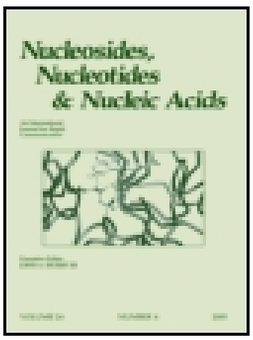

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OLIGONUCLEOTIDE LABELING: SYNTHESIS OF A NEW SPIN-LABELED 2'-DEOXYGUANOSINE ANALOGUE

Cesare Giordano[1]*, Francesco Pedone[2], Paola Fattibene[3] and Luciano Cellai[4]

[1]Centro di Chimica del Farmaco, CNR, Dipartimento di Studi Farmaceutici, Università "La Sapienza", 00185 Rome, Italy
[2]Dipartimento di Genetica e Biologia Molecolare, Università "La Sapienza", 00185 Rome, Italy
[3]Servizio di Fisica, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy
[4]Istituto di Strutturistica Chimica "G. Giacomello", CNR, CP 10, 00016 Monterotondo Stazione, Rome, Italy

ABSTRACT: in order to achieve an EPR sensitive probe for DNA, 3-carboxy-Proxyl free radical was linked to O-6 of dG through a five-atoms-tether. The modified base was incorporated into a 30-mer ODN, then annealed to its complementary DNA strand. Hydrodinamic parameters show only a slight destabilization with respect to the equivalent unlabeled hybrid. EPR could monitor the hybrid formation showing a progressive enlargement of the upfield signal in passing from the labeled ss- to the ds-30-mer.

Several non-isotopic DNA probing techniques have been developed by means of the insertion of a modified base carrying a group selected to be easily detectable by spectroscopic methods.

In recent years, Electron Paramagnetic Resonance (EPR) has been used for investigation into nucleic acid dynamics: detection of the single strand to double strand interconversion[1,2], identification of B-Z transition[3-5], and DNA bending[6] are among the most relevant problems which have been successfully treated by adopting this approach. On this purpose, the incorporation of nitroxyde-substituted nucleotides by chemical synthesis offers the possibility of preparing labeled nucleic acids of any sequence with any desiderable tether.

During the last years, several methods were published describing the preparation of oligodeoxynucleotides carrying a nitroxyde radical[7-11]. A variety of position-4 and -5 nitroxyde spin-labeled deoxyuridine derivatives as well as some 5-substituted deoxycytidine analogs have been prepared and inserted into DNA sequence both chemically[12,13] and enzymatically[14,15].

We report the synthesis of the phosphoramidite of a new 2'-deoxyguanosine analogue (**6b**, Fig. 1), i.e. 2'-deoxyguanosine with a five-atoms-tethered system carrying a cyclic five membered spin-label probe, which was then inserted into a 30-mer ODN in order to verify the possibility to monitor changes in DNA by EPR spectroscopy.

RESULTS AND DISCUSSION

In the title compound the spin-label 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3-carboxy-Proxyl) free radical has been bound to O-6 of 2'-deoxyguanosine through a propylamido tether chain and inserted at position 15 of the 30-mer 5'-d(GATCCTCTA GAGTCG*ACCTGCAGGCATGCA)-3' by authomatic synthesis according to the phosphoramidite chemistry.

The synthetic strategy (Fig. 1) started from N^2,3',5'-triisobutyroyldeoxyguanosine (Tri-*i*bu-dG) **1a**[16] which was reacted (i) with N-fmoc-3-aminopropan-1-ol by Mitsunobu procedure[17] to give **2a**. Deprotection with piperidine afforded the crude free amine which was directly acylated (ii) with 3-carboxy-Proxyl free radical by using the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) procedure to give **3a** . Brief treatment at room temperature with conc ammonia in methanol (iii) afforded the N^2-protected deoxynucleoside adduct **4a**, while the fully deprotected adduct **4b** was obtained by refluxing for prolonged period in the same solvent. Protection of the 5'-hydroxyl of **4a** (iv) as 4,4'-dimethoxytrityl (DMTr) derivative with 4,4'-dimethoxytrityl chloride in pyridine gave **5a** which was activated (v) as 3'-N,N-diisopropyl-O-β-cyanoethyl phosphoramidite affording the key reagent **6a**.

