DNA Tandem Lesions Containing 8-Oxo-7,8-dihydroguanine and Formamido Residues Arise from Intramolecular Addition of Thymine Peroxyl Radical to Guanine

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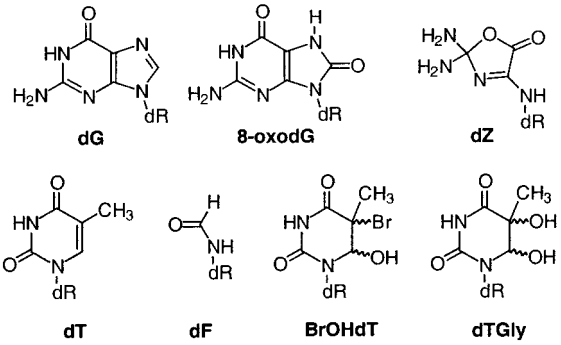
Exposure of aerated aqueous solutions of dinucleoside monophosphates bearing both a pyrimidine base and a guanine residue to ionizing radiation leads to the formation of 8-oxo-7,8-dihydroguanine/formamido tandem lesions (8-oxodG/dF). Recent evidence for the formation of the latter damage within isolated DNA emphasized the possible biological relevance of this class of lesions. Therefore, an extensive mechanistic study of the formation of 8-oxodG/dF was carried out with thymine and guanine containing dinucleoside monophosphates (dGpdT and dTpdG). First, the peroxyl radical-induced degradation of guanine within dGpdT and dTpdG was studied in order to assess the possibility of intramolecular electron transfer between guanine and thymine peroxyl radicals. Then, the formation of a series of tandem lesions involving a formamido residue, thymine glycols, 8-oxodG, and oxazolone was monitored within aerated aqueous solutions of dTpdG and dGpdT exposed to γ-radiation. The absence of formation of tandem lesions other than 8-oxodG/dF in significant yield led to us propose a new mechanism involving addition of the thymine peroxyl radical to the guanine moiety. This received support from ^{18}O labeling experiments.

Introduction

Formation of oxidative base damage within DNA is likely to be associated with deleterious cellular effects including lethality and mutagenesis. Extensive studies involving monomeric model systems such as bases and nucleosides allowed the identification of the primary produced radical intermediates (*1, 2*). In addition, isolation of the final products followed by extensive spectroscopic analyses made possible their structural assignment (*3, 4*). Altogether, oxidative degradation pathways have been proposed for the four DNA bases and nucleosides. The amount of available data on the chemistry of bases within isolated and cellular DNA is growing with the development of new analytical tools but remains to be completed. In the recent years, model systems other than single bases or nucleosides have been used. Isolation of the products generated by oxidation of dinucleoside monophosphates showed that in addition to the well-known monomeric lesions, other damage involving two adjacent bases, the so-called tandem lesions, was produced as the result of a single initial radical event (*5*). A first class of tandem lesions involves addition of the methyl-centered radical of a thymine residue to the adjacent guanine base. Under anaerobic conditions, the final product of the reaction was shown to exhibit a 8-(α-thyminyl)−guanine structure (*6*−*8*). The formation of the latter thymine−guanine adduct has also been observed within isolated DNA (*9*). Addition of the methyl-centered thymine radical to guanine may also occur in the presence of oxygen. Indeed, an adduct arising from the addition of the latter thymine radical to the C4 position of guanine followed by rearrangement of the purine ring after addition of molecular oxygen has been isolated upon type I photosensitization (*10*). The formation of other thymine−guanine lesions involving the addition of the C5 atom of a 6-hydroxy-5,6-dihydro-thymin-5-yl radical to a guanine moiety has also been reported (*11*).

Scheme 1. Chemical Structure of the Modified Bases Studied in This Work



A second class of tandem lesions consists of two adjacent monomeric base damage. A first example has been reported by Box et al. who isolated degradation products bearing both a 8-oxo-7,8-dihydroguanine (8-oxoGua) and a formamido (dF) residue (Scheme 1) as respective lesions of guanine and thymine upon exposure of short oligonucleotides to X-rays in aerated aqueous solution (*5, 12*−*14*). Evidence for the involvement of a single initial event was obtained from kinetic considerations. Recently, we reported that the two isomeric

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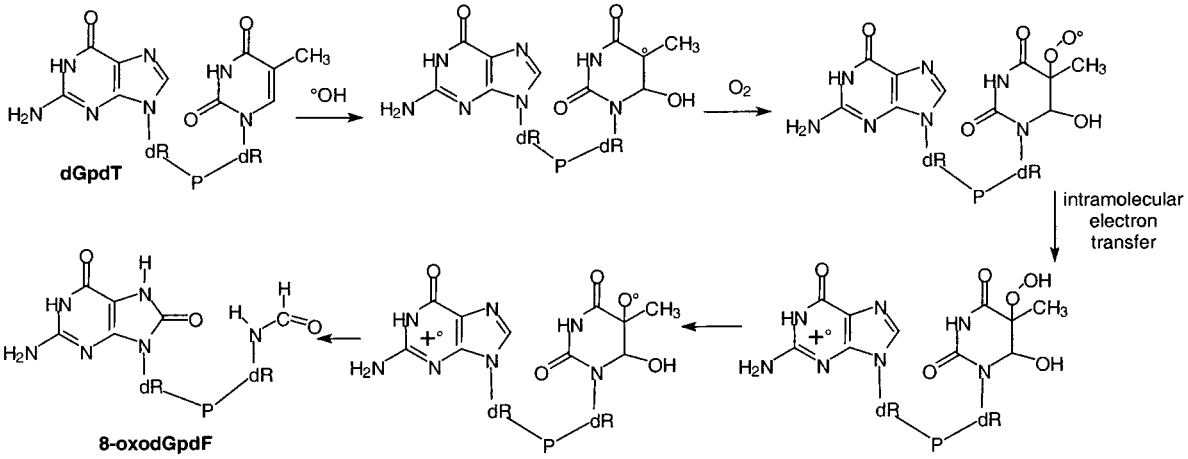
Scheme 2. Previously Proposed Mechanism for the Formation of 8-oxodG/dF Tandem Lesions[a]



[a] The key step is the intramolecular electron transfer from the guanine residue to the thymine peroxyl radical.

