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Targeting Chimeric α,β-Oligonucleotides to the Flanks of a Stem in DNA. The Enhanced Effect of an Intercalator

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3′-*O*-(4,4′-Dimethoxytrityl)-5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine (**7**) was prepared from α-thymidine by the reaction of 1-pyrenylmethylamine with an appropriate protected 4-(1,2,4-triazolyl)-α-thymidine derivative **5**. 3′-*O*-(4,4′-Dimethoxytrityl-α-thymidine (**9**) was synthesized by successive, 5′-*O*-acetylation, 3′-*O*-tritylation and 5′-*O*-deacetylation of α-thymidine. Phosphitylation of the 5′-hydroxy group of **7** and **9** afforded the amidites **8** and **10**. These amidites and commercial β-2′-deoxyribonucleoside-3′-phosphoramidites were used in the synthesis of chimeric ODNs with 3′-3′ and 5′-5′ internucleotidic phosphodiester linkages. The stability of a DNA three way junction (TWJ) was studied by observing the thermal melting when a DNA was targeted to the flanks of a hairpin. The TWJ was considerably stabilized when **7** and **9** were inserted into the junction region. The corresponding duplex obtained by deleting the hairpin, however, was destabilized on insertion of **7** and **9**.

The strategy of using synthetic oligodeoxynucleotides (ODNs) complementary to mRNA or double-stranded DNA to impair gene expression in living cells has provided the impetus to design and develop oligonucleotide analogues for therapeutic purposes.[1] As a consequence, there has been a need for synthesis of ODNs chemically modified in the nucleobase, in the carbohydrate, or in the internucleoside linkage.[2,3] In an effort to develop a class of antisense ODNs that would display affinity to complementary DNA and RNA sequences and resistance to the nucleolytic activity of nucleases, alternating α,β-ODNs with additional alternating (3′-3′) and (5′-5′) phosphodiester linkages have been proposed.[4] The antisense potential of these compounds was illustrated by their involvement in RNase H-mediated arrest of reverse transcription *in vitro*.[5] Koga *et al.*[6] have demonstrated by gel electrophoresis and UV denaturation studies that stable duplexes can be formed on targeting ODNs containing multiple polarity reversals which were generated by combination of 3′-3′ and 5′-5′ phosphodiester bonds to the complementary DNA and RNA sequences. Moreover, it has been demonstrated that a 10mer ODN, in which the α-anomeric nucleotides are in a parallel orientation via 3′-3′ and 5′-5′ phosphodiester linkages, can bind its modified β-DNA analogue forming stable duplexes which are of potential importance in the area of antisense therapy.[7] Investigations on a parallel-stranded DNA hairpin have demonstrated that unusual phosphodiester linkages 5′-5′ and 3′-3′ can force the stem to adopt a parallel configuration, featuring reverse Watson–Crick base pairing between the complementary strands.[8]

Stable hybridisation has been obtained on targeting a 17mer ODN to non-adjacent single stranded DNA containing a hairpin structure. Formation of Watson–Crick base-pairs with the two single-stranded regions flanking the hairpin structure in the DNA resulted in a three-way junction (TWJ).[9] TWJ are commonly found in naturally occurring RNA molecules, with 5*S* ribosomal RNA constituting a notable example,[10] comprised of three double helical arms that are connected in a branch point to form a Y-shaped structure. TWJ could be stabilized with the addition of unpaired or 'bulged' bases to one of the strands at the branch point,[11] while TWJs lacking unpaired bases appear to be conformationally flexible.[12] Formation of TWJ has been confirmed by NMR studies on complexes with two unpaired bases at the branch point.[13] The NMR work indicated a preferred coaxial base stacking interaction across the branch point. On the basis of this finding, we have studied[14] TWJ stability on targeting a 18mer ODN, containing an intercalating moiety as an unpaired nucleobase, to DNA fragments

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containing hairpin structures. We found improved stabilities of the TWJ when such an 18mer, hybridized to the flanks at the bottom of a hairpin, contained an intercalating moiety in the junction region.

Oligonucleotide derivatives with intercalating aromatic fragments have been used as gene probes[15] and as agents for inhibiting gene expression.[16] The synthesis and the properties of oligonucleotide–intercalator conjugates has been investigated in a number of studies.[17–19]

Considering the stability of duplex α,β chimeric ODN containing 3′-3′ and 5′-5′ phosphodiester linkages with respect to β-targets prompted us to investigate the propensity of a targeting ODN, containing an inserted α anomeric intercalating nucleoside with 3′-3′ and 5′-5′ phosphodiester linkages, to the β-sequences binding to the flanks at bottom of stem in DNA containing a hairpin structure. We now report an easy synthesis of a 2′-deoxy-5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine derivative and how insertion of this compound into a β-ODN through 3′-3′ and 5′-5′ phosphodiester bonds can stabilize a three-way junction when inserted into the junction region.