The 30-mer was synthesized at the 1 μmole scale (2 x 1 μmole) and the average coupling yield was 98%. The ODN was cleaved from the solid support by conc NH_4OH / 0.25 M β-mercaptoethanol, deprotected (17 h at 55°C) and purified by HPLC. The collection of the main peak gave 105 O.D. The ODN was further purified, analized by polyacrylamide gel electrophoresis (PAGE) and the purity of the labeled 30-mer was greater than 95%. It was then annealed to its complementary sequence and complete annealing was checked by PAGE. UV melting analysis of duplex samples with and without the spin-label yielded melting temperatures very similar, 71 and 73 °C respectively, indicating a slight destabilization introduced by the presence of the spin-label. Fluorescence polarization

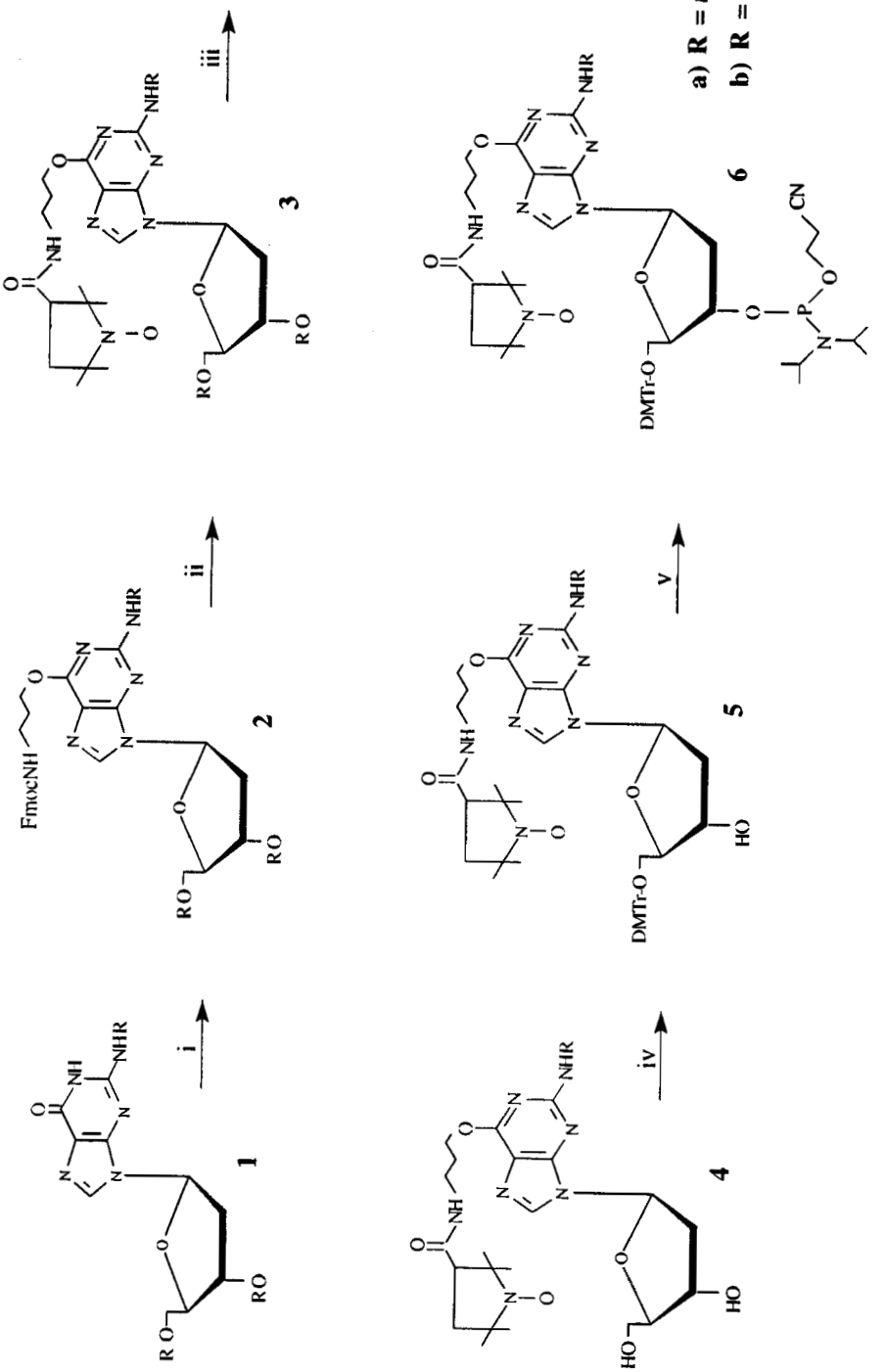

FIG. 1: Reaction scheme of the synthesis of the new spin-labeled dG phosphoramidite

analysis[18] measured by the phase method, using Ethidium Bromide (EB) as DNA fluorescent probe, gave the results reported in Table 1 for the labeled and unlabeled sample, as measured at 20 °C. These results show that the control and the spin-labeled DNAs exhibit the same hydrodynamic parameters with the exception of a slight reduction of the torsional constant value. This is probably due to the presence of the probe at the O-6 of dG, with the consequent loss of one of the three G-C hydrogen bonds (Fig. 3). This fact can account also for the reduction of the Tm.

The EPR spectra of (a) the Proxyl spin-label itself, (b) the labeled monomer 4b, (c) the Proxyl spin-label inserted into the single stranded 30-mer and (d) the Proxyl spin-label inserted into the double stranded 30-mer are reported in Fig. 2 and show a marked broadening of the high field band upon interaction of the probe with the single and double stranded oligomers, in accordance with PAGE analysis.

