Synthesis and duplex stability of oligodeoxyribonucleotides containing a 2′→5′-amide linkage

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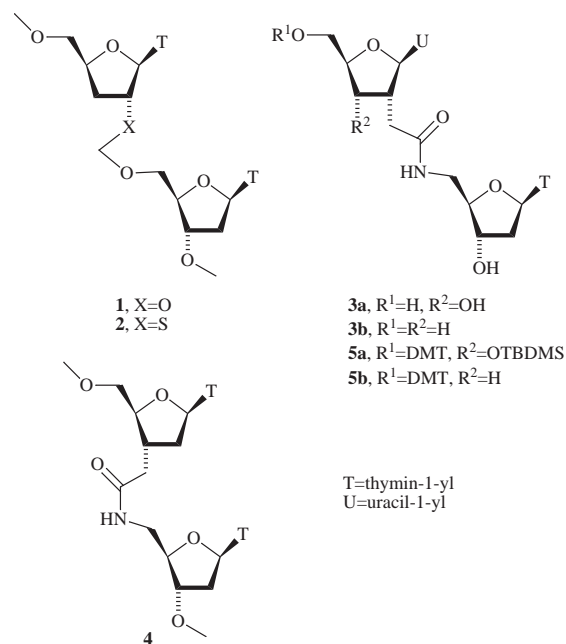[c] *School of Chemistry and* [d] *School of Biology, University of Leeds, Leeds UK LS2 9JT*

2′-Deoxy-2′-α-C-carboxymethyl-3′-O-tert-butyldimethylsilyl-5′-O-dimethoxytrityluridine (**11a**) and the corresponding 3′-deoxy derivative 2′,3′-dideoxy-2′-α-C-carboxymethyl-5′-O-dimethoxytrityluridine (**11b**) have been condensed with 5′-amino-5′-deoxythymidine to prepare dinucleotide analogues (**5a** and **5b**) which contain a 2′→5′-amide linkage. These dimer units have been incorporated into deoxynucleotide dodecamers using solid-phase phosphoramidite chemistry. Thermal melting studies show that a single 2′→5′-amide linkage in a deoxyoligonucleotide has a considerable destabilising effect on duplexes formed with both the DNA and RNA complementary sequences. Interestingly, the amide linkage has a significantly greater destabilising influence in the DNA duplexes than in the RNA hybrids.

Introduction

DNA or RNA bearing exclusively 2′→5′ phosphodiester bonds (isoDNA/RNA) has been shown to associate with itself and both DNA and RNA in a manner that is consistent with duplex formation through standard Watson–Crick base pairs.[1–6] Intriguingly, isoDNA forms a heteroduplex with RNA that is almost as stable as the comparable natural DNA–RNA duplex, yet it binds only relatively weakly with complementary DNA and isoDNA.[7,8] Although the isoDNA–RNA duplexes are not substrates for *E. coli* RNase H, the selective binding of isoDNA to RNA makes it a strong candidate for therapeutic antisense applications. Indeed, chimeric 2′→5′ linked oligonucleotides containing seven contiguous and centrally placed 3′→5′ phosphorothioate residues, as a putative RNase H recognition site, show the ability to inhibit the expression of steroid 5 alpha reductase in cell culture.[9]

To date relatively little work on oligonucleotides bearing dephospho 2′→5′ linkages has appeared in the scientific literature. Pudlo *et al.* have investigated the duplex stability of oligonucleotides containing either 2′→5′ formacetal (**1**) or thioformacetal (**2**) linkages and shown that the former linkage has RNA binding properties that are almost comparable to the 3′→5′-phosphodiester control.[10] Dinucleotide analogues have also been prepared with a 2′-O-carboxylic ester linkage[11] and previously we have prepared a 2′-C-acetamido linked analogue of a dinucleoside monophosphate (**3a**) by opening uridine 2′-C, 3′-O-γ-butyrolactone with 5′-amino-5′-deoxythymidine.[12] It is worth noting that the analogous 3′→5′-amide linkage (**4**) has been shown to be one of the most successful phosphodiester replacements in terms of a potential therapeutic antisense agent.[13] The amide linkages offer some degree of rigidity which leads to preorganisation of the backbone in a conformation that is favourably disposed for duplex formation and as a result 2′-deoxyoligonucleotides containing this linkage exhibit RNA binding properties that are very similar to the natural DNA oligomers.

We now describe the synthesis of two suitably protected 2′→5′-acetamido linked analogues (**5a**, **5b**) of UpT, the

1, X=O
2, X=S

3a, R¹=H, R²=OH
3b, R¹=R²=H
5a, R¹=DMT, R²=OTBDMS
5b, R¹=DMT, R²=H

4

T=thymin-1-yl
U=uracil-1-yl

incorporation of these dimer units into a dodecadeoxynucleotide and T_m studies on duplexes formed with complementary ribo- and deoxyribo-oligonucleotides.

Results and discussion

An initial consideration at the inception of this study was uncertainty as to whether dimer **3a** would undergo lactonisation (Scheme 1) during oligonucleotide synthesis–deprotection, resulting in chain cleavage of the oligomer at the amide linkage. Our concern originated from an earlier observation[14] that the amide **6** undergoes slow lactonisation in solution and a report by Rosenthal and Baker[15] that a related dimethyl amide was susceptible to thermal lactonisation at 60 °C. In a more recent study we have shown that in the case of dimer **3a**,
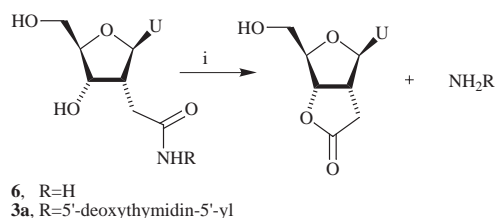
Scheme 1 *Reagents and conditions:* i, acetic acid–water (8 : 2) at 50 °C, half-life 115 and 930 min for **6** and **3a**, respectively.

the lactonisation process is slow, even when heated in aqueous acetic acid.[12] However, it was deemed desirable to also prepare the 2′→5′-acetamido linked dinucleoside without a 3′-hydroxy group (**3b**) in order to establish whether the absence of this group would have any subtle effect on duplex stability.

