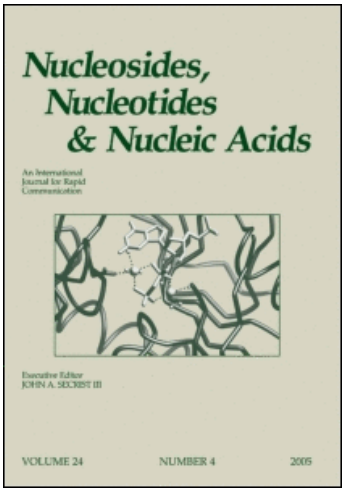

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SYNTHESIS OF OLIGODEOXYNUCLEOTIDES CONTAINING 2-SUBSTITUTED GUANINE DERIVATIVES USING 2-FLUORO-2'-DEOXYINOSINE AS COMMON NUCLEOSIDE PRECURSOR.

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Abstract: Oligonucleotides containing 2-substituted guanine derivatives with double-helix stabilizing molecules such as spermine, spermidine and propylimidazole have been prepared using protected 2-fluoro-2'-deoxyinosine phosphoramidite and two different protective strategies: the *p*-nitrophenylethyl (NPE) and the *t*-butylphenoxyacetyl groups. Melting studies show a large increase on the melting temperatures of duplexes containing these 2-substituted guanine derivatives.

Oligonucleotides carrying polyamines and imidazole groups have attracted considerable interest, owing to their potential use as antisense inhibitors of gene expression. Stabilization of double-helix has been described for oligonucleotides carrying guanine with an spermine group at position 2.[1] A similar stabilizing effect was found in oligonucleotides carrying imidazole groups linked to the amino group at position 2 of guanine.[2,3] These oligonucleotide-imidazole conjugates were designed[2,4] for sequence-specific cleavage of nucleic acids because imidazole is known to promote the catalytic hydrolysis of RNA. Recently, oligonucleotides carrying imidazole groups at position 6 of adenine were found to be useful for selective solid-phase immobilization of PCR products.[5] The stabilization of triple helix by oligonucleotides containing spermine molecules has also been described.[6]

Different methods for the preparation of oligonucleotides carrying these functional groups have been described.[1-6] Protected phosphoramidite derivatives are used for the preparation oligonucleotides containing imidazole[2-4] and spermine.[6] In these cases the imidazole moiety is protected with the 2,4-dinitrophenyl group that is cleaved with mercaptoethanol[4] and the amino groups of spermine with the trifluoroacetyl group.[6] Also, the use of a special nucleoside precursor has been described for the preparation of oligonucleotides containing imidazole and spermine groups.[1,5]

In this paper we compare two methods for the preparation of oligodeoxynucleotides carrying guanine derivatives with spermine (SPM), spermidine (SPD) and propylimidazole (API) groups at position 2 using a common precursor which is converted to the appropriate derivative during or after the deprotection of the oligonucleotide. The base-pairing properties of these oligonucleotides are described.

RESULTS AND DISCUSSION

Design of the synthetic strategy.

It has been shown that 2-fluoro-2'-deoxyinosine (dI^F) is a versatile intermediate for the preparation of 2-substituted guanine derivatives because it reacts readily with amines.[1,4,7] 2-Fluoro-2'-deoxyinosine is prepared from dG by diazonium salt formation in presence of fluoride ions, however protection of the position 6 of the base is required.[1,4,7] The use of the 2-(4-nitrophenyl)ethyl group (NPE)[8] for the protection of position 6 is convenient because this group is used for the protection of the nucleobase during oligonucleotide synthesis.[8] The use of 5'-DMT-O^6-NPE-protected dI^F phosphoramidite for the incorporation of 2-substituted guanine derivatives into synthetic oligonucleotides has been described.[1,7] After the incorporation in the desired oligonucleotide sequence, this nucleoside is converted to different derivatives at the oligonucleotide level during deprotection by small modifications during the deprotection of the oligonucleotide.[1,7e,g] Moreover, it has been shown that oligonucleotides carrying dI^F can be isolated if special deprotection protocols are used.[8] This methodology relies on the use of NPE/NPEOC-protected phosphoramidites[8,10] and oxalyl supports[11,12] which can be removed by treatment with a non-nucleophilic base (for example 1,8-diazabicyclo[5.4.0]undec-7-ene, DBU) in aprotic solvents. Therefore, two different strategies[13] could be used for the preparation of oligonucleotides carrying 2-substituted guanines (FIG. 1). First, oligonucleotides are assembled using *t*-butylphenoxyacetyl[14] and O^6-NPE-protected dI^F 2-cyanoethyl phosphoramidites followed by treatment with the appropriate amine that will perform the conversion of the 2-fluorohypoxanthine to 2-substituted guanine together with the elimination of the *t*-butylphenoxyacetyl groups. In order to avoid modification of the C residues[15] *t*-butylphenoxyacetyl groups are used instead of the standard (benzoyl and isobutyryl) groups for the protection of the natural bases. Second, oligonucleotides could be assembled using NPE,NPEOC-protected phosphoramidites as described.[9,10] Deprotection of the oligonucleotide with DBU will yield 2-fluorohypoxanthine oligonucleotides[9] which can be isolated and characterized. Subsequent treatment with the appropriate amines in aqueous solutions will yield oligonucleotides containing 2-substituted guanines.