Results

*Synthesis of phosphoramidite building blocks **8** and **10**.* The phosphoramidite **8** with a conjugated intercalator was prepared as shown in Scheme 1. Acylation of commercially available α-thymidine (**1**) was accomplished with acetic anhydride in pyridine affording **2** and **3** which were separated by column chromatography in 40 and 53% yield, respectively. Protection of the free 3′-hydroxy group was achieved with 4,4′-dimethoxytrityl chloride in anhydrous pyridine to afford **4** in 86% yield. Activation of C-4 on thymine was achieved by treating **4** with putative tris(1*H*-1,2,4-triazol-1-yl)phosphine oxide[20] in the presence of 1,2,4-triazole and triethylamine in acetonitrile at room temperature affording the triazole derivative **5** in 91% yield. Commercial 1-pyrenylmethylamine hydrochloride was allowed to react with **5** in the presence of triethylamine in DMF at 80 °C for 2 h to give **6** in 76% yield.

Deacetylation of compound **6** was carried out in 1 M sodium methoxide at room temperature to afford **7** in 96% yield. Subsequent reaction of **7** with 2-cyanoethyl-*N,N*-diisopropylphosphoramidochloridite and *N,N*-diisopropylethylamine in anhydrous CH_2Cl_2 gave the phosphoramidite **8** in 80% yield after column chromatographic purification and precipitation from petroleum ether.

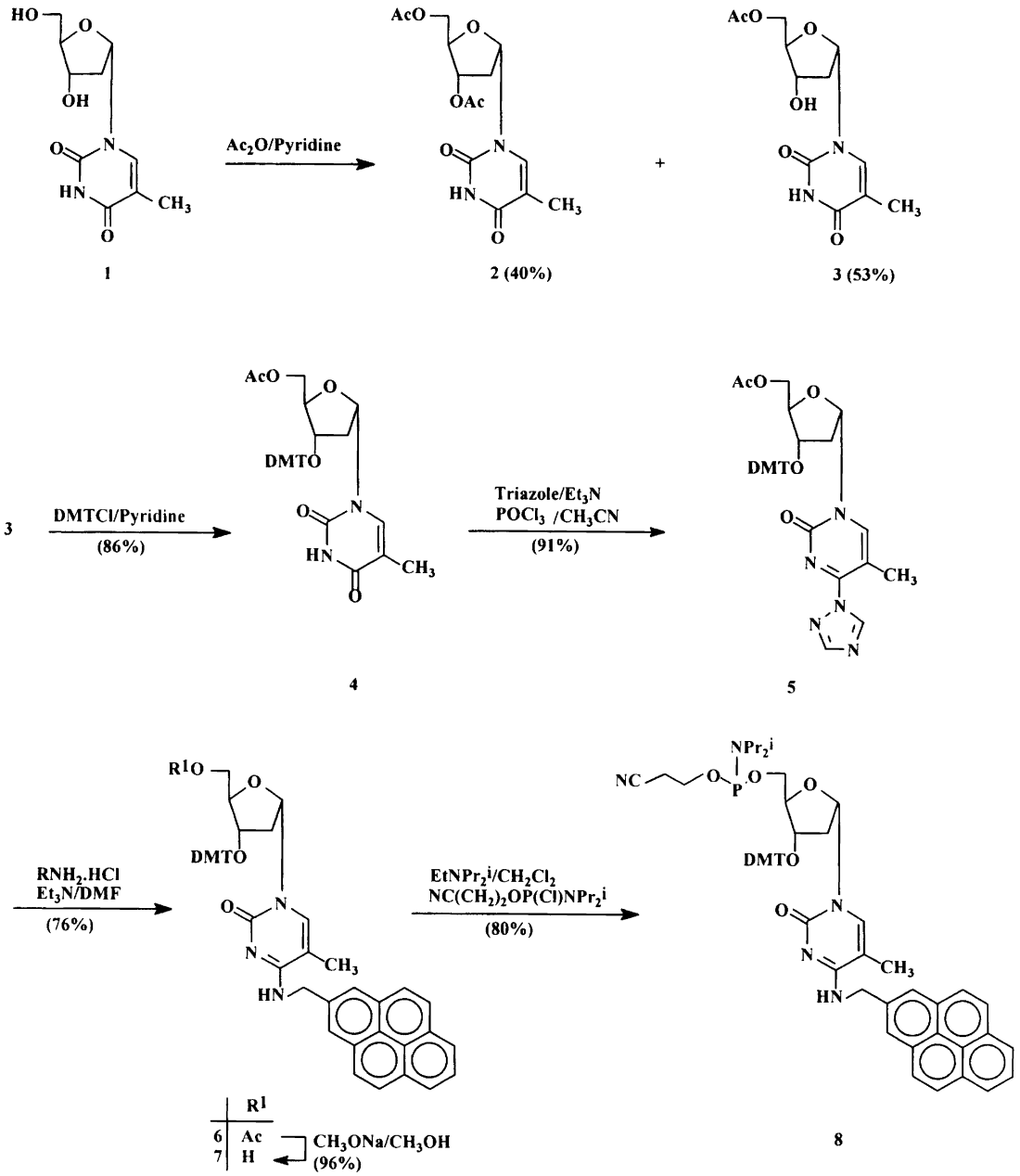
Deacetylation of the protected α-thymine (**4**) was carried out in 1 M sodium methoxide (Scheme 2) at room temperature to give **9** in 95% yield as a white foam. Phosphitylation of the 5′-hydroxy group of **9** was performed according to the reported method[7] and the product was purified by column chromatography and precipitation from petroleum ether. The phosphoramidites **8** and **10** were found to be 100% pure according to ^1H and ^{31}P NMR spectroscopy. The amidites **8** (X) and **10** (T) were used for automated synthesis of ODNs.

