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Tetrahedron
Tetrahedron 63 (2007) 3483–3490

Naphthalenyl- and anthracenyl-ethynyl dT analogues as base discriminating fluorescent nucleosides and intramolecular energy transfer donors in oligonucleotide probes

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Received 17 July 2006; revised 24 October 2006; accepted 24 October 2006
Available online 2 February 2007

Abstract—Fluorescent thymidine analogues functionalised in the 5-position with the moieties naphthalenylethynyl (**NeT**), anthracenylethynyl (**AeT**) and anthracenylbuta-1,3-diynyl (**AeeT**) have been incorporated into oligonucleotides. The modified oligonucleotides undergo significant emission enhancement when hybridised to fully complementary strands and a decrease in fluorescence emission when the modified thymine is paired with guanine. Thus these analogues are potentially useful as base discriminating fluorescent nucleosides (BDFs). When a fluorescein dT monomer is incorporated into the same oligonucleotide strand as the modified base, energy transfer enhances the fluorescein emission, particularly upon duplex formation. These dual-labelled probes may be useful for genetic analysis to detect point mutations and SNPs and could provide multiplexing capability.

1. Introduction

Fluorogenic oligonucleotide probes are currently the subject of great interest due to their widespread use in nucleic acid sequence analysis.[1] Important examples are Molecular Beacons,[2] Hairpin primers,[3] Scorpions,[4] TaqMan probes[5] and HyBeacons,[6] which are used in mutation detection and for genotyping single nucleotide polymorphisms (SNPs), the most common source of human genomic variation.[7] Fluorogenic oligonucleotides are typically labelled with a reporter fluorophore and fluorescence quencher, and discrimination between SNPs is achieved on the basis of thermodynamic differences between correct and mismatched base pairs. Fluorescence is measured at a temperature above the T_m of mismatched probe/target duplexes, but below that of the fully complementary probe/target duplex, ensuring that a signal is emitted only from the latter. Consequently discrimination can be difficult to achieve for stable mismatches such as G:T and G:A. In a different approach, suitably designed fluorescent nucleosides can be used as sensors for the sequence to which they are hybridised, as their fluorescence emission is highly dependent on the local electronic environment.[8] This enables nucleic acid detection to be carried out under non-stringent hybridisation conditions, with the added advantage that the need for a separate fluorescence quencher is obviated, simplifying probe design and synthesis. Fluorescent nucleosides that have been used in this context include benzo- and naphtho-deazapurines,[9,10] pyrrolo-dC,[11] certain pyrenyl substituted nucleosides[12–14] and 5-furanyl-2′-deoxythymidine. The latter has been used to detect abasic sites in DNA.[15] Molecular Beacons containing two fluorescent nucleosides have also been investigated.[16] Fluorescent nucleosides are now attracting attention as potential building blocks for nanodevices such as DNA logic gates[17] and optical arrays,[18] and as scaffolds for the assembly of porphyrin arrays.[19]

With the above applications in mind we were particularly interested in studying the properties of fluorophores appended to 5-alkynyl-2′-deoxythymidines, which are readily synthesised by Pd-catalysed Sonogashira coupling chemistry.[20] In this paper we describe the synthesis of oligonucleotides containing the naphthalene and anthracene derivatives **NeT**, **AeT** and **AeeT** (Fig. 1) and investigate them as base discriminating nucleosides and fluorescent antennae for energy transfer.

2. Results and discussion

2.1. Synthesis of fluorescent phosphoramidite monomers

Synthesis of the fluorescent anthracene and naphthalene derivatives of deoxythymidine used in this study was straightforward and high-yielding. The ethynyl derivatives

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doi:10.1016/j.tet.2006.10.090

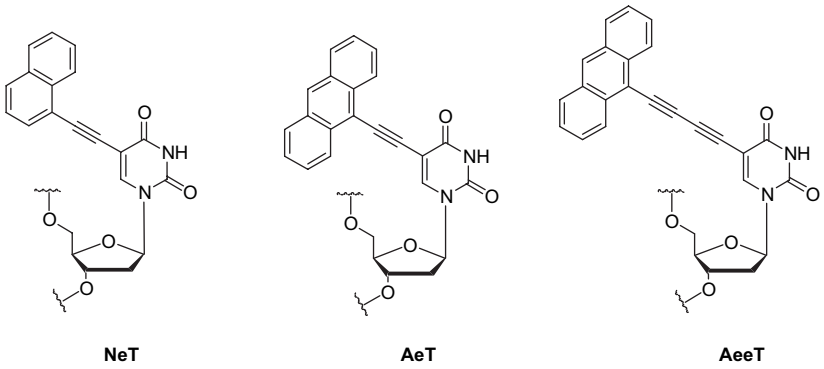
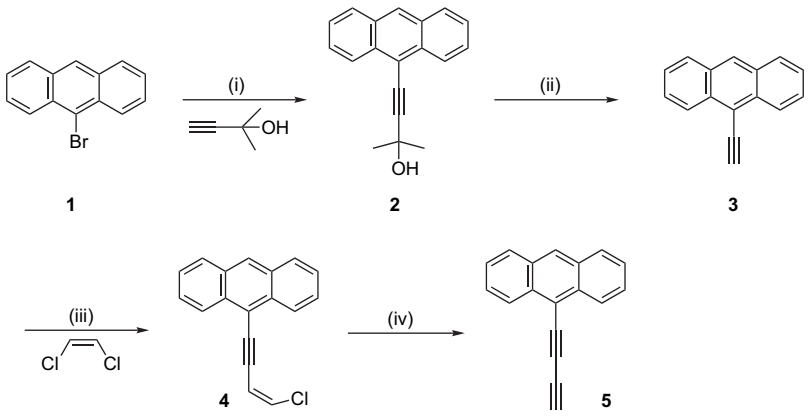


