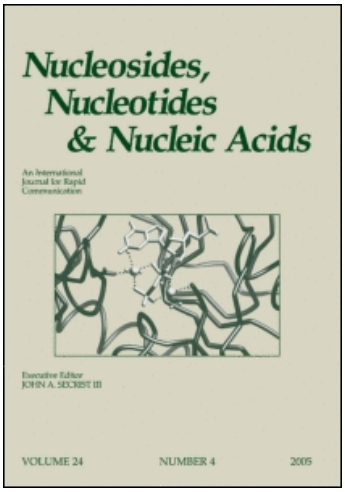

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A New Approach for the Efficient Synthesis of Oligodeoxyribonucleotides Containing the Mutagenic DNA Modification 7,8-Dihydro-8-oxo-2'-deoxyguanosine at Predefined Positions

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A NEW APPROACH FOR THE EFFICIENT SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING THE MUTAGENIC DNA MODIFICATION 7,8-DIHYDRO-8-OXO-2'-DEOXYGUANOSINE AT PREDEFINED POSITIONS

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ABSTRACT

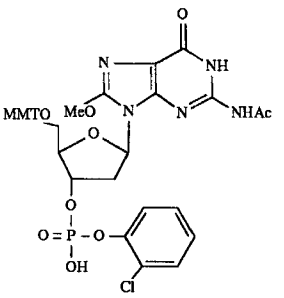
A combination of H-phoshonate and phosphoramidite chemistry has been applied for the automated solid-phase synthesis of oligodeoxyribonucleotides containing 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) residues at predefined positions. The unmodified part of the oligomers has been synthesized by using protected standard phosphoramidites, for the incorporation of 8-oxodG the synthon 2-N-acetyl-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-2'-deoxyguanosin-8-one-3'-H-phosphonate, prepared in a five step synthesis via 8-bromo-2'-deoxyguanosine, has been used. This approach combines the advantages of both DNA synthesis strategies in that a high yield of full length oligomers is obtained and unreacted, protected 8-oxodG monomers can be recycled, respectively.

INTRODUCTION

Oxygen derived free radicals, produced in the cell during aerobic metabolism[1-3], by ionizing radiation[4-6] and by chemical oxidants[7,8], can cause various types of damages in cellular DNA, including strand breaks, abasic sites and base modifications. Some of these damages formed and persisting in the DNA of target cells are suspected to play an important role in mutagenesis, tumorigenesis, ageing, and other degenerative diseases[9-11]. A frequent form of oxidative DNA damage is 7,8-dihydro-8-oxo-2'-deoxyguanosine[7,12] (8-oxodG). This DNA modification has been shown to possess miscoding potential[13]. NMR structural analyses have revealed that 8-oxodG occurs predominantly in the 6,8-diketo tautomeric form[14] and that it can adopt both the *anti*- and the *syn*-conformation[15,16]. In the *syn*-conformation 8-oxodG can form stable base pairs with dA[16]. This observation is consistent with other studies demonstrating that 8-oxodG directs the incorporation of dA in newly synthesized DNA opposite the modified nucleoside[17], and that it can induce G-C to T-A transversion mutations in biological model systems[18-20]. In both bacterial and mammalian cells 8-oxodG is efficiently eliminated[21-23], indicating that formation of this lesion in genomic DNA is deleterious to the cell.

In most of the above mentioned investigations synthetic DNA oligomers have been used which contain 8-oxodG at predefined positions. Kuchino et al.[13] have first described a method of introducing the modified nucleoside into DNA oligomers. Their method involved the incorporation of an 8-methoxy-2'-deoxyguanosine residue (1) using phosphotriester chemistry on solid supports. Conversion of the methoxy group to the oxo compound was performed post-synthetically after the ammonia deprotection step. A markedly improved strategy has been applied by Bodepudi et al.[24] and Roelen et al.[25] who recognized that