Synthesis of oligodeoxynucleotides. Oligonucleotides **A–F** (Table 1) were synthesized on a Pharmacia Gene Assembler Special DNA-synthesizer in 0.2 μmol scale following standard phosphoramidite methodology[21] using **8** and **10** and commercial 2′-deoxynucleoside β-cyanoethyl phosphoramidites. The coupling efficiencies (8 min couplings) for the modified phosphoramidites were approximately 89% for amidite **8** and approximately 90% for amidite **10** compared with approximately 99% for standard phosphoramidites (2 min couplings). The efficiency of each coupling step was monitored by release of dimethoxytrityl cation after each coupling step. Removal from the solid support and deprotection was carried out in 25% ammonia at room temperature for 72 h. All ODNs were desalted using Pharmacia NAP-10 columns. The purity of the ODNs was confirmed by HPLC using a Resource Q 1 ml ion-exchange column with gradient elution (10 mM NaOH and 10 mM NaOH – 1.8 M NaCl, 0–53 min). Concentrations of ODNs were determined by UV absorbance at 260 nm using the molar extinction coefficient for modified and unmodified nucleotides.[22] Insertion of the modified nucleosides into the strands **C–E** was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry showing *m/z* in the range 5558.20–5558.64 (calcd. *m/z* 5558.46). The samples were prepared using a 3-hydroxypicolinic acid matrix.[23] The ODNs were observed in the MALDI mass spectra as singly protonated species.

UV thermal denaturation. The ability of ODNs **A–F** to hybridize to their complementary DNA strands was examined by UV melting measurements at 260 nm. The melting points T_m were determined as the maximum of the first derivative of the melting curve. ODN TWJs (Table 1) and ODN duplexes (Table 2) were carried out in medium salt buffer, 2 mM EDTA, 20 mM Na_2HPO_4 and 280 mM NaCl at pH 7.0, using equimolar amounts (3 μM) of each strand. Before each experiment, all the samples were heated at 90 °C in water bath for 5 min and then cooled slowly to 0 °C.

For hairpin DNA we observed considerable stabilization (Table 1) upon insertion of the reversed N^4-pyrenylmethyl-α-cytidine (**8**) and reversed αT **10** in the targeting β-ODN (**C–F**) through 3′-3′ and 5′-5′ phosphodiester linkages at the middle of the TWJ. The largest increase ($\Delta T_m = 18$ °C) in melting temperature was observed for sequence **E** with the modified intercalating base inserted into the targeting β-ODN together with replacement of T with αT at the 5′ end of the modified base (Fig 1.).

The stabilization of the TWJ was compared with the stabilization of the corresponding duplex obtained by deletion of the hairpin in the TWJ. For 24mer DNA (Table 2), the modified β-strands (**C–F**) result in approximately 1.2–3.6 °C destabilization of the duplex when compared with the unmodified β-strand **A**. On insertion

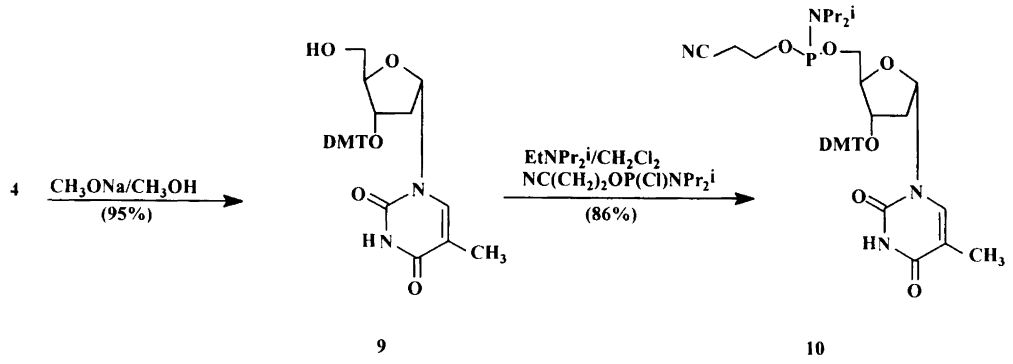


Scheme 1.

of 2-deoxy-β-cytidine, the duplex with the bulged strand **B** was considerably destabilized ($\Delta T_m = 9.2\,^{\circ}C$).

