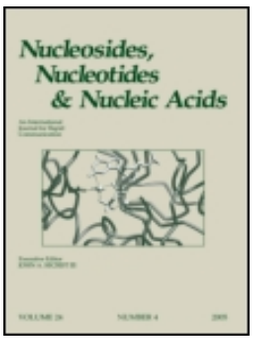

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IMPROVED TARGETING OF THE FLANKS OF A DNA STEM USING α-OLIGODEOXYNUCLEOTIDES.-THE ENHANCED EFFECT OF AN INTERCALATOR

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Dedicated to Professor Gottfried Heinisch on the occasion of his 60th birthday.

Abstract: 2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine (**5**) was prepared by reaction of 1-pyrenylmethylamine with an appropriate protected 4-(1,2,4-triazolyl)-α-thymidine derivative **3** which was synthesized from 5-*O*-DMT protected α-thymidine **1**. Aminolysis of **3** afforded 3'-*O*-acetyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (**8**). Benzoylation of **8** and removal of acetyl afforded N^4-benzoyl-2-deoxy-5-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (**10**). The amidites of compounds **5** and **10** were prepared and used in α-oligonucleotide synthesis. DNA three-way junction (TWJ) is stabilized when an α-ODN is used for targeting the dangling flanks of the stem in a DNA hairpin. Further stabilization of the TWJ is observed when **5** is inserted into the α-ODN at the junction region.

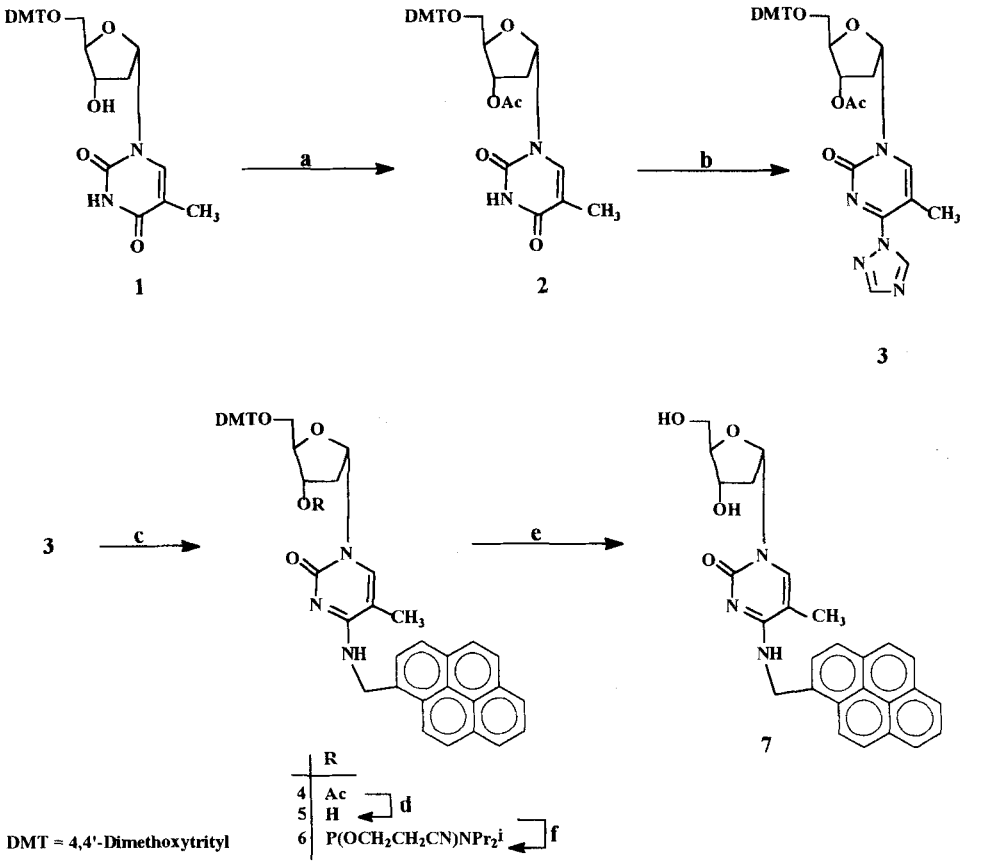
Artificial control of gene expression can be achieved from the binding of an oligonucleotide complementary to the specific messenger RNA target.[1] The specificity of such binding is ruled by Watson-Crick base-pairing interactions and, thus, provides an attractive route for the development of selective drugs. However, mRNA's are not single-stranded random coil nucleic acids but exhibit short-range and long-range internal structures such as hairpins.

