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Synthesis and enzymatic processing of oligodeoxynucleotides containing tandem base damage

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ABSTRACT

Several studies have shown that ionizing radiation generates a wide spectrum of lesions to DNA including base modifications, abasic sites, strand breaks, cross-links and tandem base damage. One example of tandem base damage induced by ·OH radical in X-irradiated DNA oligomers is *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine/8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo). In order to investigate the biological significance of such a tandem lesion, both 8-oxo-7,8-dihydroguanine and formylamine were introduced into synthetic oligonucleotides at vicinal positions using the solid phase phosphoramidite method. For this purpose, a new convenient method of synthesis of 8-oxodGuo was developed. The purity and integrity of the modified synthetic DNA fragments were assessed using different complementary techniques including HPLC, polyacrylamide gel electrophoresis, electrospray and MALDI-TOF mass spectrometry. The piperidine test applied to the double modified base-containing oligonucleotides revealed the high alkaline lability of formylamine in DNA. In addition, various enzymatic experiments aimed at determining biochemical features of such multiply damaged sites were carried out using the synthetic substrates. The processing of the vicinal lesions by nuclease P1, snake venom phosphodiesterase, calf spleen phosphodiesterase and repair enzymes including *Escherichia coli* endonuclease (endo) III and Fapy-glycosylase was studied and is reported.

INTRODUCTION

Cell killing induced by photosensitizers, ionizing radiation or radiomimetic drugs cannot be explained only by the formation of single DNA damaged sites. Thus, multiply damaged sites, including double-strand breaks, tandem base modifications, crosslinks between bases and the sugar–phosphate backbone are likely to have harmful biological consequences (1–4). Efficient repair of oxidative base damage to DNA by endonucleases and DNA glycosylases is a requisite to prevent deleterious effects occurring. Several types of tandem lesion in irradiated and photosensitized DNA oligomers have been isolated and characterized to date (5–9). Thus, it was shown that when short dinucleotides including d(TpG), d(CpG), d(GpT) and d(GpC) were exposed to X-rays in oxygenated aqueous solutions, two of the major modified products are tandem base lesions. These were identified as *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine (dβF)/8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and the opposite sequence 8-oxodGuo/dβF (Fig. 1; 10). The two clustered DNA base lesions, in which the guanine base is oxidized at the C-8 position whereas the thymine or cytosine base is degraded to a formylamine remnant, result from the action of a single ·OH radical (11). The postulated mechanism for the generation of the tandem lesions implies the initial ·OH addition to the pyrimidine base followed by oxidation of the adjacent guanine.

Investigations aimed at determining the biochemical and structural features of each of these two modified bases have been performed. 8-Oxo-7,8-dihydroguanine (8-oxoGua), which is the major oxidized guanine modification, is used as a biomarker of DNA oxidation. Thus, it was shown that 8-oxoGua is efficiently removed from the DNA duplex by *Escherichia coli* Fapy-glycosylase (Fpg) protein with the exception of when it is paired with adenine (12). This also applies to Ogg1 protein of *Saccharomyces cerevisiae* (13–15) and the corresponding protein of human cells (16–23). On the other hand, 8-oxoGua is not a substrate for *E.coli* endonuclease (endo) III. Both nuclease P1 and snake venom phosphodiesterase were found to efficiently release 8-oxodGuo nucleoside from DNA whereas digestion by calf spleen phosphodiesterase seems highly affected (24). It was shown that the 8-oxodGuo lesion has significant mutagenic potential and may ultimately result in deleterious effects if it is not repaired (25). Thus, during DNA synthesis, 8-oxoGua, due to its ability to base pair with both C and A, may be incorporated opposite the oxidized base. This was shown to lead to G:C→T:A mutations during the second round of replication within the cells.

In contrast, fewer investigations have been performed on the assessment of the biological role of the formylamine lesion. Only a few results have been reported using oligonucleotides that contain a formylamine residue. Thus, it was shown that formylamine-containing templates used in *in vitro* replication assays either promote insertion of guanine or induce a deletion (26). Conformational changes associated with the presence of a formylamine residue in a DNA fragment were delineated. It was shown that the formylamine remnant considerably affects the stability of the DNA duplex (27,28). Recently it was found that dinucleoside monophosphates that contain a formylamine residue were completely resistant to hydrolysis by nuclease P1 (29). Investigations aimed at determining the release of the related nucleotide by both 3′- and 5′-exonucleases were carried out using

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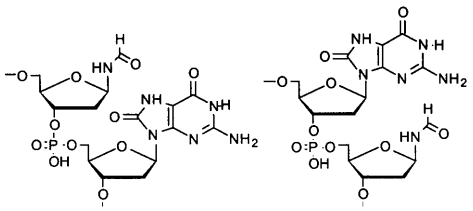


Figure 1. Structure of the two tandem base lesions chemically inserted into oligonucleotides.

modified tetranucleotides (30). No enzymatic repair study of formylamine by either Fpg or endo III has been performed up to now.

Both 8-oxoGua and formylamine were incorporated into synthetic oligonucleotides at a vicinal position in order to better assess the activity of both hydrolytic enzymes and DNA repair proteins. The major difficulty met during the preparation of such modified DNA fragments dealt with the chemical insertion of the labile dβF residue, which shows a high susceptibility to degradation and anomerization. Thus, the synthetic approach required the use of mild conditions during both the solid phase condensation and deprotection steps of the oligonucleotides. Herein, we describe the chemical synthesis of several oligonucleotides that contain both lesions at a vicinal position. The characterization of the latter DNA fragments showed the presence and integrity of the fragile tandem defect. Then, these synthetic molecules were used to determine whether or not this multiply damaged site is able to be cleaved by nucleases. Information on the specificity and mechanism of excision of the two tandem base damage by Fpg and endo III repair enzymes is also reported.

MATERIALS AND METHODS

Enzymes

Calf spleen phosphodiesterase and snake venom phophodiesterase were purchased from Boehringer Mannheim (Mannheim, Germany). Fpg and endonuclease III were kind gifts from Dr Serge Boiteux (CEA, Fontenay-aux-Roses, France). Nuclease P1 (*Penicillium citrium*) and alkaline phosphatase were from Sigma (St Louis, MO). Labeling at the 5′-end of DNA fragments was achieved by treatment with [γ-^{32}P]ATP from Amersham (Buckinghamshire, UK) and T4 polynucleotide kinase from Pharmacia Biotech (Uppsala, Sweden) using the standard procedure (31).

Capillary electrophoresis

The homogeneity of oligonucleotides was controlled by capillary gel electrophoresis on a P/ACE 5500 system (Beckman, Fullerton, CA), using a 27 cm × 100 µm capillary and ssDNA 100-R Kit (UV detection at 214 nm). Electrokinetic injection of the sample was performed for 1 s at 10 kV. Then, separation was carried out at a constant voltage of 8.1 kV leading to a constant current of 8 µA.

Oligonucleotide digestion mixtures were analyzed in the micellar electrokinetic (MEKC) mode using a standard procedure with a buffer consisting of an aqueous solution of 20 mM boric acid and 25 mM SDS, pH 10 (32).

Mass spectrometry measurements

FAB (fast atom bombardment) mass spectrometry analyses were carried out in the negative mode ionization on a VG ZAB 2-EQ apparatus (Manchester, UK). The samples to be analyzed were dissolved in either a glycerol or a nitrobenzyl alcohol (NBA) matrix.

All modified and unmodified oligonucleotides were characterized by electrospray ionization mass spectrometry measurements (ESI-MS) using a Platform 3000 model spectrophotometer from Micromass (Manchester, UK). Typically, 0.1 OD of the sample was dissolved in a solution of acetonitrile and water (50:50 v/v) that contained 1% triethylamine prior to analysis in the negative mode. Modified nucleosides were analyzed using both the negative and positive modes. For the positive mode analysis, the sample was dissolved in a solution of acetonitrile and water (50:50 v/v) that contained 0.5% formic acid.

MALDI mass spectra were obtained with a commercial time-of-flight mass spectrometer (Voyager-DE; Perseptive Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and pulsed delay source extraction. Spectra were recorded from 256 laser shots with an accelerating voltage of 25 kV in the linear and positive modes. For the matrix, a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4:1 (w/w) ratio was dissolved in 50% acetonitrile aqueous solution that contained 0.1% TFA and a small amount of Dowex-50W 50X8-200 (Sigma) cation exchange resin. Then, 1 µl of a 0.1% trifluoroacetic acid aqueous solution of the sample was added to 1 µl of the matrix and the resulting solution was stirred. The resulting sample was subsequently placed on the top of the target plate and allowed to dry by itself. The spectra were calibrated with a 1 pmol/µl solution of myoglobin (*m/z* 16 952), using the same assay conditions that were described for the oligonucleotides.