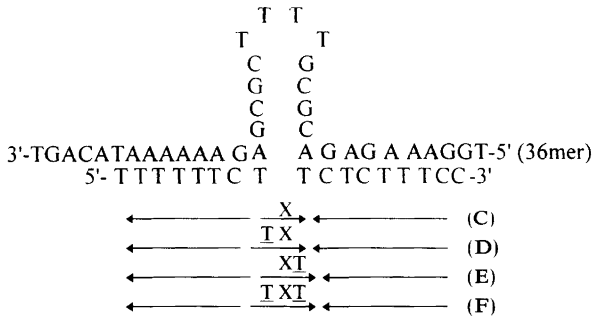
Discussion

Formation of a stable duplex that contained a parallel segment embedded in an overall antiparallel helix was found[7] upon incorporation of αT into otherwise fully β-DNA using 3′-3′ and 5′-5′ phosphodiester linkages to invert the strand polarity and to provide an α-anomeric base with a local parallel stranded environment. Bearing in mind that promising TWJ stabilities were found[14] when 5′-*O*-DMT-protected 5-methyl-N^4-(1-pyrenylmethyl)-β-cytidine was inserted into the junction region, the corresponding 3′-*O*-DMT-α-nucleoside was considered a promising intercalating nucleoside for the present



Scheme 2.

Table 1. Hybridization data (T_m/°C) for hybridization at the flanks of a stem with a complementary ODN. ΔT_m is relative to natural β-ODN **A**.



Sequence		Inserted base	T_m/°C	ΔT_m/°C
5'TTTTTTCTTCTCTTTCC3'	**(A)**	–	27.6	–
5'TTTTTTCTCTCTCTTTCC3'	**(B)**	C	30.0	2.4
5'TTTTTTCT3'-3'X5'-5'TCTCTTTCC3'	**(C)**	X	40.4	12.8
5'TTTTTTC3'-3'TX5'-5'TCTCTTTCC3'	**(D)**[a]	TX	39.2	11.6
5'TTTTTTCT3'-3'XT5'-5'CTCTTTCC3'	**(E)**[a]	XT	45.6	18.0
5'TTTTTTC3'-3'TXT5'-5'CTCTTTCC3'	**(F)**[a]	TXT	43.2	15.6

[a] T is αT.

Table 2. Hybridization data (T_m/°C) for duplexes formed when the hairpin of the 36mer is deleted. ΔT_m is relative to natural β-ODN **A**.

Sequence	T_m/°C	ΔT_m/°C
A	47.6	–
B	38.4	−9.2
C	46.4	−1.2
D	44.4	−3.2
E	45.6	−2.0
F	44.0	−3.6

work. 3'-*O*-DMT-protected 5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine **7** was synthesized in a few steps from commercially available α-thymidine. Appropriately protected α-2'-deoxyribonucleosides **7** and **9** were converted into the corresponding 5'-phosphoramidite derivatives **8** and **10**. These and commercial β-2'-deoxyribonucleoside 3'-phosphoramidites were used to form chimeric α,β-ODNs with 3'-3' and 5'-5' internucleotide phosphodiester linkages.

For our investigation of hybridization to the foot of a stem in a hairpin in ODNs by chimeric α,β-ODNs (**C–F**), we selected a sequence for the hairpin containing ODN (Table 1) which had previously been confirmed[9] by spectroscopic measurements and chemical reactions to form a TWJ when hybridized at both flanks of the stem at an ODN.

It is obvious that the hybridization of chimeric α,β-ODN (**C–F**) with the hairpin complex is stabilized more (ΔT_m = 11.6–18.0 °C) than the hybridization of the natural strand **A** with the hairpin, and this is probably due to the insertion of the α-pyrenylmethyl-modified linkage into the TWJ region. The large increase in thermal stability is remarkable when compared with previously reported results[14] for the same hairpin ODN when hybridized with the corresponding modified complementary β-ODN containing the same intercalating β nucleo-

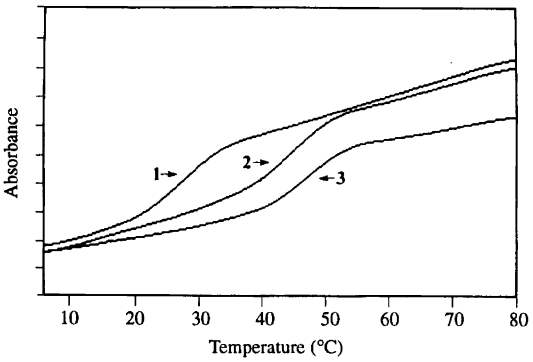


Fig. 1. Melting curves measured at 260 nm at pH 7.0 at 3 μM in each strand for **1**, natural strand (**A**) with hairpin (36mer); **2**, modified strand (**E**) with hairpin (36mer); **3**, natural strand (**A**) forming duplex with complementary strand (24mer).

