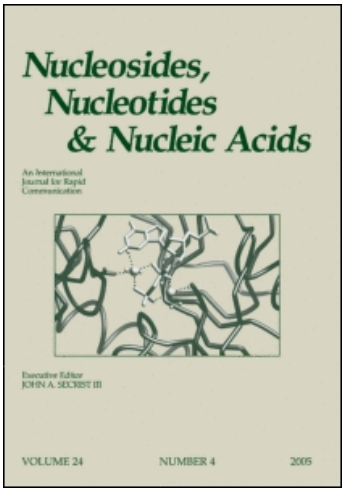

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Methylphosphonate Modified DNA Hairpin Loops

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METHYLPHOSPHONATE MODIFIED DNA HAIRPIN LOOPS

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ABSTRACT: We synthesized and analyzed DNA hairpin molecules with methylphosphonate linkages of defined stereochemistry in the loop region. Dinucleotide building blocks A*p*A and T*p*T (*p* indicating methylphosphonate linkage with either Rp or Sp configuration) were synthesized, separated into the diastereomers, and incorporated at three positions of the tetraloops 5'-CGCAAAAGCG-3' and 5'-CGCTTTTGCG-3'. The oligonucleotides were analyzed for their melting behavior. With a Tm of 67.5 °C the molecule 5'-CGCAAA*p*AGCG-3' with a Sp configurated methylphosphonate is distinctly more stable than the Rp configurated one (Tm = 60.5 °C) and the unmodified oligonucleotide (Tm = 64.5 °C). In contrast to double helical DNA where the substitution of a phosphorodiester by a Sp configurated methylphosphonate results in a lower Tm, in DNA hairpin the introduction of Sp and Rp methylphosphonates at specific positions can lead to a stabilization of the structure.

INTRODUCTION

Methylphosphonates are among the first analogs of the phosphorodiester backbone described[1]. Their resistance towards nuclease degradation makes them attractive for antisense applications. The substitution of one of the nonbinding oxygen atoms at phosphorus has two major consequences: methylphosphonates are neutral and the phosphorus center is chiral. During the synthesis via phosphoramidite approach[2] both diastereoisomers are formed in a 1 : 1 ratio. Although several diastereoselective syntheses

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In memoriam Tsujiaki Hata who showed us „Es ist der Geist, der sich den Körper schafft"

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of methylphosphonates have been described[3] the most common way of obtaining pure isomers is to separate the diastereoisomers of methylphosphonate dimers chromatographically before incorporating them into oligonucleotides. This obstacle in obtaining pure isomers is reflected in the limited number of publications referring to the hybridization properties of methylphosphonates[4].

These studies resulted in a more stable hybridization (higher Tm) for the Rp isomer in experiments with antisense setup. In those assays the melting behavior of duplexes formed by methylphosphonate oligonucleotides in one strand and phosphorodiester DNA or RNA in the second strand of a duplex was examined. The more stable hybridization of the Rp isomer in contrast to the Sp isomer is believed to be a general rule and is supposed to be independent from sequence and nature of the target (RNA or DNA). Apart from these studies diastereoisomerically pure methylphosphonates have not been investigated in structures other than Watson Crick duplexes as far as we know.

Our idea was to incorporate methylphosphonates with defined stereochemistry into the loop region of small DNA hairpins (FIG. 1). Since hairpin loops are important secondary structure elements of RNA and have been described for DNA oligonucleotides too our question was how the modification in the loop would affect the stability (Tm) of these molecules.

Knowing about the dependency of the hairpin stability on the position and configuration of the methylphosphonate linkage could be useful in case of antisense oligonucleotides with self complementary sequences. The hairpin structure of such molecules has to be unfolded before the hybridization with the target is possible. Hence a less stable self association is desirable. These studies might contribute in part to answering the question of where to modify an antisense oligonucleotide.

The structural features of DNA hairpin loops has been objective of a large number of studies[5]. NMR solution structures reveal a complex pattern of H-bonds between the nucleobases, phosphate backbone as well as stacking interactions between the nucleobases.

