ARTICLE

OBC
www.rsc.org/obc

Synthesis of fluorophore and quencher monomers for use in Scorpion primers and nucleic acid structural probes

Catherine M. McKeen,[b] Lynda J. Brown,[b] Jamie T. G. Nicol,[a] John M. Mellor[a] and Tom Brown *[a]

[a] *Department of Chemistry, University of Southampton, Highfield, Southampton, UK SO17 1BJ. E-mail: tb2@soton.ac.uk*

[b] *Oswel Research Products Ltd., Eurogentec Group, Unit 2, Winchester Hill Commercial Park, Winchester Hill, Romsey, Hampshire, UK SO51 7UT*

Received 19th February 2003, Accepted 7th May 2003
First published as an Advance Article on the web 28th May 2003

We report the syntheses of monomers to incorporate fluorescein and methyl red within the sequence and at the termini of modified oligonucleotides. These monomers have been used in the solid-phase synthesis of fluorogenic oligonucleotides for genetic analysis and the study of multi-stranded nucleic acid structures.

Introduction

Fluorescence and fluorescence quenching form the basis of the majority of detection systems currently used in nucleic acid diagnostics,[1] such as Taqman,[2] Molecular Beacons,[3] and Scorpions.[4] In this field we are developing new synthetic methodologies to extend the utility of Scorpion primers and other fluorogenic detection systems. Scorpions technology is now well established in the detection of amplicon-specific PCR products by homogenous fluorescence detection in real-time.[4–7] A Scorpion primer consists of a probe sequence held in a hairpin loop conformation by a stem at its 5′and 3′ termini. A fluorophore is attached to the 5′-end of one arm of the stem, and a quencher to the 3′ end of the other arm. The hairpin loop is attached to the 5′-end of a PCR primer by a PCR stopper to prevent undesirable read-through of the probe by *Taq* DNA polymerase. The sequence of events during one PCR cycle is shown in Fig. 1. After denaturation and cooling, the Scorpion primer anneals to the target DNA and extension of the primer occurs. The Scorpion becomes an integral part of the amplicon, and on cooling the probe element hybridises to its complement within the same DNA strand. This opens the hairpin loop, the fluorophore and quencher are no longer in close proximity, and a large increase in fluorescence is observed. Scorpion technology is used in allelic discrimination[4,6] and in SNP genotyping.[8]

Several formats are possible: Fig. 2(i) depicts the "stem-loop" format[4] described above; and 2(ii) shows the recently developed "duplex" format[7] in which the probe element, with a fluorophore at its 5′-end, is annealed to a separate complementary oligonucleotide bearing a 3′-quencher. The mode of action is essentially the same as in the stem-loop format, but the duplex format has a number of advantages.[7] Figs 2(iv), (v) and (vi) show alternative "FRET duplex" formats (FRET = Fluorescence Resonance Energy Transfer) in which the Scorpions are labelled with two fluorophores, typically FAM and ROX. Excitation at the fluorescein wavelength (FRET donor)[9] results in energy transfer to the rhodamine dye (FRET acceptor). Finally, 2(iii) depicts the FRET stem-loop format. The use of FRET is necessary to allow the simultaneous observation of multiple probing events from a monochromatic excitation source on genetic analysis platforms such as the Roche LightCycler.[7]

Fluorescence quenching and energy transfer are also used to study the structural, thermodynamic and kinetic properties of higher order nucleic acid structures.[10–15] Such studies are currently attracting a great deal of attention due to the importance of multi-stranded nucleic acid structures in biology and medicine. Triple helices are particularly interesting in this context as intramolecular triplexes can form in genomic DNA under supercoiling and may have biological significance. In the therapeutic arena triplex forming oligonucleotides that stabilize DNA triple helices in a sequence-specific manner are under investigation as antigene and anti-cancer agents.[16] In order to evaluate potential new therapeutic agents, we have devised a fluorescence quenching method to measure the effects of novel

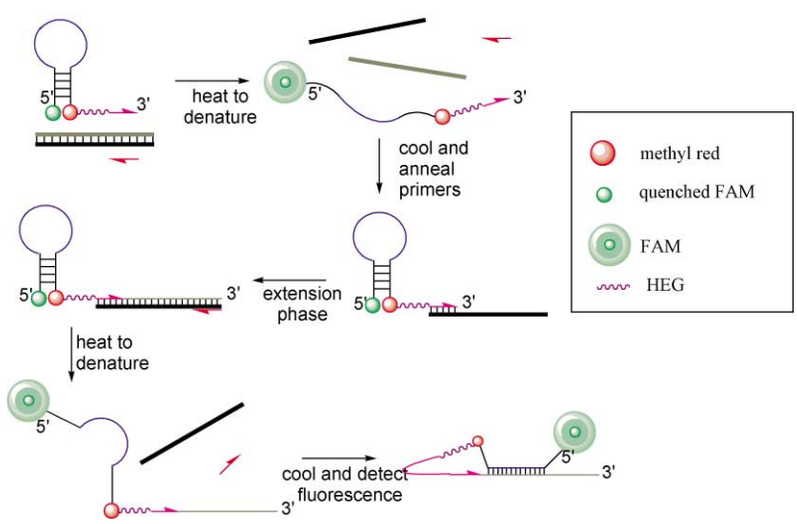


Fig. 1 Sequence of events during one cycle of PCR using a Scorpion Primer in real-time fluorescence detection.

DOI: 10.1039/b301859h

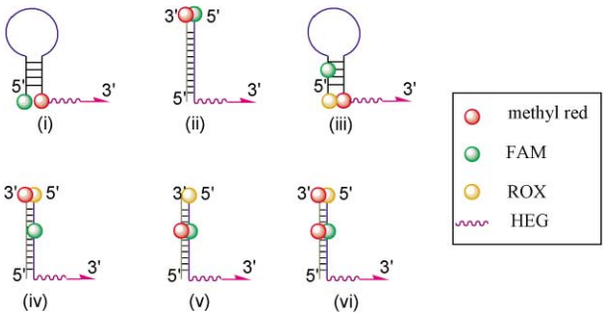


Fig. 2 Scorpion Primer formats: (i) stem-loop Scorpion, (ii) duplex Scorpion, (iii) FRET stem-loop Scorpion, (iv) FRET duplex Scorpion (quencher adjacent to ROX), (v) FRET duplex Scorpion (internal quencher adjacent to FAM), (vi) FRET duplex Scorpion (quenchers adjacent to FAM and ROX).

modified nucleosides[17] on triplex stability (Fig. 3). The technique, which depends upon the labeling of base pairs with fluorescein (the fluorophore) and methyl red (the quencher), enables us to absolutely differentiate the melting of the third strand from that of the underlying duplex. This is not possible by commonly used methods such as ultraviolet melting.

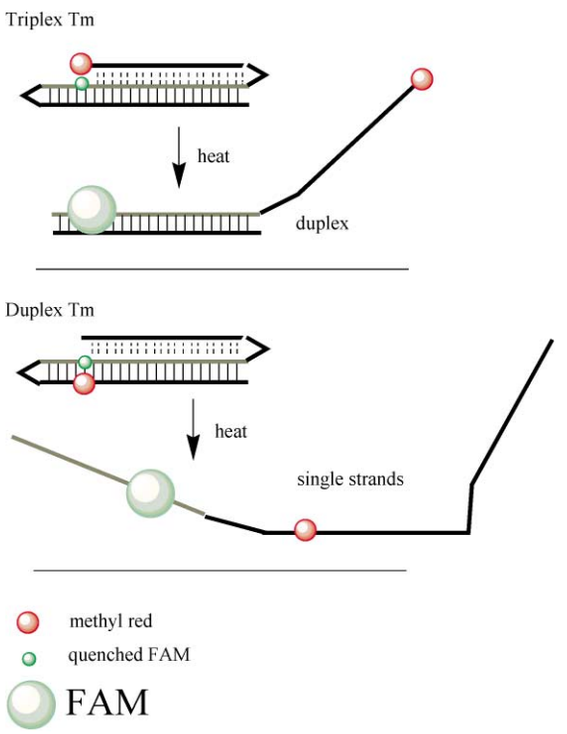


Fig. 3 The use of fluorescence to monitor the melting of an intramolecular triple helix: top; triplex to duplex, bottom; duplex to single stranded.

In order to synthesize oligonucleotides for the above applications we have developed a range of novel phosphoramidites. These monomers enable us to incorporate fluorophores and quenchers at the following loci; into A–T base pairs, on non-hybridizing sugar moieties as an integral part of PCR stoppers and linkers, and at the 3′- and 5′-termini of DNA strands. We now describe their syntheses.