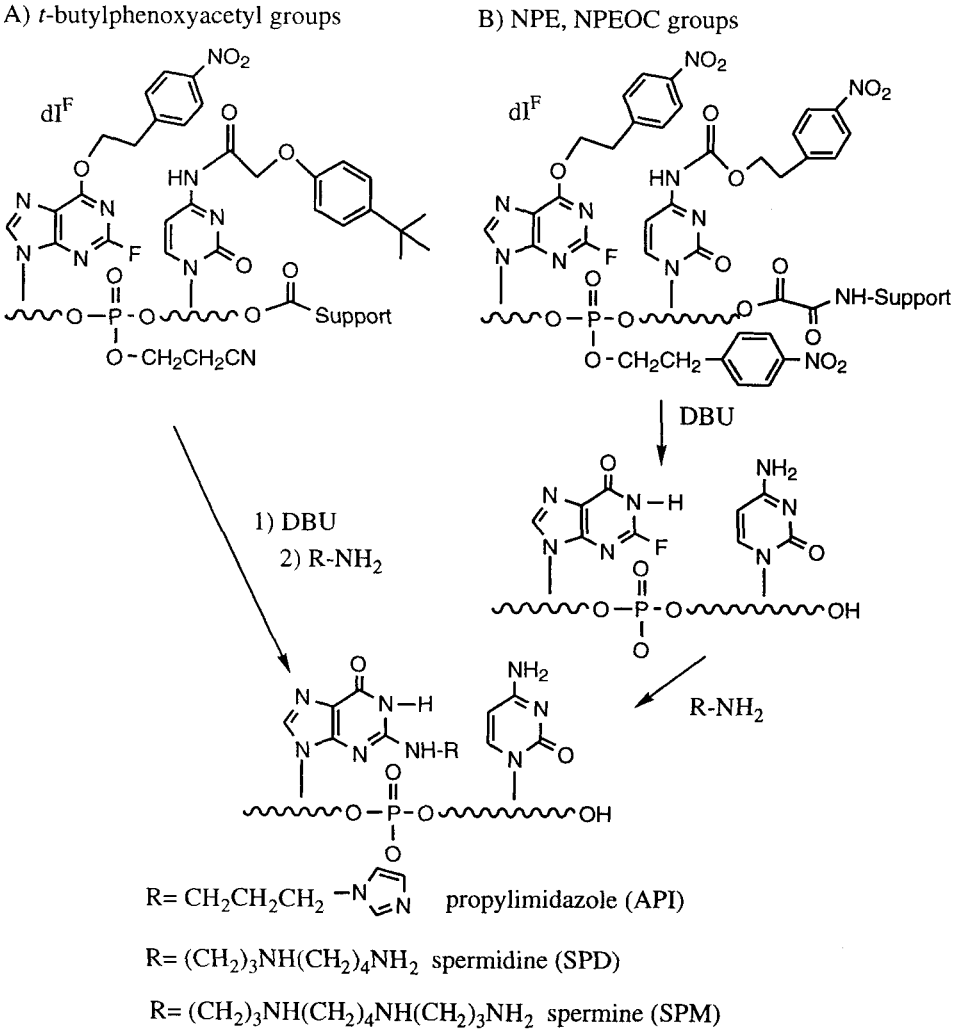

FIGURE 1: Two different protecting groups strategies used in this work for the preparation of oligonucleotides containing 2-substituted guanines.

Conversion of NPE protected 2'-deoxy-2-fluoroinosine to N^2-guanine derivatives.

First, 2'-deoxy-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine was treated with 1M solutions of spermine, spermidine and 1-(3-aminopropyl)imidazole. Complete conversion to a single polar derivative was observed after 6-12 hours at room temperature. Purification of the spermine or spermidine derivatives by silica gel chromatography was difficult due to

the polarity of these derivatives and for this reason low recoveries (25-35%) were found even after using 50% methanol and 10% triethylamine. Moreover the products were not homogeneous since ^{1}H and ^{13}C-NMR spectra of the spermine and spermidine derivatives showed the duplication of a large number of resonances. This could be explained by the different amino groups of spermine and spermidine yielding different isomers. Mass spectrometry showed the expected molecular weight together with the mass corresponding to the loss of the NPE group. On the other hand, the guanine derivative carrying the 3-(1-imidazolyl)propyl group was obtained in good yield (80%) and purity as judged by NMR and mass spectrometry. These experiments showed that the conversion of the 2-fluoro-dI to the desired 2-substituted guanine derivatives is fast and that the NPE group is stable under reaction conditions.

Preparation of the phosphoramidite derivatives.

Phosphoramidites carrying the *t*-butylphenoxyacetyl groups were obtained from commercial sources. The preparation of NPE, NPEOC-protected nucleosides has been described previously.[8] We have developed some modified protocols for the preparation of these phosphoramidites. Silylation of nucleosides was performed with hexamethyldisilazane in DMF instead of the standard trimethylsilyl chloride in pyridine. In this way, silylation of alcohol functions was faster and no pyridinium chloride was produced. After silylation, excess hexamethyldisilazane and volatile side products were easily removed in vacuum, yielding pure silylated nucleosides that were readily protected with the NPE and NPEOC groups. Guanine derivatives (O^6-NPE-dG and N^2-NPEOC-O^6-NPE-dG) were prepared directly from dG using transient protection instead of using 3',5'-diacetyl-dG.[8] Preparation of the phosphoramidite derivative of 2'-deoxy-2-fluoro-O^6-NPE-inosine was performed following previously described protocols[7] with small modifications.

Oligonucleotide synthesis using NPE,NPEOC-protected phosphoramidites and oxalyl supports.

Oligonucleotides A: 5' I^FT 3' and B: 5' GCCGI^{F}ATCTAGACG 3' were prepared using DMT-NPE,NPEOC-protected nucleoside NPE-phosphoramidites and the appropriate oxalyl supports.[12] Coupling efficiency was 99% yield per coupling. At the end of the synthesis, the support was treated with a 0.5 M DBU solution in pyridine containing thymine as scavenger. Purification of oligonucleotide B was done by gel filtration (Sephadex G-10) followed by reverse phase HPLC (DMT on and DMT off). Removal of DBU from dimer was performed by passing an aqueous solution of the dimer through a Dowex 50WX4 (Na+ form) column. Oligonucleotides were isolated in 10-15% overall yield. Mass spectrometry confirmed the expected molecular mass (TABLE 1).

TABLE 1. Oligonucleotide sequences prepared in this work.