Synthetic procedures

*8-Oxo-7,8-dihydro-2′-deoxyguanosine (**1**)*. A fresh solution of sodium benzoylate was generated by stirring benzyl alcohol (10 ml, 96.6 mmol) with small pieces of sodium (0.8 g). The resulting solution was heated at 60°C. Then, 8-bromo-2′-deoxyguanosine (3 g, 8.67 mmol) dissolved in DMSO (7 ml, 98.5 mmol) was added and the resulting mixture was heated at 170°C for 30 min before being allowed to cool down at room temperature. After neutralization with glacial acetic acid, the solvents were removed by distillation under reduced pressure. The last traces of DMSO were removed by repeated washings with acetone and ethyl ether. For this purpose, acetone was added under stirring to the oily residue. The organic layer was removed and the precipitate formed was washed three times with acetone and ethyl ether and, at last, removed by filtration. The crude residue was dissolved in CH_2Cl_2 and prepurified by chromatography on a short silica gel column with a 0–30% gradient of MeOH in CH_2Cl_2. Then, the mixture was purified by reverse phase HPLC using a Nucleosil (Macherey-Nägel, Strasbourg, France) ODS column (7 µm, 250 × 21 mm) with detection set at 290 nm. Isocratic elution was achieved using 5% CH_3CN/25 mM ammonium formiate to afford compound **1** in a 480 mg yield (20%); R_f 0.65 (CH_2Cl_2/MeOH 1/1); λ_{max} (H_2O) 210, 248 and 290 nm; ESI-MS (negative mode) *m/z* = 282.35 ($M\text{-}H^-$) (calculated $M\text{-}H^-$, 282); ^{1}H NMR (200.13 MHz, D_2O) δ: 6.31 (t, 1H, H-1′), 4.72 (m, 1H, H-3′), 4.15 (m, 1H, H-4′), 3.90 (m, 2H, H-5′, H-5″), 3.11 (m, 1H, H-2′), 2.35 (m, 1H, H-2″).

*2-N-(Phenoxyacetyl)-8-oxo-7,8-dihydro-2′-deoxyguanosine (**2**).* Compound **1** (200 mg, 700 µmol) was dried by repeated co-evaporation with dry pyridine and then dissolved in dry pyridine (10 ml) under an argon atmosphere. To the stirred solution, trimethylchlorosilan (441 µl, 3.5 mmol) was added. After 45 min, phenoxyacetyl chloride (100 µl, 706 µmol) was introduced and stirring was continued for 16 h at room temperature. In order to destroy the excess of chloride, 1 ml of water was added and the resulting mixture was concentrated to dryness. The residue suspended in water was filtered and redissolved in pyridine. The nucleoside was deprotected by addition of 1 ml of a solution of ammonia/water (1:2 v/v). Then, the reaction mixture was evaporated to dryness and the residue was resuspended in water and filtered to afford compound **2** (200 mg, 68%). λ_{max} (H_2O) 214, 267.8 and 295 nm; ESI-MS (positive mode) *m/z* = 418.07 ($M+H^+$) (calculated $M+H^+$, 418), 440.04 ($M+Na^+$); ^1H NMR (400.13 MHz, DMSO-d_6) δ: 7.3 (t, 3H arom., PAC), 6.96 (d, 2H, H arom., PAC), 6.07 (t, 1H, H-1′), 5.15 (d, 1H, 3′-OH), 4.84 (s, 2H, CH_2 PAC), 4.70 (t, 1H, 5′-OH), 4.35 (m, 1H, H-3′), 3.73 (m, 1H, H-4′), 3.57 (m, 1H, H-5′), 3.42 (m, 1H, H-5″), 3.04 (m, 1H, H-2′), 1.97 (m, 1H, H-2″). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ: 170.6 (C=O, PAC), 157.5 (C-quat., PAC), 151.5, 149.2, 146.2, 144.6 (C-6, C-8, C-2 and C-4), 129.5 (2CH arom., PAC), 121.3 (CH arom., PAC), 114.5 (2CH arom., PAC), 103.7 (C-5), 87.2 (C-4′), 81.1 (C-1′), 71.0 (C3′), 66.1 (CH_2, PAC), 62.2 (C-5′), 35.2 (C-2′).

*2-N-(Phenoxyacetyl)-5′-O-monomethoxytrityl-8-oxo-7,8-dihydro-2′-deoxyguanosine (**3**).* Compound **2** (0.48 mmol, 200 mg) was dried by repeated co-evaporations with dry CH_2Cl_2. Then, the nucleoside was dissolved in dry dichloromethane (10 ml). The resulting solution was cooled in an ice–water bath and kept under an argon atmosphere. Then, 4-methoxytriphenylchloromethane (296 mg, 0.96 mmol) was added. After 1 h, the cooling bath was removed and stirring was pursued at room temperature overnight. The mixture was diluted with ethyl acetate (30 ml) and subsequently washed with saturated $NaHCO_3$ aqueous solution and water. The solvent was evaporated under reduced pressure. Then, the crude residue was suspended in a cold ethyl ether solution and filtered in order to afford compound **3** (200 mg, 60%). R_f 0.56 (CH_2Cl_2/MeOH 9/1); ^1H NMR (400.13 MHz, acetone-d_6) δ: 7.60–6.87 (m, 17H, H arom., MMT, PAC), 6.90 (d, 2H, H arom., PAC), 6.33 (t, 1H, H-1′), 4.98 (s, 2H, CH_2Ph), 4.71 (m, 1H, OH-3′), 4.46 (m, 1H, H-3′), 4.16 (m, 1H, H-4′), 3.85 (s, 3H, OCH_3, MMT), 3.57–3.35 (m, 2H, H-5′, H-5″), 2.32 (m, 1H, H-2′), 1.50 (m, 1H, H-2″).

*2-N-(Phenoxyacetyl)-5′-O-monomethoxytrityl-8-oxo-7,8-dihydro-2′-deoxyguanosine-3′-O-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite] (**4**).* Compound **3** (0.39 mmol, 271 mg) and tetrazole diisopropylammonium (0.18 mmol, 30 mg) were co-evaporated twice with dry CH_3CN and dissolved in acetonitrile (10 ml) under an argon atmosphere. Then 2-cyanoethyl-*N,N,N′,N′*-tetraisopropyldiamidite (0.63 mmol, 200 µl) was added to the solution under stirring. The course of the reaction was monitored by TLC (CH_2Cl_2:MeOH 19:1). After 2 h 30 min at room temperature, the mixture was diluted with ethyl acetate (50 ml) and washed with saturated $NaHCO_3$ aqueous solution. The organic layer was dried (Na_2SO_4) and then concentrated under vacuum. The residue was dissolved in CH_3CN and subsequently purified by reverse phase HPLC using a Nucleosil (Macherey-Nägel) ODS column (7 µm, 250 × 21 mm) with detection at 260 nm. Elution was performed using a 50–90% linear gradient of CH_3CN in H_2O at a 10 ml/min flow rate over a period of 35 min. Then, the two appropriate fractions that correspond to the diastereoisomers were collected and lyophilized to afford compound **4** (57 mg, 55%) as a white powder. R_f 0.44 (CH_2Cl_2/MeOH 19/1); ^{31}P NMR (101.21 MHz, CD_2Cl_2) δ: 147.71 and 147.80 (two diastereoisomers). FAB-MS (negative mode) *m/z* = 888.6 ($M-H^-$) (calculated $M-H^-$, 889).

Stability studies of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine (5) under alkaline conditions

Compound **5** (1 mg) was dissolved in concentrated aqueous ammonia (500 µl) (the same reaction was performed with the α-isomer). The solutions were placed either at room temperature or at 55°C. Then, the reactions were stopped at different periods of time (0, 1, 2, 4, 8 and 16 h, respectively) by freezing in liquid nitrogen and subsequent lyophilization. Samples were analyzed by HPLC using a Hypersil (Interchim, Montluçon, France) ODS column (5 µm, 250 × 4.6 mm i.d.), which was eluted with a 25 mM ammonium formiate buffer. UV detection was at 230 nm.