Results and discussion

PCR stoppers and linkers

It is necessary to incorporate a non-nucleosidic linker between the primer and hairpin loop elements of Scorpion primers to prevent undesirable read-through of the probe by the DNA polymerase. A similar linker is also required to join the DNA strands of intramolecular triplexes. Hexaethylene glycol (HEG phosphoramidite, Fig. 4) is suitable for both purposes as it is hydrophilic and flexible and thus allows nucleic acid hybridization to occur. This monomer has been synthesized previously[18] and is readily prepared in two synthetic steps. A 2-fold excess of HEG is tritylated using 4,4′-dimethoxytrityl chloride (DMTCl) in pyridine, and the resulting DMT HEG is purified by silica gel column chromatography and converted to the monomer using 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite and DIPEA in dichloromethane. Synthetic details are not included in this paper as the phosphoramidite is now commercially available from Glen Research Inc. (www.Glenres.com).

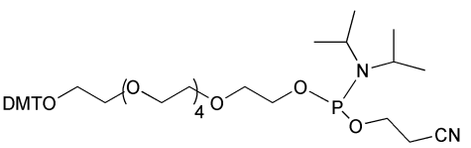


Fig. 4 HEG phosphoramidite (PCR blocker).

Octanediol can be used in place of HEG (phosphoramidite prepared in the same way), but two adjacent units must be incorporated in the oligonucleotide to allow efficient hybridization of linked DNA strands of intramolecular duplexes and triplexes. We have used octanediol rather than hexaethylene glycol as a linker in ^{1}H NMR studies on intramolecular triplexes because its spectrum does not overlap significantly with that of DNA.[19,20] If the application requires it, a quencher moiety can be placed on either side of the HEG or between the two units of octanediol during oligonucleotide synthesis.

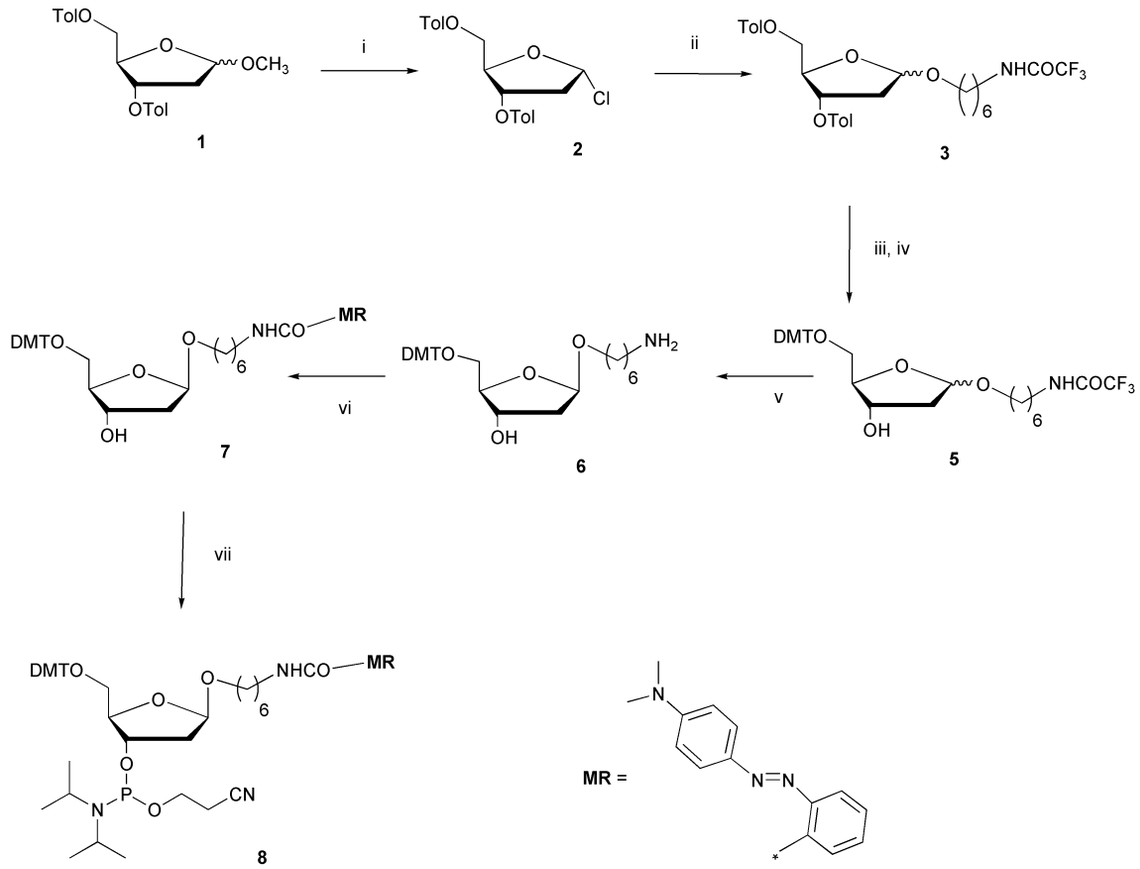
The methyl red quencher

A methyl red quencher can either be attached to a non-base pairing sugar moiety or to the adenine base of an A–T base pair. The two monomers have important uses and are not interchangeable.

The non-base pairing methyl red 2′-deoxy-β-D-ribose quencher

In this monomer the methyl red quencher is attached to the 1′-position of the sugar by means of a hexamethylene spacer. It lacks a DNA base so it cannot participate in base-pairing. It can be used to add a quencher to an oligonucleotide at the 5′-end or internally at the site of a linker or PCR stopper where base-pairing is not involved. We have used this monomer (compound **8**, Scheme 1) extensively in the synthesis of Scorpions and structural probes. It can also be used to add a quencher to the 3′-end of molecular beacons or Taqman probes by starting oligonucleotide synthesis with thymidine controlled pore glass (cpg) as the solid support, or by using a universal solid support. Alternatively compound **7** (Scheme 1) can be converted to its 3′-succinate and attached directly to cpg (not described in this paper).

The synthesis of the phosphoramidite **1** is shown in Scheme 1. The bis-toluoyl ester of 1′-*O*-methyl deoxyribose **1** was converted to the chloro-sugar **2** (α-anomer).[21] This was used to glycosylate 6-trifluoroacetamidohexan-1-ol, yielding **3** as an anomeric mixture. A small sample of the pure α-anomer was isolated by silica gel chromatography, crystallized and unambiguously characterized by X-ray crystallography.[22] Removal of the toluoyl protecting groups from **3** (anomeric mixture), followed by selective dimethoxytritylation at the primary alcohol gave **5** as a 1 : 1 mixture of anomers that were separated by silica gel column chromatography. From this point the synthesis was continued using the β-anomer only as it couples more efficiently in oligonucleotide synthesis than the corresponding α-anomer (99% compared to 96%). This is because the alkylamino side chain at the 1′-position and the



Scheme 1 *Reagents and conditions:* i, AcOH, AcCl, rt, 79%; ii, 6(trifluoroacetamido)-1-hexanol (0.9 eq.), DMAP (0.2 eq.), THF, rt, 3 h, 80%; iii, 0.5 M NaOMe–MeOH, rt, 1 h, 80%; iv, **4**, DMTCl (1.1 eq.), pyridine, rt, 1 h, 66% of a 1 : 1 α–β mixture; v, **5**, sodium hydroxide (5 eq.), MeOH : H_2O (4 : 1), rt, 15 h, 60%; vi, MR–COOH (1.2 eq.), Et_3N (2 eq.), HOBT (1.5 eq.), DCC (1.6 eq.), rt, 18 h, 88%; vii, 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine (1.1 eq.), DIPEA (4.6 eq.), THF, rt, 1 h, 74%.

phosphoramidite group on the 3′-position of the β-anomer are on opposite sides of the sugar ring, thus minimising steric hindrance during the phosphoramidite coupling reaction. In general we have found that commercially available labeling monomers based on acyclic 1,2- or 1,3-diols, and which lack the rigidity conferred by the sugar, couple relatively inefficiently during oligonucleotide synthesis. This was our major reason for utilising the β-ribofuranosyl backbone. The α-anomer of **5** is not discarded; it is a valuable intermediate in the preparation of various derivatised resins for oligonucleotide synthesis. The trifluoroacetyl protecting group was removed from **5β** to release the amine **6** which was coupled with the carboxylic acid methyl red using DCC and HOBT to give **7** in high yield. Finally, phosphitylation produced the desired methyl red labelled 2′-deoxy-D-ribofuranse phosphoramidite **8** suitable for use in DNA synthesis.