protection of the 8-oxo group is not necessary during oligomer synthesis, and who used phosphoramidite derivatives of 8-oxodG (2 and 3, respectively) for the solid phase synthesis. Though very efficient in obtaining high yields of full length oligomers, phosphoramidite chemistry is disadvantageous in that the (up to 20-fold) excess of monomer building units, and, consequently, of the costly 8-oxodG derivatives required for an efficient DNA synthesis, cannot be recycled. Here, H-phosphonate monomers are advatageous, in that unreacted dervatives can be collected, purified, and reused[26]. We have, therefore, developed an automated DNA synthesis protocol which permits to synthesize the unmodified part of DNA oligomers via phosphoramidite chemistry, and to incorporate protected 8-oxodG molecules as H-phosphonates. This strategy combines the advantages of phosphoramidite and H-phosphonate chemistry, i. e. a high coupling yield of the monomer building units and high yield recovery of the 8-oxodG synthon, respectively.



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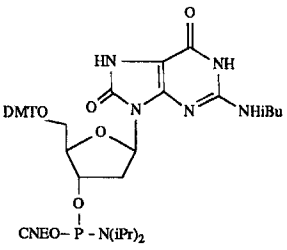

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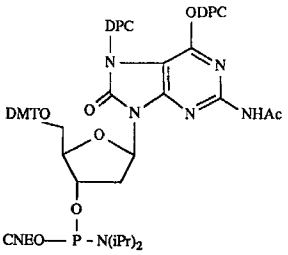


3

EXPERIMENTAL

General materials and methods

Pyridine, triethylamine (TEA, Fluka), 1,2-dichloroethane (Merck), dichloromethane (CH_2Cl_2, Merck), carbontetrachloride (CCl_4, Merck) were dried by refluxing over calcium hydride (CaH_2, 5g/l) for 4 h and then distilled. 3-Hydroxypropionitrile (Fluka) was dried by

coevaporating with dry acetonitrile (MeCN, Roth). N-Methylimidazol (NMI, Merck) was dried by refluxing over calcium hydride, and distilled under reduced pressure. Pyridine, N-methylimidazol, 3-hydroxypropionitrile were stored over molecular sieves 4Å (Merck). MeCN (Roth), used for DNA synthesis on the Gene Assembler Plus (Pharmacia) was stored over molecular sieves 3Å (Merck). Tetrazole (0.1 M) was obtained from Roth. 4,4'-Dimethoxytritylchloride (Fluka), acetic acid anhydride (Ac_2O, Fluka), 4-(dimethylamino)pyridine (DMAP, Fluka), silver acetate (AgOAc, Fluka) were of pure analytical grade and used without further purification. Sodium acetate (NaOAc, Merck) was dried in vacuo over phosphorus pentoxide. Triethylammonium bicarbonate buffer (TEAB, 1 M) was prepared by passing a stream of carbon dioxide (CO_2) gas through a cooled mixture of triethylamine (140 ml) and deionized water (860 ml) until pH 8.5. N-Acyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxynucleoside-3'-O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidites were obtained from Pharmacia and used as 0.1 M solutions in dry MeCN (Merck, DNA-grade). Aminopropyl-derivatized controlled pore glass (CPG/AP) was obtained as described[27] by using CPG-silica carrier (CPG 1400, Fluka). Oligonucleotide sequence analysis according to Maxam and Gilbert[28] was performed using Merck reagent kit. Polynucleotide kinase was obtained from New England Biolabs, [γ-^{32}P]ATP from Amersham. Macherey-Nagel Alugram Sil G/UV_{254} was used for TLC analysis in the solvent systems A: CH_2Cl_2/MeOH (4/1, v/v); B: CH_2Cl_2/MeOH (9/1, v/v). Column chromatography was performed on Kieselgel 60H (Merck) suspended in CH_2Cl_2. Sephadex G-50 was obtained from Pharmacia.

^{31}P NMR spectra were measured at 161 MHz using a BRUKER MSL 400 spectrometer equipped with an ASPECT 3000 computer and operating in the Fourier-transform mode. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard. ^{1}H NMR spectra

were measured at 400 MHz using the same instrument as for ^{31}P NMR spectra. The chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS).

High performance liquid chromatography (HPLC)-analysis was carried out on an Applied Biosystems 152A-System equipped with a gradient mixing system, an UV absorption detector(254 nm) and an integrator (Merck, D-2000).

