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Oligodeoxynucleotides with Extended 3′- and 5′-Homologous Internucleotide Linkages

T. Kofoed,[a] P. B. Rasmussen,[b] P. Valentin-Hansen[b] and E. B. Pedersen[a,*]

[a]Department of Chemistry, Odense University, DK-5230 Odense M, Denmark and [b]Department of Biochemistry, Odense University, DK-5230 Odense M, Denmark

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3′-Deoxy-3′-*C*-(hydroxymethyl)thymidine (3′-DHMT) and 5′-deoxy-5′-*C*-(hydroxymethyl)thymidine (5′-DHMT) have been prepared and used for the synthesis of novel oligodeoxynucleotides containing extended internucleotide linkages. Enzymatic stability toward exonuclease III degradation was studied and hybridization properties were tested. The extended 3′-homologous DNA causes complete cessation of exonuclease III degradation while the extended 5′-homologous DNA shows only some decrease in degradation. Duplexes having extended internucleotide linkages show a minor decrease in hybridization stability. Triplex formation with 20-mer duplexes at pH 5.5 and high salt concentration gave almost no decrease in binding affinity when compared with unmodified triplex formation. UV experiments were also performed with oligodeoxynucleotides containing hairpin structures and bulged bases. Extended linkages in the oligodeoxynucleotides gave a small increase in T_m with sequences containing a loop, but no change or only a small decrease was observed when an extra thymidine nucleotide was incorporated in the complementary strand.

The potential uses of oligodeoxynucleotides (ODNs) in the control of gene expression has received much attention over the past few years. In general, there are two possible ways to influence gene expression: at the level of translation (antisense) or at the level of transcription (antigene). The antisense strategy relies on duplex formation between the sense strand (ODNs) and mRNA, and the antigene strategy on the binding of ODNs to double-stranded DNA with the formation of a DNA triplex structure. Natural oligonucleotides have been shown to work as antisense molecules *in vitro*. Unfortunately, they have poor cell membrane permeability and low nuclease resistance, and this makes them unsuitable for use in the antisense and antigene strategy. In order to overcome these problems much effort has been made toward the development of oligonucleotides bearing modified internucleotides linkages. Several phosphate backbone modifications have been proposed and tested for antisense properties. Phosphorothioates,[1] phosphorodithioates[2] and methylphosphonates[3] are among the most studied examples and they all have interesting antisense properties. Analogues in which one of the two bridging oxygen atoms in the phosphodiester linkage is replaced by nitrogen,[4] sulfur[5] or methylene[6] have also been described in the literature. In addition, several dephospho linkages have been reported and found to fulfill at least some of the requirements of antisense oligonucleotides.[7] The use of ODNs in the antigene approach has not been investigated as intensively but is of great interest, since inhibition at the level of transcription should be more efficient than at the level of translation. Felsenfeld *et al.*[8] reported the first triple helix formation between two polypyrimidine strands hybridizing with a polypurine strand. One pyrimidine strand binds in the major groove parallel to the Watson–Crick purine strand through the formation of Hoogsteen hydrogen bonds. Recently, purine–purine–pyrimidine triple helices have been observed,[9] but most studies have been done with pyrimidine–pyrimidine–purine triple helices.[10]

There have been reported only a few examples in which the internucleotide linkages have been extended by one atom, resulting in a 5-atom connection between the two pentofuranosyl moieties. Analogues containing 3′-deoxy-[11] and 3′-*C*-*threo*-(hydroxymethyl)thymidine[12] have been incorporated into novel ODNs and reported to cause only minor decreases in thermal stability. Furthermore, these analogues were shown to be enzymatically stable toward snake venom phosphodiesterase.

Here we report on the synthesis of 3′-*C*-(hydroxymethyl) (3′-homologous) and 5′-*C*-(hydroxymethyl) (5′-homologous) nucleosides and the incorporation of these two monomers into oligodeoxynucleotides. This gives internucleotide linkages that are extended by a

* To whom correspondence should be addressed.

single atom, a methylene group, situated directly at the 3′- or 5′-carbon (Fig. 1). We tested these new oligodeoxynucleotides for both antisense and antigene properties. It has been reported that a conformational change occurs in the phosphodiester backbone as a consequence of triplex formation.[13] We thought that these extended oligonucleotides, might better accommodate this conformational change during triplex formation with duplex DNA.

