Synthesis and Hybridization Properties of Oligonucleotides Containing Polyamines at the C-2 Position of Purines: A Pre-synthetic Approach for the Incorporation of Spermine into Oligodeoxynucleotides Containing 2-(4,9,13-Triazatridecyl)-2′-deoxyguanosine

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Abstract: We have developed a synthesis of spermine-containing oligonucleotides (ODN-sper) which allows incorporation of multiple polyamine residues. This approach was based on the pertrifluoroacetylated 5′DMT-dGsper phosphoramidite synthon. Its coupling yield with resin-bound ODN decreased dramatically when close to the 3′-end. Optimization of the coupling conditions allowed 22-mer ODNs containing up to six spermine residues to be synthesized. Several ODNs of different sequences with 1–4 pendent spermines could be purified and their hybridization properties were evaluated. Duplex melting temperatures increased linearly with the number of polyamine residues (ΔT_m/sper = 3.0 ± 0.2 °C in 100 mM NaCl). This compares very favorably with values reported for duplexes of similar initial stability containing other cation-substituted bases. Moreover, the stability increase was neither sequence nor position-dependent, and even contiguous spermine residues did not cross-talk. Extrapolation based on these findings leads to the conclusion that a duplex formed with a 22-mer oligonucleotide containing seven spermine residues would be as stable as genomic DNA, which highlights its potential for DNA strand invasion.

Keywords: DNA recognition · duplex stability · minor groove · oligonucleotides · spermines

Introduction

Therapeutic and diagnostic uses of oligonucleotides (ODNs) have attracted the interest of chemists for over 20 years (For recent reviews see refs. [1–3]). Numerous modified DNA structures have been synthesized in order to improve properties such as affinity for the complementary DNA or RNA target. An extensive hybridization study including over 200 chemical modifications, however, led to only few compounds that increased thermal stability.[4] Cationic residues seem an obvious choice to favor interaction between two polyanions. This seemed to be the clue for the high melting temperature of bacteriophage ϕW-14 DNA[5] where half of the thymines are replaced by α-putrescinylthymidine.[6] Short α-putrescinylthymidine-containing ODNs, however, lost this property.[7] A similar strategy, aimed at decreasing repulsion between the phosphates of the two strands, led to the design of cationic[8] and zwitterionic[9] ODNs, and of ODNs bearing amines,[10–19] polyamines,[20–27] guanidines,[28] or S-methylthiourea.[29] In terms of stabilization of duplex structures, the results were widely spread and sometimes even opposite, clearly showing that the introduction of positive charges is not sufficient per se. Endogenous and synthetic polyamines are known to stabilize DNA against thermal denaturation.[30–32] This may be due to formation of a *trans*-strand network of ammonium hydrogen bonds involving the O-2 atoms of pyrimidines and N-3 of purines in the minor groove, thus clipping the DNA strands together.[33]

Spermine was therefore attached to an ODN via the C-2 position of purine which bisects the minor groove in duplex DNA; a strong thermal stabilization of the structure was observed.[23] Groove location indeed seems to be of importance, since alkylammonium[15] and imidazolium[13] groups within the minor groove showed similar effects, whereas spermine conjugation to the C-4 position of cytosine (which lies in the major groove) actually decreased stability.[21]

We[34] and others[26] have described the introduction of spermine into ODNs containing up to two 2-fluoro-2′-deoxyinosine residues (post-oligomerization strategy). Unfortunately, this method is not suitable for the preparation of polysubstituted oligonucleotides, (ODN-sper)[34] which may be of interest for diagnostic and gene therapy applications. Here we describe the synthesis of a guanine phosphoramidite **9** bearing a protected spermine moiety which is compatible

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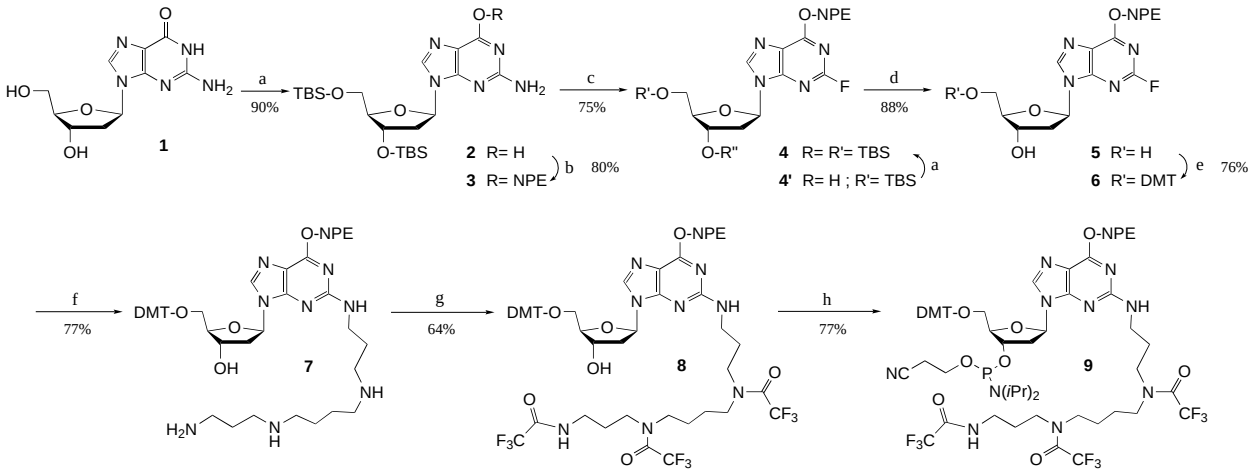

Scheme 1. Synthesis of the phosphoramidite **9**. a) TBS-Cl, imidazole, DMF; b) NPE-OH, DEAD, PPh_3, dioxane; c) PVPHF, *t*BuONO, toluene, $-5\,^{\circ}C$; d) TEA-$(HF)_3$, THF; e) DMT-Cl, pyridine; f) spermine, MeOH, $55\,^{\circ}C$; g) CF_3CO_2Et, TMG, MeOH; h) $(i\mathrm{Pr})_2P(\text{-}OCH_2CH_2CN)$, tetrazole, CH_3CN.

with oligonucleotide synthesis (a similar pre-synthetic approach is being developed for obtaining polyamine–oligonucleotide libraries[35]). Using this strategy, ODN-sper with up to six spermine residues were synthesized.