SEQUENCE (5'-3')	Yield	MS (Found) (Daltons)	(Expected) (Daltons)
I^FT	15%	574.0	574
$G^{SPD}T$	--	699.2	699
GCCGI^{F}ATCTAGACG	11%	4290.0	4291
GCCGG^{API}ATCTAGACG	--	4397.9	4396
GCCGG^{SPM}ATCTAGACG	--	4475.3	4473
GCCGG^{SPD}ATCTAGACG	--	4417.9	4416
CACCG^{API}ACGGCGC	13%	3724.0	3723
CACCG^{SPM}ACGGCGC	16%	3801.9	3800
CACCG^{SPD}ACGGCGC	14%	3744.0	3743

Treatment of dimer I^FT with an aqueous solution of spermidine at 60 °C overnight gave complete conversion to the dimer containing spermidine that was characterized by mass spectrometry. The sample was purified twice by HPLC to eliminate the excess of spermidine.

Afterwards, treatment of 14 mer B with spermine, spermidine and 1-(3-aminopropyl)imidazole were performed. In these longer oligonucleotides the excess of the amine was removed by gel filtration prior HPLC analysis. In all cases a single peak was obtained with a similar retention time to that of the initial oligonucleotide. Conversion of 2-fluoro-dI to the modified guanine derivatives was confirmed by mass spectrometry (TABLE 1). Moreover, enzyme digestion showed the disappearance of 2-fluoro-dI. In oligonucleotide with the propylimidazole group a new nucleoside peak eluting after dA appeared (FIG. 2). This product coeluted with N^2-3-(1-imidazolyl)propyl-dG prepared as described above. Conversion was quantitative and recovery after HPLC was 40-60%.

Oligonucleotide synthesis using *t*-butylphenoxyacetyl-protected phosphoramidites.

Oligonucleotide D 5' CACCI^{F}ACGGCGC 3' was prepared using *t*-butylphenoxyacetyl-protected phosphoramidites. After the assembly of the sequence, support was treated with a DBU solution to remove the NPE group. The resulting support was divided in three parts and each part was treated with the appropriate amine solution at 60 °C overnight. This treatment yielded the modified oligonucleotides that were purified and characterized as described above (TABLE 1). In some cases, double peaks were

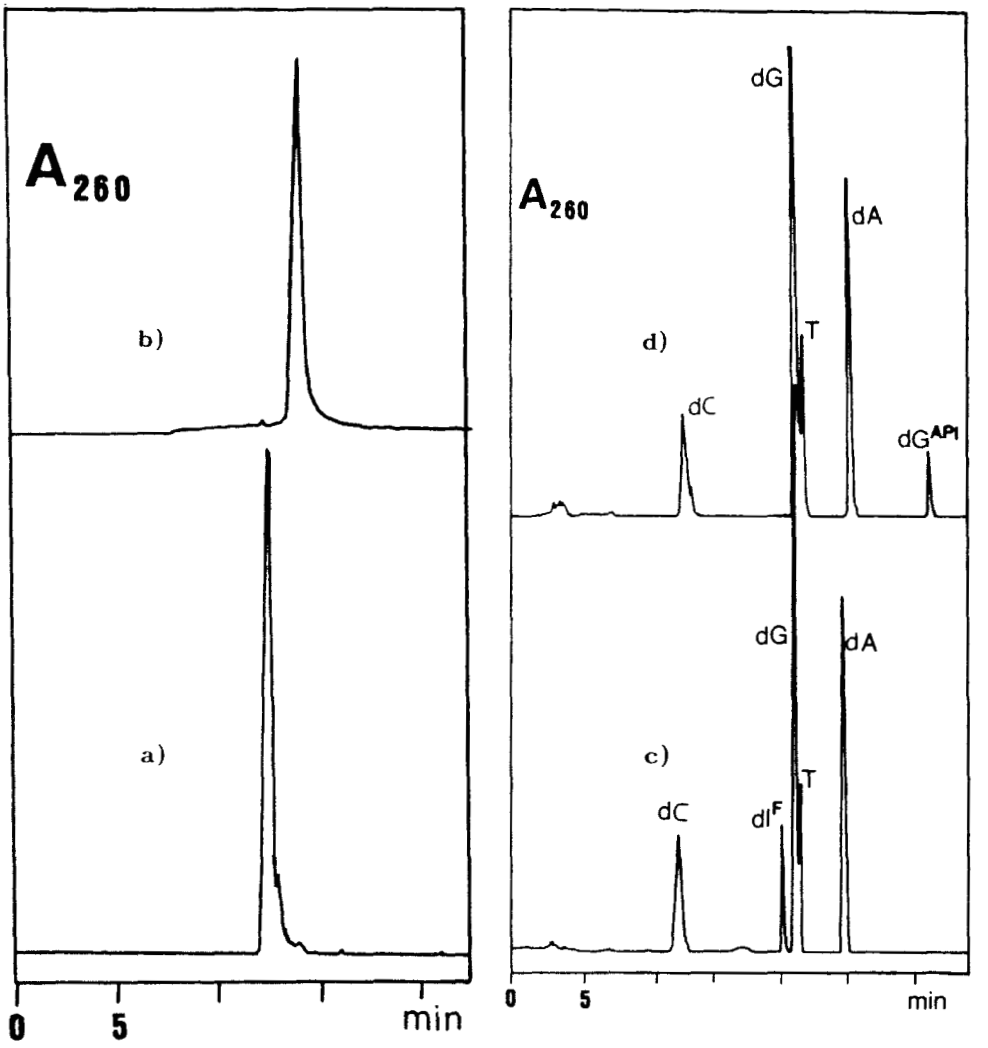

FIGURE 2: Preparation of GCCGG^{API}ATCTAGACG using NPEOC protecting groups. a) Analytical HPLC of purified GCCGI^{F}ATCTAGACG. b) Analytical HPLC after treatment of GCCGI^{F}ATCTAGACG with 1-(3-aminopropyl)imidazole. c) Enzyme digestion of purified GCCGI^{F}ATCTAGACG. d) Enzyme digestion of purified GCCGG^{API}ATCTAGACG.

observed, although mass spectra showed the correct mass for both products. These double peaks were assigned to the different isomers formed during the reaction of polyamines with dI^F. Oligonucleotides were isolated in 13-16% overall yields. In some fractions, small amounts of shorter products were observed. Peaks with higher than expected molecular mass resulting from incomplete deprotection or the addition of more than one molecule of amine were not observed. This indicates that elimination of *t*-butylphenoxyacetyl groups and the conversion to the 2-substituted guanine derivative was clean and complete.