Synthesis of acetamide-linked dinucleosides 5a and 5b

The synthesis of the suitably protected dimers (**5a** and **5b**) started from the previously reported 2′-deoxy-2′-α-*C*-(diphenyl-methoxycarbonylmethyl)uridine (**7**, Scheme 2). Tritylation of
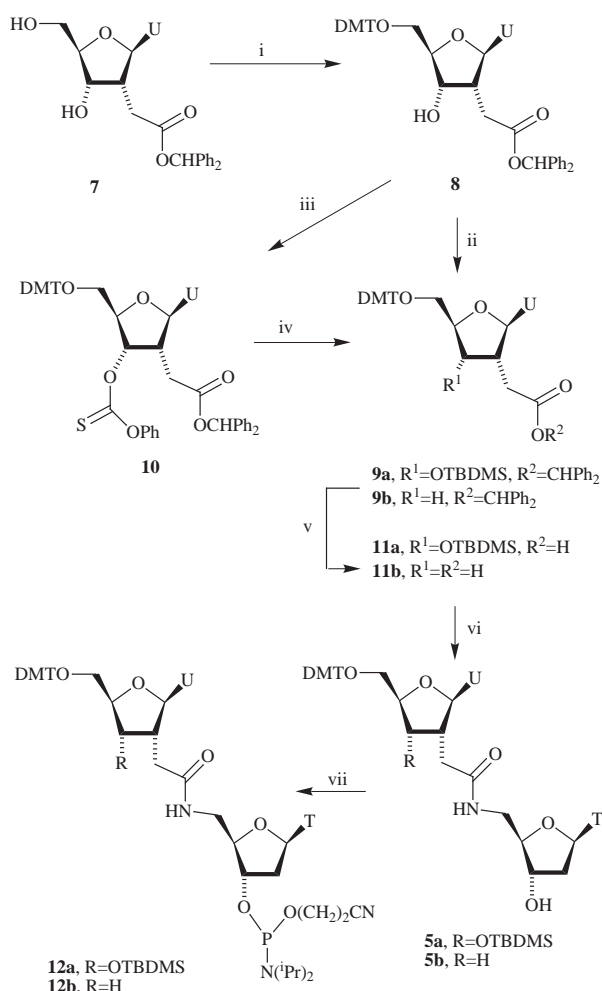
Scheme 2 *Reagents and conditions:* i, 4′,4′-dimethoxytrityl chloride, pyridine, CH₂Cl₂; ii, TBDMS triflate, pyridine; iii, PhOC(S)Cl, DMAP, NEt₃, CH₂Cl₂; iv, Bu₃SnH (3 equiv.), AIBN, toluene, 65 °C; v, H₂, Pd–C, THF–MeOH (3 : 1); vi, *N*-hydroxysuccinimide, DCC, DMAP, 5′-amino-5′-deoxythymidine, 1,4-dioxane; vii, 2-cyanoethoxy bis(*N*,*N*-diisopropylamino)phosphine, diisopropylammonium tetrazolide, CH₂Cl₂.

7 under standard conditions gave the dimethoxytrityl derivative **8** in good yield. For the preparation of dimer **5a** the *tert*-butyldimethylsilyl (TBDMS) group was chosen to protect the 3′-position of the uridine moiety. The use of this blocking group which is routinely used in RNA synthesis would enable

us to use established protocols for the deprotection of oligo-nucleotides containing **5a**. Treatment of alcohol **8** with excess TBDMS triflate (3 equiv.) in dry pyridine gave the fully protected nucleoside **9a** in a low yield of 44% following isolation by flash chromatography.

In the case of the 3′-deoxy dimer **5b** deoxygenation was achieved by a standard Barton-type procedure. Thus, alcohol **8** was converted to the thiocarbonate **10** in 67% yield by reaction with *O*-phenyl chlorothioformate and was subsequently reduced by treatment with excess tributyltin hydride and AIBN in toluene, to yield the 3′-deoxy derivative **9b** in 88% yield. The *C*3′ atom of **9b** was observed at 35.36 ppm in the ¹³C NMR spectrum and was shifted significantly upfield from its position (83.98 ppm) in the thiocarbonate **10**. Removal of the benzhydryl group from **9a** and **9b** was achieved by hydrogenolysis using 10% palladium on charcoal in MeOH–THF, to give the carboxylic acids **11a** and **11b** in yields of 77 and 67%, respectively. 5′-Amino-5′-deoxythymidine, which was required for the lower half of the dimers, was prepared in two steps from thymidine by an established procedure.[16,17]

Previous studies on 3′→5′-amide linked dinucleosides had revealed that amide formation could be achieved very efficiently from TBDMS-protected nucleoside carboxylic acids, using TBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate).[18] The condensation of **11a** with 5′-amino-5′-deoxythymidine was initially investigated using TBTU as previously described.[19] Using these conditions a poor isolated yield (27%) of the dimer **5a** was obtained and the paucity of the product was attributed to partial loss of the dimethoxytrityl (DMT) group. Subsequently much higher yields were obtained (90% for **5a** and 86% for **5b**) using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide to effect the condensation. Dimers **5a** and **5b** were made ready for solid-phase synthesis by conversion to their 3′-*O*-phosphonamidites **12a** and **12b** respectively, using 2-cyanoethoxy bis(*N*,*N*-diisopropylamino)phosphine and diisopropylammonium tetrazolide under standard conditions.[20,21] The ³¹P NMR spectra of both **12a** and **12b** showed a characteristic pair of singlets at around 149 ppm resulting from the diastereomeric phosphorus centre.

Synthesis of and studies on oligonucleotide duplexes containing 2′→5′ amide linkages

The duplexes that were selected to assess the effect of a single amide linkage on duplex stability are shown in Table 1 and are based on a previously studied non-self-complementary DNA duplex (duplex **G** in Table 1). Fawthrop *et al.*[22] have completely assigned the ¹H NMR spectrum of this duplex and these data would be of considerable assistance in providing structural information on the duplexes containing the amide linkage. As the amide linkage was constructed from a uridine nucleoside, a corresponding uracil base was incorporated into the top strand of the control duplexes (entries **A** and **D**) to enable the direct comparison of results. Oligonucleotides were assembled using procedures based on standard protocols although modifications to the coupling times and deprotection conditions were introduced (see Experimental section). Oligonucleotides containing amide linkages were characterised by MALDI-TOF mass spectrometry and gave the expected molecular ions [3529.46 for d(CCT AAA TU × T GCC) and 3545.46 for d(CCT AAA TU^OH × T GCC)].

As anticipated, melting curves for duplexes **A**–**F** showed a single transition and T_m values were determined from the average of three experiments. Comparison of the data for the two control duplexes showed that the DNA–DNA duplex (entry **D**, Table 1) had a higher melting temperature than the DNA/RNA hybrid duplex (entry **A**, Table 1). This is in agreement with data previously published for duplexes containing mixed pyrimidine–purine sequences.[23–25] Entries **A**–**C** show T_m values for duplexes containing the complementary RNA strand.