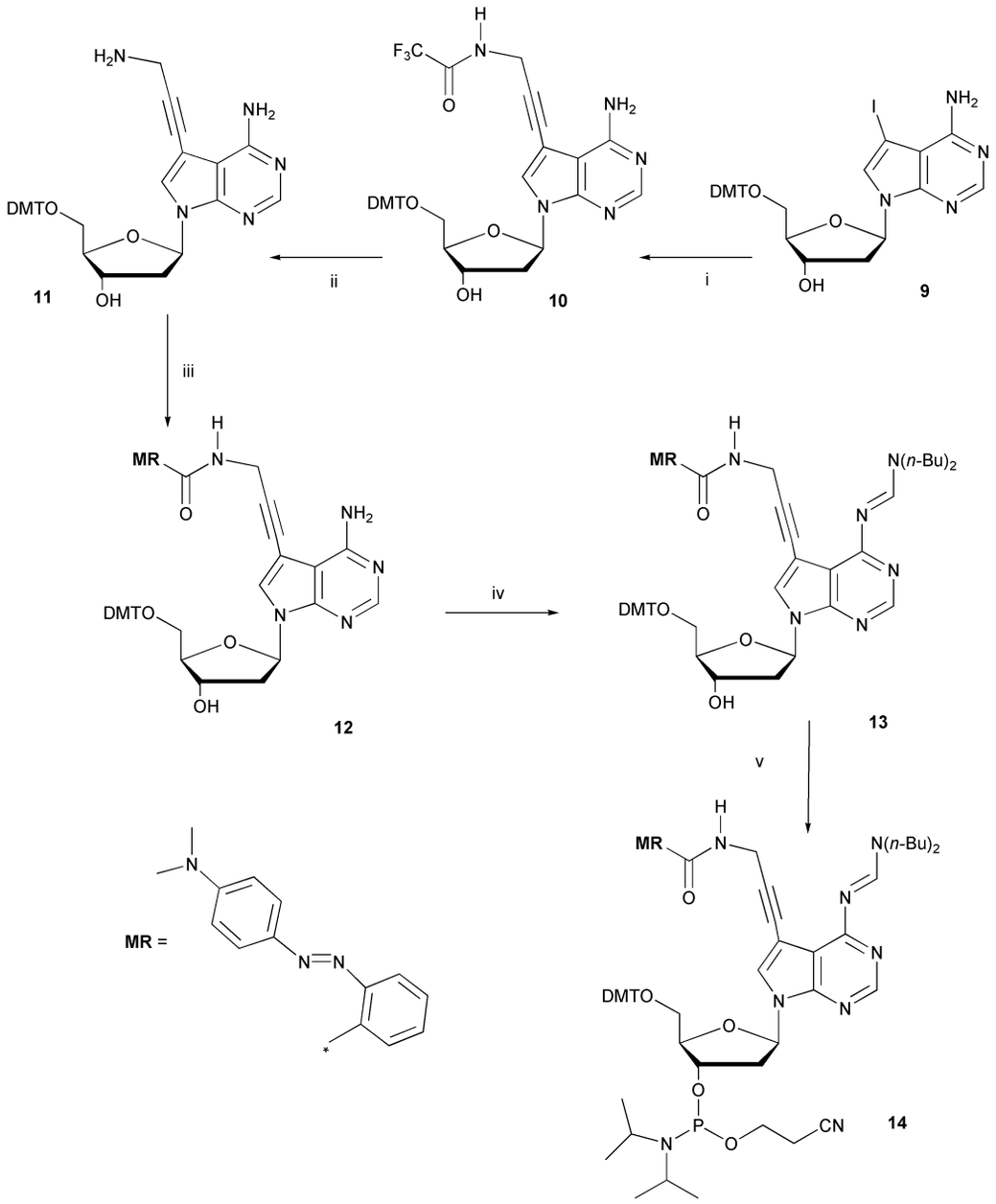
The methyl red 2′-deoxy-7-deazadenosine quencher

FRET Scorpion primers require efficient fluorescence quenching in the "closed" state (Fig. 2) and this can be achieved by placing the fluorophore and quencher moieties on the major groove side of the same base pair. Making these substitutions at an A.T base pair, and placing the quencher on adenine and the fluorophore on thymidine offered the most readily accessible target compounds. The 7-position is the only locus on adenine that can be substituted without inhibiting base pairing, so we decided to synthesize the 7-substituted-7-deazaadenosine monomer **14** (Scheme 2). Pyrrolo{2,3-*d*}pyrimidines have previously been used to prepare 7-deaza analogues of purine nucleosides,[23–25] and we used these published methods to make compound **9**. This was converted to **10** in high yield using standard Sonagashira conditions. The trifluoroacetyl-protected amine was deblocked with sodium hydroxide in aqueous methanol, and the resultant propargylamine derivative **11** was coupled with the carboxylic acid methyl red, with DCC–HOBT activation, to give **12** in 75% yield. Protection of the exocyclic amino group with di-*n*-butyl formamidine[26] gave **13** (76%) and phosphitylation of the 3′-hydroxyl function gave the phosphoramidite **14** in almost quantitative yield.

The fluorescein-labelled thymidine internal fluorophore

In order to prepare FRET Scorpion primers a method was required for the incorporation of the FAM fluorophore internally, within the stem sequence, on the thymine base of an A–T base pair (Fig. 2). The monomer of choice was a modified deoxyuridine with a fluorescein moiety linked to the 5-position of the heterocyclic base (compound **21** in Scheme 3). The rigid alkyne function, which is directly attached to the pyrimidine ring, places the fluorophore in the major groove when the DNA is hybridized. The alkynyl group is essential to prevent oligonucleotides that contain this base analogue from severely inhibiting PCR and the caproyl linker separates the fluorophore from the bases and DNA backbone to minimize collisional fluorescence quenching and steric inhibition of duplex formation. Studies on Scorpions have shown that neither of the base analogues used in this study inhibited base pairing.[7] In Scheme 3 linker **16** was prepared by protection of 6-aminocaproic acid with ethyl trifluoroacetate to give **15**, followed by reaction of **15** with propargylamine and EDC†. The alkynyl group of **16** was coupled to 5′-DMT-5-iodo-2′-deoxyuridine (**17**) under Sonogashira conditions to give the 5-substituted nucleoside **18**.[27] Removal of the amino-protecting group with sodium hydroxide afforded the free amine **19**, which was coupled, to 5-carboxyfluorescein dipivaloate[28,29] to give the FAM nucleo-

† EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.



Scheme 2 *Reagents and conditions:* i, N1-(2-propynyl)-2,2,2-trifluoroacetamide (5.4 eq.), Cu(I)I (0.36 eq.), Et_3N (18 eq.), $(Ph_3P)_4Pd$ (0.18 eq.), DMF, rt, 36 h, 97%; ii, NaOH (3 eq.), CH_3OH : H_2O (10 : 1), rt, 18 h, 87%; iii DCC (1.5 eq.), HOBT (1.5 eq.), MR–COOH (1.2 eq.), Et_3N (2 eq.), CH_2Cl_2, rt, 18 h, 88%; iv, di *n*-butylformamide dimethyl acetal (2 eq.), DMF, rt, 48 h, 76%; v, 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine (1.1 eq.), DIPEA (4 eq.), THF, rt, 1.25 h, 99%.

side **20**. The yield of **20** was limited by a side-reaction in which the free amine reacts with the pivaloyl ester of FAM, resulting in acylation of **19**. Phosphitylation of **20** produced the fully protected labelled nucleoside phosphoramidite **21**. The use of a single regioisomer of dipivaloyl fluorescein is desirable as isomeric mixtures lead to multiple oligonucleotide peaks on HPLC. The pivaloyl protecting groups of FAM are rapidly cleaved after DNA synthesis under standard oligonucleotide deprotection conditions.