Alternatively, different strategies have been considered for binding oligomers to a hairpin structure without disrupting the RNA stem and this has been exemplified by using DNA hairpins as model systems.[2,3] A stable hybridization has been obtained on targeting a natural oligopyrimidine (17mer) to non-adjacent single stranded DNA (36mer) containing a hairpin structure through the formation of Watson-Crick base-pairs with two single stranded regions flanking a hairpin structure in DNA fragment forming a three-way junction (TWJ).[4]

Unpaired nucleotides are common features of TWJ's and have been shown to stabilize junction formation.[5] Formation of the TWJ has been confirmed by NMR studies on complexes with two unpaired bases at the branch point.[6] The NMR work indicated a preferred coaxial base staking interaction across the branch point. On the basis of this finding, we have studied[7] the TWJ stability using a 18mer oligodeoxynucleotide (ODN), containing an unpaired nucleobase with an intercalating moiety, with DNA fragments containing a hairpin and we found improved stabilities of the TWJ.

α-Oligonucleotides are molecules of interest in antisence studies because they have shown improved hybridization properties to target nucleic acids with stability against nuclease degradation.[8] Olignucleotides covalently linked to intercalators have recently gained attention as sequence-specific agents having an increased ability to bind single-stranded and double-stranded DNA.[7,9] The β-deoxynucleoside units composing the natural nucleic acids were replaced by their corresponding α-anomers and were covalently linked to an intercalating agent such as an acridine[10] and/or an oxazolopyridocarbazolium[11] to increase the stability of hybridization. Considering the stability of duplexes of α-ODN's versus β target prompted us to demonstrate that the combined effects of using an α-oligodeoxynucleotide having an intercalating moiety linked to an unpaired nucleobase can bind to the flanks at the bottom of a stem in a 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 an α-ODN's can stabilize a three-way junction when inserted into the junction region. The synthesis of the nucleoside phosphoramidite **6** was performed as follows (Scheme 1). The protected nucleoside **2** was prepared in 92% yield from 5'-*O*-(4,4'-dimethoxytrityl)-α-thymidine (**1**).[12] Activation of the C-4 position on thymine was achieved by treating **2** with putative tris(1H-1,2,4-triazol-1-yl)phosphineoxide[13] in the presence of 1,2,4-triazole and triethylamine in acetonitrile at room temperature to

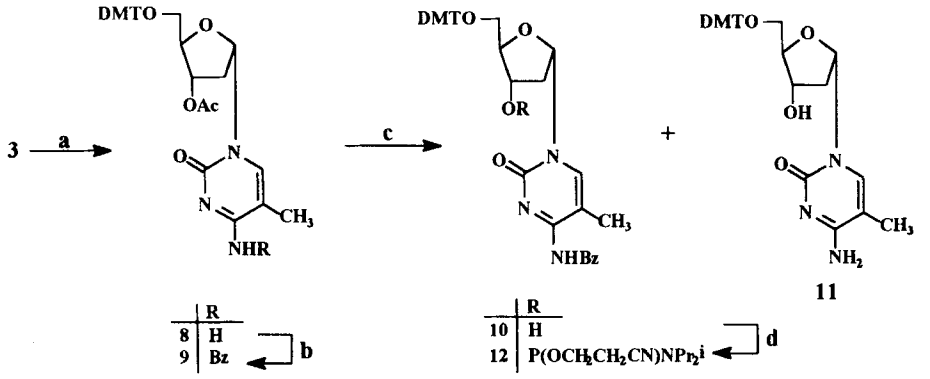
afford the triazolyl derivative **3** in 85% yield. Commercial 1-pyrenemethylamine hydrochloride was allowed to react with **3** in presence of triethylamine in DMF at 80 °C for 2 h to give **4** in 74% yield.



Scheme 1. a) Ac_2O, pyridine, b) Triazole/Et_3N, $POCl_3$/MeCN, c) 1-pyrenemethylamine hydrochloride, Et_3N/DMF, d) MeONa, MeOH, e) Cl_3CCOOH, $C_2H_4Cl_2$, f) CH_2Cl_2, $EtNPr_2^i$, $NCCH_2CH_2OP(Cl)NPr_2^i$.

Deacetylation of compound **4** was carried out in 0.2 M sodium methoxide in methanol at room temperature to afford **5** in 98% yield. Subsequent deprotection using the detritylation reagent $CCl_3COOH/C_2H_4Cl_2$ at room temperature gave the unprotected nucleoside **7** in 95% yield. Phosphitylation[14] of **5** by reaction with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloride [$NC\text{-}CH_2CH_2OP(Cl)N(Pr_2^i)$] in the presence of *N,N*-diisopropylethylamine in anhydrous dichloromethane afforded the nucleoside

phosphoramidite **6** as a white powder in 80% yield after column chromatographic purification and precipitation from petroleum ether. The phosphoramidites of 2-deoxy-5-methyl-α-cytidine was synthesized starting from the triazole derivative **3** (Scheme 2).

