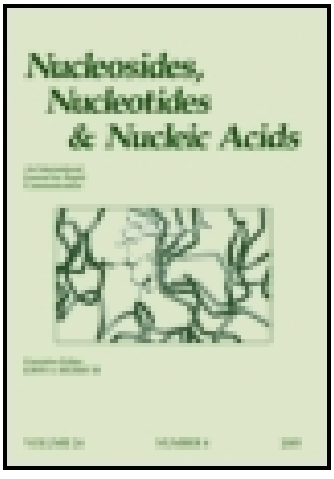

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Preparation of Oligonucleotides Containing 5-Bromouracil and 5-Methylcytidine.

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PREPARATION OF OLIGONUCLEOTIDES CONTAINING 5-BROMOURACIL AND 5-METHYLCYTIDINE.

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Abstract: A previously described side reaction on 5-bromouracil during standard oligonucleotide deprotection conditions has been studied in detail. The side product, 5-amino-2'-deoxyuridine, is isolated and characterized.The use of several 5-methylcytidine protected derivatives for the preparation of oligonucleotides containing 5-bromouracil and 5-methylcytidine free of 5-amino-2'-deoxyuridine is discussed.

Several years ago, it was shown that oligonucleotides could bind to specific sequences on double stranded DNA by forming triple helices. Although triple helix formation is restricted to homopurine-homopyrimidine sequences a large effort is being done in order to use oligonucleotides to bind specific genomic sequences. The potential applications of the triple helix formation has been recently reviewed[1,2].

Substitution of some natural bases for base analogues may improve the binding and/or change the specificity. For example it has been demonstrated that substituting 5-methylcytosine (m^5C) to cytosine increases the affinity of homopyrimidine oligodeoxynucleotides for its recognition sequence in duplex DNA and extends the pH range in where triple helix is stable to neutral pH[3,4]. A similar effect has been described for the substitution of 5-bromouracil (Br^5U) to thymine[4]. Incorporation of both m^5C and Br^5U resulted in a large increase of triple helix formation over an extended pH range[4].

For these reasons we were interested in preparing homopyrimidine oligonucleotides containing both m^5C and Br^5U. During the synthesis of an oligonucleotide containing Br^5U for NMR studies using the phosphotriester method, a partial conversion of Br^5U

was found while using the standard ammonia treatment (overnight at 55 °C) to an unknown derivative[5]. When ammonia deprotection was performed for 3 days at 37 °C, no side product was detected[5]. Recently, similar results were found using phosphoramidite chemistry[6] and the decomposition of Br^{5}U was avoided by using phosphoramidites protected with more labile groups such as phenoxyacetyl (Pac) and isobutyryl groups[7] and performing the ammonia deprotection at room temperature[6]. In this communication we will describe the characterization of the side product obtained during the ammonia treatment of Br^{5}U and the preparation of a new m^{5}C phosphoramidite derivative designed for the preparation of oligonucleotides containing both Br^{5}U and m^{5}C free of side products coming from decomposition of Br^{5}U.

RESULTS AND DISCUSSION

Characterization of the main decomposition product of 5-bromo-2'-deoxyuridine during ammonia treatment at 55°C.

Previous reports on the synthesis of oligonucleotides containing 5-bromouracil have described the formation of a side reaction during the ammonia deprotection at 55 °C[5,6]. The side reaction was detected by the appearance of a new nucleoside during the HPLC analysis of the enzyme digestion of the oligonucleotide[6]. The new nucleoside eluted near the dC peak and the integration of the peaks indicated the presence of a 10-20% of the modified oligonucleotide in which 5-bromouracil was converted to another product[5,6]. When deprotection was run at lower temperature, no side product was detected. When deprotection of oligonucleotides was done by treatment with a 1:1 ethylenendiamine/ethanol solution at room temperature for 6 hours, a different Br^{5}dU side product was formed in approx. 50% yield (data not shown). The modified oligonucleotide was easily separated from the correct sequence by gel electrophoresis and ion-exchange chromatography indicating that a ethylenediamine molecule (and subsequently a positive charge) was added to the oligonucleotide. All these data indicated that the side product arose from the addition to 5-bromouracil of one molecule of the amine used during the deprotection. This addition could only happen at positions 5 and 6 of the pyrimidine ring of 5-bromouracil. It has been described that 5-aminouridine could be isolated from the reaction of 5-bromouridine with liquid ammonia at high pressure and temperature[8]. Also 5-aminocytosine is the main product from the reaction of 5-bromocytosine with liquid ammonia together with some small amounts of 6-aminocytosine[9]. So most likely the side

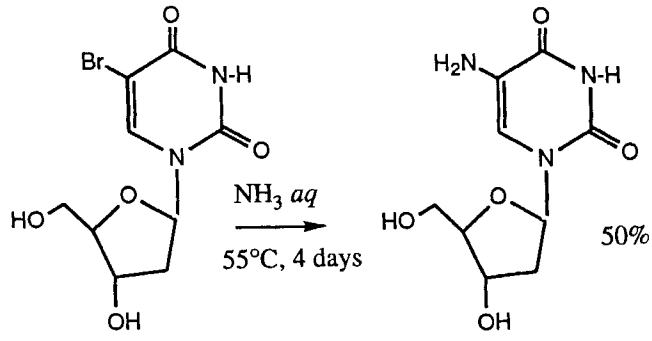


FIGURE 1: Conversion of 5-bromo-2'-deoxyuridine to 5-amino-2'-deoxyuridine in aqueous ammonia solutions.

product formed during the ammonia deprotection of the oligonucleotides containing Br^{5}U is the corresponding 5-amino-2'-deoxyuridine oligonucleotide.