side and normal 3′-5′ phosphodiester linkages. In this case only a 6.4 °C increase in the melting temperature was observed when the β-pyrenyl-modified anomer was inserted into the middle of the TWJ region. The larger increase in stability is also remarkable when compared with previous results of inserting αT with parallel orientation into a duplex, which resulted in lower thermal stabilities.[6,7,24]

We observed a positional effect in the stability of the TWJ when there was a neighbouring αT (T) to the parallel stranded 5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine (X). As seen from Table 1, the highest stabilization ($\Delta T = 18.0$ °C) was observed when T was replaced with αT at the 5′-end (**E**) of the inserted parallel strand, whereas replacement at the other side (**D**) of the intercalating nucleoside X did not result in any further stabilization. Replacements on both sides (**F**) resulted in some further stabilization but not as high as that for **E**. The positional effect may be correlated to coaxial stacking, and a determining factor for this could depend on the sequence of the base-pairs immediately flanking the junction region.[25]

The above stabilization should also be compared with the small effect ($\Delta T_m = 2.4$ °C) achieved upon insertion of extra 2′-deoxy-β-cytidine into the middle of the TWJ region and when β-thymidine (1.5 °C)[9] is inserted into the middle of the TWJ. The observed difference in the melting temperature (10.4 °C) on going from inserted β-cytidine to the pyrenemethyl-modified base is ascribed to improved recognition of the stem region by the intercalating base and the presence of unusual phosphodiester linkages (i.e., 3′-3′ and 5′-5′) which can force the α nucleosides to adopt a favourable parallel configuration featuring base pairing between the complementary strands.[8] Others have also targeted natural oligonucleotides against the hairpin structure, but only antisense oligonucleotides targeted to open regions of the loop had nearly equal affinity for transcript complement.[26]

In contrast, an extra β-cytidine, forming a bulge in the same region of the corresponding duplex (deletion of hairpin), was found to destabilize the double-helix ($\Delta T = -9.2$ °C), and this is in good agreement with previously reported results.[27] Also on the insertion of the unit **8** into the 24mer duplex, slight destabilization for the chimeric α,β-ODNs (**C–F**) ($\Delta T = -1.2$ to -3.2 °C) was observed (Table 2). It is a reasonable supposition that a bulged duplex can be stabilized by the conjugated pyrene intercalator compared with **B**. It has been reported that bulges in double-stranded DNA can be stabilized by intercalating drugs such as ethidium[28] or by a benzo-[*a*]pyrene adduct to a deoxyguanosine in the bulge.[29]

It is interesting that a TWJ can be stabilized by modifying the targeting ODN by insertion of a reversed strand containing an intercalating nucleobase. In fact, such a TWJ using strand **E** is only slightly less stable than the corresponding natural duplex of strand **A** with the 24mer (deletion of hairpin), the ΔT_m being only -2.0 °C. This may have an interesting perspective if an RNA hairpin can also form a stabilized TWJ when the targeting ODN has been modified analogously. Antisense targeting of RNA often requires disruption of the secondary structure of the RNA and a thermodynamic penalty has to be paid for this. Instead, if it is possible to design modified ODNs that can target the flanks of a stem in RNA, a large number of new targets can be envisaged for the antisense strategy. Also it should be noted that the proposed modification of the ODNs will most likely allow RNase H degradation.

Experimental

NMR spectra were recorded at 300 MHz for ^{1}H NMR, 75.5 MHz for ^{13}C NMR and 121.5 MHz for ^{31}P NMR on a Varian Gemini 2000 300 MHz spectrometer; δ-values are in ppm relative to tetramethylsilane as an internal standard (^{1}H and ^{13}C), and relative to 85% H_3PO_4 as an external standard in ^{31}P. Positive FAB mass spectra were recorded on a Kratos MS 50 RF spectrometer. Analytical silica gel TLC was performed on Merck precoated 60 F_{254} plates. The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck. Matrix-assisted laser desorption ionization mass (MALDI) mass spectra were obtained on a Bruker Reflex mass spectrometer. Melting experiments were carried out on a Perkin–Elmer UV–VIS spectrometer Lamda 2 fitted with a PTP-6 Peltier temperature programming element. The absorbance at 260 nm was increased by 1 °C min^{-1} in a 1-cm cuvette. DNA syntheses were performed on a Pharmacia Gene Assembler Special® DNA-synthesizer and HPLC of DNAs on a Waters Delta Prep 3000.

3′,5′-Di-O-acetyl-α-thymidine (**2**) *and 5′-O-acetyl-α-thymidine* (**3**). To a stirred solution of **1** (2.43 g, 10 mmol) in dry pyridine (20 ml) was added acetic anhydride (1.0 ml, 10 mmol) at 0 °C. The reaction mixture was stirred for 4 h at 0 °C and 24 h at room temperature. The solvent was removed *in vacuo* and the crude mixture purified on a silica gel column using 0–4% MeOH–Et_2O yielding

two fractions. Compound **2** was obtained from the faster eluting fraction as a white foam (1.3 g, 40%); the slower fraction yielded **3** as a white foam (1.5 g, 53%).

