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Oligonucleotides with Strongly Fluorescent Groups π-Conjugated to a Nucleobase: Syntheses, Melting Temperatures, and Conformation

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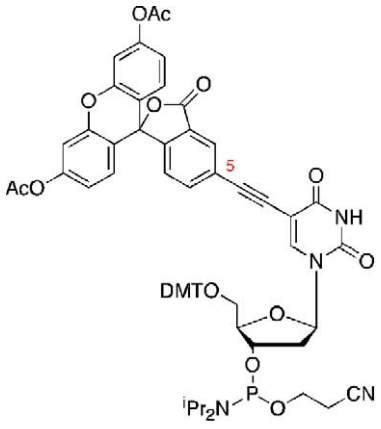
Abstract—Phosphoramidite **1** was prepared, incorporated into oligonucleotides, and these were studied via thermal denaturation and circular dichroism.

Fluorescently labelled oligonucleotides are central to high throughput sequencing of genomic materials.[1,2] Connection of dyes to DNA primers almost invariably features flexible, non-π-conjugated linkers. Many groups have prepared nucleobases with rigid, π-conjugated linkers that attach them to fluorescent entities,[3–5] metal complexes,[6–11] EPR spin labels,[12] and electrochemically active tags.[13] However, nucleobases rigidly linked to the types of strongly fluorescent dyes that are useful in DNA sequencing have not been reported to date. This is surprising because rigid, π-conjugated linkers could potentially be valuable in that area. For instance, whereas attachment of fluorescent dyes to primers via flexible, saturated linkers does not exclude the possibility that complications could arise in the sequencing process as a result of intercalation of the dyes into ds-DNA; this is less likely if the linkers are rigid and π-conjugated (Fig. 1).

Oligonucleotides π-conjugated to strongly fluorescent dyes also have potential applications outside of DNA sequencing. For instance, if energy transfer from the nucleobase to the fluorescent dye is fast relative to non-radiative processes, then emission from the fluorescent group provides a means to monitor excitation of that base, whether it occurs directly or via transmission through the DNA strand.



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Phosphoramidite **1** was conceived as a first step to exploring the characteristics of rigid, π-conjugated linkers in dye-labelled primers. This Letter describes preparation of this phosphoramidite, its incorporation into oligonucleotides via solid-phase syntheses, melting temperatures of the modified ds-DNA oligomers containing this special nucleobase, and circular dichroism spectra to probe conformational effects. The 6-linked fluorescein isomer of phosphoramidite **1** was also examined in the same way.

Scheme 1 outlines a synthesis of the phosphoramidite **1**.[14] Regioisomerically pure 5-iodofluorescein was required for this synthesis. To obtain it, commercially available 5-aminofluorescein was diazotized and treated with potassium iodide. The product was then coupled

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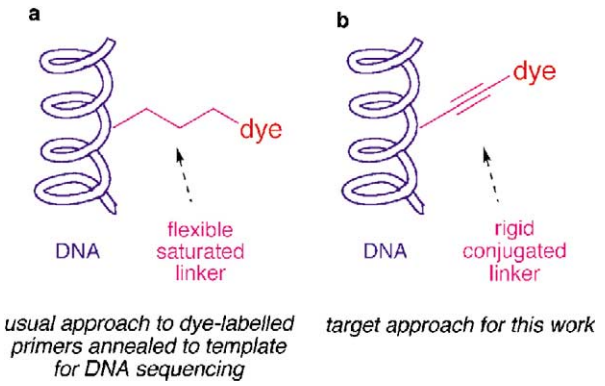
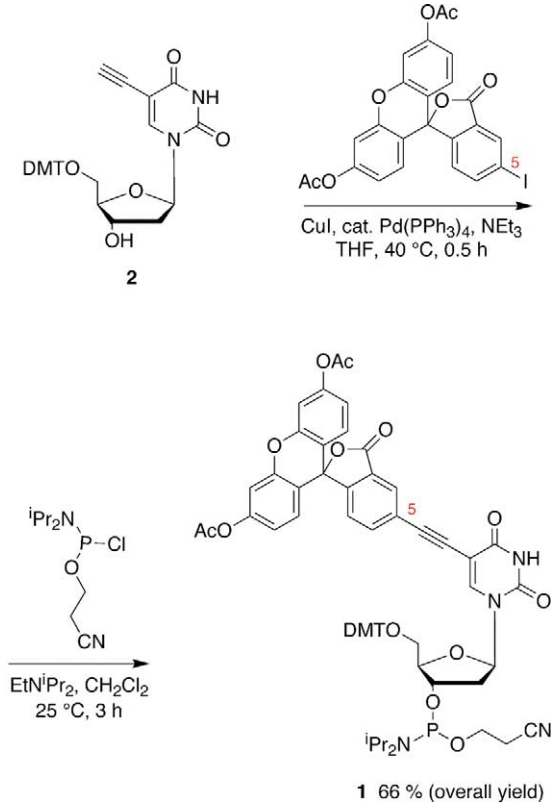