Results and Discussion

Synthetic strategy: The introduction of molecular groups into ODNs can be carried out either before (the pre-synthetic approach) or after (the post-synthetic, or convertible nucleotide approach[36]) the stepwise ODN synthesis. Both methods have been used to achieve conjugation of polyamines to ODNs. For C-2-spermine-derivatized purines, only the latter strategy has been described.[23, 26] The pre-synthetic approach requires synthesis of a protected phosphoramidite which is compatible with automated DNA synthesis. As a frequent consequence of decreased solubility and reactivity, it also needs screening and optimization of the automated DNA synthesis parameters (coupling efficiencies $<90\,\%$ for the non-natural nucleotide would lead to inseparable ODN mixtures).

The post-synthetic strategy is more versatile. It relies on the introduction of a nucleotide bearing a small reactive group such as a halogen atom. ODN synthesis thus requires no optimization and so a single tagged ODN can serve for the introduction of various molecular groups. 2-Fluoroinosine is an ideal precursor for the preparation of guanine N-2 adducts.[37, 38] ODN-sper with up to two spermine-derivatized guanines (Gsper) have initially been obtained from oligonucleotides containing this halogenated nucleotide.[23, 26] ODNs with up to seven fluoroinosines were obtained subsequently,[34] but attempts to convert them to spermine conjugates led to complex mixtures. We were therefore bound to synthesize the spermine-deoxyguanosine phosphoramidite **9** and to optimize its incorporation into an oligonucleotide.

Synthesis of phosphoramidite 9: Fluorine displacement is an efficient method for introducing nucleophilic residues at the C-2 position of purine nucleosides.[39–45] We followed a similar route, using the key 2-fluoro-2′-deoxyinosine intermediate **5** (Scheme 1). Deoxyribose protection of **1** was achieved using *tert*-butyldimethylsilylchloride. The O-6 was protected to increase subsequent fluorination yields. Compound **2** was thus treated with *p*-nitrophenylethyl alcohol using Pfleiderer's procedure[46] to yield compound **3**. As described earlier, several methods were tested for diazotation and fluorination of deoxyguanosine.[34] The best results were obtained in nonaqueous medium using *tert*-butylnitrite and polyvinylpyridinium/HF[47] as source of fluorine. The reaction proceeded so smoothly that the silyl groups remained in place: the main product was the disilylated 2-fluoro-2′-deoxyinosine (**4**), with monoprotected derivative **4′** as by-product. The latter compound was resilylated to afford the protected fluoro-2′-

Abstract in French: *Nous avons développé une méthode de synthèse d'oligonucléotides contenant des résidus spermine (ODN-sper) permettant l'incorporation de résidus multiples. Cette approche est basée sur le phosphoramidite du synthon per-trifluoroacetyl 5′DMT-dGsper. Son rendement de couplage avec un oligonucléotide (ODN) porté par la résine décroît dramatiquement au fur et à mesure que l'on se rapproche de l'extrémité 3′. L'optimisation des conditions de couplage a néanmoins permis de synthétiser des ODN 22-mères contenant jusqu'à six résidus spermine. Plusieurs ODN's de séquences variées, contenant de une à quatre pendigouillantes spermines ont été purifiés et étudiés. Les températures de fusion des hybrides bicaténaires correspondants augmentent proportionnellement au nombre de résidus polyamine (ΔT_m/sper = 3.0 ± 0.2 °C dans 100 mM NaCl). Ces valeurs se comparent avantageusement à celles décrites pour des séquences de stabilités initiales comparables, comprenant d'autres substitutions cationiques sur les bases. De plus, le gain de stabilité ne dépend ni de la séquence ni de la position, et même des spermines contiguës ne se gênent pas. Une extrapolation basée sur ces résultats conduit à la conclusion qu'un oligonucléotide 22 mère contenant sept résidus spermine serait aussi stable que l'ADN génomique, révélant ainsi le potentiel de cette approche pour l'invasion de brin d'ADN.*

deoxyinosine (**4**) with an overall yield of 75%. The lipophilic TBS groups allowed straightforward purification of **4**. The silyl groups were gently removed with triethylamine tris(hydrogen fluoride)[48] (**5**) and the primary alcohol was selectively protected with DMT chloride, providing the fluorodeoxyinosine derivative **6**.

Spermine was readily incorporated into the purine nucleoside through its primary amines by heating the reaction mixture in methanol,[49] leading to the 2-spermino-2′-deoxyinosine (**7**). The excess of hydrophilic spermine was removed by extraction in a ternary solvent system (CH_2Cl_2/*i*PrOH/water) and no further purification was necessary. The trifluoroacetyl group (TFA) seemed to be a good polyamine protecting group for ODN synthesis[21, 24, 27] but pertrifluoroacetylation of **7** proved to be tedious. Trifluoroacetic anhydride could not be used as it causes glycosidic bond cleavage. Smoother methods using ethyl trifluoroacetate or *S*-ethyl trifluorothioacetate in the presence of triethylamine gave rather poor yields. Replacement of triethylamine by other bases has been shown to significantly improve the protection of some amino acids.[50] Using tetramethylguanidine (TMG) as base, ethyl trifluoroacetate converted **7** into the tris protected compound **8** with 64% yield. TMG also reacted with ethyl trifluoroacetate, so the reagents had to be used in large excess. Prior to chromatography, the TMG–TFA adduct had to be removed by washing the crude material with diethyl ether. Phosphitylation using a standard protocol[51] afforded the phosphoramidite **9**. During chromatographic purification, the two phosphorus diastereoisomers **9a** (fast migrating) and **9b** (slow migrating) were separated incidentally (see ^{31}P NMR in Figure 1), although mixed for the coupling reaction.

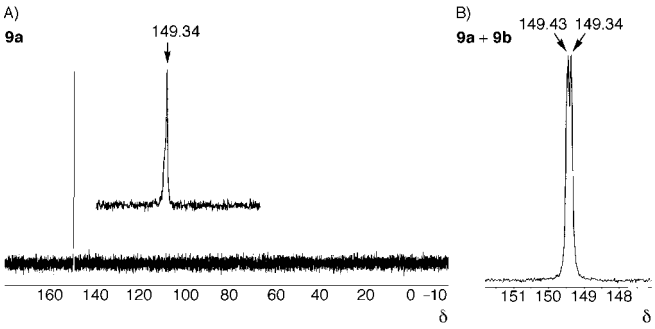

Figure 1. ^{31}P NMR spectra of A) isomer **9a**, B) mixture of isomers **9a** and **9b**.

