Incorporation of Oxidized Guanine Nucleoside 5′-Triphosphates in DNA with DNA Polymerases and Preparation of Single-Lesion Carrying DNA†

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ABSTRACT: We investigated the incorporation of oxidatively modified guanine residues in DNA using three DNA polymerases, *Escherichia coli* Kf exo+, Kf exo−, and *Taq* DNA polymerase. We prepared nucleoside 5′-triphosphates with modified bases ($dN^{ox}TP$) including imidazolone associated with oxazolone (dIzTP/dZTP), dehydroguanidinohydantoin (dOGhTP), and oxaluric acid (dOxaTP). We showed that the single-nucleotide incorporation of these $dN^{ox}TP$ at the 3′-end of a primer DNA strand was possible opposite C or G for dIzTP/dZTP, opposite C for dOGhTP using the Klenow fragment, and opposite C for dOxaTP using *Taq*. The efficiency of these misincorporations was compared to that of the nucleoside 5′-triphosphate modified with the mutagenic guanine lesion 8-oxo-G opposite A or C as well as to that of the natural dNTPs. The reaction was found not competitive. However, the ability of Kf exo− to further copy the whole template DNA strand from the primer carrying one modified residue at the 3′-end proved to be easy and rapid. The two-step polymerization process consisting of the single-nucleotide extension followed by the full extension of a primer afforded a method for the preparation of tailored double-stranded DNA oligonucleotides carrying a single modified base at a precise site on any sequence. This very rapid method allowed the incorporation of unique residues in DNA that were not available before due to their unstable character.

Free radicals, ionizing radiations, chemicals, and cellular metabolism have been implicated in oxidatively mediated DNA damage. Oxidation of DNA results in strand breaks, DNA–protein cross-links, and base damage mainly at guanines (G) (*1–4*). This process is harmful to the cells and is at the origin of mutagenesis, carcinogenesis, or aging. These effects are most probably due to failures in DNA repair or to mistakes made by polymerases in addition to inhibition of DNA-dependent enzymes or proteins.

Until now, the most studied lesion resulting from guanine oxidation is 8-oxo-7,8-dihydroguanine (8-oxo-G). It is known to be mutagenic although a powerful set of repair pathways exists and is now well documented (*5–8*). The excision of this residue from DNA is performed by two DNA glycosylases, Fpg or MutM, in *Escherichia coli* (or hOGG1 in humans) when the lesion is detected opposite C. MutY (or MUTYH in humans) excises the A base when the lesion is detected opposite A. The genotoxicity of 8-oxo-G is not only due to its formation in chromosomic DNA but also due to its misincorporation in DNA opposite A or C by DNA polymerases due the possibility of stable base pairing 8-oxo-G•C and 8-oxoG•A (*9–16*). This process is highlighted by the existence of a third enzyme, MutT (*6, 17, 18*) (or MTH1 in humans) (*19, 20*), which is in charge of the hydrolysis of the modified nucleoside 5′-triphosphate, 8-oxo-dGTP, together with NUDT5 (*21*). For this classical guanine oxidation product it is now clear that the genotoxicity due to its presence in the nucleoside 5′-triphosphate pool cannot be ignored.

Beyond the intensively studied 8-oxo-G, other lesions due to guanine or 8-oxo-G oxidation have emerged in recent years, e.g., imidazolone (*22–24*), guanidinohydantoin, spiroiminodihydantoin (*25–30*), dehydroguanidinohydantoin (*31–35*), or oxaluric acid (*31, 36, 37*). Spiroiminodihydantoin was recently observed in vivo (*38*). The interest of these new oxidation products of G is illustrated by the large number of studies dealing with their mutagenic potential (by misinsertion opposite lesions by polymerases) that has been studied in vitro and in vivo (*39–46*) and their repair by the glycosylases of the base excision repair (BER) pathway (*8, 41, 42, 47–52*). The stability of the corresponding base pair mismatches became also a recent subject of investigation (*53, 54*).

However, only one paper (*40*) reported on the possibility of incorporating Iz into DNA from the oxidation of the dNTP pool. This is why we investigated in the present work the incorporation of nucleoside 5′-triphosphates with oxidatively modified guanine residues (Figure 1). In this work we studied two of them, imidazolone (Iz) and dehydroguanidinohydantoin (OGh), together with their hydrolysis products oxazolone (Z) and oxaluric acid (Oxa). The results show that the tested oxidized guanine lesions were incorporated during in vitro primer extension using Klenow fragment and *Taq* DNA

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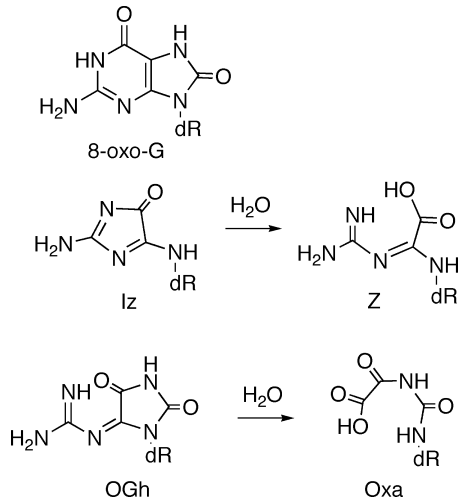


FIGURE 1: Structure of the oxidized guanine residues tested in this work. The 2-deoxyribose unit is noted dR.

polymerase but with a low efficiency. This reaction is thus not expected to affect DNA polymerization in vivo. However, it was used as a tool for the preparation of modified DNA substrates carrying one modified base at a selected position on a sequence for further investigation on the chemical and biological features of these important oxidized DNA base lesions.

MATERIALS AND METHODS

Materials. Potassium monopersulfate, $KHSO_5$ (triple salt $2KHSO_5 \cdot K_2SO_4 \cdot KHSO_4$, Curox) was from Interox. The manganese porphyrin Mn-TMPyP was prepared as previously described (*55*). Oligonucleotides were synthesized by standard solid-phase β-cyanoethyl phosphoramidite chemistry. Purification was performed by electrophoresis on 20% polyacrylamide gels. DNA concentration was determined by UV measurements at 260 nm. The 12-mer oligonucleotide primer was 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP using standard procedures. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham, T4 polynucleotide kinase, *Taq* DNA polymerase, and Klenow fragment exo+ were from Sigma, and dNTP and Klenow fragment exo− were purchased from New England Biolabs.

HPLC Analysis. The reaction media were analyzed by HPLC on an analytical reverse-phase column (nucleosil C_{18}, 10 μm, 250 × 4.6 mm; Interchrom, Montluçon, France), eluted in the isocratic mode with triethylammonium acetate (TEAA)/CH_3CN (99/1) for 5 min followed by a linear gradient (eluents: A = 10 mM TEAA, pH = 6.5, B = CH_3CN, 1–10% B over 25 min; flow rate, 1 mL/min). The products eluting from the column were detected by UV absorbance with a diode array detector. The most usual single wavelength detections were set at 260 or 236 nm.

LC/ESI-MS Analysis. In the case of the LC/ESI-MS analysis, the same column was used with a quaternary pump under the same conditions as described above. The ESI-MS spectrometer was a Perkin-Elmer SCIEX API 365. The ionization voltage was 4.7 kV, and the orifice voltage was 50 V. The analyses were performed in the negative mode.

Preparative Oxidation of dGTP by Mn-TMPyP/KHSO$_5$. 2′-Deoxyguanosine 5′-triphosphate (dGTP) (6 mM) was incubated at 0 °C in TEAA buffer, pH = 6.5 (100 mM), with Mn-TMPyP (300 μM). After preincubation (20 min), $KHSO_5$ (22 mM) was added. The final volume was 100 μL; final concentrations are indicated in parentheses. After 5 min of reaction at 0 °C, the reaction was stopped by the addition of Hepes buffer, pH = 8 (100 mM). The reaction medium was separated by HPLC under the above chromatographic conditions (Figure S1; see Supporting Information). The collected fractions were stored at −80 °C before lyophilization that was performed as soon as possible. Desalting was performed by a second chromatography on the same HPLC column with pure water as eluent. Under these conditions elution occurred within 5 min. The concentrations of oxidized nucleoside 5′-triphosphates in aqueous solutions were determined by UV absorbance measurement based on the following extinction coefficients: dIzTP, ϵ = 5000 and 2300 M^{-1} cm^{-1} at λ = 254 and 234 nm, respectively; dZTP, ϵ = 1200 and 2300 M^{-1} cm^{-1} at λ = 254 and 234 nm, respectively; and dOGhTP, ϵ = 7500 M^{-1} cm^{-1} at λ = 236 nm. Each collected modified nucleoside 5′-triphosphate was analyzed by LC/ESI-MS (Figure S3; see Supporting Information).