Determination of the deprotection half-life times of 2-*N*-(phenoxyacetyl)-8-oxo-7,8-dihydro-2′-deoxyguanosine (2) and 2-*N*-(isobutyryl)-8-oxo-7,8-dihydro-2′-deoxyguanosine

Deprotection half-life times of the exocyclic amino groups of **1** were determined by incubating 0.2 A_{260} units of each title compound in concentrated ammonia at room temperature. Deprotection reactions were stopped at various times (0, 1, 2, 4, 6, 8 and 16 h) by freezing in liquid nitrogen and subsequent lyophilization. Then, samples were analyzed by HPLC. This was carried out on a Hypersil (5 µm C_{18}, 250 × 4.6 mm) column with a 0–50% linear gradient of CH_3CN in 25 mM ammonium formiate. UV detection was at 260 nm.

Solid phase synthesis of oligodeoxyribonucleotides

Unmodified 2′-deoxyribonucleoside phosphoramidites protected with phenoxyacetyl for dAdo, isopropyl phenoxyacetyl for dGuo and acetyl for dCyd were purchased from Glen Research (Sterling, VA). Functionalized CPG supports, using a phenoxyacetyl protective group for dAdo and dGuo and an isobutyryl protective group for dCyd, were purchased from Sigma (St Louis, MO). The synthesis of tandem base damage-containing oligodeoxyribonucleotides was performed at the 1 µmol scale using an Applied Biosystems 392 DNA synthesizer, with elimination of the 5′-terminal DMTr goup (trityl-off mode). The standard 1 µmol DNA cycle was used, however with a modification. Thus, the duration of condensation of the modified nucleoside phosphoramidites was 4-fold increased (120 s instead of 30 s for normal nucleoside phosphoramidites). This allowed a coupling efficiency of >90% for each modified monomer. The detritylation, oxidation and capping steps were carried out with 3% DCA in CH_2Cl_2, a 0.02 M iodine solution and a solution of phenoxyacetic anhydride in THF, respectively.

Deprotection and purification of oligodeoxyribonucleotides

Upon completion of the synthesis, alkali-labile protecting groups of the oligodeoxyribonucleotides were removed upon treatment with concentrated aqueous ammonia (32%), in the presence of 0.25 M β-mercaptoethanol, at room temperature for 4 h. The synthetic dinucleotides and the tetranucleotides were purified by

reverse phase HPLC on a Hypersil (5 µm C_{18}, 250 × 4.6 mm) column using a 0–12% linear gradient of CH_3CN in 25 mM ammonium formiate over 30 min. UV detection was at 260 nm. The 25mer oligodeoxynucleotides were purified by preparative polyacrylamide gel electrophoresis and then desalted using NAP-25 sephadex columns (Pharmacia).

Acidic stability of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine (5) inserted in oligodeoxyribonucleotides

The 5′-protected dinucleotide MMTd(FpT) was prepared by phosphoramidite solid phase synthesis at a 0.2 µmol scale following the conditions described above. However, the resulting dinucleotide was not cleaved from the support by a final ammonia treatment. The crude product was used to study the percentage of dβF anomerization versus time under three different acidic conditions: 3% TCA, CH_2Cl_2; 3% DCA, CH_2Cl_2; 1% TFA, CH_2Cl_2. For each assay, 150 µl of the acid solution was added to the dinucleotide bound to the support. Then, the reactions were quenched at various times (1, 10, 30 and 60 min, respectively) by removing 30 µl of the mixture, filtering and washing the CPG support with CH_2Cl_2. Then, the dinucleotides were cleaved from the support by treatment with 150 µl concentrated aqueous ammonia at room temperature for 1 h. After evaporation to dryness, each sample was analyzed by HPLC on a 5 µm C_{18} (250 × 4.6 mm, Hypersil) column using a 0–10% linear gradient of CH_3CN in 25 mM ammonium formiate.

Lability studies of modified oligodeoxyribonucleotides in piperidine solution

Alkali-labile lesions were revealed by treatment with a freshly made 1 M piperidine aqueous solution, at 90°C for 15 and 30 min, respectively. Typically, the reactions were carried out on 0.01 OD of 5′-^{32}P-labeled modified oligodeoxyribonucleotides in 100 µl of piperidine solution in sealed tubes. After cooling, the samples were co-evaporated twice and then loaded onto a 20% polyacrylamide denaturing gel. The electrophoresis was carried out for 3 h at a voltage of 1300 V, before the gel was exposed to X-ray film. In addition, the piperidine treatments were performed with 0.05 OD of unlabeled modified oligodeoxyribonucleotides. The resulting DNA fragments were desalted by several 3 M ammonium acetate:ethanol (1:3 v/v) precipitations at –20°C, prior to analysis by MALDI-TOF mass spectrometry.

Digestion of modified oligonucleotides by nuclease P1 and alkaline phosphatase

Modified oligonucleotides (0.1 or 0.2 $AU_{260\ nm}$ for dinucleotides or tetranucleotides, respectively) were digested into nucleosides by incubation for 15 min, 1 h or 2 h 30 min at 37°C with 5 U of nuclease P1 in an aqueous solution of 30 mM NaOAc and 0.1 mM $ZnSO_4$, pH 5.5, in a total volume of 50 µl. Then, 10 U of calf intestinal alkaline phosphatase in buffer that contained 500 mM Tris, 1 mM EDTA, pH 8.5 (5 µl), were added. The mixture was subsequently incubated for 2 h prior to injection onto a C_{18} reverse HPLC column. Elution was achieved with a 0–10% linear gradient of CH_3CN in 0.25 mM ammonium formiate buffer, at a 1 ml/min flow rate over a period of 35 min. UV detection of the compounds was at 260 nm. The different products generated were identified by both co-injection with synthetic standards and electrospray ionization mass spectrometry analysis in the negative mode of the collected peaks. Oligodeoxyribonucleotide digestion mixtures were also analyzed by capillary electrophoresis in the MEKC mode with UV detection at 214 nm.

Enzymatic digestion of oligodeoxyribonucleotides by 3′- or 5′-exonuclease and subsequent MALDI-TOF mass spectrometry analysis

Modified oligodeoxyribonucleotides, $\mathbf{A_{25}}$, $\mathbf{B_{25}}$, $\mathbf{C_{25}}$, $\mathbf{D_{25}}$, $\mathbf{E_{25}}$ and $\mathbf{F_{25}}$, were precipitated twice with 3 M ammonium acetate:ethanol (1:3 v/v) prior to the start of the enzymatic digestions.

Digestion by calf spleen phosphodiesterase (5′-exo). Modified oligodeoxyribonucleotides (0.2 $AU_{260\ nm}$) were incubated at 37°C with 10^{-3} U of calf spleen phosphodiesterase (2 U/ml) in 30 µl of 0.02 M ammonium citrate (pH 5). Aliquots (1.5 µl) were withdrawn at increasing periods of time and the reactions were quenched by addition of 50 µl of water. Then, the solution was frozen in liquid nitrogen and lyophilized. The resulting samples were analyzed by MALDI-TOF spectrometry following the conditions described above.

Digestion by snake venom phosphodiesterase (3′-exo). Similar enzymatic reaction and MALDI-TOF spectrometry analysis were performed with 3×10^{-4} U of snake venom phosphodiesterase (3 U/ml) in 0.02 M ammonium citrate buffer (pH 9) (*vide supra*).

Fpg and endonuclease III repair studies

The reactions of Fpg (1.5 µg/µl) and endo III (2 µg/µl) proteins with a duplex that contained a simple or a tandem lesion were carried out in a buffer (A) consisting of 20 mM Tris–HCl, 1 mM EDTA, 100 mM KCl, pH 7.5. The products of the reactions were analyzed by both polyacrylamide gel electrophoresis (PAGE) and MALDI-TOF mass spectrometry.

Analysis by polyacrylamide gel electrophoresis. Modified oligodeoxyribonucleotides (10 pmol) were 5′-end-labeled using [γ-^{32}P]ATP and purified on MicroSpin™ G-25 columns. Then, the non-labeled oligodeoxyribonucleotide (95 pmol) and the complementary strand (150 pmol) were added to form a double-stranded duplex that contained either a simple or a tandem lesion. Hybridization was performed in 10 µl of 1× buffer A by heating for 5 min at 80°C and subsequent slow cooling to 4°C. Water (40 µl) was added and the resulting solution was aliquoted (5 µl/sample). Then, 5 µl of either Fpg or endo III at a 2–100 ng/µl final concentration in 2× buffer A was added to the aliquoted solution of double-stranded oligodeoxyribonucleotide. Reactions using increasing amounts of enzyme (2, 5, 10, 15, 25, 50, 75 and 100 ng/µl, respectively) were performed at 37°C for 30 min and stopped by addition of formamide (15 µl). Samples were denatured by heating at 80°C and electrophoresed on a 20% polyacrylamide–7 M urea gel at 1300 V for 30 min in TBE buffer (50 mM Tris, 50 mM boric acid, 50 mM EDTA, pH 8). The reaction products were visualized using a Bio-Rad Molecular Imager (Hercules, CA) and quantified with ImageQuant software.