CONCLUSIONS

The present synthetic procedure used to obtain the labeled phosphoramidite was successful and provides a facile route for the synthesis of a chemically modified 2'-dG containing a linker arm terminating in an alifatic amine group. This can be used to covalently attach through an amide bond a variety of labeling ligands such as fluorescent molecules and groups for affinity labeling.

Furthermore, since it is a property of the tether nature how well the spin-label reflects DNA motions, the theter can be selected and used by this method on the base of its lentgh and flexibility: in fact, although the coupling between the nitroxyde and the base to which it is attached is better with shorter tether (2-4 atoms) in respect of those with long tethers (5-11 atoms), the latter can still consistently detect the magnitude of base motion[4,5].

Finally, since the probe bound to O-6 of dG protrudes into the central space of the major groove (Fig. 3), it is expected to cause only a minor perturbation of the duplex DNA structure.

EXPERIMENTALS

Melting points (Buchi oil apparatus) are uncorrected. UV absorption spectrum of **6a** was measured with a Shimadzu UV-2101PC recording spectrophotometer. ^{1}H-NMR (300 MHz, TMS as internal standard) and ^{31}P-NMR (121.4 MHz, H_3PO_4 as external standard) spectra were recorded on a Varian XL-300 spectrometer; samples containing nitroxyde group were treated with phenylhydrazine (1.5 eq) as reducing agent. Fast atom bombardment (FAB) mass spectra were recorded on a VG 70-70 EQ-MF instrument equipped with a standard FAB source (8 keV, Xe atoms, glycerol/1-thioglicerol solution). Silica gel for column chromatography and TLC silica gel plates were from Merck AG

TABLE 1: Fluorescence polarization anaysis measured by the phase method using Ethidium Bromide (EB) as DNA fluorescent probe.

