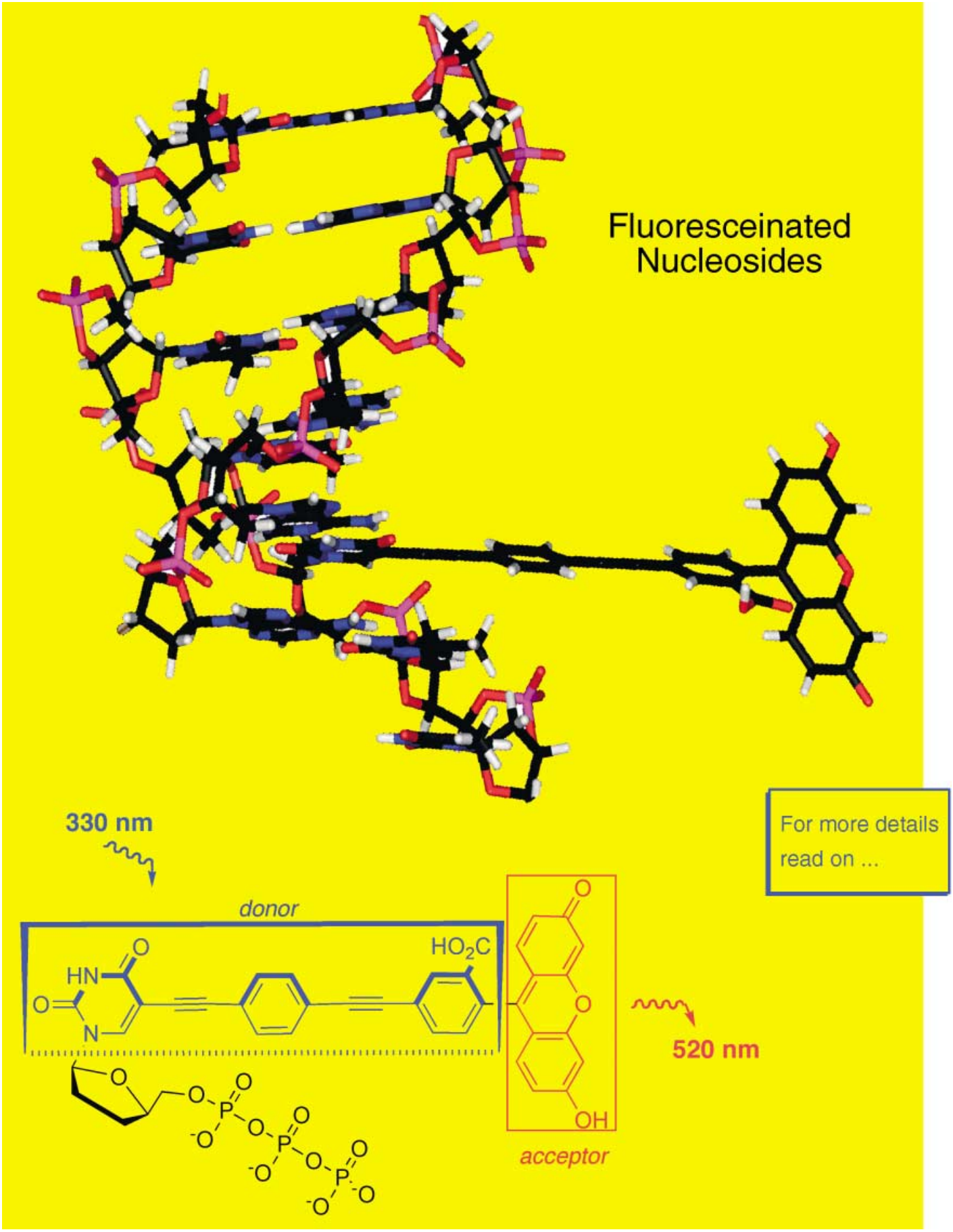

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Rigid, Conjugated, Fluoresceinated Thymidine Triphosphates: Syntheses and Polymerase Mediated Incorporation into DNA Analogues