Results and discussion

Synthesis of 3′-deoxy-3′-C-(hydroxymethyl) thymidine (3′-DHMT) (**5**). The synthesis of 3′-*C*-(hydroxymethyl)-2′,3′-dideoxynucleosides has previously been achieved by condensation of 3-*C*-(hydroxymethyl) sugars with protected nucleobases.[14] However, such approaches require multistep syntheses and suffer from the lack of stereospecificity. Recently two strategies using nucleosides as starting material have been developed. Bamford *et al.*[15] synthesized 3′-*C*-(hydroxymethyl) nucleosides by ring opening of 2′,3′-epoxide with lithium methylthioformaldine, followed by reaction with $HgO–HgCl_2$. This gave 1-(3-deoxy-3-*C*-formyl-β-D-*arabino*-pentofuranosyl)-thymine and the corresponding uracil analogues. Reduction with sodium borohydride gave the 3′-deoxy-3′-*C*-(hydroxymethyl) nucleosides and the 2′-deoxy analogue was obtained by Barton deoxygenation. The second synthesis[16] starting from a nucleoside makes use of a stereoselective radical addition reaction of a 3′-radical thymidine to β-tributylstannylstyrene giving 3′-deoxy-3′-*C*-styrylthymidine. Cleavage of the C=C double bond was achieved with OsO_4 and $NaIO_4$, followed by reduction of the aldehyde function with sodium borohydride, to give 3′-deoxy-3′-*C*-(hydroxymethyl)thymidine (**5**). We chose to introduce the carbon–carbon bond through the formation of a 3′-cyano nucleoside. A recent report by Parkes and Taylor[17] describes a new and short synthetic route to 3′-cyano-3′-deoxy-5′-*O*-tritylthymidine. The nitrile derivative **2** is synthesized in three steps from thymidine in an overall yield of 33% (Scheme 1). The nitrile function is introduced by a free radical reaction, a modification of the Stork radical cyclization–cyanation

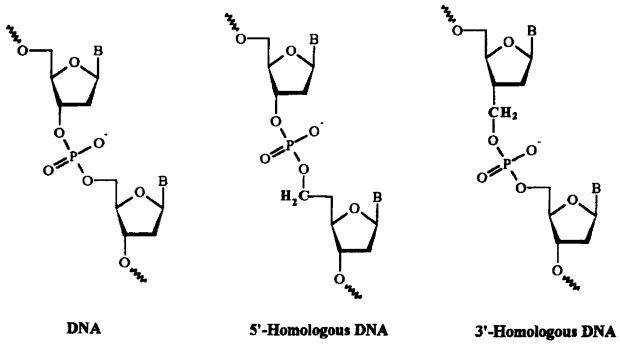
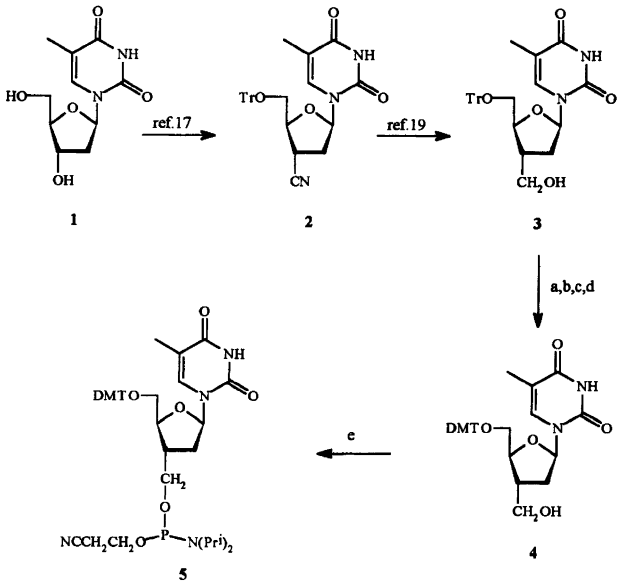


Fig. 1. Stuctures of DNA, 5′-homologous DNA and 3′-homologous DNA.



Scheme 1. Preparation of building blocks used for the synthesis of 5′-homologous DNA: a, Ac_2O, pyridine, DMAP; b, AcOH, reflux; c, DMTCl, pyridine, DMAP; d, NH_3, MeOH, 71% for four steps; e, $NCCH_2CH_2OP(Cl)N(Pr^i)_2$, *N,N*-diisopropylethylamine, CH_2Cl_2, 90% yield.

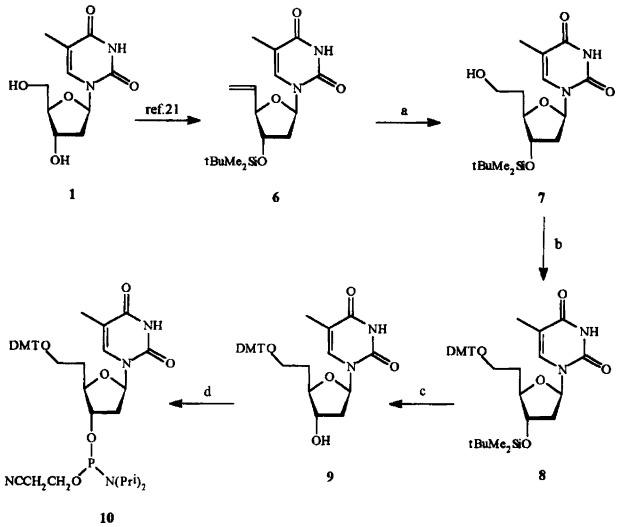
reaction.[18] An attempt to use 3′-deoxy-3′-iodo-5′-*O*-(4,4′-dimethoxytrityl)thymidine in this step was unsuccessful.