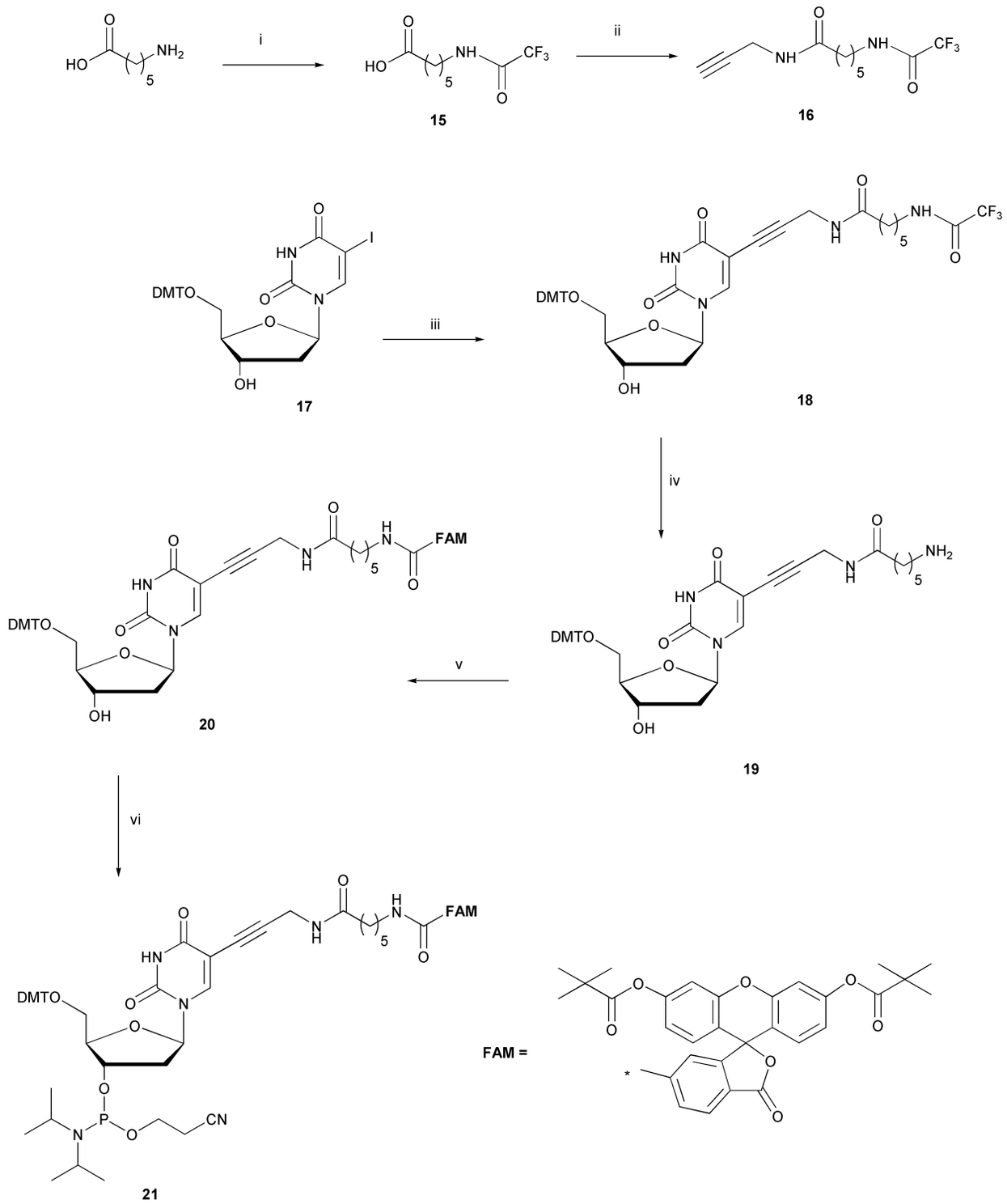
Conclusions

The phosphoramidites **8**, **14** and **21** described in this paper have all been successfully incorporated into oligonucleotides on an ABI 394 DNA synthesizer with high coupling efficiencies (>98%). Large numbers of DNA structural probes and Scorpion primers, in both"stem-loop" and "duplex forms", have been synthesized and used successfully in normal and FRET applications.[4–7] These phosphoramidites also have potential applications in the synthesis of modified Taqman probes, hybridization probes, Molecular Beacons and fluorogenic nucleic acid structure probes and work in these areas is currently in progress.[30,31]

Experimental

General

All reactions requiring anhydrous conditions were performed in oven-dried glassware under an atmosphere of argon. CH_2Cl_2, pyridine and Et_3N were distilled from calcium hydride, THF was freshly distilled from sodium wire and benzophenone, MeOH was distilled from Mg and I_2 and diisopropylethylamine (DIPEA) was distilled from KOH. Anhydrous DMF was purchased from Aldrich. Other reagents were purchased from Aldrich/Sigma or Lancaster Ltd. Proton NMR spectra were recorded at 300 MHz and carbon NMR spectra at 75.5 MHz on a Bruker AM 300 spectrometer. Mass Spectra were recorded on a Fisons VG platform instrument or Dynamo MALDI TOF. All oligonucleotides were synthesized on an Applied



Scheme 3 *Reagents and conditions:* i, ethyl trifluoroacetate (1.5 eq), Et_3N (5 eq.), CH_3OH, rt, 18 h, 95%; ii, 2-propynylamine (1.05 eq.), EDC (1.1 eq.), DMF, rt, 18 h, 89%; iii, Cu(I)I (0.25 eq.), Et_3N (5 eq.), $(Ph_3P)_4Pd$ (0.1 eq.), **16** (1.2 eq.), DMF, rt, 4 h, 89%; iv, NaOH (4.0 eq.), CH_3OH : H_2O (10 : 1), rt, 48 h, 79%; v, EDC (1.1 eq.), HOBT (1.1 eq.), 5-carboxyfluorescein dipivalate (1.1 eq.), pyridine, rt, 2 h, 55%; vi, 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine (1.2 eq.), DIPEA (2.0 eq.), THF, rt, 1 h, 93%.

Biosystems 394 solid phase DNA/RNA synthesizer on the 0.2 μmole scale using the standard cycle.[32] Stepwise coupling efficiencies were recorded by the automated trityl cation conductivity monitoring system. Standard DNA phosphoramidites, solid supports and additional reagents were purchased from Applied Biosystems Ltd except anhydrous CH_3CN wash solvent (Rathburn Ltd). All phosphoramidites were stored under argon and dissolved in anhydrous MeCN immediately prior to use. Oligonucleotides were cleaved from the solid support by dissolving in concentrated aqueous ammonia and heating in a sealed tube at 60 °C for 4 hours prior to HPLC purification.[33] Oligonucleotides that did not contain A, G and C monomers were not heated. The sequences of several Scorpions and DNA structure probes synthesized by the methods described in this paper have been published.[4,–7,30,31]

1′-chloro-2′-deoxy-3′,5′-ditoluoyl-D-ribose (2). This was prepared from (**1**) by the method of Rolland.[21]

1′-(6-Trifluoroacetamidohexyl)-3′,5′-ditoluoyl-2′-deoxy-α- and β-D-ribofuranose (3). 6-(Trifluoroacetamido)-1-hexanol (1.70 g, 8.00 mmol) was dissolved in anhydrous THF (20 ml)

and DMAP (0.22 g, 1.80 mmol) was added, followed by compound **2** (3.40 g, 8.70 mmol). The reaction was stirred for three hours at room temperature then the solvent was evaporated under reduced pressure and the resultant gum was co-evaporated three times with CH_2Cl_2. The residue was purified by flash silica gel column chromatography, eluting with CH_2Cl_2 : EtOAc (9 : 1), to give a colourless oil (3.60 g, 80%, 1 : 1, α : β mixture); R_f = 0.70, 0.60 (CH_2Cl_2 : EtOAc, 9 : 1); ^{1}H NMR ($CDCl_3$): 8.05–7.85 (m, 4H, CH Ar^{Tol}), 7.30–7.15 (m, 4H, CH Ar^{Tol}), 6.56 (s, 0.5H, NH α), 6.21 (s, 0.5H, NH β), 5.54–5.49 (m, 0.5H, $H^{1'}$ α), 5.35 (dt, J = 5.0, 8.8 Hz, 0.5H, $H^{1'}$ β), 5.28–5.18 (m, 1H, $H^{3'}$), 4.60–4.15 (m, 3H, $H^{4'}$, $H^{5'}$), 3.72–3.61 (m, 1H, CH_2O), 3.41–3.19 (m, 3H, CH_2O, CH_2N), 2.52–2.19 (m + 2s, 8H, $H^{2',2''}$, CH_3^{Tol}), 1.60–1.15 (m, 8H, CH_2); MS: ES^+ 566 $(M + H)^+$, HRMS: $C_{29}H_{34}NO_7F_3$: $(M + Na)^+$ requires 588.2179, found: 588.2185. A sample of the anomeric mixture (1.0 g) was subjected to silica gel column chromatography eluting with a gradient of 1 : 2 to 2 : 1 EtOAc : *n*-hexane and the anomers were carefully separated. The α-anomer crystallised after storage of the gum at room temperature for several months; mp 103 °C. An X-ray crystal structure confirmed its stereochemistry.[22]

1′-(6-Trifluoroacetamidohexyl)-2′-deoxy-α/β-D-ribofuranose (4). Compound **3** (3.55 g, 6.30 mmol) was dissolved in a freshly prepared 0.5 M solution of NaOMe in MeOH (50 ml). The reaction was stirred for 1 hour at room temperature then the solvent was removed under reduced pressure. The residue was pre-absorbed on silica and purified by elution with CH_2Cl_2 : MeOH (9 : 1) to yield the title compound as a white solid (1.7 g, 80%), α/β mixture; R_f = 0.40 (EtOAc); ^{1}H NMR ($CDCl_3$): 6.61 (s, 0.4H, NH), 6.42 (s, 0.6H, NH), 5.16–5.12 (m, 1H, $H^{3'}$α,β), 4.50–4.46 (m, 0.6H, $H^{1'}$β), 4.13–4.07 (m, 1H, OH), 4.03–3.97 (m, 0.4H, $H^{1'}$α), 3.72–3.49 (m, 3H, $H^{4'}$, CH_2O), 3.35–3.18 (m, 4H, $H^{5'}$, CH_2N), 2.39–1.88 (m, 2H, $H^{2',2''}$), 1.55–1.46 (m, 4H, CH_2), 1.33–1.27 (m, 4H, CH_2); MS: ES^+ 352.1 $(M + Na)^+$ $C_{13}H_{22}NO_5F_3$: $(M + K)^+$ requires: 368.1082 found: 368.1080.