Transformation of dOGhTP into dOxaTP. The collected dOGhTP fractions were pooled together (5 OD at 236 nm), subjected to lyophilization, dissolved in 1 mL of H_2O, and heated at 65 °C for 30 min. The hydrolysis of dOGhTP was 90% complete under these conditions. The oxaluric acid derivative, dOxaTP, was purified by HPLC and desalted as described above. The purity of the sample was confirmed by LC/ESI-MS analysis.

Hydrolysis of dIzTP and dOGhTP. The collected modified nucleoside 5′-triphosphates (dIzTP and dOGhTP) were incubated separately at 37 °C during 20 and 30 min, respectively, in the Kf buffer [10 mM Tris-HCl, 5 mM $MgCl_2$, 7.5 mM dithiothreitol (DTT), pH = 7.5]. The hydrolysis was followed by HPLC at 260 nm for dIzTP and 236 nm for dOGhTP.

Preparation of 8-Oxo-dGTP. The triphosphate 8-oxo-dGTP was prepared as described in ref *56* with some modifications. A reaction mixture containing 6 mM dGTP, 30 mM ascorbic acid, and 100 mM H_2O_2 in phosphate buffer (100 mM, pH = 6.5) was incubated at 37 °C for 2 h in the dark. The final volume was 100 μL. The 8-oxo-dGTP was purified by HPLC with an analytical nucleosil C_{18} reverse-phase column as described above (detection at 260 nm). 8-Oxo-dGTP eluted with a retention time 1 min later than dGTP. The pooled fractions of 8-oxo-dGTP were concentrated and desalted on the C_{18} column as described above. The 8-oxo-dGTP was lyophilized and stored at –80 °C. The product was quantified by UV absorbance measurements based on the following extinction coefficients: ϵ = 12300 M^{-1} cm^{-1} at λ = 245 nm and ϵ = 10300 M^{-1} cm^{-1} at λ = 293 nm (*57*). The identity and purity of 8-oxo-dGTP were confirmed by LC/ESI-MS analysis.

Preparation of the Primer/Template Duplex for Primer Extension. The 5′-end-labeled primer was annealed to the template at a final concentration of 250 nM of each strand in 10 μL of optimal buffer for each polymerase. The buffer used contained 50 μg/mL bovine serum albumin (BSA) in addition to the components listed as follows: 10 mM Tris-HCl, 5 mM $MgCl_2$, and 7.5 mM DTT, pH = 7.5, for Kf and 10 mM Tris-HCl, pH = 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.012% gelatin for the *Taq* DNA polymerase.

Single-Nucleotide Primer Extension. Each primer extension reaction contained 2.5 pmol of annealed primer/template in the polymerase buffer as detailed in the preceding paragraph. The 12-mer primer was extended using 0.5 unit of Kf exo− or Kf exo+ and 1 unit of *Taq* DNA polymerase in the presence of 50 μM dIzTP, dOGhTP, or dOxaTP (or 100 μM dOxaTP using *Taq* or Kf exo+) in a final volume of 50 μL. Reactions lasted 10 min at 37 °C for Kf or 1 h for *Taq* and were stopped by adding 2 μL of 200 mM EDTA. Samples were ethanol precipitated, and the dry DNA pellet was dissolved in 3 μL of formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol (marker dyes). The samples were incubated for 2 min at 90 °C, chilled in ice, and run on a 20% denaturing polyacrylamide gel (7 M urea) for 4–5 h at 2200 V. Subsequently, analysis of the radiolabeled bands was achieved by phosphorimaging using Image Quant software (Molecular Dynamics model 400E phosphorimager, image software version 3.3).

C_{50} Determination. The 5′-end-labeled 12-mer was annealed separately to the different 25-mer templates as described above. Duplex (2.5 pmol, 50 nM) was incubated with increasing amounts of normal or oxidized dNTP and Kf exo− (0.5 unit, 7 nM) in 50 μL of the polymerase buffer for 10 min at 37 °C. Reactions were quenched by adding 2 μL of 200 mM EDTA. Samples were ethanol precipitated, and the DNA pellet was dissolved in 3 μL of formamide with marker dyes. The samples were incubated for 2 min at 90 °C, chilled in ice, and run on a 20% denaturing polyacrylamide gel (7 M urea) for 4–5 h at 2200 V. The amount of extended primer was quantitated by phosphorimaging.

Enzymatic Synthesis of an Oligonucleotide Containing a Single Residue of Oxidized Guanine. The 5′-end-labeled 12-mer was annealed to the template 25-mer (25-mer C or 25-mer G) in the polymerase buffer. The reaction (50 μL) was carried out in two steps. In the first step, only the oxidized dNTPs were present for a single-nucleotide extension at the +1 position opposite the G or C nucleotide of the corresponding 25-mer template. The first step of the reaction was performed in the presence of 30 μM dIzTP/dZTP for 5 min at 37 °C with 2 units of Kf exo−. This was followed, in the second step, by the full copy of the 25-mer template by the addition of the four natural dNTPs (100 μM each) for 2 min at 37 °C.

In the case of the preparation of OGh-containing duplexes, the single-nucleoside extension of the 12-mer/25-mer C primer/template was catalyzed by 2 units of Kf exo− in the presence of 200 μM dOGhTP for 20 min at 37 °C. Subsequently, the addition of the four normal dNTPs (100 μM each) in the reaction medium allowed the complete extension of the 25-mer C during 5 min at 37 °C.

Enzymatic Synthesis of a Modified Oligonucleotide with Capping of the Nonmodified Primer. The 5′-end-labeled 12-mer was annealed to the template 25-mer C. The duplex was incubated with dOGhTP at a concentration of 200 μM and in the presence of 2 units of Kf exo− for 20 min at 37 °C. 2′,3′-Dideoxyguanosine 5′-triphosphate (ddGTP) was added in a second step at a concentration of 10 μM and incubated for 10 min at 37 °C. Subsequently, the final extension of the primer was performed by the addition of the four natural dNTPs (1 mM each) during 10 min at 37 °C. The reaction was stopped with 2 μL of 200 mM EDTA. Samples were ethanol precipitated, and the DNA pellet was dissolved in 3 μL of formamide with marker dyes. The samples were incubated for 2 min at 90 °C, chilled in ice, and run on a 20% denaturing polyacrylamide gel (7 M urea) for 4−5 h at 2200 V. The gel was analyzed by phosphorimagery.

MALDI-TOF Mass Analysis. MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA) equipped with an N_2 laser (337 nm). MALDI conditions were as follows: accelerating potential, 24000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns. Spectra were obtained in the negative mode and were not smoothed. The oligonucleotides (100 pmol) were suspended in 10 μL of water and desalted using drop dialysis through a membrane filter, WSWP, 0.025 μm, 13 mm (Millipore), floating on 0.1 M ammonium citrate solution. After 30 min dialysis, 0.5 μL of the samples was withdrawn and mixed with 0.5 μL of the matrix 2,4,6-trihydroxyacetophenone [45 mg of THAP and 4 mg of ammonium citrate in 500 μL of acetonitrile/water (1:1 v/v)], and the mixtures were spotted on the stainless steel MALDI target and left to dry under air before MALDI analysis. When piperidine was applied to the oligonucleotides, the dialysis was followed with a treatment with a few beads of Dowex 50W-X8 resin (ammonium form) before spotting them on the MALDI target.