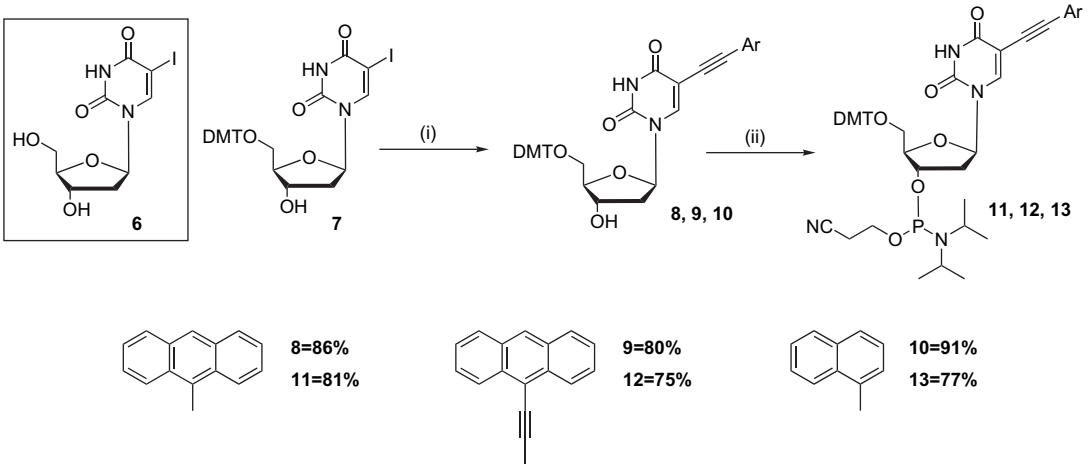
Figure 1. 5-Substituted dT nucleosides 5-(1-naphthalenylethynyl)dT (**NeT**), (10-anthracenylethynyl)dT (**AeT**) and 10-(buta-1,3-diynyl)anthracenyl dT (**AeeT**).

of anthracene were synthesised according to Scheme 1. Reaction of the commercially available bromoanthracene **1** with 2-methylbut-3-yn-2-ol in the presence of $Pd^{(0)}$ produced the protected ethynylanthracene **2**, which was converted to alkyne **3** by treatment with potassium hydroxide in refluxing toluene. Reaction of **3** with *cis*-1,2-dichloroethylene in the presence of *n*-butylamine and $Cu^{(I)}$ iodide gave 10-((*Z*)-4-chlorobut-3-en-1-ynyl)anthracene **4**, which was converted to the bis-ethynylanthracene **5** by treatment with tetrabutylammonium fluoride in tetrahydrofuran.[21]

All 5-substituted thymidine nucleosides were synthesised in good yield by Sonogashira coupling of 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine **7** with the relevant alkyne (Scheme 2). A successful Sonogashira coupling reaction was also carried out between unprotected 5-iodo-2′-deoxyuridine **6** and alkyne **3** prior to 5′-dimethoxytritylation but this route did not offer any advantage and was not pursued further. Phosphitylation of DMT-protected nucleosides **8**, **9** and **10** under standard conditions followed by silica-gel column chromatography gave phosphoramidite monomers



Scheme 1. (i) $Pd(PPh_3)_2Cl_2$, CuI, PPh_3, Et_3N, 91%; (ii) KOH, toluene, 86%; (iii) $Pd(Ph_3P)_4$, CuI, *n*-butylamine, toluene, 62%; (iv) $^nBu_4NF$, THF, 62%.



Scheme 2. (i) $Pd(Ph_3P)_4$, Et_3N, CuI, DMF; (ii) *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, THF.

11, **12** and **13** in good yield. These monomers were incorporated into oligodeoxynucleotides by automated solid-phase synthesis with no changes to the standard procedures. All coupling efficiencies (stepwise yields) were >98% and purification of the oligomers was carried out by reversed-phase HPLC (C8). MALDI-TOF mass spectrometry was used to characterise key modified oligonucleotides.

2.2. Thermal stabilities of oligonucleotides containing fluorescent nucleosides

To evaluate the effects of the modified nucleosides on duplex stability we incorporated them into 18mer oligonucleotides bearing a 3′-dabcyl quencher (**MNeT**, **MAeT** and **MAeeT**) and conducted fluorescence melting experiments with a series of complementary oligonucleotides functionalised at the 5′-position with fluorescein (Table 1). This fluorescence melting technique provides higher throughput than conventional UV-melting and is an established technique for measuring the stability of duplexes, triplexes and quadruplexes.[22]

The fully matched duplexes formed between **MNeT**, **MAeT** and **MAeeT** and **CompA** are destabilised relative to the unmodified duplex by 2.9, 3.4 and 6.1 °C, respectively. These effects are typical for duplexes in which a large hydrophobic moiety is projected into the major groove, and the fact that the smallest carbocycle **NeT** is less destabilising than **AeT** reflects this. The longer hydrophobic buta-1,3-diynyl anthracene in **AeeT** leads to significant destabilisation, probably because the aromatic moiety is fully exposed to the aqueous environment. In general, mismatches opposite the fluorescent nucleosides (T*) give only a small drop in T_m relative to T*:A since the modified nucleoside is a point of duplex instability. Interestingly the T*:C mismatches are more stable than expected and for the anthracene-containing duplexes the T*:C mismatch is more stable than the T*:A Watson–Crick analogue. In addition, the T*:C mismatched duplexes have similar photophysical properties to the fully matched duplexes (Section 2.3). High-field NMR, X-ray crystallographic and modelling studies on the relevant duplexes are needed to provide a structural rationale for these observations.

Table 1. Thermal stabilities of duplexes incorporating nucleoside analogues **NeT**, **AeT** and **AeeT**

Oligonucleotide	Complement oligonucleotide	Mismatch	T_m (°C)	ΔT_m (°C)
MT	**CompA**	—	66.5	—
MT	**CompG**	T:G	60.7	−5.8
MT	**CompC**	T:C	58.6	−7.9
MT	**CompT**	T:T	59.2	−7.3
MNeT	**CompA**	—	63.6	—
MNeT	**CompG**	T*:G	60.4	−3.2
MNeT	**CompC**	T*:C	63.0	−0.6
MNeT	**CompT**	T*:T	60.6	−3.0
MAeT	**CompA**	—	63.1	—
MAeT	**CompG**	T*:G	61.4	−1.7
MAeT	**CompC**	T*:C	64.7	1.5
MAeT	**CompT**	T*:T	62.1	−1.0
MAeeT	**CompA**	—	60.4	—
MAeeT	**CompG**	T*:G	59.5	−1.0
MAeeT	**CompC**	T*:C	62.6	2.2
MAeeT	**CompT**	T*:T	60.2	−0.2

Sequences of oligonucleotides: CGTCTGAT**Y**TGATCGCAG-Dabcyl, **MT** (**Y**=T), **MNeT** (**Y**=**NeT**), **MAeT** (**Y**=**AeT**) and **MAeeT** (**Y**=**AeeT**); FAM-CTGCGATCA**X**ATCAGACG where **X**=A (**CompA**), **X**=G (**CompG**), **X**=C (**CompC**), **X**=T (**CompT**).