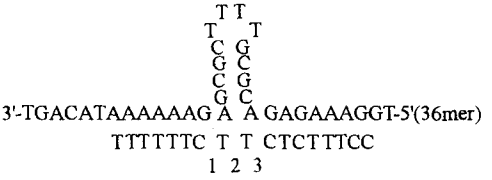


Scheme 2. a) NH_3/dioxan, b) $(PhCO)_2O$, pyridine, c) MeONa, MeOH, d) CH_2Cl_2, $EtNPr^i_2$, $NCCH_2CH_2OP(Cl)NPr^i_2$.

Aminolysis[12,15] of **3** with aqueous ammonia in dioxane gave **8** in 92% yield. After benzoylation of the exocyclic amino group with benzoic anhydride in pyridine, the deprotection of **9** with 1M $NaOCH_3$ afforded a mixture of **10** and **11** which was separated by column chromatography. Phosphoramidite **12** was obtained from **10** as a white powder in 88% yield after column chromatographic purification and precipitation from petroleum ether according to the published procedure [12] The phosphoramidites **6** and **12** were found to be 100% pure according ^{1}H-NMR and ^{31}P-NMR.

Synthesis of α-oligomers **B-H** (Table 1) was performed using standard phosphoramidite methodology[16] on a Pharmacia Gene Assembler Special DNA synthesizer using **6**,**12** and α-thymidinephosphoramidite.[16] The coupling efficiencies for the modified phosphoramidite **6** (8 min couplings) was approximately 88%. The efficiency of each coupling step was monitored by release of the 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 ODN's were desalted using Pharmacia NAP-10 columns. The purity of the ODN's was confirmed by HPLC[17] using Pharmacia Resource™ Q, 1 mL ion exchange column with gradient elution (10 mM NaOH and 10 mM NaOH/1.8 M NaCl, 0-53 min.). The extinction coefficient of compound **7** was used to calculate the molar extinction

Table 1: Hybridization data (T_m/°C) when hybridized at the flanks of a stem with a complementary α-ODN which has insertion of 7 at positions 1-3. ΔT_m is relative to natural β-ODN **A** and ΔΔT is relative to α-ODN **B**.



Sequence	Position of Insertion	Inserted Base	T_m	ΔT_m	$\Delta\Delta T_m$	MS Calcd	MS Found
5'-β (TTTTTTTCTTCTCTTTCC)-3' **(A)**			30.0				
3'-βT-α (TTTTTTCTTCTCTTTCC)-5' **(B)**[a]			45.2	15.2		5409.6	5410.0
3'-βT-α (TTTTTTCXTTCTCTTTCC)-5' **(C)**[a]	1	X = 7	53.2	23.2	8.0	5932.8	5931.9
3'-βT-α (TTTTTTCTXTCTCTTTCC)-5' **(D)**[a]	2	X = 7	56.4	26.4	11.2	5932.8	5932.1
3'-βT-α (TTTTTTCTTXCTCTTTCC)-5' **(E)**[a]	3	X = 7	48.4	18.4	3.2	5932.8	5932.2
3'-βT-α (TTTTTTCXTTCTCTTTCC)-5' **(F)**[a]	1	X = C	45.2	15.2	0.0		
3'-βT-α (TTTTTTCTXTCTCTTTCC)-5' **(G)**[a]	2	X = C	46.4	16.4	1.2		
3'-βT-α (TTTTTTCTTXCTCTTTCC)-5' **(H)**[a]	3	X = C	44.4	14.4	-0.8		

[a]/C is 5-methyl-α-cytidine

coefficient for the modified nucleotides (**B, C** and **D**) when measuring the strand concentration. It was observed that compound **7** has a 3.2 times higher absorbance than the one of 2'-deoxycytidine at 260 nm due to the presence of the pyrene moiety. The composition of the ODN's was verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The samples were prepared using a 3-hydroxypicolinic acid matrix.[18] The ODN's were observed in the MALDI mass spectra as singly protonated species. Good correlation was found between the expected and measured masses.

Hybridization studies of DNA TWJ (Table 1) and DNA duplexes (Table 2) were carried out in medium salt buffer, 1 mM EDTA, 10 mM Na_2HPO_4 and 140 mM NaCl at pH 7.0, using equimolar amounts (3 μM) from each strand. Before each experiment, all the samples were heated in a water bath at 90 °C for 5 min and then cooled slowly to 0 °C. The increase in the UV absorbance at 260 nm as a function of time was recorded while the temperature was raised linearly from 20-70 °C at a rate of 1°C/min.

