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Rapid and Efficient DNA Strand Cross-Linking by Click Chemistry

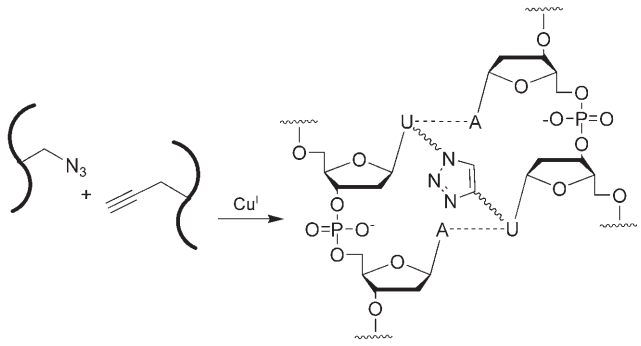
Petr Kočalka, Afaf H. El-Sagheer, and Tom Brown*[a]

Click chemistry has been used to covalently cross-link complementary DNA strands between bases to form very stable duplexes. Several alkyne- and azide-modified uracil monomers were used to evaluate the effect of the linkers on the efficiency of the click reaction. All cross-linked duplexes had much higher thermal stabilities than non-cross-linked ones, with increases in melting temperature of up to 30 °C. In some cases, the conversion was near-quantitative, and the reaction was complete in 5 min.

Introduction

The Huisgen [3+2] cycloaddition reaction has been known to chemists for many decades.[1–3] The copper-catalysed variant (CuAAC), the best example of click chemistry,[4] was recently discovered by Meldal[5] and Sharpless.[6,7] Cu^{I} catalysis increases the reaction rate dramatically and exclusively affords the 1,4-regioisomer of the 1,2,3-triazole. Reactions are usually very efficient at ambient temperature, are tolerant of other functional groups and require minimal work-up and purification.[8] Moreover the reaction can be performed in a variety of solvents, including water. For these reasons the CuAAC reaction has been widely exploited by synthetic and biological chemists. In the nucleic acids field it has been used to attach DNA to microarrays,[9] to couple oligonucleotides to self-assembled monolayers,[10] to immobilise oligonucleotides on glass substrates for microcontact printing,[11] for the preparation of protein–oligonucleotide conjugates,[12] for labelling oligonucleotides with various reporter groups,[13–21] for intrastrand cross-linking, cyclisation and catenation of DNA duplexes,[22,23] and for ligating single stranded oligonucleotides and PNA to peptides.[24]

Cross-linked DNA duplexes have applications in many fields including DNA repair,[25–30] gene regulation,[31] reversible control of DNA hybridisation[32] and as aptamers and decoys to sequester DNA-binding proteins.[33,34] In addition, they have potential in nanotechnology for the assembly of stable DNA nanoarrays.[35] Recently we have shown that the CuAAC reaction can be used to synthesise very stable cyclic DNA miniduplexes by forming triazole linkages between the sugar–phosphate backbones at the termini.[23] In this paper we demonstrate the use of click chemistry to cross-link complementary DNA strands internally through modified nucleobases (Scheme 1). The method described here is very efficient, simple and compatible with standard methods of oligonucleotide synthesis.



Scheme 1. Interstrand cross-linking between azide and alkyne-modified uracil bases in complementary oligonucleotides.

Results and Discussion

Synthesis of oligonucleotides containing alkyne or azide groups attached to deoxyuridine

The objective of this study was to investigate the utility of the CuAAC reaction for the cross-linking of two DNA strands between modified uracil bases (Scheme 1). For this purpose, a single deoxyuridine (dU) nucleoside modified with a terminal alkyne was incorporated into one strand, and an azide-modified dU was inserted in its complement. Two different 5-alkyne-modified deoxyuridines were evaluated. The first, 5-ethynyl-2′-deoxyuridine has the closest possible attachment of the alkyne to the nucleobase, and the other, 5-(octa-1,7-diynyl)-2′-deoxyuridine, has a flexible linker between the base and the terminal alkyne. The phosphoramidite building blocks **1** and **2**) were synthesised by published procedures[13,14,19,36] and introduced into the oligonucleotides during solid-phase synthesis. The coupling efficiencies of the alkynyl dU monomers during oligonucleotide assembly were greater than 98%.