Analysis by MALDI-TOF mass spectrometry. The modified oligodeoxyribonucleotide (40 pmol) and the complementary strand (60 pmol) were hybridized in 10 µl 2× buffer A by heating for 5 min at 80°C and subsequent slow cooling down to 4°C. The final volume was completed to 20 µl by addition of water. The enzyme, either Fpg or endo III (20 µl, 150 or 200 ng/µl) in 1× buffer A, was added and the resulting mixture was incubated at

37°C for 30 min. Reactions were quenched by freezing the solution in liquid nitrogen followed by lyophilization. The size of generated DNA fragments was analyzed by MALDI-TOF-MS upon desalting by two 3 M ammonium acetate:ethanol (1:3 v/v) precipitations.

RESULTS AND DISCUSSION

Synthesis of the modified phosphoramidite building blocks and condensation on a solid support

N-(2-deoxy-β-D-erythro-pentofuranosyl)-formylamine. Synthesis of dβF and its chemical incorporation into ODNs have already been reported in the literature (26–28,33). However, dβF is likely to anomerize easily under basic and acid conditions. Thus, the stability of dβF under standard conditions of automated oligodeoxyribonucleotides synthesis was assessed. Emphasis was placed on the anomeric configuration stability. Thus, when dβF was treated with concentrated ammonia at room temperature overnight, HPLC analysis of the reaction mixture revealed the presence of two compounds (data not shown). They were assigned as the β- and α-anomers of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine (dβF and dαF) on the basis of ES-MS and NMR analyses together by comparison with previously reported data (34). Further studies, performed with the β-anomer in concentrated ammonia at 55°C, showed that anomerization occurs readily in 40% yield within 4 h. Moreover, the formation of additional degradation products was observed under these conditions. However, <5% of dβF was converted into the corresponding α-anomer using concentrated ammonia at room temperature for 4 h. Consequently, the latter conditions were chosen to release the dβF-containing oligodeoxyribonucleotides from the support and to remove the alkali-labile protecting groups from the exocyclic amino functions. The stability of dβF and the conditions of deprotection applied are compatible with the 'Pac phosphoramidite' chemistry (35) which was used in the present study. Mono-*p*-methoxytritylated thymidine (MMT-dT) was oxidized, in the presence of $KMnO_4$ and $Pb(OAc)_4$, to obtain the dβF product (26). Then, the resulting MMT-dβF compound was phosphitylated with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-diamidite yielding the desired phosphoramidite building block in good yield (90%).

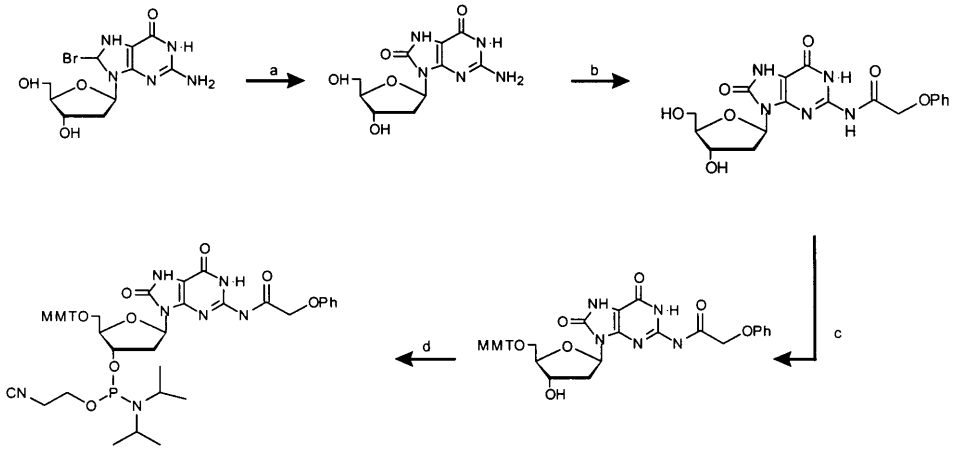
The MMTd(βFpT) dinucleoside monophosphate was synthesized in order to delineate the anomeric stability of dβF during the acid detritylation steps. Treatment of the resulting dinucleoside still attached to the support with three acid solutions in a non-protic solvent (1% TFA, CH_2Cl_2, 3% TCA, CH_2Cl_2 or 3% DCA, CH_2Cl_2) for 30 min led to a low conversion of dβF into dαF (7.7, 8.7 and 3.8% anomerization, respectively). Incubation for 30 min in acid solution corresponds to ~25 cycles of detritylation. According to these results, the 3% DCA, CH_2Cl_2 treatment, which induced the lowest rate of anomerization, was used to remove the trityl groups during the oligodeoxyribonucleotide synthesis cycle. It should be added that all the oligodeoxyribonucleotides that contained the dβF residue were prepared in the trityl-off mode. This was chosen with regard to the high level of anomerization observed during post-detritylation of the RP-HPLC purified trityl-on DNA fragments in an 80% aqueous solution of acetic acid. Under the latter conditions, 42% anomerization was found to occur upon 45 min treatment at room temperature.

The β configuration of the inserted *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine lesion in oligodeoxyribonucleotides was checked upon digestion of d(CpGpTpApGpGp**F**pGpC) (**B_9**) with nuclease P1 (0.05 UE for 10 nmol of substrate) and alkaline phosphatase. The resulting d(FpG) were analyzed by HPLC co-injection with d(βFpG) and d(αFpG) standards. The peak area calculation revealed that 7% anomerization occurred under the present conditions of synthesis and deprotection. This low level appeared acceptable for the preparation of oligonucleotides containing tandem base damage and their subsequent use for biochemical studies.

8-Oxo-7,8-dihydro-2′-deoxyguanosine. Several syntheses of 8-oxodGuo-containing oligonucleotides have already been reported (36–39). Moreover, the 2-*N*-(isobutyryl)-8-oxo-7,8-dihydro-2′-deoxyguanosine phosphoramidite monomer is commercially available from Glen Research (Sterling, VA). However, the deprotection half-life time of the isobutyryl protecting group is 60 min at 55°C. This made unsuitable the use of the latter protecting group for the synthesis of oligodeoxyribonucleotides that contain the tandem lesion, taking into account the *N*-glycosidic instability of dβF. Thus, a phosphoramidite building block of 8-oxodGuo which contains the highly alkali-labile phenoxyacetyl protective group was prepared as a better alternative.

8-OxodGuo was prepared first by Lin *et al*. (40). However, we developed a more convenient synthesis of 8-oxodGuo providing a slightly higher yield. Thus, 8-oxodGuo was obtained in one step in 20% yield by heating 8-bromo-2′-deoxyguanosine with sodium and alcohol benzoylate at 170°C for 30 min in DMSO (Scheme 1). The 2-*N*-(phenoxyacetyl)-8-oxo-7,8-dihydro-2′-deoxyguanosine building block (**2**) was then synthesized according to the reported procedures (35,39) (yield 68%). The deprotection half-life time for the phenoxyacetyl was found to be 30 min at room temperature. Thus, the use of the latter protecting group is compatible with the chemical incorporation of the formylamine residue into oligodeoxynucleotides. Therefore, building block **4** was prepared and used for further insertion into the DNA fragment. The 4-monomethoxytrityl group was added under standard conditions giving compound **3** in 60% yield. Then, phosphitylation of **3** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-diamidite afforded the desired compound **4** in 54% yield after RP-HPLC purification.

Synthesis and characterization of the oligonucleotides. Several oligodeoxyribonucleotides (Table 1) bearing one or the two modified lesions, namely 8-oxodGuo and dβF were synthesized on a solid support using the phosphoramidite chemistry with some adaptations (*vide supra*). This allowed a coupling efficiency of >90% for each of the modified nucleosides. The purity and the homogeneity of the material were assessed by applying several methods including analytical PAGE of 5′-^{32}P-labeled fragments, capillary gel electrophoresis and mass spectrometry measurements. Thus, the molecular weights of the oligonucleotides were inferred from electrospray ionization mass spectrometry analyses in the negative mode (Table 1). The obtained results confirmed the incorporation and the integrity of the modified nucleosides into the oligomers.



Scheme 1. (**a**) DMSO, $PhCH_2ONa$, 170°C, 30 min; (**b**) TMS-Cl, pyridine, 20°C, 45 min; PAC-Cl, 20°C, 16 h; NH_4OH/H_2O 1/2 v/v, 20°C, 10 min; (**c**) MMT-Cl, CH_2Cl_2, 20°C, 16 h; (**d**) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyldiamidite, tetrazole diisopropylammonium, CH_3CN, 20°C, 2 h 30 min.