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

3`-TGAC ATAAAAAAGAAGAGAAAGGT-5`(24mer)

Sequence	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
T_m/°C	48.0	58.4	55.2	55.2	54.4	54.0	50.8	50.8
ΔT_m/°C	-	10.4	7.2	7.2	6.4	6.0	2.8	2.8
$\Delta\Delta T_m$/°C	-	-	-3.2	-3.2	-4.0	-4.0	-7.6	-7.6

For hairpin stranded DNA (Table 1) we observed a considerable stabilization as measured by the change of melting point (ΔT = 15.2 °C) when ODN **A** with natural β anomers were replaced by **B** containing synthetic α anomers. Even more promising results were obtained (Table 1) when the pyrenylmethyl modified nucleoside **7** was inserted into the targeting α-strand, (**C, D** and **E**) at the middle of the TWJ or at the adjacent sites. The largest increase (ΔT = 26.4 °C) in melting temperature was obtained when the modified base was inserted into the targeting α-ODN at the middle of the TWJ.

The stabilization of TWJ was compared with the stabilization of the corresponding duplex obtained by deletion of the hairpin in the TWJ. For duplex stranded 24mer DNA (Table 2) the unmodified α-ODN **B** hybridizes better than the corresponding complementary β-strand **A** with ΔT_m = 10.4 °C. Insertion of the pyrenylmethyl nucleoside **7** into the α-strand (**C**, **D** and **H**) results in destabilization of approximately 3.2-4.0 °C when compared with **B**. Further destabilization (4.4-7.6 °C) was observed (Table 2) when 5-methyl-α-deoxycytidine was inserted instead of the intercalating nucleoside **7** into the α-strand (**F**, **G** and **H**).

For our investigation of hybridization to the foot of a stem in a hairpin in ODN's by α-oligodeoxy-nucleotides (**B-H**) we selected a sequence for the DNA containing a hairpin (Table 1) which previously has been investigated[4] by spectroscopic measurements and chemical reactions with osmium tetroxide and copper phenanthroline. Therefore we are sure that our reference ODN can form a TWJ. We assumed that the α-ODN's would bind in a parallel orientation with respect to their complementary β target sequence[19] at the flanks of the stem of the hairpin. It was convenient to use commercial βT-support at the 3'-end of the α-ODN's (**B-H**) for the ODN synthesis. This extra mismatching nucleoside at the 3'-end

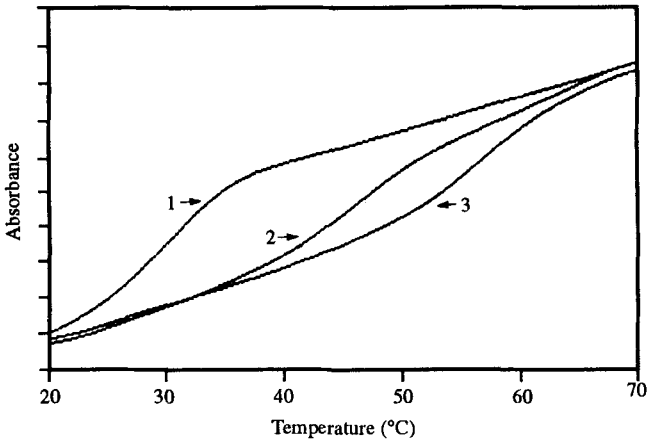


Figure 1: Melting curves of hairpin (36 mer) + ODN's measured at 260 nm at pH 7 at 3 μM in each strand. **1**: β-ODN **A**. **2**: α-ODN **B**. **3**: Intercalating α-ODN **D**.

in all the ODN's is believed not to contribute to the differences in stabilities of the TWJ's in the present investigation. In addition, we used 5-methyl substituted cytidines because of their easy synthesis. A further advantage is that the presence of 5-methylcytosine in an α-sequence is known to enhance the stability of duplex structures.[12]

When compared to the natural strand **A**, a considerable increase in the TWJ stability (ΔT = 15.2 °C) was observed using the α-ODN **B** (Table 1). This stabilization was even higher than the one observed for the corresponding duplex (24mer) (ΔT = 10.4 °C) when compared with the hybridization of the natural strand **A** (Table 2). We observed a positional effect in the TWJ stabilization when 5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine was inserted. As seen from Table 1, the highest stabilization occurred when the base was inserted into the middle of the three-way junction at position 2.

The further stabilization ($\Delta\Delta T$ = 11.2 °C) due to insertion of an α- pyrenylmethyl modified base **6** into the middle of TWJ region is rather remarkable when compared to the previously reported results[7] for the same hairpin DNA when hybridized with the corresponding modified complementary β-ODN. In this case only a 6.4 °C increase in the melting temperature was observed when the pyrene modified β-anomer was inserted into the middle of the TWJ region.

In this paper we reported the highest stabilization observed so far for an ODN three-way junction. The stabilization was achieved by replacing a targeting β-ODN with an α-

ODN containing in the junction region an inserted α-cytidine conjugated with an N^4-pyrenylmethyl intercalating moiety. The stability was even higher than for the corresponding duplex of two β strands where the hairpin had been deleted and comparable to the stability of the corresponding duplex using a targeting α strand. Even a higher stabilization for the three-way junction is to be foreseen when the structure in the junction region has been determined, e.g. by NMR. This will be helpful in construction of linkers to give correctly positioned intercalators which in turn should be selected among non-mutagenic ones. If the same strategy can be used for targeting stems in RNA, a large number of new targets in stem regions will be useful in the antisense approach of antiviral therapy.