Sample	Lifetime of EB ($s \times 10^{-9}$)	Torsional constant ($erg \times 10^{-12}$)	Hydration radius (Å)	X^2
Unlabeled	22.6 ± 0.2	5.7 ± 0.2	10.7 ± 0.1	1.10
Spin-labeled	22.6 ± 0.2	5.5 ± 0.2	10.7 ± 0.1	1.05

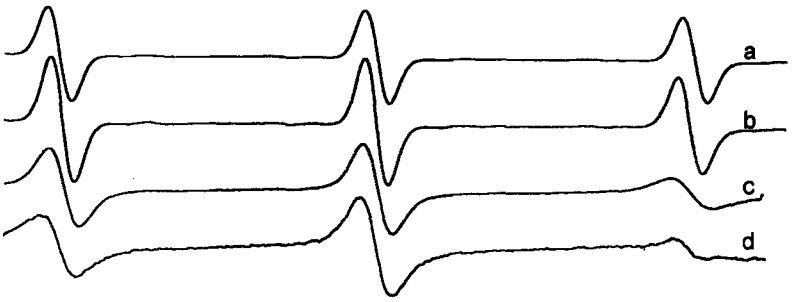

FIG. 2: EPR spectra of (a) 3-carboxy-Proxyl, (b) the labeled monomer **4b**, (c) the labeled single-stranded 30-mer and (d) the labeled double-stranded 30-mer.

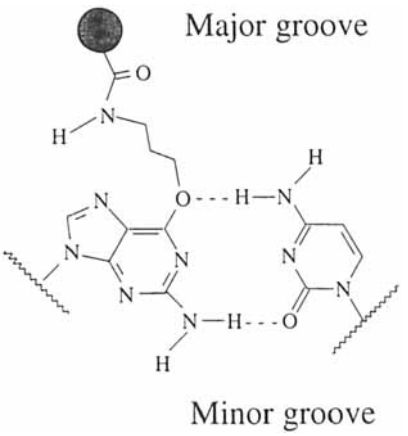

FIG. 3: Location of the spin-label group in the duplex

(Darmstadt, Germany). EPR spectra were performed with a Bruker ESP 300 spectrometer, X band, modulation amplitude of 0.4 gauss, time constant of 320 ms, field scan time of 80 s, 20 scans, microwave power of 1 mV. The sample holder was a glass capillary tube and the measurement was performed in DMSO solution at 20 °C. Lifetime and Fluorescence Polarization Anisotropy measurements were performed on a K2-ISS phase-shift fluorometer instrument (Urbana IL, USA) using the 514-nm 0.5-W output of a Coherent Innova 90C Argon laser.

N-fmoc-3-aminopropan-1-ol

To an ice-cooled aqueous solution (10ml) of Na_2CO_3 (583 mg, 5.5 mmol) and 3-amino-1-propanol (375 mg, 5.0 mmol), 9-fluorenylmethyl chloroformate (1.29 g, 5.0 mmol) in CH_2Cl_2 (15 ml) was added dropwise under vigorous stirring. The reaction mixture was allowed to warm to room temperature and stirred overnight. CH_2Cl_2 (50 ml) was added, the organic phase was separated, washed with brine (2 x 20 ml), dried over Na_2SO_4, filtered and concentrated to about half volume. The product was crystallized by addition of *n*-hexane and recovered as white crystals (1.25 g, 84%). mp: 128-30 °C; ^{1}H-NMR ($CDCl_3$) δ 1.50-1.85 (m, 2H, CH_2-C*H*$_2$-CH_2), 2.48 (bs, 1H, OH), 3.14-3.44 (m, 2H, CH_2N), 3.50-3.75 (m, 2H, C*H*$_2$OH), 4.00-4.25 (m, 1H, CH), 4.95 (bs, 1H, NH), 7.03-7.35 and 7.40-7.68 (two m, 8H, aryl).