5′-(4,4″-Dimethoxytrityl-1′-(6-trifluoroacetamidohexyl)-2′-deoxy-α and β-D-ribofuranose (5). Compound **4** (3.00 g, 9.10 mmol) was co-evaporated three times with anhydrous pyridine then dissolved in pyridine (30 ml). 4,4′-Dimethoxytrityl chloride (3.40 g, 10.00 mmol) was added and the reaction was stirred under argon for one hour at room temperature. Pyridine was removed by evaporation at high vacuum then the gum was dissolved in DCM (50 ml) and extracted with saturated aqueous $NaHCO_3$. The organic layer was then dried (Na_2SO_4) and evaporated to dryness. The residue was co-evaporated with toluene then dissolved in the minimum of DCM and purified by flash column chromatography on pre-equilibrated silica gel (hexane–EtOAc–Et_3N 1 : 1 : 0.05) eluting with hexane–EtOAc 1 : 1 to separate the anomers. This gave the products as pale yellow foams in 66% yield overall (α-anomer1.9 g, 33%, β-anomer 1.9 g, 33%); R_f α-anomer 0.34; β-anomer 0.11 in hexane–EtOAc 2 : 1; R_f α-anomer 0.39, β-anomer 0.22 in DCM–EtOAc 9 : 1. Only the β-anomer was used as a synthetic intermediate in the current study;^{1}H NMR ($CDCl_3$, β-anomer): 7.49–7.20 (m, 9H, CH Ar), 6.90–6.80 (m, 4H, CH Ar), 6.40 (br s, 1H, NH), 5.25–5.15 (m, 1H, $H^{1'}$), 4.60–4.30 (m, 1H, $H^{4'}$), 4.10–3.90 (m, 1H, $H^{3'}$), 3.50 (s, 6H, OCH_3), 3.80–3.20 (m, 6H, $H^{5'}$, CH_2O, CH_2N), 2.40–1.90 (m, 2H, $H^{2',2''}$), 1.65–1.50 (m, 4H, CH_2), 1.35–1.28 (m, 4H, CH_2); MS: ES^+: 654.0 $(M + Na)^+$ HRMS requires: 631.2757 found: 631.2783.

5′-(4,4″-Dimethoxytrityl-1′-(6-aminohexyl))-2′-deoxy-β-D-ribofuranose (6). The β-anomer of compound **5** (0.63 g, 1.00 mmol) was dissolved in a solution of NaOH (0.20 g, 5.00 mmol) in water (1.5 ml) and MeOH (6 ml). The mixture was stirred at room temperature for 15 h, then evaporated to dryness under reduced pressure and purified by flash silica gel column chromatography eluting with CH_2Cl_2 : MeOH : Et_3N (9 : 1 : 1) to yield the title product as a pale yellow foam (0.32 g, 60%); R_f = 0.22 (CH_2Cl_2 : MeOH, 9 : 1); ^{1}H NMR ($CDCl_3$): 7.40–7.10 (m, 9H, CH Ar), 6.80–6.70 (m, 4H, CH Ar), 5.10–5.00 (m, 1H, $H^{1'}$), 4.40–4.30 (m, 1H, $H^{3'}$), 3.90–3.80 (m, 1H, $H^{4'}$), 3.70 (s, 6H, OCH_3), 3.55–3.05 (m, 2H, CH_2O), 3.30–3.10 (m, 2H, $H^{5'}$), 2.50 (t, J = 6.0 Hz, 2H, CH_2N), 2.00–1.90 (br s, 3H, OH, NH_2), 2.15–1.85 (m, 2H, $H^{2',2''}$), 1.45–1.10 (m, 8H, CH_2); MS: ES^+ 536.3 $(M + H)^+$ HRMS M + Na^+ requires: 558.28316 found: 558.2837.

5′-*O*-(4,4′-Dimethoxytrityl)-1′-β-(6-amido(2-(4-*N*,*N*′-dimethylaminophenylazo)phenyl)hexyl)-2′-deoxy-D-ribofuranose (7). The amine **6** (1.02 g, 1.90 mmol) was dissolved in CH_2Cl_2 (5 ml) and anhydrous Et_3N (0.36 g, 0.50 ml, 3.6 mmol), methyl red (0.58 g, 2.16 mmol), HOBT (0.38 g, 2.81 mmol), DCC (0.60 g, 2.91 mmol) and a further volume of CH_2Cl_2 (2 ml) were added. The reaction was stirred under argon overnight at room temperature, then diluted with CH_2Cl_2 (50 ml), filtered, the filtrate washed with sat. $NaHCO_3$ (100 ml), dried (anhydrous Na_2SO_4) and the solvent removed under reduced pressure. The residue was purified by flash silica gel column chromatography eluting with CH_2Cl_2 : EtOAc : NEt_3 (6 : 5 : 0.5) and the resultant red foam was further purified on silica, eluting with CH_2Cl_2 : EtOAc (3 : 2) and dried over P_2O_5 (1.40 g, 88%); R_f = 0.71 (EtOAc); ^{1}H NMR ($CDCl_3$): 9.05–8.95 (m, 1H, CH Ar), 8.35–8.25 (m, 1H, CH Ar), 7.80–7.60 (m, 3H, CH Ar), 7.80–7.60 (m, 11H, CH Ar), 6.80–6.60 (m, 5H, CH Ar), 5.05–5.00 (m, 1H, $H^{1'}$), 3.92–3.85 (m, 1H, $H^{4'}$), 3.65–3.55 (m, 7H, OCH_3, $H^{3'}$), 3.55–3.02 (m, 6H, CH_2, $H^{5'}$), 3.00 (s, 6H, $N(CH_3)_2$), 2.15–1.85 (m, 2H, $H^{2',2''}$), 1.65–1.45 (m, 2H, CH_2), 1.40–1.10 (m, 6H, CH_2); MS ES^+: 809.0 $(M + Na)^+$, 787.0 $(M + H)^+$. HRMS requires: 786.399 found: 786.398.

5′-*O*-(4,4′-Dimethoxytrityl)-1′-β-(6-amido(2-(4-*N*,*N*′-dimethylaminophenylazo) phenyl)hexyl)-2′-deoxy-D-ribofuranose-3′-(2-cyanoethyl-*N*,*N*-(diisopropyl))phosphoramidite (8). Compound **7** (1.00 g, 1.27 mmol) was dissolved in anhydrous THF (6 ml) and *N*,*N*-diisopropylethylamine (0.75 g, 1.0 ml, 5.80 mmol) was added followed by 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.35 g, 0.35 ml, 1.48 mmol). After stirring at room temperature for 1 hour the reaction mixture was diluted with anhydrous CH_2Cl_2 (100 ml) and sat. KCl (100 ml) was added with vigorous stirring. The organic layer was removed by cannula and dried (anhydrous Na_2SO_4). Evaporation under reduced pressure gave an oily residue that was dissolved in anhydrous CH_2Cl_2 (5 ml) and precipitated from cold hexane at −78 °C. The precipitate was collected by filtration under argon, dissolved in anhydrous MeCN (6 ml), filtered, and the solvent removed on a rotary evaporator. The product was dried in a vacuum desiccator over P_2O_5 to yield the title compound as a red foam (0.93 g, 74%); R_f of diastereomers= 0.88, 0.85 (CH_2Cl_2 : EtOAc, 1 : 1); MS ES^+ 1009 $(M + H)^+$; The phosphoramidite (**8**) was used to prepare the oligonucleotide 8GAAAAAACTTGGATCC as a test sequence and coupling efficiencies >98.5% were observed; MS ES^-: requires: 5442.1 found: 5443.0.

4-Amino-5-(trifluoroacetamidoprop-2-ynyl)-7-(5′-(4,4′-dimethoxytrityl)-2′-deoxy-1′-β-D-ribofuranosyl)pyrrolo{2,3-*d*}pyrimidine (10). 4-Amino-5-iodo-7-(5′-(4,4′-dimethoxytrityl)-2′-deoxy-1′-β-D-ribofuranosyl)pyrrolo{2,3-*d*}pyrimidine (**9**),[23–25] (1.00 g, 1.47 mmol), $Pd(PPh_4)_3$ (0.30 g, 0.27 mmol) and copper(I) iodide (0.1 g, 0.53 mmol) were dissolved in anhydrous DMF (10 ml) and Et_3N (3.70 ml, 26.60 mmol) was added. *N*-Trifluoroacetyl 2-propynylamine (1.20 g, 7.98 mmol) was added and the mixture was stirred for 36 hours at room temperature. The reaction was diluted with Et_2O (150 ml), washed with water (50 ml), and the organic phase was dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo*. The residue was purified by flash silica gel

column chromatography eluting with CH_2Cl_2 : MeOH : Et_3N (94 : 4 : 2). The resulting solid was precipitated from hexane at room temperature and dried over P_2O_5 in a desiccator to afford an amorphous solid (1.00 g, 97%); mp: 130 °C; R_f 0.29 (CH_2Cl_2 : MeOH, 95 : 5 + 2% Et_3N); ^{1}H NMR ($CDCl_3$): 8.15 (s, 1H, CH^2), 7.38 (s, 1H, CH^6), 7.35–7.10 (m, 9H, CH Ar), 6.80–6.69 (m, 4H, CH Ar), 6.70–6.50 (m, 1H, $H^{1'}$), 5.65 (br s, 2H, NH_2), 4.60–4.48 (m, 1H, $H^{3'}$), 3.98–3.10 (m, 1H, $H^{4'}$), 3.27–3.23 (m, 2H, CH_2NH), 3.70 (s, 6H, OCH_3), 3.43–3.39 (m, 2H, $H^{5'}$), 2.50–2.30 (m, 2H, $H^{2',2''}$); MS ES$^+$: 702.0 (M + H)$^+$, 724.0 (M + Na)$^+$, 740.0 (M + K)$^+$; HRMS M + H$^+$ requires: 702.2534 found: 702.2682.