Oligodeoxynucleotide synthesis: ODNs were synthesized on controlled pore glass using standard phosphoramidite chemistry.[52] The behavior of synthon **9** in the ODN synthesis conditions was checked by TLC. The inertness of the trifluoroacetyl group was tested, as it was reported that it could undergo transamidation during the capping step.[53] Mass spectrometry showed no trifluoroacetyl/acetyl exchange in the capping conditions. The phosphoramidite **9** appeared to be less reactive than the natural base phosphoramidites, possibly because of steric hindrance due to the large tris-TFA-spermino moiety. We optimized the coupling reaction (Table 1) using several solvents (CH_3CN, CH_2Cl_2 and CH_2Cl_2/CH_3CN mixtures), coupling times and concentrations. Dichloromethane, which allowed higher concentrations to be used, let to decreased yields possibly because of reduced acidity of tetrazole. Coupling times larger than 10 min did not improve yields. The best results were obtained with two coupling steps, using a 0.09 M solution of **9** in acetonitrile. We also observed variable coupling yields for **9** along the ODN synthesis, in otherwise identical conditions. A plot of yield versus coupling step (Figure 2) clearly shows that the closer to the 3′-end, the worse the yield. The simplest explanation is restricted approach of the large phosphoramidite **9** to the growing oligonucleotide chain when close to the CGP-resin. We still were able to incorporate up to six Gsper with good yields (Table 2), yet subsequent work-up was unsuccessful up to now for ODN-sper with more than four Gsper.

Table 1. Optimization of the coupling yield for reaction of **9** with a nascent ODN.

Conditions	**I**	**II**	**III**	**IV**	**V**
solvent	CH_2Cl_2/CH_3CN	CH_3CN	CH_3CN	CH_3CN	CH_3CN
concentration	0.1 M	0.09 M	0.09 M	0.09 M	0.09 M
number of steps	1	1	1	1	2
coupling time	10 min	10 min	15 min	20 min	10 min
coupling yield	61.0%	81.8%	82.9%	83.0%	94.8%

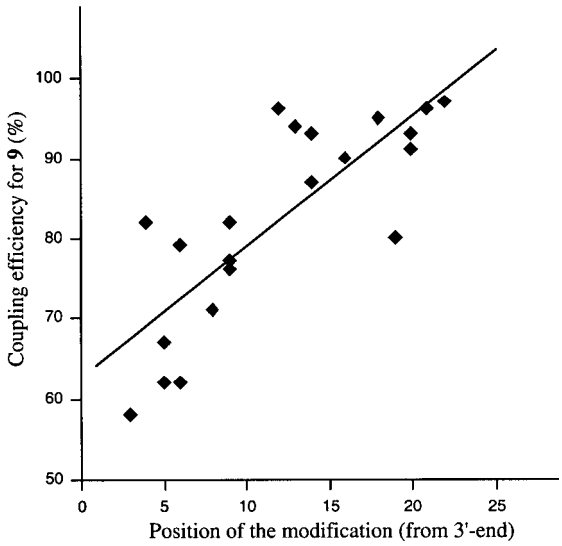

Figure 2. Coupling yields decrease with decreasing distance resin/compound.

Table 2. Oligonucleotide sequences. X: 2-spermino-2′-deoxyinosine.

Name	Sequence
22dGsp0-a	5′-ATG AGA TGT GAC GAA CGT GTA C-3′
22dGsp1-a	5′-ATG AGA TGT GAC GAA CGT **X**TA C-3′
22dGsp3-a	5′-ATG A**X**A TGT GAC **X**AA C**X**T GTA C-3′
22dGsp6-a	5′-ATG A**X**A T**X**T **X**AC **X**AA C**X**T **X**TA C-3′
22compl-a	5′-GTA CAC GTT CGT CAC ATC TCA T-3′
22dGsp0-b	5′-TGG TAA AAT GGA AGA CGC CAA A-3′
22dGsp3-b	5′-TG**X** TAA AAT **X**GA A**X**A CGC CAA A-3′
22compl-b	5′-TTT GGC GTC TTC CAT TTT ACC A-3′
22dGsp0-c	5′-ATG AAG AGA TAC GCC CTG GTT C-3′
22dGsp4-c	5′-AT**X** AA**X** AGA TAC GCC CT**X** **X**TT C-3′
22compl-c	5′-GAA CCA GGG CGT ATC TCT TCA T-3′

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Removal of NPE cannot be performed by standard ammonia ODN deprotection conditions,[54] so the solid support was first treated with DBU in hot pyridine. Thymine was added as a scavenger, as acrylonitrile which was simultaneously liberated from the phosphate cyanoethylester groups damages DNA.[55] ODN-sper were then cleaved and further deprotected in hot aqueous ammonia. The DMT-on ODN-sper were purified by reverse-phase HPLC. After DMT removal, ODN-sper showed a single HPLC peak and were characterized by MALDI-TOF mass spectrometry. The mass spectrum of 22dGsp3-a showed a second peak that was attributed to sequences containing only two Gsper (i.e., 21dGsp2-a). Indeed, 22dGsp3-a was synthesized with non-optimal conditions **I** (Table 1) which led to 21dGsp2-a as a consequence of incomplete capping reactions.

PAGE electrophoresis of ODN-sper: ODN-sper were 5′-radiolabeled with ^{32}P(ATP). The kinase activity was not inhibited by the pendent polyamine, even for ODN-sper with Gsper close to the 5′-end (i.e., 22dGsp3-b and 22dGsp4-c). Purity was confirmed by denaturating PAGE (Figure 3). ODN-sper had lower electrophoretic mobility than their control ODNs. The relative retardation was proportional to the number of spermine substitutions (Figure 3). The mobilities of 22dGsp3-a and 21dGsp2-a were very different (Figure 3A, lane 3) and allowed final purification to be performed.

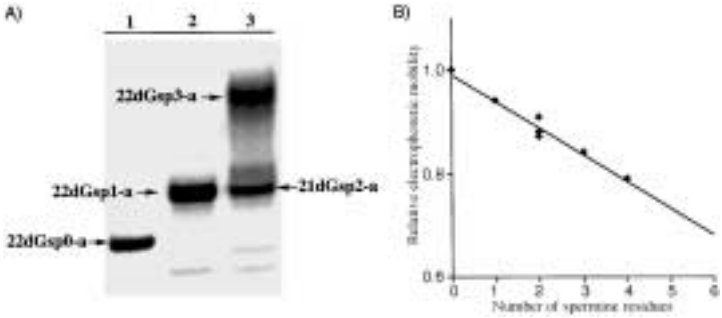

Figure 3. Electrophoretic mobilities of ODN-sper′s. A) Denaturating PAGE. Lane 1: 22dGsp0-a, 2: 22dGsp1-a, 3: 22dGsp3-a. B) Relative mobilities decrease with increasing number of spermine modifications.

Duplex formation of ODN-sper: The presence of several spermine residues linked to the nucleic bases may interfere with proper duplex formation. Spectroscopic titration of 22dGsp4-c (Figure 4) and of control 22dGsp0-c with their complementary strand 22compl-c was therefore undertaken. The maximum hypochromicities of 22dGsp0-c and 22dGsp4-c were similar (91.1 and 89.6 %, respectively) and went through a minimum for a 1:1 molar ratio; this confirms that ODN-sper form predominantly duplex structures.