The synthesis of oligonucleotides containing azide functionalities involves incorporation of amino-modified nucleosides **3a** and **4a** by using phosphoramidites **3c** and **4c**, respectively

[a] *Dr. P. Kočalka, Dr. A. H. El-Sagheer, Prof. T. Brown*
School of Chemistry, University of Southampton
Highfield, Southampton SO17 1BJ (UK)
Fax: (+44) 2380-592991
E-mail: tb2@soton.ac.uk

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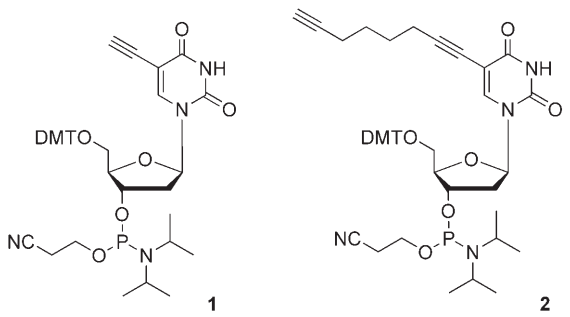
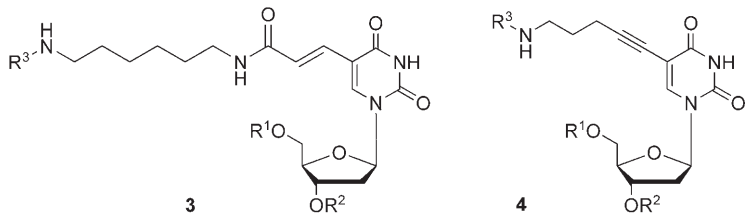
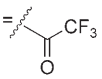
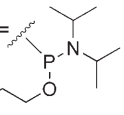
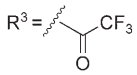


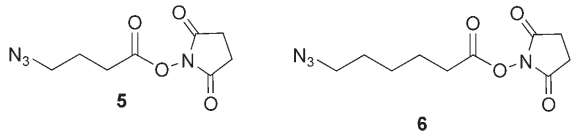


3a, **4a**: $R^1 = R^2 = R^3 = H$

3b, **4b**: R^1 = DMT, R^2 = H, R^3 = 

3c, **4c**: R^1 = DMT, R^2 =  R^3 = 

(Scheme 2) followed by postsynthetic derivatisation of the amines with the NHS ester of 4-azidobutyric acid (**5**)[22] or 6-azidohexanoic acid **6**.[37] This provided DNA strands containing



four different azides with varying linker lengths (10, 12, 15 and 17 atoms). This postsynthetic labelling procedure avoids exposure of the azide to the conditions of solid-phase oligonucleotide synthesis and deprotection, and gives good yields of the desired azide-labelled oligonucleotides **ON4**-**ON7** (Table 1).

RP-HPLC of the oligonucleotides and mass spectrometry showed complete derivatisation of the amino groups.

The CuAAC reaction between complementary DNA strands

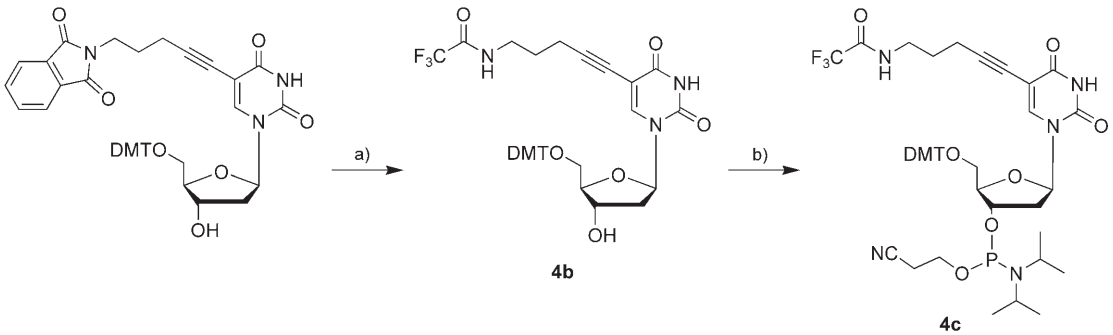
The above building blocks provide a useful toolkit to explore the effects of the various linkers on the efficiency of the CuAAC reaction between complementary DNA strands. Experiments were carried out to determine the nature and location of the alkyne–azide pairs necessary for efficient reaction. The relevant alkyne (**Y**) was inserted as the fifth nucleotide from the 5′-end of the 14-mer (**ON1** and **ON2**), or at the third position (**ON3**), and the azide (**Z**) was located four nucleotides from the 3′-end in **ON4**–**ON7**. Complementary pairs of alkyne- and azide-labelled oligonucleotides were studied as described below.