Reduction of the nitrile group to the alcohol was achieved by a two-step procedure.[19] Reduction with diisobutylaluminium hydride (DIBAL) followed by hydrolysis of the imine formed gave, after column chromatography, 3′-deoxy-3′-*C*-formyl-5′-*O*-tritylthymidine as a white foam (yield; 57%). The aldehyde was reduced to the alcohol with sodium borohydride in ethanol. After column chromatography 3′-deoxy-3′-*C*-(hydroxymethyl)-5′-*O*-tritylthymidine (**3**) was obtained as a foam (yield; 89%). To be able to use this nucleoside in the synthesis of oligodeoxynucleotides it was necessary to replace the 5′-trityl protecting group with 4,4′-dimethoxytrityl (DMT). The trityl group is considered too stable toward standard deprotection conditions used on an automated solid-phase DNA synthesizer. This was done by a four-step synthesis. First the 3′-*C*-(hydroxymethyl) group was protected using acetic anhydride in pyridine, followed by detritylation with acetic acid at reflux. After concentration and purification by column chromatography, the 5′-hydroxy function was protected by treatment with 4,4′-dimethoxytrityl chloride (DMTCl) in pyridine and a catalytic amount of 4-dimethylaminopyridine (DMAP). Finally the acetyl group was removed with methanolic ammonia. This gave, after column chromatography, **4** as a white foam (the overall yield for all four steps was 71%). The phosphoramidite **5** was obtained by the reaction of 3′-deoxy-5′-*O*-dimethoxytrityl-3′-*C*-(hydroxymethyl)-thymidine (**4**) with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite $[NCCH_2CH_2OP(Cl)N(Pr^i)_2]$ in the presence of *N,N*-diisopropylethylamine. This gave **5** in

90% yield after silica gel column chromatography and precipitation from petroleum ether.

Synthesis of 5'-deoxy-3'-C-(hydroxymethyl) thymidine (5'-DHMT) (**10**). As with 3'-*C*-(hydroxymethyl) nucleosides there are two general strategies used in the synthesis of 5'-*C*-(hydroxymethyl) nucleosides. One uses hexofuranoses[20] as starting material and affords nucleosides by coupling reactions with nucleobases. The other route uses nucleosides as starting material. The former methods involve, as mentioned above, multistep procedures and suffer from lack of stereoselectivity. The introduction of an extra carbon atom at the 5'-position of nucleosides has been achieved by a nucleophilic displacement reaction of cyanide with 5'-deoxy-5'-iodo nucleosides.[21] It was not possible to reduce the nitrile function to the aldehyde with DIBAL. Instead the cyano group was reduced to the amine with Raney nickel, followed by nitrous acid treatment, which gave the 5'-*C*-(hydroxymethyl) nucleoside. Since the yield of the last step was only 33% we decided to use, instead, a method developed by Weis *et al.*[22] which involved Wittig methylenation for the introduction of an extra carbon atom at the 5'-position in thymidine. 3'-*O*-*tert*-Butyldimethylsilylthymidine was prepared in three steps from thymidine (**1**) (Scheme 2).

The primary hydroxy group in thymidine was oxidized under Swern conditions (oxalyl chloride and DMSO in dichloromethane) to give the aldehyde analogue. This was reacted directly, as the crude product, with methyltriphenylphosphonium bromide to give the 4'-vinyl nucleoside **6** in 35% yield. The vinyl compound was hydroborated with borane–oxathiane complex, and after oxidation with alkaline hydrogen peroxide 5'-deoxy-5'-*C*-(hydroxymethyl)-3'-*O*-*tert*-butyldimethylsilylthymidine (**7**) was obtained in 61% yield. The introduction of DMT was achieved under standard conditions giving **8** in 79% yield after column chromatography. The 3'-*tert*-Butyldimethylsilyl group was removed by treatment with tetrabutylammonium fluoride (TBAF) in THF which gave **9** in 81% yield. The final phosphoramidite **10** was prepared by the same method as used for the 3'-*C*-(hydroxymethyl) analogue **5** (yield: 89%). The purity of both **5** and **10** exceeded 90%. According to ^{31}P NMR spectroscopy, the only impurities were oxidized phosphorus compounds.



Scheme 2. Preparation of building blocks used in the synthesis of 3'-homologous DNA: a, BH_3·oxathiane, THF, NaOH, H_2O_2, yield 61%; b, DMTCl, pyridine, yield 79%; c, TBAF, THF, yield 81%; d, $NCCH_2CH_2OP(Cl)N(Pr^i)_2$, *N,N*-diisopropylethylamine, CH_2Cl_2, 89% yield.

DNA synthesis and melting profiles. Solutions of **5** and **10** in dry acetonitrile were applied directly to an automatic solid-phase DNA synthesizer to prepare the oligodeoxynucleotides **B**—**G** (Table 1) using standard phosphoramidite methodology, and purified as described in the Experimental. The ability to hybridize to their complementary DNA strands was examined by UV melting point measurements. The results are given in Table 1. The melting points of modified 14-mer duplexes are seen to be lowered by 3–5 °C per modification, owing to the extension of the internucleotide linkages.

The ability of these modified oligodeoxynucleotides to hybridize to a DNA duplex (triplex formation) was also examined by UV melting point measurements. As the target double-strand DNA we used a 20-mer double helix DNA,[23] and as seen in Table 2 there was only a small decrease in T_m (triplex). Only the oligonucleotide **G**, containing modification in two successive internucleotide linkages, showed a significant decrease in T_m.