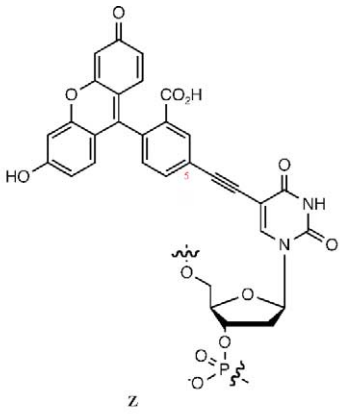
Figure 1. (a) Flexible linkers used in conventional attachment methods permit intercalation and exclude through-bond energy transfer, whereas (b) intercalation is disfavored and through-bond energy transfer is possible if rigid linkers are used.



Scheme. 1. Preparation of the phosphoramidite **1**.

with 5-ethynyl thymidine **21**[15] via Sonogashira's procedure.[16] Conversion of the protected-fluorescein-labelled thymidine derivative into the corresponding phosphoramidite was performed using standard methods.[15,17]

In the solid-phase syntheses of the oligonucleotides **3–5**, controlled pore glass was used as the support in the conventional phosphoramidite method.[17] *O*-Acylation of the fluorescein label protects it from iodination, and this masking is conveniently removed in the course of the standard resin cleavage procedure (treatment with aqueous ammonia). The product oligonucleotides contain the modified nucleobase derived from **1**, here given the symbol '**Z**' (Fig. 2). These were analyzed by HPLC



5'-TCA ACG **Z**AG CTG A-3'
3

5'-TCA ACG TAG **Z**CG ATG CAT-3'
4

5'-**Z**CA ACG TAG TCG ATG CAT-3'
5

5'-TCA ACG **X**AG CTG A-3'
3'-AGT TGC **A**TC GAC T-5'
6, X = **Z** (T_m = 47.0 °C); **7**, X = **T** (51.1 °C); **8**, X = **A** (38.5 °C)

5'-TCA ACG TAG **X**CG ATG CAT-3'
3'-AGT TGC ATC **A**GC TAC GTA-5'
9, X = **Z** (T_m = 59.0 °C); **10**, X = **T** (63.0 °C); **11**, X = **A** (56.1 °C)

5'-**Z**CA ACG TAG TCG ATG CAT-3'
3'-**A**GT TGC ATC AGC TAC GTA-5'
12 (T_m = 60.4 °C)

Figure 2. Modified oligonucleotides and corresponding ds-DNA.

to assess purity, phosphodiesterase hydrolysis followed by HPLC to assess nucleobase composition, and electrospray mass spectrometry for confirmation of their molecular masses.[18]

Stability changes caused by incorporating the **Z** residue into ds-oligonucleotides were assayed by monitoring thermal denaturation via UV. Representative data for derivatives of oligonucleotide **4** and some control sequences are shown in Figure 3. This shows there are relatively small differences in stabilities, and that the modified oligo **9** is less stable than the natural ds-oligo **10** but is more stable than the A-A mismatched oligo **11** (T_m = 59.0, 63.0, 56.1 °C, respectively). When the **Z** residue was instead placed at the 5′-terminus in **12**, then the melting temperature was almost the same as when it was centrally situated (60.4 vs 59.0 °C). The shorter oligos **6–8** have similar but more profound stability trends, as do oligos **9–11** (T_m = 47.0, 51.1, 38.5 °C, respectively).

A parallel study was carried out to determine if the position of the fluorescein linker is influential (two isomers of substituted fluoresceins are common, the 5-isomer featured above, and the 6-isomer). 6-Aminofluorescein thus was converted into regioisomerically pure 6-iodofluorescein using the methods developed for the

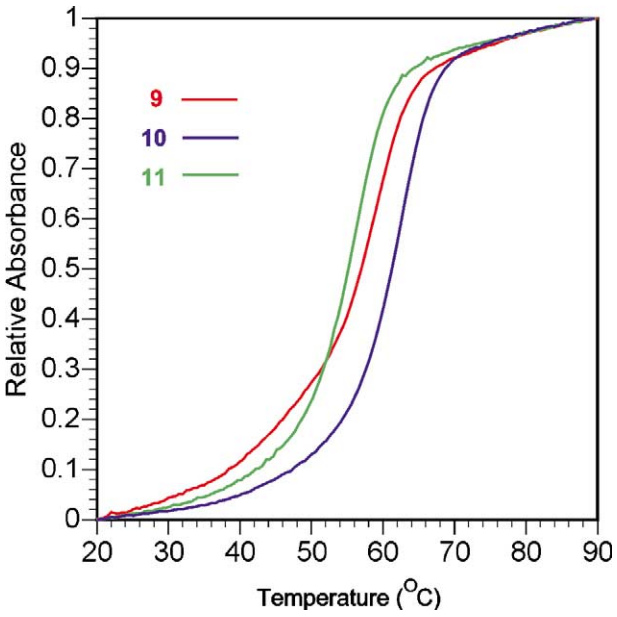


Figure 3. Thermal denaturation curves for the natural and modified ds-DNA strands **9**–**11**.