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Abstract: Syntheses of a unique set of energy transfer dye labeled nucleoside triphosphates, compounds **1**–**3**, are described. Attempts to prepare these compounds were only successful if the triphosphorylation reaction was performed before coupling the dye to the nucleobase, and not the other way around. Compounds were prepared as both the 2′-deoxy (**a**) and 2′,3′-dideoxy- (**b**) forms. They feature progressively longer rigid conjugated linkers connecting the nucleobase and the hydroxyxanthone moiety. UV spectra of the parent nucleosides **12**–**14** show that as the length of the linker increases so does the absorption of the donor in the 320–330 nm region, but with relatively little red-shift of the maxima. Fluorescence spectra of the same compounds show that radiation in the 320–330 nm region results in predominant emission from the fluorescein. When the linker is irradiated at 320 nm, the only significant emission observed corresponds to the hydroxyxanthone part of the molecules at 520 nm; this corresponds to an effective Stokes' shift of 200 nm. As the absorption at 320–330 nm by the linker increases with length, so does the intensity of the fluorescein emission. A gel assay was used to gauge relative incorporation efficiencies of compounds **1**–**3**, dTTP, ddTTP, and 6-TAMRA-ddTTP. Throughout, the thermostable polymerase *Taq*FS was used, as it is the one most widely applied in high throughput DNA sequencing. This assay showed that only compounds **3** were incorporated efficiently; these have the longest linkers. Of these, the 2′-deoxy nucleoside **3a** was incorporated and did not prevent the polymerase from extending the chain further. The 2′,3′-dideoxy nucleoside **3b** was incorporated only about 430 times less efficiently than ddTTP under the same conditions, and caused chain termination. The implications of these studies on modified sequencing protocols are discussed.

Keywords: DNA sequencing · dyes/pigments · fluorescence spectroscopy · fluorescent probes · UV/Vis spectroscopy

Introduction

Many situations in biotechnology and biomedicine require that DNA be labeled with highly fluorescent labels. Almost invariably, this is done by connecting nucleosides to labels like fluorescein and rhodamine via aliphatic linkers. The nucleoside triphosphates used in DNA sequencing are typical examples of this, such as 6-TAMRA-ddTTP **A**; Figure 1.[1] Rigid, non-aliphatic linkers have been investigated, but only to link other labels. The types of probes that have been connected in this way include weakly fluorescent, hydrophobic groups (e.g. **B** and **C**),[2–4] metal complexes (e.g. **D**),[5–10] EPR spin labels (**E**),[11] and electrochemically conspicuous groups (**F**)[12] to nucleobases, but never the types of fluorescent labels that give the sensitivity required for application in DNA sequencing and some areas of diagnostics.

The lack of research into nucleobases labeled with strongly fluorescent compounds via rigid conjugated linkers is unfortunate because systems like this could be useful. Relatively inflexible linkers have the potential to limit intercalation or other non-covalent interactions of the dye with the DNA framework. They could also be used to keep the dye oriented away from the enzyme active site of polymerases. Moreover, conjugated linkers could electronically couple with the fluorescent label and the nucleobase in ways that affect their UV absorption and fluorescent properties in potentially useful ways. For instance, this coupling could increase the

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Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author. Complete experimental details for the preparation of the compounds, the spectroscopic measurements (with some additional UV/fluorescence data for compounds **12**–**14**), and protocols for the polymerase mediated incorporation studies.

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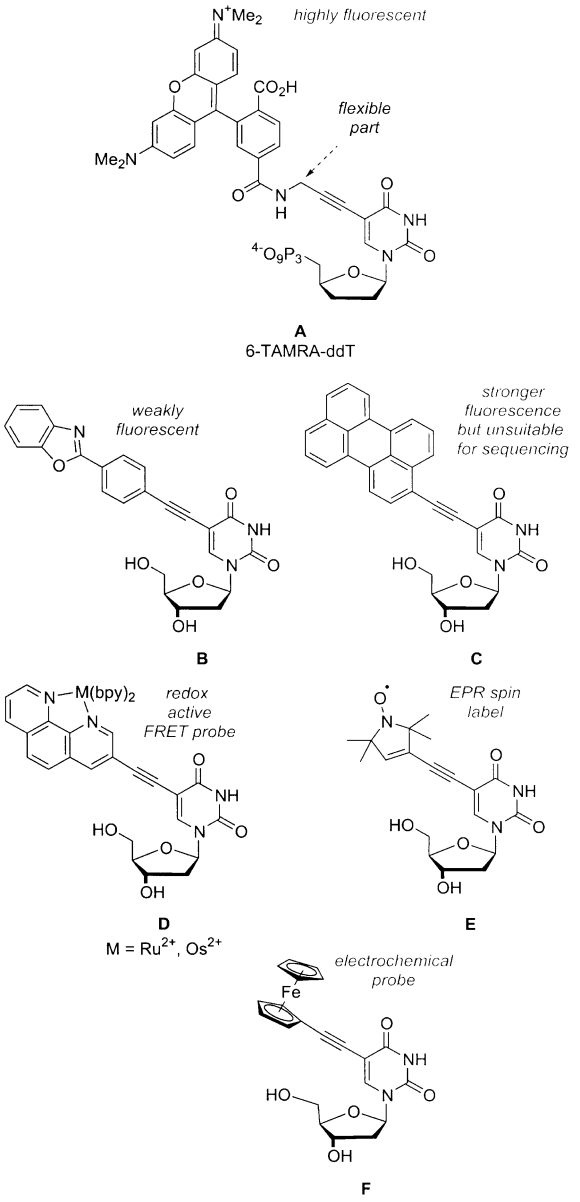
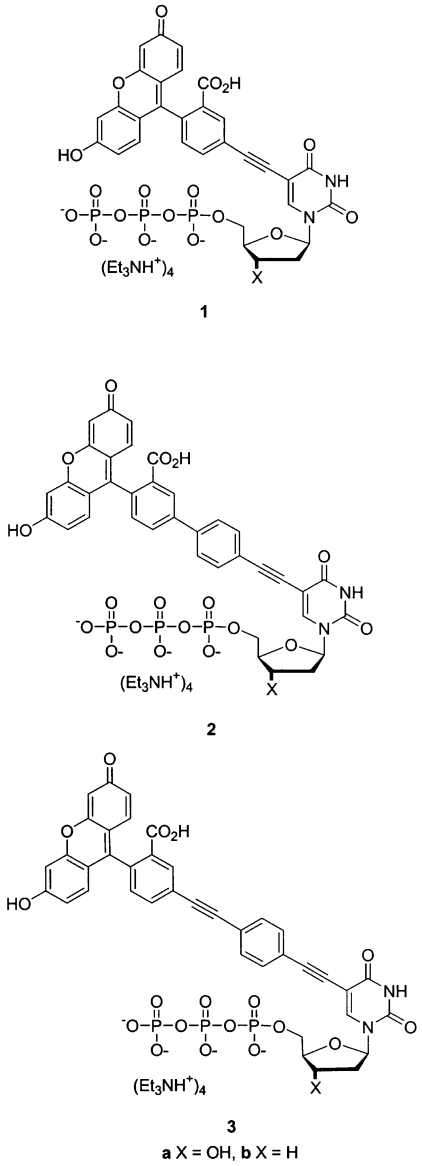