According to our concept a methylphosphonate in the loop should have two main effects: First the substitution of one oxygen atom by a methyl group should destroy the formation of H-bonds in this center. Second we believe that Rp configurated methylphosphonate dimers prefer helical conformations with stacked nucleobases

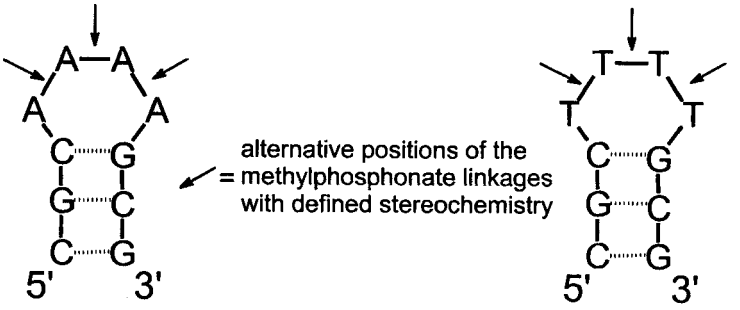

FIG. 1. Position of the methylphosphonate modifications.

whereas dimers with Sp configuration at the phosphorus tend to adopt more elongated or turned orientations. According to this hypothesis Sp-methylphosphonates should stabilize turn like conformations. These favored orientations of dinucleotide methylphosphonates are also reflected in the x-ray structures[6] of Rp-C*p*G which crystallizes as a Watson Crick paired dimer whereas the Sp-isomers of A*p*T and T*p*sT (sT is 4-thiothymidine) both adopt extended conformations in the crystal. A further evidence for the preference of helical conformations by the Rp isomer is the more stable hybridization of Rp-methylphosphonates in Watson Crick duplexes[4].

RESULTS AND DISCUSSION

The synthesis of oligonucleotides containing diastereoisomerically pure methylphosphonate linkages was accomplished via a block coupling strategy (FIG. 2). Starting from 5'- and 3'- protected thymidine and dichloromethylphosphine dinucleotide methylphosphonates T*p*T were obtained via a one pot reaction[7]. This synthetic route is more effective than the amidite chemistry applied in the synthesis of the A*p*A dimers[8]. It is fast, simple, requires no isolation and purification of trivalent intermediates, and is applicable for all base combinations. When carried out at -78 °C a remarkable diastereoselectivity in favor of the Rp-isomer (up to 8 : 1) is observed. Here we carried out the coupling reaction at 0 °C since we were interested in the Sp isomer as well. Under these conditions the two isomers were formed in a 3 : 1 (Rp : Sp) ratio as judged from ^{31}P NMR of the crude reaction mixture.

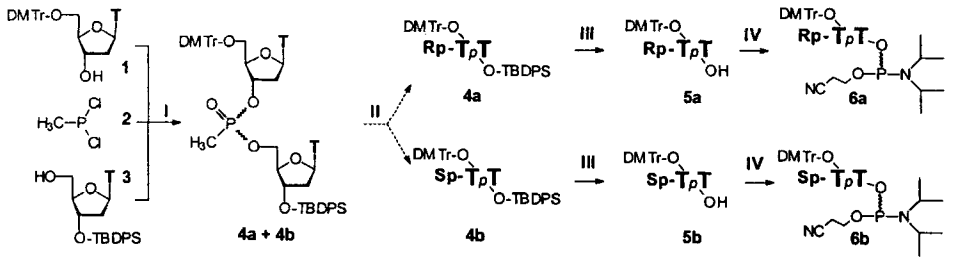

FIG. 2. Synthesis of the T*p*T dimer building blocks. I: collidine, tetrahydrofurane (THF), **2**, RT, 10 min; then addition of **1** in THF, RT, 10 min; then cooling to 0 °C and addition of **3** in THF, 0 °C, 15 min; then addition of *tert*-butylhydroperoxide, 0 °C, 15 min. II: Chromatographic separation of diastereomers. III: tetrabutylammonium fluoride (TBAF), THF, RT, 30 min. IV: diisopropylethylamine, chloro-2-cyanoethoxy-diisopropylamine-phosphine, CH_2Cl_2, RT, 2-5 h.