Table 1. Sequences and molecular weights (Da) of the oligonucleotides synthesized and used in the present study

Oligo	Sequences	calc. MW	found MW
1	5'- **F** T -3'	465	465,03
2	5'- **F** A -3'	474	474,21
3	5'- **F** C -3'	450	449,99
4	5'- **F** G -3'	490	489,02
A_4	5'- A **F** **G°** C -3'	1109	1108,35 ±0,14
B_4	5'- A **F** G C -3'	1093	1092,32 ±0,31
C_4	5'- A **G°** **F** C -3'	1109	1108,29 ±0,17
D_4	5'- A **G°** T C -3'	1190	1189,33 ±0,43
F_4	5'- A T G C -3'	1174	1173,26 ±0,35
A_{25}	5'- C A G T A C G T A G G A G **F** **G°** C C A T C G A T A G -3'	7666	7665,44 ±0,72
B_{25}	5'- C A G T A C G T A G G A G **F** G C C A T C G A T A G -3'	7650	7648,03 ±1,13
C_{25}	5'- C A G T A C G T A G G A **G°** **F** G C C A T C G A T A G -3'	7666	7661,59 ±7,35
D_{25}	5'- C A G T A C G T A G G A G T **G°** C C A T C G A T A G -3'	7747	7744,83 ±0,89
E_{25}	5'- C A G T A C G T A G G A **G°** T G C C A T C G A T A G -3'	7747	7745,01 ±1,15
F_{25}	5'- C A G T A C G T A G G A G T G C C A T C G A T A G -3'	7731	7729,08 ±0,97
G_{25}	5'- C T A T C G A T G G C A C T C C T A C G T A C T G -3'	7593	7592,28 ±0,88

G°, 8-oxodGuo; F, formylamine. The oligonucleotide molecular masses were obtained by electrospray ionization mass spectrometry measurements in the negative mode.

Piperidine lability of base damage inserted into oligodeoxyribonucleotides

The alkaline lability of tandem base lesion-containing oligonucleotides was investigated using the usual piperidine treatment. The piperidine test allowed us to reveal the presence of alkali-labile DNA modifications at the sequence level in oxidized oligonucleotides after appropriate gel electrophoresis analyses. The stability experiment was also carried out on a single 8-oxodGuo or dβF lesion incorporated into defined sequence DNA fragments. The comparative study of the stability of modified nucleosides was performed by treating 5′-^{32}P-labeled oligonucleotides $\mathbf{A_{25}}$, $\mathbf{B_{25}}$ and $\mathbf{D_{25}}$ with piperidine at 90°C for 15 and 30 min, respectively. In a subsequent step, the resulting DNA fragments were analyzed by denaturing PAGE (data not shown). It was found, in agreement with other reported studies, that the 8-oxodGuo-containing oligonucleotide $\mathbf{D_{25}}$ was stable under piperidine treatment, since only a few breaks were observed after 30 min incubation. In contrast, a similar treatment led to a nearly quantitative cleavage at the formylamine site of single or double modified oligonucleotides $\mathbf{A_{25}}$ and $\mathbf{B_{25}}$, respectively. It should be added that the PAGE mobility of the released labeled DNA fragments was consistent with the cleavage of oligonucleotides $\mathbf{A_{25}}$ and $\mathbf{B_{25}}$ at the formylamine sites.

Further information on the mechanism of piperidine-mediated release of the formylamine residue was gained from a MALDI-TOF mass spectrometry study. For this purpose, the three unlabeled modified oligodeoxyribonucleotides $\mathbf{A_{25}}$, $\mathbf{B_{25}}$ and $\mathbf{E_{25}}$ were heated at 90°C for 30 min in 1 M piperidine solution. Then, the desalted reaction mixtures were analyzed by MALDI-TOF mass spectrometry. Piperidine treatment of the modified oligodeoxyribonucleotide $\mathbf{B_{25}}$, which contains a single formylamine lesion, gave rise to two fragments which result from a β–δ-elimination reaction of the dβF residue. Thus, the peak at m/z = 3420.7 ($M+H^+$) corresponds to the d(pGCCATCGATAG) residue (calculated $M+H^+$, 3422) whereas the second peak at m/z = 4103.2 ($M+H^+$) is accounted for by the d(CAGTACGTAGGAGp) fragment (calculated $M+H^+$, 4105). Piperidine treatment of the dβF/8-oxodGuo tandem base modification-containing oligonucleotide $\mathbf{A_{25}}$ was found to induce three degradation fragments as revealed by MALDI-TOF-MS analyses. Two of them, with molecular weights

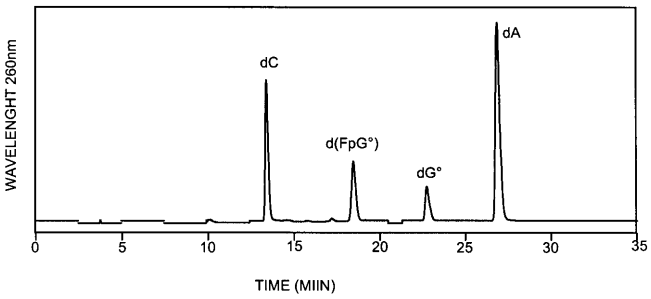


Figure 2. HPLC chromatogram showing the partial digestion of d(ApFpG°pC). The tetranucleotide was incubated with 5 EU of nuclease P1 at 37° for 1 h and then with 10 EU of alkaline phosphatase at 37°C for 2 h. The resulting mixture was analyzed by reverse phase HPLC (the conditions of the analysis are reported in the Materials and Methods). Detection of the nucleoside dF was unsuccesful because of its lack of significant absorption above 260 nm.

of 4103.4 and 3437.4 ($M+H^+$), correspond to cleavage of the 3′- and 5′-phosphodiester bonds of dβF through a β–δ-elimination mechanism (calculated $M+H^+$, 4105 and 3438, respectively). The third one (detected $M+H^+$, 3091.6) results from cleavage of the phosphodiester bond 3′ of the 8-oxodGuo residue (calculated $M+H^+$, 3093), as previously observed in oligonucleotide **E_{25}**.

Enzymatic hydrolysis of modified oligonucleotides by nuclease P1

Oligonucleotides, namely d(FpT), d(FpG), d(FpC), d(FpA), d(TpT), d(ApGpFpC), d(ApG°pTpC), d(ApG°pFpC), d(ApFpG°pC) and d(ApGpTpC), were incubated at 37°C in the presence of nuclease P1 and alkaline phosphatase. The dβF-containing oligonucleotides were found to be partly hydrolyzed. These data are not consistent with previous reported results which showed a total inhibition of nuclease P1 activity toward hydrolysis of the phosphodiester bond 3′ of the dβF lesion (29,30). The efficiency of cleavage of the formylamine lesion inserted into DNA fragments appears to be sequence dependent. Thus, the level of hydrolysis of the dinucleoside monphosphate d(FpC) reached 60% instead of 40% for the other dinucleoside monophosphates d(FpA), d(FpG) and d(FpT), using 5 U of nuclease P1 for 60 min. It may be concluded that the formylamine lesion slowed down the hydrolytic activity of nuclease P1. In contrast, 8-oxodGuo was not found to affect the enzymatic activity of nuclease P1 since d(ApG°pTpC) was completely hydrolyzed upon incubation with 5 U of the enzyme for 60 min. Interestingly, the dinucleoside monophosphate d(FpC) was observed in the reaction mixture of d(ApG°pFpC) with 5 U of nuclease P1 at 37°C after 1 h incubation. Under the same conditions, the partial digestion of d(ApFpG°pC) gave rise to the dinucleoside monophosphate d(FpG°) which was characterized by ESI-MS (calculated $M-H^-$, 505; found $M-H^-$, 505.2) (Fig. 2).

MALDI-MS analysis of the 3′- and 5′-exonuclease-mediated digestions of the oligonucleotides

Enzymatic digestions by snake venom phosphodiesterase (VPD) (3′-exonuclease) and calf spleen phosphodiesterase (SPD) (5′-exonuclease) were carried out on the modified oligonucleotides **A_{25}**, **B_{25}**, **C_{25}**, **D_{25}** and **E_{25}** and the normal oligodeoxyribonucleotide **F_{25}**. To follow enzymatic hydrolysis along the DNA strands, aliquots were withdrawn from the digestion mixtures at increasing periods of time and the products directly analyzed by MALDI-TOF mass spectrometry (41). Thus, the different molecular ions observed correspond to the digested DNA fragments which differ in mass by successive loss of nucleotides. The difference in mass between two adjacent products allows to identify the released nucleoside and therefore to determine the overall sequence.