4-Amino-5-(prop-2-ynylamino)-7-(5′-(4,4′-dimethoxytrityl)-2′-deoxy-1′-β-D-ribofuranosyl)pyrrolo(2,3-*d*)pyrimidine (11). Compound **10** (1.21 g, 1.76 mmol) was dissolved in MeOH (10 ml) to which a solution of NaOH (0.21 g, 5.28 mmol) in H_2O (1 ml) was added dropwise, with stirring. The reaction was stirred overnight at room temperature, and then diluted with CH_2Cl_2 (80 ml) and washed with saturated brine (100 ml). The organic phase was dried (anhydrous Na_2SO_4), evaporated to dryness, and purified by flash silica gel column chromatography eluting with CH_2Cl_2 : MeOH : Et_3N (91.8 : 8 : 0.2) to give the product as a white foam which was dried *in vacuo* over P_2O_5 (0.92 g, 87%). R_f 0.29 (CH_2Cl_2 : MeOH, 9 : 1 + 4% $NH_4OH_{(conc.)}$); ^{1}H NMR ($CDCl_3$): 8.15 (s, 1H, CH^2), 7.38 (s, 1H, CH^6), 7.35–6.69 (m, 13H, CH Ar), 6.70–6.50 (m, 1H, $H^{1'}$), 5.60 (br s, 2H, NH_2), 4.52–4.42 (m, 1H, $H^{3'}$), 4.05–3.98 (m, 1H, $H^{4'}$), 3.70 (s, 6H, OCH_3), 3.56 (s, 2H, CH_2NH), 3.28–3.18 (m, 2H, $H^{5'}$), 2.52–2.35 (m, 2H, $H^{2',2''}$).

4-Amino-5-(2-(4-*N*,*N*′-dimethylaminophenylazo)phenyl)-amidoprop-2-ynyl)-7-(5′-(4,4′-dimethoxytrityl))-2′-deoxy-1′-β-D-ribofuranosyl)pyrrolo{2,3-*d*}pyrimidine (12). Compound **11** (0.96 g, 1.59 mmol) was placed in a flask with methyl red (0.51 g, 1.91 mmol), HOBT (0.32 g, 2.39 mmol) and DCC (0.49 g, 2.39 mmol). Anhydrous CH_2Cl_2 (12 ml) was added, followed by anhydrous Et_3N (0.44 ml, 3.18 mmol) and the reaction was stirred overnight at room temperature. The reaction mixture was then diluted with CH_2Cl_2 (80 ml), washed with saturated brine (80 ml) and dried (anhydrous Na_2SO_4). Evaporation to dryness *in vacuo* gave an orange foam that was purified by flash silica gel column chromatography eluting with CH_2Cl_2 : MeOH : NH_3 (92 : 4 : 4). The material was further purified by silica gel column chromatography, eluting with EtOAc. This afforded the desired compound as orange solid that was dried over P_2O_5 *in vacuo* (1.02 g, 88%); mp 126–129; R_f 0.61 (CH_2Cl_2 : MeOH, 9 : 1 + 4% $NH_4OH_{(conc.)}$); ^{1}H NMR ($CDCl_3$): 8.46–8.39 (m, 1H, CH Ar), 8.15 (s, 1H, H^2), 7.75 (d, J = 8.0 Hz, 2H, CH Ar), 7.50–7.35 (m, 3H, CH Ar), 7.35 (s, 1H, CH^6), 7.35–7.10 (m, 9H, CH Ar), 6.80–6.69 (m, 4H, CH Ar), 6.79–6.59 (m, 1H, $H^{1'}$), 6.30 (d, J = 8.0 Hz, 2H, CH Ar), 5.65 (br s, 2H, NH_2), 4.50–4.41 (m, 1H, $H^{3'}$), 4.46–4.40 (m, 2H, CH_2NH), 4.02–3.95 (m, 1H, $H^{4'}$), 3.66 (s, 6H, OCH_3), 3.40–3.18 (m, 2H, $H^{5'}$), 2.68 (s, 6H, Me_2N), 2.50–2.33 (m, 2H, $H^{2',2''}$); MS ES$^+$: 857.0 (M + H)$^+$, 879.0 (M + Na)$^+$, 895.0 (M + K)$^+$; HRMS (M + H)$^+$requires: 857.3770 found: 857.3762.

4-(*N*,*N*-di-*n*-butylformamidinyl)-5-(2-(4-*N*,*N*′-dimethylamino-phenylazo)phenyl)amidoprop-2-ynyl)-7-(5′-(4,4′-dimethoxy-trityl))-2′-deoxy -1′-β-D-ribofuranosyl)-pyrrolo{2,3-*d*}pyrimidine (13). Compound **12** (0.8 g, 0.93 mmol) was dissolved in anhydrous DMF (60 ml), *N*,*N*-di-*n*-butylformamide dimethylacetal (0.38 g, 0.44 ml 1.87 mmol) was added dropwise and the reaction was stirred at room temperature for 48 hours. The mixture was then diluted with Et_2O (250 ml) and washed with water (100 ml, ×3) and the organic layer dried (anhydrous Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was purified by flash silica gel column chromatography using pre-equilibrated silica (Et_3N : hexane, 99 : 1), eluting with a gradient of hexane to EtOAc. This afforded an orange solid that was dried *in vacuo* over P_2O_5 (0.71 g, 76%). R_f 0.50 (EtOAc); ^{1}H NMR ($CDCl_3$): 9.99 (br s, 1H, CONH), 8.52 (s, 1H, CH^2), 8.30 (s, 1H, CH^6), 8.32–8.28 (m, 1H, CH Ar), 7.80 (s, 0.5 H, CHN, *Z*–*E* pair), 7.75 (d, J = 8.0 Hz, 2H, CH Ar), 7.74 (s, 0.5 H, CHN, *Z*–*E* pair), 7.50–7.30 (m, 3H, CH Ar), 7.35–7.10 (m, 9H, CH Ar), 6.72–6.64 (m, 4H, CH Ar), 6.70–6.60 (m, 1H, $H^{1'}$), 6.07 (d, J = 8.0 Hz, 2H, CH Ar), 5.60 (br s, 1H, OH), 4.60–4.40 (m, 3H, $H^{3'}$, CH_2NH), 4.03–4.00 (m, 1H, $H^{4'}$), 3.62 (s, 6H, OCH_3), 3.56–3.07 (m, 4H, CH_2), 3.33–3.12 (m, 2H, $H^{5'}$), 2.50 (s, 6H, $N(CH_3)_2$), 2.40–2.32 (m, 2H, $H^{2',2''}$), 1.49–1.28 (m, 4H, CH_2), 1.17–1.00 (m, 4H, CH_2), 0.76 (t, J = 9.0 Hz, 3H, CH_3), 0.57 (t, J = 9.0 Hz, 3H, CH_3); MS ES$^+$: 996.0 (M + H)$^+$, 1018.0 (M + Na)$^+$ HRMS requires: (M + H) 996.5131 found: 996.5131.

