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Synthesis of a modified thymidine monomer for site-specific incorporation of reporter groups into oligonucleotides

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Abstract—The efficient incorporation of reporter groups into oligonucleotides at specific sites has been facilitated by the synthesis of a novel modified thymidine monomer with an FMOC-protected hydroxyl group on a linker. The primary hydroxyl group can be deprotected during or after solid-phase oligonucleotide synthesis and reacted with any reporter phosphoramidite. © 2001 Elsevier Science Ltd. All rights reserved.

Oligonucleotides possessing fluorescent labels attached to the heterocyclic bases are of increasing importance in molecular diagnostics.[1] There are currently two main methods for introducing such labels. The first involves the reaction of functional groups attached to the bases (e.g. alkylamino groups) with activated dye molecules (e.g. *N*-hydroxysuccinimide esters) after oligonucleotide synthesis.[2] However, this post-synthetic method does not readily allow the incorporation of different dyes at separate sites, additional purification steps are required and the yields are often low. The second is to construct a labelled nucleoside unit that can be incorporated as a phosphoramidite during automated DNA synthesis. This method is site-specific but is constrained by the availability of expensive monomers, each of which has to be individually prepared.[3]

In this report we describe the synthesis of a new thymidine-based monomer that can be used to introduce any phosphoramidite monomer into an oligomer during solid-phase synthesis. The molecule has a side-chain on the five-position of thymine which consists of a triple bond (essential for efficient PCR[4]), an additional spacer and a terminal FMOC-protected hydroxyl group. Once the monomer is incorporated into an oligonucleotide during routine automated synthesis, the FMOC protection can be removed and the hydroxyl group coupled with any chosen phosphoramidite (monomer). FMOC deprotection can be performed immediately after incorporation of the monomer, or at the end of oligonucleotide synthesis.

The monomer **8** was prepared as follows (Fig. 1): 6-aminohexan-1-ol was protected using BOC anhydride. The alcohol **1** was reacted with FMOC chloride in pyridine and following prompt acidic deprotection the amine **3** was obtained in a yield of 65% for steps (i) to (iii). 5-Iodo-2′-deoxyuridine was protected as its 4,4-dimethoxytrityl ether to give **4** in good yield (87%). The nucleoside was then reacted with propargylamine under Sonagashira conditions, without need for prior protection of the amine.[5] Treatment of the product **5** with succinic anhydride, and subsequent coupling of amine **3** gave the nucleoside **7**. Finally phosphitylation of the 3′ hydroxyl group in an argon atmosphere using 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine afforded the phosphoramidite **8** in 70% yield.

Monomer **8** was incorporated into a 13-mer oligonucleotide (TCTCAC**8**ACTATC, Fig. 2) which was assembled using standard phosphoramidite chemistry[6] on an ABI 394 DNA synthesiser (coupling time of 6 minutes for monomer **8**, Fig. 2). Coupling efficiencies of >98% were observed throughout. The column with the attached, fully protected oligonucleotide was removed from the synthesiser and a solution of piperidine in DMF (20%) was passed through the column by syringe. A reaction time of 5 minutes was sufficient to remove the FMOC group; significantly longer reaction times causing partial cleavage of the oligonucleotide from the resin (a reaction time of 5 minutes gave a 96% yield of oligonucleotide when compared to a standard synthesis). The column was washed thoroughly with anhydrous DMF and acetonitrile before being returned to the DNA synthesiser. The support-bound oligonucleo-