2-N,3'-O,5'-O-triacetyl-7,8-dihydro-2'-deoxyguanosin-8-one (5)

Dry 8-bromo-2'-deoxyguanosine[29] (**4**) (3.46 g, 10.0 mmol) was suspended in pyridine (100 ml) and Ac_2O (10 ml). To this mixture dry NaOAc (8.20 g, 100 mmol), AgOAc (1.67g, 10 mmol) and N-methylimidazol (0.3 ml) was added. After refluxing for 24 h, the reaction was quenched by the addition of an aqueous NH_4HCO_3 solution (40 ml, 5%), and the mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (120 ml) and washed three times with the above mentioned NH_4HCO_3 solution. The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography (100g) using a 0 to 8% gradient of MeOH in CH_2Cl_2. Yield 1.540 g (37.7 %); R_f 0.49 (B); ^1H NMR ($CDCl_3$): δ 12.13 (s, 1H, NH), 10.50 (s, 1H, NH), 9.72 (s, 1H, NH), 6.33 (t, J=7.2 Hz, 1H, H-1'), 5.36 (m, 1H, H-3'), 4.75 (m, 1H, H-4'), 4.44-4.25 (m, 2H, H-5"), 3.03 (m, 1H, H-2"), 2.35 (m, 1H, H-2'), 2.31 (s, 3H, CH_3, NHAc), 2.09 (s, 6H, 2x CH_3, 2x OAc); FAB-MS (NBA / +): 410 (M+H), 409 (M), 210 (B+Ac); FAB-MS (NBA / -): 408 (M-H), 366 (M-Ac), 208 (B+Ac), 166 (B)

2-N-Acetyl-7,8-dihydro-2'-deoxyguanosin-8-one (6)

Compound 5 (1.227 g, 3 mmol) was dissolved in conc. aqueous NH_3 (10 ml) and stirred for 1h at room temperature. NH_3 was removed under reduced pressure and the reaction mixture was concentrated.

The crude product was recrystallized from hot MeOH. Yield 762 mg, (78.2%), R_f 0.58 (A), ^1H NMR (DMSO-D_6): δ 12.10 (s, 1H, NH), 11.64 (s, 1H, NH), 11.25 (s, 1H, NH), 6.07 (t, J=7.2 Hz, 1H, H-1'), 5.15 (d, J=4.3 Hz, 1H, OH-3'), 4.72 (m, 1H, OH-5'), 4.36 (m, 1H, H-3'), 3.74 (m, 1H, H-4'), 3.68-3.20 (m, 2H, H-5', H-5"), 3.06 (m, 1H, H-2"), 2.13 (s, 3H, CH_3, NHAc), 1.98 (m, 1H, H-2'); FAB-MS (NBA / +): 326 (M+H), 325 (M); FAB-MS (NBA / -): 324 (M-H)

2-N-Acetyl-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-2'-deoxy-guanosin-8-one (7)

Compound **6** (650 mg, 2 mmol) was dried by repeated evaporation with pyridine and dissolved in pyridine (15 ml). DMTr-Cl (744 mg, 2.2 mmol) and DMAP (12.2 mg 0.05 eq) were added, and the reaction mixture was stirred for 4 h at room temperature. After the addition of MeOH (2 ml) the mixture was concentrated to dryness. The residue was dissolved in CH_2Cl_2 (100 ml) and washed twice with water (25 ml). The organic layer was dryed over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography (20 g). Elution with a 0-8% gradient of MeOH in CH_2Cl_2 gave compound **7** as white foam. Yield 852 mg (68 %), R_f 0.41 (B), ^1H NMR ($CDCl_3$): δ 12.13 (s, 1H, NH), 10.54 (s, 1H, NH), 9.49 (s, 1H, NH), 7.45-7.05 (m, 9H arom. H's, DMT), 6.80-6.60 (m, 4H, arom. H's, DMT, 6.17 (dd, 1H, H-1'), 4.65 (m, 1H, H-3'), 3.98 (m, 1H, H-4'), 3.78 (s, 6H, 2x OCH_3, DMT), 3.45-3.05 (m, 3H, H-5', H-5", H-2"), 2.20 (s, 3H, CH_3, NHAc), 1.94 (m, 1H, H-2'); FAB-MS (NBA / +): 650 (M+Na), 627 (M), 303 (DMT), 210 (B+Ac+H); FAB-MS (NBA / -): 626 (M-H), 584 (M-Ac), 208 (B+Ac)