4-(*N*,*N*-di-*n*-butylformamidinyl)-5-(2-(4-*N*,*N*′-dimethylamino-phenylazo)phenyl)amidoprop-2-ynyl)-7-(5′-(4,4′-dimethoxy-trityl))-3′-cyanoethoxy-*N*,*N*-(diisopropylamino)phosphinyl-2′-deoxy -1′-β-D-ribofuranosyl)pyrrolo(2,3-*d*)pyrimidine (14). Compound **13** (425 mg, 0.44 mmol) was dissolved in anhydrous THF (3.0 ml) and DIPEA (0.31 ml, 1.75 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.114 ml, 0.48 mmol) were added. The reaction was stirred at room temperature for 1 hour, after which a further portion of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.02 ml, 0.09 mmol) was added with further stirring (15 minutes). The mixture was diluted with CH_2Cl_2 (60 ml), sat. KCl (60 ml) was added with vigorous stirring and the organic layer was removed by cannula, dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo*. The residue was dissolved in anhydrous CH_2Cl_2 (2 ml) and precipitated by dropwise addition to stirred hexane at −78 °C. The precipitate was collected by filtration under argon, and co-evaporated with anhydrous CH_2Cl_2 to give a red foam which was dissolved in anhydrous MeCN (3 ml), filtered and then dried *in vacuo* over P_2O_5 for 5 hours. This afforded the title compound as a red foam (0.52 g, 99%). R_f 0.70, 0.60 (EtOAc : hexane, 1 : 2); ^{1}H NMR ($CDCl_3$): 9.99 (br s, 1H, CONH), 8.52 (s, 1H, CH^2), 8.30 (s, 1H, CH^6), 8.32–8.28 (m, 1H, CH Ar), 7.80 (s, 0.5 H, CHN, *Z*–*E* pair), 7.75 (d, J = 8.0 Hz, 2H, CH Ar), 7.74 (s, 0.5 H, CHN, *Z*–*E* pair), 7.50–7.30 (m, 3H, CH Ar), 7.35–7.10 (m, 9H, CH Ar), 6.72–6.65 (m, 4H, CH Ar), 6.70–6.60 (m, 1H, $H^{1'}$), 6.07 (d, J = 8.0 Hz, 2H, CH Ar), 5.60 (br s, 1H, OH), 4.60–4.40 (m, 3H, $H^{3'}$, CH_2NH), 4.03–4.00 (m, 1H, $H^{4'}$), 3.62 (s, 6H, OCH_3), 3.56–3.07 (m, 4H, CH_2), 3.45–3.30 (m, 2H, CH_2), 3.33–3.12 (m, 2H, $H^{5'}$), 2.50 (s, 6H, $N(CH_3)_2$), 2.40–2.32 (m, 2H, $H^{2',2''}$), 1.60–1.50 (m, 2H, CH_2), 1.49–1.28 (m, 4H, CH_2), 1.25–1.15 (m, 2H, CH_2), 1.17–1.10 (m, 16H, CH_3CH, CH_2), 0.76 (t, J = 9.0 Hz, 3H, CH_3), 0.57 (t, J = 9.0 Hz, 3H, CH_3); MS ES$^+$: 1197.0 (M + H)$^+$; The phosphoramidite (14) was used to prepare the oligonucleotide TTTTT(14)TTTTT as a test sequence. Coupling efficiencies >98.5% were observed; ES$^-$: calc. 3734.8 found: 3735.0.

6-(Trifluoroacetamido)caproic acid (15). 6-Aminocaproic acid (20 g, 0.15 mol) was suspended in anhydrous methanol (200 ml) and Et_3N (106 ml, 0.76 mol) was added. The mixture was stirred with gentle heating to aid dissolution (*nb*. the acid did not fully dissolve at this stage) and ethyl trifluoroacetate (32.5 g, 27.00 ml, 0.23 mol) was added dropwise to the stirring suspension. The reaction became clear after one hour and was stirred under argon overnight at room temperature. Evaporation under reduced pressure gave a yellow oil that was dissolved in EtOAc (250 ml), washed with 1 M HCl (250 ml, ×3), sat. KCl (250 ml) and then dried (anhydrous Na_2SO_4). Filtration and evaporation of the solvent under reduced pressure gave a pale yellow solid which was recrystallised from hot Et_2O, washed with cold hexane and dried *in vacuo* over P_2O_5 to give a white solid. (33 g, 95%); R_f = 0.37 (CH_2Cl_2 : MeOH, 9 : 1); ^{1}H NMR (DMSO-d6): 9.40 (s, 1H, NH), 3.45 (br s, 1H, OH), 3.16 (q, J = 6.6 Hz, 2H,

CH_2NH), 2.20 (t, J = 6.6 Hz, 2H, CH_2CO), 1.58–1.43 (m, 4H, CH_2), 1.32–1.20 (m, 2H, CH_2); MS ES$^+$: 129.9 (M − CF_3CO)$^+$.

6-(Trifluoroacetamido)caproic acid prop-2-ynylamide (16). Compound **15** (4.00 g, 17.70 mmol) was dissolved in anhydrous DMF (20 ml). To the stirred solution was added 2-propynylamine (1.02 g, 18.60 mmol) in DMF (10 ml) and EDC (3.73 g, 19.40 mmol). The reaction was stirred under argon overnight at room temperature after which the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (200 ml), washed with H_2O (200 ml), sat. $NaHCO_3$ (200 ml), brine (200 ml) and dried (anhydrous Na_2SO_4). Evaporation gave a creamy white foam which was recrystallised from a minimal amount of hot Et_2O to give white crystals which were dried *in vacuo* over P_2O_5 overnight (4.10 g, 89%); mp 68–70 °C; R_f = 0.60 (CH_2Cl_2 : MeOH, 9 : 1); ^{1}H NMR ($CDCl_3$): 7.42 (s, 1H, NH), 7.35 (s, 1H, NH), 4.05–3.96 (m, 2H, $CHCCH_2$), 4.01 (q, J = 7.3 Hz, 2H, CH_2NHCO), 2.25–2.18 (m, 3H, CH_2CO, CCH), 1.72–1.54 (m, 4H, CH_2), 1.42–1.30 (m, 2H, CH_2); MS ES$^+$: 265.0 (M + H)$^+$, 287.0 (M + Na)$^+$, 303.0 (M + K)$^+$ HRMS: $C_{11}H_{15}N_2O_2F_3$ (M + Na)$^+$ requires: 287.0978 found 287.0973.

5′-(4,4′-Dimethoxytrityl)-5-(6-(2,2,2-trifluoroacetamido)-caproamidoprop-2-ynyl)-2′-deoxyuridine (18). 6-(Trifluoroacetamido)caproic acid prop-2-ynylamide (2.42 g, 9.15 mmol) was dissolved in anhydrous DMF (30 ml) and anhydrous Et_3N (3.86 g, 5.30 ml, 38.00 mol) was added followed by Cu(I) iodide (0.36 g, 1.90 mmol). To the stirred yellow solution was added 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine[27] (5.00 g, 7.62 mmol) followed by $(Ph_3P)_4Pd$ (0.88 g, 0.76 mmol). The pale orange solution was stirred in the absence of light for 4 hours at room temperature then concentrated *in vacuo* to give a deep red oil which was dissolved in CH_2Cl_2 (200 ml), washed with aqueous 5% EDTA (200 ml), 5% $NaHCO_3$ (200 ml), sat. KCl (200 ml) and dried (anhydrous Na_2SO_4). Evaporation of the solvent gave an orange foam which was purified by flash silica gel column chromatography on pre-equilibrated silica gel (CH_2Cl_2 : Et_3N, 95 : 5) eluting with CH_2Cl_2 : MeOH (100 : 0 to 95 : 5 to 90 : 10). The title compound was obtained as a pale yellow foam (5.32 g, 89%); R_f = 0.31 (CH_2Cl_2 : MeOH, 9 : 1); ^{1}H NMR ($CDCl_3$): 9.70 (s, 1H, NH), 8.20 (s, 1H, H^6), 7.53–7.15 (m, 10H, CH Ar, NH), 6.85 (d, J = 8.8 Hz, 4H, CH Ar), 6.35–6.25 (m, 1H, H$^{1'}$), 6.07 (br s, 1H, NH), 4.55 (s, 1H, H$^{3'}$), 4.19–4.08 (m, 2H, $CCCH_2$), 3.90–3.80 (m, 1H, H$^{4'}$), 3.75 (s, 6H, OCH_3), 3.42–3.22 (m, 4H, H$^{5'}$, CH_2NHCO) 2.60–2.21 (m, 2H, H$^{2',2''}$), 2.00–1.90 (m, 2H, CH_2CO), 1.60–1.47 (m, 4H, CH_2), 1.33–1.20 (m, 2H, CH_2); MS: ES$^+$ 303.1 (DMT$^+$), 810.0 (M + NH_4)$^+$, 814.9 (M + Na)$^+$ HRMS: $C_{41}H_{43}N_4O_9F_3$ (M + Na)$^+$ requires: 815.2874, found: 815.2894.

5′-(4,4′-Dimethoxytrityl)-5-(6-aminocaproamidoprop-2-ynyl)-2′-deoxyuridine (19). Sodium hydroxide (1.20 g, 30 mmol) was dissolved in water (2 ml) with gentle heating and added to compound **18** (6.00 g, 7.56 mmol) in methanol (20 ml). The reaction was stirred at room temperature for 48 hours then concentrated *in vacuo* to give an oily solid. Silica gel (10 ml) and methanol (30 ml) were added and the mixture evaporated under reduced pressure to give a powder. This was loaded onto a column and purified by flash silica gel column chromatography eluting with CH_2Cl_2 : CH_3OH : Et_3N (90 : 5 : 5 then 80 : 10 : 10), to give the title product as a pale yellow foam (4.18 g, 79%); R_f = 0.31 (EtOAc : MeOH : $NH_4OH_{(conc.)}$, 9 : 1); ^{1}H NMR (DMSO-d6): 8.25 (s, 1H, H^6), 7.68 (s, 1H, NH), 7.45–7.18 (m, 9H, CH Ar), 6.95–6.81 (m, 4H, CH Ar), 6.25 (t, J = 6.6 Hz, 1H, H$^{1'}$), 4.25 (s, 1H, H$^{3'}$), 4.00–3.90 (m, 3H, $CCCH_2$, H$^{4'}$), 3.78 (s, 6H, OCH_3), 3.37–3.09 (m, 4H, H$^{5'}$, CH_2NH_2) 2.23–2.03 (m, 4H, H$^{2',2''}$, CH_2CO), 1.56–1.45 (m, 2H, CH_2), 1.43–1.17 (m, 4H, CH_2); MS ES$^+$: 697.6 (M + H)$^+$ HRMS: $C_{39}H_{45}N_4O_8$ (M + H)$^+$ requires: 697.32374 found: 697.32319.