In order to confirm the formation of this product, 5-bromo-2'-deoxyuridine (Br^{5}dU) was treated with 33% concentrated ammonia solution at 55°C for 4 days. The crude mixture was analysed by HPLC and a product with the same retention time and UV absorption properties than the side product observed in the Br^{5}U oligonucleotide enzyme digestions was found. The area of the peak of the side product was found to be equal to the area of peak of the remaining Br^{5}dU. The side product was isolated by semi-preparative HPLC in sufficient amounts for characterization. Mass spectrometry confirmed a molecular weight in agreement with the structure of 5- or 6-amino-2'-deoxyuridine (M+ found 244.2, expected 244.2) and spectroscopic data (U.V. absorption spectra, ^{1}H and ^{13}C-NMR) were in agreement with published data for 5-aminouridine[8] and different from data described for 6-aminouridine[9,10]. So, similarly to what has been described for the reaction of Br^{5}dU with liquid ammonia[8], 5-amino-2'-deoxyuridine was the modified nucleoside obtained in 10-20% yield during the deprotection of Br^{5}dU oligonucleotides (FIGURE 1). Also when 5-iodo-2'-deoxyuridine was treated with 33% ammonia solution at 55 °C, the same side product was obtained (data not shown). On the other hand, it has been described that oligonucleotides carrying 5-fluorouracil does not produce any side product during the ammonia deprotection[6]. All this data are in agreement with the addition-displacement-elimination mechanism postulated by Goldman and Kalman to explain the formation of 5-aminocytidine from 5-bromocytidine[9].

Table 1: Preparation of (Br^5dU-m^5dC)$_9$ using commercially available phosphoramidites.

Phosphoramidites	Ammonia treatment	% Modification
Br^5dU, m^5dC^{bz}	conc. NH_3, 55°C, 16hr	15% Br^5dU->$NH_2{}^5dU$
Br^5dU, m^5dC^{bz}	conc. NH_3/EtOH, 55°C, 16hr	10% Br^5dU->$NH_2{}^5dU$
Br^5dU, m^5dC^{bz}	conc. NH_3, R.T., 3 days	5% Br^5dU->$NH_2{}^5dU$
Br^5dU, T^{tri}	conc. NH_3, R.T., 16hr	12% T^{tri}->T

Preparation of oligonucleotides with both 5-bromouracil and 5-methylcytidine.

It has been described that the use of phosphoramidites with more labile phenoxyacetyl (Pac) and isobutyryl (ibu) protecting groups allowed the deprotection of oligonucleotides at room temperature[7] and, for that reason, oligonucleotides carrying Br^5dU could be prepared without apparent decomposition of Br^5dU[6]. Unfortunately, neither phenoxyacetyl or isobutyryl groups have been described for the protection of m^5dC. The only commercially available me^5dC phosphoramidite derivative was protected with the rather stable benzoyl group. In preliminary experiments, the octadecamer A (Br^5dU-m^5dC)$_9$ was prepared using the commercially available m^5dC^{bz} phosphoramidite and the 4-(1,2,4-triazolyl)thymidine phosphoramidite (T^{tri})[12,13] as precursors of me^5dC. Milder deprotection conditions were assayed to obtain oligonucleotides with both 5-bromouracil and 5-methylcytidine. Results are summarised in table 1. The percentage of modification was calculated from the enzyme digestion of the HPLC-purified oligonucleotide.

As described previously[5,6], the use of standard ammonia deprotection conditions (conc. NH_3, 55°C, 16hr) yielded a mixture of oligonucleotides in which 15% of the Br^5dU molecules were converted to $NH_2{}^5dU$. Because there are nine Br^5dU molecules in the oligonucleotide, a 15% modification implies that only a few molecules are the desired (Br^5dU-m^5dC)$_9$ oligonucleotide. On the contrary, the product obtained was a mixture of oligonucleotides that instead of Br^5dU contained one or two NH_2dU molecules . It has been suggested[11] that performing the ammonia treatment with a 3:1 mixture of conc. NH_3 / ethanol, could improve the quality of the product and we have found a reduction of the formation of the side product from 15% to 10%. Unfortunately, in our case a 10% side reaction implied that the octadecamer was only 10-20% pure. When ammonia treatment was performed at room temperature for 3 days, a cleaner product was obtained (only 5%

side product), but with low yield, because some new products appeared at the back of the main peak that made the separation difficult. These products could be oligonucleotides containing still some of the benzoyl groups but there was not enough material to characterize them. Finally, the use of the triazolyl derivative of thymidine[12,13] as a source of me^{5}dC was not satisfactory because 12% of T was obtained. This product could be obtained as side product by hydrolysis instead of the desired ammonolysis, but also T phosphoramidite could be present as minor contamination of the triazolyl T phosphoramidite. All this preliminary data shows the need to find a more labile protecting groups for m^{5}dC for the preparation of oligonucleotides containing several molecules of Br^{5}dU together with m^{5}dC.