Single-Nucleotide Extension of the Modified Primer. After the single-nucleotide primer extension step that extended the initial 5′-end-labeled 12-mer primer with dIzTP/dZTP to a 13-mer using 2 units of Kf exo−, only one of the four natural nucleoside 5′-triphosphates (100 μM) was added to the reaction mixture. Reactions lasted 2 min at 37 °C. The reactions were stopped by EDTA, subjected to ethanol precipitation, and analyzed by PAGE, and the bands were quantified as described above.

RESULTS

Preparation of the Modified Nucleoside 5′-Triphosphates. The nucleoside 5′-triphosphates carrying one oxidized guanine lesion ($dN^{ox}TP$) were prepared by oxidation of 2′-deoxyguanosine 5′-triphosphate (dGTP) with a manganese porphyrin, manganese(III) bis(aqua)-*meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (Mn-TMPyP) in the presence of $KHSO_5$. The guanine oxidation products formed with this oxidant have been extensively characterized in previous studies (*24, 32–34, 37*). The oxidation of dGTP was complete within 5 min at 0 °C and afforded a mixture of the products, dIzTP and dOGhTP, under the experimental conditions used. The products were identified in a first step by their in-line UV spectra; dIzTP has two λ_{max} at 254 and 320 nm (*23*), and the λ_{max} of dOGhTP is at 236 nm (*58*) (Figure S1; see Supporting Information). Purification was performed by HPLC, fractions were collected and desalted, and the products were dissolved in H_2O. The collected samples were analyzed by LC/ESI-MS (Figure S2; see Supporting Information). Due to the large difference in the retention times during the chromatography, $dN^{ox}TP$s were pure and did not contain trace amounts of dGTP.

Imidazolone is slowly hydrolyzed to oxazolone (Z in Figure 1), which is likely present as the ring-opened structure shown in Figure 1 rather than the heterocyclic structure originally proposed. The half-life of Iz in H_2O was reported

to be 24 h at 0 °C or 2.5 h at 37 °C (*23*) whereas Z was stable under the same conditions. Due to this hydrolysis process, the collected dIzTP was obtained as a mixture of dIzTP together with its hydrolysis product dZTP (Figure S1b; see Supporting Information). The in-line mass spectra of the collected triphosphate showed two signals at $m/z = 466.95$ amu ($z = 1$) and $m/z = 441.05$ amu ($z = 1$), the former corresponding to dIzTP. The expected m/z ($z = 1$) signal for dZTP at 485.05 (467 + 18) amu was observed with a very low intensity (Figure S3; see Supporting Information). The signal at 441.05 amu corresponds to the decarboxylation of the oxazolone residue that occurred in the mass spectrometer as observed previously (*59, 60*).

The molar ratio of dIzTP:dZTP was determined by the ratio of the HPLC peak areas, taking into account the relative ϵ values for the two products. At $\lambda = 254$ nm the ϵ value of Iz is 5000 M^{-1} cm^{-1} (*24*). The isosbestic point on the UV spectra obtained for the transformation of dIz into dZ (*23*) provides the ϵ value of Iz and Z at $\lambda = 234$ nm ($\epsilon \sim 2300$ M^{-1} cm^{-1}). From the absorbance ratio (254/234) on the in-line UV spectrum of dZTP the ϵ value for Z was estimated to be ~1200 M^{-1} cm^{-1} at $\lambda = 254$ nm. The dIzTP sample contained between 60% and 80% dIzTP and thus 20−40% dZTP depending on the preparations. These compounds were used as a mixture. Despite the storage of the aqueous solution of dIzTP/dZTP at –80 °C, further hydrolysis could not be avoided. The samples containing more than 40% dZTP were discarded.

In contrast, no hydrolysis could be detected during the isolation process of dOGhTP under the experimental conditions used (Figure S2b; see Supporting Information). The LC/ESI-MS analysis of the collected dOGhTP showed a single HPLC peak with $m/z = 510.05$ amu ($z = 1$). This signal was associated with cleavage fragments with $m/z = 466.95$ amu ($z = 1$) (−43 amu) and 414.95 amu ($z = 1$) (−96 amu). The concentration of dOGhTP was measured by UV absorbance using $\lambda = 7500$ M^{-1} cm^{-1} at $\lambda = 236$ nm (measured in this laboratory for the triphosphate derivative). The aqueous solution of dOGhTP was kept at –80 °C. The stability of the product under these storage conditions was checked after 6 months, and the sample contained 50% dOxaTP. Due to the observed lack of stability in both cases, the preparation of fresh samples of modified nucleoside 5′-triphosphate was undertaken regularly.

The OGh lesion is also prone to hydrolysis to an oxaluric acid derivative (Oxa in Figure 1) (*31, 37*). The half-life of OGh in water was reported to be 5 h at 37 °C (*31*) or 8 h at 0 °C (*33*). Further hydrolysis of Oxa into a urea derivative is also possible (*46, 61*); however, the half-life of Oxa was reported to be 40 h at 37 °C under physiological conditions, pH = 7.4; thus, it was considered as stable under the present experimental conditions (*46*).

Hydrolysis of dOGhTP in H_2O at 65 °C for 30 min afforded dOxaTP with good yield (80–90% conversion). The dOxaTP was subsequently further purified by HPLC due to minor amounts of dOGhTP remaining in the reaction medium. The purity of the sample was confirmed by LC/ESI-MS analysis (Figure S2c; see Supporting Information). The observed m/z value corresponding to dOxaTP was at 487.15 amu ($z = 1$). MS fragmentation included fragments at $m/z = 442.15$ and 415.15 amu corresponding to the loss of 44 and 72 amu, respectively. The concentration of dOxaTP was measured by UV absorbance, taking an ϵ value of 750 M^{-1} cm^{-1} at $\lambda = 236$ nm. The ϵ value of dOxaTP was determined by the measurement of the ratio of HPLC peak areas in the hydrolysis reaction transforming pure dOGhTP in dOxaTP in H_2O followed by HPLC at 236 nm.

Considering that these products are hydrolytically unstable, their stability under the experimental conditions of DNA polymerization was also followed. The half-life of the two oxidized residues (dIzTP and dOGhTP) was found to be very short, $t_{1/2} \sim 30$ min.

The last nucleoside 5′-triphosphate prepared in this series was the derivative carrying an 8-oxo-G residue, 8-oxo-dGTP. It was prepared by oxidation of dGTP in the presence of H_2O_2 and a reducing agent (*56*). As for the previous modified nucleoside 5′-triphosphates, this compound was purified and desalted by HPLC. The product was quantified by UV absorbance measurement, considering $\epsilon = 12300$ M^{-1} cm^{-1} at $\lambda = 245$ nm and $\epsilon = 10300$ M^{-1} cm^{-1} at $\lambda = 293$ nm (*57*). The purity of 8-oxo-dGTP was confirmed by LC/ESI-MS analysis. The 8-oxo-dGTP showed a signal at $m/z = 522.05$ amu ($z = 1$). It was stable in aqueous solution at –80 °C for up to 5 months.

In summary, four nucleoside 5′-triphosphates carrying residues resulting from guanine oxidation were prepared: imidazolone/oxazolone (dIzTP/dZTP), dehydroguanidinohydantoin (dOGhTP), oxaluric acid (dOxaTP), and 8-oxo-7,8-dihydroguanine (8-oxo-dGTP).