After chromatographic separation of the diastereomers **4a** (23% yield) and **4b** (15% yield) the 3'-TBDPS protecting group was cleaved by treatment with fluoride (60-90% yield) and the released hydroxyl group reacted with chloro-2-cyanoethoxy-diisopropylamine-phosphine to obtain the amidites **6a** and **6b** (60-70% yield). These were employed in solid phase oligonucleotide synthesis and incorporated into three possible positions of the loop sequence.

Oligonucleotides were purified via PAGE and melting curves were recorded at 10 μM oligonucleotide concentration in 140 mM NaCl, 10 mM Phosphate pH 7.0. The results are shown in TABLE 1. No concentration dependency of the Tm was observed for the unmodified reference compounds between 5 μM to 50 μM oligonucleotide concentration as expected for an unimolecular hairpin to coil transition.

In FIG. 3 the difference of the Tm in comparison to the melting temperature of the unmodified reference molecules is plotted. The modified molecules show a remarkable dependence of the Tm from the position and the configuration of the methylphosphonate linkage.

In the case of the T_4 loop a stabilization of the hairpin is observed for TT*p*TT and TTT*p*T which is independent from the configuration of the methylphosphonate. This observation reflects the structural flexibility of the T_4 loop which has been observed previously by NMR studies of similar DNA hairpins[9]. The loop residues can escape the structural constraints exerted by the methylphosphonate. NMR solution structures of

TABLE 1. Melting temperatures of modified hairpin oligonucleotides (*p* indicates the position of the methylphosphonate linkage). The Tm values for the unmodified reference compounds are 64.5 °C for 5'-CGCAAAAGCG-3' and 65.0 °C for 5'-CGCTTTTGCG-3'. All melting curves were recorded 140 mM NaCl, 10 mM phosphate, pH 7.0 and 10 μM oligonucleotide concentration. Values are estimated to be accurate within ± 1 °C.

Position of MP-linkage	Rp	Sp	Position of MP-linkage	Rp	Sp
5'-CGC**Ap**AAAGCG-3'	67.0	65.0	5'-CGC**Tp**TTTGCG-3'	66.0	63.5
5'-CGCA**Ap**AAGCG-3'	66.5	67.5	5'-CGCT**Tp**TTGCG-3'	70.5	69.0
5'-CGCAA**Ap**AGCG-3'	60.5	67.5	5'-CGCTT**Tp**TGCG-3'	69.0	69.0

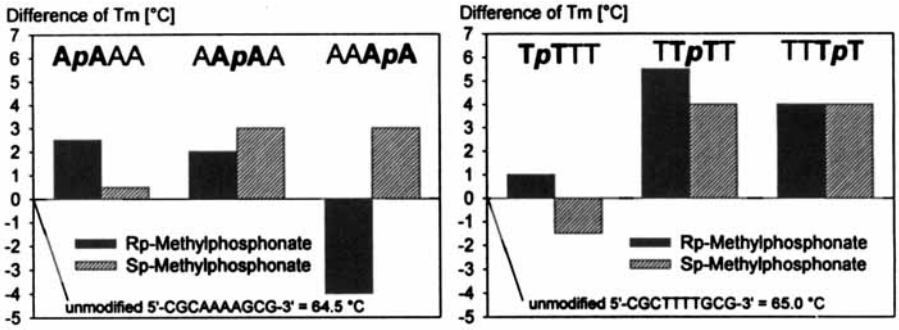

FIG. 3. Difference of melting temperature [°C] of the methylphosphonate modified oligonucleotides in comparison to the unmodified molecules. *p* indicates the position of the modification within the loop.

DNA hairpins show that these molecules adopt a B-DNA stem at moderate salt concentration. The structural features of the loop are dependent on loop size, base sequence, and the loop closing base pair. Hydrophobic stacking interactions and hydrogen bonding are important forces involved in the stabilization of the loop.