Figure 1. Highly fluorescent labels linked to nucleobases via flexible linkers, as in 6-TAMRA-dTTP, are the norm in DNA sequencing protocols. For other applications, rigid conjugated linkers have been employed to link weakly fluorescent groups, redox, EPR, and electrochemical probes, but mostly to nucleotides for incorporation into synthetic DNA and never for DNA sequencing.

UV absorption of the labeled nucleoside so that the fluorescence intensity of the label is increased. Alternatively, conjugated linkers might be designed to absorb in UV regions where it could be advantageous to excite the fluorescent probe, but in which the probe without the linker has relatively low extinction coefficients. Finally, attaching nucleobases to fluorescent probes via conjugated linkers facilitates fast fluorescence energy transfer between them, and potentially provides a probe for energy transfer to that base from neighboring nucleotides.[13, 14]

For the reasons described above, we have begun to study nucleobases connected to highly fluorescent labels via rigid conjugated linkers. This paper describes syntheses of deoxy- and dideoxynucleoside triphosphates **1**–**3** in which fluorescein fragments are connected to thymidine via progressively longer, alkyne-based linkers. The linkers in **1**–**3** moves the relatively bulky fluorescein unit further away from the

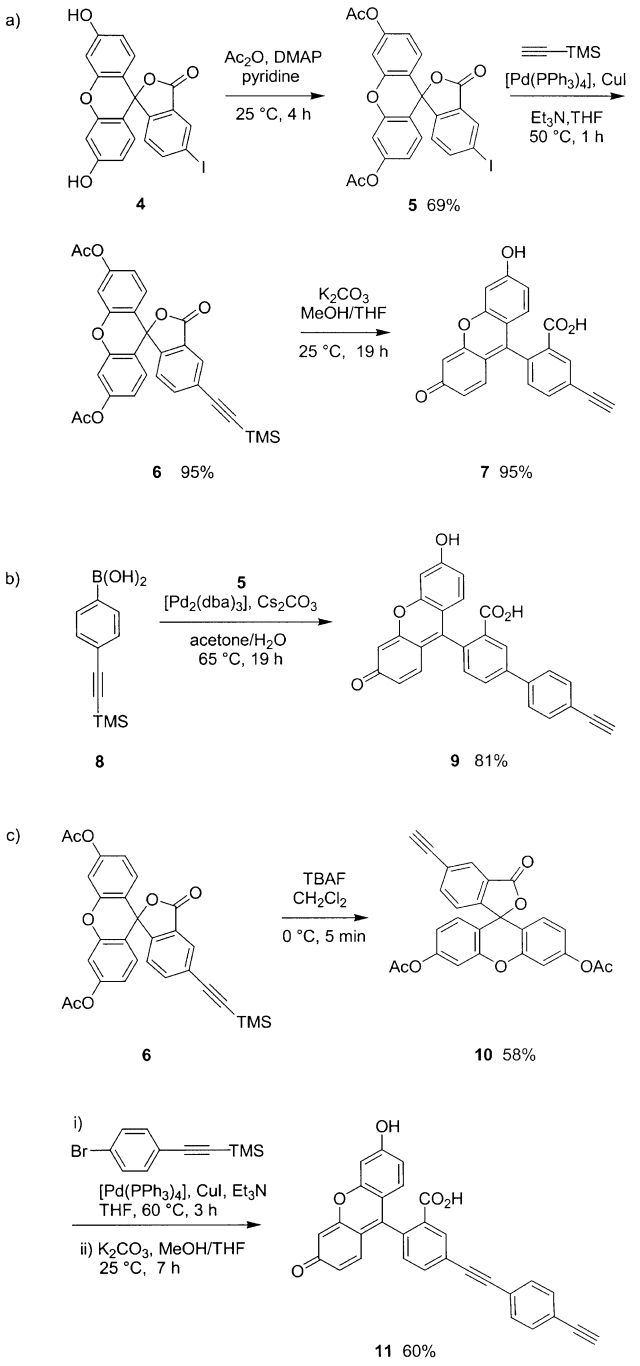