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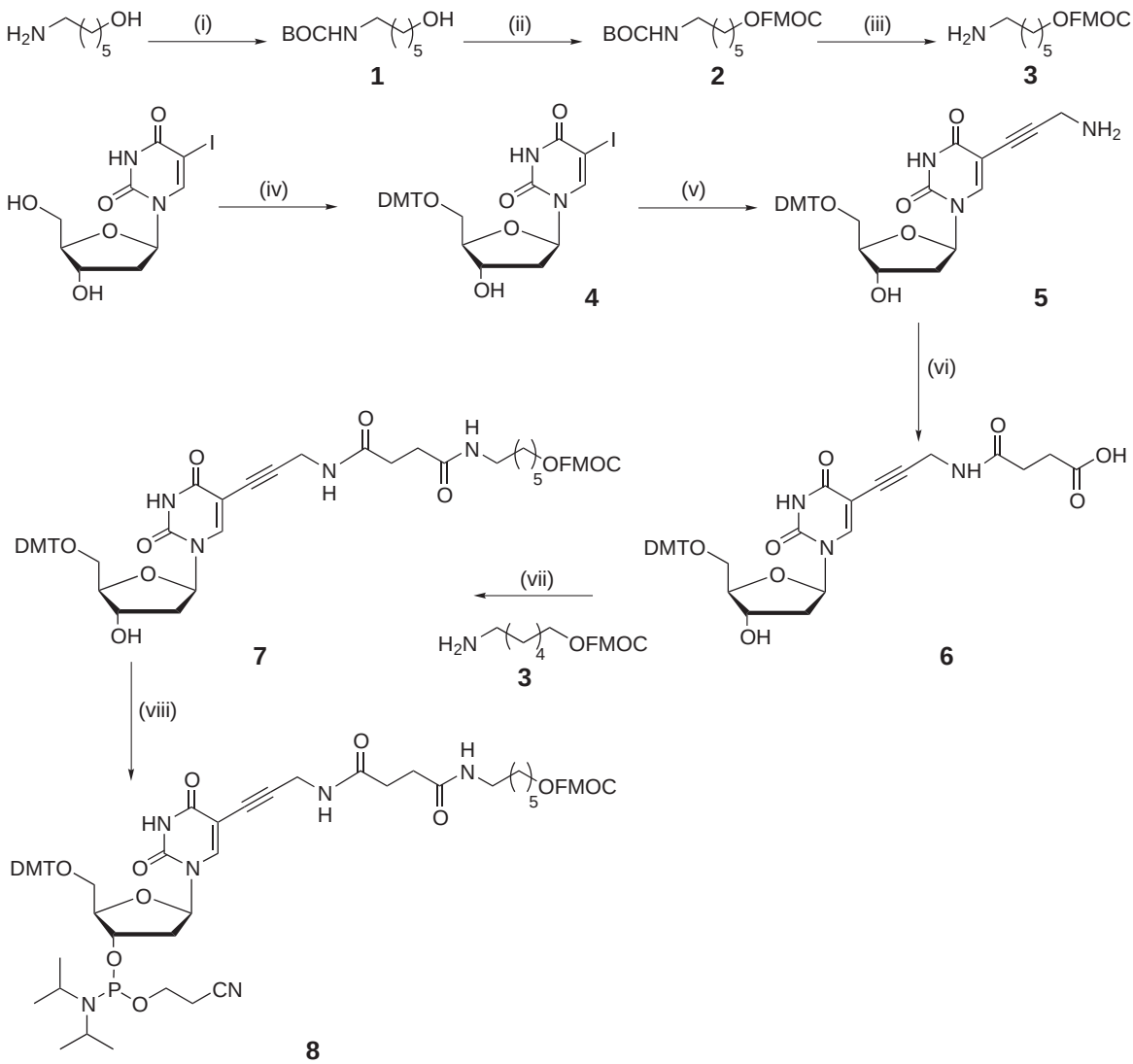


Figure 1. *Reagents and conditions*: i, BOC anhydride (1 equiv.), DMF, rt, 97%, ii, FMOC chloride (1.1 equiv.), pyridine, rt; iii, trifluoroacetic acid, CH_2Cl_2 (2:1), rt, 67% (two steps); iv, 4,4′-dimethoxytrityl chloride (1.1 equiv.), pyridine, rt, 87%; v, propargylamine (2 equiv.), Cu(I) iodide (0.25 equiv.), $Pd(Ph_3)_4$ (0.1 equiv.), triethylamine (2 equiv.), DMF, rt, dark, 87%; vi, succinic anhydride (1.05 equiv.), pyridine; vii, EDC (1.2 equiv.), pyridine, rt, 57% (two steps); viii, 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine (1.1 equiv.), DIPEA (1.5 equiv.), CH_2Cl_2, rt, 70%.

tide was treated with tetrachloro-fluorescein phosphoramidite (TET, PE Biosystems) and tetrazole using a standard synthesis cycle, and then cleaved from the support and deprotected using standard conditions (conc. aq. ammonia, 5 h, 55°C, Fig. 2). Reversed-phase HPLC analysis showed the presence of one main peak, which was shown to be the correct labelled product by electrospray mass spectrometry.

In order to add the dye phosphoramidite to the hydroxyl of monomer **8** selectively, the 5′-hydroxyl group of the oligonucleotide requires protection. In the previous experiment this 5′-OH was conveniently masked by addition of a trifluoroacetylaminohexyl phosphoramidite (R = i, Fig. 2). Alternatively, the oligonucleotide can be synthesised 'trityl-on' and for the addition of the dye phosphoramidite only, a modified cycle can be used that does not involve acid treatment prior to addition of the dye monomer (R = ii, Fig. 2). In a third method, after 'trityl-off' DNA synthesis, the detritylated 5′-end can be capped with acetic anhydride using a special synthesis cycle before removal of the FMOC group and addition of the dye (R = iii, Fig. 2).

