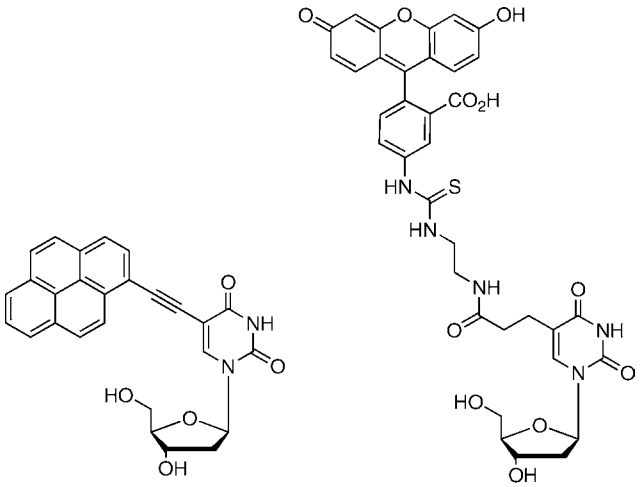

irradiated at 320 nm. Consequently, fluorescence of the cassette could be used as a marker for events that occur when oligonucleotides containing it are irradiated at 320 nm, very close to the absorption of the natural DNA bases.[12]

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Supporting Information Available: Experimental procedures for the preparation of **1** and **3**−**9**; absorption and fluorescence spectra of **2**, **7**, and **8**; fluorescence spectra of DNAs containing the modified nucleoside; tabulated photophysical data of **1**−**3**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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