For the DNA interstrand cross-linking (reactions **R1**, **R2** and **R3** in Figure 1), the Cu^I catalyst was prepared in situ from Cu^{II} sulfate (200 equiv relative to DNA) and sodium ascorbate (10 equiv relative to Cu^{II} sulfate). The water-soluble *tris*-hydroxypropyltriazolylamine Cu^I-binding ligand[38] was used in sevenfold excess relative to Cu^{II} sulfate. All ligation reactions were carried out in 0.2 M aqueous NaCl to ensure

Table 1. List of oligonucleotides

Code	Oligonucleotide	Building Block	Label	Linker length[a]
ON1	3′-TGTCTTAAG**Y**ATAA-5′	**1**	–	**0**
ON2	3′-TGTCTTAAG**Y**ATAA-5′	**2**	–	**6**
ON3	3′-TGTCTTAAGTA**Y**AA-5′	**2**	–	**6**
ON4	5′-ACAGAATTCA**Z**ATT-3′	**3a**	**5**	**15**
ON5	5′-ACAGAATTCA**Z**ATT-3′	**3a**	**6**	**17**
ON6	5′-ACAGAATTCA**Z**ATT-3′	**4a**	**6**	**12**
ON7	5′-ACAGAATTCA**Z**ATT-3′	**4a**	**5**	**10**
ON8	3′-TGTCTTAAGTATAA-5′	–	–	–
ON9	5′-ACAGAATTCATATT-3′	–	–	–

Y = alkyne moiety, **Z** = azide moiety. [a] Calculated from the functional group to the 5-position of the uracil base.



Scheme 2. Synthesis of protected aminopentynyl dU phosphoramidite monomer. a) Hydrazine hydrate, EtOH, then CF_3COOEt, DMAP, THF; 38%. b) DCM, DIPEA, 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine; 60%.

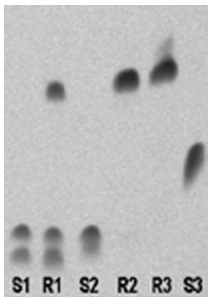


Figure 1. Denaturing 8% PAGE; S = mixture of alkyne and azide non-cross-linked oligonucleotide starting materials, R = reaction mixture: **R1** = **ON1** + **ON4**, **R2** = **ON2** + **ON4**, **R3** = **ON3** + **ON4.**

complete formation of the duplex, and a standard reaction time of 2 h was used. The reaction was quenched by eluting down a NAP-25 gel-filtration column, and the ligated DNA duplexes were purified by RP-HPLC. The efficiency of cross-linking was evaluated by denaturing 8% PAGE (Figure 1).

The click reaction **R1** between **ON1** and **ON4** (ethynyl dU + azide on the 15-atom side chain) was not efficient, probably due to steric hindrance at the alkyne. A similar observation was made by Carell et al. when using the CuAAC reaction for postsynthetic oligonucleotide labelling.[19] Reactions **R2** (**ON2** + **ON4**) and **R3** (**ON3** + **ON4**), that is, octadiynyl dU and azide with a 15 atom spacer, went smoothly to completion in 2 h. Thus, in these cases, the length of the linkers and the steric environment were suitable.

Next the effects of reducing quantities of reagents on the efficiency of the CuAAC reaction were determined. The successful reaction between **ON3** and **ON4** was repeated with significantly smaller excesses of reagents. In the new reaction (**R4** in Figures 2 and 3), 5 equiv of Cu^{II} sulfate, relative to oligonucleotide, were used at a concentration of 125 μM instead of the previous 200 equiv at 2 mM, keeping the same ratios of Cu^{I}/ascorbate/tris-hydroxypropyltriazolylamine ligand[38] as previously, that is, 1:10:7.