Duplex melting temperatures (T_m) of ODN-sper with their complementary sequences were measured and compared with the corresponding natural duplexes (Table 3). A single, clear-cut transition was observed in all cases. Spermine conjugation always increased T_m, although alkyl substitution at N-2 interferes with GC base pairing. Unexpectedly, melting

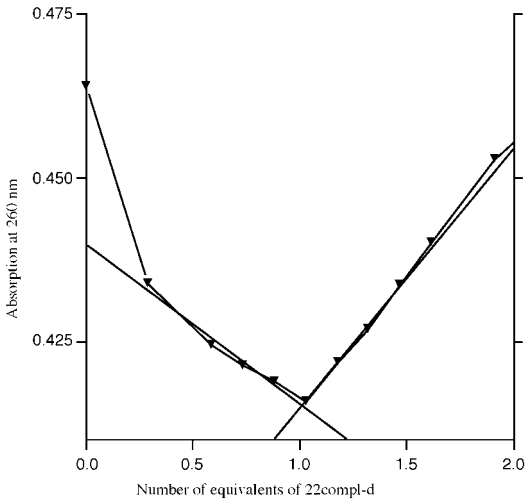

Figure 4. Duplex formation of 22dGsp4-c with the complementary strand 22compl-c.

Table 3. Melting temperatures [°C] of duplexes formed by ODN and by ODN-sper with their complementary strands in 10 mM phosphate buffer (pH 7.4), 100 mM NaCl.

ODN-sper	ODN T_m	ODN-sper T_m	ΔT_m	ΔT_m/sper
22dGsp1-a	66.4	69.4	+3.0	+3.0
22dGsp3-a	66.4	76.1	+9.7	+3.2
22dGsp3-b	64.0	72.8	+8.8	+2.9
22dGsp4-c	64.5	76.0	+11.5	+2.9

temperatures increased linearly with the number of spermines (up to four) borne by the oligonucleotide, with an increment of ΔT_m/sper $= 3.0 \pm 0.2$ °C. This value compares very favorable with the values reported in similar salt conditions for ODNs containing other cation-substituted bases. Moreover (Table 3), the stability increase was neither sequence nor position-dependent with respect to the ODN extremities. Even contiguous Gsper in 22dGsp4-c did not decrease their individual contribution to duplex stability. This remarkable behavior may be a consequence of minor groove location of the pendent spermine residues, where multiple bidentate hydrogen-bonding acceptor sites are available (a purine N-3, a pyrimidine O-2, and two O-4′ per base pair) regardless of the sequence and in both directions. Extrapolation to multiple spermine substitutions is therefore justified. Stability similar to that of a large DNA fragment ($T_m = 85$ °C in 100 mM NaCl) would be reached with seven spermine residues.

Conclusion

We have succeeded in extending the number of ODN-conjugated polyamines from two, with the convertible nucleotide approach, to four with the optimized post-synthetic strategy. The remarkable hybridization properties of ODN-sper a posteriori justify the choice for purine N-2 conjugation, which was in turn guided by our initial finding that spermine was located in the B-DNA minor groove. A reasonable extrapolation shows that a duplex formed with a 22-mer

oligonucleotide bearing seven spermine residues would be as stable as genomic DNA. Besides potential solubility problems (it would be an overall neutral molecule), such an oligonucleotide may be capable of sequence-selective recognition of double-stranded DNA by strand invasion. The underlying Watson–Crick pairing is general (as opposed to triple helix formation by Hoogsteen pairing with homopurine sequences) and may find wide applications in diagnosis and therapy.

Experimental Section

Abbreviations: EtOH: ethanol, TBS: *tert*-butyldimethylsilyl, DMF: *N*,*N*-dimethylformamide, DBU: 1,8-diaza-bicyclo[5.4.0]undec-7-ene, NPE: 2-(4-nitrophenyl)ethyl, DEAD: diethyl azodicaboxylate, PVPHF: poly[4-vinylpyridinium poly(hydrogen fluoride)], *t*BuONO: *tert*-butylnitrite, TEA: triethylamine, TEA-$(HF)_3$: triethylamine tris(hydrogen fluoride), DMT: 4,4′-dimethoxytrityl, spermino: -NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2, TFA: trifluoroacetate, TMG: 1,1,3,3-tetramethylguanidine, $(i\mathrm{Pr})_2$P(-OCH_2CH_2CN): 2-cyanoethyl tetraisopropylphosphorodiamidite, TEAA: triethylammoniumacetat, MALDI-TOF: matrix-assisted laser desorption/ionization–time-of-flight, PAGE: polyacrylamide gel electrophoresis.

General: Chemicals and solvents were purchased from Fluka, Aldrich, Merck, Janssen, Carlo Erba and SDS. TLC analysis was performed on precoated Merck silica gel 60 F_{254} plates using phosphomolybdic acid as indicator. Flash silica gel chromatography was performed with silica Si60 (40–63 μm, Merck) and the indicated solvent system. ^{1}H, ^{31}P NMR spectra were recorded on a Bruker DPX-300 spectrometer (300 MHz) and ^{19}F on a Bruker WP-200 SY (200 MHz).

3′,5′-*O*-Di-*tert*-butyldimethylsilyl-2′-deoxyguanosine (2): 2′-deoxyguanosine (**1**) (1 g, 3.5 mmol), *tert*-butyldimethylsilyl chloride (2.1 g, 13.9 mmol), and imidazole (1.68 g, 24.7 mmol) were dissolved in DMF (2 mL) and stirred at room temperature. After 30 min the solution was clear and a white solid began to precipitate. After 24 h the reaction was quenched with ethanol (10 mL) and stirred for an additional 30 min. The precipitate was filtered, washed with cold ethanol, crystallized in ethanol at −20 °C and dried under vacuum to give **2** as a fine white powder (1.56 g, 90 %). R_f (EtOH/CH_2Cl_2 1:9): 0.50; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 8.1$ (d, 2H, Ar), 8.0 (s, 1H, H-8), 7.6 (d, 2H, Ar), 6.3 (t, 1H, H-1′), 4.85 (br s, 2H, -NH_2), 4.7 (t, 2H, -CH_2-O), 4.6 (m, 1H, H-3′), 3.9 (m, 1H, H-4′), 3.8 (t, 2H, H-5′), 3.3 (t, 2H, -CH_2-Ar), 2.6 (m, 1H, H-2′), 2.4 (m, 1H, H-2′), 0.92, 0.88 (2s, 2 × 9H, 2 × SiC-CH_3), 0.07, 0.05 (2s, 2 × 6H, 2 × Si-CH_3); FAB-MS (positive mode): *m*/*z* (%): calcd for $C_{22}H_{41}N_5O_4Si_2$ (495.27); found 1013.5 (13) [2*M*+Na]$^+$, 518.3 (35) [*M*+Na]$^+$, 496.3 (11) [*M*+H]$^+$, 174.1 (85) [*M* − ribose+Na]$^+$, 152.1 (100) [*M* − ribose + H]$^+$.