An important application of this monomer is the synthesis of Cyanine Dye™ (CyDye™) labelled oligonucleotides. The commercially available non-nucleosidic Cy5 phosphoramidite (Amersham-Pharmacia Biotech, Fig. 3a) can be incorporated within the oligonucleotide backbone (Fig. 3b) or at the 5′ end.

Ultraviolet thermal melting studies using the oligonucleotides **O1**–**O3** and their complementary strand **O4** (Fig. 4) showed that an oligonucleotide containing the Cy5 monomer internally caused significant destabilisa-

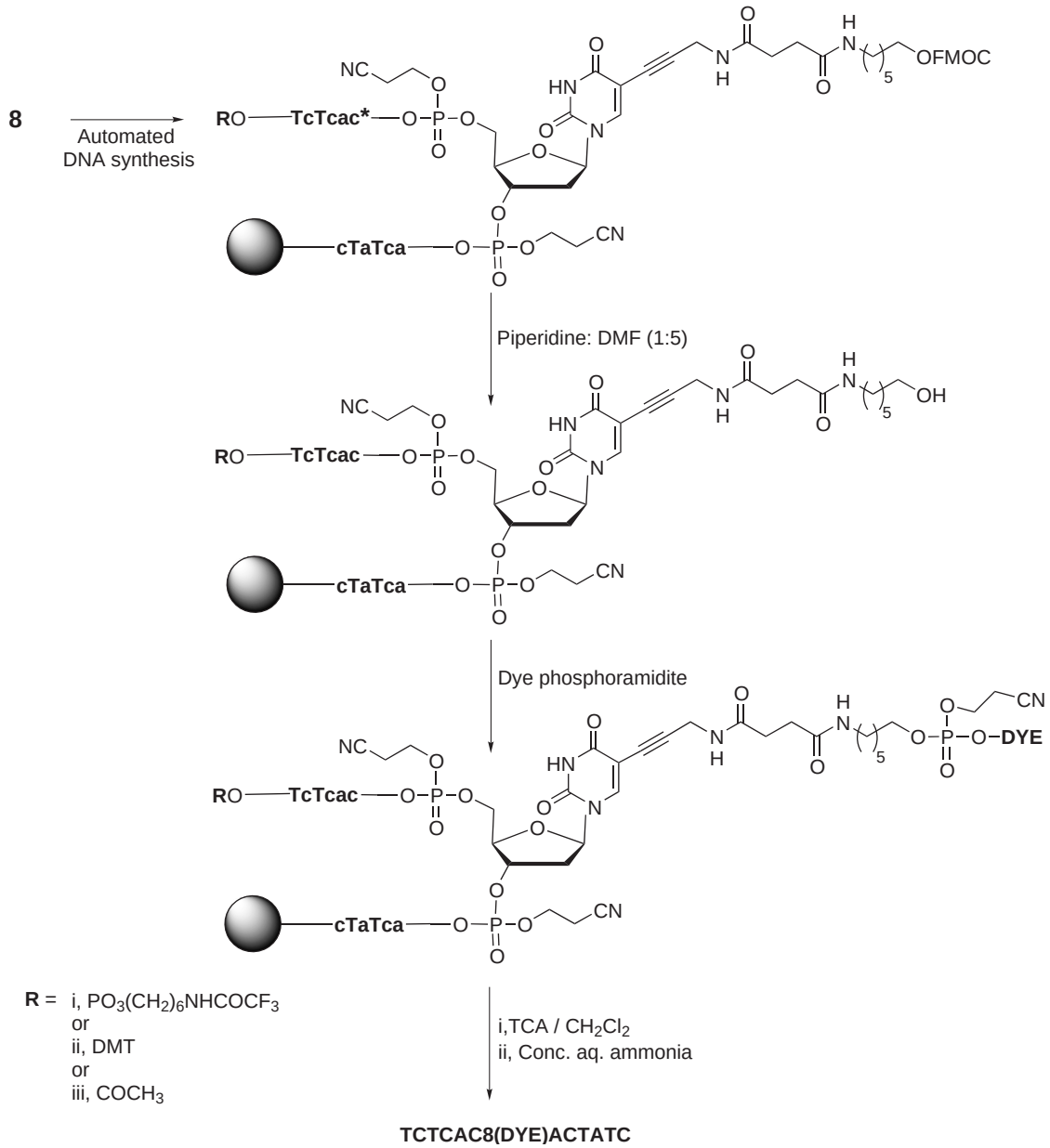


Figure 2. * Bases in lower case are protected, and upper case are unprotected.