Melting experiments.

Thermal denaturation of duplexes containing sequence D and its complementary sequence (5' GCGCCGTXGGTG 3' X = C, A, T, G) were studied in 0.15 M NaCl, 50 mM tris.HCl pH 7.5. In all cases a clear cooperative curve was observed. Melting temperatures are shown in TABLE 2. A large difference in melting temperature (21-23 °C) was observed with the three modified guanine derivatives. The increase of the melting temperature found in these duplexes are higher than previously described[1,2] especially for the duplex containing the aminopropyl derivative[2] which might be due to differences in the sequence. In the present case a G.C rich sequence is used while in the previous case it was a A.T rich duplex. Melting temperatures of single mismatches were also increased, specially G.T mismatch but differences between perfect match and mismatch are kept similar or higher (Tm differences from -11 to -20 °C) than natural mismatches (from -13, to -15 °C).

Discussion

In this paper, we have shown that oligonucleotides containing 2-substituted guanine derivatives with duplex stabilizing properties can be prepared from a common precursor using two different routes. In the first approach, commercially available *t*-butylphenoxyacetyl (Expedite) protected phosphoramidites can be used together with a modified deprotection protocol yielding directly the desired oligonucleotides. Alternatively, the use of NPE,NPEOC-protected phosphoramidites allows the isolation of intermediate oligonucleotides containing 2-fluoro-dI which can be converted to the desired oligonucleotides. This is the first time that oligonucleotides containing the four natural bases and these 2-substituted guanine derivatives have been prepared. Previous to this work, an oligonucleotide containing the spermine derivative of guanine was described but the oligonucleotide did not contain C and G residues.[1] Depending on the group used for the protection of the amino function of C, extra polyamine groups could be added to position 4 of C.[15] In the present work care was taken to avoid this side reaction by using more labile protecting groups (*t*-butylphenoxyacetyl strategy) or performing the substitution after

TABLE 2. Melting temperatures (°C) of duplexes containing N-2-substituted guanine derivatives in 0.15 M NaCl, 0.05 M tris.HCl pH 7.4.

5' CAC CXA CGG CGC 3'
3' GTG GYT GCC GCG 5'

		X		
Y	G	G^{API}	G^{SPM}	G^{SPD}
C	51	74	73	72
A	38	58	53	53
T	36	63	61	61
G	37	54	57	59

deprotection (NPE strategy). In the present case, the first approach is more convenient because the amine derivatives used for the conversion of 2-fluorohypoxanthine are efficient in the removal of *t*-butylphenoxyacetyl groups, but the NPEOC strategy could be of advantage in other cases where the reactivity of the incoming group is not enough to deprotect *t*-butylphenoxyacetyl groups such as azido groups.[16] In this work, evidence for the formation of different isomers during the conversion of 2-fluorohypoxanthine is presented. This result is different from the results obtained during the preparation of the spermine derivative of 5-methyl-dC where only one isomer is formed.[6] This difference could be explained by the steric effect of the methyl group at position 5. On the other hand the reaction with 3-(1-imidazole)propylamine yields only one product facilitating the purification and characterization. Moreover, melting temperatures of duplexes containing one modified guanine residues showed an enhanced duplex stability with similar mismatch discrimination properties. Surprisingly all three guanine derivatives have similar stabilization properties. Some of the stabilizing properties observed were expected from previous studies[1-3] especially from spermine and spermidine oligonucleotides. But in our case, the melting temperature increases are very large and provide the possibility of using these modified oligonucleotides for antisense studies and clinical diagnosis. The study of the biological properties of these modified oligonucleotides are in progress. The methodology presented here could be used for the rapid synthesis of series of oligonucleotides carrying different duplex stabilizing molecules.

EXPERIMENTAL SECTION

Abbreviations used: A_{260}: absorbance at 260 nm, API: 3-(1-imidazolyl)propyl, Ar: aromatic, Cquat: quaternary C, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DCM:

dichloromethane, dI^F: 2-fluoro-2'-deoxyinosine, DMF: N,N-dimethylformamide, DMT: dimethoxytrityl, Et_2O: diethyl eter, Et_3N: triethylamine, G^{API}: guanine residue containing the propylimidazolyl group, G^{SPM}: guanine residue containing spermine group, G^{SPD}: guanine residue containing spermidine group.I^F: 2-fluorohypoxanthine, MeOH: methanol, NPE: 2-(4-nitrophenyl)ethyl, NPEOC: 2-(4-nitrophenyl)ethoxycarbonyl, OD: optical density units at 260 nm, pyr: pyridine, SPD: spermidine, SPM: spermine, THF: tetrahydrofuran. *t*-Butylphenoxyacetyl (Expedite) protected phosphoramidites and *t*-butylphenoxyacetic anhydride were from PerSeptive Biosystems (Framingham, MA, USA).

^1H, ^{13}C, ^{31}P NMR spectra were recorded on a Bruker AM250 spectrometer. ^{31}P NMR spectra were recorded using broad proton decoupling. ^{13}C NMR data are reported below with broad band proton decoupling, however assignments were made with the aid of the off-resonance data and DEPT experiments. Assigment of the resonances of carbons 1' and 4' have been made using bibliography data but they have not been rigorously determined. Therefore values could be interchanged.

General methods and standard work-up. All reactions were carried out in oven-dried glassware, under a nitrogen or argon atmosphere, unless specified otherwise. Before use, starting materials were dried by evaporation with the dry solvent that will be used for the reaction. Once the reaction was completed, solutions were concentrated to dryness and the residues were dissolved in DCM or $CHCl_3$ and washed with 5% aqueous $NaHCO_3$ and saturated aqueous NaCl. The organic phase was dried (Na_2SO_4) and the solvent was removed.