In order to obtain m^{5}dC phosphoramidite derivatives, m^{5}dC and DMT-me^{5}dC were prepared from T and DMT-T following the method described by Sung[15]. The method involved protection of the alcohol functions with trimethylsilyl or acetyl groups and formation of the 4-(1,2,4-triazolyl) thymidine derivative. Treatment of this intermediate with ammonia, yield the desired m^{5}dC. A similar protocol was applied to 5'-O-DMT-T to obtain 5'-O-DMT-m^{5}dC. Protection of the 3'-OH was done by reaction with trimethylsilyl-1,2,4-triazole followed by the formation of the 1,2,4-triazolyl derivative. Ammonolysis with ammonia in dioxane gave 5'O-DMT-m^{5}dC in 76 % yield.

It has been described[14] that T could be transformed in a one pot reaction to me^{5}dC by heating T with hexamethyldisilazane, formamide and ammonium sulphate at 140 °C for 60 hours. We found this protocol not suitable, because low recoveries of the product were obtained and the product was impurified with large amounts of the depyrimidation product : 5-methylcytosine (data not shown).

Protection of position 4 of m^{5}dC with isobutyryl (ibu) groups was studied. 5'-O-DMT-m^{5}dC was reacted with trimethylsilyl chloride in pyridine and isobutyric anhydride obtaining after purification the desired isobutyryl derivative in a moderate yield (42%).

A small part of 5'-O-DMT-N^4-ibu-m^{5}dC was detritylated to obtain N^4-ibu-m^{5}dC. Stability of the isobutyryl m^{5}dC derivative to ammonia was studied by HPLC. At 50 °C, N^4-ibu-m^{5}dC was converted to m^{5}dC with a half-life of 4 hours. At room temperature the half-life was approx. 10 hours. When this result was compared with the 30 minutes half-time described for the isobutyryl derivative of dC at room temperature[7], we concluded that in our case the isobutyryl group was too stable for the protection of m^{5}dC. Also protection of position 4 with the phenoxyacetyl (pac) group was attempted. As described for the pac derivative of dC[7], low yields were obtained indicating that decomposition was occurring during silica gel chromatography (data not shown).

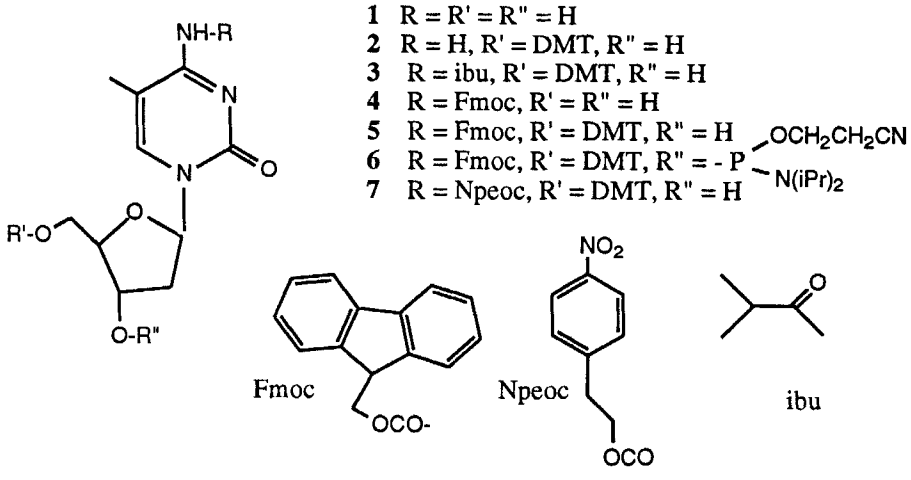


FIGURE 2: 5-Methyl-2'-deoxycytidine derivatives prepared in this work.

Afterwards, the base labile fluorenylmethoxycarbonyl (Fmoc)[16] and *p*-nitrophenylethoxycarbonyl (Npeoc)[17] groups were tested for the protection of the amino group of m^{5}dC. These groups could be removed with non-nucleophillic bases through a β-elimination mechanism. When a strong non-nucleophillic base like DBU is used for the removal of this group no side reaction should happen at Br^{5}dU moiety. Fmoc and Npeoc groups were introduced at position 4 of m^{5}dC using the transient protection method[16] obtaining the desired Fmoc and Npeoc derivatives of m^{5}dC with moderate yields (59% for Npeoc and 20% for Fmoc). The protection of the 5'-OH function with the dimethoxytrityl group was accomplished using standard protocols[18]. Small aliquots of DMT-protected nucleosides were treated with a 0.5 M DBU solution in dioxane in order to investigate the deprotection reaction. It was found that the Fmoc group was quantitatively removed in 5 minutes and the Npeoc group in 4 hours. For this reason, the Fmoc group was selected. The preparation of the phosphoramidite derivative of DMT-N^4-Fmoc-dC needed for the DNA synthesis was performed following standard protocols[18].