Compound **2**: ^{1}H NMR ($CDCl_3$): δ 1.97 (s, 3 H, CH_3), 2.06 (s, 3 H, Ac), 2.14 (s, 3 H, Ac), 2.26 (d, 1 H, *J* 15.2 Hz, 2′-H), 2.80–2.87 (m, 1 H, 2′-H), 4.23 (d, 2 H, *J* 4.3 Hz, 5′-H), 4.62 (t, 1 H, *J* 4.3 Hz, 4′-H), 5.25–5.27 (m, 1 H, 3′-H), 6.30 (dd, 1 H, *J* 2.4 and 7.3 Hz, 1′-H), 7.34 (s, 1 H, 6-H). ^{13}C NMR ($CDCl_3$): δ 12.53 (CH_3), 20.66 (Ac), 20.74 (Ac), 38.29 (C-2′), 63.71 (C-5′), 74.29 (C-3′), 84.32 (C-1′), 86.77 (C-4′), 110.36 (C-5), 135.32 (C-6), 150.63 (C-2), 164.29 (C-4), 170.05 (*C*OCH_3), 170.56 (*C*OCH_3). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 327 ($M+H^+$).

Compound **3**: ^{1}H NMR ($CDCl_3$): δ 1.82 (s, 3 H, CH_3), 2.11 (s, 3 H, Ac), 2.53 (d, 1 H, *J* 14.9 Hz, 2′-H), 2.59–2.66 (m, 1 H, 2′-H), 4.11 (d, 2 H, *J* 5.3 Hz, 5′-H), 4.42 (m, 1 H, 3′-H), 4.60 (t, 1 H, *J* 5.1 Hz, 4′-H), 6.14 (dd, 1 H, *J* 2.0 and 6.5 Hz 1′-H), 7.53 (s, 1 H, 6-H). ^{13}C NMR ($CDCl_3$): δ 12.82 (CH_3), 21.32 (Ac), 41.05 (C-2′), 64.61 (C-5′), 72.38 (C-3′), 87.55 (C-1′), 88.66 (C-4′), 109.79 (C-5), 138.19 (C-6), 151.77 (C-2), 165.55 (C-4), 171.46 (*C*OCH_3). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 285 ($M+H^+$).

5′-O-Acetyl-3′-O-(4,4′-dimethoxytrityl)-α-thymidine (**4**). Dimethoxytrityl chloride (1.88 g, 5.5 mmol) was added to a stirred solution of **3** (1.42 g, 5 mmol) in dry pyridine (20 ml) at 0 °C. After 24 h at room temperature, the excess dimethoxytrityl chloride was quenched by addition of methanol (1 ml). The mixture was concentrated *in vacuo*, the residue was dissolved in chloroform (150 ml) and the organic solution was washed with satd. aq. $NaHCO_3$, dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified on a silica gel column using 0–2% MeOH–Et_2O to give **4** (2.51 g, 86%) as a white foam. ^{1}H NMR ($CDCl_3$): δ 1.81 (d, 1 H, *J* 14.9 Hz, 2′-H), 1.89 (s, 3 H, CH_3), 2.00 (s, 3 H, Ac), 2.36–2.45 (m, 1 H, 2′-H), 3.47–3.58 (m, 1 H, 5′-H), 3.72–3.82 (m, 7 H, 2 × OCH_3, 5′-H), 4.04–4.07 (m, 1 H, 4′-H), 4.25 (d, 1 H, *J* 6.0 Hz, 3′-H), 6.20 (dd, 1 H, *J* 1.7 and 7.6 Hz, 1′-H), 6.84 (d, 4 H, *J* 9.1 Hz, arom), 7.20–7.42 (m, 9 H, arom), 7.65 (s, 1 H, 6-H). ^{13}C NMR ($CDCl_3$): δ 12.53 (CH_3), 20.70 (Ac), 39.86 (C-2′), 55.20 (2 × OCH_3), 64.23 (C-5′), 74.57 (C-3′), 85.59 (C-1′), 86.68 (C-4′), 88.29 (trityl), 110.26 (C-5), 135.70 (C-6), 150.79 (C-2), 164.45 (C-4), 113.64, 127.23, 127.93, 128.29, 129.96, 135.79, 136.71, 144.75, 159.01 (arom), 170.51 (*C*OCH_3). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 587 ($M+H^+$).