The shift from pyrimidine to purine bases leads to more conformational constraints within the loop. These molecules should exhibit an increased sensitivity towards modification. Hence the effect of the methylphosphonate modification on the stability of the A_4 hairpin is different from those observed for the T_4 loop. At middle position AA*p*AA a stabilization of 2 °C to 3 °C is observed which is independent of the

configuration at the methylphosphonate linkage. However in the case of the AAA*p*A loop a Rp configuration at the methylphosphonate destabilizes the loop whereas the molecule with the Sp methylphosphonate at this position shows a higher Tm value. Since Sp methylphosphonates prefer extended conformations this effect may suggest a turn in the phosphate backbone at this position of the loop. NMR studies on T_4 and A_4 DNA hairpins[10] support this assumption. NOE connectivities between the first and the last base of a tetraloop could be observed. Additionally the 3' loop residue exhibits connectivities with the adjacent stem residue suggesting a stacking interaction between these two bases. Another possible explanation for the dependence of the Tm on the configuration of the methylphosphonate at the AAA*p*A position is the formation of a hydrogen bond to this phosphate. The substitution of the H-bond accepting oxygen by a methyl group should have a significant effect on the stability of the hairpin.

At the 5'-end of the loop region the effects of the modification on the Tm are generally smaller. Only the Rp-A*p*AAA molecule shows a stabilization compared to the unmodified compound.

The results presented here allow three conclusions: First there is a dependence of the melting temperature of the DNA hairpin loop on the position and the configuration of the methylphosphonate linkage introduced. Second no general rule is observed for the examined sequences so that each system has to be examined for its own when the effect of a modification is to be determined. Thermal stabilization of a Sp configurated methylphosphonate in a loop region may indicate a turn position. And third the incorporation of Sp configurated methylphosphonate linkages into certain positions of DNA hairpin loops can stabilize these structures whereas Sp methylphosphonates in double helical systems in general tend to destabilize.

EXPERIMENTAL

All reactions were carried out under dry argon. Acetonitrile and diisopropylethylamine were distilled from calcium hydride. Other solvents were purchased dry over molecular sieves. Flash chromatography was performed using Merck 230-400 mesh silica gel 60. ^{1}H NMR and ^{31}P NMR spectra were recorded at 400 MHz using a Bruker AMX 400 spectrometer. Chemical shifts are reported as δ units using $CHCl_3$ (δ = 7,26) as internal standard for ^{1}H NMR spectra and 85% phosphoric acid as external standard for ^{31}P spectra. Electrospray mass spectra (ESI-MS) were measured on a Micromass Platform II instrument in negative mode. The synthesis of the phosphoramidites of Rp- and Sp-A*p*A has been previously described[8].