3′,5′-*O*-Di-*tert*-butyldimethylsilyl-6-*O*-[2-(4-nitrophenyl)ethyl]-2′-deoxyguanosine (3): 3′,5′-*O*-Di-TBS-2′-deoxyguanosine (**2**) (5.8 g, 11.7 mmol), PPh_3 (6.15 g, 23.4 mmol), and NPE alcohol (3.90 g, 23.3 mmol) were suspended in dry dioxane (100 mL). The mixture was stirred for 15 min under argon and DEAD (3.7 mL, 23.4 mmol) were added. The suspension went pink and turned to a clear yellow solution. After 24 h the solvent was evaporated and the product was purified by silica gel chromatography (CH_2Cl_2/hexane 9:1). The fraction containing the protected compound was further chromatographed with 1–5% acetone in CH_2Cl_2. The desired product was obtained as yellowish foam (6.01 g, 80 %). R_f (EtOH/CH_2Cl_2 1:9): 0.69; ^{1}H NMR (200 MHz, [D$_4$]MeOH): $\delta = 8.1$ (d, 2H, Ar), 8.0 (s, 1H, H-8), 7.6 (d, 2H, Ar), 6.3 (t, 1H, H-1′), 4.85 (br s, 2H, -NH_2), 4.7 (t, 2H, -CH_2-O), 4.6 (m, 1H, H-3′), 3.9 (m, 1H, H-4′), 3.8 (t, 2H, H-5′), 3.3 (t, 2H, -CH_2-Ar), 2.6 (m, 1H, H-2′), 2.4 (m, 1H, H-2′), 0.92, 0.88 (2s, 2 × 9H, 2 × SiC-CH_3), 0.07, 0.05 (2s, 2 × 6H, 2 × Si-CH_3); elemental analysis calcd (%) for $C_{30}H_{48}N_6O_6Si_2$(644.32): C 55.87, H 7.50, N 13.03; found C 55.71, H 7.31, N 12.98; FAB-MS (positive mode): *m*/*z* (%): 1289.6 (3) [2*M*+H]$^+$, 645.3 (22) [*M*+H]$^+$, 301.1 (100) [*M* − ribose+H]$^+$.

3′,5′-*O*-Di-*tert*-butyldimethylsilyl-6-*O*-[2-(4-nitrophenyl)ethyl]-2′-deoxy-2-fluoroinosine (4): 3′,5′-*O*-Di-TBS-6-*O*-NPE-2′-deoxyguanosine (**3**) (2.6 g, 4.03 mmol) was dissolved in anhydrous toluene (30 mL) and cooled to −65 °C. This solution was added to a suspension of PVPHF (8 g) in toluene (30 mL, also at −65 °C) in a Teflon flask. *t*BuONO (1.9 mL, 16 mmol) was gradually added to the mixture under vigorous stirring and the temperature was slowly allowed to rise to −5 °C. The reaction was followed by disappearance of the starting material on TLC. The polymer was filtered off and the solution was neutralized with a 0.1 M $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, evaporated, and the residual oil was purified on a silica gel column eluted with 1–20 % MeOH in CH_2Cl_2. The by-products were re-silylated (TBS-Cl, imidazole, DMF) and purified by chromatography as described above. All fractions containing the protected fluoroinosine (**4**) were collected, affording the desired product with 75 % overall yield. R_f (MeOH/CH_2Cl_2 1:99): 0.70; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 8.25$ (s, 1H, H-8), 8.15 (d, 2H, Ar), 7.5 (d, 2H, Ar), 6.4 (t, 1H, H-1′), 4.85 (t, 2H, -CH_2-O), 4.6 (m, 1H, H-3′), 4.0 (m, 1H, H-4′), 3.8 (t, 2H, H-5′), 3.3 (t, 2H, -CH_2-Ar), 2.6 (m, 1H, H-2′), 2.4 (m, 1H, H-2′), 0.92, 0.88 (2s, 2 × 9H, 2 × SiC-CH_3), 0.07, 0.05 (2s, 2 × 6H, 2 × Si-CH_3); ^{19}F NMR (188.3 MHz, CD_3CN, TFA as reference): $\delta = 29.9$ (s); FAB-MS (positive mode): *m*/*z* (%): calcd for $C_{30}H_{46}FN_5O_6Si_2$ (647.30); found 648.5 (20) [*M*+H]$^+$, 304.2 (100) [*M* − ribose+H]$^+$.