nucleoside base in defined increments. We anticipated that increasing the linker conjugation would influence the spectral properties of the dye, and the ease of incorporation of the triphosphates. Consequently, this paper also describes some fundamental fluorescence properties of the parent nucleosides, and tests for their incorporation by the thermostable DNA polymerase *Taq*FS. *Taq*FS was chosen for this study to test compounds **1**–**3** because of its wide use in fluorescent dye-terminator based DNA sequencing.[1, 15, 16] Both the 2′,3′-dideoxy (**b**) and the 2′-deoxy forms (**a**) were assayed, the latter to gauge for the possibility of multiple incorporation.

Synthesis of the modified thymidine triphosphates 1–3: Scheme 1 describes how the fluorescein derivatives required for this work were made from 5-iodofluorescein **4**. Compounds **4** and **5**, in regioisomerically pure form, were prepared

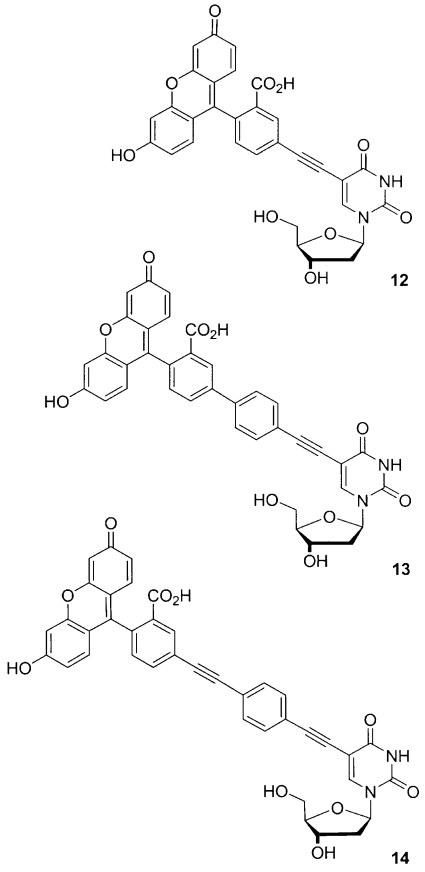
via fractional crystallization of a mixture of 5- and 6-iodofluorescein from acetic anhydride.[17] Acylation and Sonogashira coupling[18] of this material with trimethylsilylethyne gave the protected alkyne **6** and the terminal alkyne **7** after deprotection.

The critical fluorescein-containing fragment **9** for preparation of compounds **2** was formed via a Suzuki coupling,[19] as shown in Scheme 1b; fortunately, desilylation and hydrolysis of the acetate protecting group also occurs in this step. Synthesis of the diyne **11**, a key fragment in the formation of compounds **3**, was made via a Sonogashira/deprotection sequence as shown in Scheme 1c.



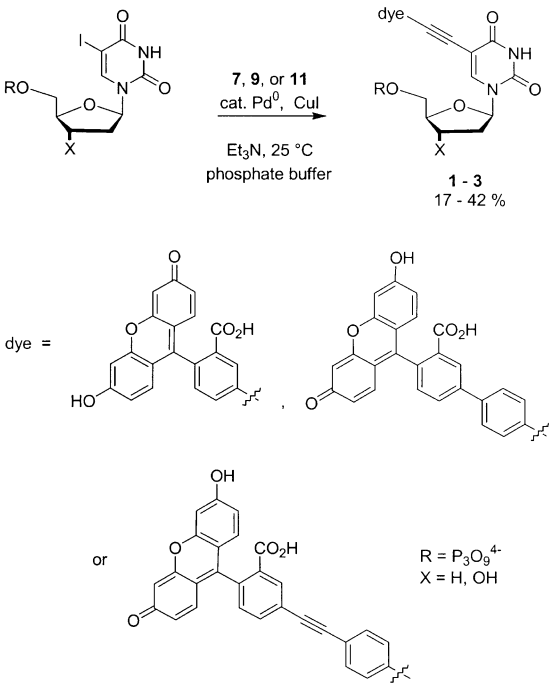
Scheme 1. Preparation of the fluorescein-derived synthons **7**, **9**, and **11**.