The reaction went smoothly to completion within 2 h (Figure 3) as did all other reactions **R5**–**R8** with these lower quantities of reagents (Figure 2). Neither the length of the azide linker (**ON4**–**ON7**) nor the position of octadiynyl group in the oligonucleotide (**ON2, ON3**) affected the yield or the rate of reaction. A space-filling model of one of these products (**R8**) is shown in Supporting Information.

This shows that cross-linking of these complementary DNA strands requires no more than five equivalents of Cu^{I} and is not sensitive to linker length in the range between 16 and 23 atoms. The presence of the linker in the major groove of the cross-linked duplex has no significant effect on DNA conformation, and the CD spectrum of the double-stranded products confirmed the presence of helically stacked nucleotides, consistent with the B-family of conformations (Figure 4 and Supporting Information).

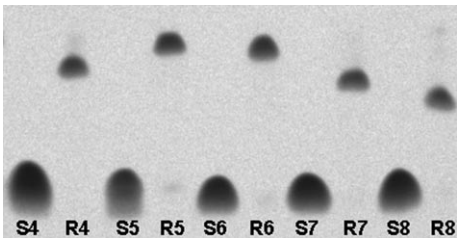


Figure 2. Denaturing 8% PAGE; S = mixture of non-cross-linked alkyne and azide oligonucleotides, 0.5 OD, R = reaction mixture, 0.2 OD: **R4** = **ON3** + **ON4**, **R5** = **ON2** + **ON5**, **R6** = **ON2** + **ON6**, **R7** = **ON3** + **ON6**, **R8** = **ON3** + **ON7.**

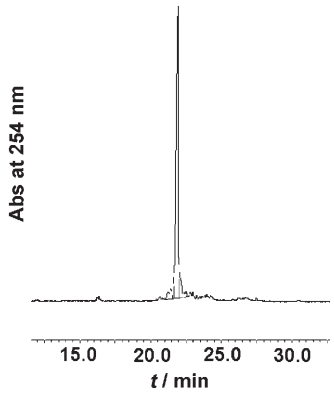


Figure 3. Capillary electrophoresis of reaction mixture **R4** (**ON3** + **ON4**) after 2 h.

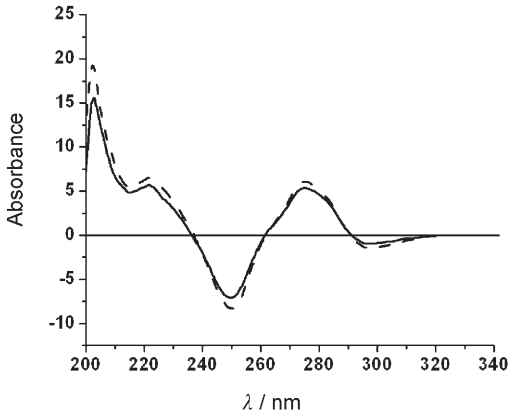


Figure 4. CD spectra of cross-linked (-----) and non-cross-linked (——) DNA duplex (reaction **R3** = **ON3** + **ON4**).

The above reactions were carried out for 2 h. Short reaction times have obvious advantages, so the minimum time required for completion of the CuAAC reaction between complementary DNA strands was investigated. Reactions were performed under an argon atmosphere in capped vials from which samples were collected at specific times, desalted and analysed by MALDI-TOF-MS, capillary electrophoresis and denaturing 8% PAGE. Analysis of the results for **ON2** + **ON6** = **R6** indicated that the reaction was essentially complete in 5 min (gel-electrophoresis in Figure 5 and mass spectrum in the Supporting Information). The same results were also obtained for **R4** (**ON3** + **ON4**) and **R5** (**ON2** + **ON5**; data not shown). These transformations are remarkably fast and illustrate the power of the CuAAC reaction.

The cross-linked DNA duplexes from reactions **R4**–**R8** were purified by RP-HPLC, characterised by mass spectrometry and analysed by both PAGE (denaturing 8%) and capillary electrophoresis (CE). Duplex stability was then measured by UV melting and confirmed by fluorescence melting to determine the effects of the various triazole cross-links. Importantly all cross-