6-*O*-[2-(4-Nitrophenyl)ethyl]-2′-deoxy-2-fluoroinosine (5): 3′,5′-*O*-Di-TBS-6-*O*-NPE-2′-deoxy-2-fluoroinosine (**4**) (700 mg, 1.08 mmol) was dissolved in THF (6 mL) and mixed with TEA-$(HF)_3$ (1.06 mL, 6.48 mmol). The mixture was stirred under argon for 17 h at room temperature. The solvent was evaporated, the residue was dissolved in CH_2Cl_2 and purified by silica gel column chromatography with 0–5 % MeOH in CH_2Cl_2. The solvent was evaporated in vacuo to give a beige foam (400 mg, 88 %). R_f (MeOH/CH_2Cl_2 5:95): 0.45; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.18$ (d, 2H, Ar), 8.00 (s, 1H, H-8), 7.50 (d, 2H, Ar), 6.33 (dd, 1H, H-1′), 4.86 (t, 2H, -CH_2-O), 4.80 (m, 1H, H-3′), 4.20 (m, 1H, H-4′), 3.9 (m, 2H, H-5′), 3.33 (t, 2H, -CH_2-Ar), 2.96 (m, 1H, H-2′), 2.37 (m, 1H, H-2′); FAB-MS (positive mode): *m*/*z* (%): calcd for $C_{18}H_{18}FN_5O_6$ (419.12); found 420.2 (55) [*M*+H]$^+$, 304.1 (100) [*M* − ribose + H]$^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-[2-(4-nitrophenyl)ethyl]-2′-deoxy-2-fluoroinosine (6): 6-*O*-NPE-2′-deoxy-2-fluoroinosine (**5**) (400 mg, 0.95 mmol) was coevaporated with dry pyridine and the residue was dissolved in pyridine (10 mL). DMT chloride (520 mg, 1.53 mmol) was added and the solution was kept under argon. After 17 h the reaction was quenched with MeOH (10 mL) and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and purified by chromatography (silica gel pretreated with 1 % of TEA in CH_2Cl_2) with 1–4 % MeOH in CH_2Cl_2 containing 1 % TEA to yield the title compound (520 mg, 76 %). R_f (TEA/MeOH/CH_2Cl_2 1:5:94): 0.50; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.17$ (d, 2H, Ar), 8.05 (s, 1H, H-8), 7.48 (d, 2H, Ar), 7.38 (d, 2H, Ar), 7.25 (m, Ar), 6.79 (dd, 4H, Ar), 6.38 (t, 1H, H-1′), 4.83 (t, 2H, -CH_2-O), 4.66 (m, 1H, H-3′), 4.15 (m, 1H, H-4′), 3.77 (s, 6H, -O-CH_3), 3.4 (m, 2H, H-5′), 3.31 (t, 2H, -CH_2-Ar), 2.7 (m, 1H, H-2′), 2.5 (m, 1H, H-2′); ^{19}F NMR (188.3 MHz, CD_3CN, TFA as reference): $\delta =$ 30.3 (s); FAB-MS (positive mode): *m*/*z* (%): calcd for $C_{39}H_{36}FN_5O_8$ (721.25); found 722.2 (2) [*M*+H]$^+$, 303.1 (100) [DMT]$^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-[2-(4-nitrophenyl)ethyl]-2-(3,9,13-triazatridecyl)-2′-deoxyguanosine (7): 5′-*O*-DMT-6-*O*-NPE-2′-deoxy-2-fluoroinosine (**6**) (715 mg, 0.99 mmol) and spermine (1 g, 4.95 mmol) were dissolved in MeOH (40 mL, distilled over magnesium). The reaction mixture was stirred at 55 °C under argon for 4 h, then CH_2Cl_2 (70 mL) and *i*PrOH (10 mL) were added. The organic layer was washed with water until its pH was neutral. When separation of the phases was too slow, small amounts of *i*PrOH were added. The organic layer was dried over $MgSO_4$ and evaporated in vacuo. The residue was lyophilized with a benzene/MeOH 9:1 mixture providing a white powder (685 mg, 77 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.15$ (d, 2H, Ar), 7.71 (s, 1H, H-8), 7.47 (d, 2H, Ar), 7.3 (m, Ar), 6.76 (dd, 4H, Ar), 6.26 (t, 1H, H-1′), 4.86 (m, 1H, H-3′), 4.72 (t, 2H, -CH_2-O), 4.07 (m, 1H, H-4′), 3.79 (s, 6H, -O-CH_3), 3.4 (m, 2H, H-5′), 3.27 (t, 2H, -CH_2-Ar), 2.7 (m, H-2′, C-CH_2-NH-), 1.7 (m, C-CH_2-C); FAB-MS (positive mode): *m*/*z* (%): calcd for $C_{49}H_{61}N_9O_8P$ (903.46); found 904.1 (2) [*M*+H]$^+$, 602.0 (5) [*M* − DMT+H]$^+$, 303.0 (100) [DMT]$^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-[2-(4-nitrophenyl)ethyl]-2-[tris-*N*,*N*′,*N*″-trifluoroacetamido-(3,9,13-triazatridecyl)]-2′-deoxyguanosine (8): 1,1,3,3-Tetramethylguanidine (1.4 mL, 11 mmol) and ethyl trifluoroacetate (2.7 mL, 22 mmol) were added to a solution of 5′-*O*-DMT-6-*O*-NPE-2-spermino-2′-deoxyinosine (**7**) (680 mg, 0.75 mmol) in MeOH (25 mL). The reaction was stirred under argon at room temperature for 17 h, AcOEt (12 mL) was added and the solvent was evaporated in vacuo (repeated twice). The residue was washed with Et_2O and purified on a neutral

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aluminum oxide column with 0–15% EtOH in CH_2Cl_2 (containing 0.2% of TEA) to yield the title compound (575 mg, 64%). R_f ($MeOH/CH_2Cl_2$ 5:95+1% TEA): 0.55; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.15$ (dd, 2H, Ar), 7.72 (d, 1H, H-8), 7.47 (d, 2H, Ar), 7.38 (d, 2H, Ar), 7.3 (m, Ar), 6.79 (dd, 4H, Ar), 6.33 (t, 1H, H-1′), 4.72 (t, 2H, -CH_2-O), 4.64 (m, 1H, H-3′), 4.11 (m, 1H, H-4′), 3.79 (s, 6H, -O-CH_3), 3.4 (m, 16H, H-5′ + C-CH_2-N-CO- + -CH_2-Ar), 2.7 (m, 1H, H-2″), 2.5 (m, 1H, H-2′), 1.9 (m, 4H, C-CH_2-C), 1.6 (m, 4H, C-CH_2-C); FAB-MS (positive mode): *m/z* (%): calcd for $C_{55}H_{58}F_9N_9O_{11}P$ (1191.41); 1192.0 (4) $[M+H]^+$, 890.0 (3) $[M-DMT+H]^+$, 774.0 (13) $[M-ribose+H]^+$, 303.1 (100) $[DMT]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-[2-(4-nitrophenyl)ethyl]-2-[tris-*N*,*N*′,*N*″-trifluoroacetamido-(4,9,13-triazatridecyl)]-2′-deoxyguanosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl phosphoramididite (9): Compound **8** (220 mg, 0.18 mmol) and tetrazole (12 mg, 0.17 mmol) were dissolved in CH_2Cl_2 (3 mL) and 2-cyanoethyl-tetraisopropylphosphorodiamidite (170 μL, 0.54 mmol) was added. The solution was stirred under argon at room temperature. After 2 h, the reaction was quenched with EtOH (2 mL) and the mixture was immediately evaporated in vacuo. The residue was dissolved in CH_2Cl_2 (50 mL) and washed twice with 0.2 M $NaHCO_3$ (15 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under vacuum. The residue was purified on a silica gel column with AcOEt/hexane 1:1 to AcOEt. The products were precipitated from cold hexane and were lyophilized from benzene to give the diastereoisomers **9a** and **9b** as a beige powder (total 209 mg, 77%). Before ODN synthesis, both isomers were dried under vacuum over P_2O_5 for one week and then dissolved in the dry solvents indicated below. R_f (AcOEt/hexane 3:1+1% of TEA) **9a**: 0.48, **9b**: 0.32; ^1H NMR (300 MHz, $CDCl_3$): **9a**: $\delta =$ 2.50, **9b**: $\delta = 2.63$ (t, 2H, O-C-CH_2-CN); ^{31}P NMR (121.5 MHz, CD_3CN): **9a**: $\delta = 149.35$, **9b**: $\delta = 149.43$ (s); FAB-MS (positive mode): *m/z* (%): calcd for $C_{64}H_{75}F_9N_{11}O_{12}P$ (1391.52); 1392.5 (1) $[M+H]^+$, 774.2 (4) $[M-ribose+H]^+$, 303.1 (100) $[DMT]^+$.