2.3. Luminescence properties

2.3.1. Suitability as base discriminating fluorescent nucleosides (BDFs). Fluorescent nucleosides in DNA that can be used to distinguish between bases in the complementary strand by base pairing can in principle be used to detect point mutations and SNPs under non-stringent hybridisation conditions. In this context the thermal denaturation results in Table 1 indicate that for nucleosides **NeT**, **AeT** and **AeeT** differences in T_m are unlikely to be sufficiently large to be useful indicators of the nature of the partner base. However, discrimination due to changes in fluorescence properties on duplex formation is a potentially valuable alternative. To investigate this possibility **NeT** and **AeT** were incorporated into 12mer oligonucleotides **FNeT** and **FAeT** (Table 2). The longer 18mer oligomer **FAeeT** was used in the case of the most destabilising analogue **AeeT** to ensure efficient hybridisation. Typically, chromophores appended to C5-position of pyrimidines display enhanced emission upon duplex formation due to a change from a hydrophobic environment in the single stranded form to an aqueous environment in the solvated B-DNA major groove.[15] Indeed, the naphthalene-derivatised oligonucleotide **FNeT** undergoes significant (4.91-fold) emission enhancement when hybridised to its complement **Comp2A** (Table 2). The duplexes formed with **Comp2C** and **Comp2T**, which give T*:C and

Table 2. Steady state fluorescence measurements of nucleoside analogues **NeT**, **AeT** and **AeeT** incorporated into oligonucleotides

Fluorescent oligonucleotide sequence	Complement oligonucleotide	Mismatch	λ_{ex} (nm)	λ_{em} (nm)	I_{ds}:I_{ss}
FNeT=GTCTAT(**NeT**)TATCG	**Comp2A**	—	340	426	4.91
FNeT=GTCTAT(**NeT**)TATCG	**Comp2G**	T*:G	340	435	0.59
FNeT=GTCTAT(**NeT**)TATCG	**Comp2C**	T*:C	340	417	4.17
FNeT=GTCTAT(**NeT**)TATCG	**Comp2T**	T*:T	340	424	2.10
FAeT=GTCTAT(**AeT**)TATCG	**Comp2A**	—	410	440	2.49
FAeT=GTCTAT(**AeT**)TATCG	**Comp2G**	T*:G	410	445	0.46
FAeT=GTCTAT(**AeT**)TATCG	**Comp2C**	T*:C	410	442	2.22
FAeT=GTCTAT(**AeT**)TATCG	**Comp2T**	T*:T	410	442	1.05
FAeeT=CGTCTGAT(**AeeT**)TGATCGCAG	**Comp3A**	—	410	456	2.13
FAeeT=CGTCTGAT(**AeeT**)TGATCGCAG	**Comp3G**	T*:G	410	456	1.41
FAeeT=CGTCTGAT(**AeeT**)TGATCGCAG	**Comp3C**	T*:C	410	456	4.47
FAeeT=CGTCTGAT(**AeeT**)TGATCGCAG	**Comp3T**	T*:T	410	454	3.86

Sequences of target oligonucleotides: CGATA**X**ATAGAC, where **X**=A (**Comp2A**), **X**=G (**Comp2G**), **X**=C (**Comp2C**), **X**=T (**Comp2T**); CTGCGATCA**X**-ATCAGACG, where **X**=A (**Comp3A**), **X**=G (**Comp3G**), **X**=C (**Comp3C**), **X**=T (**Comp3T**).

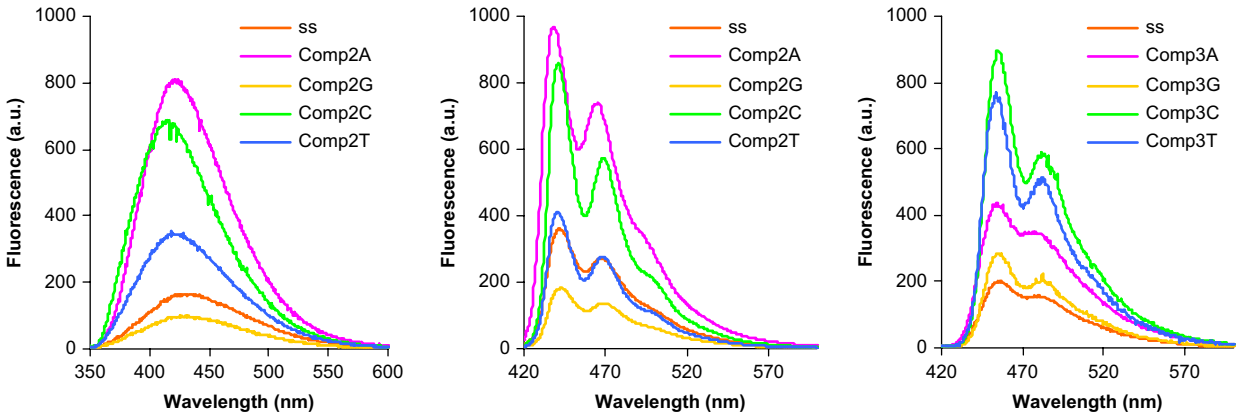


Figure 2. Steady state emission spectra of oligonucleotides, **FNeT** (left, excitation 340 nm), **FAeT** (centre, excitation 410 nm) and **FAeeT** (right, excitation 410 nm) and when hybridised to complements **Comp2A**, **G**, **C** and **T** or **Comp3A**, **G**, **C** and **T**.

T*:T mismatches, also exhibit increased fluorescence upon hybridisation, though to a lesser extent (Fig. 2). Most interestingly, hybridisation to **Comp2G**, in which the modified thymine is paired with a guanine base, gives a *decrease* in fluorescence emission of approximately 2-fold. There is therefore a 10-fold differentiation in fluorescence intensity between the T*:A and T*:G base pairs. This could be due to the fluorescence quenching properties of guanine[23] or the effect of the T*:G wobble pair on the location of the chromophore in the major groove. However, it is not possible to draw firm conclusions on the basis of thermal denaturation studies alone. Regardless of its cause, this is a highly desirable property, since it enables detection of any of the four transition mutations (A to G, G to A, T to C and C to T) simply by using suitably designed probes. This is particularly important, as transition mutations can give rise to stable G:T mismatches in probe:target duplexes, and these are difficult to discriminate from Watson–Crick base pairs on the basis of T_m alone.