2-(4-Nitrophenyl)ethyl chloroformate.

To a 1 l round-bottom flask 15g of bis(trichloromethyl)carbonate (triphosgene, 30 mmol) were dissolved with 400 ml of anhydrous Et_2O and the solution was cooled with an ice-bath. A mixture containing 15 g (90 mmol) of 2-(4-nitrophenyl)ethanol and 12.5 ml (90 mmol) of Et_3N in 100 ml of a Et_2O: THF (2:3) was added slowly with stirring. Caution: if addition is too fast overheating and overpressure caused by the boiling of ether could happen. After the addition, the solution was stirred at room temperature for 4 hours. The white precipitate of $Et_3N.HCl$ was filtered and the filtrate was concentrated to dryness yielding 17.2 g (74.9 mmol, 83%) of a waxy product that was used without further purification. Physical and spectroscopic data as previously described.[8] ^{13}C-NMR (Cl_3CD) δ (ppm): 150.3 (C=O), 147.0 (C-NO_2), 144.1 (Cquat, Ar) , 129.7 (*o*-Ar), 123.7 (*m*-Ar), 70.9 (CH_2), 34.3 (CH_2).

2'-Deoxy-5'-O-dimethoxytrityl-N^4-[2-(4-nitrophenyl)ethoxycarbonyl]-cytidine.

To a solution of 2.27 g (10 mmol) of dC in 50 ml of DMF, 4.3 ml (20 mmol) of hexamethyldisilazane was added. After 30 min of stirring at room temperature, TLC analysis (20% MeOH in DCM) showed complete silylation (Rf silylated nucleoside 0.63). The solution was concentrated to dryness and the residue was dissolved in 10 ml toluene and concentrated (3 times). Finally, the residue was dissolved in 30 ml of dry pyridine, the solution was cooled with ice and 2.7 g (12 mmol) of 2-(4-nitrophenyl)ethyl chloroformate were added. After 1 h of magnetic stirring, the solution was concentrated to dryness and the residue was dissolved in $CHCl_3$. After standard work-up the product was dissolved in 100 ml of dioxane/MeOH (1:1) and 100 ml of 25% ammonia were added. After 15 min of magnetic stirring solvents were removed and the residue was purified by column chromatography on silica gel (0-15% MeOH gradient in $CHCl_3$). Yield 4.1 g (9.6 mmol, 96%). Physical and spectroscopic data as previously described.[8]

The product prepared above was dried (3 x 10 ml pyr) and the residue was dissolved in 100 ml of dry pyridine and 3.6 g (10.6 mmol) of dimethoxytrityl chloride were added. After 2 h of magnetic stirring, solvents were concentrated to dryness. The residue was dissolved in $CHCl_3$ and, after standard work-up, the residue was purified by column chromatography on silica gel (0-10% MeOH gradient in $CHCl_3$). Yield: 5.6 g (7.7 mmol, 80%). Physical and spectroscopic data as previously described.[8] ^{13}C-NMR ($CDCl_3$) δ (ppm): 162.3 (C4), 158.4 (DMT), 154.7 (CO, NPEOC), 152.4 (C2), 146.7 (C-NO_2), 145.1 (Cquat, Ar), 144.1(C6, Cquat, Ar), 135.4 (Cquat, Ar), 135.2 (Cquat, Ar), 129.8 (CH, Ar), 129.6 (CH, Ar), 127.9 (CH, Ar), 127.7 (CH, Ar), 126.8 (CH, Ar), 123.4 (CH, Ar), 113.0 (CH, Ar), 94.8 (C5), 86.9 (C4'), 86.6 (Cquat, DMT), 86.4 (C1'), 70.4 (C3'), 65.1 (CH_2), 62.7 (C5'), 54.9 (OCH_3), 41.7 (C2'), 34.6 (CH_2).

2'-Deoxy-5'-O-dimethoxytrityl-N^2-[2-(4-nitrophenyl)ethoxycarbonyl]-O^6-[2-(4-nitrophenyl)ethyl]guanosine.

To a solution of 5.34 (20 mmol) of dG in 100 ml of dry DMF, 8.4 ml of hexamethyldisilazane were added. After 30 min of magnetic stirring, the mixture was concentrated to dryness and the residual hexamethyldisilazane was eliminated by evaporation of DMF (5 x 10 ml) and dry ethanol (20 ml). The residue was dissolved in 200 ml of dry dioxane and 5.01 g (30 mmol) of 2-(4-nitrophenyl)ethanol and 8.39 g (32 mmol) of triphenylphosphine were added. To the solution, 5 ml (32 mmol) of diethyl azodicarboxylate were added dropwise. After 90 min of magnetic stirring at room temperature the mixture was concentrated to dryness. The residue was dissolved in 100 ml of dry pyr and the solution was cooled with ice. To the cooled solution 13.7 g (60 mmol)

2-(4-nitrophenyl)ethyl chloroformate were added. After 2 hours of magnetic stirring at room temperature the mixture was concentrated to dryness. The residue was dissolved in DCM and washed following the standard work-up. The resulting product was dissolved in 100 ml of dioxane / MeOH (1:1) and 100 ml of 25% aqueous ammonia were added. After 30 min of magnetic stirring the mixture was concentrated to dryness. The desired nucleoside (Rf 0.45, 10% MeOH in DCM) was purified by column chromatography on silica gel (5-15% MeOH in $CHCl_3$). Yield: 9.12 g (15 mmol, 75%). Physical and spectroscopic data as previously described.[8]

The NPE/NPEOC-protected nucleoside was reacted with DMT-Cl as described for the cytidine derivative. Yield after silica gel purification 10.2 g (11.2 mmol, 74%). Physical and spectroscopic data as previously described.[8] ^{13}C-NMR ($CDCl_3$) δ (ppm): 160.1 (C6), 158.1 (Cquat, Ar), 152.4 (C2), 151.3 (CO), 151.0 (C5), 146.3 (C-NO_2), 145.4 (Cquat, Ar), 145.2 (Cquat, Ar), 144.3 (Cquat, Ar), 139.9 (C8), 135.4 (Cquat, Ar), 129.6 (CH, Ar), 129.3 (CH, Ar), 127.7 (CH, Ar), 127.4 (CH, Ar), 126.5 (CH, Ar), 123.2 (CH, Ar), 117.8 (C5), 112.7 (CH, Ar), 86.4 (C4'), 86.0 (Cquat, DMT), 83.9 (C1'), 71.7 (C3'), 66.4 (CH_2), 64.4 (CH_2), 63.7 (C5'), 54.2 (OCH_3), 53.2 (OCH_3), 40.1(C2'), 34.5 (CH_2).