1-[5-O-Acetyl-2-deoxy-3-O-(4,4′-dimethoxytrityl)-α-D-pentofuranosyl]-5-methyl-4-(1,2,4-triazol-1-yl)pyrimidine-2(1H)-one (**5**). 1,2,4-Triazole (3.40 g, 48 mmol) was suspended in dry CH_3CN (28 ml) at 0 °C after which $POCl_3$ (0.79 ml, 10 mmol) was added with rapid stirring. Triethylamine (6.48 g, 46 mmol) was then added dropwise with stirring of the slurry at 0 °C for 30 min. A solution of **4** (2.93 g, 5 mmol) in dry CH_3CN was added dropwise at 0 °C. The ice–water bath was removed and the stirred mixture was allowed to stand for 3 h at room temperature. Triethylamine (4.5 ml, 32 mmol) and water (1.2 ml) were added and the mixture was allowed to stand for 10 min before the solvent was removed *in vacuo*. The residue was partitioned between $CHCl_3$ (150 ml) and satd. aq. $NaHCO_3$ (100 ml). The organic layer was dried (Na_2SO_4), evaporated *in vacuo* and chromatographed on a silica gel column using 0–4% MeOH–Et_2O to give **5** (2.88 g, 91%) as a pale yellow foam. ^{1}H NMR ($CDCl_3$): δ 2.03 (s, 3 H, CH_3), 2.34 (d, 1 H, *J* 15.1 Hz, 2′-H), 2.42 (s, 3 H, Ac), 2.55–2.65 (m, 1 H, 2′-H), 3.53 (dd, 1 H, *J* 4.6 and 12.0 Hz, 5′-H), 3.72–3.79 (m, 7 H, 2 × OCH_3, 5′-H), 3.84–3.93 (m, 1 H, 4′-H), 4.28 (d, 1 H, *J* 5.7 Hz, 3′-H), 6.22 (d, 1 H, *J* 6.5 Hz, 1′-H), 6.79 (d, 2 H, *J* 8.8 Hz, arom), 6.81 (d, 2 H, *J* 8.7 Hz, arom), 7.17–7.33 (m, 9 H, arom), 8.07 (s, 1 H, 6-H), 8.23 (s, 1 H, triazole 3-H), 9.40 (s, 1 H, triazole 5-H). ^{13}C NMR ($CDCl_3$): δ 16.83 (CH_3), 20.26 (Ac), 39.98 (C-2′), 54.77 (2 × OCH_3), 63.62 (C-5′), 74.00 (C-3′), 86.31 (C-1′), 87.91 (C-4′), 89.56 (trityl), 104.77 (C-5), 113.18, 113.23, 126.95, 127.37, 127.85, 129.33, (arom), 135.08 (C-6), 135.29, 144.07, 144.84, 158.59, (arom), 153.92 (C-2), 147.49, 153.12 (triazole), 158.60 (C-4), 170.12 (*C*OCH_3). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 638 ($M+H^+$).

2′-Deoxy-3′-O-(4,4′-dimethoxytrityl)-5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine (**7**). A solution of **5** (1.27 g, 2 mmol) and 1-pyrenylmethylamine hydrochloride (1.34 g, 5 mmol) in triethylamine (20 ml) and DMF (40 ml) was stirred at 80 °C for 2 h. The solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column using $CHCl_3$ to give **6** (1.21 g, 76%) as a pale yellow foam. A stirred solution of **6** (1.21 g) in dry MeOH (20 ml) was treated with 1 M sodium methoxide (5 ml). Stirring was continued at room temperature for 1 h after which deacetylation was complete according to TLC, and ammonium chloride (1.0 g) was added. The solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column using 0–1% MeOH in $CHCl_3$ to afford **7** (1.07 g, 96%) as a pale yellow foam. ^{1}H NMR ($CDCl_3$): δ 1.71 (s, 3 H, CH_3), 2.12 (d, 1 H, *J* 15.1 Hz, 2′-H), 2.50–2.59 (m, 1 H, 2′-H), 3.06–3.12(m, 1 H, 5′-H), 3.23–3.28 (m, 1 H, 5′-H), 3.68 (s, 3 H, OCH_3), 3.69 (s, 3 H, OCH_3), 3.77–3.79 (m, 1 H, 4′-H), 4.29 (d, 1 H, *J* 6.2 Hz, 3′-H), 5.25 (br.s, 1 H, NH), 5.34–5.53 (m, 2 H, CH_2), 6.32 (d, 1 H, *J* 6.3 Hz, 1′-H), 6.76–6.81(m, 4 H, arom), 7.15–7.39 (m, 9 H, arom), 7.59 (s, 1 H, 6-H), 7.94–8.30 (m, 9 H, arom). ^{13}C NMR ($CDCl_3$): δ 13.08 (CH_3), 40.84 (C-2′), 45.89 (CH_2NH), 55.13 (2 × OCH_3), 63.07 (C-5′), 74.79 (C-3′), 87.88 (C-1′), 88.05 (C-4′), 88.89 (trityl), 100.85 (C-5), 113.43–131.44 (arom), 136.22 (C-6), 136.36, 138.97, 145.17, 158.83 (arom), 156.83 (C-2), 162.97 (C-4). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 758 ($M+H^+$).