Octadecamer A [5'(Br^{5}dU-m^{5}dC)$_9$3'] and elevenmer B (5' m^{5}dC-Br^{5}dU-Br^{5}dU-m^{5}dC-m^{5}dC-Br^{5}dU-m^{5}dC-m^{5}dC-Br^{5}dU-m^{5}dC-Br^{5}dU 3') sequences were made on an automated DNA synthesiser using the commercially available Br^{5}dU phosphoramidite and N^4-Fmoc-m^{5}dC phosphoramidite prepared in this work. Average coupling yield of the m^{5}dC phosphoramidite measured by DMT absorbance was 95% while Br^{5}dU coupling yield was 99%. After the synthesis, the solid supports were treated with a 0.5M DBU

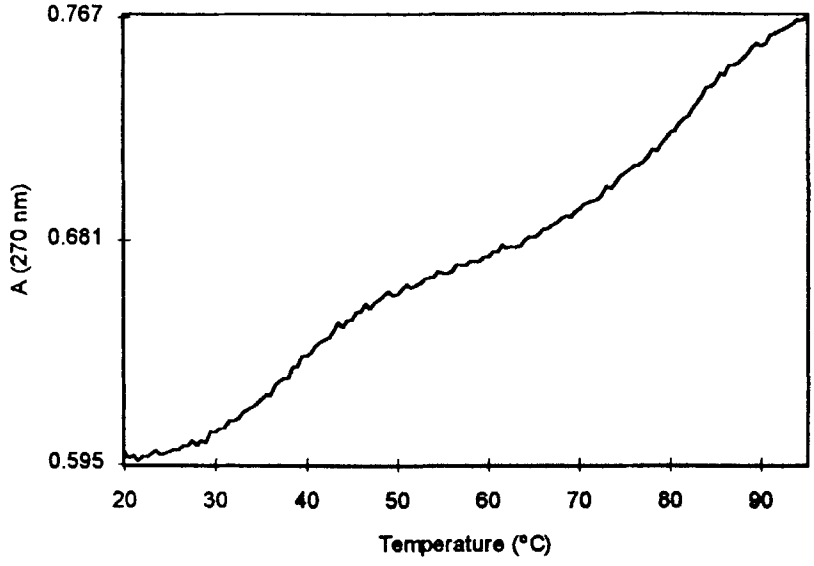
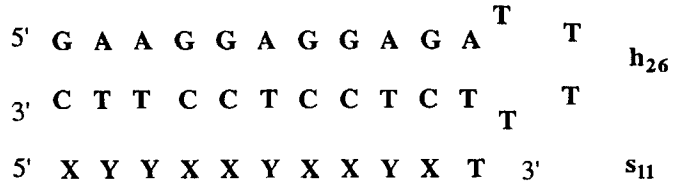


FIGURE 3 : Absorvance vs temperature plot for the $h_{26}.s_{11}$-m^{5}CBr^{5}U triplex (see table 2) in 1M NaCl, 100 mM sodium phosphate / citric acid buffer at pH 6.5.

solution in dichloromethane for 15 minutes to remove the Fmoc group. After subsequent washings, the supports were treated with concentrated ammonia for 16 hours at room temperature. The resulting oligonucleotides with the DMT group at the 5'-end were purified by using COP™ cartridges. Purified oligonucleotides were analysed by HPLC and enzyme digestion showed a correct nucleoside composition and the absence of 5-amino-dU.

The influence of the presence of Br^{5}dU and m^{5}dC on triple helix formation was studied with the sequence described by Xodo *et al.*[3] Triple helix were formed by mixing the hairpin molecule (h_{26}) with different single stranded 11-mers (s_{11}) which contain T, C, Br^{5}dU and m^{5}C. The ultraviolet absorbance at 270nm was then followed as a function of temperature at different pH values[3]. At pH 5.5-6.5, two clear transitions were observed (FIGURE 3). As described by Xodo *et al*[3], the first transition is due to the dissociation of s_{11} from h_{26} (triplex to duplex) and the second transition to the denaturation of h_{26} (duplex to single strand). The results in table 2 show that the triplex dissociation is strongly dependent on the pH, because C and m^{5}C need to be protonated in order to form triple helix. As previously described,[3,4,19] m^{5}C stabilize the triple helix. We observed that melting temperatures of triple helix dissociation in $h_{26}.s_{11}$-m^{5}CT are 20-30 degrees higher

Table 2: Melting temperatures for the triplex $h_{26} : s_{11}$ in 1M NaCl, 100 mM sodium phosphate/ citric acid buffer.