The unmodified oligodeoxyribonucleotide **F_{25}** was totally hydrolyzed by both 5′- and 3′-exonucleases allowing the determination of the complete sequence by MALDI-TOF-MS measurement. For example, the mass spectrum corresponding to the reaction with SPD after 15 min incubation showed the release of 19 nucleoside 3′-monophosphates. Hydrolysis of the fragment was complete after 30 min. The reaction of the 3′-exonuclease enzymatic digestion was found to be even more efficient. Interestingly, after 1 min hydrolysis, the VPD reached the nucleotide located at position 12 from the 5′-side and digestion was complete upon 15 min incubation. The relative intensity of the ladder peaks showed, as was previously described (42), that the digestion rate was sequence dependent, suggesting a base specificity for the hydrolytic action of both PDases.

The presence of the formylamine lesion in **B_{25}** was found to induce a partial resistance toward digestion by SPD, in agreement with previous findings (24). Thus, the 5′-exonuclease activity was strongly slowed down at the phosphodiester bonds both before and after the lesion. After incubation with the enzyme for 1 h, the two fragments d(GFGCCATCGATAG) (found $M+H^+$, 3891.8; calculated $M+H^+$, 3894) and d(FGCCATCGATAG) (found $M+H^+$, 3565.2; calculated $M+H^+$, 3565) were digested with great difficulty. Since the enzyme was able to bypass the formylamine remnant, the resulting strand was then rapidly digested. On the other hand, the 3′-exonuclease activity of VPD was completely inhibited by the modified nucleoside. These results were not consistent with previously reported data obtained on a dF-containing tetramer (30). However, after a longer period of incubation (60 min), a few digested fragments were observed. Surprisingly, these corresponded to oligomers resulting from an endonucleolytic digestion activity. The strands thus had either a formylamine at the 3′-end or a normal nucleoside. The latter fragment was then quickly digested by the 3′-exonuclease. Concerning the other generated fragment, the formylamine remnant located at the 3′-end inhibited the exonuclease activity of VPD. Nevertheless, this fragment will be slowly digested due to the slight endonuclease activity exhibited by VPD. As a consequence, the modified DNA fragment was hydrolyzed upon incubation for longer periods of time up to 8 h due to both the endonuclease and exonuclease activities.

The ability for the 5′-exonuclease to digest the 8-oxodGuo modification inserted in **D_{25}** was also assessed. After 60 min incubation, the activity of the enzyme was strongly slowed down one nucleoside before the lesion. The mass spectrum of the digestion products after 4 h reaction clearly shows that the enzyme was completely inhibited by 8-oxodGuo. This was inferred from the lack of any additional digestion of 8-oxodGuo at the 5′-end even upon a longer period of incubation. In addition, the 3′-exonuclease was inhibited by 8-oxodGuo upon incubation of oligonucleotide **E_{25}** with VPD. Thus, the MALDI-TOF mass spectrum after 8 min of enzymatic incubation exhibited a single peak at m/z = 4040.5 ($M+H^+$) corresponding to d(CAGTACG-TAGGAG°) (calculated $M+H^+$, 4041). VPD was found to induce

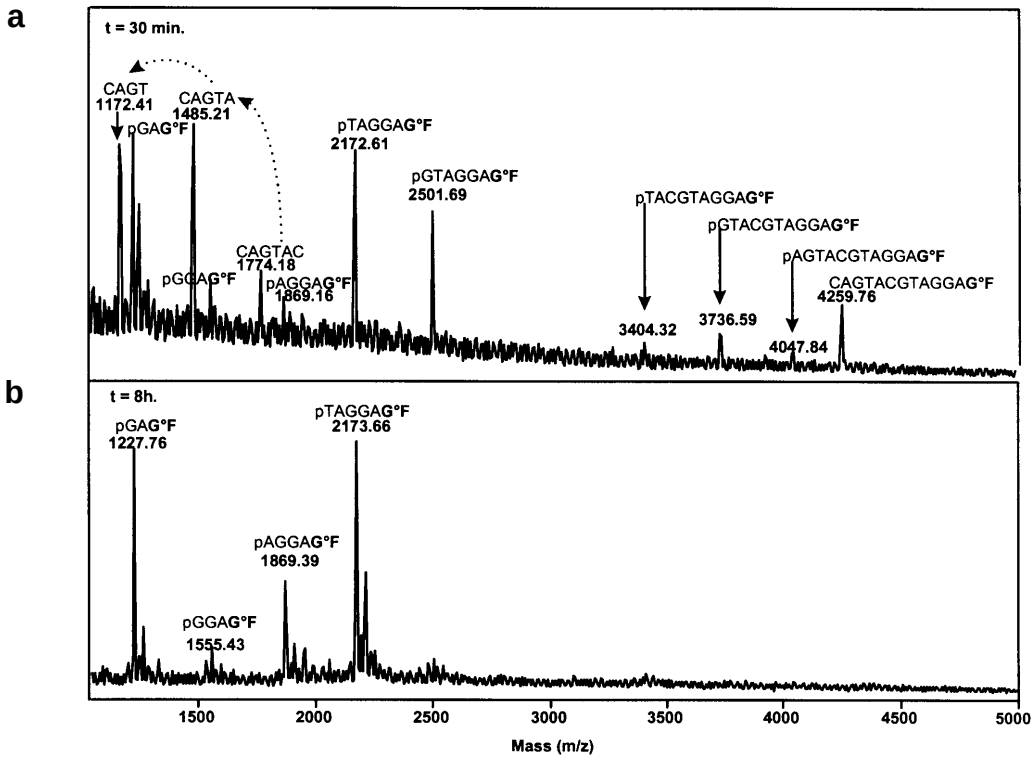


Figure 3. MALDI-TOF mass spectra of products resulting from the digestion of the modified oligonucleotide CAGTACGTAGGA**G**°**F**GCCATCGATAG (C_{25}) by the 3′-exonuclease after 30 min (**a**) and 8 h (**b**). Unmarked peaks were attributed to salt adducts (K^+ and Na^+).

the release of the normal nucleotides at the 3′-end of the oligonucleotide. However, no 3′-exonuclease cleavage of the phosphodiester linkage between 8-oxodGuo and dAdo was observed. The present results are also not consistent with the findings obtained by Box *et al*. using short oligonucleotides (30). Nevertheless, with longer incubation periods, the same slow endonuclease activity that was previous observed (*vide supra*) gave rise to a nick in the ODN fragment. At the end, the oligonucleotide was hydrolyzed due to both exonuclease and endonuclease VPD activities.

The same enzymatic reactions were performed on tandem lesion-containing oligonucleotides. Firstly, the modified oligonucleotide **A_{25}**, which contains the dβF/8-oxodGuo double modification, was incubated with SPD. It was found that the 5′-exonuclease activity of SPD slowed down one nucleotide before the tandem lesion and then was completely inhibited by the double modification at the formylamine site. In contrast, the 3′-exonuclease acted normally until reaching the lesion and was then stopped by the double modification. With longer times of incubation, a secondary endonuclease activity allowed hydrolysis into nucleosides as described above. Up to 8 h, a few fragments are detectable using MALDI-TOF-MS. The modified oligonucleotide **C_{25}**, bearing the 8-oxodGuo/dβF lesion, was also used as a substrate for SPD. The enzyme activity was slowed down one nucleoside before the lesion, as was previously observed for **A_{25}**. The MS analyses of the reaction products obtained upon 8 h incubation with the 5′-exonuclease revealed that the enzyme was completely blocked by the tandem lesion. SPD generated a DNA fragment, namely d(G°FGCCATCGATAG), bearing the tandem lesion modification at the 5′-end. Incubation of **C_{25}** with VPD led to similar findings to those obtained with the other tandem base damage. In particular, fragments resulting from an endonucleolytic action were observed (Fig. 3). As an example, the fragment d(CAGTACGTAGGAG°F) (found $M+H^+$, 4259.8) was cleaved by VPD generating d(CAGTAC) and d(GTAGGAG°F) (found $M+H^+$, 1774.2 and 2501.7, respectively). The latter fragment, which contains the tandem lesion at the 3′-end, namely d(GTAGGAG°F), inhibited the 3′-exonuclease activity . However, due to the slow endonucleolytic activity of the enzyme, the fragment was completely hydrolyzed into nucleosides after a long period of incubation. In contrast, the other fragment d(CAGTAC) (5′ of the site of digestion) was efficiently digested by the exonuclease giving rise to ladder peaks. After 8 h incubation, only a few fragments which bear the tandem lesion at the 3′-end and which result from an endonuclease activity were detected.