2'-Deoxy-5'-O-dimethoxytrityl-N^6-[2-(4-nitrophenyl)ethoxycarbonyl]adenosine.

To a solution of 5.02 g (20 mmol) of dA in 100 ml of DMF, 8.4 ml of hexamethyldisilazane (40 mmol) were added. After 30 of magnetic stirring, TLC analysis (20% MeOH/ DCM) showed complete silylation (Rf 0.46). The solution was concentrated to dryness and the residue was dried by evaporation of toluene (2 x 10 ml). The residue was dissolved in dry DCM and 25 mmol of 2-(4-nitrophenyl)ethyloxycarbonyl imidazolium chloride[8] were added. After 4 hours of magnetic stirring at room temperature the solution was concentrated to dryness. The residue was dissolved in 100 ml dioxane / MeOH (1:1) and 100 ml of 25% aqueous ammonia were added. After 30 min of magnetic stirring the solution was concentrated to dryness. TLC analysis (20% MeOH in DCM) showed one single nucleoside product (Rf 0.32) and the resulting product was used without further purification. The Npeoc protected nucleoside was reacted with DMT-Cl as described for the cytidine derivative. Yield after silica gel purification 11.22 g (15.0 mmol, 75%). Physical and spectroscopic data as previously described.[8] ^{13}C-NMR ($CDCl_3$) δ (ppm): 158.3 (Cquat, Ar), 152.2 (C2), 151.0 (C=O), 150.7 (C4), 149.0 (C6), 146.5 (Cquat, Ar), 145.1 (Cquat, Ar), 144.2 (Cquat, Ar), 141.3 (C8), 135.4 (Cquat, Ar), 129.7 (CH, Ar), 129.4 (CH, Ar), 127.8 (CH, Ar), 127.5 (CH, Ar), 126.6 (CH, Ar), 123.3

(CH, Ar), 122.1 (C5), 112.9 (CH, Ar), 86.3 (C4', Cquat DMT), 84.6 (C1'), 71.8 (C3'), 64.9 (CH_2), 63.5 (C5'), 54.8 (OCH_3), 40.1 (C2'), 34.6 (CH_2).

2'-Deoxy-O^6-[2-(4-nitrophenyl)ethyl]guanosine.

To a solution of 3 g (11 mmol) of dG in 100 ml of DMF, 6,64 ml of hexamethyldisilazane (33 mmol) were added. After 1 hour of magnetic stirring, TLC analysis (10% EtOH/DCM) showed complete silylation (Rf 0.85). The solution was concentrated to dryness and dried by coevaporation with dry acetonitrile. The residue was dissolved in 20 ml of dry dioxane and 1.75 g (10 mmol) of 2-(4-nitrophenyl)ethanol, 2.94 g (11.2 mmol) of triphenylphosphine, and 1.76 ml (11.2 mmol) of diethyl azodicarboxylate were added. After 1 hour of magnetic stirring the solution was concentrated to dryness and dried by coevaporation with dry pyr. The residue was dissolved in 80 ml of dioxane/MeOH 1:1 and 80 ml of 25% aqueous ammonia were added. After 20 min of magnetic stirring the solution was concentrated to dryness, dissolved in DCM and purified by column chromatography on silica gel with a 0-15% MeOH gradient in DCM. Yield 3.69 g, 76%. TLC (10% EtOH in DCM): Rf 0.48. Physical and spectroscopic data as previously described.[8] ^{13}C-NMR ($CDCl_3$) δ (ppm): 161.4 (C6), 158.6 (C2), 152.5 (C4), 146.9 (C-NO_2), 145.8 (Cquat Ar), 139.2 (C8), 129.9 (CH, Ar), 123.7 (CH, Ar), 117.0 (C6), 89.3 (C4'), 87.4 (C1'), 73.3 (CH_2), 66.3 (C3'), 63.5 (C5'), 40.4 (C2'), 35.1 (CH_2).

2'-Deoxy-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine.

To a precooled (-30° C) mixture of 32 ml HF 52.5% in pyridine and 8 ml THF, 1 g of 2'-deoxy-O^6-[2-(4-nitrophenyl)ethyl]guanosine (2.3 mmol) dissolved in 16 ml of THF, and 1 ml of *t*-butyl nitrite were added. The mixture was stirred under nitrogen atmosphere for 20 min keeping the bath temperature at -30° C. To the reaction mixture, a 50% aqueous solution of NaOH was added slowly until neutralization was reached (approx. 50 ml, pH=5). TLC analysis (10% EtOH in DCM) showed a single nucleoside product (Rf 0.61). The mixture was filtered and the precipitate was washed first with water, collecting an aqueous phase, and then with DCM collecting an organic phase. The aqueous phase was concentrated to dryness and the residue was treated with DCM. The organic solution was combined with the washings with DCM and the mixture was concentrated to dryness. The residue was purified by column chromatography on silica gel using a 0-10% MeOH gradient in DCM. Yield 0.62 g (61%) Physical and spectroscopic data as previously described.[7] ^{13}C-NMR ($CDCl_3$) δ (ppm): 163.4 (J_{C-F}= 19 Hz) (C6), 159.0 (J_{C-F}= 220 Hz) (C2), 153.8 (J_{C-F}= 19 Hz) (C4), 148.2 (C-NO_2), 147.1 (Cquat Ar), 143.7 (C8), 131.4 (CH, Ar), 124.9 (CH, Ar), 121.2 (C5), 89.8 (C4'), 87.1 (C1'), 72.7 (CH_2), 69.1 (C3'), 63.6 (C5'), 41.9 (C2'), 36.1 (CH_2).