	Triplex Tm values (°C)[a]			
Sequence	pH 5.5	pH 6.0	pH 6.5	pH 7.0
X = C, Y = T	53	20	--	--
X =m^{5}C, Y = T	58	38	30	--
X = C, Y = Br^{5}U	55	35	28	--
X =m^{5}C, Y = Br^{5}U	74	45	39	20

[a]The duplex Tm values occurred between 80°C (pH 7.0) and 75°C (pH 5.5).

than $h_{26}.s_{11}$-CT. Substitution of T for Br^{5}U has a similar effect . Substitution of both C and T for m^{5}C and Br^{5}U has a strong stabilizing effect and therefore triple helix dissociation could be observed at pH 7.0. These results are similar to what has been described by Povsic and Dervan.[4]

In conclusion, we have isolated and characterized a side product observed during the preparation of oligonucleotides containing Br^{5}dU. Although the side product is formed in small amounts (10-20%), it could become important if the number of Br^{5}dU molecules in the oligonucleotide is high. Due to the triple helix stabilizing properties of Br^{5}U and m^{5}C, we have developed protected derivatives of m^{5}dC that can be used for the preparation of oligonucleotides containing Br^{5}dU and m^{5}dC free of side products. The method described here exploits the use β-elimination groups for the protection of the amino function of m^{5}dC. As described by Pfleiderer,[17] these groups can be removed by non-nucleophillic bases that makes them suitable for the preparation of oligonucleotides containing ammonia-sensitive bases. Although in this work we have used the Fmoc derivative because it is more labile, the Npeoc derivative could also be advantageous because it can be isolated in better yields and still be removed in a reasonable time. The method described here could be of interest for other oligonucleotide derivatives that contain 5-bromouracil[20].

EXPERIMENTAL SECTION

Abbreviations used: AcOEt: ethyl acetate, Br^5U: 5-bromouracil, Br^5dU: 5-bromo-2'-deoxyuridine, CPG: controlled-pore glass, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DCM: dichloromethane, DMF : N,N-dimethylformamide, DMT: dimethoxytrityl, EtOH: ethanol, Fmoc: 9-fluorenylmethoxycarbonyl; ibu: isobutyryl, LCAA: long chain amino alkyl, m^5C: 5-methyluracil, m^5dC: 5-methyl-2'-deoxyuridine, MeOH: methanol, Npeoc: 2-(4-nitrophenyl)ethoxycarbonyl, $NH_2{}^5dU$: 5-amino-2'-deoxyuridine, pac: phenoxyacetyl, THF: tetrahydrofuran, T^{tri}: 4-(1,2,4-triazol-1-yl)pyrimid-2-one 2'-deoxyriboside.

Isolation of 5-amino-2'-deoxyuridine by treatment of 5-bromo-2'-deoxyuridine with aqueous ammonia.

5-Bromo-2'-deoxyuridine (20 mg, 0,065 mmol) was treated with concentrated aqueous ammonia (32%, 1 ml) at 55 °C for 4 days in a screw-cap vial. The reaction mixture was cooled and concentrated to dryness. The residue was dissolved in water and purified by analytical HPLC. Column: Lichrospher 100 RP-18, 10 μm, 250 x 4 mm. Solvent A: water, solvent B: acetonitrile / water (1:1). A 20 minutes linear gradient from 5 to 50% B. A new product was observed with a lower retention time. Retention times: peak 1 (characterised as 5-amino-dU) 4.9 min, peak 2 (characterised as the starting material, 5-bromo-dU) 12 min. The area ratio between the two peaks was approximately 1:1. The newly formed product was isolated after repeated injections obtaining 34 absorbance units at 290 nm. Taking into account the extinction coefficient described by Roberts and Visser[8] for 5-amino-dU (8.600), the amount isolated was 0.96 mg (6%). UV(water / acetonitrile) λ_{max} = 292.9 nm. ^{1}H-NMR (D_2O, 300 MHz): 7.24 (1H, s), 6.25 (1H, t, J= 5.8 Hz), 4.38 (1H, m), 3.94 (1H, m), 3.71 (2H, m), 2.28 (2H, m). ^{13}C-NMR (D_2O, 75 MHz): 160.4, 142.2, 114.0, 112.5, 78.2, 76.7, 62.3, 53.0, 38.4. Mass spectrum (electrospray): 244.2 (M + 1), 266.3 (M + Na), 282.3 (M + K).

5-Methyl-2'-deoxycytidine (1).

Thymidine (1.41 g, 5 mmol) was suspended in 50 ml of DCM and 8.4 ml (60 mmol) of triethylamine were added followed by 6.2 ml (50 mmol) of trimethylsilyl chloride. After 30 min of magnetic stirring, the reaction mixture was poured with stirring in cold 1M aqueous $NaHCO_3$. Phases were separated, the organic phase was dried and evaporated to dryness. The residual brown oil was dried with evaporation of toluene three times and the residue was used without further purification.

1,2,4-Triazole (3.2 g, 46.3 mmol) was suspended in 100 ml of acetonitrile and the mixture was placed on an ice-bath. 1 ml of $POCl_3$ (10.7 mmol) was added followed by 10 ml of triethylamine (72 mmol). After 30 min of magnetic stirring, previously prepared bis(trimethylsilyl)thymidine was dissolved in dry acetonitrile and added to the phosphoryl tris(1,2,4-triazolide) solution. The reaction was stirred two hours at room temperature and solvents were concentrated. The residue was dissolved in DCM and the solution was washed with 1 M aqueous $NaHCO_3$. The organic phase was dried and concentrated to dryness. The presence of the triazolyl derivative was observed by its fluorescence properties on TLC plates. The residue was dissolved in THF (200 ml) and the solution was treated with ammonia in a stainless steel bomb for three days at 70 °C. The cooled bomb was opened carefully and solvent was evaporated. A mixture of 40% MeOH in $CHCl_3$ was used to dissolve the residual product left in the bomb and the washings were combined with the product previously obtained. The product was purified by column chromatography on silica gel, eluting with a gradient from 6% to 25% MeOH in $CHCl_3$. Yield: 1.1 g (4.6 mmol, 92%). TLC (20% EtOH / DCM): Rf 0.2. ^{1}H-NMR (CD_3OD, 200 MHz): 7.6 (1H, s), 6.25 (1H, dd), 5.25 (1H, m), 4.4-4.2 (3H, m), 2.6-2.2 (2H, m), 1.95 (3H, s). ^{13}C-NMR (DMSO-d_6, 62.9 MHz): 165.3, 155.2, 138.3, 101.3, 87.1, 84.7, 70.4, 61.3, 40, 13.2.