2-N-Acetyl-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-2'-deoxy-guanosin-8-one-3'-H-phosphonate (8)

To a stirred solution of PCl_3 (686.5 mg, 436 ml, 5 mmol) and N-methylmorpholine (5.057 g, 3.53 ml, 50 mmol) in anhydrous CH_2Cl_2

(50 ml) 1,2,4-triazol (1.115 g, 16.67 mmol) was added at room temperature. After 30 min. the reaction mixture was cooled to 0°C, and **7** (627 mg, 1 mmol, dried by coevaporation from CH_3CN) in CH_2Cl_2 (15 ml) was added dropwise within 10 min., stirred for 30 min., poured into aqueous triethylammonium bicarbonate (TEAB, 40 ml, 1.0 M, pH 8.5), shaken and separated. The aqueous layer was extracted twice with CH_2Cl_2 (50 ml) and the combined organic layers were dried over Na_2SO_4 and evaporated to a foam. The crude product was purified by silica gel column chromatography (10 g, elution gradient 2% Et_3N/CH_2Cl_2 to 2% Et_3N/ 10% MeOH/ CH_2Cl_2) followed by TEAB extraction and evaporation. Yield 514 mg (65 %), ^{31}P NMR ($CDCl_3$): δ 0.22 (dd, $^1J(P-H)$=620.4 Hz, $^3J(P-H)$=9.2 Hz); FAB-MS (NBA / +) 303 (DMT), 102 (TEA+H); FAB-MS (NBA / -): 690 (anion), 388 (anion-DMT), 208 (B+Ac), 166 (B)

Functionalization of the CPG solid support

CPG 1400 (Fluka) was functionalized according to Atkinson and Smith[27]. As first monomers 3'-O-(4',4'-dimethoxytrityl)-N-acyl-nucleosides were used.

Synthesis of oligodeoxynucleotides on CPG

Solid phase oligodeoxynucleotide synthesis was performed on a fully automated synthesizer (Pharmacia, Gene Assembler Plus) using commercially available protected deoxynucleoside 2-cyanoethyl-phosphoramidites and compound **8**. CPG, covalently linked to the appropriate deoxynucleoside (loading 20 μmol/g), was used as solid phase. The individual steps for the H-phosphonate elongation cycle are reported in Table 1. After each step, the column was washed to remove impurities and excess of reagents.

Cleavage from resin, deprotection and purification

A saturated aqueous ammonia solution (1 ml) which contained 0.25 M 2-mercaptoethanol to avoid oxidative degradation of 8-oxodG[30], was

Table 1: Chemical steps involved in H-phosphonate elongation cycle

Step	Manipulation	Solvents and reagents[a]
1	detritylation	2% TCA in DCE; 1.6 min
2	coupling	compound 8[b], pivaloyl chloride[c]; 1.0 min
3	oxidation	10% HPCN in CCL_4/DCE/MeCN/TEA/NMI 90:10:10:5:5 (v/v/v/v/v); 15.0 min
4	capping	available

[a] Reactions were performed on 10 mg (0.2 μmol) of support; [b] 0.05 M TEA H-phosphonate (125 μl) in Py/MeCN 1:1 (v/v); [c] 0.25 M pivaloyl chloride (125 μl) in Py/MeCN 1:1 (v/v).
Abbreviations: TCA, trichloroacetic acid; DCE, 1,2-dichloroethane; Py, pyridine; MeCN, acetonitrile; HPCN, 3-hydroxypropionitrile; TEA, triethylamine; NMI, N-methylimidazole.

added to the protected DNA oligomers bound to the resin. After incubation for 1 h at room temperature the solid support was removed. Deprotection of the oligomers in the same solvent was complete after incubation at 45°C for 20h. The solution was then evaporated under reduced pressure and the DNA oligomers were purified by preparative polyacrylamide gel electrophoresis.