2'-Deoxy-5'-O-dimethoxytrityl-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]-inosine.

To a solution of 1 g (2.3 mmol) of 2'-deoxy-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine dissolved in 10 ml of dry pyr, 1.5 g of DMT-Cl (4.6 mmoles) were added. The mixture was stirred at 40°C for 3 hours. After this time, TLC analysis showed a single nucleoside product (Rf 0.87, 10% MeOH id DCM). The reaction was stopped by addition of 5 ml of MeOH, and the mixture was concentrated to dryness, dissolved in DCM, and purified on silica gel using a 0-5% MeOH gradient in DCM. Yield 1.35 g (80%). Physical and spectroscopic data as previously described.[7] ^{13}C-NMR ($CDCl_3$) δ (ppm): 161.5 (J_{C-F}= 19 Hz) (C6), 157.2 (J_{C-F}= 214 Hz) (C2), 158.0 (Cquat, Ar), 152.4 (J_{C-F}= 19 Hz) (C4), 146.3 , 145.0, 144.2, 141.2 (C8), 135.2, 129.5, 127.6, 127.3, 126.4, 123.2, 119.4 (C5), 112.6, 86.4 (Cquat DMT), 86.0 (C4'), 84.5 (C1'), 71.3 (CH_2), 67.1 (C3'), 63.4 (C5'), 54.6 (OCH_3), 39.7 (C2'), 34.4 (CH_2).

N,N-Diisopropylamino-[2-(4-nitrophenyl)ethoxy]-chlorophosphine.

To a 1l round-bottom flask 17.5 ml of phosphorous trichloride (200 mmol) were dissolved in 500 ml of anhydrous acetonitrile. The mixture was cooled with an ice-bath and a solution 3.34 g (20 mmol) of 2-(4-nitrophenyl)ethanol in 100 ml of anhydrous acetonitrile was added slowly and with stirring. After the addition the mixture was stirred at room temperature for 1 hour and concentrated to dryness to eliminate the solvent and the excess of PCl_3.

The resulting yellowish oil was dissolved with 500 ml of anhydrous acetonitrile and the solution was cooled with an ice bath. Diisopropylamine (6.2 ml, 44 mmol) was added slowly. After 6 hour of magnetic stirring at room temperature, the precipitate was filtered out and the filtrates were concentrated to dryness yielding a yellowish oil that was used without further purification. Yield 5 g (75%). ^{31}P-NMR (Cl_3CD): 179.4 ppm.

5'-O-DMT-N,O-(NPEOC, NPE) protected-2'-deoxyribonucleoside 3'-N,N-diisopropyl-O-[2-(4-nitrophenyl)ethyl] phosphoramidite.

DMT-N,O-(NPEOC, NPE) protected 2'-deoxyribonucleoside (2 mmol) and diisopropylethylamine (8 mmol) were dissolved in acetonitrile and reacted with N,N-diisopropylamino-[2-(4-nitrophenyl)ethoxy]-chlorophosphine (2.5 mmol). After standard work-up, products were purified by column chromatography (AcOEt / *n*-hexane 1:2 + 10% Et_3N). DMT-T, yield 1.41 g (1.67 mmol, 83%); DMT-dA^{NPEOC}, yield 1.61 g (1.54 mmol, 77%); DMT-dC^{NPEOC}, yield 1.55 g (1.52 mmol, 76%); DMT-dG$^{NPE,\ NPEOC}$, yield 1.9 g (1.57 mmol, 79%); DMT-dI$^{F,\ NPE}$, yield 1.0 g (1 mmol, 50 %). Spectroscopic data as previously described.[10] ^{31}P-NMR for DMT-dI$^{F,\ NPE}$ NPE-phosphoramidite (Cl_3CD) δ (ppm) 148.3, 148.1.

Conversion of 2-fluorohypoxantine to 2-substituted guanine derivatives.

2'-Deoxy-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]inosine (0.15 g, 0.35 mmol) was treated with 1 ml of 1M solution of the corresponding amine compound (spermine, spermidine and 1-(3-aminopropyl)imidazole) in DMF / water (1:1) at room temperature. After 6-12 hours of magnetic stirring, TLC analysis (10% EtOH in DCM) showed complete formation of a polar compound. The reaction mixture was concentrated to dryness and the residue was purified by column chromatography using a 2-50% MeOH gradient in DCM + 10% Et_3N for the spermine and spermidine derivatives. The imidazole derivative was purified using a 2-10 % MeOH gradient in DCM + 1% Et_3N.

2'-Deoxy-2-spermidine-O^6-[2-(4-nitrophenyl)ethyl]inosine: Yield 47 mg (0.08 mmol, 25%). Proton and ^{13}C-NMR showed the signals corresponding to the nucleoside, NPE and spermidine groups. Some spermidine signals were duplicated probably due to the presence of the three possible isomers resulting from the reaction of the different amino groups. Mass spectra (MALDI): Molecular mass 545.2 (expected 545.2), other minor peaks: 396.2 (loss of NPE group).

2'-Deoxy-2-spermine-O^6-[2-(4-nitrophenyl)ethyl]inosine: Yield 73 mg (0.12 mmol, 35%). Proton and ^{13}C-NMR showed the signals corresponding to the nucleoside, NPE and spermine groups. Spermine signals were duplicated as explained before. Mass spectra (MALDI): Molecular mass 602.2 (expected 602.3).