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-2'-deoxycytidine (2).

DMT-T (1 g, 1.83 mmol) was dissolved in 42 ml of dry acetonitrile and 0.52 g (3.8 mmol) of N-trimethylsilyl-1,2,4-triazole were added. After 1 hour of magnetic stirring at 55 °C, the solution was cooled at room temperature and added to a solution of phosphoryl tris(1,2,4-triazolide) previously prepared as follows: To an ice-cooled suspension of 1.28 g (18.5 mmol) of 1,2,4-triazole in acetonitrile (20 ml), 615 mg (0.36 ml, 3.96 mmol) of $POCl_3$ and 2.81 ml (20 mmol) of triethylamine were added. The mixture was kept on ice for 30 minutes with stirring. After the addition of the solution of DMT-nucleoside, the mixture was kept at room temperature for 4 hours. The progress of the reaction was monitored by TLC for the appearance of a highly fluorescent derivative with a slightly higher Rf. Afterwards, the reaction mixture was concentrated to dryness. The residue was treated with 5% $NaHCO_3$ solution and DCM. The organic phase was separated and dried. The product was used without further purification. Yield : 0.9 g (72%). TLC (5% MeOH in DCM) Rf 0.4. ^{1}H-NMR ($CDCl_3$, 300 MHz): 8.3 (1H, s), 8.02 (1H, s), 7.6 (1H, s), 7.55-6.88 (9H, m), 6.75 (4H, d, J=8.7), 6.30 (1H, t, J=6.9), 4.42 (1H, m), 4.02 (1H, m), 3.72 (6H, s), 3.46 (2H, m), 2.6 (1H, m), 1.9 (3H, s), 0 (9H, s). ^{13}C-NMR ($CDCl_3$,

75 MHz): 163.7, 158.6, 158.0, 154.0, 144.1, 135.7, 135.5, 135.1, 129.9, 128.0, 127.9, 127.1, 127.0, 113.2, 105.6, 87.5, 87.0, 86.5, 71.6, 62.7, 55.1, 41.3, 16.4, 0.

5'-O-(4,4'-Dimethoxytrityl)-3'-O-trimethylsilyl-4-(1,2,4-triazol-1-yl)pyrimid-2-one 2'-deoxyriboside (0.9 g, 1.31 mmol) was dissolved in dioxane and the solution was placed into a steel bomb. Ammonia was destilled on the bomb and the reaction was left overnight at 60 °C. The reaction mixture was treated as described for compound **1**. The product was purified by column chromatography on silica gel (0% to 15% MeOH in DCM). Yield: 0.55 g (76%). TLC (10% MeOH in DCM) Rf 0.3. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.78 (1H, s), 7.43-7.23 (9H, m), 6.84 (4H, d, J=13.2 Hz), 6.30 (1H, t, J= 10.2 Hz), 4.48 (1H, m), 4.07 (1H, m), 3.80 (6H, s), 3.38 (2H, m), 2.46 (1H, m), 2.23 (1H, m), 1.46 (3H, s). ^{13}C-NMR ($CDCl_3$, 75 MHz): 164.8, 158.3, 138.1, 135.8-135.2, 129.8, 127.8, 127.6, 126.7, 112.9, 102.6, 86.4, 86.0, 85.6, 71.1, 63.1, 54.9, 41.3, 12.0.

5'-O-(4,4'-Dimethoxytrityl)-N^4-isobutyryl-5-methyl-2'-deoxycytidine (3).

5'-O-(4,4'-Dimethoxytrityl)-5-methyl-2'-deoxycytidine (490 mg, 0.9 mmol) were dissolved in 5 ml of pyridine and 167 mg of trimethylsilyl chloride (1.53 mmol) were added. After one hour of magnetic stirring at room temperature, 212 mg of isobutyric anhydride (1.34 mmol) were added. The reaction was stirred for 3 hours at room temperature and 0.5 ml of MeOH were added followed by 0.5 ml of water. Solvents were evaporated and the residue was purified by column chromatography on silica gel eluted with a gradient from 0 to 15% MeOH in DCM. Yield 350 mg (63%). TLC (15% MeOH in DCM) Rf 0.87. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.63 (1H, s), 6.77-7.42 (13H, m), 6.45 (1H, t, J= 3 Hz), 4.57 (1H, m), 4.10 (1H, m), 3.7 (6H, s), 3.41 (2H, m), 2.48 (1H, m), 2.22 (2H, m), 1.43 (3H, s), 1.17 (6H, d, J=8.5 Hz).