Most ODNs have been designed to form duplexes with regions of mRNA expected to be single-stranded, but hairpin structures are often formed between neighbouring complementary sequences and it would be of interest to design ODNs that could recognize hairpins. As a model system we investigated the hybridization of an ODN at the free ends at the bottom of the stem in a DNA hairpin. Francois *et al.*[24] have tested the hybridization of unmodified oligonucleotides with two single-stranded regions flanking a hairpin structure. An oligodeoxynucleotide–phenanthroline conjugate gave, in the presence of

Table 1. Sequences synthesized and hybridization data for duplex formation.[a]

	Sequence	T_m(duplex)/°C	ΔT_m
A	5'-(CCCCTTTCTTTTTT)-3'	51.4	
B	5'-(CCCCTTXCTTTTTT)-3'	48.0	−3.4
C	5'-(CCCCTTXCXTTTTT)-3'	43.2	−4.1
D	5'-(CCCCTTYCTTTTTT)-3'	46.4	−5.0
E	5'-(CCCCTTYCYTTTTT)-3'	44.8	−3.3
F	5'-(CCCCTTXCYTTTTT)-3'	44.4	−3.5
G	5'-(CCCCTTYCXTTTTT)-3'	44.0	−3.7

[a] Duplex formation with complementary sequence (5'-AAAAAAGAAAGGGG-3'), 10 mM phosphate buffer, pH = 7.0, 140 mM NaCl, 1 mM EDTA. ΔT_m represents the decrease in T_m per modification. X = 5'-homologous DNA, Y = 3'-homologous DNA.

Table 2. Sequences synthesized and hybridization data for triplex formation.[a]

	Sequence	T_m(triplex)/°C	ΔT_m
A	5′-(CCCCTTTCTTTTTT)-3′	34.0	
B	5′-(CCCCTTXCTTTTTT)-3′	33.6	−0.4
C	5′-(CCCCTTXCXTTTTT)-3′	31.6	−1.2
D	5′-(CCCCTTYCTTTTTT)-3′	32.4	−1.6
E	5′-(CCCCTTYCYTTTTT)-3′	30.8	−1.6
F	5′-(CCCCTTXCYTTTTT)-3′	32.0	−1.0
G	5′-(CCCCTTYCXTTTTT)-3′	27.6	−3.2

[a] Triplex formation with 20-mer duplex,[21] 10 mM phosphate buffer, pH = 5.5, 0.5 M NaCl. ΔT_m represents the decrease in T_m per modification. X = 5′-homologous DNA, Y = 3′-homologous DNA.

Cu^{2+} ions, two main cleavage sites. Competition experiments showed that both parts of the ODN must be bound to observe sequence-specific cleavage. We synthesized two 29-mer oligodeoxynucleotides (loop 1 and loop 2) containing a hairpin structure, as described by Francois *et al.* so that the modified ODNs were complementary to sequences on both sides of the hairpin (Fig. 2).

As seen in Table 3 both loop 1 and loop 2 gave, with the oligonucleotide **B** and **D**, a small increase in T_m (one modification introduced into the ODN). This indicates that an extended linkage in this area leads to better

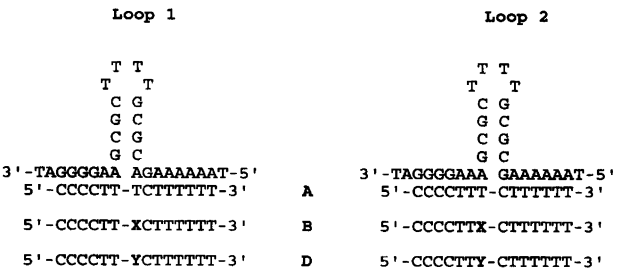


Fig. 2. Graphical presentation of the duplex formed between unmodified (**A**) or modified (**B** and **D**) oligonucleotide with a 29-mer oligonucleotide containing hairpin structure. X = 5′-homologous DNA. Y = 3′-homologous DNA.

Table 3. Hybridization data for duplex formation with non-adjacent sequences.[a]

		Loop 1		Loop 2	
	Sequence	T_m	ΔT_m	T_m	ΔT_m
A	5′-(CCCCTTTCTTTTTT)-3′	22.8		21.2	
B	5′-(CCCCTTXCTTTTTT)-3′	24.0	+1.2	22.0	+0.8
C	5′-(CCCCTTXCXTTTTT)-3′	20.8	−1.0	20.0	−0.6
D	5′-(CCCCTTYCTTTTTT)-3′	23.6	+0.8	22.0	+0.8
E	5′-(CCCCTTYCYTTTTT)-3′	19.2	−1.8	18.4	−1.4
F	5′-(CCCCTTXCYTTTTT)-3′	19.6	−1.6	18.8	−1.2

[a] Duplex formation with complementary sequence containing hairpin structure (see Fig. 2), 10 mM phosphate buffer, pH = 7.0, 140 mM NaCl, 1 mM EDTA. ΔT_m represents the change in T_m per modification. X = 5′-homologous DNA, Y = 3′-homologous DNA.

hybridization of the strand. Additional modifications in the T region of the sequences **C**, **E** and **F** gave decreases in T_m, compared with the sequences **B** and **D** with a modification across the hairpin region only. This decrease is similar to that found for simple duplexes as shown in Table 1. This is a further proof that hybridization actually takes place in the weaker binding T region as claimed by Francois *et al.*[24] Finally we investigated the case of a bulge, using a complementary sequence containing an extra thymidine residue (Fig. 3), but no change, or only a small decrease, in the melting points was observed (Table 4).