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 external standard (^{1}H-NMR and ^{13}C-NMR), relative to 85 % H_3PO_4 as external standard in ^{31}P-NMR. Positive FAB mass spectra (HRMS) 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 (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 260 nm was increased 1 °C/min in 1 cm cuvette. DNA syntheses were performed on a Pharmacia Gene Assembler Special® DNA-synthesizer. HPLC was performed on a Waters Delta Prep 3000.

3'-*O*-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-α-thymidine (2). To a stirred solution of **1** (2.72 g, 5 mmol) in dry pyridine was added acetic anhydride (1.1 mL, 11 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 8 h at room temperature. The solvent was removed *in vacuo* and the resulting gum was coevaporated with dry toluene (2 × 20 mL). The product was purified by silica gel column using 0-1% MeOH/$CHCl_3$ to yield 2.7 g, (92%) of **2** as a white foam ^{1}H-NMR ($CDCl_3$): δ 9.40 (s, br, 1H, NH), 7.14-7.43 (m, 10H, arom, 6-H), 6.84 (d, 4H, J = 8.9 Hz, arom), 6.37 (d, 1H, J = 6.5 Hz, 1'-H), 5.26 (d, 1H, J

= 6.0 Hz, 3'-H), 4.48 (m, 1H, 4'-H), 3.78 (s, 6H, 2 × OCH_3), 3.24 (m, 2H, 5'-H), 2.90 (1H, m, 2'-H), 2.18 (d, 1H, J = 15.3 Hz, 2'-H), 2.00 (s, 3H, Ac), 1.95 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$): δ 169.66 ($COCH_3$), 164.07 (C-4), 150.35 (C-2), 158.27, 144.27, 135.54, 135.49, 129.92, 128.94, 128.13, 128.00, 127.89, 126.90, 125.21, 113.22, (arom, C-6), 109.85 (C-5), 86.96, 86.76, 86.39 (C-1', C-4', trityl), 75.09 (C-3'), 63.89 (C-5'), 55.13 (OCH_3), 39.01 (C-2'), 20.85 (Ac), 12.57 (CH_3). MS (FAB, $CHCl_3$ + 3-nitrobenzyl alcohol) *m/z*: 587 ($M + H^+$).

1-[3'-*O*-Acetyl-2-deoxy-5-*O*-(4,4'-dimethoxytrityl)-α-D-pentofuranosyl]-5-methyl-4-(1,2,4-triazolyl)pyrimidine-2(1H)-one (3). 1,2,4-Triazole (3.40 g, 48 mmol) was suspended in anhydrous MeCN (28 mL) at 0 °C followed by addition of $POCl_3$ (0.97 mL, 10 mmol) with rapid stirring. Triethylamine (6.48 g, 46 mmol) was then added dropwise while stirring the slurry at 0 °C for 30 min. A solution of **2** (2.93 g, 5 mmol) in MeCN 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 before adding triethylamine (4.5 mL, 32 mmol) and water (1.2 mL). After 10 min the solvent was evaporated *in vacuo*. The residue was partitioned between $CHCl_3$ (150 mL) and saturated aqueous $NaHCO_3$ (100 mL). The organic layer was dried (Na_2SO_4), concentrated till dryness and chromatographed on a silica gel column using 0-1% MeOH/$CHCl_3$ to give **3** (2.71 g, 85%) as a pale yellow foam. ^{1}H-NMR ($CDCl_3$): δ 9.13 (s, 1H, 5-H, triazole), 8.13 (s, 1H, 3-H, triazole), 7.96 (s, 1H, 6-H), 7.23-7.44 (m, 9H, arom), 6.86 (d, 4H, J = 9.1 Hz, arom), 6.38 (d, 1H, J = 6.4 Hz, 1'-H), 5.31 (d, 1H, J = 5.6 Hz, 3'-H), 4.62 (t, 1H, J = 3.7 Hz, 4'-H), 3.79 (s, 6H, 2 × OCH_3), 3.26-3.42 (m, 2H, 5'-H), 2.99-3.09 (m, 1H, 2'-H), 2.50 (s, 3H, Ac), 2.44 (d, 1H, J = 15.5 Hz, 2'-H), 1.89 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$): δ 169.29 ($COCH_3$), 158.50 (C-4), 153.73, 146.32 (triazole), 153.29 (C-2), 135.19 (C-6), 158.50, 144.91, 144.11, 135.32, 129.82, 127.88, 127.81, 126.86, 113.14 (arom), 104.45 (C-5), 89.56 (trityl), 87.53 (C-4'), 86.71 (C-1'), 74.87 (C-3'), 63.64 (C-5'), 55.05 (OCH_3), 39.01 (C-2'), 20.65 ($COCH_3$), 17.03 (CH_3). MS (FAB, $CHCl_3$ + 3-nitrobenzyl alcohol) *m/z*: 638 ($M + H^+$).