2'-Deoxy-N^2-[propyl-3-(1-imidazole)]-O^6-[2-(4-nitrophenyl)ethyl]guanosine: Yield 80%. ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.17 (d, J= 8.4 Hz, 2H, Ar NPE), 7.69 (s, 2H, H-8 and H imidazole), 7.48 (d, J= 8.5Hz, 2H, Ar NPE), 7.09 (s, 1H, imidazole), 6.94 (s, 1H. imidazole), 6.26 (dd, 1H, H-1'), 4.97 (t, 1H, OH), 4.70 (t, 4H, $2CH_2$), 4.19 (m, 1H, H-3'), 4.10 (m, 1H, H-4'), 3.85 (m, 2H, H-5'), 3.37 (dd, 2H, CH_2), 3.28 (t, 2H, CH_2), 2.75 (m, 1H, H-2'), 2.3 (m, 1H, H-2'), 2.2 (m, 2H, CH_2). ^{13}C-NMR ($CDCl_3$) δ (ppm): 160.4 (C6), 158.4 (C2), 153.0 (C4), 146.4 (Cquat, Ar), 145.9 (Cquat, Ar), 138.5 (C8), 136.9 (C2 imidazole), 129.7 (CH, Ar, NPE), 128.5 (C4 imidazole), 123.3 (CH, Ar, NPE), 118.9 (C5 imidazole), 115.4 (C5), 88.4 (C4'), 85.5 (C1'), 71.8 (CH_2, NPE), 65.9 (C3'), 62.6 (C5'), 45.6 (CH_2, propyl) , 44.1 (CH_2, propyl), 40.0 (C2'), 38.5 (CH_2, propyl), 34.7 (CH_2, NPE). Mass spectra (MALDI): Molecular mass 525.2 (expected 525.2).

In order to remove the NPE group, aliquots of these derivatives were treated with a 33% dimethylamine solution in ethanol and the resulting mixtures were heated at 60 °C overnight. The solutions were cooled and concentrated to dryness. The resulting products were analyzed by reverse phase HPLC (see conditions below). Only the propylimidazole derivative (dG^{API}) gave a peak in the conditions used for the analysis of nucleoside composition. This product eluted after the peak of dA. The spermine and spermidine derivatives were retained by the reverse phase column and for this reason were not eluted.

Oligonucleotide synthesis and purification.

Sequences A: 5' I^{F}T 3' and B: 5' GCCGI^{F}ATCTAGACG 3' were assembled using 35 mg (1 μmol) of the appropriate oxalyl supports[11,12] and DMT-NPE,NPEOC-protected nucleoside NPE-phosphoramidites on a DNA synthesizer (Applied Biosystems Mod. 394). NPE-protected phosphoramidites were dissolved in dry DCM. Standard 1 μmol scale synthesis cycles were used. Coupling efficiencies were higher than 99%.

After the assembly of the sequences, supports were treated with a 0.5 M DBU solution in *anhydrous* pyridine (2 ml) containing 5 mg of thymine[9] at room temperature for 16 hours. Deprotection solutions were neutralized with a 50% acetic acid aqueous solution, filtered and the supports were washed with pyridine and water. The combined filtrates were concentrated to dryness. The residues were dissolved in 20 mM triethylammonium acetate buffer and the solutions were desalted on a Sephadex G-10 column with the exception of the dimer that was desalted with Dowex 50wx4 (Na^+ form). The oligonucleotide containing fractions were analyzed and purified by 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: sequence A (4 μmol scale) : 6 OD (0.6 μmol, 15%) and sequence B (1 μmol scale): 11 O.D. (0.11μmol, 11%). Oligonucleotides were analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis. Also, oligonucleotides were analyzed by mass spectrometry (electrospray). Results are shown in TABLE 1.

Treatment of oligonucleotides containing 2-fluoro-dI with spermine, spermidine and 1-(3-aminopropyl)imidazole was performed as follows: Oligonucleotides (1 OD) were treated with 1M aqueous solutions of the appropriate amine at 60 °C overnight and the solution was concentrated to dryness. The residue was dissolved in 20 mM triethylammonium acetate buffer and the solution was desalted on a Sephadex G-10 column with the exception of the dimer that was injected directly to the HPLC. The oligonucleotide containing fractions were analyzed and purified by HPLC. In all cases a single peak was observed that was collected, recovering 0.3-0.6 OD units of the desired oligonucleotide. Purified oligonucleotides were analyzed by enzyme digestion and by mass spectrometry (electrospray). Results are shown in TABLE 1.

Sequence D 5' CACCI^{F}ACGGCGC 3' was assembled using 35 mg (1 μmol) of the appropriate supports and DMT-*t*-butylphenoxyacetyl-protected nucleoside 2-cyanoethyl phosphoramidites on a DNA synthesizer. Standard 1 μmol scale synthesis cycles were used. Coupling efficiencies were higher than 98%. Oligonucleotide-support was treated with 1 ml of 0.5M DBU solution in acetonitrile for 30 min at room temperature. The support was washed with acetonitrile, 1% triethylamine in acetonitrile and acetonitrile and

dried. The resulting supports were treated with 1 ml of 1M aqueous solutions of the appropriate amine at 60 °C overnight and the solution was filtered and concentrated to dryness. The residue was dissolved in 20 mM triethylammonium acetate buffer and the solution was desalted on a Sephadex G-10 column. The oligonucleotide containing fractions were analyzed and purified by HPLC. Overall (synthesis and purification) yields were: sequence D with spermine (0.5 μmol scale) : 10 OD (0.08 μmol, 16%) , sequence D with spermidine (0.5 μmol scale) : 9 OD (0.07 μmol, 14%), sequence D with aminopropylimidazole (0.5 μmol scale): 8 O.D. (0.06 μmol, 13%). Purified oligonucleotides were analyzed by enzyme digestion and by mass spectrometry (MALDI). Results are shown in TABLE 1.

Melting experiments. Melting experiments of dodecamer duplexes were made by mixing equimolar amounts of two dodecamer strands dissolved in a solution that contains 0.15 M NaCl, 0.05 M tris-HCl buffer pH 7.4. Duplex were annealed by slow cooling from 80 °C to 4 °C. UV absorption spectra and melting curves (absorbance vs temperature) were recorded in 1-cm path-length cells using a Varian Cary 13 spectrophotometer with a temperature controller with a programmed temperature increase of 0.5 °C/min. Melts were run by triplicate on duplex concentrations of 4 μM at 260 nm. Results are shown in TABLE 2.

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