N^4-(9-Fluorenylmethoxycarbonyl)-5-methyl-2'-deoxycytidine (4).

5-Methyl-2'-deoxycytidine (1 g, 4.14 mmol) was dissolved with 20 ml of pyridine and 1.8 ml (14.5 mmol) of trimethylsilyl chloride were added. After 1 hr of magnetic stirring at room temperature, 1.30 g (4.9 mmol) of Fmoc-Cl were added. After two hours, 10 ml of water were added and the mixture was stirred overnight. The reaction mixture was concentrated to dryness and the residue was purified by column chromatography on silica gel (0 % to 10% MeOH in DCM). Yield: 370 mg (20%). TLC (10% MeOH in DCM) Rf 0.3. ^{1}H-NMR (DMSO-d_6, 250 MHz): 8.62 (1H, m), 7.30-8.0 (8H, m), 6.32 (1H, t, J=6.7Hz), 5.3 (1H, t), 5.2 (1H, t), 4.5 (2H, d), 4.25 (2H, m), 3.9 (1H, m), 3.68 (2H, m), 2.47 (2H, m), 1.90 (3H, s). ^{13}C-NMR (DMSO-d_6, 63 MHz): 143.7, 140.6, 127.5, 127.0, 124.9, 120.0, 87.6, 84.9, 69.9, 66.5, 60.9, 54.8, 46.4, 13.2.

5'-O-(4,4'-Dimethoxytrityl)-N^4-(9-fluorenylmethoxycarbonyl)-5-methyl-2'-deoxycytidine (5).

N^4-Fmoc-5-methyl-2'-deoxycytidine (370 mg, 0.8 mmol) was dissolved in 8 ml of pyridine and 324 mg of DMT chloride (0.96 mmol) were added. After 3 hours of magnetic stirring at room temperature, 2 ml of MeOH were added. Solvents were evaporated and the residue was dissolved in DCM and washed twice with a 1M $NaHCO_3$ aqueous solution. The organic phase was dried and concentrated to dryness. The residue was purified by column chromatography on silica gel (0% to 10 % MeOH in DCM). Yield: 250 mg (41%). TLC (5% MeOH in DCM) Rf 0.87. ^{1}H-NMR (DMSO-d_6, 250 MHz): 8.5 (1H, m), 6.77-7.73 (m), 6.35 (1H, t, J= 6.6 Hz), 4.56 (1H, m), 4.35 (2H, m), 4.06 (1H, m), 3.7 (7H, s), 3.34 (2H, m), 2.35 (2H, m), 1.52 (3H, s).

5'-O-(4,4'-Dimethoxytrityl)-N^4-(9-fluorenylmethoxycarbonyl)-5-methyl-2'-deoxycytidine 3'-O-(N,N-diisopropyl)-2-cyanoethyl phosphoramidite (6).

5'-O-DMT-N^4-Fmoc-5-methyl-2'- deoxycytidine (1.23 g, 1.61 mmol) was dissolved in 2 ml of dry DCM and the solution was kept under argon on an ice-water bath. To the solution, 0.84 ml (6.44 mmol) of N,N-diisopropylethylamine were added dropwise with a syringe followed by 0.53 ml (2.4 mmol) of 2-cyanoethoxy-N,N-diisopropylaminochloro-phosphine. After 1hour of magnetic stirring at room temperature, the reaction mixture was diluted with more DCM and washed with 1M $NaHCO_3$ aqueous solution. The organic phase was dried and concentrated to dryness. The residue was purified by column chromatography on silica gel eluted with hexane-AcOEt (2:1) containing 1% of triethylamine. Yield: 920 mg (60%). TLC (hexane: AcOEt 1:2 containing 1% triethylamine) Rf 0.29. ^{31}P-NMR (CH_3CN, 101 MHz): 145.6 ppm.

5'-O-(4,4'-Dimethoxytrityl)-N^4-[2-(4-nitrophenyl)ethoxycarbonyl]-5-methyl-2'-deoxycytidine (7).

5-Methyl-2'-deoxycytidine (1.1 g, 4.6 mmol) was dissolved in dry DMF and 1.9 ml of hexamethyldisilazane (9.2 mmol) were added. After 30 min of magnetic stirring at room temperature solvents were evaporated to dryness, the residual oil was dissolved in 50 ml of MeOH / DMF (1 : 1) and again solvents were concentrated. The product was dried twice with 20 ml of dry pyridine and the residue was dissolved in 100 ml of dry pyridine. The solution was cooled in an ice bath and 1.58 g (6.9 mmol) of Npeoc-Cl[17] were added. After two hours, 20 ml of MeOH were added and the mixture was evaporated to dryness. The residual oil was dissolved with $CHCl_3$ (300 ml) and the organic solution was washed with 300 ml of 1M $NaHCO_3$ aqueous solution. The organic phase was dried and

concentrated to dryness. The product was dissolved in DCM and a solution of 2.28 g (11.5 mmol) of *p*-toluensulfonic acid in 100 ml of THF was added to remove the trimethylsilyl groups. After 20 min, 2.8 ml (20 mmol) of triethylamine were added and the mixture was stirred for 5 min. Solvent was removed and the residual oil was purified by column chromatography on silica gel (7-20 % MeOH gradient in $CHCl_3$). The fractions containing the product were pooled and evaporated to dryness obtaining 1.19 g (2.7 mmol, 59%) of an oil that contained the desired product (judged by NMR) with some impurities.