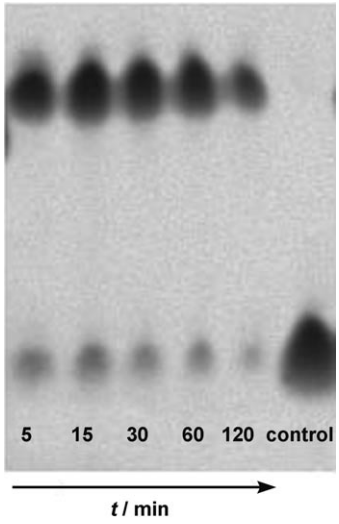


Figure 5. Monitoring of the click reaction between oligonucleotides **ON2** and **ON6** on denaturing 8% PAGE. Control is mixture of **ON2** and **ON6** without Cu^{I}.

linked duplexes had essentially the same ultraviolet melting temperatures at 0.25 and 4.0 μM concentrations, in contrast to the non-cross-linked duplexes, thus confirming the intramolecular nature of the former (Table 2). Close contact between complementary DNA strands favours base-pair formation relative to interactions between the unpaired bases and their surrounding water molecules, and these studies confirm the expectation that interstrand cross-linking leads to a significant increase in duplex stability. The magnitude of the increase (in some cases $>30\,^{\circ}C$) varies slightly according to the nature of the linker and position of the alkyne in the oligonucleotide. Locating the octadiynyl group at position 3 close to the 5′-end of the duplex at a TpA step (**ON3**+**ON4**, **ON3**+**ON6**, that is, **R4**, **R7**) yields slightly more stable DNA duplexes than when it is at position 5 (ApT step, **ON2**+**ON4**, **ON2**+**ON6**, that is, **R2**, **R6**). Clearly an alkyne at position 3 is located in a less-stable A·T-rich region of the duplex than one in position 5, as it is closer to the end where fraying occurs. Adding stability to this region by covalent cross-linking has a particularly large effect on duplex stability. There is also a difference in the base-stacking environment of the alkyne in the two cases (A**Y**A at position 3 vs. A**Y**G at position 5).

Table 2. Characterisation of duplexes.

Oligonucleotides	Fluorescence melting temperatures [°C] T_m	ΔT_m	UV melting temperatures [°C] [DNA duplex] 0.25 μM T_m	ΔT_m	[DNA duplex] 4.0 μM T_m	ΔT_m
ON8/**ON9**	53.0	–	41.2	–	46.5	–
ON1/**ON4**	n/a	–	n/a	–	n/a	–
ON1/**ON4**^{CL}	n/a	–	n/a	–	68.6	22.1
ON2/**ON4**	51.8	−1.2	n/a	–	43.9	−2.6
ON2/**ON4**^{CL}	70.8	17.8	66.3	25.1	67.7	21.2
ON3/**ON4**	53.3	0.3	n/a	–	45.7	−0.8
ON3/**ON4**^{CL}	77.2	24.2	70.9	29.7	71.7	25.2
ON2/**ON5**	52.7	−0.3	38.6	−2.6	44.2	−2.3
ON2/**ON5**^{CL}	74.2	21.2	66.7	25.5	67.6	21.1
ON2/**ON6**	54.7	1.7	39.9	−1.3	45.6	−0.9
ON2/**ON6**^{CL}	75.1	22.1	67.8	26.6	68.3	21.8
ON3/**ON6**	54.1	1.1	41.0	−0.2	45.7	−0.8
ON3/**ON6**^{CL}	78.2	25.2	72.7	31.5	72.3	25.8
ON3/**ON7**	54.2	1.2	41.1	−0.1	45.4	−1.1
ON3/**ON7**^{CL}	79.0	26.0	>72	>30	>72	>25

^{CL}: cross-linked DNA duplexes. Details of procedures in the Experimental Section. ΔT_m columns show the changes in T_m values per modification compared with the reference duplex **ON8**/**ON9**.

Conclusions

Oligonucleotides have been synthesised with ethynyl and octadiynyl groups attached to uracil bases and cross-linked to complementary azide-labelled oligonucleotide strands to yield stable DNA duplexes. Octadiynyl dU, in contrast to ethynyl dU, gave near-quantitative conversion to the cross-linked triazole products in two different base-stacking environments. In all cases, the cross-linked duplexes were thermally very stable and displayed conformational properties typical of B-DNA. The CuAAC reaction is a powerful method for building covalently cross-linked DNA constructs and could be a useful tool in nanotechnology and in biological applications.