Single-Nucleotide Primer Extension. Attempts were made to determine the insertion potential of selected guanine oxidation products in the form of nucleoside 5′-triphosphates ($dN^{ox}TP$) into a neosynthesized DNA strand. The first step was to test the single-nucleotide extension of each selected modified dNTP opposite each of the four natural nucleobases. For this purpose, the template DNA sequence at the +1 position (with respect to the 3′-end of the primer) was varied (X = A, C, G, or T). The primer/template oligonucleotides chosen for the assay are shown in Figure 2A. The primer was extended using three DNA polymerases, Kf exo−, Kf exo+, and *Taq* DNA polymerase. The 5′-end-labeled 12-mer primer was annealed separately to each template 25-mer in the appropriate polymerase buffer. Primer extension was catalyzed by each tested polymerase in the presence of a mixture of all four natural dNTPs or the unique $dN^{ox}TP$. The results of the experiment carried out with Kf exo− and *Taq* are shown in panels B and C of Figure 2, respectively. The polymerase reaction lasted 10 min for Kf exo− and 1 h for *Taq* at 37 °C. The production of the full-length DNA was complete in the presence of the four dNTPs, ensuring that the enzymes were active under the experimental conditions used (control not shown). The polymerase Kf exo− was able to extend by one nucleotide the 12-mer primer with dIzTP/dZTP modified triphosphate when the primer was annealed to the 25-mer C or the 25-mer G templates (X = C or G in Figure 2B); i.e., the dIzTP/dZTP was inserted opposite C or G. On the contrary, the primer was not extended opposite A or T. Interestingly, the migration of the two 13-mer oligonucleotides was not identical in the case of dIzTP/dZTP inserted opposite C or G. This may suggest that dIzTP in one case and dZTP in the other may be selected for incorporation. Kino and Sugiyama (*40*) reported that dIzTP was incorporated opposite G using Kf; consequently, dIzTP may be preferentially inserted opposite G whereas

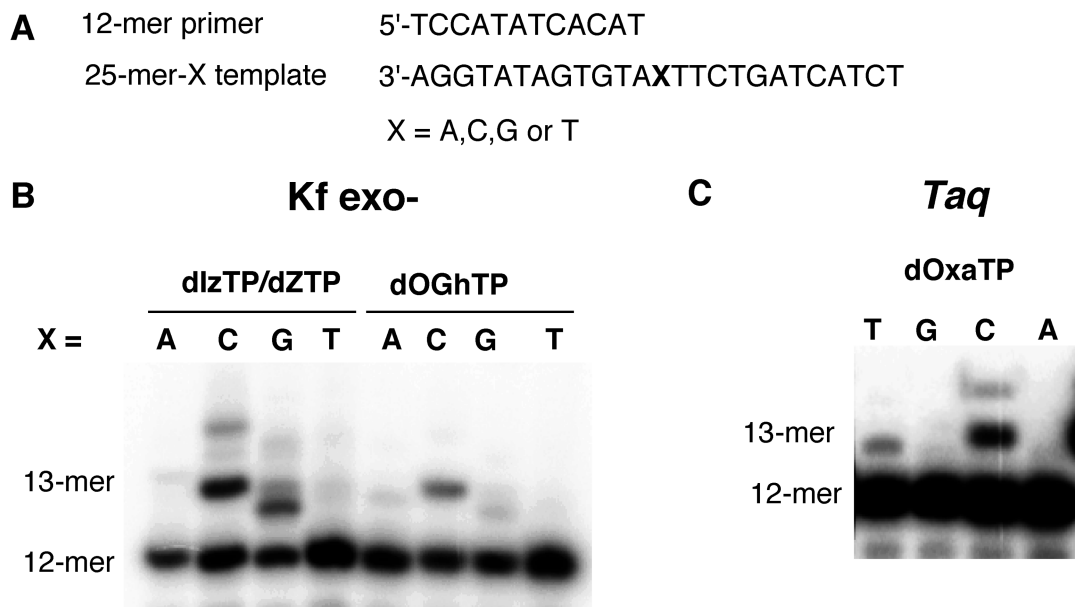


FIGURE 2: Single-nucleotide insertion opposite A, C, G, or T of the modified nucleoside 5′-triphosphates with Kf exo− and *Taq* polymerases: (A) the template/primer duplexes used in this study, (B) PAGE analysis of the incorporation of a single modified dNTP dIzTP/dZTP, dOGhTP using 0.5 unit of Kf exo−, or (C) the incorporation of dOxaTP using 1 unit of *Taq*. The concentration of the added dNTP was 50 μM in (B) and 100 μM in (C). The reactions were performed at 37 °C in the appropriate enzyme buffers (see Materials and Methods) for 10 min in (B) and 1 h in (C).

dZTP may be inserted opposite C. In addition, the Kf exo− was able to insert one nucleoside dOGh opposite C while it was inactive in the presence of the three other 25-mer templates. The dOxaTP derivative was not considered as a substrate for Kf exo−; no reaction could be observed when it was tested (data not shown). Since *Taq* DNA polymerase was described in the literature to be able to insert dA or dG opposite an oxaluric acid residue (present on the template strand) (*42*), this enzyme was also tested in this work. However, under the experimental conditions used, it was not efficient in incorporating dOxaTP in the single-nucleotide insertion assay (Figure 2C). Only the 25-mer C template allowed minor incorporation after 1 h of reaction. The Kf exo+ gave the same results of single-nucleotide insertion of dN^{ox}TP as the Kf exo− (Figure S6; see Supporting Information).

The 13-mer elongated primers were analyzed by MALDI-TOF from larger scale reactions. The incorporation of dIzTP/dZTP opposite G or C and dOGhTP opposite C by Kf exo− was checked. The reaction medium of dIzTP/dZTP opposite G showed two signals at m/z = 3851.7 and 3831.6 amu (z = 1). These two peaks are in accordance with the calculated masses of the monocharged ions of the 13-mer oligonucleotide carrying one Iz residue (m/z = 3853.4 amu) or one Z residue after decarboxylation (3827.4 amu) (Figure S4a; see Supporting Information). The decarboxylation of oxazolone was observed for the nucleoside 5′-triphosphate in the present work and in the literature (*59, 60*). The reaction medium of the incorporation of dIzTP/dZTP opposite C assay showed two peaks at m/z = 3871.8 and 3831.8 amu (z = 1) that were assigned to the monocharged ions of the 13-mer oligonucleotide carrying one Z residue (calculated m/z = 3871.4 amu, z = 1) and one Z residue after decarboxylation (calculated m/z = 3827.4 amu, z = 1) (Figure S4b; see Supporting Information). The 13-mer containing one Iz residue was observed only in the case of single-nucleotide incorporation opposite G. The addition of one nucleoside unit of dOGhTP at the 3′-end of the 12-mer primer annealed to the 25-mer C was confirmed by a signal at m/z = 3896.3 amu (z = 1), which corresponds to the calculated m/z value of 3896.6 for the monocharged species (Figure S4c; see Supporting Information). Moreover, depending on the template used, the 25-mer G or 25-mer C strands were also observed at m/z = 7700.8 or 7660.8 amu (z = 1). The calculated masses of the monocharged species were at m/z = 7701 and 7662 amu for the 25-mer G and 25-mer C, respectively.

Piperidine treatment of the 13-mer oligonucleotides carrying the modified residue at the 3′-end was also performed. The three lesions were alkali labile. The 13-mer was totally degraded, and the expected 3′-phosphate-ending 12-mer was observed in all cases. It was confirmed by MALDI-TOF analysis with a peak at m/z = 3641 amu (z = 1) corresponding to its calculated monocharged ion (3643 amu) (Figure S5; see Supporting Information).

Comparison of the Efficiency of Incorporation of the Modified dN^{ox}TP with Respect to the Normal dNTPs in the Single-Nucleotide Extension Assay Using Kf exo−. The previous experiments have shown that Kf exo− was able to accept mixed dIzTP/dZTP and dOGhTP as nucleoside 5′-triphosphate monomers for single-nucleotide primer extension. Attempts were made to determine the relative efficiency of each dN^{ox}TP to be inserted opposite C or G using this enzyme with respect to normal dNTP and to 8-oxo-dGTP. Since the 8-oxo-G/A mispair is biologically relevant, the insertion of 8-oxo-dGTP opposite A was also included in the series of experiments. We determined and compared, under the same experimental conditions, the concentrations of dNTP and dN^{ox}TP that were necessary to observe 50% of single-nucleotide extension of the 12-mer primer annealed to the different 25-mer templates. These concentrations are referred to as C_{50}. The same 12-mer primer/25-mer template system was used (Figure 2). The Kf exo− (0.5 unit) was