Enzymatic hydrolysis. In order to examine the enzymatic stability of the 3′- and 5′-homologous modified ODNs, exonuclease III susceptibility studies were performed on duplexes formed between unmodified (**A**) or modified (**B–E**) 14-mer oligodeoxynucleotides (5′-end labelled with ^{32}P) and the complementary strand. The duplexes were incubated with nuclease for varying lengths of time and the resulting oligodeoxynucleotides were electrophoresed on 20% PAG–50% urea gels and visualized by autoradiography (Fig. 4) as described previously.[25]

The results clearly show that the presence of the modified nucleosides induces an increase in the stability toward exonuclease III. Hydrolysis of a duplex by exo III proceeds to a 50% limit because at this point no duplex DNA remains. The unmodified oligodeoxynucleotide (**A**) was degraded to a major product six base pairs (bp)

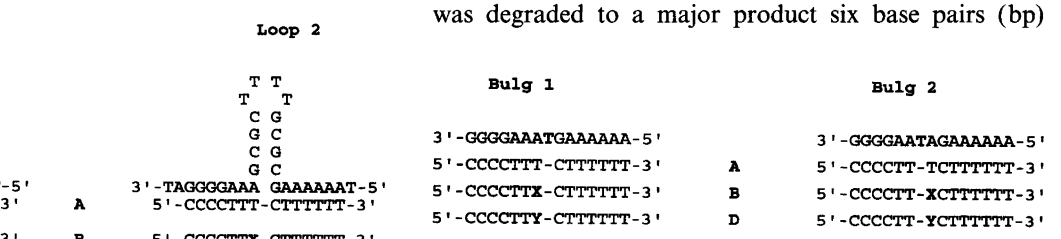


Fig. 3. Graphical presentation of hybridization between unmodified (**A**) or modified (**B** and **D**) oligonucleotides with the complementary strand containing one extra (bulge) nucleobase residue. X = 5′-homologous DNA. Y = 3′-homologous DNA.

Table 4. Hybridization data for duplex formation with non-adjacent sequences.[a]

		Bulg 1		Bulg 2	
	Sequence	T_m	ΔT_m	T_m	ΔT_m
A	5′-(CCCCTTTCTTTTTT)-3′	33.3		34.5	
B	5′-(CCCCTTXCTTTTTT)-3′	33.3	0	32.1	−2.4
C	5′-(CCCCTTXCXTTTTT)-3′	31.2	−1.0	30.0	−2.2
D	5′-(CCCCTTYCTTTTTT)-3′	31.7	−1.6	33.3	−1.2
E	5′-(CCCCTTYCYTTTTT)-3′	29.6	−1.8	30.0	−2.2
F	5′-(CCCCTTXCYTTTTT)-3′	30.0	−1.6	29.2	−2.6
G	5′-(CCCCTTYCXTTTTT)-3′	20.4	−6.4	19.2	−7.6

[a] Duplex formation with complementary sequence containing bulging thymidine residues (see Fig. 3), 10 mM phosphate buffer, pH = 7.0, 140 mM NaCl, 1 mM EDTA. ΔT_m represents the decrease in T_m per modification. X = 5′-homologous DNA, Y = 3′-homologous DNA.

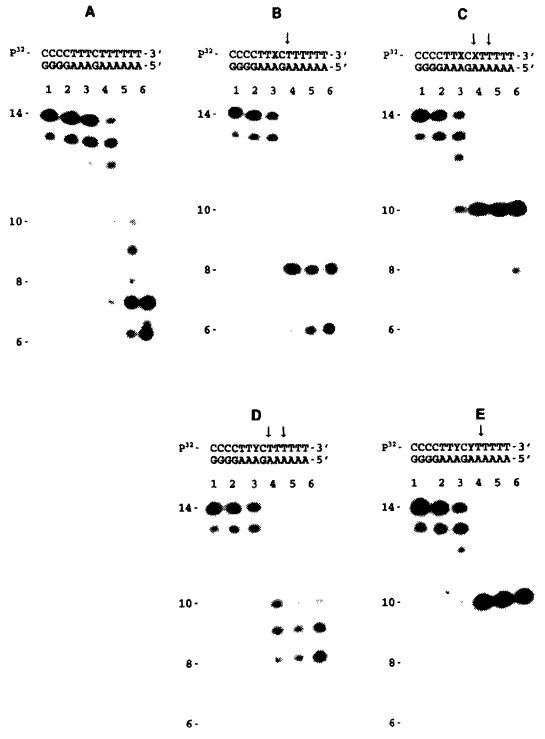


Fig. 4. Exonuclease III digests of duplexes formed between unmodified (**A**) or modified (**B–E**) 14-mer oligonucleotides and the complementary DNA strand. Exonuclease III treatment at 30 °C for 10, 30, 240, 600 and 1200 s, respectively. Arrows indicate Exo III stop sites in the modified duplex. Numbers in between figures refer to the size of oligodeoxynucleotides. X = 5′-homologous DNA. Y = 3′-homologous DNA.

long, whereas the introduction of one (**D**) or two (**E**) 3′-homologous residues caused complete inhibition of exonuclease III cleavage. The effect of 5′-homologous was not that pronounced, but exonuclease III was capable of removing only 4–6 bp of the modified ODN containing two modified nucleosides (**C**).