(*Rp*) and (*Sp*) **5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidylyl-(3'→5')-3'-*O*-[(1,1-dimethylethyl)diphenylsilyl]-2'-deoxythymidine 3'-Methylphosphonate** (**4a** and **4b**): The coupling reaction was performed following the procedure of Jäger and Löschner[7]. At RT 6.02 g (11.05 mmol) 5'-dimethoxytrityl protected thymidine **1** were reacted with 1 mL (11.15 mmol) dichloromethylphosphine **2** and coupled at 0 °C with 4.97 g (10.14 mmol) 3'-*tert*-butyldiphenylsilyl protected thymidine **3**. Oxidation with *tert*-butylhydroperoxide at 0 °C and aqueous workup yielded crude product containing 49.3% Rp product **4a** and 19.2% Sp dimer **4b** along with 15.1% (5'-5') and 16.4% (3'-3') symmetric linked sideproducts as judged from ^{31}P-NMR spectroscopy. Chromatography on silica gel with a step gradient of methanol (0 to 10%) in ethylacetate / CH_2Cl_2 (3:1) afforded 2.55 g (23%) **4a** containing 0,53 g (5%) 5'-5' isomer and 1.70 g (15%) **4b** along with several other fractions containing variable amounts of the isomers. $C_{58}H_{65}N_4O_{13}PSi$; **4a**: R_f(CH_2Cl_2/ methanol 9:1): 0.57, R_f(ethylacetate / CH_2Cl_2/ methanol 67:23:10): 0.54; ^{1}H NMR ($CDCl_3$) 9.02, 9.00 (2 H, N-H), 7.63-7.60 (m, 4 H, arom.) 7.48 (s, 1 H, 6-H), 7.45-7.20 (m, 16 H, arom.), 6.83-6.77 (m, 4 H, arom), 6.37-6.32 (m, 2 H, 1'-H), 5.11-5.08 (m, 1 H), 4.33-4.30 (m, 1 H), 4.10-4.07 (m, 2 H), 3.95-3.88 (m, 1 H), 3.77 (s, 6 H, O-CH_3), 3.60-3.53 (m, 1 H), 3.49-3.46 (m, 1 H), 3.32-3.29 (m, 1 H), 2.38-2.23 (m, 3 H), 1.93-1.86 (m, 1 H), 1.82 (s, 3 H, CH_3 thymine), 1.38 (s, 3 H, CH_3 thymine), 1.29 (d, 3 H, $^2J_{P,H}$=17.5 Hz, P-CH_3), 1.07 (s, 9 H, *t*-Bu); ^{31}P NMR ($CDCl_3$) 31.70; ESI-MS [M-H]$^-$ found: 1083.5, calc.: 1084.2; **4b**: R_f(CH_2Cl_2 / methanol 9:1): 0.48, R_f(ethylacetate / CH_2Cl_2 / methanol 67:23:10): 0.43; ^{1}H NMR ($CDCl_3$) 9.10, 8.97 (2 H, N-H), 7.71-7.70 (m, 4 H, arom.) 7.68 (s, 1 H, 6-H), 7.66-7.22 (m, 16 H, arom.), 6.90-6.86 (m, 4 H, arom), 6.47-6.40 (m, 2 H, 1'-H), 5.23-5.19 (m, 1 H), 4.29-4.26 (m, 1 H), 4.19-4.15 (m, 1 H), 4.07-4.05 (m, 1 H), 3.83 (s, 6 H, O-CH_3), 3.81-3.73 (m, 1 H), 3.67-3.58 (m, 1 H), 3.51-3.47 (m, 1 H), 3.36-3.33 (m, 1 H), 2.57-2.52 (m, 1 H), 2.47-2.32 (m, 2 H), 1.90 (s, 3 H, CH_3 thymine), 1.88-1.81 (m, 1 H), 1.46 (d, 3 H, $^2J_{P,H}$=17.6 Hz, P-CH_3), 1.45 (s, 3 H, CH_3 thymine), 1.13 (s, 9 H, *t*-Bu); ^{31}P NMR ($CDCl_3$) 32.56; ESI-MS [M-H]$^-$ found: 1083.5, calc.: 1084.2

5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidylyl(3'→5')deoxythymidine 3'-(*Rp*) Methylphosphonate (**5a**): 2.46 g mixture of **4a** and (5'-5')-isomer containing 2.04 g (1.88 mmol) **4a** were dissolved in THF (50 mL) and stirred at RT. 5.6 mL tetrabutylammonium fluoride (TBAF, 1 M in THF) were added and stirring was continued for 30 min. Chloroform (500 mL) was added and the mixture was washed with 5% aqueous $NaHCO_3$ and water. The aqueous layers were extracted twice with $CHCl_3$. The organic layers were combined, dried with Na_2SO_4 and evaporated to dryness. Chromatography on slica gel and elution with CH_2Cl_2 / methanol (9:1) afforded 1.05 g (66%) **5a** (colorless foam). No traces of deprotected 5'-5' isomer could be detected after chromatography. $C_{42}H_{47}N_4O_{13}P$; R_f(CH_2Cl_2 / methanol 9:1): 0.35, R_f(ethylacetate / methanol 9:1): 0.19; ^{1}H NMR ($CDCl_3$) 10.11, 9.80 (2 H, N-H), 7.48 (s, 1 H, 6-H), 7.30 (m, 3 H, arom and 6-H), 7.23-7.13 (m, 7 H, arom.), 6.77-6.75 (m, 4 H, arom), 6.32-6.28 (m, 1 H, 1'-H), 6.17-6.14 (m, 1 H, 1'-H), 5.12 (m, 1 H), 4.43-4.40 (m, 1 H), 4.31-4.25 (m, 1 H), 4.21-4.17 (m, 2 H), 4.12 (m, 1 H), 4.05-4.04 (m, 1 H), 3.71 (s, 6 H, O-CH_3),