2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-N^4-(1-pyrenylmethyl)-α-cytidine (5). A solution of **3** (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 **4** (1.18 g, 74%) as a yellow foam. A stirred solution of **4** (1.18 g, 2.25 mmol) in dry MeOH (20 mL) was treated with 1 M sodium methoxide (5 mL). After stirring at room temperature for 30 min deacetylation was complete according to TLC and ammonium chloride (1.0 g) was added. The solvent was removed *in vacuo* and the residue chromatographed on a silica gel column using 0-5 % MeOH in $CHCl_3$ to give **5** (1.1 g, 98%) as a pale yellow foam. ^{1}H-NMR ($CDCl_3$): δ 7.86-8.27 (m, 10H, arom), 7.17-7.45 (m, 9H, arom), 6.81 (d, 4H, J = 8.7Hz, arom), 6.08 (dd, 1H, J_1 = 2.4, J_2 = 7.8 Hz, 1'-H), 5.29-5.32 (m, 1H, NH), 5.24 (d, 2H, J = 4.0 Hz, CH_2 NH), 4.49 (m, 1H, 3'-H), 4.42 (br. s, 1H, OH), 4.25 (d, 1H, J = 3.6 Hz, 4'-H), 3.75 (s, 6H, 2 × OCH_3), 3.12-3.24 (m, 2H, 5'-H), 2.75-2.83 (m, 1H, 2'-H), 2.29 (d, 1H, J = 14.8 Hz, 2'-H), 1.69 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$): δ 162.68 (C-4), 156.18 (C-2), 158.36, 144.59, 139.56, 135.77 (arom), 135.68 (C-6), 131.02-113.04 (arom), 101.07 (C-5), 90.34 (trityl), 88.50 (C-4'), 86.22 (C-1'), 72.94 (C-3'), 64.34 (C-5'), 55.03 (2 × OCH_3), 43.39 (CH_2NH), 41.20 (C-2'), 12.75 (CH_3). HRMS (FAB, $CHCl_3$ + 3-nitrobenzyl alcohol) calcd 758.323 ($M + H^+$), found 758.326.

3'-*O*-(2-Cyanoethyldiisopropylphosphoramidite)-2'-deoxy-5'-*O*-(4,4'-dimethoxyltrityl)-5-methyl-*N*^{4}-(1-pyrenylmethyl)-α-cytidine (6). Compound **5** (320 mg, 0.42 mmol) was coevaporated with dry MeCN 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-*N,N*-diisopropyl chlorophosphoramidite (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 saturated solution of $NaHCO_3$ (3 × 15 mL), dried (Na_2SO_4) and evaporated *in vacuo*. The product was purified by silica gel column chromatography (EtOAc/CH_2Cl_2/Et_2N 48:48:4). The resulting residue was redissolved in anhydrous toluene (2 mL) and precipitated by addition of ice-cooled petroleum ether (b.p. 60-80 °C). The solid product was collected by filtration and dried under reduced pressure to give **6** (280 mg, 80%) as a colourless powder. ^{1}H-NMR ($CDCl_3$): δ 8.01-8.36 (m, 9H, arom), 7.14-7.48 (m, 10H, arom and 6-H), 6.86-6.95 (m, 4H, arom), 6.36 (2 × d, 1H, J = 8.2 Hz, 1'-H), 5.21-5.48 (m, 3H, CH_2, NH), 4.54-4.66 (m, 2H, 4'-H and 3'-H), 3.78 (s, 6H, 2 × OCH_3), 3.56-3.74 (m, 2H, CH_2O), 3.48- 3.52 (m, 2H, NCH(Me)$_2$), 3.17-3.31 (m, 2H, 5'-H), 2.68-2.95

(m, 1H, 2'-H), 2.34-2.48 (m, 3H, 2'-H, CH_2CN), 1.73 (2 × s, 3H, CH_3), 0.86-1.19 (m, 12H, 4 × CH_3). ^{31}P-NMR ($CDCl_3$): δ 148.73 and 149.17.