*N^2,3',5'-tri-ibu-O^6-(N-fmoc-3-aminopropyl)-2'-dG (**2a**)*

To an ice-cooled solution of **1a** (890 mg, 1.86 mmol), triphenylphosphine (736 mg, 2.80 mmol) and N-fmoc-3-aminopropan-1-ol (555 mg, 1.86 mmol) in dry tetrahydrofuran (10.0 ml), diethyl azodicarboxylate (488 mg, 2.80 mmol) was added by syringe under N_2 atmosphere and the mixture was allowed at room temperature for 3 hr. After evaporation to small volume and dilution in $CHCl_3$ (50 ml) , the solution was washed with brine (2 x 30 ml). The organic layer was separated, dried over Na_2SO_4 and evaporated under reduced pressure. The crude mixture was loaded on a silica gel column and eluted first with CH_2Cl_2, then with CH_2Cl_2/MeOH (99:1). The pure **2a** was recovered as a white foam which was tritured with *n*-hexane (858 mg, 61%). R_f: 0.25 (EtOAc/$CHCl_3$ 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.10-1.35 (m, 18H, 6 x CH_3), 1.80-2.19 (m, 2H, CH_2-C*H*$_2$-CH_2), 2.30-2.66 [m, 3H, 3 x C*H*(CH_3)$_2$], 2.71-3.00 (m, 2H, H-2', H-2"), 3.10-3.44 (m, 2H, CH_2N), 4.00-4.40 (m, 5H, CH_2O, H-5', H-5" and CH_{fmoc}), 4.47-4.73 (m, 3H, H-4', CH_{2fmoc}), 5.17-5.37 (m, 1H, H-3') 5.82 (bs, 1H, NH), 6.12 (t, 1H, H-1', J = 7.5 Hz), 6.94-7.24 and 7.33-7.58 (two m, 8H, aryl), 7.70 (s, 1H, NH), 8.00 (s, 1H, H-8); FAB-MS = 765 m/z (M^+).

N^2,3',5'-tri-ibu-O^6-[3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamido)-propyl]-dG (3a)

To an ice-cooled solution of **2a** (700 mg, 0.93 mmol) in CH_2Cl_2 (5 ml) piperidine (1 ml) was added and the mixture was allowed to warm to room temperature. After 1 hr the solvent and the piperidine excess was removed under vacuum, the residue was tritured three times with *n*-hexane and dried under high vacuum. To a solution of the crude product in dry dioxane (15 ml), 3-carboxy-Proxyl (173 mg, 0.93 mmol), HOBT (12 mg, 0.09 mmol) and solid DCC (210 mg, 1.02 mmol) were consecutively added at room temperature and the mixture stirred overnight. Dicyclohexylurea was filtered off and the solvent evaporated. The pure **3a** was isolated by a silica gel column (EtOAc) as a pale yellow foam and was used without further purification (457 mg; 70%). R_f: 0.13 (EtOAc); ^{1}H-NMR ($CDCl_3$) δ 0.80-1.18 (m, 30 H, 10 x CH_3), 1.41-1.69 (m, 2H, CH_2-C*H*$_2$-CH_2), 1.76-1.85 (m, 2H, $CH_{2pyrrolidine}$), 2.24-2.61 [m, 5H, H-2', H-2", 3 x C*H*$(CH_3)_2$], 2.64-2.90 (t, 1H, $CH_{pyrrolidine}$, J = 7.5 MHz), 2.96-3.65 (m, 2H, CH_2N), 4.00-4.34 (m, 4H, H-5', H-5" and CH_2O), 4.32-4.57 (m, 1H, H-4'), 5.16-5.35 (m, 1H, H-3'), 6.15 (t, 1H, H-1', J = 7.5 Hz), 7.60 (bs, 1H, NH), 8.10 (s, 1H, H-8), 10.00 (s, 1H, NH); FAB-MS = 702 m/z (M^+).