Oligonucleotide synthesis: Oligonucleotides were assembled on an automated DNA synthesizer (Applied Biosystems 380B, CA, USA) using standard phosphoramidite chemistry. The reagents and phosphoramidites were purchased from PerSeptive Biosystems and the solid supports from MilliGen/Biosearch (LCAA-CPG, 1000 Å, 1 μmol). Commercial phosphoramidites were dissolved in dry acetonitrile (0.1 M solutions) and 3 min standard coupling times were used. Several solvents and coupling times were tested for phosphoramidite **9**. Finally, oligonucleotides 22dGsp1-a and 22dGsp3-a were obtained with 0.1 M of **9** in 1:1 CH_2Cl_2/CH_3CN but the best results were obtained using a 0.09 M solution in dry acetonitrile. During the synthesis, the phosphoramidite **9** solution was kept on activated 4 Å molecular sieves. After introduction of the modified base, the capping step was repeated and, in some cases, the coupling step as well. Resin-supported ODN-sper were treated with 0.5 M DBU in pyridine (containing 2×10^{-2} M thymine) for 16 h at 55 °C, washed twice with dry pyridine, then twice with water. ODN-sper were cleaved and further deprotected with 32% aqueous ammonia for 15 min at room temperature and for 16 h at 55 °C. DMT-protected oligonucleotides were purified by HPLC on a Uptisphere C18 reverse phase column eluted with increasing concentrations of CH_3CN in 0.1 M TEAA buffer (pH 7.0). The desired fractions were evaporated and the residue was treated with 80% aqueous AcOH for 15 min. The solution was quickly evaporated, 1% aqueous TEA was added and concentrated to give the fully deprotected oligonucleotides. Samples were further purified by HPLC (with the same eluent) to remove minor impurities. MALDI-TOF MS (negative-ion mode) *m/z*: 22dGsp1-a: calcd 7009, found 7008; 22dGsp3-a: calcd 7379, found 7376; 21dGsp2-a: calcd 6866, found 6865; 22dGsp3-b: calcd 7381, found 7380; 22dGsp4-c: calcd 7495, found 7488.

PAGE electrophoresis: ODNs were labeled with ^{32}P using T4 polynucleotide kinase (BioLabs)[56] and run (52 V cm^{-1} for 2 h at room temperature) on a denaturating gel (18.5%/0.5% acrylamide/*N*,*N*-methylenebisacrylamide, 90 mM Tris borate, 2 mM EDTA, 7 M urea). Labeled ODNs were visualized with a PhosphoImager 425 (Molecular Dynamics). Relative mobilities (m_R) were measured using the unmodified sequence 22dGsp0-a as reference. 22dGsp1-a: $m_R = 0.91$; 21dGsp2-a: $m_R = 0.91$; 22dGsp3-a: $m_R = 0.84$. Oligonucleotides 21dGsp2-a and 22dGsp3-a were cut out from the gel, extracted with 0.5 M $AcONH_4$, 1 mM EDTA pH 8.0 buffer and precipitated with three volumes of cold ethanol.

Duplex formation experiments: 22dGsp0-c (2.22 nmol) and 22c-dGsp4 (1.72 nmol) were each dissolved in 10 mM phosphate buffer (1 mL) containing 100 mM NaCl (pH 7.5). Solutions were titrated by adding 1–4 μL aliquots of 125 μM 22compl-c. After each addition the mixture was heated 5 min at 90 °C and slowly cooled to RT. UV absorption was recorded at 260 nm with a UVIKON 930 spectrophotometer (Kontron Instruments). Values were corrected and the concentration of single stranded DNA was normalized after each measurement.

Melting temperatures: Hybridization experiments were carried out on a CARY3 UV-visible spectrophotometer (Varian). Absorption at 260 nm was recorded versus temperature at a heating rate of 0.5 °C min^{-1}. Duplex concentrations were 2 μM in a 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl. Transition temperatures were obtained from first-order derivative plots of absorbance *versus* temperature. Decane was added on the top of each sample to avoid evaporation.

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[1] K. J. Myers, N. M. Dean, *Trends Pharmacol. Sci.* **2000**, *21*, 19–23.
[2] S. Agrawal, R. P. Iyer, *Pharmacol. Ther.* **1997**, *76*, 151–160.
[3] A. Alama, F. Barbieri, M. Cagnoli, G. Schettini, *Pharmacol. Res.* **1997**, *36*, 171–178.
[4] S. M. Freier, K. H. Altmann, *Nucleic Acids Res.* **1997**, *25*, 4429–4443.
[5] A. M. Kropinski, R. J. Bose, R. A. Warren, *Biochemistry* **1973**, *12*, 151–157.
[6] J. S. Wiberg, *J. Biol. Chem.* **1967**, *242*, 5824–5829.
[7] T. Takeda, K. Ikeda, Y. Mizuno, T. Ueda, *Chem. Pharm. Bull.* **1987**, *35*, 3558–3567.
[8] R. L. Letsinger, C. N. Sigman, G. Histand, M. Salunkhe, *J. Am. Chem. Soc.* **1988**, *110*, 4470–4471.
[9] H. Hashimoto, M. G. Nelson, C. Switzer, *J. Am. Chem. Soc.* **1993**, *115*, 7128–7134.
[10] J. T. Goodwin, D. G. Lynn, *J. Am. Chem. Soc.* **1992**, *114*, 9197–9198.
[11] G. Y. Wang, D. E. Bergstrom, *Tetrahedron Lett.* **1993**, *34*, 6725–6728.
[12] R. Fathi, Q. Huang, J. L. Syi, W. Delaney, A. F. Cook, *Bioconjugate Chem.* **1994**, *5*, 47–57.
[13] K. S. Ramasamy, M. Zounes, C. Gonzalez, S. M. Freier, E. A. Lesnik, L. L. Cummins, R. H. Griffey, B. P. Monia, P. D. Cook, *Tetrahedron Lett.* **1994**, *35*, 215–218.
[14] M. Manoharan, K. Tivel, L. K. Andrade, P. Dan Cook, *Tetrahedron Lett.* **1995**, *36*, 3647–3650.
[15] M. Manoharan, K. S. Ramasamy, V. Mohan, P. D. Cook, *Tetrahedron Lett.* **1996**, *37*, 7675–7678.
[16] R. H. Griffey, B. P. Monia, L. L. Cummins, S. Freier, M. J. Greig, C. J. Guinosso, E. Lesnik, S. M. Manalili, V. Mohan, S. Owens, B. R. Ross, H. Sasmor, E. Wancewicz, K. Weiler, P. D. Wheeler, P. Dan Cook, *J. Med. Chem.* **1996**, *39*, 5100–5109.
[17] N. Haginoya, A. Ono, Y. Nomura, Y. Ueno, A. Matsuda, *Bioconjugate Chem.* **1997**, *8*, 271–280.
[18] Y. Ueno, Y. Nagasawa, I. Sugimoto, N. Kojima, M. Kanazaki, S. Shuto, A. Matsuda, *J. Org. Chem.* **1998**, *63*, 1660–1667.
[19] N. Mignet, S. M. Gryaznov, *Nucleic Acids Res.* **1998**, *26*, 431–438.
[20] C. H. Tung, K. J. Breslauer, S. Stein, *Nucleic Acids Res.* **1993**, *21*, 5489–5494.
[21] T. P. Prakash, D. A. Barawkar, V. Kumar, K. N. Ganesh, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1733–1738.
[22] P. Bigey, G. Pratviel, B. Meunier, *Chem. Commun.* **1995**, 181–182.
[23] N. Schmid, J.-P. Behr, *Tetrahedron Lett.* **1995**, *36*, 1447–1450.
[24] H. Nara, A. Ono, A. Matsuda, *Bioconjugate Chem.* **1995**, *6*, 54–61.
[25] C. Sund, N. Puri, J. Chattopadhyaya, *Tetrahedron* **1996**, *52*, 12275–12290.
[26] A. R. Diaz, R. Eritja, R. G. Garcia, *Nucleosides Nucleotides* **1997**, *16*, 2035–2051.
[27] Y. Ueno, M. Mikawa, A. Matsuda, *Bioconjugate Chem.* **1998**, *9*, 33–39.