5-Methyl-*N*^{4}-(1-pyrenylmethyl)-α-cytidine (7). Detritylation solution ($C_2H_2Cl_2$/Cl_3CCOOH, Cruachem) (5 mL) was added to a stirred solution of **5** (0.38 g, 0.5 mmol) in $CHCl_3$ (10 mL). The reaction mixture was stirred for 10 min. at room temperature. The solvent was removed by evaporation *in vacuo* and the resulting reddish residue was purified on silica gel column using 0-20% MeOH in Et_2O yielding **7** (0.215 g, 95%) as a pale yellow foam. ^{1}H-NMR (DMSO-d_6): δ 7.71-8.51 (m, 10H, arom, 6-H), 6.11-6.14 (m, 1H, 1'-H), 5.30-5.34 (m, 3H, CH_2 and NH), 4.9 (s. br. 1H, OH), 4.19-4.24 (m, 2H, 3'-H, 4'-H), 3.35-3.42 (m, 2H, 5'-H), 2.41-2.48 (m, 1H, 2'-H), 2.02 (s, 3H, CH_3), 1.92-1.96 (m, 1H, 2'-H). ^{13}C-NMR (DMSO-d_6): δ 163.38 (C-4), 155.53 (C-2), 138.94 (C-6), 133.24-123.44 (arom), 101.28 (C-5), 89.07 (C-4'), 86.36 (C-1'), 70.88 (C-3'), 62.04 (C-5'), 45.89 (CH_2NH), 13.55 (CH_3). HRMS (3-nitrobenzyl alcohol) calcd 456.192 ($M + H^+$), found 456.185.

3'-*O*-Acetyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (8). A solution of triazole compound **3** (3.20 g, 5 mmol) in 20% aqueous ammonia (20 mL) and 1,4-dioxane (50 mL) was stirred at room temperature. After 2 h the mixture was evaporated under reduced pressure. The residue was partitioned between $CHCl_3$ (200 mL) and saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified on a silica gel column using 0-4% MeOH/EtOAc to yield **8** (2.7 g, 92%) as a pale yellow foam. ^{1}H-NMR (DMSO-d_6): δ 7.49 (s, 1H, 6-H), 7.25-7.43 (m, 11H, NH_2 and arom), 6.91 (d, 4H, J = 8.8 Hz, arom), 6.25 (d, 1H, J = 3.2 Hz, 1'-H), 5.10-5.13 (m, 1H, 3'-H), 4.61-4.64 (m, 1H, 4'-H), 3.75 (s, 6H, 2 × OCH_3), 3.10-3.36 (m, 2H, 5'-H), 2.75-2.88 (m, 1H, 2'-H), 2.13 (d, 1H, J = 15.2 Hz, 2'-H), 1.93 (s, 3H, Ac), 1.91 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6): δ 169.78 (COCH$_3$), 165.47 (C-4), 155.09 (C-2), 158.09, 144.61, 138.12, 135.37 (arom), 135.27 (C-6), 129.63, 127.87, 127.62, 126.73, 113.22 (arom), 100.45 (C-5), 86.62 (trityl), 85.78 (C-4'), 84.94 (C-1'), 74.63 (C-3'), 63.76 (C-5'), 54.98 (OCH_3), 38.08 (C-2'), 20.67 (COCH_3), 13.11 (CH_3). MS (FAB, $CHCl_3$ + 3-nitrobenzyl alcohol) *m/z*: 586 ($M + H^+$).

3'-*O*-Acetyl-N^4-benzoyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (9). To a suspension of **8** (2.34 g, 4 mmol) in dry pyridine (25 mL) was added 1.0 g (4.42 mmol) of benzoic anhydride and the mixture was stirred at room temperature for 12 h. The pyridine was removed *in vacuo* to leave a gum, which was chromatographed on a silica gel column using MeOH/Et_2O (0-3%) to afford **9** (2.34 g, 88%) as a pale yellow foam. ^{1}H-NMR ($CDCl_3$): δ 8.31-8.34 (m, 2H, arom), 7.23-7.55 (m, 13H, arom, 6-H), 6.83-6.87 (m, 4H, arom), 6.37-6.40 (m, 1H, 1'-H), 5.28-5.29 (m, 1H, 3'-H), 4.52-4.54 (m, 1H, 4'-H), 3.79 (s, 6H, 2 × OCH_3), 3.22-3.39 (m, 2H, 5'-H), 2.92-3.03 (m, 1H, 2'-H), 2.25 (d, 1H, J = 15.2 Hz, 2'-H), 2.15 (s, 3H, Ac), 1.99 (s, 3H, CH_3). ^{13}C- NMR ($CDCl_3$): δ 179.40 (*C*OPh), 169.54 (*C*OCH_3), 159.79 (C-2), 158.54, 147.78, 144.20, 137.09, 136.69, 135.41 (arom), 135.28 (C-6), 132.26-126.88, 113.18, 113.11(arom), 110.55 (C-5), 87.63 (trityl), 86.76 (C-1), 77.41 (C-4'), 75.01 (C-3'), 63.81 (C-5'), 55.09 (2 × OCH_3), 39.10 (C-2'), 20.81 (Ac), 13.58 (CH_3). MS (FAB, $CHCl_3$ + 3-nitrobenzyl alcohol) *m/z*: 690 (M + H^+).