N^2-ibu-O^6-[3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamido)-propyl]-dG (4a)

To a solution of **3a** (400 mg, 0.56 mmol) in MeOH (10.0 ml) 25% aqueous ammonia (6.0 ml) was added and the mixture allowed for 4 hr at room temperature. After evaporation to dryness under reduced pressure, the residue was loaded on a silica gel column and eluted with a mixture of EtOAc/MeOH 9:1. The pure **4a** was recovered as a pale yellow foam (275 mg, 86%). R_f: 0.15 (EtOAc/MeOH 90:10); ^{1}H-NMR (d_6-DMSO) δ 0.85-1.19 (m, 18H, 6 x CH_3), 1.38-2.04 (m, 4H, CH_2-C*H*$_2$-CH_2 and $CH_{2pyrrolidine}$), 2.13-2.69 [m, 3H, H-2', H-2" and C*H*$(CH_3)_2$], 2.73-2.92 (t, 1H, $CH_{pyrrolidine}$, J = 7.5 Hz), 2.95-3.25 (m, 2H, CH_2N), 3.32-3.55 (m, 2H, H-5', H-5"), 3.61-3.82 (m, 1H, H-4'), 4.15-4.51 (m, 3H, CH_2O and H-3'), 6.12 (t, 1H, H-1', J = 7.5 Hz), 7.63 (bs, 1H, NH), 8.05 (s, 1H, H-8), 9.94 (s, 1H, NH); FAB-MS = 562 m/z (M^+).

O^6-[3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamido)-propyl]-dG (4b)

A sample of **3a** (80 mg, 0.11 mmol) dissolved in a mixture of 2.0 ml MeOH and 1.5 ml 25% aqueous ammonia was refluxed overnight. After evaporation to dryness, the residue was purified by preparative TLC on a silica gel plate using EtOAc/MeOH 80:20 as developing mixture. The main band was scraped and recovered from silica with $CHCl_3$/MeOH 95:5 affording on evaporation **4b** as an amorphous pale yellow solid which was lyofilized from a mixture of water/dioxane 1:1 (48 mg, 85%). R_f: 0.15 (EtOAc/MeOH

90:10); ^{1}H-NMR (d_6-DMSO) δ 0.75, 0.94, 1.00 and 1.05 (four s, 12 H, 4 x CH_3), 1.42-1.97 (m, 4H, CH_2-CH_2-CH_2 and $CH_{2pyrrolidine}$), 2.01-2.75 (m, 3H, H-2', H-2" and $CH_{pyrrolidine}$), 2.90-3.27 (m, 2H, CH_2N), 3.35-3.59 (m, 2H, H-5' and H-5"), 3.65-3.78 (m, 1H, H-4'), 4.11-4.51 (m, 3H, CH_2O and H-3'), 4.75-5.44 (bs, 2H, 2 x OH), 5.99-6.41 (m, 3H, H-1' and NH_2), 7.85 (bs, 1H, NH), 8.30 (s, 1H, H-8); FAB-MS = 492 m/z (M^+).

N^2- ibu-5'-O-DMTr-O^6-[3 - (2 , 2 , 5 , 5 - tetramethyl -1- pyrrolidinyloxy-3-carboxamido)-propyl]-dG (5a)

The N^2-protected **4a** (268 mg, 0.48 mmol), previously coevaporated in abs pyridine (2 x 3 ml) and dried under high vacuum overnight, was solubilized in abs pyridine (5 ml) and then 4,4'-dimethoxytrityl chloride (325 mg, 0.96 mmol) was added at room temperature. After 3 hr the reaction was quenched with MeOH (0.2 ml) and the solution evaporated to small volume and diluted with CH_2Cl_2 (25 ml). The CH_2Cl_2 layer was washed with $NaHCO_3$ saturated solution (2 x 15 ml), brine (15 ml), dried over Na_2SO_4, evaporated and finally coevaporated with toluene. The residue was chromatographed on a silica gel column (EtOAC/MeOH/triethylamine 94:5:1) and the 5'-O-protected deoxynucleoside was recovered as a pale yellow amorphous powder after precipitation from $CHCl_3$ into *n*-hexane (303 mg, 73%). R_f: 0.25 (EtOAc/MeOH 9:1); ^{1}H-NMR (d_6-DMSO) δ 0.85-1.38 (m, 18H, 6 x CH_3), 1.50-1.72 (m, 2H, CH_2-CH_2-CH_2), 1.77-2.05 (m, 2H, $CH_{2pyrrolidine}$), 2.10-2.53 [m, 3H, H-2', H-2" and CH(CH_3)$_2$], 2.64-2.92 (bs, 1H, $CH_{pyrrolidine}$), 2.99-3.51 (m, 4H, CH_2N, H-5' and H-5"), 3.76-4.04 (m, 1H, H-4'), 3.65 (s, 6H, 2 x CH_3O), 3.76-4.04 (m, 1H, H-4'), 4.25-4.63 (m, 3H, CH_2O and H-3'), 6.19 (t, 1H, H-1', J = 7.5 Hz), 6.41-6.66 and 6.83-7.25 (two m, 13H, aryl), 7.70 (bs, 1H, NH), 8.08 (s, 1H, H-8), 9.95 (s, 1H, NH); FAB-MS = 865 m/z (M^+).