The product obtained above was dissolved in 20 ml of dry pyridine and reacted with 2.2 g (6.5 mmol) of DMT chloride as described for compound **5**. Yield 40% from m^{5}dC (1.32 g, 1.8 mmol). TLC (5% EtOH / DCM) Rf: 0.5. ^{1}H-NMR ($CDCl_3$, 300 MHz) : 12.2 (1H, s), 8.1 (2H, d, J = 8.7 Hz), 7.71 (1H, s), 7.42 (4H, d, J = 9 Hz), 7.1 (7H, m), 6.84 (4H, d, J = 9 Hz), 6.37 (1H, t, J = 6 Hz), 4.58 (1H, m), 4.39 (2H, t, J = 7.2 Hz), 4.06 (1H, m), 3.79 (6H, s), 3.50 (1H, dd J = 10.5 and 3 Hz), 3.37 (1H, dd, J 10.5 and 3 Hz), 3.13 (2H, t, J = 7.2 Hz), 2.45 (1H, m), 2.32 (1H, m), 1.5 (3H, s). ^{13}C-NMR (DMSO-d_6, 63 MHz): 163.4, 160.8, 158.6, 147.8, 146.7, 145.6, 144.1, 136.8, 135.3, 129.9, 129.6, 128.0, 127.8, 127.0, 123.5, 113.1, 110.9, 86.6, 86.4, 85.3, 71.8, 65.1, 63.3, 55.0, 41.1, 34.8, 12.6.

Oligonucleotide synthesis and purification.

Sequences A: [5'(Br^{5}U-m^{5}C)$_9$ 3']; B (s_{11}-m^{5}CBr^{5}U): 5' m^{5}C-Br^{5}U-Br^{5}U-m^{5}C-m^{5}C-Br^{5}dUmdC-m^{5}C-Br^{5}U-m^{5}C-Br^{5}U 3'); C (h_{26}): 5'GAAGGAGGAGATTTTTCTCCTC CTTC 3'; D (s_{11}-CT): 5' CTTCCTCCTCT 3'; E (s_{11}-m^{5}CT): m^{5}C-T-T-m^{5}C-m^{5}C-T-m^{5}C-m^{5}C-T-m^{5}C-T 3'; and F (s_{11}-CBr^{5}U): C-Br^{5}U-Br^{5}U-C-C-Br^{5}U-C-C-Br^{5}U-C-Br^{5}U 3' were assembled using 35 mg of solid support and the appropriate protected nucleoside phosphoramidites on an automatic DNA synthesizer (Applied Biosystems Mod. 392). T^{tri} phosphoramidite was prepared as described[12, 13] and was also obtained from Glen Research. Both products gave identical results. The standard 1 μmol scale synthesis cycle was used. In all cases the last DMT group was left to help purification. Coupling efficiencies were 99% except for the Fmoc-m^{5}C phosphoramidite that was 95%.

Sequences A and B prepared using Fmoc-m^{5}dC phosphoramidite were deprotected as follows : the supports were treated with a 0.5 M DBU solution in *anhydrous* DCM (2-3 ml) at room temperature for 15 minutes (5 treatments of 3 minutes each). The support was washed with acetonitrile, water, 2% ammonium acetate aqueous solution and acetonitrile. The supports were dried at vacuum and transferred to a screw-cap flask. 1.5 ml of concentrated ammonia were added and the treatment was prolonged overnight at room

temperature. The support was filtered and the solutions were used directly for purification using COP™ cartridges.

Sequence F was prepared using C phosphoramidite protected with isobutyryl group and the ammonia deprotection was performed at room temperature overnight.

The rest of the sequences were deprotected using standard protocols[18] or as described in table 1.

Oligonucleotides with the DMT group at the 5'-end were purified using COP™ cartridges following the instructions of the manufacters. Further purification was achieved by reversed-phase HPLC. HPLC conditions were as follows : Column : Nucleosil 120C18 (200 x 4 mm), flow rate 1 ml / min, a 20 min linear gradient from 2 to 25% acetonitrile over 20 mM aqueous triethylammonium acetate. Overall (synthesis and purification) yields were around 40-50%.

Nucleoside composition were analyzed by enzyme digestion followed by HPLC analysis. The polymers (20-40 μg) were incubated in 50 mM tris HCl pH 8.0 and 10 mM magnesium chloride with snake venom phosphodiesterase (Boehringer-Mannheim, 1 μl) and alkaline phosphatase (Boehringer-Mannheim, 1 μl) in a total volume of 20 μl at 37 °C overnight. The resulting mixture was analysed by HPLC using the conditions described above.

Melting studies.

Solutions of equimolar amounts of the hairpin oligonucleotide (h_{26}) and the appropriate elevenmer (s_{11}) were mixed in the appropiate buffer. The solutions were heated at 80 °C and allowed to cool slowly to room temperature and samples were kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-lenght cells a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5 deg min^{-1}. Melts were run on duplex concentration of 4 μM at 270 nm.

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