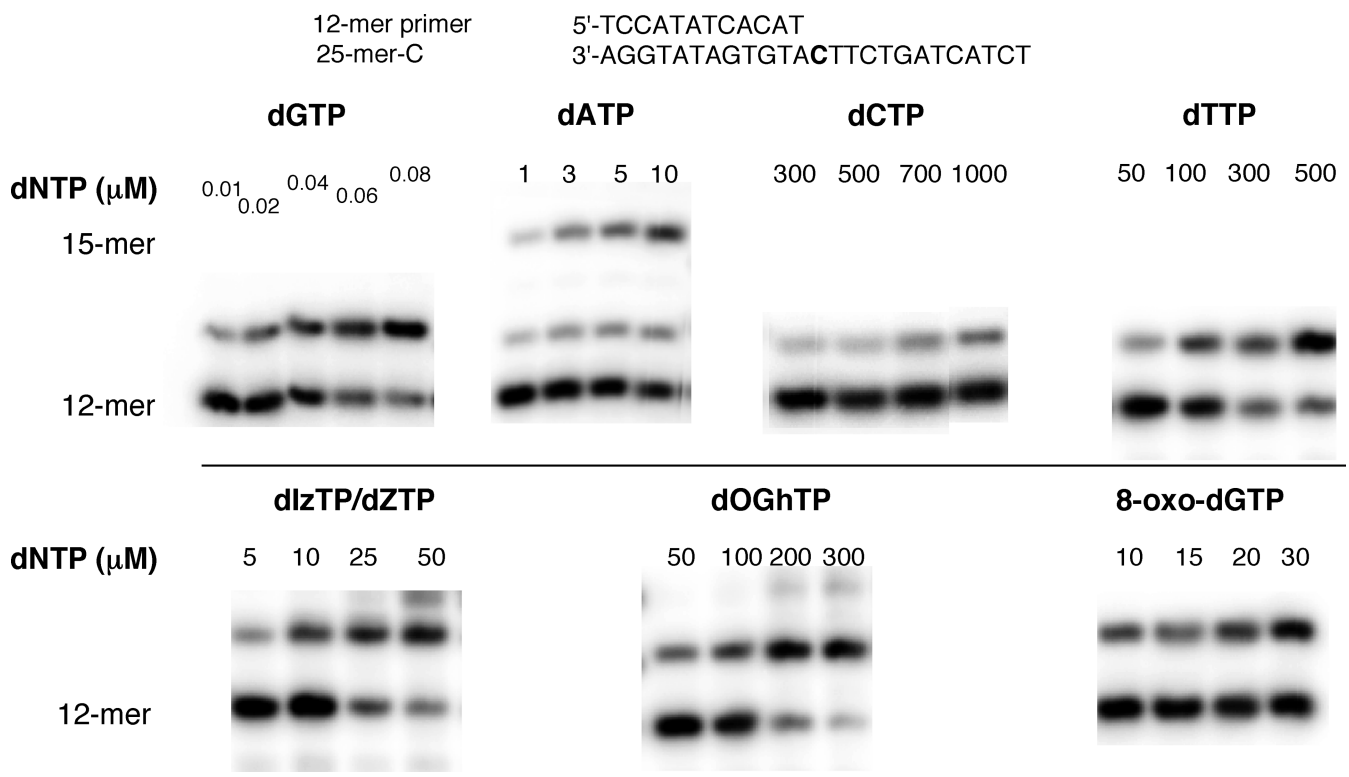


FIGURE 3: Comparison of the single-nucleotide extension efficiencies with the 25-mer C template and using Kf exo− (0.5 unit) and single dNTP. The concentrations of the normal and modified nucleoside 5′-triphosphates were varied in order to determine the concentration that afforded 50% extension after 10 min at 37 °C. The sequence of the primer/template is shown in the upper part of the figure.

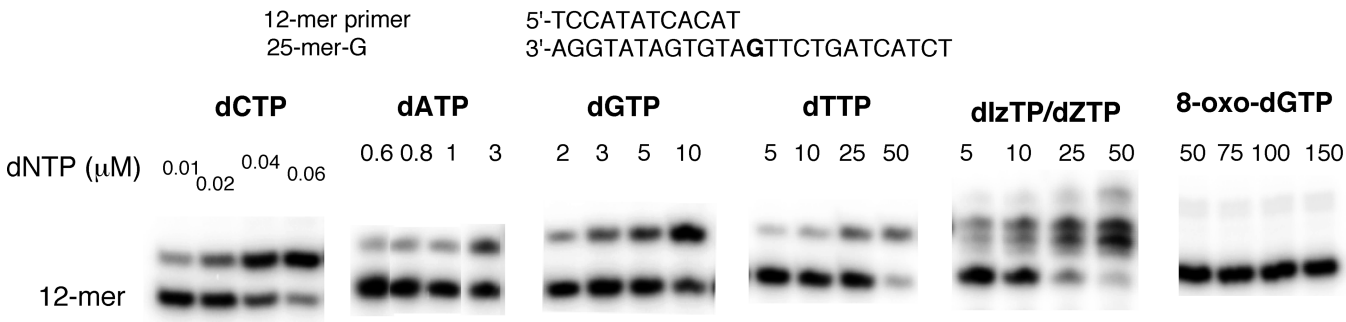


FIGURE 4: Comparison of the single-nucleotide extension efficiencies with the 25-mer G template and using Kf exo− (0.5 unit) and single dNTP. The concentrations of the normal and modified nucleoside 5′-triphosphates were varied in order to determine the concentration that afforded 50% extension after 10 min at 37 °C. The sequence of the primer/template is shown in the upper part of the figure.

Table 1: C_{50} Concentrations (μM) for the Single-Nucleotide Extension Using Kf exo− in the Presence of Normal dNTP or Modified dN^{ox}TP and opposite C or G (or A for 8-Oxo-dGTP)[a]

	C_{50} (μM)						
X	dGTP	dATP	dCTP	dTTP	dIzTP/dZTP	dOGhTP	8-oxo-dGTP
C	0.03 ± 0.01	8 ± 4	ni[b]	150 ± 50	22±12	200 ± 50	25[c]
G	4 ± 0.6	3[c]	0.03 ± 0.01	27 ± 6	17± 6	ni	ni
A	nd[b]	nd	nd	nd	ni	ni	0.8±0.3

[a] These values correspond to the concentration of dNTP necessary to extend 50% of the primer under the experimental conditions used. [b] ni, nonincorporated; nd, not determined. [c] Only two experiments with similar result.

incubated, at 37 °C for 10 min, with 2.5 pmol of labeled 12-mer/25-mer duplex in the presence of increasing concentrations of dN^{ox}TP. The determination of the C_{50} concentrations was done by quantification of the bands related to the 13-mer and 12-mer oligonucleotides after PAGE analysis of the reactions followed by plotting the percentage of 12-mer versus dN^{ox}TP concentration. Figures 3 and 4 show selected examples of electrophoresis gels from which the C_{50} were determined in the case of the 25-mer C and 25-mer G template, respectively. For each different dNTP or dN^{ox}TP at least three series of experiments were performed (unless otherwise specified). The results are summarized in Table 1. The incorporation of dOGhTP opposite C using Kf exo− was 10 times less efficient than the incorporation of dIzTP/dZTP opposite G or C. However, these dN^{ox}TP could be incorporated with an efficiency of the same order of magnitude compared to that of a normal dNTP in a mismatch under the experimental conditions used. In addition, incorporation of 8-oxo-dGTP opposite A was found to be relatively efficient.

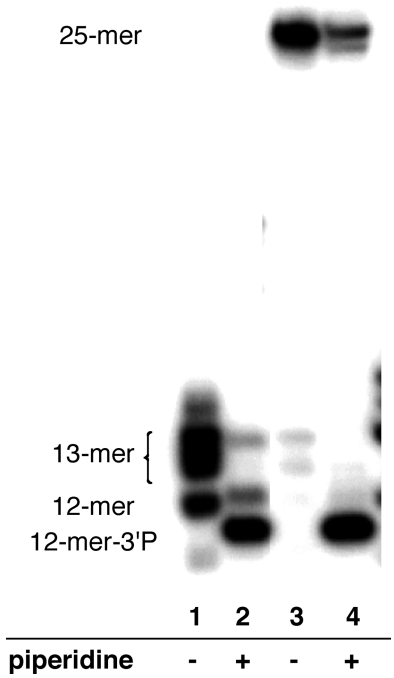


FIGURE 5: Single-nucleoside insertion of the modified nucleoside 5′-triphosphate dIzTP/dZTP opposite G followed by full extension of the 25-mer G template in the presence of the four natural dNTPs using Kf exo−. Lanes: 1, single-nucleotide insertion at the +1 position with the modified nucleoside 5′-triphosphate (30 μM) during 5 min at 37 °C; 2, lane 1 reaction after piperidine treatment; 3, extension of the modified 13-mer in the presence of 100 μM of the four natural dNTPs for 2 min at 37 °C; 4, lane 3 reaction after piperidine treatment.