Table 1 T_m values for duplexes prepared in this study

Entry	Duplexes with RNA	$T_m/°C^a$	$\Delta T_m/°C$
A (control)	5′-d(CCT AAA TUT GCC) 3′-r(GGA UUU AAA CGG)	38.9	—
B	5′-d(CCT AAA TUT × GCC) 3′-r(GGA UUU AA A CGG)	31.2	−7.7
C	5′-d(CCT AAA TU^{OH} × T GCC) 3′-r(GGA UUU AA A CGG)	30.9	−8.0
	Duplexes with DNA		
D (control)	5′-d(CCT AAA TUT GCC) 3′-d(GCA TTT AAA CGG)	45.4	—
E	5′-d(CCT AAA TU × T GCC) 3′-d(GGA TTT AA A CGG)	32.7	−12.7
F	5′-d(CCT AAA TU^{OH} × T GCC) 3′-d(GGA TTT AA A CGG)	32.0	−13.4
G	5′-d(CCT AAA TTT GCC) 3′-d(GGA TTT AAA CGG)	—	—

U×T   U^{OH}×T
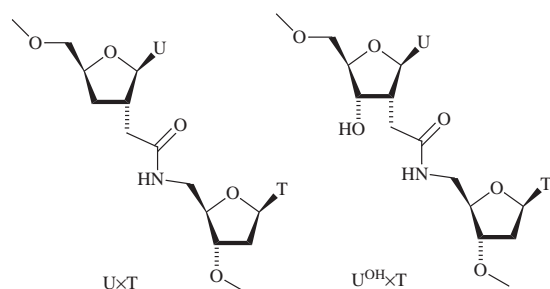
^{a} For details of experimental conditions see Experimental section.

Comparison of the data for these three duplexes reveals the following: i) considerable destabilisation of the duplex structure results from the incorporation of a single $2′→5′$ amide linkage; ii) the presence (entry **C**, T_m 30.9 °C) or absence (entry **B**, T_m 31.2 °C) of a 3′-hydroxy group adjacent to the amide linkage does not have a significant influence on duplex stability.

For the duplexes formed with the DNA complement (entries **D**–**F**) the trends in the T_m data are similar to those observed with the RNA hybrids. Interestingly, comparison of entries **B** and **E** and also **C** and **F** reveal that the amide linkage has a significantly greater destabilising influence in the DNA duplexes than in the RNA hybrids. Comparison of entries **E** and **F** suggests that the presence of the 3′-hydroxy group adjacent to the amide exerts a small destabilising effect (−0.7 °C) and could result from the hydroxy group inducing unfavourable steric interactions and/or adversely affecting the sugar conformation. Indeed molecular modelling studies performed by Pudlo *et al.*[10] suggest that in comparison to the $3′→5′$ linkage, there is reduced steric space for a $2′→5′$ connection and the introduction of a 3′-OH group may result in unfavourable steric interactions. The results discussed in this study are necessarily derived from a restricted set of oligonucleotides and it is therefore difficult to make general assumptions about the effect of the amide modifications. However, preliminary studies incorporating the dimer **3a** into other DNA sequences suggest that the extent of the destabilisation is dependent on the sequence and, as expected, the reduction in the T_m per modification is reduced in longer duplexes.[26] In the case of double substitution the destabilisation was approximately additive.

In order to improve the design of future antisense constructs it is important to recognise specific features that cause a modification to either stabilise or destabilise the duplex structure. Comparison of ^{1}H NMR data previously obtained[22] for duplex

G with preliminary spectra acquired for the analogous amide-containing duplex **E** indicates that the two structures are almost identical in the regions flanking the modification. However, at the immediate site of the modification the spectra show that there is some degree of structural heterogeneity consistent with the amide-linkage producing increased conformational flexibility. A more detailed structural analysis of duplex **E** will be reported at a later date.

Experimental

General experimental procedures and spectroscopic instrumentation are as previously reported.[12] Peaks in NMR spectra displaying obvious diastereomeric splitting are denoted with an asterisk.

2′-Deoxy-2′-α-C-(diphenylmethoxycarbonylmethyl)-5′-O-dimethoxytrityluridine (8)

2′-Deoxy-2′-α-C-(diphenylmethoxycarbonylmethyl)uridine (3.18 g, 7.03 mmol) was dried by coevaporation with dry pyridine and dissolved in the same solvent (32 cm³). Dimethoxytrityl chloride (4.04 g, 11.93 mmol) was dissolved in a mixture of dry pyridine and dry dichloromethane (4.5 cm³ : 16.1 cm³ respectively), added to the nucleoside solution dropwise over 30 min and the mixture stirred for a further 1 hour. Methanol (0.5 cm³) was added and after a further 15 min saturated aq. NaHCO$_3$ (1.0 cm³) was also added and the mixture was then concentrated *in vacuo* to a thick oil. The residue was coevaporated twice with toluene (2 × 100 cm³), diluted with CH$_2$Cl$_2$ (100 cm³) and washed with saturated aq. NaHCO$_3$ (2 × 50 cm³) and brine (50 cm³). The aqueous layers were combined and back extracted with CH$_2$Cl$_2$ (100 cm³) and the organic layers dried (MgSO$_4$) and evaporated. The crude product was purified by column chromatography (CH$_2$Cl$_2$ containing an increasing gradient of MeOH from 0–2%) to yield the required DMT derivative as a white foam (4.35 g, 82%). δ_H (400 MHz, CDCl$_3$) 2.62–2.77 (2H, m, H2′, H6′), 2.94 (1H, dd, $J_{6″-2′}$ 11.5, $J_{6″-6′}$ 17.6, H6″), 3.40 (2H, m, H5′, H5″), 3.79 (6H, s, OCH$_3$), 4.10 (1H, m, H4′), 4.54 (1H, dd, J 2.2, 5.2, H3′), 5.39 (1H, dd, J 2.2, J_{5-6} 8.2, H5), 6.05 (1H, d, $J_{1′-2′}$ 8.1, H1′), 6.82 (4H, d, J 8.7, ArH *o*-OCH$_3$), 6.88 (1H, s, OC*H*Ph$_2$), 7.24–7.35 (19H, m, ArH), 7.64 (1H, d, J_{6-5} 8.2), 8.20 (1H, br s, NH); δ_C (75.5 MHz, CDCl$_3$) 29.88 (C6′), 45.96 (C2′), 55.12 (OCH$_3$), 63.42 (C5′), 72.98 (C3′), 77.95 (*C*HPh$_2$), 85.65 (O*C*Ar$_3$), 86.96 (C4′), 87.64 (C1′), 102.80 (C5), 113.31 (ArC), 126.91 (ArC), 127.18 (ArC), 128.01 (ArC), 128.17 (ArC), 128.64 (ArC), 130.11 (ArC), 134.78 (ArC), 139.44 (C6), 139.63 (ArC), 144.10 (ArC), 150.31 (C2), 158.51 (ArC), 162.23 (C4), 171.12 (C7′); *m/z* (FAB$^+$) 777 (M + Na$^+$, 0.4%), 755 (M + H$^+$, 1.9%), 754 (M$^+$, 2.2%), 303 (DMT$^+$, 91.8%), 167 (Ph$_2$CH$^+$, 100%) (Found: C, 71.17; H, 5.61; N, 3.68. C$_{45}$H$_{42}$N$_2$O$_9$ requires C, 71.60; H, 5.61; N, 3.76%).