The anthracenylethynyl nucleoside **AeT** behaves similarly to **NeT**, with the fluorescence intensity of the **FAeT** duplexes following the order **Comp2A** (T*:A)>**Comp2C** (T*:C)>**Comp2T** (T*:T)>**Comp2G** (T*:G). The intensity difference between T*:A and T*:G (5.4-fold) is still significant, and the optimum excitation wavelengths of the anthracene and naphthalene moieties are sufficiently resolved to suggest that the two fluorophores could be used in tandem to detect two different DNA sequences, mutations or SNPs in a single tube PCR reaction.

Compounds **NeT** and **AeT** exhibit the dual sensing/reporting capabilities required of a base discriminating fluorophore. They are useful additions to the range of fluorescent 5-alkynyl-2′-deoxythymidine derivatives, which was very recently extended by the description of a series of benzoxazole- and perylene-functionalised nucleosides with interesting fluorescence properties.[24]

When the anthracene chromophore is introduced via a buta-1,3-diynyl linker (**AeeT**) the fluorescence and base discriminating properties are affected. The emission spectra are red-shifted by ~25 nm, suggesting that the longer rigid linker relieves steric hindrance in the major groove, allowing the anthracene to lie in the plane of the nucleobase, thus producing greater conjugation. The base discriminating properties are also altered so that the fluorescence intensity of duplexes formed by oligonucleotide **FAeeT** follows the order **Comp3C** (T*:C)>**Comp3T** (T*:T)>**Comp3A** (T*:A)>**Comp3G** (T*:G). The anthracene bis-ethynyl nucleoside **AeeT** is the most destabilising of the three modifications, possibly due to the fact that the anthracene moiety protrudes into the aqueous environment surrounding the DNA to produce an unfavourable entropic effect.

2.3.2. Intramolecular energy transfer. The C5-position of pyrimidines has long been used to introduce dye molecules into DNA for energy transfer applications.[25,26] The rigid ethynyl linker separates the donor molecule from the nucleobases, reducing quenching, leading to efficient fluorescence resonance energy transfer (FRET). The naphthalenyl- and anthracenyl-modified nucleosides used in this study are good candidates as FRET donors to fluorescein due to the overlap of their emission spectra with the excitation spectrum of fluorescein. Therefore, we investigated the possibility of energy transfer between fluorescent nucleosides **NeT**, **AeT** and **AeeT** and a fluorescein dT acceptor located three nucleotides away in the same strand of the 18mers **FFNeT**, **FFAeT** and **FFAeeT**.

The results (Table 3, Fig. 3) demonstrate not only that energy transfer occurs from all the fluorescent nucleosides to

Table 3. Energy transfer and fluorescence enhancement of fluorescein/BDF-labelled probes

Fluorescent oligonucleotide sequence	λ_{ex} (nm)	I_{ds}:I_{ss}, FRET excitation	I_{ds}:I_{ss}, 495 nm excitation	I_{FRET}:$I_{ex\ 495\ nm}$, ds
FFNeT=CGTC(**TF**)GAT(**NeT**)TGATCGCAG	340	2.64	1.59	0.30
FFAeT=CGTC(**TF**)GAT(**AeT**)TGATCGCAG	410	2.38	1.79	0.26
FFAeeT=CGTC(**TF**)GAT(**AeeT**)TGATCGCAG	410	2.21	1.93	0.28

TF=fluorescein dT.
Sequence of target oligonucleotide **Comp3A**=CTGCGATCAAATCAGACG.

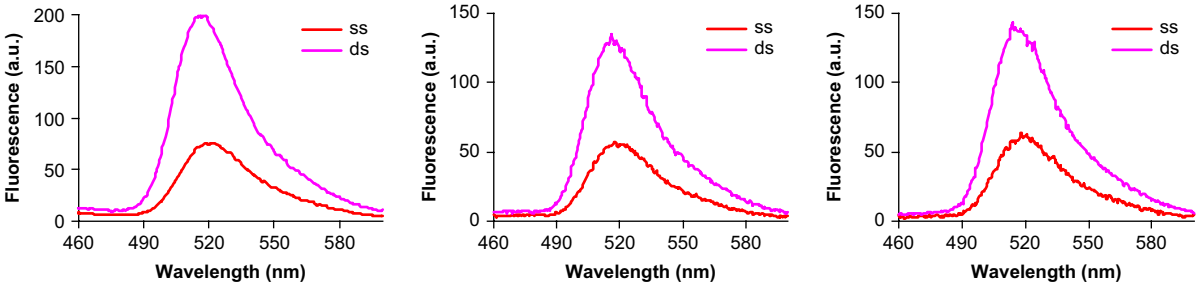


Figure 3. FRET emission from fluorescein-labelled oligonucleotides **FFNeT** (left, excitation 340 nm), **FFAeT** (centre, excitation 410 nm) and **FFAeeT** (right, excitation 410 nm) in the absence (ss) and presence (ds) of 1 equiv of complementary oligonucleotide **Comp3A**.

fluorescein, but also that the fluorescence intensity of the probes increases upon hybridisation to the complementary strand **Comp3A**. In the single stranded and duplex forms, the emission from the donor moieties was completely suppressed, indicating efficient energy transfer (data not shown). The signal obtained from fluorescein was enhanced upon hybridisation, whether excited by FRET or by direct irradiation at 495 nm, but the enhancement was greater when FRET excitation was used, presumably due to a combined de-quenching effect from both dyes. These dual-labelled probes may be very useful for genetic analysis. Firstly, the base discriminating capability of the fluorescent nucleosides should allow facile distinction of point mutations and SNPs and secondly, energy transfer from several different fluorophores to fluorescein could provide multiplexing capability if multiple excitation sources are used to produce emission that can be detected in a single channel.