Full Extension of DNA after Misincorporation of One dN^{ox}TP. The next step was to assess whether the Kf exo− polymerase would be able to carry out the extension of the 3′-end modified 13-mer. Figure 5 shows the data in the case of dIzTP/dZTP inserted opposite G. The Kf exo− (2 units) was incubated in the presence of the single dIzTP/dZTP mixture of triphosphates and incorporated one dN^{ox}TP at the 3′-end of the ^{32}P-labeled 12-mer primer (lane 1). The band corresponding to the 13-mer was large, indicating probably a mixture of incorporation of dIzTP and dZTP (or the transformation of Iz to Z after incorporation). Under the experimental conditions used the yield of the reaction of the 12-mer single-nucleotide extension was ∼80%. Piperidine treatment (lane 2) showed that the modified 13-mer contained an alkali-labile residue at the 3′-end. The initial 12-mer which was not elongated was not sensitive to piperidine treatment whereas the 13-mer oligonucleotides were transformed into the 3′-phosphate-ending 12-mer. The addition of all four natural dNTPs (100 μM) in the reaction medium allowed the polymerase to copy the 25-mer template to the end. The reaction was 95% complete within 5 min at 37 °C (lane 3). The piperidine treatment of this reaction medium showed that the 25-mer elongated from the initial 12-mer, with the incorporation of a C opposite G, was resistant whereas the modified 25-mer was sensitive to piperidine (lane 4). At the end of this two-step polymerization procedure, a 25-mer duplex was synthesized which consisted of a mixture of two species, a double-stranded 25-mer with one modified residue (Iz/Z)/G mispair at the 13th position of the 25-mer sequence (∼80%) and a double-stranded 25-mer with a normal C/G base pair at that position (∼20%). The efficiency of the first step (lane 1) determined the purity of the final fully extended duplex.

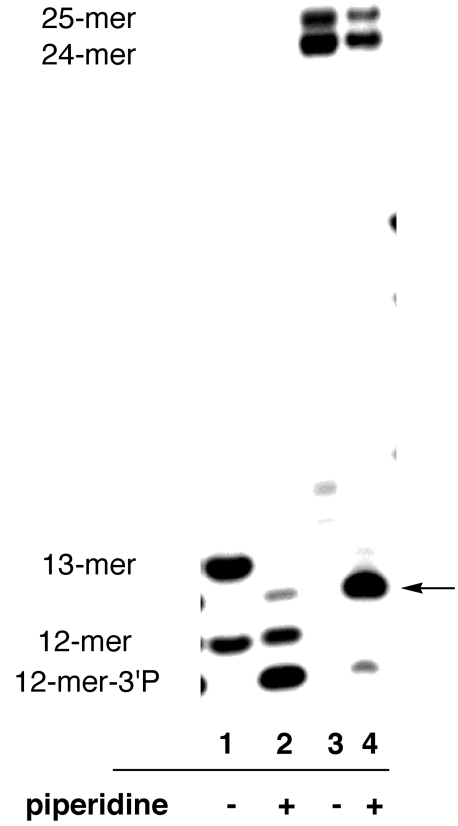


FIGURE 6: Single-nucleoside insertion of the modified nucleoside 5′-triphosphate dOGhTP opposite C followed by full extension of the 25-mer C template in the presence of the four natural dNTPs using Kf exo−. Lanes: 1, single-nucleotide insertion at the +1 position with the modified nucleoside 5′-triphosphate during 20 min at 37 °C; 2, lane 1 after piperidine treatment; 3, extension of the modified 13-mer in the presence of 100 μM of the four natural dNTPs for 5 min at 37 °C; 4, lane 3 after piperidine treatment. The arrow indicates the migration of a nonidentified alkali-labile fragment.

In the same way, the full extension of the modified primer with a OGh moiety as the last residue at the 3′-end of the primer gave similar results (Figure 6). Under the experimental conditions used, the polymerase catalyzed the incorporation of dOGhTP opposite C with ∼80% yield (lane 1). Piperidine treatment gave rise to the 3′-phosphate-ending 12-mer (lane 2) but also to a minor unidentified product that showed an intermediate migration on the electrophoresis gel (marked by an arrow in Figure 6). The full-length double-stranded duplex was obtained in quantitative yield (∼100%) (lane 3) although, in this case, the incorporation of the last nucleoside unit was slowed down. The cleavage of the alkali-labile site in the middle of the sequence, upon piperidine treatment, led to the formation of the same product as observed in lane 2 (arrow in Figure 6), but this time it was the major product of strand cleavage (lane 4). The structural determination of this product was not undertaken. It may correspond to a special chemical reaction of the OGh lesion upon piperidine treatment. The most important step in this study is again the first one (lane 1). The yield of the final modified double-stranded 25-mer was dependent on the yield of the addition of the modified nucleoside 5′-triphosphate and not on the further extension by the polymerase.

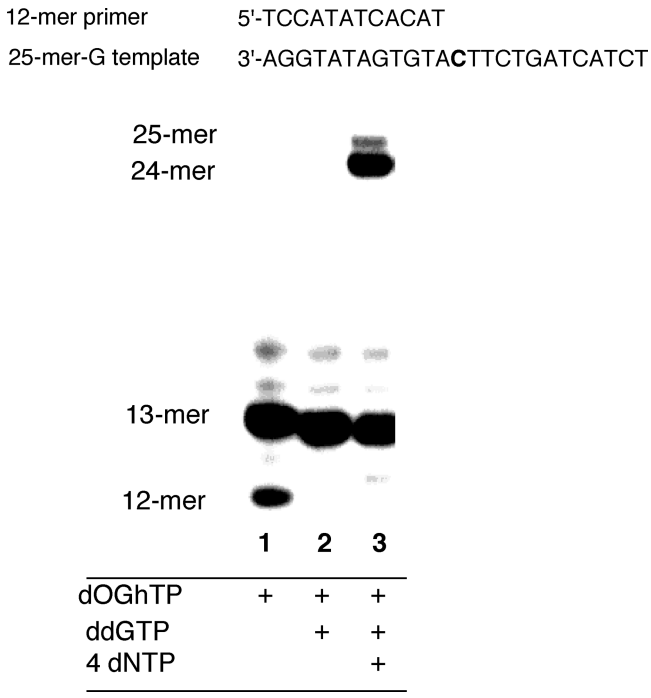
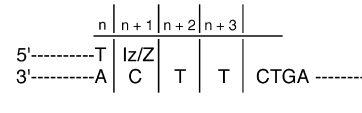


FIGURE 7: Single-nucleoside insertion of the modified nucleoside 5′-triphosphate dOGhTP opposite C followed by capping of the residual primer with ddGTP and, finally, full extension of the 25-mer C template in the presence of the four natural dNTPs using Kf exo−. Lanes: 1, single-nucleotide insertion at the +1 position and the modified nucleoside 5′-triphosphate during 20 min at 37 °C; 2, lane 1 reaction followed by incubation with ddGTP (10 μM) for 10 min at 37 °C; 3, reactions of lane 2 followed by extension of the modified 13-mer in the presence of 1 mM of the four natural dNTPs for 10 min at 37 °C.

In order to prepare a sample containing only the modified double-stranded 25-mer, the remaining 3′-OH-ending 12-mer was capped after the first step so that it was not elongated during the full-length extension step. We introduced a third step in the preparation procedure. After the single-nucleotide extension of the 12-mer opposite C, 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP) was added to the reaction medium to abort the polymerization of the 12-mer by the formation a 13-mer ending with a 2,3-dideoxyribose unit. This experiment is shown in Figure 7. It is illustrated in the case of a single-nucleotide extension with dOGhTP opposite C and can be compared with the data shown in Figure 6. The first step of the preparation is shown in lane 1; ∼20% of the 12-mer is still present after 20 min of reaction with Kf exo− (2 units) at 37 °C. In lane 2, the band corresponding to the 12-mer disappeared, and all of the radiolabeled material was in the form of a 13-mer corresponding to the incorporation of either one dOGhTP or one ddGTP. The capping with ddGTP (10 μM) lasted 10 min at 37 °C. Subsequently, the third step consisted of the addition of an excess of the four normal dNTPs (1 mM) to complete the polymerization (lane 3) to the full-length DNA containing one lesion.