2′-Deoxy-2′-α-C-(diphenylmethoxycarbonylmethyl)-3′-O-tert-butyldimethylsilyl-5′-O-dimethoxytrityluridine (9a)

2′-Deoxy-2′-α-C-(diphenylmethoxycarbonylmethyl)-5′-O-dimethoxytrityluridine (100 mg, 0.13 mmol) was coevaporated with dry pyridine (2 × 5 cm³) and then dissolved in the same solvent (1 cm³). To a stirred solution of the nucleoside, *tert*-butyldimethylsilyl triflate (90 μl, 0.40 mmol) was added under a nitrogen atmosphere. The mixture was left stirring for 5 hours, then the solvent was removed *in vacuo* to leave an oily residue. The residue was diluted with CH$_2$Cl$_2$ (10 cm³) and washed with saturated aq. NaHCO$_3$ (2 × 5 cm³), H$_2$O (5 cm³), dried (MgSO$_4$) and evaporated *in vacuo* to yield the crude product which was purified by column chromatography (CH$_2$Cl$_2$ containing an increasing gradient of MeOH from 0–1%) to yield a white foam (52 mg, 44%). δ_H (400 MHz, CDCl$_3$) −0.16 (3H, s, SiCH$_3$), −0.10 (3H, s, SiCH$_3$), 0.80 (9H, s, *t*-Bu), 2.53 (1H, dd, $J_{6′-2′}$ 4.3, $J_{6′-6″}$ 17.8, H6′), 2.73 (1H, m, H2′), 2.88 (1H, dd, $J_{6″-2′}$

Left column:

9.0, $J_{6''-6'}$ 17.7, H6''), 3.36 (2H, m, H5', H5''), 3.78 (6H, s, OCH$_3$), 4.04 (1H, m, H4'), 4.52 (1H, d, J 5.1, H3'), 5.41 (1H, d, J_{5-6} 8.0, H5), 6.06 (1H, d, $J_{1'-2'}$ 9.3, H1'), 6.81 (4H, d, J 8.7, ArH o-CH$_3$), 6.88 (1H, s, OCHPh$_2$), 7.18–7.42 (19H, m, ArH), 7.59 (1H, d, J_{6-5} 8.0, H6), 8.52 (1H, br s, NH); δ_{C} (100.6 MHz, CDCl$_3$) −5.35 (SiCH$_3$), −4.66 (SiCH$_3$), 17.83 (Si-C(CH$_3$)$_2$), 25.64 (C(CH$_3$)$_3$), 29.66 (C6'), 45.84 (C2'), 55.22 (OCH$_3$), 63.63 (C5'), 74.11 (C3'), 77.30 (CHPh$_2$), 87.08 (OCAr$_3$), 87.14 (C4'), 87.57 (C1'), 102.72 (C5), 113.30 (ArC), 127.07 (ArC), 127.17 (ArC), 127.92 (ArC), 128.11 (ArC), 128.50 (ArC), 130.04 (ArC), 135.29 (ArC), 139.89 (C6), 140.08 (ArC), 144.08 (ArC), 150.53 (C2), 158.67 (ArC), 162.70 (C4), 170.82 (C7') (Found: FAB HRMS m/z (M − H)$^-$, 867.3702. C$_{51}$H$_{55}$N$_2$O$_9$Si requires (M − H)$^-$, 867.3677).

2'-Deoxy-2'-α-C-(diphenylmethoxycarbonylmethyl)-3'-O-phenoxythiocarbonyl-5'-O-dimethoxytrityluridine (10)

To a stirred, ice-cold, nitrogen-flushed solution of 2'-deoxy-2'-α-C-(diphenylmethoxycarbonylmethyl)-5'-O-dimethoxytrityl-uridine (8) (133 mg, 0.18 mmol) in dry CH$_2$Cl$_2$ (1.86 cm^3) was added triethylamine (27 µl, 0.19 mmol) and 4-(N,N-dimethylamino)pyridine (24 mg, 0.19 mmol). O-Phenyl chlorothioformate (37 µl, 0.26 mmol) was subsequently added dropwise under nitrogen and the mixture was then left to react at room temperature for 16 hours. The solvent was evaporated to leave an oily residue which was dissolved in ethyl acetate (5 cm^3). The organic solution was washed with saturated aq. NaHCO$_3$ (3 cm^3) and brine (3 cm^3), dried (MgSO$_4$) and evaporated in vacuo to yield the crude product as a yellow oil. The required compound was obtained following purification by column chromatography (CH$_2$Cl$_2$ containing an increasing gradient of MeOH from 0–0.5%) yielding the pure product as a pale yellow foam (105 mg, 67%). δ_{H} (400 MHz, CDCl$_3$) 2.75 (1H, dd, $J_{6'-2'}$ 5.78, $J_{6'-6''}$ 17.6, H6'), 2.97 (1H, dd, $J_{6''-2'}$ 7.87, $J_{6''-6'}$ 17.6, H6''), 3.24 (1H, m, H2'), 3.40 (2H, m, H5', H5''), 3.77 (6H, s, OCH$_3$), 4.41 (1H, m, H4'), 5.37 (1H, d, J_{5-6} 8.03, H5), 6.06 (1H, d, $J_{1'-2'}$ 5.46, H1'), 6.20 (1H, d, J 9.15, H3'), 6.80 (4H, d, J 8.77, ArH, o-CH$_3$), 6.88 (1H, s, OCHPh$_2$), 6.93 (2H, m, o-ArH), 7.19–7.41 (22H, m, ArH), 7.65 (1H, d, J_{6-5} 8.19, H6), 8.28 (1H, br s, NH); δ_{C} (75.5 MHz, CDCl$_3$) 28.61 (C6'), 43.63 (C2'), 54.48 (OCH$_3$), 63.48 (C5'), 78.00 (CHPh$_2$), 83.47 (OCAr$_3$), 83.70 (C4'), 83.98 (C3'), 86.74 (C1'), 102.33 (C5), 112.76 (ArC), 121.03 (ArC), 126.19 (ArC), 126.41 (p-ArC), 126.52 (ArC), 126.68 (ArC), 127.36 (ArC), 127.46 (ArC), 127.97 (ArC), 128.99 (ArC), 129.46 (m-Ar), 134.32 (ArC), 139.06 (C6), 143.44 (ArC), 152.61 (C2), 158.20 (ArC), 162.26 (C4), 169.52 (C7'), 193.39 (C=S); m/z (FAB$^+$) 891 (M + H$^+$, 0.73%), 890 (M$^+$, 1.86%), 303 (DMT$^+$, 100%), 167 (Ph$_2$CH$^+$, 93.61%), 154 (OC(S)OPh$^+$, 4.21%) (Found: C, 69.70; H, 5.05; N, 2.94. C$_{52}$H$_{46}$N$_2$O$_{10}$S requires C, 70.10; H, 5.20; N, 3.14%).