3. Conclusion

Oligonucleotides containing naphthalenylethynyl, anthracenylethynyl and anthracenylbuta-1,3-diynyl dT undergo significant fluorescence emission enhancement when hybridised to fully complementary strands. When the modified thymine is paired with guanine a decrease in fluorescence emission is observed suggesting that these analogues could be useful as base discriminating fluorescent nucleotides.

When a fluorescein dT monomer is incorporated into the same oligonucleotide strand as the modified base, energy transfer enhances the fluorescein emission, particularly upon duplex formation. Energy transfer to other fluorophores should also be possible. These dual-labelled probes are potentially useful for genetic analysis to detect point mutations and SNPs and could provide multiplexing capability.

4. Experimental

4.1. General

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker WP-400 or Bruker WP-300 spectrometer (at ambient temperature). All spectra were referenced to $CDCl_3$ or DMSO-d_6. Melting points were measured on a Gallenkamp electrothermal melting point apparatus and are uncorrected. Mass spectra were recorded on a Fisons VG platform instrument. Column chromatography was carried out under positive pressure on Merck 60-mesh silica. TLC was carried out using Merck Kieselgel 60 F_{254} sheets. Dichloromethane, *N*,*N*-diisopropylethylamine and triethylamine were distilled from CaH_2. *N*,*N*-Dimethylformamide was purchased from Aldrich. Tetrahydrofuran was distilled from sodium wire and benzophenone immediately before use. 1-Ethynylnaphthalene was purchased from the Aldrich Chemical Company.

4.1.1. 4-(Anthracen-10-yl)-2-methylbut-3-yn-2-ol (2). 9-Bromoanthracene **1** (10.0 g, 40.0 mmol) and 2-methylbut-3-yn-2-ol (7.72 g, 80.0 mmol) were dissolved in triethylamine (50 mL) in the presence of $Pd(PPh_3)_2Cl_2$ (75 mg), CuI (100 mg) and PPh_3 (155 mg). The mixture was heated to reflux for 4 h under argon, then cooled to room temperature and filtered. After removal of the solvent in vacuo, the residue was dissolved in dichloromethane (150 mL) and washed with distilled water followed by satd aqueous NaCl. The organic layer was dried (anhydrous Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The crude product was purified by flash column chromatography (dichloromethane), which provided **2** (9.46 g, 91%). R_f 0.30 (dichloromethane); mp (dichloromethane): 94–95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (d, J=8.4 Hz, 2H), 8.28 (s, 1H), 7.87 (d, J=8.4 Hz, 2H), 7.48–7.35 (m, 4H), 1.75 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 132.5, 131.1, 128.6 (2×*CH*), 127.6, 126.6 (2×*CH*), 126.5 (2×*CH*), 125.7 (2×*CH*), 116.6, 105.2, 78.8, 66.2, 31.8 (2×CH_3); MS (ES$^+$) *m*/*z*: 283.0 [M+Na]$^+$.

4.1.2. 10-Ethynylanthracene (3). A mixture of 4-(anthracen-10-yl)-2-methylbut-3-yn-2-ol **2** (5.20 g, 6.46 mmol) and KOH (1.32 g, 9.70 mmol) in toluene (150 mL) was heated under reflux for 25 min at 115 °C under argon. The reaction mixture was cooled and filtered and after removal of the solvent under reduced pressure the residue was purified by flash column chromatography (hexane), which provided **3** (3.50 g, 87%) as a light yellow crystalline solid. Compound **3** was not stable and was used in the next reaction directly. R_f 0.25 (hexane); mp (dichloromethane): 68–70 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.2 (d, J=8.4 Hz, 2H), 8.44 (s, 1H), 7.80 (d, J=8.4 Hz, 2H), 7.64–7.49 (m, 4H), 4.01 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 133.1 (2×*C*), 131.0 (2×*C*), 128.6 (2×*CH*), 128.2, 126.8 (2×*CH*), 126.5 (2×*CH*), 125.6 (2×*CH*), 116.0, 88.2, 80.3; MS (ES$^+$) *m*/*z*: 225.0 [M+Na]$^+$.

4.1.3. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[10-ethynylanthracenyl]-2′-deoxyuridine (8). 5′-*O*-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine **7** (1.43 g, 2.19 mmol), 10-ethynylanthracene **3** (663 mg, 3.28 mmol) and CuI (83 mg,

0.44 mmol) were dried in vacuo for 1 h. Anhydrous *N*,*N*-dimethylformamide (5 mL) and anhydrous triethylamine (0.7 mL, 4.38 mmol) were added under argon. The reaction mixture was stirred for 10 min and $(Ph_3P)_4Pd^{(0)}$ (252 mg, 0.219 mmol) was added in one portion. The reaction mixture was stirred at room temperature with exclusion of light overnight, concentrated in vacuo and purified by flash column chromatography (EtOAc/toluene, 3:2, containing 1% pyridine), which provided 4.16 g (87%) of **8** as a yellow foam. R_f 0.26 (EtOAc/toluene, 3:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.91 (s, 1H, N*H*-3), 8.63 (s, 1H, *CH*-6), 8.40 (d, J=8.5 Hz, 2H), 8.31 (s, 1H), 8.62 (d, J=8.5 Hz, 2H), 7.52–7.01 (m, 13H), 6.76 (d, J=9.0 Hz, 1H), 6.23 (t, J=6.6 Hz, 1H, $CH^{1'}$), 5.34 (m, 1H, $OH^{3'}$), 4.28 (m, 1H, $CH^{3'}$), 3.97 (m, 1H, $CH^{4'}$), 3.48 (s, 3H, CH_3^{DMT}), 3.46 (s, 3H, CH_3^{DMT}), 3.27 (m, 2H, $CH_2^{5'}$), 2.50 (m, 1H, $CH_2^{2'a}$), 2.29 (m, 1H, $CH_2^{2'b}$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.5 (C^4), 157.8, 149.3 (C^2), 144.6, 142.5 (*CH*-6), 135.4, 135.3, 131.4, 130.5, 129.5, 128.7, 128.5, 128.1, 127.7, 127.6, 127.5, 126.8, 126.5, 125.7, 125.2, 116.1, 113.0, 98.8 (C^5), 93.2, 89.4, 85.9 ($CH^{4'}$), 85.7, 85.4 ($CH^{1'}$), 70.4 ($CH^{3'}$), 63.6 ($CH_2^{5'}$), 54.6 (2×OCH_3), 40.2 ($CH_2^{2'}$); MS (ES$^+$) *m*/*z*: 753.0 [M+Na]$^+$; HR-ESIMS calcd for $C_{46}H_{38}N_2NaO_7$: 753.2571, found: 753.2564.