Analysis of DNA oligomers containing 8-oxodG

The presence of 8-oxodG in an oligodeoxynucleotide was verified by enzymatic hydrolysis[31] of the DNA oligomer d-[CGCCA(8-oxoG)GGTTTTCCCAGTCACGAC] and subsequent HPLC analysis of the corresponding deoxyribonucleosides. To 80 μl of a solution containing 1 OD (4200 pmol) of the oligodeoxynucleotide, 10 μl of buffer (500 mM Tris-HCl, 1 mM EDTA, pH 8.5), 10 μl of alkaline phosphatase (CIP, 10 units/μl), and 10 μl of snake venom phosphodiesterase (100 μg/10 μl) were added and incubated at 37°C over night. The resulting mixture of deoxyribonucleosides was

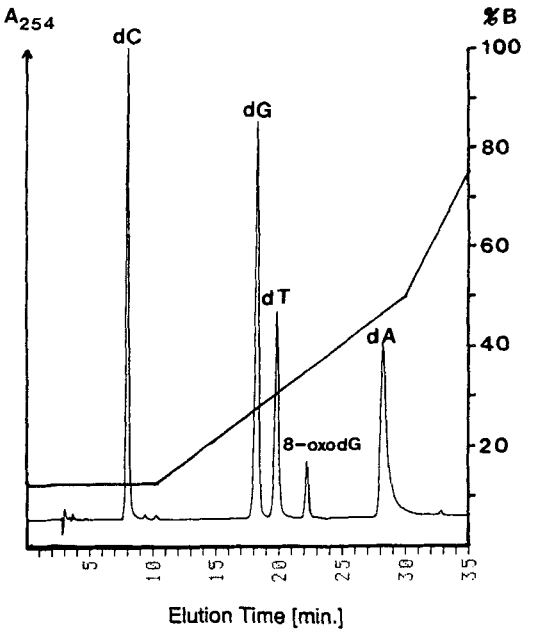


Fig.1: HPLC elution profile of deoxynucleosides obtained by enzymatic digestion of the modified oligonucleotide d-[CGCCAG^{8oxo}GGTTTTCCCAGTCACGAC]. Separation of deoxynucleosides was performed on a RP-18 column by using the gradient outlined in the diagram. Solvent A, 50 mM potassium phosphate buffer, pH 7.0; solvent B, 50 mM potassium phosphate buffer, pH 7.0, 40% MeOH.

separated on a Merck RP-18 reversed phase column. Elution was performed as indicated in Fig.1. In addition, the base sequence of the oligodeoxynucleotide was confirmed by sequence analysis according to Maxam and Gilbert[28].

Recovery and regeneration of the 8-oxodG H-phosphonate

During the H-phosphonate elongation cycle the unreacted H-phosphonate was collected in 5 ml 1M triethylammonium bicarbonate buffer (pH 8.5). After extraction of the aqueous layer with 20 ml CH_2Cl_2 the organic layer was dried over Na_2SO_4 and

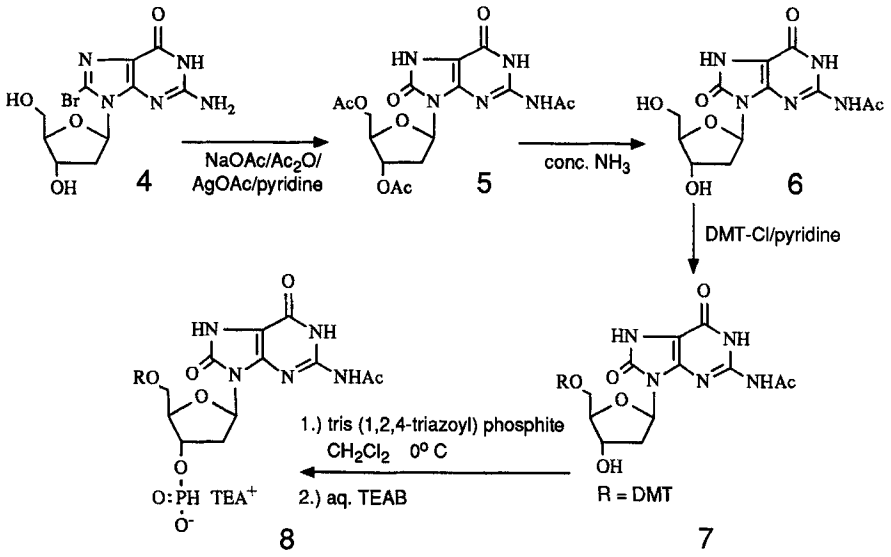
evaporated. The crude mixture was purified by silica gel column chromatography (2g, elution gradient 2% Et_3N/ CH_2Cl_2 to 2% Et_3N/ 10% MeOH/CH_2Cl_2) followed by TEAB extraction and evaporation.