Experimental

All commercial compounds were used as received and solvents were purified by standard methods. NMR spectra were recorded at 25 °C at 250 MHz for ^{1}H, 62.9 MHz for ^{13}C and 101.3 MHz for ^{31}P. Chemical shifts are given in ppm (δ) relative to tetramethylsilane for ^{1}H, relative to $CDCl_3$ (77.0) for ^{13}C both as internal standards, and relative to 85% H_3PO_4 for ^{31}P as an external standard. FAB mass spectra were obtained on a Kratos MS 50 RF spectrometer, for samples dissolved in $CHCl_3$ with 3-nitrobenzyl alcohol as the matrix. Column chromatography was performed on silica gel 230–400 mesh (Merck) and precoated silica gel TLC plates were supplied by Merck (Silica gel F_{254}). Detection was achieved by UV (254 nm) and/or by spraying with 10% H_2SO_4 in methanol and heating. Microanalyses were carried out at the H. C. Ørsted Institute, Universitetsparken 5, DK-2100 Copenhagen. HPLC was done on Water Delta Prep 3000 HPLC system (C18 column), using an ammonium acetate buffer (pH = 9) with a linear gradient of acetonitrile (5–80%).

*3′-Deoxy-3′-*C*-(hydroxymethyl)-5′-*O*-(4,4′-dimethoxytrityl) thymidine* (**4**). 3′-Deoxy-3′-*C*-(hydroxymethyl)-5′-*O*-tritylthymidine (**3**) (820 mg, 1.64 mmol) was dissolved in a mixture of dry pyridine (10 ml) and acetic anhydride (2 ml) containing a catalytic amount of 4-dimethylaminopyridine (DMAP). After 2 h at room temperature the solution was concentrated under reduced pressure, and the crude acetylated product was redissolved in 80% aqueous acetic acid (20 ml) and refluxed for 30 min. After evaporation to dryness the 3′-deoxy-3′-*C*-(acetoxymethyl)thymidine was purified by column chromatography (100 g, $CHCl_3$–CH_3OH, 19:1). The product was coevaporated with dry pyridine (2 × 10 ml) and dissolved in pyridine (10 ml). Triethylamine (0.25 ml) and 4,4′-dimethoxytrityl chloride (610 mg, 1.80 mmol) were added and the solution stirred at room temperature for 2 h. Water (5 ml) was added and the solution extracted with diethyl ether (2 × 20 ml). The organic layer was concentrated to dryness and the residue dissolved in 10 M ammonia in methanol and left at room temperature overnight. After concentration the product was purified by silica gel column chromatography using a stepwise gradient of methanol in dichloromethane (0–5%) as the eluent. This gave **4** (650 mg, 71%) as a colourless foam. ^{1}H NMR ($CDCl_3$): δ 1.53 (s, 3 H, 5-CH_3), 2.20–2.35 (m, 2 H, H-2′), 2.60 (m, 1 H, H-3′), 3.35 (dd, *J* 3.4 and 10.5 Hz, 1 H, H-5′a), 3.52 (dd, *J* 3.3 and 10.4 Hz, 1 H, H-5′b) 3.62 (d, *J* 5.8 Hz, 2 H, CH_2), 3.78 (s, 6 H, 2 × OCH_3), 3.98 (m, 1 H, H-4′), 6.13 (t, *J* 5.9 Hz, 1 H, H-1′), 6.82–6.85 (m, 4 H, DMT), 7.23–7.44 (m, 9 H, DMT), 7.59 (s, 1 H, H-6); ^{13}C NMR ($CDCl_3$): δ 11.30 (CH_3), 35.85 (C-2′), 41.55 (C-3′), 55.29 (OCH_3), 63.08 (CH_2), 64.16 (C-5′), 82.38 (C-4′), 85.08 (C-1′), 86.89 (DMT), 110.65 (C-5), 113.27, 127.10, 127.97, 128.16, 130.09, 135..47, 135.51, 144.38, 158.72 (DMT), 135.64 (C-6), 150.21 (C-2), 163.63 (C-4); FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z:* 559 ($M+H^+$). Anal. $C_{32}H_{34}N_2O_7 \cdot 0.25\ H_2O$: C, H, N.