4.1.4. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[10-ethynylanthracenyl]-2′-deoxyuridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite (11). 5′-*O*-(4,4′-Dimethoxytrityl)-5-[10-ethynylanthracenyl]-2′-deoxyuridine **8** (730 mg, 1.0 mmol) was evaporated with freshly distilled tetrahydrofuran (2×5 mL). The resulting syrup was dissolved in freshly distilled tetrahydrofuran (5 mL). Anhydrous *N*,*N*-diisopropylethylamine (387 mg, 3.0 mmol) was then added to the solution by syringe under a weak flow of argon. The phosphitylating reagent, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (295 mg, 1.3 mmol), was added slowly via syringe and a white precipitate quickly formed. The reaction mixture was stirred at room temperature for 2 h and diluted with dichloromethane (20 mL), then satd aqueous KCl solution (5 mL) was added by syringe with vigorous stirring under argon. The organic phase was dried over anhydrous Na_2SO_4 under argon for 2 h, then filtered, evaporated to dryness under reduced pressure and purified by flash chromatography (EtOAc/toluene, 1:1, containing 1% pyridine). The desired phosphoramidite monomer was obtained as a yellow solid (760 mg, 82%). R_f 0.35 (EtOAc/toluene, 1:1); ^{31}P NMR (121 MHz, DMSO-d_6) δ 148.88, 148.53; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.92 (s, 1H, NH-3), 8.63 (s, 1H, *CH*-6), 8.40 (d, J=8.5 Hz, 1H), 8.38 (m, 2H), 8.11 (d, J=8.4 Hz, 3H), 7.56–7.03 (m, 13H), 6.76 (d, J=9.0 Hz, 4H), 6.29 (m, 1H, $CH^{1'}$), 4.50 ($CH^{3'}$), 4.10 (m, 1H, $CH^{4'}$), 3.73–3.26 (m, 12H, 2×OCH_3, OCH_2CH_2CN, $CH_2^{5'}$), 2.58–2.27 (m, 4H, $CH_2^{2'}$, $((CH_3)_2CH)_2$N), 1.13–0.97 (m, 12H, $((CH_3)_2CH)_2$N); MS (ES$^+$) *m*/*z*: 931.4 [M+Na]$^+$; HR-ESIMS calcd for $C_{55}H_{55}N_4NaO_8P$: 953.3650, found: 953.3646.

4.1.5. 10-((*Z*)-4-Chlorobut-3-en-1-ynyl)anthracene (4). 10-Ethynylanthracene **3** (2.10 g, 10.4 mmol) was dissolved in anhydrous toluene (100 mL). *cis*-1,2-Dichloroethylene (2.04 g, 21 mmol) and anhydrous *n*-butylamine (52 mmol, 5.2 mL) were introduced to the solution under argon followed by CuI (200 mg, 1.04 mmol) and $(Ph_3P)_4Pd^{(0)}$ (598 mg, 0.52 mmol). The reaction mixture was then stirred at room temperature with exclusion of light overnight after which the organic phase was washed with brine (2× 30 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed in vacuo and the residue purified by flash column chromatography (hexane) to give **4** as a yellow solid (1.70 g, 63%). R_f 0.20 (hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (d, J=8.5 Hz, 2H), 8.45 (s, 1H), 8.80 (d, J=8.5 Hz, 2H), 7.64–7.49 (m, 4H), 6.60 (d, J=7.2 Hz, 1H), 8.66 (d, J=7.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 132.7, 131.1, 128.6 (2×*CH*), 128.4 (*CH*), 128.2 (*CH*), 126.9 (2×*CH*), 126.7 (2×*CH*), 125.7 (2×*CH*), 112.5 (*CH*), 94.6, 94.5.

4.1.6. 10-(Buta-1,3-diynyl)anthracene (5). To a stirred solution of **4** (100 mg, 0.38 mmol) in anhydrous tetrahydrofuran (10 mL) was added 1.0 M ^{n}Bu$_4$NF in tetrahydrofuran (0.95 mL, 0.95 mmol) under argon. After stirring for 24 h the organic phase was diluted with diethyl ether (50 mL), washed with satd aqueous NH_4Cl (2×15 mL) followed by satd aqueous NaCl (2×15 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed in vacuo and the residue purified by flash column chromatography (hexane) to give **5** (65 mg, 63%) as a yellow solid. R_f 0.23 (hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.52 (d, J=8.6 Hz, 2H), 8.44 (s, 1H), 8.00 (d, J=8.4 Hz, 2H), 7.64–7.49 (m, 4H), 2.82 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 134.3, 130.9, 129.2, 128.8 (2×*CH*), 127.3 (2×*CH*), 126.3 (2×*CH*), 125.9 (2×*CH*), 84.2, 74.0 (*CH*), 72.7, 68.5.