2'-Deoxy-3'-O-(4,4'-dimethoxytrityl)-5-methyl-N⁴-(1-pyrenylmethyl)-α-cytidine 5'-O-(2-cyanoethyl diisopropylphosphoramidite) (**8**). Compound **7** (320 mg, 0.42 mmol) was coevaporated with dry CH_3CN and dissolved under N_2 in anhydrous CH_2Cl_2 (5 ml). *N,N*-Diisopropylethylamine (0.4 ml) was added followed by dropwise addition of 2-cyanoethyl diisopropylphosphoramidochloride (0.17 ml, 0.76 mmol). After 2 h the reaction mixture was quenched with MeOH (0.5 ml) and diluted with EtOAc (20 ml). The solution was washed with a satd. solution of $NaHCO_3$ (3 × 15 ml), dried (Na_2SO_4) and evaporated *in vacuo*, and the residue was purified on a silica gel column (EtOAc–CH_2Cl_2–Et_3N 49:49:2). The product **8** was dissolved in anhydrous toluene (2 ml) and precipitated from ice-cooled petroleum ether (b.p. 60–80 °C). The solid product was collected by filtration and dried under reduced pressure to give **8** in 80% yield as a pale yellow powder. ^1H NMR ($CDCl_3$): δ 0.84–1.33 (m, 12 H, 4 × CH_3), 1.74 (s, 3 H, 6-CH_3), 2.13 (m, 1 H, 2'-H), 2.48–2.63 (m, 3 H, 2'-H, CH_2CN), 3.18–3.90 (m, 13 H, 5'-H, NCH, OCH_3, OCH_2, 4'-H), 4.29–4.32 (m, 1 H, 3'-H), 5.12–5.14 (m, 1 H, NH), 5.36–5.54 (m, 2 H, CH_2NH), 6.32 (m, 1 H, 1'-H), 6.77–6.8 (m, 4 H, arom), 7.16–7.39 (m, 9 H, arom), 7.59 (s, 1 H, 6-H), 8.15–8.31 (m, 9 H, arom). ^{31}P NMR ($CDCl_3$): δ 148.22 and 148.48.

3'-O-(4,4'-Dimethoxytrityl)-α-thymidine (**9**). To a stirred solution of **4** (1.5 g, 2.6 mmol) in dry MeOH (20 ml) was added 1 M sodium methoxide and the mixture was stirred at room temperature for 30 min followed by addition of ammonium chloride (1.0 g). The solvent was evaporated *in vacuo* and the crude mixture was purified by column chromatography (MeOH–Et_2O 0–5%) yielding **9** (1.32 g, 95%) as a white foam. ^{1} H NMR ($CDCl_3$): δ 1.65 (d, 1 H, *J* 14.9 Hz, 2'-H), 1.86 (s, 3 H, CH_3), 2.33–2.43 (m, 1 H, 2'-H), 3.12–3.16 (m, 1 H, 5'-H), 3.35–3.39 (m, 1 H, 5'-H), 3.78 (s, 6 H, 2 × OCH_3), 3.99–4.01 (m, 1 H, 4'-H), 4.30 (d, 1 H, *J* 6.5 Hz, 3'-H), 6.24 (dd, 1 H, *J* 1.3 and 7.7 Hz, 1'-H), 6.82 (d, 4 H, *J* 8.8 Hz, arom), 7.18–7.42 (m, 9 H, arom), 7.72 (s, 1 H, 6-H). ^{13}C NMR ($CDCl_3$): δ 12.46 (CH_3), 39. 97 (C-2'), 55.21 (2 × OCH_3), 63.04 (C-5'), 74.68 (C-3'), 86.59 (C-1'), 88.04 (C-4'), 88.82 (trityl), 110.39 (C-5), 113.56, 127.25, 128.02, 128.21, 130.03, 137.11, 144.92, 158.92 (arom), 136.01 (C-6), 150.93 (C-2), 164.37 (C-4). FAB MS ($CHCl_3$, 3-nitrobenzyl alcohol): *m/z* 545 ($M+H^+$).

3'-O-(4,4'-Dimethoxytrityl)-α-thymidine 5'-O-(2-cyanoethyl) diisopropylphosphoramidite (**10**). Compound **10** was obtained from **9** (0.87 g, 1.6 mmol) according the published procedure[7] as a white powder (86%) after chromatography (CH_2Cl_2–petroleum ether–Et_3N 49:49:2) and precipitation in ice-cooled petroleum ether. ^1H NMR ($CDCl_3$): δ 0.83–1.34 (m, 12 H, 4 × CH_3), 1.66–1.74 (m, 1 H, 2'-H), 1.89 (s, 3 H, CH_3), 2.42–2.59 (m, 3 H, 2'-H, CH_2CN), 3.12–3.79 [m, 12 H, 5'-H, $NCH(CH_3)_2$, CH_2O, 2 × OCH_3], 3.98–4.02 (m, 1 H, 4'-H), 4.31–4.35 (m, 1 H, 3'-H), 6.21–6.27 (m, 1 H, 1'-H), 6.82–6.86 (m, 4 H, arom), 7.18–7.51 (m, 9 H, arom), 7.70 (2 × s, 1 H, 6-H). ^{31}P NMR ($CDCl_3$): δ 148.42 (quintet, *J* 7.5 Hz) and 148.71 (quintet, *J* 7.1 Hz).

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