2',3'-Dideoxy-2'-α-C-(diphenylmethoxycarbonylmethyl)-5'-O-dimethoxytrityluridine (9b)

2'-Deoxy-2'-α-C-(diphenylmethoxycarbonylmethyl)-3'-O-phenoxythiocarbonyl-5'-O-dimethoxytrityluridine (100 mg, 0.11 mmol) was dissolved in dry toluene (3.5 cm^3). Tributyltin hydride (0.15 cm^3, 0.56 mmol) and AIBN (15 mg, 0.09 mmol) were added to the solution and the flask was degassed with N$_2$. The mixture was heated for 8 hours at 65 °C. The solvent was removed in vacuo and the crude product purified by column chromatography (100% petroleum ether (bp 60–80 °C) then 60% petroleum ether–40% ethyl acetate) to give the required product as a white foam (73 mg, 88%). δ_{H} (200 MHz, CDCl$_3$) 1.77 (1H, m, H3'), 2.30 (1H, m, H3''), 2.56–2.89 (3H, m, H2', H6', H6''), 3.21 (1H, dd, $J_{5'-4'}$ 3.85, $J_{5'-5'}$ 10.72, H5'), 3.36 (1H, dd, $J_{5''-4'}$ 2.75, $J_{5''-5'}$ 10.45, H5''), 3.78 (6H, s, OCH$_3$), 4.29 (1H, m, H4'), 5.35 (1H, d, J_{5-6} 8.24, H5), 5.80 (1H, d, $J_{1'-2'}$ 4.95, H1'), 6.80 (4H, d, J 8.8, ArH, o-OCH$_3$), 6.87 (1H, s, OCHPh$_2$), 7.18–7.45 (19H, m, ArH), 7.73 (1H, d, J_{6-5} 8.25, H6), 8.46 (1H, br s,

NH); δ_{C} (75.5 MHz, CDCl$_3$) 30.94 (C6'), 35.36 (C3'), 40.12 (C2'), 54.40 (OCH$_3$), 63.49 (C5'), 78.48 (CHPh$_2$), 83.80 (OCAr$_3$), 88.70 (C4'), 89.84 (C1'), 101.81 (C5), 112.62 (ArC), 126.38 (ArC), 126.52 (ArC), 127.17 (ArC), 127.40 (ArC), 127.53 (ArC), 128.38 (ArC), 128.52 (ArC), 129.46 (ArC), 130.02 (ArC), 135.85 (ArC), 139.20 (C6), 139.95 (ArC), 150.03 (C2), 158.10 (ArC), 162.30 (C4), 170.03 (C7'); m/z (FAB$^+$) 739 (M + H$^+$, 2.58%), 738 (M$^+$, 2.39%), 303 (DMT$^+$, 96.15%), 167 (Ph$_2$CH$^+$, 100%) (Found: HRMS (FAB$^+$) m/z (M + H$^+$), 739.3038. C$_{45}$H$_{43}$N$_2$O$_8$ requires (M + H$^+$), 739.3019).

General procedure for the removal of the diphenylmethyl protecting group

The ester (0.27 mmol) was dissolved in MeOH–THF (9 : 3 cm^3) contained in a flask (50 cm^3) with a side arm. The mixture was degassed with N$_2$ before addition of (10%) Pd–C (≈10 mg). The flask was evacuated using the water pump, and then charged with H$_2$; this operation was repeated twice and after the third time the flask was left stirring under a H$_2$ atmosphere. After 4 hours, the flask was evacuated and flushed with N$_2$. The charcoal was filtered through Celite and the solvent removed in vacuo to give a clear oil which was purified by column chromatography (CH$_2$Cl$_2$ containing an increasing gradient of MeOH from 0–5%).

2'-Deoxy-2'-α-C-carboxymethyl-3'-O-tert-butyldimethylsilyl-5'-O-dimethoxytrityluridine (11a).

White foam (77%); δ_{H} (400 MHz, CD$_3$OD) −0.12 (3H, s, SiCH$_3$), −0.05 (3H, s, SiCH$_3$), 0.77 (9H, s, t-Bu), 2.26 (1H, dd, $J_{6''-2'}$ 4.69, $J_{6''-6'}$ 17.0, H6''), 2.52 (1H, dd, $J_{6'-2'}$ 8.81, $J_{6'-6''}$ 17.0, H6'), 2.77 (1H, m, H2'), 3.28 (2H, m, H5', H5''), 3.65 (6H, s, OCH$_3$), 3.91 (1H, m, H4'), 4.45 (1H, d, J 4.11, H3'), 5.30 (1H, d, J_{5-6} 8.22, H5), 5.88 (1H, d, $J_{1'-2'}$ 9.39, H1'), 6.75 (4H, d, J 8.22, ArH, o-OCH$_3$), 7.11–7.34 (9H, m, ArH), 7.65 (1H, d, J_{6-5} 8.22, H6); δ_{C} (75.5 MHz, CDCl$_3$) −5.25 (SiCH$_3$), 17.85 (SiC(CH$_3$)$_2$), 25.62 (C(CH$_3$)$_3$), 29.58 (C6'), 46.05 (C2'), 55.20 (OCH$_3$), 63.27 (C5'), 75.24 (C3'), 86.56 (OCAr$_3$), 87.17 (C4'), 89.04 (C1'), 103.36 (C5), 113.34 (ArC), 127.19 (ArC), 128.03 (ArC), 128.16 (ArC), 130.04 (ArC), 135.26 (ArC), 139.69 (C6), 144.02 (ArC), 152.50 (C2), 158.79 (ArC), 163.15 (C4), 177.50 (C7'); m/z (FAB$^-$) 701 (M − H$^-$, 5.2%), 569 (M − H − HTBDMSO$^-$, 8.92%), 111 (uracil$^-$) (Found: HRMS (FAB$^-$) m/z (M − H$^-$), 701.2911. C$_{38}$H$_{45}$N$_2$O$_9$Si requires (M − H$^-$), 701.2894).

2',3'-Dideoxy-2'-α-C-carboxymethyl-5'-O-dimethoxytrityluridine (11b).