*3′-Deoxy-3′-*C*-[2-cyanoethoxy(diisopropylamino)phosphinooxymethyl]-5′-*O*-(4,4′-dimethoxytrityl) thymidine* (**5**). Nucleoside **4** (180 mg, 0.32 mmol) was coevaporated with dry CH_3CN and dissolved in a mixture of *N,N*-diisopropylethylamine (0.28 ml) and dry CH_2Cl_2 (0.9 ml). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.13 ml, 0.58 mmol) was added dropwise and, after 90 min at room temperature, the mixture was quenched by addition of CH_3OH (0.7 ml). To this solution was added ethyl acetate (10 ml) and the mixture was washed with aquous saturated $NaHCO_3$ (3 × 10 ml), and then H_2O (3 × 10 ml), and dried over Na_2SO_4. The crude product was purified by silica gel column chromatography (75 g, EtOAc–CH_2Cl_2–Et_3N 45:45:10). The

resulting white foam was redissolved in dry toluene (1 ml) and added dropwise to cold petroleum ether (50 ml). After filtration, **5** was obtained as a white powder (220 mg, 90%). ^{31}P NMR ($CDCl_3$): δ 149.17.

5′-Deoxy-5′-C-(hydroxymethyl)-3′-O-tert-butyldimethylsilylthymidine (**7**). A solution of **6** (500 mg, 1.42 mmol) in anhydrous THF (3 ml) was added dropwise to a stirred solution of BH_3·oxathiane (0.19 ml of a 7.8 M solution in oxathiane; 1.5 mmol) in anhydrous THF (4 ml). After 30 min at room temperature, a 2 M solution of NaOH (0.8 ml) was added. The mixture was cooled to 0 °C and 35% H_2O_2 (0.15 ml, 1.5 mmol) was added dropwise. After stirring at room temperature for 45 min the mixture was poured into water–ice (20 ml) and extracted with diethyl ether (2 × 20 ml). The organic phase was washed with aq. sat. $NaHCO_3$ (2 × 10 ml), dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (100 g) using a gradient of ethyl acetate–petroleum ether (1:4–4:1) giving **7** (320 mg, 61%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 0.08 (s, 6 H, 2 × CH_3), 0.89 (s, 9 H, *tert*-butyl), 1.87–2.26 (m, 4 H, H-2′, H-5′), 1.92 (s, 3 H, CH_3), 2.75 (br s, 1 H, OH), 3.76–3.98 (m, 3 H, H-3′, CH_2), 4.13 (m, 1 H, H-4′), 6.17 (t, *J* 6.5 Hz, 1 H, H-1′), 7.17 (d, *J* 0.9 Hz, 1 H, H-6), 9.85 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$): δ −4.94, −4.70 (2 × CH_3), 12.47 (5-CH_3), 17.79, 25.58 (*tert*-butyl), 35.61 (C-5′), 40.08 (C-2′), 59.87 (CH_2), 74.87 (C-3′), 84.66, 85.05 (C-1′, C-4′), 111.06 (C-5), 135.51 (C-6), 150.38 (C-2), 164.05 (C-4); FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z*: 371 ($M+H^+$), 393 ($M+Na^+$).

5′-Deoxy-5′-C-(4,4′-dimethoxytrityloxymethyl)-3′-O-tert-butyldimethylsilylthymidine (**8**). A mixture of 3′-*O*-*tert*-butyldimethylsilyl-5-deoxy-5′-*C*-(hydroxymethyl)thymidine (550 mg, 1.49 mmol) and 4,4′-dimethoxytrityl chloride (600 mg, 1.77 mmol) in dry pyridine (5 ml) was stirred at room temperature for 2 h. After concentration under reduced pressure and column chromatography (100 g, CH_2Cl_2–MeOH, 98:2) **8** was obtained as a white foam. Yield: 792 mg (79%). ^{1}H NMR ($CDCl_3$): δ 0.04, 0.05 (2 × s, 6 H, 2 × CH_3), 0.89 (s, 9 H, *tert*-butyl), 1.73–2.30 (m, 7 H, H-2′, H-5′, 5-CH_3), 3.24 (t, *J* 6.5 Hz, 2 H, CH_2), 3.78 (s, 6 H, 2 × OCH_3), 3.97 (m, 1 H, H-3′), 4.11 (m, 1 H, H-4′), 6.12 (t, *J* 6.6 Hz, 1 H, H-1′), 6.78–7.44 (m, 14 H, H-6, DMT), 8.75 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$): δ −4.79, −4.59 (2 × CH_3), 12.61 (5-CH_3), 17.91, 25.70 (*tert*-butyl), 34.22 (C-5′), 40.60 (C-2′), 55.17 (OCH_3), 60.33 (CH_2), 75.09 (C-3′), 84.28, 84.89, 86.25 (C-4′, C-1′, DMT), 110.78 (C-5), 113.07, 126.73, 127.75, 128.12, 129.96, 135.94, 136.26, 145.02, 158.46 (DMT), 135.10 (C-6), 150.07 (C-2), 163.59 (C-4); FAB$^+$ MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z*: 673 ($M+H^+$), 695 ($M+Na^+$).