Experimental Section

General experimental: Unless stated otherwise, all reagents obtained from commercial suppliers were used without further purification. The following solvents were purified by distillation: dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIPEA), Et_3N and pyridine (over calcium hydride). All reactions were carried out under an argon atmosphere in oven-dried glassware with purified and distilled solvents. Disposable Sephadex G25 NAP columns were purchased from GE Healthcare. Water-soluble *tris*-hydroxypropyltriazolylamine Cu^{I}-binding ligand was synthesised according to the method reported ref. [38]. Column chromatography was carried out under argon pressure by using Fisher Scientific DAVISIL 60A (35–70 micron) silica. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AV300 or a Bruker DPX400 spectrometer. Chemical shifts are given in ppm relative to tetramethylsilane; *J* values are given in Hz and are correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined by using the DEPT spectral-editing technique. ^{31}P NMR spectra were recorded on a Bruker AV300 spectrometer at 121 MHz and were externally referenced to 85% phosphoric acid in deuterated water. Low-resolution mass spectra were recorded by using the electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in acetonitrile (HPLC grade). High-resolution mass spectra were recorded in acetonitrile, methanol or water (HPLC grade) by using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded by using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive-ion mode with oligonucleotide $d(T)_n$ standards.[39] Thin-layer chromatography (TLC) was carried out on aluminium-backed silica-gel plates (60 F_{254}, 0.2 mm, Merck) with the following solvent systems: A) ethyl acetate/methanol/ammonia (5:1:1) for nucleosides; B) chloroform/ethanol (9:1) for compounds containing the DMT group. Products were visualised on TLC by UV absorption

at 264 nm, by staining with anisaldehyde or potassium permanganate solution, or, for compounds containing a DMT group, by exposure to 10% sulfuric acid in ethanol.

5′-*O*-(4,4′-Dimethoxytrityl)-5-(5′′-trifluoroacetamido-1′′-pentynyl)-2′-deoxyuridine (4b): Hydrazine monohydrate (73 μL, 1.5 mmol) was added to a solution of 5′-O-(4,4′-dimethoxytrityl)-5-(5′′-phthalimido-1′′-pentynyl)-2′-deoxyuridine[40] (519 mg, 0.7 mmol) in a mixture of ethanol (6 mL) and water (3 mL), and the solution was stirred at room temperature overnight. Water (6 mL) was then added, and the suspension was washed with DCM (3×15 mL). The organic layers were combined and concentrated in vacuo, and the crude product was purified by silica-gel flash column chromatography (pre-equilibrated with 1% Et_3N in DCM), eluting with methanol in DCM (0–30%+1% Et_3N). This yielded the amine intermediate (200 mg, 0.33 mmol), which was dissolved in THF (5 mL). DMAP (40 mg, 0.3 mmol) and ethyl trifluoroacetate (0.66 mmol) were added to the solution, and the reaction mixture was stirred overnight at room temperature, then concentrated and purified by silica-gel flash column chromatography (pre-equilibrated with 1% Et_3N in chloroform) eluting with ethanol in chloroform (0–10%). This yielded the product **4b** as a foam (188 mg, 38% over 2 steps). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=11.60 (s, 1H; NH), 9.39 (t, J=5.3 Hz, 1H; *NH*COCF$_3$), 7.87 (s, 1H; H-6), 7.42–7.20 (m, 9H; DMT), 6.89–6.87 (m, 4H; DMT), 6.13 (t, J=6.6 Hz, 1H; H-1′), 5.32–5.31 (d, J=4.4 Hz, 1H; 3′-OH), 4.29 (m, 1H; H-3′), 3.92 (m, 1H; H-4′), 3.74 (s, 6H; OCH$_3$), 3.26–3.10 (m, 4H; H-5′+H-5′′), 2.29–2.17 (m, 4H; H-3′′+H-2′), 1.57–1.50 (m, 2H; H-4′′); ^{13}C NMR (100.6 MHz, [D$_6$]DMSO): δ=162.15 (uracil C-4), 158.55 (uracil C-2), 156.69 (*C*OCF$_3$), 149.81, 145.22 (DMT), 142.64 (uracil C-6), 136.03, 135.81, 130.14, 130.11, 128.31, 128.05, 127.08 (DMT), 116.38 (CO*C*F$_3$), 113.65 (DMT), 99.60 (uracil C-5), 92.76 (C-2′′), 86.32 (C-4′), 85.26 (C-1′), 73.15 (C-1′′), 70.90 (C-3′), 64.14 (C-5′), 55.46 (O*C*H$_3$), 40.44, 38.92 (C-2′, C-5′′), 27.75 (C-4′′), 16.83 (C-3′′); LRMS (ES$^+$, MeCN) *m/z* 730 [*M*+Na]$^+$; HRMS calcd for $C_{37}H_{36}F_3N_3NaO_8$: 730.2347 [*M*+Na]$^+$, found 730.2343.