White foam (67%); δ_{H} (400 MHz, CDCl$_3$) 1.83 (1H, m, H3'), 2.30 (1H, m, H3'), 2.59–2.80 (3H, m, H2', H6', H6''), 3.10 (1H, dd, $J_{5'-4'}$ 3.58, $J_{5'-5''}$ 10.45, H5'), 3.29 (1H, dd, $J_{5''-4'}$ 2.75, $J_{5''-5'}$ 10.45, H5''), 4.29 (1H, m, H4'), 5.35 (1H, d, J_{5-6} 8.25, H5), 5.80 (1H, d, $J_{1'-2'}$ 4.95, H1'), 6.80 (4H, d, J 8.8, ArH, o-OCH$_3$), 7.25–7.36 (9H, m, ArH), 7.74 (1H, d, J_{6-5} 8.25, H6), 8.35 (1H, br s, NH); δ_{C} (75.5 MHz, CDCl$_3$) 31.74 (C6'), 36.16 (C3'), 40.92 (C2'), 55.20 (OCH$_3$), 64.29 (C5'), 84.60 (OCAr$_3$), 87.50 (C4'), 88.64 (C1'), 101.51 (C5), 113.36 (ArC), 126.91 (ArC), 127.71 (ArC), 128.10 (ArC), 128.28 (ArC), 130.20 (ArC), 135.96 (ArC), 136.14 (ArC), 141.02 (C6), 144.28 (ArC), 152.36 (C2), 158.84 (ArC), 162.23 (C4), 172.12 (C7'); m/z (FAB$^+$) 595 (M + Na$^+$, 0.75%), 573 (M + H$^+$, 2.31%), 572 (M$^+$, 2.91%), 303 (DMT$^+$, 100%) (Found: HRMS (FAB$^+$) m/z (M + H$^+$), 573.2246. C$_{32}$H$_{33}$N$_2$O$_8$ requires (M + H$^+$), 573.2237).

General procedure for the synthesis of the 2'→5'-linked amide dimers

The carboxylic acid (0.65 mmol), 1,3-dicyclohexylcarbodiimide (0.85 mmol), DMAP (0.2 mmol), and N-hydroxysuccinimide (0.85 mmol) were dissolved in dry dioxane (11 cm^3) and stirred overnight under N$_2$. The dicyclohexylurea was removed by filtration and washed with dioxane. To the filtrate was added

5′-amino-5′-deoxythymidine (0.98 mmol) dissolved in pyridine (2 cm³) and the mixture stirred. After 2.5 hours the mixture was diluted with CH_2Cl_2 (50 cm³), and the solution washed with aq. $NaHCO_3$ (75 cm³), brine (50 cm³) and dried ($MgSO_4$). The aqueous layers were back extracted with CH_2Cl_2 (2 × 50 cm³) and dried ($MgSO_4$). The organic solvent was removed *in vacuo* and coevaporated with toluene. The crude amide dimer was purified by column chromatography (CH_2Cl_2 containing an increasing gradient of MeOH 0–3%).

5′-O-(Dimethoxytrityl)-3′-(O-*tert*-butyldimethylsilyl)-2′-deoxy-2′-α-C-[N-(5′-deoxythymidin-5′-yl)carbamoylmethyl]uridine (5a).

White foam (90%); δ_H (400 MHz, CD_3OD) −0.11 (3H, s, $SiCH_3$), 0.05 (3H, s, $SiCH_3$), 0.77 (9H, s, *t*-Bu), 1.84 (3H, s, CH_3), 2.24 (2H, m, H2′, H2″T), 2.38 (1H, dd, $J_{6′-2′}$ 6.4, $J_{6′-6″}$ 15.4, H6′U), 2.42 (1H, dd, $J_{6′-2′}$ 6.9, $J_{6′-6′}$ 15.4, H6″U), 2.96 (1H, m, H2′U), 3.39 (2H, m, H5′, H5″T), 3.76 (6H, s, OCH_3), 3.84 (3H, m, H4′T, H5′U, H5″U), 4.01 (1H, m, H4′), 4.22 (1H, m, H3′T), 4.52 (1H, m, H3′U), 5.38 (1H, d, J_{5-6} 8.17, H5U), 6.02 (1H, d, $J_{1′-2′}$ 8.75, H1′U), 6.16 (1H, t, $J_{1′-2′}$ 6.74, H1′T), 6.80 (4H, d, J 8.9, Ar*H*, *o*-OCH_3), 7.20–7.50 (9H, m, Ar*H*) 7.52 (1H, d, J 1.2, H6T), 7.76 (1H, d, J_{6-5} 8.16, H6U); δ_C (75.5 MHz, $CDCl_3$) −5.00 ($SiCH_3$), −5.37 ($SiCH_3$), 12.21 (CH_3T), 17.94 (Si*C*(CH_3)$_3$), 25.76 (C(CH_3)$_3$), 31.47 (C6′U), 38.80 (C2″T), 40.50 (C5″T), 46.03 (C2′U), 55.38 (OCH_3), 63.81 (C5′U), 70.62 (C3′), 75.20 (C3′), 85.10 (C4′), 86.08 (C4′), 87.18 (O*C*Ar$_3$), 87.32 (C1′), 88.60 (C1′), 102.82 (C5U), 111.18 (C5T), 113.62 (ArC), 128.30 (ArC), 128.43 (ArC), 130.34 (ArC), 135.56 (ArC), 138.90 (ArC), 140.87 (C6), 144.49 (C6), 151.23 (C2), 151.40 (C2), 159.02 (ArC), 164.17 (C4), 164.31 (C4), 172.02 (C7′U); *m/z* (FAB⁺) 948 (M + Na⁺, 3.79%), 926 (M + H⁺, 7.15%) (Found: HRMS (FAB⁺) *m/z* (M + H⁺), 926.4031. $C_{48}H_{60}N_5O_{12}Si$ requires (M + H⁺), 926.4008).

5′-O-(Dimethoxytrityl)-2′,3′-dideoxy-2′-α-C-[N-(5′-deoxy-thymidin-5′-yl)carbamoylmethyl]uridine (5b).