To obtain the target materials, compounds **7**, **9**, and **11** must be coupled to 5-iodo-U, and the 5′-hydroxyl group must converted to a triphosphate. Initially, we tried to achieve this via a route in which the coupling preceded the triphosphorylation. After much experimentation, and a considerable amount of time, we concluded that it is extremely hard to phosphorylate nucleosides with large, reasonably lypophilic dyes attached. There are probably several reasons for this, but paramount are solubility issues that impact purification using conventional ion exchange chromatography on Sephadex or various HPLC column purifications. The compounds may aggregate under these conditions and stick to the support. Lack of 3′/5′-selectivity in the triphosphorylation protocols[20] may also have been a factor contributing to the difficulties with this approach. This set of experiments did, however, provide samples of the nucleoside intermediates **12**–**14** and these were useful for spectroscopic studies (see below).



The successful approach to the target triphosphates **1**–**3** was to form 2′-deoxy-5-iodouridine triphosphate, and 2′,3′-dideoxy-5-iodouridine triphosphate (prepared via the protocol outlined in the Supporting Information), then coupling the dye fragments to these. These triphosphates are only soluble in aqueous media, so it was important to find a water-soluble catalyst for this transformation. Sonogashira couplings in aqueous media have been performed using preformed $[Pd(PPh_2Ar)_4]$ where $Ar = 3\text{-}(NaO_3SC_6H_4)$,[21] and by mixing $Pd(OAc)_2$ and PAr_3 in aqueous buffer and "aging" the solution (Method A in the Experimental Section).[22] That

proved to be less convenient than preparing a crude sample of catalyst by reacting Na_2PdCl_4, $NaBH_4$, H_2O with PAr_3 at 25 °C, isolating the solid material, then using this in the coupling reaction (Method B). Lithium salts of the phosphates were used as the substrates in the coupling process, the products were isolated as triethylammonium salts after HPLC purification (Scheme 2).



Scheme 2. Coupling of triphosphates with the dye fragments **7**, **9**, and **11** to give the target products **1**–**3**.

UV absorption and fluorescence spectra of nucleosides 12–14: Figure 2 shows the absorption and fluorescence spectra for nucleosides **12**–**14** corresponding to the triphosphates **1**–**3**. The dye-nucleosides have absorption maxima at 320–330 and 492 nm, probably corresponding to uptake of radiation by the nucleobase in conjugation with its 5-substituent, and the fluorescein, as illustrated in Figure 2 for compound **12** (though similar diagrams could be drawn for **13** and **14**).

Experiments with control compounds show that the absorption spectra of these molecules resemble that which would be obtained simply by adding their donor and acceptor parts (a typical overlay of control compounds is given in the Supporting Information). This is indicative of a lack of conjugation between them, reflecting a twist between the donor and acceptor planes imposed by steric effects, as implied in Figure 2a. As the donor part of the molecules becomes more extended on going from **12**–**14**, so the absorption in the 320–330 nm region becomes more intense. This is consistent with observations for poly- or oligo-phenylethyne materials $\{(-C_6H_4CC-)_n\}$, where the UV absorption becomes stronger in this region, but with little shift to the red, as more repeating units are added.[23–26] No emission from the donor part of these molecules was detected in their fluorescence spectra. Instead, rapid energy transfer seems to