The 5′-exonuclease activity of SPD is strongly slowed down by the formylamine remnant. Thus, complete digestion of the DNA fragment bearing a formylamine residue requires an 8-fold longer incubation period than for the related unmodified oligonucleotide. In contrast, SPD is completely inhibited by the 8-oxodGuo and both tandem lesions. The 3′-exonuclease activity of VPD is inhibited by the different lesions inserted into the oligonucleotides. Nevertheless, an unexpected slow endonucleolytic activity was observed upon longer periods of incubation. In this respect, a 32-fold increase in the incubation allowed degradation of the oligonucleotides. However, no information on the site of cleavage can be provided by the MALDI-TOF analyses since fragments of molecular weight <500 Da cannot be observed by this method. Interestingly, Box *et al*. observed successful VPD-mediated hydrolysis of the modified DNA. The endonuclease activity was

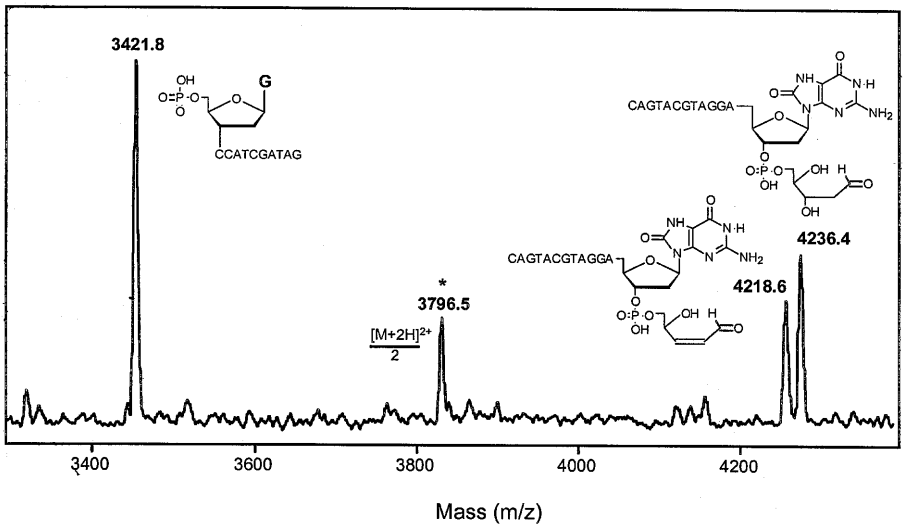


Figure 4. MALDI-TOF mass spectrum of products resulting from the incubation of CAGTACGTAGGA**G**°**F**GCCATCGATAG (C_{25}) with endonuclease III. The peak marked with an asterisk represents doubly charged ions belonging to complementary oligonucleotide G_{25}.

not mentioned by the authors who used di- and tetranucleotides to perform their enzymatic reactions and analyzed only the final mixtures of generated nucleotides and nucleosides.

Enzymatic cleavage of tandem base damage-containing oligonucleotides with Fpg and endo III proteins

Duplex substrates (25 bp), obtained by annealing modified oligodeoxyribonucleotides **A_{25}**, **B_{25}**, **C_{25}**, **D_{25}** and **E_{25}** with normal complementary strand **G_{25}**, were incubated with either Fpg or endo III proteins. The products were analyzed by both PAGE and MALDI-TOF mass spectrometry. As can be observed in the mass spectra, the oligonucleotides are present in the protonated form, together with K^+ or Na^+ adducts as counter ions of the phosphate groups leading to a distribution of peaks. The oligodeoxyribonucleotide **B_{25}**, which contains a dβF residue, was cleaved by both endo III and Fpg proteins. The size of the Fpg-generated fragments (found M+H$^+$, 4105.7 and 3422.7; calculated M+H$^+$, 4105 and 3422, respectively) as inferred from MALDI-TOF-MS indicated that the formylamine nucleoside was excised. It was shown that Fpg cleaved both the 3′- and the 5′-phosphodiester bonds at the site of the formylamine lesion. The latter observation is in agreement with previously reported data which suggested that Fpg operates through a β–δ-elimination mechanism (43). Cleavage by endo III protein gave rise to two major fragments with molecular weights M+H$^+$ of 3425.0 and 4223.9 (calculated M+H$^+$, 3421 and 4221, respectively). Moreover, it was found that the enzyme cleaves mainly the phosphodiester bond 3′ of a formylamine residue by a hydrolysis reaction and not by a β-elimination reaction as previously reported (44).

In agreement with previous findings (45–48), Fpg protein was found to excise 8-oxoGua in the duplex **E_{25}**, whereas no cleavage was observed when the oligodeoxyribonucleotide was incubated with endo III.

The occurrence of strand breaks within duplex **A_{25}**, in which the tandem lesion dβF/8-oxodGuo was inserted, was visualized by PAGE upon incubation with either Fpg or endo III (data not shown). When endo III was used at a concentration of 100 ng/µl, 80% cleavage was observed for a 1 µM oligonucleotide concentration. Incubation with Fpg at a concentration of 75 ng/µl led to 90% cleavage for the same oligonucleotide concentration. The MALDI-TOF-MS analysis revealed that only 8-oxoGua was excised by Fpg. Clearly, two strands corresponding to cleavage of the phosphodiester bonds 3′- and 5′- of 8-oxodGuo were observed (found M+H$^+$, 4330.2 and 3095.5; calculated M+H$^+$, 4328 and 3093, respectively) (data not shown). The endo III protein was found to mainly hydrolyze the phosphodiester bond 3′ of the formylamine residue (found M+H$^+$, 4223.8 and 3441.2; calculated M+H$^+$, 4221 and 3438, respectively), whereas the phosphodiester bond 3′ of the 8-oxoGua residue was not affected by the enzyme.

Oligodeoxyribonucleotide **C_{25}**, which contains the tandem lesion of opposite sequence, 8-oxodGuo/dβF, was found to be incised by both the Fpg and endo III enzymes (data not shown). The experimental conditions allowed cleavage of 90 and 70% of the oligonucleotide with Fpg and endo III, respectively, as determined by PAGE. In contrast to what was observed with **A_{25}**, three products and not two are present in the MALDI-TOF-MS spectrum (data not shown) upon incubation of the duplex with Fpg. The phosphodiester bond 5′ of the 8-oxoGua residue was completely cleaved by the enzyme (found M+H$^+$, 3422.5; calculated M+H$^+$, 3422). Interestingly, two fragments (found M+H$^+$, 3422.4 and 3645.4) were observed on the 3′-side of the tandem lesion. The major fragment (found M+H$^+$, 3645.4; calculated M+H$^+$, 3644) corresponds to hydrolysis of the phosphodiester bond 3′ of the 8-oxodGuo residue, whereas the other minor one resulted from cleavage of the phosphodiester bond 3′ of the formylamine remnant (found M+H$^+$, 3422.4, calculated M+H$^+$, 3422). Clearly, Fpg protein was found to excise both formylamine and 8-oxoGua residues to generate the latter minor DNA fragment. The reason why this reaction did not occur with the duplex that contains the other tandem lesion (**A_{25}**) is not clear. Reaction with endo III gave rise to three products (found M+H$^+$, 4236.4, 4218.8 and 3421.8) (Fig. 4). The peak marked with an asterisk represents a doubly charged ion $[M+2H]^{2+}/2$ belonging to the complementary strand. These observations may be rationalized in terms of the occurrence of two mechanisms of

action for endo III. The major reaction observed was cleavage of the phosphodiester bond 3′ of the formylamine via a hydrolytic reaction giving rise to two fragments with molecular weights of 4236.4 (5′-side $M+H^+$) (calculated $M+H^+$, 4237) and 3421.8 (3′-side $M+H^+$) (calculated $M+H^+$, 3422). In addition, a third fragment of lower intensity (found $M+H^+$, 4218.8) was found to be present. This may be accounted for by a β-elimination reaction (calculated $M+H^+$, 4219) which represents only a less important pathway of endo III mediated-processing of the 8-oxodGuo-dβF lesion.