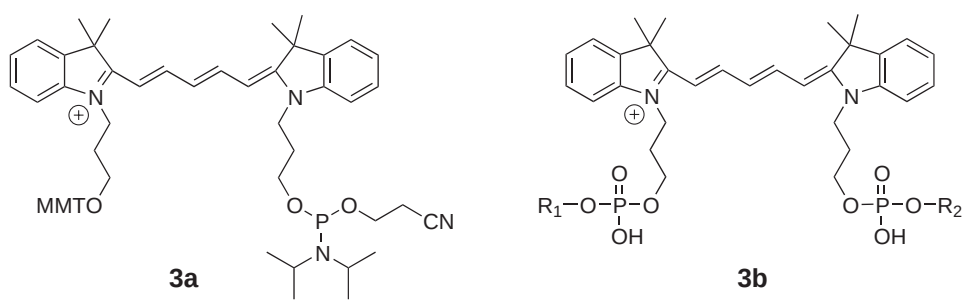
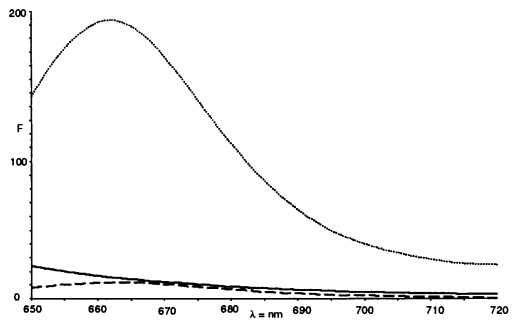


Figure 3.

Base pair	Oligonucleotides	Description	Tm (K)	Tm
A.T	**O1 / O4**	Control	319.0	-----
A.Cy5	**O2 / O4**	Cy5 in backbone	301.6	-17.4
A.X(Cy5)	**O3 / O4**	Cy5 on monomer 8	316.5	-2.5

O1 ATTAGTAAAAAAC **O3** ATTAG**X(Cy5)**AAAAAAC
O2 ATTAG**Cy5**AAAAAAC **O4** GTTTTTTACTAAT

Figure 4.



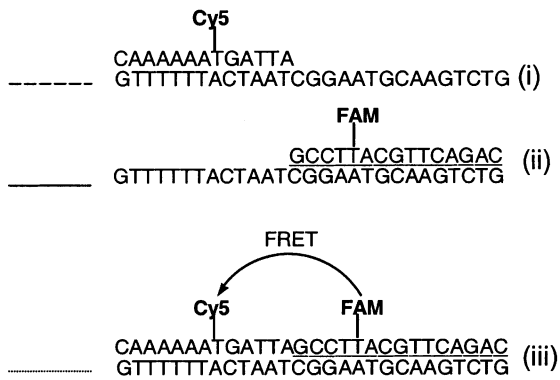


Figure 5.

tion of the duplex (–17.4 K) in comparison to the normal A.T base pair. However, when the Cy5 phosphoramidite was attached to the modified thymidine monomer **8** the destabilising effect was very small (–2.5 K). This has important implications in the design of probes containing these dyes.

Fluorescence resonance energy transfer (FRET) is an important phenomenon used extensively to allow dyes of various emission wavelengths to be excited with a single light source on fluorescence-based genetic analysers.[7] We synthesised three oligonucleotides; two labelled internally, one with fluorescein (FAM, PE Biosystems), one with Cy5 (both on monomer **8**) and a complementary strand (Fig. 5). Cy5 has an excitation wavelength of 650 nm and emits at 665 nm, therefore using a light source of 490 nm the Cy5 labelled oligonucleotide was not excited (Fig. 5, i). The FAM labelled oligonucleotide was excited and emits at 520 nm, thus no emission was observed at the wavelength associated with Cy5 emission (Fig. 5, ii). However, when the two labelled oligonucleotides hybridise adjacent to each other on a complement, the FAM is excited at 490 nm and can transfer its energy to the Cy5 causing it to emit at 665 nm (Fig. 5, iii). In this example, the ability to label oligonucleotides internally is crucial as, if the dyes are placed at the end of the oligonucleotides, they are too close and fluorescence quenching occurs.

Monomer **8** has also been used to couple standard A, T, G and C phosphoramidites thus allowing oligonucleotide side chains to be grown from a single strand (branched oligonucleotides). The monomer **8** can also be used repeatedly for the addition of different dye labels. It is important to note that to prevent any unwanted chain growth, phosphoramidites added to the side-chain of monomer **8** that possess acid-labile protecting groups (e.g. MMT on Cy5) require detritylation and capping before synthesis of the oligonucleotide can continue. This is easily achieved on an automated DNA synthesiser.

In conclusion, the modified thymidine monomer **8** permits the facile incorporation of phosphoramidite at a specific location along an oligonucleotide chain. The ability to synthesise oligonucleotides of this nature is particularly important in the field of DNA diagnostics.[8]

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