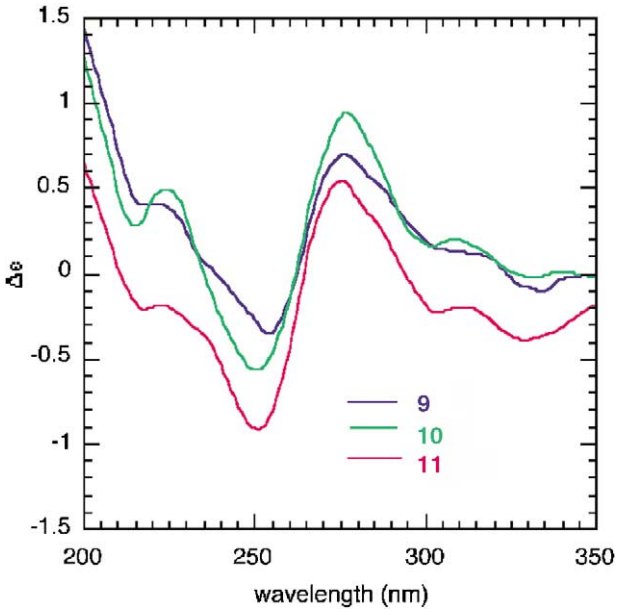
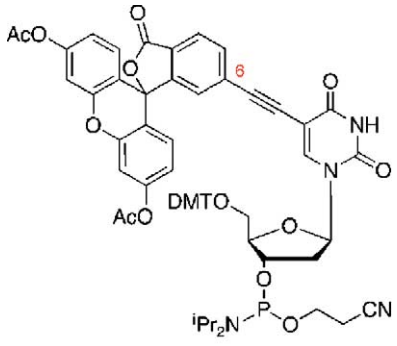
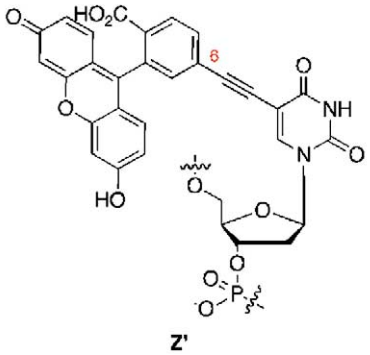


Figure 4. CD spectra of oligonucleotide duplexes **9**, **10**, and **12**.

5-isomer. The sequence shown in Scheme 1 was repeated, but using 6-iodofluorescein to give an isomeric phosphoramidite **1′**, then oligonucleotide **4′** containing the fragment **Z′**. In the series of ds-DNA **9′**, **10**, and **11**, the T_m values obtained were 58.0, 63.0, and 56.1 °C, respectively. Thus with identical sequences but containing **Z** and **Z′** (i.e., **9** and **9′**) the T_m values measured were very similar (59.0 and 58.0 °C), and the order of stabilities (natural > **9** or **9′** > A–A mismatch) is the same.



1′



Z′

Circular dichroism (CD) studies were performed to probe if incorporation of fragment **Z** disrupts the DNA conformation. Figure 4 shows the CD spectra obtained. Oligos **9**, **10**, and **12** give similar spectra, and all resemble that expected for the B-DNA conformation.[19]

In summary, T-derivatives with protected fluorescein residues directly π-conjugated via rigid linkers are accessible via a relatively direct synthesis. 3′-Phosphoramidites of these can be formed, and di-*O*-acetate protection of the fluorescein moiety renders it compatible with solid phase methods to produce modified DNA oligomers (containing **Z** and **Z′**). Thermal denaturation studies prove that the modified nucleobases do not decrease the stability of the ds-DNA significantly, less, in fact, than the corresponding A–A mismatch. The CD studies indicate that incorporation of the **Z** fragment does not perturb the ds-DNA B-form conformation. Ds-DNA from the modified oligonucleotides with the fluorescein π-conjugated in the 5- or 6-position have similar stabilities. These studies lay the foundations for experiments to probe the applications of rigid dye-labelled primers in DNA sequencing, and in depth studies of fluorescence energy transfer along DNA strands.[20]

Acknowledgements

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