3.44-3.41 (m, 1 H), 3.29-3.26 (m, 1 H), 2.64-2.59 (m, 1 H), 2.37-2.30 (m, 2 H), 2.17-2.10 (m, 1 H), 1.80 (s, 3 H, CH_3 thymine), 1.43 (d, 3 H, $^2J_{P,H}$=17.6 Hz, P-CH_3), 1.34 (s, 3 H, CH_3 thymine); ^{31}P NMR ($CDCl_3$) 32.60; ESI-MS $[M-H]^-$ found: 845.5, calc.: 845.8

5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidylyl(3'→5')deoxythymidine 3'-(*Sp*) Methylphosphonate (5b): From 1.30 g (1.19 mmol) **4b** and 3.0 mL 1 M TBAF a colorless foam containing 940 mg (93%) **5b** was obtained according to the above described procedure. $C_{42}H_{47}N_4O_{13}P$; R_f(CH_2Cl_2 / methanol 9:1): 0.32, R_f (ethylacetate / methanol 9:1): 0.16; ^1H NMR ($CDCl_3$) 9.95 (b, 2 H, N-H), 7.56 (s, 1 H, 6-H), 7.40-7.38 (m, 2 H, arom and 6-H), 7.31-7.22 (m, 8 H, arom.), 6.87-6.84 (m, 4 H, arom), 6.43-6.40 (m, 1 H, 1'-H), 6.27-6.24 (m, 1 H, 1'-H), 5.22 (m, 1 H), 4.38 (m, 1 H), 4.28-4.12 (m, 3 H), 4.08-4.01 (m, 2 H), 3.79 (s, 6 H, O-CH_3), 3.54-3.52 (m, 1 H), 3.43-3.41 (m, 1 H), 2.63-2.59 (m, 1 H), 2.51-2.46 (m, 1 H), 2.40-2.37 (m, 1 H), 2.18-2.12 (m, 1 H), 1.89 (s, 3 H, CH_3 thymine), 1.59 (d, 3 H, $^2J_{P,H}$=17.6 Hz, P-CH_3), 1.46 (s, 3 H, CH_3 thymine); ^{31}P NMR ($CDCl_3$) 33.05; ESI-MS $[M-H]^-$ found: 845.4, calc.: 845.8

5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidylyl-(3'→5')-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylamido-phosphoramidite]-2'-deoxythymidine 3'-(*Rp*)-Methylphosphonate (6a): 260 mg (0.307 mmol) 3'-deprotected methylphosphonate dimer **5a** were dissolved in 30 mL dry CH_2Cl_2, 260 µL (1.53 mmol) diisopropylethylamine and 90 µL (0.403 mmol) chloro-(2-cyanethoxy)-diisopropylamine-phosphine were added with stirring. After 2 h at RT TLC showed incomplete reaction. 50 µL (0.224 mmol) phosphitylating agent were added and stirring was continued for 3 h. 200 mL CH_2Cl_2 were added and the solution was washed twice with 5% aqueous $NaHCO_3$ and with brine. The aqueous layers were extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4 and evaporated to dryness and applied to a flash chromatography column. Elution with gradient of methanol (0 to 10%) in ethylacetate / acetonitrile / triethylamine (64:34:2) afforded 210 mg (65%) **6a** as colorless foam (mixture of two diasteroisomers). $C_{51}H_{64}N_6O_{14}P_2$; R_f(CH_2Cl_2 / methanol / triethylamine 89:10:1): 0.75, R_f(ethylacetate / methanol / triethylamine 89:10:1): 0.61; ^1H NMR ($CDCl_3$) 7.53-7.52 (m, 1 H), 7.37-7.23 (m, 10 H, arom.), 6.84-6.81 (m, 4 H, arom.), 6.42-6.38 (m, 1 H, 1'-H), 6.22-6.19 (m, 1 H, 1'-H), 5.23-5.19 (m, 1 H), 4.58-4.51 (m, 1 H), 4.37-4.15 (m, 4 H), 3.90-3.76 (m, 1 H), 3.78 (s, 6 H, O-CH_3), 3.74-3.65 (m, 1 H), 3.63-3.54 (m, 1 H), 3.51-3.48 (m, 1 H), 3.42-3.30 (m, 2 H), 2.68-2.55 (m, 3 H), 2.50-2.38 (m, 2 H), 2.35-2.19 (m, 1 H), 1.89 (s, 3 H, CH_3 thymine), 1.45 (m, 3 H, P-CH_3), 1.24 (s, 3 H, CH_3 thymine), 1.18-1.11 (m, 12 H, CH_3 diisopropylamide); ^{31}P NMR ($CDCl_3$) 150.03 149.85 (phosphoramidite), 32.32 32.24 (methylphosphonate); ESI-MS $[M-H]^-$ found: 1045.9, calc.: 1046.0