White foam, (86%); δ_H (400 MHz, CD_3OD) 1.87 (3H, s, CH_3), 1.91 (1H, m, H3′U), 2.24 (2H, m, H2′, H2″T), 2.31 (1H, m, H3″U), 2.42 (1H, dd, $J_{6′-2′}$ 8.14, $J_{6′-6″}$ 14.61, H6′U), 2.52 (1H, dd, $J_{6″-2′}$ 8.00, $J_{6″-6′}$ 14.61, H6″U), 2.83 (1H, m, H2′U), 3.29 (2H, m, H5′, H5″T), 3.46 (2H, m, H5′, H5″U), 3.76 (6H, s, OCH_3), 3.90 (1H, m, H4′T), 4.25 (1H, m, H4′U), 4.32 (1H, m, H3′T), 5.29 (1H, d, J_{5-6} 8.10, H5U), 5.81 (1H, d, $J_{1′-2′}$ 5.31, H1′U), 6.14 (1H, t, $J_{1′-2′}$ 6.91, H1′T), 6.84 (4H, d, J 8.9, Ar*H*, *o*-OCH_3), 7.21–7.43 (9H, m, ArH), 7.51 (1H, d, J 1.2, H6T), 7.88 (1H, d, J_{6-5} 8.10, H6U); δ_C (101.6 MHz, $CDCl_3$) 12.27 (CH_3), 31.82 (C6′U), 37.56 (C3′U), 39.44 (C2′T), 40.90 (C5′T), 41.97 (C2′U), 55.16 (OCH_3), 64.74 (C5′U), 71.44 (C3′T), 83.51 (C4′), 84.91 (C4′), 86.55 (O*C*Ar$_3$), 88.75 (C1′), 89.77 (C1′), 102.35 (C5U), 111.13 (C5T), 111.45 (ArC), 113.18 (ArC), 126.94 (ArC), 127.90 (ArC), 128.09 (ArC), 130.04 (ArC), 135.37 (ArC), 137.10 (C6), 140.35 (C6), 144.41 (ArC), 150.89 (C2), 151.27 (C2), 158.52 (ArC), 163.92 (C4), 164.20 (C4), 171.55 (C7′U); *m/z* (FAB⁺) 796 (M + H⁺, 1.45%), 303 (DMT⁺, 95.68%) (Found: HRMS (FAB⁺) *m/z* (M + H⁺), 796.3176. $C_{42}H_{46}N_5O_{11}$ requires (M + H⁺), 796.3194).

General procedure for the preparation of dinucleoside-3′-O-phosphonamidites

2-Cyanoethoxy bis(*N,N*-diisopropylamino)phosphine (1.59 mmol) was added to a solution of the dinucleoside (0.53 mmol) and diisopropylammonium tetrazolide (2.65 mmol) in CH_2Cl_2 (16 cm³) at 0 °C. The reaction mixture was stirred overnight at room temperature. The mixture was diluted with CH_2Cl_2 (20 cm³) and washed with saturated aq. $NaHCO_3$ (2 × 10 cm³), the aqueous layers were combined and back extracted with CH_2Cl_2 (2 × 10 cm³) and the organic layers dried ($MgSO_4$) and evaporated. The crude nucleoside phosphoramidite was purified by column chromatography (ethyl acetate containing 0.1% triethylamine) to yield the product as a white foam. The product

was dissolved in CH_2Cl_2 (20 cm³) and washed with saturated aq. $NaHCO_3$ (10 cm³) and the organic layer dried ($MgSO_4$). The product was precipitated by addition of the CH_2Cl_2 solution to pentane (60 cm³) and isolated by filtration.

5′-O-(Dimethoxytrityl)-3′-O-*tert*-butyldimethylsilyl-2′-deoxy-2′-α-C-{N-[5′-deoxy-3′-O-(2-cyanoethoxy-*N,N*-diisopropyl-amino)phosphinothymidin-5′-yl]carbamoylmethyl}uridine (12a).

White amorphous solid (91%); δ_H (400 MHz, d_6-acetone) 0.05 (3H, s, $SiCH_3$), 0.08 (3H, s, $SiCH_3$), 0.83 (9H, s, *t*-Bu), 1.12 (12H, m, 4 × CH_3), 1.22 (2H, m, CH_2CN), 1.77 (3H, s, CH_3), 2.05 (4H, m, 2 × Me$_2$C*H*N, NCC*H*$_2$C*H*$_2$O), 2.32 (2H, m, H2′T, H2″T), 2.58 (2H, m, H6′U, H6″U), 2.90 (1H, m, H2′U), 3.32 (2H, m, H5′T, H5″T), 3.45 (1H, m, H2′U), 3.65 (2H, m, H5′U, H5″U), 3.76 (6H, s, OCH_3), 4.01 (1H, m, H4′T), 4.49 (1H, m, H3′T), 4.62 (1H, m, H3′U), 5.35 (1H, d, J_{5-6} 8.04, H5U), 5.86*, 5.92* (1H, t, J 6.91, 7.92, H1′T), 6.16 (1H, d, $J_{1′-2′}$ 6.75, H1′U), 6.87 (4H, d, J 8.66, Ar*H*, *o*-OCH_3), 7.21–7.48 (9H, m, ArH), 7.16*, 7.18* (1H, s, H6T), 7.60*, 7.62* (1H, d, J 8.2, H6U); δ_P (162 MHz, d_6-acetone) 149.60, 149.67; *m/z* (ES⁺) 1149 (M + Na⁺, 12.1%), 1126 (M + H⁺, 100%), 303 (DMT⁺, 11%) (Found: C, 59.57; H, 6.82; N, 8.48. $C_{57}H_{76}N_7O_{13}PSi·H_2O$ requires C, 59.82; H, 6.88; N, 8.57%).

5′-O-(Dimethoxytrityl)-2′,3′-dideoxy-2′-α-C-{N-[5′-deoxy-3′-O-(2-cyanoethoxy-*N,N*-diisopropylamino)phosphinothy-midin-5′-yl]carbamoylmethyl}uridine (12b).

White amorphous solid (82%); δ_H (300 MHz, $CDCl_3$) 1.18 (12H, m, 4 × CH_3), 1.22 (2H, m, CH_2CN), 1.82 (3H, s, CH_3T), 2.03 (5H, m, 2 × Me$_2$C*H*N, NCC*H*$_2$C*H*$_2$O, H3′U), 2.35 (3H, m, H2′T, H2″T, H3″U), 2.54 (2H, m, H6′U, H6″U), 2.86 (1H, m, H2′U), 3.36 (2H, m, H5′T, H5″T), 3.54 (2H, m, H5′U, H5″U), 3.81 (6H, s, OCH_3), 3.87 (1H, m, H4′T), 4.08 (1H, m, H4′U), 4.38 (1H, m, H3′T), 5.39 (1H, d, J_{5-6} 8.10, H5U), 5.70*, 5.78* (1H, d, J 4.8, J 3, H1′U), 6.20*, 6.26* (1H, t, J 7.2, H1′T), 6.82 (4H, d, J 9.0, Ar*H*, *o*-OCH_3), 7.09*, 7.14* (1H, s, H6T), 7.19–7.42 (9H, m, ArH), 7.80*, 7.83* (1H, d, J 8.2, H6U); δ_P (162 MHz, d_6-acetone) 149.94, 150.21; *m/z* (FAB⁺) 996 (M + H⁺, 13%), 895 (M − $^iPr_2N^+$, 29%) (Found: C, 60.41; H, 6.38; N, 9.65. $C_{51}H_{62}N_7O_{12}P·H_2O$ requires C, 60.40; H, 6.36; N, 9.67%).