5′-(4,4′-Dimethoxytrityl)-5-{6-(dipivaloyl-5-carboxyfluoresceinamido)caproamidoprop-2-ynyl}-2′-deoxyuridine (20). 5-Carboxyfluorescein dipivalate[28,29] (3.44 g, 6.32 mmol) and HOBT (0.85 g, 6.32 mmol) were dissolved in anhydrous pyridine (50 ml) and the solution was stirred at room temperature for 20 minutes. EDC (1.21 g, 6.32 mmol) was then added with more pyridine (20 ml) to aid dissolution, followed by compound **19** (4.00 g, 5.75 mmol). The reaction was stirred at room temperature under argon for 2 hours after which the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (200 ml), washed with sat. $NaHCO_3$ (200 ml) followed by sat. KCl (200 ml, ×2), then dried (anhydrous Na_2SO_4). The resultant pale yellow foam was purified by flash silica gel column chromatography, eluting with hexane–EtOAc (7: 3 to 1 : 1 to 4 : 6 to 2 : 8 to 0 : 1). The product was obtained as a creamy white foam that was dried *in vacuo* over P_2O_5 (3.8 g, 55%); R_f = 0.51 (EtOAc : MeOH : $NH_4OH_{(conc.)}$, 9 : 1); ^{1}H NMR ($CDCl_3$): 9.55 (s, 1H, NH), 8.10 (s, 1H, H^6), 8.05–7.95 (m, 2H, CH Ar), 7.48 (s, 1H, CH Ar), 7.38–7.17 (m, 10H, CH Ar, NH), 6.91 (s, 2H, CH Ar), 6.78–6.65 (m, 8H, CH Ar), 6.25–6.15 (m, 1H, H$^{1'}$), 7.78–5.83 (m, 1H, NH), 3.96–3.85 (m, 1H, H$^{3'}$), 3.78–3.70 (m, 3H, $CCCH_2$, H$^{4'}$), 3.68 (s, 6H, OCH_3), 3.35–3.18 (m, 4H, H$^{5'}$, CH_2NHCO), 2.48–2.12 (m, 2H, H$^{2',2''}$), 1.87–1.76 (m, 2H, CH_2CO), 1.50–1.30 (m, 6H, CH_2), 1.25 (s, 18H, CH_3); MS ES$^+$: 1245.7 (M + Na)$^+$.

5′-(4,4′-Dimethoxytrityl)-5-{6-(dipivaloyl-5-carboxyfluoresceinamido)caproamidoprop-2-ynyl}-2′-deoxyuridine-3′-(2-cyanoethyl-*N*,*N*-(diisopropyl)phosphoramidite (21). To a solution of compound **20** (3.80 g, 3.11 mmol) in anhydrous THF (20 ml) under argon gas was added *N*,*N*-diisopropylethylamine (1.1 ml, 0.80 g, 6.22 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.83 ml, 0.88 g, 3.73 mmol). After stirring at room temperature for 1 hour the solution was diluted with EtOAc (100 ml) washed with sat. KCl (100 ml), dried (anhydrous Na_2SO_4) and the solvent removed under reduced pressure under an atmosphere of argon. The resultant white foam was purified by flash silica gel column chromatography, under argon, eluting with EtOAc. After evaporation of the solvent and thorough drying in a vacuum desiccator, the compound was dissolved in anhydrous MeCN (10 ml), filtered, and the solvent was removed under reduced pressure. The title compound was obtained as a white foam (4.1 g, 93%); R_f = 0.67 (EtOAc) and was used to prepare the oligonucleotide CAGACTTGCA(21)TCCG as a test sequence. Coupling efficiencies >98.5% were obtained; MS/MALDI TOF: requires: 5038.5 found: 5038.0.

Acknowledgements

We thank BBSRC and JREI (UK Joint Research Equipment Initiative) for funding for the Roche LightCycler and EPSRC and the UK Teaching Company Directorate for providing funding for J. T. G. N.

References

1 I. G. Gut, *Hum. Mutat.*, 2001, **17**, 475.
2 K. J. Livak, S. A. J. Flood, J. Marmaro, W. Giusti and K. Deetz, *PCR Methods Appl.*, 1995, 357.
3 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1995, **14**, 303.
4 D. Whitcombe, J. Theaker, S. P. Guy, T. Brown and S. Little, *Nat. Biotechnol.*, 1999, **17**, 804.
5 D. Whitcombe, S. Kelly, J. Mann, J. Theaker, C. Jones and S. Little, *Amer. J. Human Genet.*, 1999, **65**, 2333.
6 N. Thelwell, T. Millington, A. Solinas, J. Booth and T. Brown, *Nucleic Acids Res.*, 2000, **28**, 3752.
7 A. Solinas, L. J. Brown, C. McKeen, J. M. Mellor, J. T. G. Nicol, N. Thelwell and T. Brown, *Nucleic Acids Res.*, 2001, **29**, e96.
8 L. Roberts, *Science*, 2000, **287**, 1898.
9 P. R. Selvin, *Methods Enzymol.*, 1995, **246**, 300.

10 J.-L. Mergny, L. Lacroix, M. P. Teulade-Fichou, C. Houndsou, L. Guittat, M. Hoarau, P. B. Arimondo, J.-P. Vigneron, J.-M. Lehn, J.-F. Riou, T. Garestier and C. Helene, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 3062.
11 J.-L. Mergny, T. Garestier, M. Rougee, A. V. Lebedev, M. Chassignol, N. T. Thuong and C. Helene, *Biochemistry*, 1994, **33**, 15321.
12 J. L. Mergny, S. A. Boutorine, T. Garestier, F. Belloc, M. Rougee, N. V. Bulychev, A. A. Koshkin, J. Bourson, A. V. Lebedev, B. Valeur, N. T. Thuong and C. Helene, *Nucleic Acids Res.*, 1994, **22**, 920.
13 C. Ellouse, F. Piot and M. Takahashi, *J. Biochem.*, 1997, **121**, 521.
14 M. Yang, S. S. Ghosh and D. P. Millar, *Biochemistry*, 1994, **33**, 15329.
15 M. Yang and D. P. Millar, *Methods Enzymol.*, 1997, **278**, 417.
16 K. R. Fox, *Curr. Med. Chem.*, 2000, **7**, 17.
17 M. Sollogoub, B. Dominguez, K. R. Fox and T. Brown, *Chem. Commun.*, 2000, 2315.
18 B. Sojka, P. A. E. Piunno, C. C. Wust and U. J. Krull, *Appl. Biochem. Biotechnol.*, 2000, **89**, 85.
19 J. P. Bartley, T. Brown and A. N. Lane, *Biochemistry*, 1997, **36**, 14502.
20 L. Haq, B. Z. Chowdhry, T. Brown and A. N. Lane, *Biophys. J.*, 1997, **72**, TH447.
21 V. Rolland, K. Mitsuhara and J. Lhomme, *Synth. Commun.*, 1997, **27**, 3505.
22 J. T. G. Nicol, PhD thesis, University of Southampton, 2002.
23 J. Davoll, *J. Chem. Soc.*, 1960, 131.
24 Z. Kazimierczuk, H. B. Cottam, G. R. Revanker and R. K. Robins, *J. Am. Chem. Soc.*, 1984, **106**, 6379.
25 C. A. Buhr, R. W. Wagner, D. Grant and B. C. Froehler, *Nucleic Acids Res.*, 1996, **24**, 2974.
26 B. C. Froehler and M. D. Matteucci, *Nucleic Acids Res.*, 1983, **11**, 8031.
27 A. Davison, G. Duckworth, M. Vaman-Rao, J. McClean, J. Grzybowski and T. Brown, *Nucleosides Nucleotides*, 1995, **14**, 1049.
28 G. V. Dreschsler and S. Smagin, *J. Prakt. Chem.*, 1965, **28**, 315.
29 F. M. Rossi and P. Y. Kao, *Bioconjugate Chem.*, 1997, **8**, 495.
30 R. A. J. Darby, M. Sollogoub, C. McKeen, L. J. Brown, A. Risitano, N. Brown, C. Barton, T. Brown and K. R. Fox, *Nucleic Acids Res.*, 2002, **30**, e39.
31 A. Solinas, N. Thelwell and T. Brown, *Chem. Commun.*, 2002, 2272.
32 T. Brown and J. Grzybowski, Preparation of synthetic oligodeoxynucleotide probes, in *Gene Probes 1*, ed. B. D. Hames and S. J. Higgins, Oxford University Press, 1995.
33 T. Brown and D. J. S. Brown, *Methods Enzymol.*, 1992, **211**, 20.