5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidylyl-(3'→5')-3'-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylamido-phosphoramidite]-2'-deoxythymidine 3'-(*Sp*)-Methylphosphonate (6b) From 300 mg (0.354 mmol) **5b**, 244 µL (1,43 mmol) diisopropylethylamine and 96 µL (0.430 mmol) chloro-(2-cyanethoxy)-diisopropylamine-phosphine, 220 mg (73%) **6b** (mixture of two diasteroisomers) were obtained as colorless foam as described above. In this case (Sp-Isomer) the reaction was complete after 2 h and no extra

addition of phosphitylation agent was necessary. $C_{51}H_{64}N_6O_{14}P_2$; R_f(CH_2Cl_2 / methanol / triethylamine 89:10:1): 0.72, R_f(ethylacetate / methanol / triethylamine 89:10:1): 0.55; ^{1}H NMR ($CDCl_3$) 8.23-8.19 (b, 2 H, N-H), 7.55 (s, 1 H), 7.38-7.35 (m, 2 H, arom.), 7.31-7.22 (m, 8 H, arom.), 6.85-6.82 (m, 4 H, arom.), 6.44-6.40 (m, 1 H, 1'-H), 6.26-6.22 (m, 1 H, 1'-H), 5.23-5.21 (m, 1 H), 4.48-4.44 (m, 1 H), 4.28-4.11 (m, 2 H), 4.07-3.94 (m, 2 H), 3.88-3.80 (m, 1 H, 2-cyanoethyl), 3.79 (s, 6 H, O-CH_3), 3.74-3.66 (m, 1 H, 2-cyanoethyl), 3.64-3.55 (m, 2 H, C-H diisopropylamide), 3.50-3.46 (m, 1 H), 3.41-3.36 (m, 1 H), 2.64-2.57 (m, 2 H, 2-cyanoethyl), 2.55-2.49 (m, 1 H), 2.48-2.39 (m, 2 H), 2.18-2.06 (m, 1 H), 1.90 (s, 3 H, CH_3 thymine), 1.56 1.55 (2 d, 3 H, $^2J_{P,H}$=17.6 Hz, P-CH_3), 1.40 (s, 3 H, CH_3 thymine), 1.19-1.16 (m, 12 H, CH_3 diisopropylamide); ^{31}P NMR ($CDCl_3$) 150.04 149.93 (phosphoramidite), 32.94 32.77 (methylphosphonate); ESI-MS $[M-H]^-$ found: 1045.8, calc.: 1046.0

Oligonucleotides were synthesized in a 1 µM scale on an Applied Biosystems 380 B DNA synthesizer using standard protocols. The coupling time was increased to 10 min for the methylphosphonate building block. Cleavage from the CPG-support was achieved by treatment with aqueous ammonia and deprotection was completed by incubation with ethylendiamine / ethanol[11]. The crude oligonucleotides were purified via polyacrylamide gel electrophoresis (PAGE) (TBE pH 8.0; 8 M urea). Product containing bands were visualized by UV-shadowing and excised. The gel pieces were crushed and eluted twice with 4 mL 10 mM aqueous ammonium acetate, concentrated in vacuo, and desalted by passage over Pharmacia PD 10 columns (sephadex). The purity of the obtained oligonucleotides was checked by RP-HPLC and analytical PAGE with silver staining.

Melting curves were recorded at 274 nm on a Varian Cary 1 Instrument equipped with a thermostated cell holder. Samples were prepared in 10 mm cells at an oligonucleotide concentration of 10 µm in 10 mM phosphate buffer pH 7.0 containing 140 mM NaCl. Tm values were extracted form the absorbance versus temperature curves by fitting to a two state model[12].

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