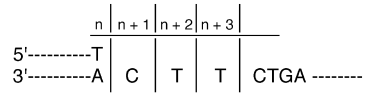
The sequence of the elongated DNA strand after the misincorporation of one $dN^{ox}TP$ is a key point. We performed the following test to check that the Kf exo− was able to copy the template strand in a correct way after the lesion. In the case of dIzTP/dZTP inserted opposite G, the Kf exo− (2 unit) was incubated in the presence of only one of the four dNTPs (100 μM concentration), and the capacity of the polymerase to insert the expected nucleotide at the +2 position was tested. As shown in Figure 8A, two A residues were incorporated opposite T. The pyrimidine nucleoside 5′-triphosphates, TTP and dCTP, did not act as a substrate of the enzyme opposite T at the modified 3′-end. Only dGTP was partly incorporated opposite T immediately after the Iz/Z lesion. This incorporation was less efficient than the correct one, namely, A opposite T. The control experiment (case of the nonmodified primer, Figure 8B) showed that, in the absence of dATP, the Kf exo− inserted also dGTP opposite T. These data allowed us to conclude that, under competition conditions, when the four dNTPs were present in the reaction mixture, full extension of DNA after the misincorporation of one dIzTP/dZTP unit was error free with respect to the template sequence since the first two bases were correct. This conclusion is to be considered as valid only in the particular case of the sequence of the present work. The design of the template sequence with one or two T residues on the template strand immediately after the modified position proved certainly a good choice, considering that in most cases of difficulty the polymerase is known to choose preferentially dATP.

A



Quantification of (n + i) band on gel (%)	n+ 2	n+ 3	n + 4
dATP	-	100	-
TTP	5	-	-
dGTP	60	-	-
dCTP	-	-	-

B



Quantification of (n + i) band on gel (%)	n+ 1	n+ 2	n+ 3	n + 4
dGTP	-	100	-	-

FIGURE 8: (A) Percentage of elongation at each position after the 3′-end of the (n + 1)-modified primer in the presence of 2 units of DNA polymerase Kf exo− and only one natural deoxynucleoside 5′-triphosphate (100 μM). (B) Control: percentage of elongation at each position after the 3′-end of the nonmodified primer in the presence of 2 units of DNA polymerase Kf exo− and dGTP only.

DISCUSSION

In the present work, modified nucleoside 5′-triphosphates ($dN^{ox}TP$) with an oxidized guanine residue were tested as substrates for DNA polymerase, principally Kf exo− (Kf exo+ showed a similar reactivity). The studied guanine oxidation products consisted of a mixture of imidazolone with its hydrolysis product, oxazolone (dIzTP/dZTP), de-

hydroguanidinohydantoin (dOGhTP), and oxaluric acid (dOxaTP). These products are likely to be formed in vivo either from dGTP or from 8-oxo-dGTP oxidation in the nucleotide pool. The biological consequences of the oxidation of the dGTP pool within one cell are well documented only in the case of 8-oxo-dGTP. We addressed the question of the incorporation of these dN^{ox}TPs into the neosynthesized DNA strand during DNA polymerization.

A single-nucleotide insertion assay was set up to determine at which position (i.e., opposite what base on the template) the DNA polymerase would incorporate the dN^{ox}TP. It was shown that, in the presence of the mixture dIzTP/dZTP, Kf was able to incorporate one modified nucleotide on the primer strand opposite C or G. The electrophoretic migration of the new one-nucleotide elongated primers opposite C or G was different, suggesting that one nucleoside 5′-triphosphate from the mix was selected opposite C while the other one was incorporated as a major pathway opposite G. The mass analysis of the reaction opposite G showed the presence of two extended primers modified with an Iz or a Z residue, respectively. It is difficult to conclude in this case whether dIzTP was incorporated opposite G and slowly hydrolyzed to Z after incorporation or whether these two species came from the fact that both dN^{ox}TP were recognized as substrates by the polymerase at that site. On the other hand, the mass analysis of the reaction opposite C showed only the incorporation of dZTP into the primer strand. It may be proposed that the dZTP was incorporated preferentially opposite C while dIzTP (or both dIzTPx + dZTP) would be incorporated opposite G by Kf. This result is in agreement with the previously reported insertion of dIzTP opposite G using Kf (*40*). Kf was also able to perform the insertion of dOGhTP, but only opposite C. In this case the reaction was less efficient since a higher concentration of dOGhTP was necessary. Finally, dOxaTP was not a substrate for Kf opposite any base but proved to be incorporated opposite C using *Taq* polymerase with very low efficiency with respect to the other tests performed using Kf.

The preferential incorporation of the modified bases opposite C does not parallel the usual introduction of dATP or dGTP during the bypass of these lesions by polymerases (*40–42, 44*). This is reminiscent of the reported asymmetry of the efficiency of incorporation, which differs depending on whether the lesion is the templating base or the nucleotide being incorporated (*9, 14*). Our results are in concordance with literature data in the case of the dIzTP/dZTP insertion opposite G but not in the case of dZTP and dOxaTP insertion opposite C. According to modeling studies the Iz·G base pair should be as stable as a GC base pair (*40*). It was previously reported that (i) the Kf polymerase inserted dGTP and (ii) Iz was efficiently bypassed in vivo and 91% mutagenic, producing almost G to C transversion mutations (*44*). On the other hand, previous work described that (i) Kf exo− and *Taq* incorporated A, or A > G, respectively opposite Z and Oxa, whereas pol β was blocked by both lesions (*41, 42*), and (ii) Z and Oxa were readily bypassed in vivo, causing G to T transversion at frequencies higher than 8-oxo-G (*43*). We found that Kf and *Taq* incorporated dZTP and dOxaTP opposite C, respectively. Furthermore, the newly investigated lesion, dOGhTP, was also preferentially inserted opposite C.

The relative efficiency of Kf to incorporate the modified dN^{ox}TP compared to the natural dNTPs was evaluated. The 8-oxo-dGTP was included in the series of experiments. The experimental conditions used for the single-nucleotide extension described in the preceding paragraph proved not to correspond to steady-state conditions. Furthermore, the tested dN^{ox}TPs were shown not to be stable in the polymerization buffer at the selected temperature, 37 °C. Thus, we decided to restrict the comparison to the determination of the concentrations of dNTP or dN^{ox}TP able to produce 50% of single-nucleotide extension upon increasing concentrations of dNTP or dN^{ox}TP and under the experimental conditions set up in the first part of the work. The misincorporation of 8-oxo-dGTP triphosphate by Kf exo− was assessed opposite A and opposite C. The tested dN^{ox}TP showed C_{50} concentrations that, for some of them, were in the same range as those of the natural dNTP tested at a mismatch position. Only two polymerases were tested in the present work, and the question of these dN^{ox}TPs as being recognized as better substrates by other polymerases is open. However, from these results, it does not seem likely that, under oxidative stress, the concentration of dN^{ox}TP could allow their competition with the natural dNTP. The case of 8-oxo-dGTP was different. The C_{50} concentration for the incorporation of 8-oxo-dGTP opposite A was only 1 order of magnitude higher than the C_{50} concentrations of complementary dNTP measured in the present work. This result was not surprising, taking into account the reported data for the incorporation of 8-oxo-dGTP into DNA opposite A or C by polymerases (*9–16*) and the reported activity of a repair enzyme whose function is the sanitization of the nucleoside 5′-triphosphates pool from 8-oxo-dGTP, namely, MutT protein in *E. coli* (*17, 18*) or its human homologue, hMTH1 (*19, 20*), that works in concert with the human Nudix type 5 protein (*21*).