3′-*O*-(2-cyanoethoxy-*N*,*N*-diisopropylaminophosphinyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-(5′′-trifluoroacetamido-1′′-pentynyl)-2′-deoxyuridine (4c): Compound **4b** (520 mg, 0.74 mmol) was dried in an evacuated heating pistol over KOH at 40 °C overnight then dissolved in DCM (7 mL) under argon. DIPEA (645 μL, 3.7 mmol) and 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (198 μL, 0.9 mmol) were added, and the reaction mixture was stirred for 1 h under argon, diluted with DCM (15 mL) and washed with saturated aqueous KCl (20 mL). The organic layer was separated and dried over Na_2SO_4, and the solvent was removed in vacuo to give an oil. This was purified by silica-gel column chromatography (pre-equilibrated with 1% Et_3N in chloroform) by using a gradient of ethylacetate in chloroform (0–100%, *v/v*) to afford the product as a white form, which was directly dissolved in DCM (10 mL) and precipitated from cold hexane (200 mL) to afford **4c** as a white solid (400 mg, 60%). ^{31}P NMR (121.6 MHz, CDCl$_3$): δ=148.8, 148.4; LRMS (ES$^+$, MeCN) *m/z* 930 [*M*+Na]$^+$.

Oligonucleotide synthesis: Standard DNA phosphoramidites, solid supports and additional reagents including the C7-aminoalkyl CPG were purchased from Link Technologies or Applied Biosystems. All oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser by using a standard 1.0 μmol phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling times were 25 s for normal (A,G,C,T) monomers and 10 min for all alkyne and amino phosphoramidites. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. MALDI-TOF-MS [*M*−H]$^+$ gave following results (calcd/found): **ON1** (4287/4289), **ON2** (4367/4369), **ON3** (4367/4369), **ON4** (4511/4513), **ON5** (4539/4541), **ON6** (4454/4454), **ON7** (4426/4428), **ON8** (4277/4279), **ON9** (4246/4249).[39]

Azide labelling: NHS esters of 4-azidobutyrate (**5**; 2 mg) or 6-azidohexanoate (**6**; 2 μL) were added postsynthetically to 1.0 μmol syntheses of the amino-modified oligonucleotides in DMSO:0.5 M Na_2CO_3/$NaHCO_3$ buffer (1:2, 120 μL, pH 8.75) for 4 h at room temperature. The fully labelled oligonucleotides were desalted on NAP-25 Sephadex columns (GE Healthcare) and purified by RP-HPLC prior to cross-linking.

General method for interstrand cross-linking of DNA duplexes: Reactions R1, R2 and R3 (Figure 1): The solution containing alkyne oligonucleotide (25 nmol) and azide oligonucleotide (25 nmol) in the appropriate amount of aqueous NaCl (0.2 M) was heated at 80 °C for 5 min, then cooled down slowly to 20 °C, degassed using argon for 5 min and added to the degassed solution of tris-hydroxypropyltriazolylamine ligand (35 μmol), sodium ascorbate (50 μmol) and $CuSO_4$·5H$_2$O (5 μmol). The total volume of the reaction mixture was 2.5 mL; the concentrations of aqueous stock solutions were 100 μg μL^{-1} sodium ascorbate and 25 μg μL^{-1} $CuSO_4$·5H$_2$O. The reaction mixture was kept under argon at room temperature for 2 h, and a disposable NAP-25 gel-filtration column was used to remove reagents. The cross-linked DNA duplexes were purified by RP-HPLC, as described below.

Reactions R4 to R8 (Figure 2): The same procedure was applied with tris-hydroxypropyltriazolylamine ligand (875 nmol), sodium ascorbate (1.25 μmol) and $CuSO_4$·5H$_2$O (125 nmol). The total volume of the reaction mixture was 1.0 mL.