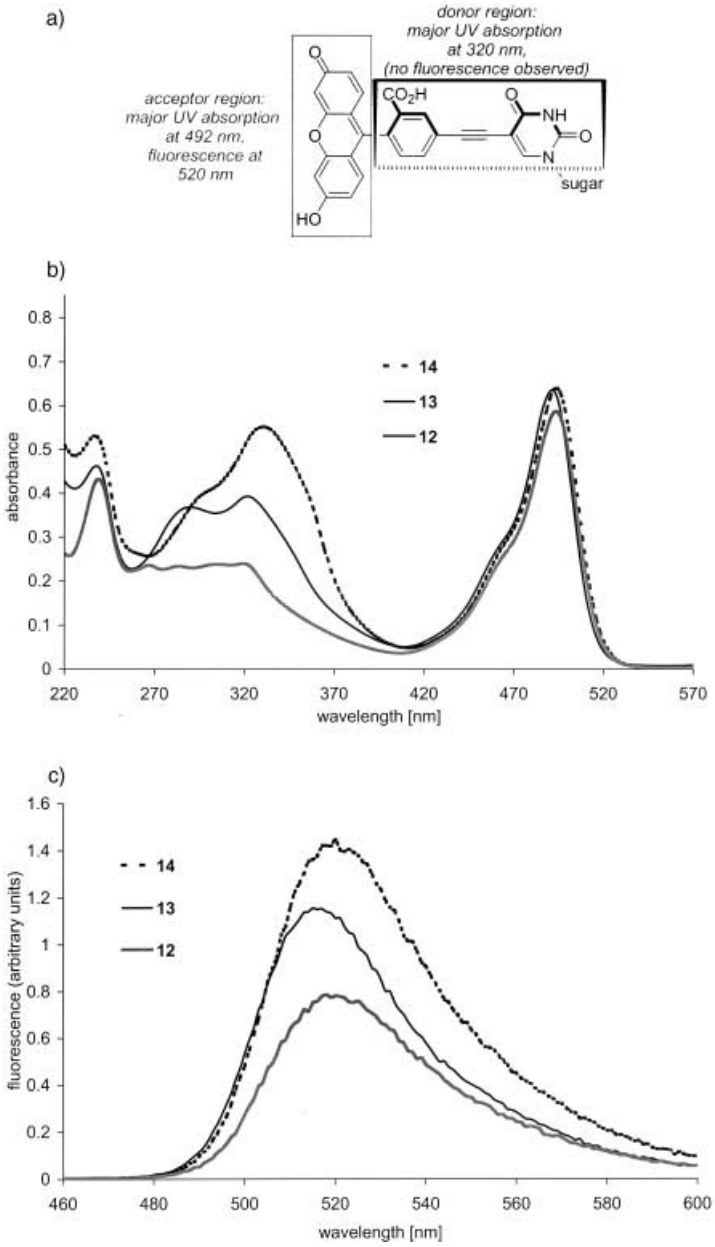


Figure 2. a) Representative assignment of donor and acceptor parts in one of the nucleosides **12**; b) absorption spectra (all at 10 μM); c) fluorescence spectra of the nucleosides in 3.2 mM phosphate buffer at pH 7.2 at equimolar concentrations (all at 1.0 μM).

prevail and fluorescence is observed from the hydroxyxanthanone acceptor unit. The fluorescence intensity of the cassettes when irradiated at 320–330 nm nearly doubles from compound **12** to **14** due to the increased UV absorption by the latter.

Incorporation of the modified thymidine triphosphates 1–3: The performance of compounds **1**–**3** was tested using the "oligo-template assay" using *Taq*FS as previously described,[27] and was analyzed using an ABI model 377 DNA sequencer. Here, the BODIPY-R6G labeled R931 universal sequencing primer[28] was used to determine incorporation of the fluorescein derivatives **1**–**3**, 6-TAMRA-ddTTP, or unlabeled ddTTP or dTTP. The sequencer run module was

modified to collect fluorescent blue, green, and yellow fluorescent light to discriminate signals emitted by the fluorescein derivatives **1**–**3**, BODIPY-R6G, and 6-TAMRA dyes, respectively. Moreover, we have previously shown that incorporation of modified-dNTPs tend to give DNA fragments with slower electrophoretic mobilities than natural substrates because of their high molecular weights.[27] The attachment of fluorescein moieties to dTTP and ddTTP caused similar mobility effects. Thus, the assignment of incorporation events was based on the color and mobility pattern of termination products relative to the unlabeled ddTTP and dTTP controls.

Each of the substrates **1**–**3** was tested at a series of concentration ranges to determine an IC_{50} value (effective concentration at 50% incorporation) in the absence of the corresponding natural nucleotide. Both the deoxy- and dideoxy-thymidine analogues (**a** and **b**) were tested individually and compared with dTTP and ddTTP to assess *Taq*FS bias incorporation effects. For the dideoxy-compounds (**b** series), there are two possible outcomes: the compounds could be incorporated causing chain termination or they are not recognized by *Taq*FS. For the latter case, DNA synthesis is halted in the absence of natural thymidine, shown as "unincorporated" in Figure 3. Incorporation of the deoxy-compounds (**a** series) would cause continued DNA synthesis or "read-through" to the "incorporated" end-point. We have also observed chain termination of series **a** compounds and hypothesize their incorporation distorts the DNA substrate for subsequent DNA synthesis. Alternatively, *Taq*FS might not recognize the substrate. Incorporation data for compounds **2b** and **3b** are shown in Figure 3.