N^4-Benzoyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (10). To a stirred solution of **9** (2.00 g, 2.9 mmol) in dry MeOH 20 mL, was added 1 M sodium methoxide in MeOH (10 mL) and the mixture was stirred at room temperature for 20 min. Ammonium chloride (1 g) was added. The solvent was removed *in vacuo* and the crude mixture purified by chromatography (0-8 % MeOH/Et_2O) yielding two fractions. Compound **10** was obtained from the faster eluting fraction as a pale yellow foam (1.13 g, 60%); the slower eluting fraction yielded compound **11** as a colourless powder (0.35 g, 22%). Compound **10**: ^{1}H-NMR ($CDCl_3$): δ 8.26-8.29 (m, 2H, arom), 7.72 (s, 1H, 6-H), 7.23-7.52 (m, 12H, arom), 6.85 (d, 4H, J = 8.9 Hz, arom), 6.24 (dd, 1H, J_1 = 1.7, J_2 = 7.4 Hz, 1'-H), 4.44-4.48 (m, 1H, 3'-H), 4.39 (d, 1H, J = 5.6 Hz, 4'-H), 3.77 (s, 6H, 2 × OCH_3), 3.12-3.26 (m, 2H, 5'-H), 2.75-2.84 (m, 1H, 2'-H), 2.15 (d, 1H, J = 14.7 Hz, 2'-H), 2.06 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$): δ 179.27 (*C*OPh), 160.00 (C-2), 158.47, 148.07, 144.39, 138.55, 137.07, 135.59 (arom), 135.48 (C-6), 132.2, 1129.86, 129.71, 127.79, 126.79, 113.12 (arom), 110.75 (C-5), 88.85 (trityl), 88.34 (C-3'), 86.46 (C-4'), 72.37 (C-3'), 63.99 (C-5'), 55.08 (OCH_3), 41.28 (C-2'), 13.45 (CH_3). HRMS ($CHCl_3$ + 3-nitrobenzyl alcohol) calcd 670.2529 (M +Na^+), found 670.256. Compound **11**: ^{1}H-NMR(DMSO-d_6): δ 7.63 (s, 1H, 6-H), 7.25-7.42 (m, 11H, arom and NH_2), 6.90 (d, 4H, J = 9.0 Hz, arom), 6.15 (dd, 1H, J_1 = 3.3, J_2 = 7.3 Hz, 1'-H),

4.30 (br.s, 1H, OH), 4.19 (br.s, 1H, 3'-H), 4.01-4.14 (m, 1H, 4'-H), 3.74 (s, 6H, 2 × OCH_3), 2.96-3.12 (m, 2H, 5'-H), 2.53-2.65 (m, 1H, 2'-H), 1.87-1.96 (m, 4H, 2'-H, CH_3). ^{13}C-NMR (DMSO-d_6): δ 165.34 (C-4), 155.21 (C-2), 158.03, 144.75, 138.88, 135.52 (arom), 135.44 (C-6), 129.61, 127.62, 126.62, 113.16 (arom), 100.58 (C-5), 86.80 (trityl), 86.16, 85.53 (C-1', C-4'), 71.00 (C-3'), 64.00 (C-5'), 54.95 (OCH_3), 40.58 (C-2'), 13.32 (CH_3).

N^4-Benzoyl-3'-*O*-(2-cyanoethyldiisopropylphosphoramidite)-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-5-methyl-α-cytidine (12). Compound **12** was obtained from **10** (345 mg, 0.5 mmol) according to the published procedure[12] as a white powder (88%), after chromatography (Et_2O/Et_3N 10:0.5) and redissolving the residue in dry toluene (1.0 mL) and precipitation with ice-cooled petroleum ether (b.p 60-80 °C, 200 mL). ^{1}H-NMR ($CDCl_3$): δ 8.33 (d, 2H, J = 7.8 Hz, arom), 7.69 (s, 1H, 6-H), 7.24-7.56 (m, 12H, arom), 6.85-6.88 (m, 4H, arom), 6.32-6.34 (m, 1H, 1'-H), 4.58-4.68 (m, 1H, 3'-H), 4.54-4.56 (m, 1H, 4'-H), 3.82 (s, 6H, 2 × OCH_3), 3.64-3.78 (m, 2H, CH_2O), 3.48-3.57 (m, 2H, $HC(CH_3)_2$), 3.17-3.31 (m, 2H, 5'-H), 2.68-2.92 (m, 1H, 2'-H), 2.25-2.56 (m, 3H, 2'-H, CH_2CN), 2.15 (s, 3H, CH_3), 0.85-1.19 (12H, m, 6 × CH_3). ^{31}P-NMR ($CDCl_3$): δ 148.97 and 149.70.

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