Monitoring cross-linking reaction: The solution containing the alkyne oligonucleotide (25 nmol) and azide oligonucleotide (25 nmol) in the appropriate amount of aqueous NaCl (0.2 M) was heated at 80 °C for 5 min, then cooled down slowly to 20 °C, purged with argon for 5 min and added to a degassed solution of tris-hydroxypropyl triazolylamine ligand (875 nmol), sodium ascorbate (1.25 μmol) and $CuSO_4$·5H$_2$O (125 nmol). The total volume of the reaction mixture was 1.0 mL. This was kept under argon at room temperature, and after 5, 15, 30, 60 and 120 min, aliquots were collected. Each was loaded directly onto a disposable NAP-10 gel-filtration column and analysed by denaturing 8% PAGE, MALDI-TOF MS and CE.

RP-HPLC analysis and purification: The oligonucleotides and cross-linked DNA duplexes were analysed and purified on a Gilson HPLC system by using a Brownlee Aquapore RP-HPLC column (8 mm×25 cm, Perkin–Elmer). The HPLC system was controlled by Gilson 7.12 software, and the following protocol was used: run time, 24 min; integration time, 21 min; flow rate, 4 mL per min; binary system. Gradient: time in minutes (% buffer B): 0 (0), 3 (0), 4 (10), 17 (40), 19 (100), 20 (100), 21 (0), 24 (0). Elution buffers: A) 0.1 M aqueous NH_4OAc, pH 7; B) 0.1 M aqueous NH_4OAc with 50% acetonitrile, pH 7. The elution of the oligonucleotides was monitored by ultraviolet absorption at 270 nm (analytical) and 297 nm (preparative). Isolated yields of reactions involving alkyne **2** were greater than 50%.

Capillary electrophoresis (CE) conditions: Oligonucleotides and cross-linked DNA duplexes were analysed by injection (0.2–0.4 OD/100 μL) of each sample. A ssDNA 100-R Gel, Tris-borate and 7 M urea were used (Kit No 477480) on a Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System with 32 Karat software. UV, 254 nm; inject voltage, 10.0 KV and separate voltage, 9.0 KV (45.0 min duration).

Analysis of cross-linked DNA duplexes: Analysis of reactions R1-R8 was carried out by gel electrophoresis (8% polyacrylamide/7 M urea gel) at constant power of 20 W for 2 h, using 0.09 M Tris-borate-EDTA buffer (pH 8.0). Following electrophoresis the plates were wrapped with cling-film, placed on a fluorescent TLC plate, and illuminated with a UV lamp (254 nm) to visualise the bands and photographed.

Circular dichroism: CD spectra were measured on a Jasco J-720 spectropolarimeter at 4.0 μM concentration of oligonucleotide in phosphate buffer (10 mM), NaCl (200 mM), pH 7.0. Spectra were recorded at 100 nm min^{-1} with a response time of 1 s and a bandwidth of 1 nm. A buffer baseline was subtracted from each spectrum to give zero ellipticity at 320 nm.

Fluorescence melting: The experiments were performed on a Roche LightCycler 1.5. Each capillary had a volume of 20 μL. Concentrations of cross-linked DNA duplexes and non-cross-linked oligonucleotides were 0.25 μM. Melting studies were carried out at pH 7.0 in sodium phosphate buffer (0.01 M) with aqueous NaCl (0.2 M) and EDTA-Na_2 (0.001 M); SYBR Green I fluorescent DNA-binding dye (Roche, 2 μL) was added to each capillary. Each sample was measured in triplicate, and the average T_m is given. Following initial rapid denaturation from 30 to 95 °C at 20 °C s^{-1}, fluorescence was measured in step-mode cooling to 35 °C, then melting from 35 °C to 95 °C at 0.5 °C intervals with a 30 s equilibration interval at each step.

Ultraviolet melting: UV melting curves were measured on Cary 400 Scan UV–Visible Spectrophotometer (Varian) at 0.25 or 4 μM concentration of oligonucleotide in phosphate buffer (10 mM), NaCl (200 mM), pH 7.0. Spectra were recorded at 260 nm. The samples were initially denatured by heating to 83 °C at 10 °C min^{-1} then cooled to 15 °C at 1 °C min^{-1} and heated to 83 °C at 1 °C min^{-1}. Three successive melting curves were measured, and T_m values were calculated with Cary Win UV Thermal application Software.

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