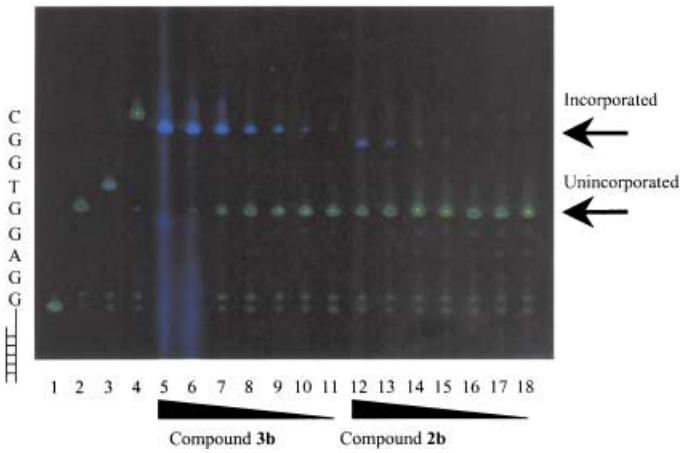


Figure 3. Incorporation of compounds **3b** and **2b** by *Taq*FS DNA polymerase. All reactions contained *Taq*FS, BODIPY-R6G labeled R931 primer, oligo-template and reaction buffer. Lane 1 contained only the primer; lanes 2–18 contained 50 nM each of dCTP, dATP, and ddGTP. Additionally, lanes 3 and 4 contained 10 nM ddTTP or 10 nM dTTP, respectively and lanes 5–11 contained **3b**, lanes 12–18 contained **2b** at 500, 250, 100, 50, 25, 10, or 5 nM, respectively.

The linker had a profound effect on the incorporation of compounds **1**–**3**. For compounds **1a** and **b**, there was no evidence of incorporation (data not shown). On the other hand, compounds **2b** and **3b** showed concentration dependent incorporation in the oligo-template assay (Figure 3). Fluorescent intensities of incorporated and unincorporated termination bands were quantitated and expressed as the percentage incorporated ($I_{\%}$). Tests for incorporation at different concentrations of the ddNTPs (performed in triplicate) are plotted in Figure 4. The relative order of preferred incorporation for TaqFS was compounds ddTTP > 6-TAMRA-ddTTP > **3b** > **2b** > **1b**.

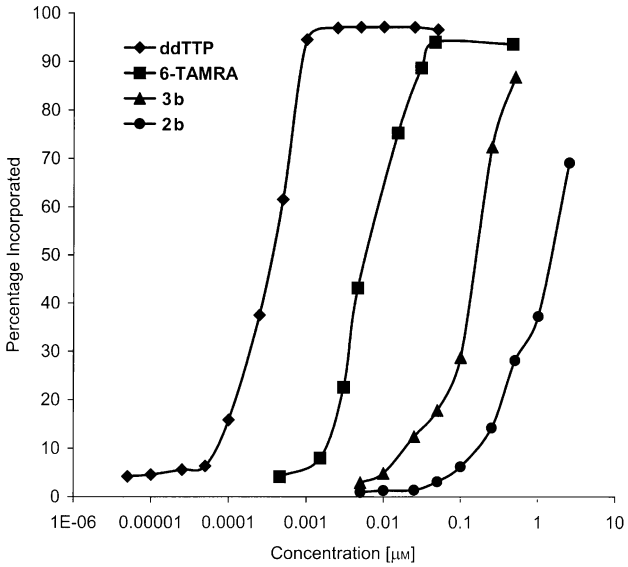


Figure 4. Incorporation curves for ddTTP, 6-TAMRA-ddTTP, and compounds **3b** and **2b**. Dilution series for each compound were performed in triplicate: the range of standard deviations were 0.2–4.7% for ddTTP, 1.6–15.6% for 6-TAMRA-ddTTP, 0.5–10.7% for **3b**, and 0.8–6.8% for **2b**.

From the titration plots, IC_{50} values were derived for compounds **2b**, **3a**, and **3b**, ddTTP, dTTP, and 6-TAMRA-ddTTP (Table 1). To compare the relative performance of nucleotide pairs, incorporation ratios (I_R) were determined. These experiments show that *Taq*FS prefers ddTTP to dTTP or 6-TAMRA-ddTTP approximately 7.6-fold and 18-fold, respectively. The IC_{50} values for compounds **2b** and **3b**, however, suggested a greater bias against incorporation compared to ddTTP (≈4300-fold and ≈430-fold, respectively). The 10-fold improvement of compound **3b** over compound **2b** can most probably be attributed to the additional length of the linker. Compared with 6-TAMRA-ddTTP, compound **3b** showed less bias against incorporation (≈25-fold) than other reported fluorescein-labeled dTTP analogues (≈50-fold). *Taq*FS seems to have an inherent bias in favor of rhodamines as opposed to fluoresceins,[1] but this is less evident for **3b**.