N^2-ibu - 5'- O - DMTr - O^6 - [3 -(2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamido)-propyl]-dG -3'-(O-cyanoethyl-N,N-diisopropyl) phosphoramidite (6a)

Derivative **5a** (250 mg, 0.29 mmol) was dried under high vacuum overnight after consecutively coevaporation with pyridine, toluene and tetrahydrofuran. To a stirred solution of the nucleoside and N,N-diisopropylethylamine (132 mg, 1.17 mmol) in anhydrous CH_2Cl_2 (5 ml) 2-cyanoethyl-diisopropylchlorophosphoramidite (137 mg, 0.58 mmol) was added by siringe over 2 min under N_2 atmosphere. The reaction mixture was allowed to react for 35 min at room temperature, then was filtered to remove the precipitated amine hydrochloride and diluted with CH_2Cl_2 to 30 ml. The solution was extracted with ice-cooled brine (2 x 15 ml) and the organic phase was dried over Na_2SO_4, filtered, concentrated to small volume at low pressure and added to stirred *n*-hexane (100

ml) at -78 °C. The precipitated **6a** was collected (as mixture of diastereoisomers) by filtration as a pale yellow amorphous powder and was dried under high vacuum (293 mg, 95%). R_f: 0.50 and 0.58 (EtOAc/MeOH 90:10); mp: 68-70 °C; UV (EtOH): λ_{max} 271 and 235 nm; ^{1}H-NMR (d_6-DMSO) δ 0.75-1.35 (m, 3H, 10 x CH_3), 1.80-2.11 (m, 4H, CH_2-CH_2-CH_2 and $CH_{2pyrrolidine}$), 2.42-2.62 [m, 6H, H-2', H-2", CH_2CN, $CH(CH_3)_2$ and $CH_{pyrrolidine}$], 2.65-2.98 (m, 2H, CH_2N), 3.05-3.60 (m, 6H, 2 x CHN, CH_2OP, H-5' and H-5"), 3.75 (s, 6H, 2 x OCH_3), 3.98-4.31 (m, 2H, H-3' and H-4'), 4.45-4.75 (m, 2H, CH_2O), 6.22-6.55 (m, 1H, H-1'), 6.60-6.95 and 7.08-7.48 (two m, 13H, aryl), 7.76-8.15 (bs, 1H, NH), 8.41 (s, 1H, H-8), 10.41 (s, 1H, NH); ^{31}P-NMR (121.4 MHz, $CDCl_3$) δ 148.31 and 148.74; FAB-MS = 1065 m/z (M^+).

Synthesis and purification of the 30-mer ODN containing the spin labeled dG analogue

The synthesis was performed on an ABI (Applied Biosystems, Inc., Foster City, CA) 392 authomatic synthesizer. HPLC was carried out on a "Pure DNA" Dynamax column module (C4), 300 Å, 5 μM, 21.4 x 50 mm. The terminal 5'-O-DMT protecting group was removed on column by 0.5% trifluoroacetic acid. The free ODN was then eluted with a gradient of acetonitrile (3-30%) in triethylammonium acetate 0.1 M, pH 7.0. PAGE was performed on 20% polyacrylamide 7 M urea, in the case of denaturing conditions, gels (1.5 mm) containing 50 mM Tris-borate pH 8.0, 0.1 mM EDTA.

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