5′-Deoxy-5′-C-(4,4′-dimethoxytrityloxymethyl) thymidine (**9**). Nucleoside **8** (570 mg, 0.85 mmol) was dissolved in 1.1 M TBAF in THF (5 ml). After stirring at room temperature for 2 h the mixture was concentrated *in vacuo*, dissolved in $CHCl_3$ (20 ml) and washed with H_2O (5 ml). The product **9** was purified by silica gel column chromatography (100 g, CH_2Cl_2–CH_3OH, 95:5). Yield: 380 mg (81%). ^{1}H NMR ($CDCl_3$): δ 1.82–2.01 (m, 6 H, H-5′, THF), 1.93 (d, *J* 1.0 Hz, 3 H, 5-CH_3), 2.17–2.25 (m, 1 H, H-2′a), 2.34–2.45 (m, 1 H, H-2′b), 3.15–3.46 (m, 3 H, OH, CH_2), 3.71–3.83 (m, 11 H, 2 × OCH_3, H-4′, THF), 4.24 (m, 1 H, H-3′), 6.14 (dd, *J* 5.4 and 7.0 Hz, 1 H, H-1′), 6.85 (m, 4 H, DMT), 7.09 (d, *J* 1.1 Hz, 1 H, H-6), 7.21–7.43 (m, 9 H, DMT), 8.69 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$): δ 12.64 (CH_3), 25.28 (THF), 33.78 (C-5′), 39.74 (C-2′), 55.20 (OCH_3), 60.90 (CH_2), 67.93 (THF), 74.20 (C-3′), 84.13, 85.11, 87.22 (C-1′, C-4′, DMT), 111.01 (C-5), 113.33, 126.99, 127.89, 128.01, 129.75, 129.86, 135.17, 135.47, 144.36, 158.64 (DMT), 135.78 (C-6), 150.08 (C-2), 163.51 (C-4); FAB$^+$ MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z: 559* ($M+H^+$). Anal $C_{32}H_{34}N_2O_7$·1 THF: C, H, N.

5′-Deoxy-5′-C-(4,4′-dimethoxytrityloxymethyl)-3′-O-[2-cyanoethoxy(diisopropylamino)phosphino] thymidine (**10**). Nucleoside **9** (280 mg, 0.50 mmol) was dried by coevaporation with anhydrous CH_3CN (2 × 2 ml) and dissolved under nitrogen in dry CH_2Cl_2 (1.4 ml) and *N,N*-diisopropylethylamine (0.44 ml). 2-Cyanoethyl *N,N*-diisopropylaminophosphoramidochloridite (0.2 ml, 0.93 mmol) was added dropwise. After 90 min the reaction mixture was quenched by addition of CH_3OH (1.1 ml) followed by addition of EtOAc (15 ml). The solution was washed with sat. aq. $NaHCO_3$ (3 × 15 ml) and then water (3 × 15 ml), dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (100 g, EtOAc–CH_2Cl_2–Et_3N 45:45:10). The resulting foam was redissolved in anhydrous toluene (1 ml) and added dropwise to cold (−20 °C) petroleum ether (200 ml). After filtration **10** (338 mg, 89%) was obtained as a white powder. ^{31}P NMR ($CDCl_3$): δ 149.35, 149.84.

Synthesis of oligodeoxynucleotides. General procedure. Synthesis of oligomers **A–H** (Table 1) was achieved using standard phosphoramidite methodology on a Pharmacia Gene Assembler Special DNA synthesizer using **5**, **10** and commercial 2′-deoxynucleoside β-cyanoethyl phosphoramidites. The coupling efficiency of **5** and **10** was similar to those of the commercial amidites (>98%). The oligonucleotides were cleaved from the support and deblocked with concentrated ammonia at room temperature for 48 h and purified on oligodeoxynucleotide purification cartridges (COP, Cruachem). The purity of all oligonucleotides was checked by HPLC.

Melting experiments. All UV melting experiments were performed on a Perkin–Elmer UV–VIS spectrometer equipped with a PTP-6 Peltiér temperature programming element. The absorbance at 260 nm was measured from

10 °C to 80 °C at 1 °C intervals (1 cm cuvettes). The thermal stability was determined as the maximum of the first derivative plots of the melting curves. The duplexes (T_m duplex) formed between the modified ODNs and their complementary strand were dissolved in a medium salt buffer (pH = 7.0, 1 mM EDTA, 10 mM Na_2HPO_4, 140 mM NaCl) to a concentration of 2.5 μM for each strand. The triplexes (T_m triplex) formed between the modified ODNs and a 20-mer unmodified duplex were studied in a high salt buffer (pH = 5.5, 10 mM Na_2HPO_4, 0.5 M NaCl) at a concentration of 2.5 μM for each strand (Table 2).

Enzymatic stability. Exonuclease III was purchased from Boehringer, Mannheim. Duplex DNA was isolated by gel electrophoresis on 20% PAG–8M urea gels and purified as described by Valentin-Hansen.[26] Duplex DNA (100 pmol) was incubated in 0.1 ml exonuclease III buffer at 30 °C. The reaction was started by addition of 10 units exonuclease III. Aliquots (10 μl) were withdrawn after increasing digestion times and reactions were terminated by addition of gel loading dye solution (formamide–EDTA). Finally, the digests were analysed on a denaturing polyacrylamide gel.

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