CONCLUSION

We report here the first synthesis of oligonucleotides bearing fragile tandem base damage. The preparation of such substrates required the use of gentle chemical conditions for the condensation and deprotection steps. The piperidine stability experiments performed with the modified DNA fragment prove the high lability of the formylamine lesion inserted into the oligomers, whereas 8-oxodGuo is confirmed as a rather stable damage. The effect of the latter tandem lesions on different enzyme functions was evaluated. Both the 3′- and 5′-exonuclease activities of SPD and VPD, respectively, are inhibited by the tandem lesions. On the other hand, both the Fpg and the endo III proteins are able to cleave the oligonucleotide chain that contains the dβF/8-oxodGuo lesions. Interestingly, insights into the mechanisms of action of Fpg and endo III were gained from MALDI-TOF-MS measurements of the generated fragments. They were found to depend on the tandem base damage inserted. The present study will be extended to other substrates which include pairs of damaged bases not immediately adjacent to each other on either the same or the opposite strands. The kinetic parameters governing the cleavage of tandem base lesion-containing oligo-nucleotides by the Fpg, endo III and yOgg1 enzymes will be investigated.

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See supplementary material available in NAR Online.

REFERENCES

1 Ward,J.F. (1995) *Radiat. Res.*, **142**, 362–368.
2 Ward,J.F., Blakely,W.F. and Joner,E.I. (1985) *Radiat. Res.*, **103**, 383–392.
3 Lindahl,T. (1993) *Nature*, **362**, 709–715.
4 Goodhead,D.T. (1994) *Int. J. Radiat. Biol.*, **65**, 7–17.
5 Box,H.C., Schroder,E.A. and Budzinski,E.E. (1995) In Fuciarelli,A.F. (ed.), *Radiation Damage to DNA*. Battelle Press, Columbus, OH, pp. 285–290.
6 Box,H.C., Budzinski,E.E., Dawidzik,J.B., Gobey,J.S. and Freund,H.G. (1997) *Free Radical Biol. Med.*, **23**, 1021–1030.
7 Box,H.C., Budzinski,E.E., Dawidzik,J.B., Wallace,J.C. and Iijima,H. (1998) *Radiat. Res.*, **149**, 433–439.
8 Delatour,T., Douki,T., Gasparutto,D., Brochier,M.C. and Cadet,J. (1998) *Chem. Res. Toxicol.*, **11**, 1005–1013.
9 Mariaggi,N., Cadet,J. and Téoule,R. (1976) *Tetrahedron*, **32**, 2385–2387.
10 Box,H.C., Freund,H.G., Budzinski,E.E., Wallace,J.C. and Maccubbin,A.E. (1995) *Radiat. Res.*, **141**, 91–94.
11 Box,H.C., Budzinski,E.E., Freund,H.G., Evans,M.S., Patrzyc,H.B., Wallace,J.C. and Maccubin,A.E. (1993) *Int. J. Radiat. Biol.*, **64**, 261–263.
12 Bulychev,N.V., Varaprasad,C.V., Dorman,G., Miller,J.H., Eisenberg,M., Grollman,A.P. and Johnson,F. (1996) *Biochemistry*, **35**, 13147–13156.
13 Nash,H.M., Bruner,S.D., Schaerer,O.D., Kawate,T., Addona,T.A., Spooner,E., Lane,W.S. and Verdine,G.L. (1996) *Curr. Biol.*, **6**, 968–980.
14 van der Kemp,P.A., Thomas,D., Barbey,R., de Oliveira,R. and Boiteux,S. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 5197–5202.
15 Karahalil,B., Girard,P.-M., Boiteux,S. and Dizdaroglu,M. (1998) *Nucleic Acids Res.*, **26**, 1228–1232.
16 Aburatani,H., Hippo,Y., Ishida,T., Takashima,R., Matsuba,C., Kodama,T., Takao,M., Yasui,A., Yamamoto,K., Asano,M., Fukasawa,K., Yoshinari,T., Inoue,H., Ohtsuka,E. and Nishimura,S. (1997) *Cancer Res.*, **57**, 2151–2156.
17 Arai,K., Morishita,K., Shinmura,K., Kohno,T., Kim,S.R., Nohmi,T., Taniwaki,S., Ohwada,S. and Yokota,J. (1997) *Oncogene*, **14**, 2857–2861.
18 Radicella,J.P., Dherin,C., Desmaze,C., Fox,M.S. and Boiteux,S. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 8010–8015.
19 Bjoras,M., Luna,L., Johnsen,B., Hoff,E., Haug,T., Rognes,T. and Seeberg,E. (1997) *EMBO J.*, **16**, 6314–6322.
20 Roldàn-Arjona,T., Anselmino,C. and Lindahl,T. (1996) *Nucleic Acids Res.*, **24**, 3307–3312.
21 Rosenquist,T.A., Zharkov,D.O. and Grollman,A.P. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 7429–7434.
22 Lu,R., Nash,H.M. and Verdine,G.L. (1997) *Curr. Biol.*, **7**, 397–407.
23 Shinmura,K., Kasai,H., Sasaki,A., Sigumura,H. and Yokota,J. (1997) *Mutat. Res.*, **385**, 75–82.
24 Maccubbin,A., Evans,M., Paul,C.R., Budzinski,E.E., Przybyszewski,J. and Box,H.C. (1991) *Radiat. Res.*, **126**, 21–26.
25 Grollman,A.P. and Moriya,M. (1993) *Trends Genet.*, **9**, 246–249.
26 Guy,A., Duplaa,A.M., Ulrich,J. and Téoule,R. (1991) *Nucleic Acids Res.*, **19**, 5815–5820.
27 Shida,T., Iwaori,H., Arakawa,M. and Sekiguchi,J. (1993) *Chem. Pharm. Bull.*, **41**, 961–964.
28 Shida,T., Arakawa,M. and Sekiguchi,J. (1994) *Nucleosides Nucleotides*, **13**, 1319–1326.
29 Falcone,J.M. and Box,H.C. (1997) *Biochim. Biophys. Acta*, **1337**, 267–275.
30 Schroder,E., Budzinski,E.E., Wallace,J.C., Zimbrick,J.C. and Box,H.C. (1995) *Int. J. Radiat. Biol.*, **68**, 509–523.
31 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32 Berthod,T., Petillot,Y., Guy,A., Cadet,J. and Molko,D. (1996) *J. Org. Chem.*, **61**, 6075–6078.
33 Baillet,S. and Behr,J.-P. (1995) *Tetrahedron Lett.*, **36**, 8981–8984.
34 Cadet,J., Nardin,R., Voituriez,L., Remin,R. and Hruska,F.E. (1981) *Can. J. Chem.*, **59**, 3313–3318.
35 Schulhof,J.C., Molko,D. and Téoule,R. (1987) *Nucleic Acids Res.*, **15**, 397–416.
36 Bodepudi,V., Iden,C.R. and Johnson,F. (1991) *Nucleosides Nucleotides*, **10**, 755–761.
37 Bodepudi,V., Shibutani,S. and Johnson,F. (1992) *Chem. Res. Toxicol.*, **5**, 608–617.
38 Koizume,S., Kamiya,H., Inoue,H. and Ohtsuka,E. (1994) *Nucleosides Nucleotides*, **13**, 1517–1534.
39 Roelen,H.C.P.F., Saris,C.P., Brugghe,H.F., van den Elst,H., Westra,J.G., van der Marel,G.A. and van Boom,J.H. (1991) *Nucleic Acids Res.*, **19**, 4361–4369.
40 Lin,T.S., Cheng,J.C., Ishiguro,K. and Sartorelli,A.C. (1985) *J. Med. Chem.*, **28**, 1194–1198.
41 Pieles,U., Zurcher,W., Schar,M. and Moser,H.E. (1993) *Nucleic Acids Res.*, **21**, 3191–3196.
42 Bentzley,C.M., Johnston,M.V. and Larsen,B.S. (1998) *Anal. Biochem.*, **258**, 31–37.
43 Bhagwat,M. and Gerlt,J.A. (1996) *Biochemistry*, **35**, 659–665.
44 Doetsch,P.W. and Cunningham,R.P. (1990) *Mutat. Res.*, **236**, 173–201.
45 Tchou,J., Kasai,H., Shibutani,S., Chung,M.H., Laval,J., Grollman,A.P. and Nishimura,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 4690–4694.
46 Tchou,J., Bodepudi,V., Shibutani,S., Antoshechkin,I., Miller,J., Grollman,A.P. and Johnson,F. (1994) *J. Biol. Chem.*, **269**, 15318–15324.
47 Boiteux,S., Gajewski,E., Laval,J. and Dizdaroglu,M. (1992) *Biochemistry*, **31**, 106–110.
48 Castaing,B., Geiger,A., Seliger,H., Nehls,P., Laval,J., Zelwer,C. and Boiteux,S. (1993) *Nucleic Acids Res.*, **21**, 2899–2905.