RESULTS AND DISCUSSION

Conversion of 8-bromo-2'-deoxyguanosine to the respective 8-oxo compound is very ineffective[25,32] and represents the limiting step for an efficient synthesis of protected 8-oxodG. Though we could increase the yield of the oxo compound significantly (about three-fold) by using pyridine as a solvent and by the addition of silver ions, still less than 40% of the 8-bromo compound were converted into this intermediate. To avoid loss of protected 8-oxodG during automated DNA synthesis, the respective H-phosphonate derivative was, therefore, synthesized. Unlike phosphoramidites, unreacted excess of H-phosphonates can be collected and recycled[26].

Synthesis of compound **8** (Scheme 1) was started with the preparation of 8-bromo-2'-deoxyguanosine (**4**) as described[29]. Following full acetylation of this compound, the 8-bromo substituent was converted to the 8-oxo-derivative with sodium acetate in pyridine. The addition of silver acetate resulted in a significantly increased yield (37.7%) of the acetylated 7,8-dihydro-8-oxo-2'-deoxyguanosine derivative (**5**). Selective de-O-acetylation of **5** was achieved by treatment with conc. aqueous ammonia for at least one hour (**6**, 78% yield). Tritylation[33] of **6** with DMT-Cl gave **7** as a white foam in good yields (68%). The target compound **8** was obtained by phosphonylation[34] (65% yield).

Synthesis of DNA oligomers was carried out on a fully automated DNA synthesizer. The unmodified part of the oligomers was synthesized by using protected standard phosphoramidites. For the incorporation of 8-oxodG synthon **8** was used. To avoid the



Scheme 1

formation of a phosphodiester bond which may cause side reactions during the subsequent activation steps of phosphoramidite elongation, the H-phosphonate group was oxidized[35] resulting then in a phosphotriester bond containing a 2-cyanoethyl group (Table 1). Between step 2 and 3 of the H-phosphonate cycle unreacted material was collected in 1 M triethylammonium bicarbonate buffer (pH 8.5) and purified on a silica gel column. The purified product was identical to compound **8**. The yield of full length oligonucleotides containing 8-oxodG obtained by the combined phosphoramidite/H-phosphonate chemistry (70.7 %, 34-mer) was very similar to that of unmodified full length oligonucleotides obtained by phosphoramidite chemistry alone (71.8 %, same 34-mer as above containing dG instead of 8-oxodG).

The presence of 8-oxodG in synthetic DNA oligomers (d-[CGCCA(8-oxoG)GGTTTTCCCAGTCACGAC]) was verified by HPLC analysis of the deoxynucleosides released after enzymatic hydrolysis (Fig.1). The

peak fractions were collected and identified via UV spectral analysis (not shown). Integration of the peaks reflected the correct molar ratios of the nucleosides contained in the DNA oligomer. Additionally, 8-oxodG was identified by comigration with the respective reference substance synthesized as described. Sequence analysis of the same oligomer according to the method of Maxam and Gilbert[28] revealed unspecific cleavage of the DNA molecules at the position of 8-oxodG (not shown). This phenomenon is typical for the pronounced instability of 8-oxodG in DNA during treatment with hot piperidine[21] which is part of the sequencing protocol.

In conclusion, protected H-phosphonate derivatives can be used for the solid phase synthesis of oligodeoxynucleotides containing 8-oxodG residues at predetermined positions via a combined phosphoramidite/H-phosphonate chemistry. This approach combines the advantages of both strategies in that a high yield of full length oligodeoxynucleotides is obtained and unreacted, protected 8-oxodG monomers can be recycled, respectively.

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