As mentioned above, all of these experiments were carried out with dN^{ox}TPs that were prone to hydrolysis, particularly under the polymerization conditions used. As a first consequence, the imidazolone derivative dIzTP could not be prepared as a pure sample. From the literature the half-life of an aqueous solution of this compound in the form of a nucleoside, dIz, is 2.5 h at 37 °C or 24 h at 20 °C (*23*). We observed that the half-life of dIzTP was 30 min at 37 °C under the polymerase reaction conditions. The incorporation of dIzTP/dZTP was, as a second consequence, always biased by the fact that the exact ratio of dIzTP:dZTP in the polymerase reaction medium was not known with certainty. At the initial time of the reaction the molar ratio of dIzTP:dZTP was at least equal to 60:40. After 10 min of polymerization it reached at least ∼50:50. After dIzTP incorporation into DNA this residue may be more stable due to its reported half-life in a double-stranded DNA as being 20.4 h at 37 °C (*62*). The stability of an Iz residue in double-stranded DNA was not evaluated under the experimental conditions of this work.

In the case of dOGhTP, a pure sample could be isolated. Additionally, a pure sample of its corresponding hydrolysis product, dOxaTP, was prepared. From what is known from the literature, the half-life of a dehydroguanidinohydantoin residue in H_2O at 37 °C is 5 h (*31*). However, this half-life was reduced to 30 min under the polymerase reaction conditions used in this work. The further slow hydrolysis of an oxaluric acid residue into a urea residue was reported to

occur with a half-life of 40 h at 37 °C and physiological conditions (pH = 7.4) (*46*) and was thus considered as not relevant under the experimental conditions of this work. The Kf exo− polymerase was shown to be able to incorporate dOGhTP opposite C but did not recognize dOxaTP as a substrate. Furthermore, the stability of an OGh residue in a double-stranded DNA structure is unknown.

From these preliminary experiments it could be concluded that dIzTP/dZTP and dOGhTP were not good substrates for Kf exo− in the single-nucleotide extension assay. However, this opened the possibility of using this reaction as a tool for the preparation of single-modified double-stranded DNA. Single-lesion-containing oligonucleotides are suitable tools for the evaluation of the DNA repair enzymes or any enzyme that interacts with DNA and which function may be disturbed by the presence of a lesion.

The next step was to check the extension of the primer strand after the incorporation of one $dN^{ox}TP$. The mispairs Iz/Z•C or Iz/Z•G and OGh•C at the 3′-end of the primer may inhibit the polymerization (*63*), but this was not the case. Kf exo− was able to catalyze the formation of the second phosphodiester bond (position +2) and to copy the template strand until the end in all cases. The full extension was easy, rapid, and total for all of the tested mispairs. The two-step polymerization process, i.e., single-nucleotide extension in the presence of a unique $dN^{ox}TP$ and full extension in the presence of the four natural dNTPs, led to the preparation of a double-stranded DNA bearing a single oxidized guanine residue at the +1 position opposite G or C. Introduction of a guanine oxidation product opposite C mimics the case of the formation of a guanine lesion at a GC base pair. The biological relevance of the mispair between a guanine lesion and G is less likely. The position of the lesion was determined by the location of the primer on the template strand that is in turn dependent upon the availability of a C (or a G) base on the template strand opposite which the $dN^{ox}TP$ would be inserted. The efficiency of the first step controlled the percentage of the modified DNA at the end of the polymerization reaction since the elongation of the nonmodified primer was concomitant with the elongation of the modified one. Optimization of the reaction conditions allowed the preparation of single-modified double-stranded DNA in 80–90% yield. The impurity consisted of the double-stranded DNA without modification. The whole process lasted 10−30 min.

The modified oligonucleotides prepared in this work carried one unstable Iz/Z residue opposite G or C or one OGh residue opposite C. These modified oligonucleotides contained a mixture of one given lesion together with its hydrolysis product, namely, Iz/Z and OGh/Oxa. These DNAs may be used as a mixture of DNA substrates for testing one lesion and its related hydrolysis products using a selected protein or enzyme. Alternatively, they can be transformed into samples of pure DNAs carrying a unique defined stable lesion. Incubation of these oligonucleotides under appropriate conditions should afford almost 100% of the oligonucleotide with the corresponding hydrolysis product. Incubation for 20 h at ambient temperature or 30 min at 65 °C in H_2O should give rise to Z opposite C (or G) and Oxa opposite C, respectively. Moreover, a reduction of OGh in the presence of $NaBH_4$ (30 min at ambient temperature) should give rise to an oligonucleotide modified with the stable lesion guanidinohydantoin opposite C.

This method extends the available methods for the preparation of DNA substrates carrying one oxidized guanine residue opposite C. A few examples of site-specific modification of oligonucleotides with guanine lesions were reported previously in the literature. A single Z•C mispair was prepared by the oxidation of an oligonucleotide containing only one G (*41*). This method is thus restricted to AT sequences. Oxidation of an oligonucleotide carrying one 8-oxo-G residue in the sequence, with an appropriate oxidant able to oxidize 8-oxo-G but not G, can lead to an oligonucleotide modified with one oxidized guanine residue at the site of the initial 8-oxo-G residue (*42, 43, 46, 64, 65*). Similar oxidation of an oligonucleotide with one 8-methoxyguanine residue was also described (*62*). Oligonucleotides synthesized with an 8-oxo-G-modified phosphoramidite (*66*) are commercially available but expensive. Furthermore, it must be noted that the selected oxidant and the experimental conditions of the oxidation of DNA must be carefully chosen in order to form only one guanine oxidation product. The purification of long oligonucleotides differing in only one base residue is difficult. Incorporation of a modified phosphoramidite during oligonucleotide synthesis was also described (*67, 68*), but it is only applicable to lesions that are stable during oligonucleotide synthesis. The use of a template-independent DNA polymerase, terminal deoxynucleotidyl transferase, was also tried in the presence of modified 2′-deoxynucleoside 5′-triphosphates. The 3′-termini of the reaction product were suitable substrates for ligation by phage T4 ligase (*69*). The yield of the preparation of the final single-modified DNA is dependent upon the efficiency of the ligase reaction, which is low.

Previous attempts to introduce $dN^{ox}TP$ into DNA using polymerases in vitro were perfomed with 8-oxo-dGTP (*9–16*), formamidopyrimidine nucleoside 5′-triphosphate, FapydGTP (*70*), and dIzTP (*40*). It was found in this work that the studied guanine lesions in the form of nucleoside 5′-triphosphates (dIzTP/dZTP, dOGhTP, and dOxaTP) were not incorporated efficiently by the DNA polymerase used. Nevertheless, this reaction allowed the preparation of single base modified DNA from individual $dN^{ox}TP$.

The advantage of the present method is to afford a very rapid preparation of oligonucleotides carrying one oxidized guanine lesion. The rapidity of the procedure allows the preparation and immediate subsequent test of fragile lesions such as imidazolone or dehydroguanidinohydantoin. Additionally, the lesions resulting from the complete hydrolysis of the primary ones and that show higher stability can also be prepared. This incorporation can be performed in almost any type of sequence context for further more intensive investigation on both chemical and biological features of the new oxidized guanine lesions.

ACKNOWLEDGMENT

We thank Dr. Catherine Claparols for LC/ESI-MS measurements performed at the Service de Spectrométrie de Masse de la FR2599 de l'Université Paul Sabatier-LCC-CNRS de Toulouse.

SUPPORTING INFORMATION AVAILABLE

Six figures showing an example of a reverse-phase HPLC chromatogram of the reaction medium from which the dN^{ox}TPs used in this study were isolated, examples of reverse-phase HPLC chromatograms of the isolated dN^{ox}TPs used in this study, LC/ESI-MS analysis of the purified nucleoside 5′-triphosphates shown in Figure S2, MALDI MS analysis of the 13-mer oligonucleotides after the incorporation of one lesion dIzTP/dZTP opposite G, dIzTP/dZTP opposite C, and dOGhTP opposite C, MALDI MS analysis of the 13-mer oligonucleotides containing one lesion dOGhTP opposite C after piperidine treatment, and single-nucleotide insertion of the dN^{ox}TPs opposite A, C, G, or T with Kf exo+. This material is available free of charge via the Internet at http://pubs.acs.org.

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