  0947-6539/00/0622-4193 $ 17.50+.50/0

[28] R. O. Dempcy, K. A. Browne, T. C. Bruice, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6097 – 6101.
[29] D. P. Arya, T. C. Bruice, *J. Am. Chem. Soc.* **1998**, *120*, 6619 – 6620.
[30] H. Tabor, *Biochemistry* **1962**, *1*, 496 – 501.
[31] H. S. Basu, H. C. Schwietert, B. G. Feuerstein, L. J. Marton, *Biochem. J.* **1990**, *269*, 329 – 334.
[32] D. Esposito, P. Delvecchio, G. Barone, *J. Am. Chem. Soc.* **1997**, *119*, 2606 – 2613.
[33] N. Schmid, J. P. Behr, *Biochemistry* **1991**, *30*, 4357 – 4361.
[34] A. Abdennaji, P. F. Potier, S. Doronina, I. Huc, J. P. Behr, *Tetrahedron Lett.* **1997**, *38*, 2989 – 2992.
[35] W. T. Markiewicz, P. Godzina, M. Markiewicz, *Nucleosides Nucleotides* **1999**, *18*, 1449 – 1454.
[36] A. M. MacMillan, G. L. Verdine, *J. Org. Chem.* **1990**, *55*, 5931 – 5933.
[37] C. M. Harris, L. Zhou, E. A. Strand, T. M. Harris, *J. Am. Chem. Soc.* **1991**, *113*, 4328 – 4329.
[38] D. A. Erlanson, L. Chen, G. L. Verdine, *J. Am. Chem. Soc.* **1993**, *115*, 12583 – 12584.
[39] J. F. Gerster, R. K. Robins, *J. Am. Chem. Soc.* **1965**, *87*, 3752.
[40] H. Lee, M. Hinz, J. J. Stezowski, R. G. Harvey, *Tetrahedron Lett.* **1990**, *31*, 6773 – 6776.
[41] B. Zajc, M. K. Lakshman, J. M. Sayer, D. M. Jerina, *Tetrahedron Lett.* **1992**, *33*, 3409 – 3412.
[42] J. Woo, S. T. Sigurdsson, P. B. Hopkins, *J. Am. Chem. Soc.* **1993**, *115*, 3407 – 3415.
[43] D. Tsarouhtsis, S. Kuchimanchi, B. L. DeCorte, C. M. Harris, T. M. Harris, *J. Am. Chem. Soc.* **1995**, *117*, 11013 – 11014.
[44] H. Lee, E. Luna, M. Hinz, J. J. Stezowski, A. S. Kiselyov, R. G. Harvey, *J. Org. Chem.* **1995**, *60*, 5604 – 5613.
[45] G. X. He, S. H. Krawczyk, S. Swaminathan, R. G. Shea, J. P. Dougherty, T. Terhorst, V. S. Law, L. C. Griffin, S. Coutre, N. Bischofberger, *J. Med. Chem.* **1998**, *41*, 2234 – 2242.
[46] F. Himmelsbach, B. S. Schulz, T. Trichtinger, R. Charubala, W. Pfleiderer, *Tetrahedron* **1984**, *40*, 59 – 72.
[47] G. A. Olah, X.-Y. Li, *Synlett* **1990**, 267 – 269.
[48] M. A. McClinton, *Aldrichimica Acta* **1995**, *28*, 31 – 35.
[49] P. Godzina, K. Adrych-Rozek, W. T. Markiewicz, *Nucleosides Nucleotides* **1999**, *18*, 2397 – 2414.
[50] T. J. Curphey, *J. Org. Chem.* **1979**, *44*, 2805 – 2807.
[51] J. Nielsen, M. Taagaard, J. E. Marugg, J. H. van Boom, O. Dahl, *Nucleic Acids Res.* **1986**, *14*, 7391 – 7403.
[52] F. Eckstein in *Oligonucleotides and analogues*: *a practical approach* (Eds.: D. Rickwood, B. D. Hames), IRL Press, Oxford **1991**.
[53] E. Ferrer, G. Neubauer, M. Mann, R. Eritja, *J. Chem. Soc. Perkin Trans. 1* **1997**, 2051 – 2057.
[54] M. Acedo, C. Fabrega, A. Avino, M. Goodman, P. Fagan, D. Wemmer, R. Eritja, *Nucleic Acids Res.* **1994**, *22*, 2982 – 2989.
[55] R. Eritja, J. Robles, A. Avino, F. Albericio, E. Pedroso, *Tetrahedron* **1992**, *48*, 4171 – 4182.
[56] T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular cloning*: *a laboratory manual*, Cold Spring Harbor Laboratory, New York, **1989**.

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  0947-6539/00/0622-4194 $ 17.50+.50/0