4.1.7. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[10-(buta-1,3-diynyl)-anthracenyl]-2′-deoxyuridine (9). 5′-*O*-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine **7** (656 mg, 1.0 mmol), 10-(buta-1,3-diynyl)anthracene **5** (270 mg, 1.2 mmol) and CuI (38 mg, 0.20 mmol) were dried in vacuo for 1 h. Anhydrous *N*,*N*-dimethylformamide (5 mL) and anhydrous triethylamine (0.45 mL, 3.0 mmol) were then added under argon. The mixture was stirred for 10 min and $(Ph_3P)_4Pd^{(0)}$ (115 mg, 0.10 mmol) was added in one portion. After stirring at room temperature overnight with exclusion of light, the reaction mixture was concentrated in vacuo and purified by flash column chromatography (EtOAc/toluene, 3:2, containing 1% pyridine) to provide **9** (588 mg, 80%) as a yellow foam. R_f 0.25 (EtOAc/toluene, 3:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.89 (s, 1H, *NH*-3), 8.74 (s, 1H, *CH*-6), 8.33 (m, 3H), 8.18 (d, J=7.8 Hz, 2H), 7.66–7.15 (m, 13H), 6.85 (d, J=8.7 Hz, 2H), 6.87 (d, J=8.7 Hz, 2H), 6.17 (t, J=6.6 Hz, 1H, $CH^{1'}$), 5.38 (d, J=4.2 Hz, 1H, $OH^{3'}$), 4.39 (m, 1H, $CH^{3'}$), 4.00 (m, 1H, $CH^{4'}$), 3.66 (s, 3H, OCH_3^{DMT}), 3.65 (s, 3H, OCH_3^{DMT}), 3.38–3.18 (m, 2H, $CH_2^{5'}$), 2.35 (m, 2H, $CH_2^{2'}$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.4 (C^4), 158.0, 149.1 (C^2), 145.6 (*CH*-6), 144.6, 135.5, 135.2, 133.0, 130.5, 129.6, 129.5, 129.3, 129.1, 128.7, 128.1, 128.1, 127.8, 127.5, 126.6, 126.1, 125.3, 125.2, 113.9, 113.1, 97.0 (C^5), 86.0 ($CH^{4'}$), 85.9 ($CH^{1'}$), 85.4, 84.5, 78.2, 78.1, 76.5, 70.1 ($CH^{3'}$), 63.5 ($CH_2^{5'}$), 54.9 (2×OCH_3), 40.2 ($CH_2^{2'}$); MS (ES$^+$) *m*/*z*: 777.2 [M+Na]$^+$; HR-ESIMS calcd for $C_{48}H_{38}N_2NaO_7$: 777.2571, found: 777.2559.

4.1.8. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[10-(buta-1,3-diynyl)anthracenyl]-2′-deoxyuridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite (12). 5′-*O*-

(4,4′-Dimethoxytrityl)-5-[10-(buta-1,3-diynyl)anthracenyl]-2′-deoxyuridine **9** (200 mg, 0.27 mmol) was evaporated with freshly distilled tetrahydrofuran (2×5 mL). The resulting syrup was dissolved in freshly distilled tetrahydrofuran (5 mL) under argon. Anhydrous *N*,*N*-diisopropylethylamine (103 mg, 0.8 mmol) was injected to the above solution under a weak flow of argon after which the phosphitylating reagent, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (73.2 mg, 0.32 mmol), was added slowly via syringe. A white precipitate quickly formed and the reaction mixture was stirred at room temperature for 2 h and diluted with CH_2Cl_2 (20 mL). Satd aqueous KCl solution (5 mL) was added with vigorous stirring via syringe under argon and the organic phase was dried over anhydrous Na_2SO_4 under argon for 2 h. The solution was then filtered and evaporated to dryness under reduced pressure and the residue was purified by flash chromatography (EtOAc/toluene, 1:1, containing 1% pyridine). The desired phosphoramidite **12** was obtained as a yellow solid (190 mg, 75%). R_f 0.35 (EtOAc/toluene, 1:1); ^{31}P NMR (121 MHz, DMSO-d_6) δ 148.91, 148.61; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.92 (br, 1H, *NH*-3), 8.78 (s, 1H, *CH*-6), 8.33 (m, 3H), 8.18 (d, J=7.8 Hz, 1H), 7.56–7.03 (m, 13H), 6.87 (m, 4H), 6.16 (m, 1H, $CH^{1'}$), 4.58 ($CH^{3'}$), 4.10 (m, 1H, $CH^{4'}$), 3.73–3.26 (m, 12H, 2×OCH_3, OCH_2CH_2CN, $CH_2^{5'}$), 2.77 (t, J=6.0 Hz, $((CH_3)_2CH)_2$N), 2.65 (t, J=6.0 Hz, $((CH_3)_2CH)_2$N), 2.48 (m, 2H, $CH_2^{2'}$), 1.13–0.97 (m, 12H, $((CH_3)_2CH)_2$N); MS (ES$^+$) *m*/*z*: 977.2 [M+Na]$^+$; HR-ESIMS calcd for $C_{57}H_{55}N_4NaO_8P$: 977.3650, found: 977.3644.

4.1.9. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[1-ethynylnaphthalenyl]-2′-deoxyuridine (10). 5′-*O*-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine **7** (1.55 g, 2.36 mmol), 1-ethynylnaphthalene (1.08 g, 7.08 mmol) and CuI (90.0 mg, 0.472 mmol) were dried in vacuo for 1 h. Anhydrous dimethylformamide (5 mL) and anhydrous triethylamine (0.66 mL, 4.72 mmol) were then added under argon. The mixture was stirred for 10 min and $(Ph_3P)_4Pd^{(0)}$ (271 mg, 0.236 mmol) was added in one portion. The reaction mixture was stirred at room temperature overnight with exclusion of light and then concentrated in vacuo. The residue was purified by flash column chromatography (EtOAc/toluene, 1:1, containing 1% pyridine), which provided 1.4 g (91%) of **10** as a colourless foam. R_f 0.25 (EtOAc/toluene, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.82 (s, 1H, *NH*-3), 8.26 (d, J=5.1 Hz, 1H), 8.18 (s, 1H, *CH*-6), 7.94 (m, 3H), 7.59–7.09 (m, 12H), 6.82 (d, J=5.7 Hz, 2H), 6.80 (d, J=5.7 Hz, 2H), 6.19 (t, J=6.6 Hz, 1H, $CH^{1'}$), 5.35 (d, J=4.2 Hz, 1H, $OH^{3'}$), 4.33 (m, 1H, $CH^{3'}$), 3.99 (m, 1H, $CH^{4'}$), 3.58 (s, 6H, CH_3^{DMT}), 3.25 (m, 2H, $CH_2^{5'}$), 2.35 (m, 1H, $CH_2^{2'}$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.5 (C^4), 157.9, 149.2 (C^2), 144.5, 142.5 (*CH*-6), 135.4, 135.2, 132.5, 132.2, 129.6, 129.5, 128.7, 128.6, 128.1, 128.0, 127.7, 127.5, 126.8, 126.4, 125.7, 125.1, 119.7, 113.0, 98.5 (C^5), 90.0, 86.7, 85.9 ($CH^{4'}$), 85.7, 85.2 ($CH^{1'}$), 70.3 ($CH^{3'}$), 63.5 ($CH_2^{5'}$), 54.7 (2×OCH_3), 40.2 ($CH_2^{2'}$); MS (ES$^+$) *m*/*z*: 703.3 [M+Na]$^+$; HR-ESMS calcd for $C_{42}H_{36}N_2NaO_7$: 703.2415, found: 703.2416.