The IC_{50} values determined for the deoxythymidine compound **3a** and its corresponding dideoxy-analogue **3b** were both 0.16 μM; these values are less than for the compounds **1** and **2** suggesting that the longer nucleobase–linker–dye structure exerts a major influence on *Taq*FS incorporation. DNA polymerase *Taq*FS showed less preference for dTTP over the deoxythymidine analogue **3a** than it did to ddTTP over the dideoxythymidine analogue **3b** (I_R = ≈57-fold). The oligo-template assay also showed that compound **3a** was

Table 1. Summary of IC_{50} values and incorporation ratios (I_R) for test compounds **1**–**3** and controls dTTP, ddTTP, and 6-TAMRA-ddTTP.

Compounds	IC_{50} [nM][a]	I_R values
dTTP	2.8	–
ddTTP	0.37	[dTTP]/[ddTTP] = 7.6
1a	NT	–
1b	NT	–
2a	NS	–
2b	1.6×10^3	[**2b**]/[ddTTP] = 4.3×10^3
3a	160	[**3a**]/[dTTP] = 57
3b	160	[**3b**]/[ddTTP] = 4.3×10^2
6-TAMRA-ddTTP	6.5	[6-TAMRA]/[ddTTP] = 18

[a] "NT" means no termination event was observed up to a final concentration of 5 μM, and "NS" means the observed termination products were not sufficient to determine an IC_{50} value.

incorporated and extended the R931 primer to the end-point nucleotide, although a second minor product was revealed, which corresponded to a thymidine termination product (data not shown); that is, **3a** was incorporated and only partially impeded subsequent DNA synthesis, hence the enzyme was able to "read through" to the end of the strand.

Conclusion

The synthetic chemistry described here shows how fluorescein derivatives can be functionalized with phenylethyne linkers to give nucleobase–fluorescein energy transfer cassettes. These particular linkers absorption in the 320–330 nm range, and excitation in that region leads to efficient fluorescence emission at the fluorescein dye. To understand the implications of these observations in areas where the intensity of fluorescence detection is critical, it is important to keep in mind the differences between the concepts of quantum yield and fluorescence intensity. Fluorescence intensity is the critical factor that determines how much radiation is emitted from a fluorescent probe. High quantum yields are necessary to obtain strong fluorescence intensities, but they are not the sole criteria: extinction coefficients of the dye at the wavelength of the excitation source are also critical. For a dye like fluorescein, it is not possible to significantly increase the quantum yield because it is near unity. However, by conjugating other groups onto fluorescein it is possible to increase the amount of energy that is channeled into a dye and tune the wavelengths at which the exciting photons are best absorbed.

This study illustrates that rigid conjugated linkers have absorptions that increase with linker length, that is the longer the linker the more energy that is harvested and fed into the dye. Predictably, long conjugated linkers have higher extinction coefficients than shorter ones. They also have absorption λ_{max} values that shift to the red, away from the intense absorption of DNA bases that would otherwise compete for the incident photons (ca. 250–300 nm). Our work also shows that, fortunately, long conjugated linkers are *also* desirable from the perspective of enzyme incorporation. While the acceptor dye in compounds **1** and **2** is too close to the nucleobase, and *Taq*FS does not tolerate them well. Compound **3**, however, seems to have dimensions that are close to the minimum separation which this polymerase needs for efficient incorporation.

The prognosis for future work in this area seems good. Modified nucleobase triphosphates with rigid conjugated linkers that are even longer than those in **3** will absorb even more strongly in the 320–330 nm range. Such compounds could be designed to relay this energy to the terminal dye with very large effective Stokes' shifts and result in increased fluorescence intensities. The triphosphates should tend to be incorporated more efficiently by polymerase enzymes like *Taq*FS, and the rigid separation of the dye from the polymerase active site should also increase the range of fluorescent label structures that these enzymes will tolerate. One of the reasons for the dominance of rhodamine-based dye-terminator chemistries in DNA sequencing, for instance, is that other dye types tend not be incorporated as efficiently.[1] Rigid active site-dye separation could alleviate this restriction. In DNA sequencing methodologies, this is of immediate interest because there are several advantages to fluorescein-based terminator chemistries that could be exploited if they were incorporated more efficiently. Specifically, dye mobility patterns are more predictable when fluorescein dyes are used, hence they can be corrected more accurately, and the unincorporated and extension by-products can be removed more readily. Moreover, if single molecule detection methods do become practical for DNA sequencing, then the chemical nature of the linker moiety may be more critical. Single molecule methods may be based on incorporation of labeled terminators, or on multiple incorporations of labeled 2′-deoxynucleoside triphosphates.[29] In the latter case, optimization of the linker geometry for clean and efficient incorporation may be essential. Similar considerations apply to some diagnostic protocols featuring incorporation of labeled bases in the PCR reaction.[30] Overall, the prospects for research in this area appear to be as bright as the labels developed.

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