Synthesis and purification of oligonucleotides

Solid-phase synthesis of the oligodeoxynucleotides and oligo-ribonucleotides were carried out on a 1 µmol scale using procedures based on those previously reported[22] although modifications to the coupling times and deprotection conditions were introduced. Coupling times for the dimer phosphonamidites 12a,b were extended to 10 min. For the removal of the 2′-O-TBDMS groups from the RNA oligonucleotides the dried oligomers were dissolved in DMSO (150 µl) and $Et_3N·3HF$ (750 µl) and heated at 50 °C for 4 h. The RNA was precipitated by the addition of a few drops of 1 M NH_4Cl and after removal of the supernatant, the precipitate was dissolved in water (1 cm³) in preparation for ion-exchange chromatography.

For the removal of the 3′-O-TBDMS group from the oligo-nucleotide derived from amidite 12a, the dried oligomer was dissolved in DMSO (150 µl) and $Et_3N·3HF$ (150 µl) and heated at 50 °C for 4 h. Triethylammonium salts were removed by reversed-phase HPLC prior to purification.

All oligonucleotides were purified by ion-exchange chromatography using a Dionex Nucleopac PA-100 column maintained at 55 °C, eluting with a gradient of 0.05–0.6 M aqueous NH_4Cl (pH 5.3) over 20 min with a flow rate of 1 cm³ min⁻¹. Oligonucleotides were desalted by filtration through Centricon filters (1000 Da cut-off). The final concentration of oligonucleotide solutions was determined by UV absorption and from calculated extinction coefficients.[27]

The modified oligonucleotides were characterised by MALDI-TOF mass spectrometry using a Hewlett Packard

G2025A LD TOF mass spectrometer and diammonium hydrogen citrate–2,6-dihydroxyacetophenone as an ionisation matrix; m/z 3529.46 for d(CCT AAA TU × T GCC) ($C_{117}H_{151}N_{41}O_{69}P_{10}$) and 3545.46 for d(CCT AAA TU^{OH} × T GCC) ($C_{117}H_{151}N_{41}O_{68}P_{10}$).

Melting studies

UV melting curves were determined using a Hewlett Packard HPUV8452A diode array spectrophotometer, with attached HP89090A Peltier unit, controlled *via* a PC. The sample temperature was monitored by a probe immersed in the sample. The sample was contained in a quartz cell of path length 1 cm and volume of 1300 µl. Average heating and cooling rates were around $0.2\,°C\ min^{-1}$. The melts were performed at a duplex concentration of 3.0 µM in 20 mM phosphate buffer (pH 6.8) and 100 mM KCl. All values are averages from at least 3 experiments. The absolute error in the T_m values is $±0.5\,°C$.

Acknowledgements

References

1 R. Kierzek, L. He and D. H. Turner, *Nucleic Acids Res.*, 1992, **20**, 1685.
2 J. P. Dougherty, C. J. Rizzo and R. Breslow, *J. Am. Chem. Soc.*, 1992, **114**, 6254.
3 H. Hashimoto and C. Switzer, *J. Am. Chem. Soc.*, 1992, **114**, 6255.
4 R. Z. Jin, W. H. Chapman, A. R. Srinivasan, W. K. Olson, R. Breslow and K. J. Breslauer, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 10568.
5 K.-E. Jung and C. Switzer, *J. Am. Chem. Soc.*, 1994, **116**, 6059.
6 H. Robinson, K.-E. Jung, C. Switzer and A. H.-J. Wang, *J. Am. Chem. Soc.*, 1994, **116**, 6059.
7 T. L. Sheppard and R. Breslow, *J. Am. Chem. Soc.*, 1996, **118**, 9811.
8 T. P. Prakash, K.-E. Jung and C. Switzer, *Chem. Commun.*, 1996, 1793.
9 P. Bhan, A. Bhan, M. K. Hong, J. G. Hartwell, J. M. Saunders and G. D. Hoke, *Nucleic Acids Res.*, 1997, **25**, 3310.
10 J. S. Pudlo, X. Cao, S. Swaminathan and M. D. Matteucci, *Tetrahedron Lett.*, 1994, **35**, 9315.
11 C. R. Noe, N. Windhab and G. Haberhauer, *Archiv. Pharm. Weinheim Ger.*, 1995, **328**, 743.
12 A. J. Lawrence, J. B. J. Pavey, M.-Y. Chan, R. A. Fairhurst, S. P. Collingwood, J. Fisher, R. Cosstick and I. A. O'Neil, *J. Chem. Soc., Perkin Trans. 1*, 1997, 2761.
13 A. De Mesmaeker, R. Häner, P. Martin and H. E. Moser, *Acc. Chem. Res.*, 1995, **28**, 366.
14 A. J. Lawrence, J. B. J. Pavey, R. Cosstick and I. A. O'Neil, *Tetrahedron Lett.*, 1995, **36**, 6341.
15 A. Rosenthal and D. A. Baker, *J. Org. Chem.*, 1973, **38**, 198.
16 I. Yamamoto, M. Sekine and T. Hata, *J. Chem. Soc., Perkin Trans. 1*, 1980, 306.
17 W. S. Mungall, G. L. Greene, G. A. Heavner and R. L. Letsinger, *J. Org. Chem.*, 1975, **40**, 1659.
18 A. De Mesmaeker, A. Waldner, J. Lebreton, P. Hoffmann, V. Fritsch and R. M. Wolf, *Angew. Chem., Int. Ed. Engl.*, 1994, **33**, 226.
19 R. Knorr, A. Trezeciak, W. Bannwarth and D. Gillessen, *Tetrahedron Lett.*, 1989, **30**, 733.
20 J. Nielsen, M. Taagaard, J. E. Marugg, J. H. van Boom and O. Dahl, *Nucleic Acids Res.*, 1986, **14**, 7391.
21 R. Kierzek, M. H. Caruthers, C. E. Longfellow, D. Swinton, D. H. Turner and S. M. Frier, *Biochemistry*, 1986, **25**, 7840.
22 S. A. Fawthrop, J. C. Yang and J. Fisher, *Nucleic Acids Res.*, 1993, **21**, 4860.
23 K. B. Hall and L. W. McLaughlin, *Biochemistry*, 1991, **30**, 10606.
24 L. Ratmeyer, R. Vinyak, Y. Y. Zhong, G. Zon and W. D. Wilson, *Biochemistry*, 1994, **33**, 5298.
25 S. Wang and E. T. Kool, *Biochemistry*, 1995, **34**, 4125.
26 M.-Y. Chan, R. A. Fairhurst, S. P. Collingwood, R. Cosstick and I. A. O'Neil, unpublished observations.
27 J. D. Puglisi and I Tinoco, *Methods Enzymol.*, 1989, **180**, 304.

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