4.1.10. 5′-*O*-(4,4′-Dimethoxytrityl)-5-[1-ethynylnaphthalenyl]-2′-deoxyuridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite (13). 5′-*O*-(4,4′-Dimethoxytrityl)-5-[1-ethynylnaphthalenyl]-2′-deoxyuridine **10** (820 mg, 1.25 mmol) was evaporated with freshly distilled tetrahydrofuran (2×5 mL). The resulting syrup was dissolved in freshly distilled tetrahydrofuran (5 mL) under argon and anhydrous *N*,*N*-diisopropylethylamine (484 mg, 3.75 mmol) was added to the solution under a weak flow of argon. The phosphitylating reagent, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (340 mg, 1.50 mmol), was added slowly via syringe, whereupon a white precipitate formed. The reaction mixture was stirred at room temperature for 2 h and diluted with CH_2Cl_2 (20 mL). Satd aqueous KCl solution (5 mL) was added with vigorous stirring via syringe under argon and the organic phase was dried over anhydrous Na_2SO_4 under argon for 2 h. The solution was then filtered and evaporated to dryness under reduced pressure. The residue was purified by flash chromatography (EtOAc/toluene, 1:1, containing 1% pyridine). Phosphoramidite monomer **13** was obtained as a light yellow solid (850 mg, 77%). R_f 0.33 (EtOAc/toluene, 1:1); ^{31}P NMR (121 MHz, DMSO-d_6) δ 148.86, 148.51; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.83 (s, 1H, *NH*-3), 8.25 (m, 2H), 7.95 (m, 2H), 7.56–7.03 (m, 12H), 6.80 (m, 4H), 6.17 (m, 1H, $CH^{1'}$), 4.54 ($CH^{3'}$), 4.10 (m, 1H, $CH^{4'}$), 3.73–3.45 (m, 12H, 2×OCH_3, OCH_2CH_2CN), 3.28 (m, 2H, $CH_2^{5'}$), 2.77 (t, J=5.7 Hz, 1H, $((CH_3)_2CH)_2$N), 2.65 (t, J=5.7 Hz, 1H, $((CH_3)_2CH)_2$N), 2.40 (m, 2H, $CH_2^{2'}$), 1.14–0.99 (m, 12H, $((CH_3)_2CH)_2$N); MS (ES$^+$) *m*/*z*: 903.1 [M+Na]$^+$; HR-ESMS calcd for $C_{51}H_{53}N_4NaO_8P$: 903.3493, found: 903.3477.

4.2. Oligonucleotide synthesis

Standard DNA phosphoramidites, solid supports and additional reagents were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser using a 1.0 μmol phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. Normal monomers (A, G, C and T) were allowed to couple for 25 s and all other monomers for an additional 300 s. Stepwise coupling efficiencies and overall yields of monomers with DMT protection were determined by measuring trityl cation conductivity and in all cases were >98.0%. Cleavage of the oligonucleotides from the solid support was carried out in concentrated aqueous ammonia in a sealed tube for 5 h at 55 °C.

4.2.1. Oligonucleotide purification. Purification of oligonucleotides was carried out by reversed-phase HPLC on a Gilson system using an ABI Aquapore column (C8), 8 mm× 250 mm, pore size 300 Å controlled by Gilson 7.12 software. The following protocol was used: run time 30 min, flow rate 3 mL min^{-1}, binary system: time in minutes (% buffer B); 0 (0); 3 (0); 5 (20); 22 (100); 25 (100); 27 (0); 30 (0). Elution buffer A: 0.1 M NH_4OAc, pH 7.0, buffer B: 0.1 M NH_4OAc with 50% acetonitrile pH 7.0 (modified oligomers), buffer B: 0.1 M NH_4OAc with 25% acetonitrile pH 7.0 (unmodified oligomers). Elution was monitored by ultraviolet absorption at 295 nm (unmodified oligomers) or 305 nm (oligomers containing 5-substituted deoxyuridine nucleosides). After HPLC purification oligonucleotides were desalted using disposable NAP 10 Sephadex columns (Pharmacia) using the manufacturer's instructions, aliquoted into Eppendorf tubes and stored at −20 °C in distilled deionised water. The integrity of oligonucleotides containing the

modified bases was verified by positive ion MALDI-TOF mass spectrometry on the following sequences: GTCTAT**X**-TATCG, X=**AeT**, found, 3814.1; calcd, 3814.6; X=**NeT**, found, 3764.6; calcd, 3764.6; CGTCTGAT**X**TGATCGC, X=**AeeT**, found, 5073.4; calcd, 5075.5. Data were obtained on a ThermoBioAnalysis Dynamo MALDI-TOF instrument in positive ion mode using delayed extraction and an initial accelerating voltage of 20 kV. Spectra were recorded from a matrix containing 4:1 3-hydroxypicolinic acid/picolinic acid in 1:1 acetonitrile/water in the presence of Dowex 50WX8-200 ion exchange beads according to the method of Langley et al.[27] All mass spectra were externally calibrated with T_{10}, T_{20} and T_{25}, and were within instrumental error (0.1%).

4.3. Thermal denaturation experiments

Fluorescent melting profiles were recorded using a Roche LightCycler® as previously described.[21] All duplexes were prepared in 10 mM sodium phosphate, 150 mM NaCl, pH 7.6, at a concentration of 0.5 μM. Melting and annealing curves were collected at a temperature gradient of 1 °C min^{-1} whilst monitoring fluorescence in Channel 1 of the LightCycler® (λ_{obsd}~520 nm). T_m values were determined from the first derivatives of the melting curves using Roche LightCycler® software. Each experiment was performed in triplicate, and the T_ms of replicates differed by less than 0.5 °C.

4.3.1. Steady state fluorescence measurements. Fluorescence spectra were recorded on LS-50B luminescence spectrophotometer (Perkin Elmer), with excitation and emission slit widths set to 5 nm, and a scan rate of 50 nm min^{-1}. Oligonucleotides and duplexes were dissolved in buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.6), to a concentration of 1 μM, except probes labelled with fluorescein, where concentrations of 0.1 μM were used.

Acknowledgements

We are grateful to the UK Engineering and Physical Sciences Research Council for funding this research. T.B. was a Royal Society Leverhulme Research Fellow for the duration of the project.

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