8-oxoGua/formamido tandem lesions (8-oxodGpdF and dFp8-oxodG), arising from °OH-mediated oxidation of either guanine−pyrimidine or pyrimidine−guanine sequences, were also produced in isolated calf thymus DNA. For this purpose, an assay involving an enzymatic hydrolysis step followed by high-performance liquid chromatography (HPLC)/tandem mass spectrometry analysis was designed (*15*). The formation of dFp8-oxodG has also been quantified within DNA using a ^{32}P-post labeling method (*16*). However, the reliability and the high throughput of the HPLC-MS/MS assay together with the possibility of detecting both dFp8-oxodG and 8-oxodGpdF allowed a more thorough investigation. Using the latter method, the 8-oxodG/dF lesions were also found to be produced in significant yield upon oxidation of DNA under Fenton reaction conditions (*15*). Similar results were recently obtained in short single-stranded oligonucleotides (*17*). Dose−course study of the formation of the lesions within DNA and comparison of their yield of formation under various oxidative conditions including γ-radiolysis, Fenton reaction, and type I photosensitization (*15*) provided partial support for the mechanism (Scheme 2) initially proposed by Box et al. (*5*). In the initial step, an hydroxyl radical adds to the C5−C6 double bond of thymine. The resulting radical further reacts with oxygen to produce a peroxyl radical. The latter intermediate was then proposed to undergo an intramolecular electron transfer from the guanine moiety, leading to the formation of a thymine hydroperoxide on one hand and the guanine radical cation on the other hand. These transient species would then give rise to the formamido residue and 8-oxoGua, respectively. This mechanism predicts that a wide range of thymine and guanine lesions might be involved in tandem lesions. The latter type of damage may represent complex lesions for the cell, resulting in a poor repair and a potentially high mutagenicity. It thus appears necessary to get further insights into the mechanism of formation of the tandem lesions, especially of 8-oxodG/dF. Dinucleoside monophosphates were chosen as the substrates for this investigation. Indeed, detection of tandem lesions within isolated DNA is hampered by the necessity of quantitative enzymatic release of the damage. In the first part of the present work, thermal production of alkylperoxyl radicals able to react with guanine and photochemical generation of thymine peroxyl radicals within dinucleoside monophosphates were used as model reactions of the currently accepted mechanism. Then, the formation of new tandem lesions was monitored. The low level of the latter degradation products led to the proposition of a new mechanism for the formation of 8-oxodG/dF. This involves the addition of the thymine peroxyl radical to the C8 position of guanine, which was confirmed by $^{18}O_2$ labeling experiments.

Experimental Procedure

Chemicals. 2,2′-Azobis(2-methylpropionamidine) hydrochloride (ABAP) and riboflavin were from Aldrich (Milwaukee, WI). Bromine and potassium phosphate were purchased from Merck (Darmstadt, Germany). Thymidylyl-(3′-5′)-2′-deoxyguanosine (dTpdG) and 2′-deoxyguanosylyl-(3′-5′)-thymidine (dGpdT) were prepared by liquid phase synthesis using a phosphotriester approach. Water was deionized using a Milli-Q system. *N*-(2-Deoxy-β-D-*erythro*-pentofuranosyl)formylamine-(3′-5′)-8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGpdF) and 8-oxo-7,8-dihydro-2′-deoxyguanosylyl-(3′-5′)-*N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formylamine (dFp8-oxodG) were synthesized as previously reported (*15*).

HPLC. Purification of the modified dinucleoside monophosphates was carried out on a Uptisphere ODB (250 mm × 4.6 mm i.d., 5 μm particle size) octadecylsilyl silica gel column connected to a 7125 Rheodyne injector. The mobile phase was delivered by a L6200 Merck-Hitachi intelligent pump in the gradient mode. The initial eluent consisted of a 10 mM triethylammonium acetate (pH 7) aqueous solution. After 5 min, the proportion of acetonitrile was raised to 20% over a period of 40 min. The flow rate was set at 1 mL/min. The detection was provided by a UV spectrophotometer set at 230 nm. HPLC coupled to tandem mass spectrometry involved the use of an Uptisphere ODB column (2 mm × 150 mm i.d., 3 μm particle size) connected to a Shimadzu autosampler. A gradient of acetonitrile in 2 mM ammonium formate was used at a flow rate of 0.2 mL/min. The proportion of organic solvent rose from 0 to 20% within 25 min. The latter composition was maintained for 5 min. The negative ion mass spectrometry detection was provided by an API 3000 apparatus (Perkin-Elmer-Sciex, Thornhill, Canada). For characterization purposes, the spectrometer was used in either the single mass spectrometry (mass range of 200−650) or the product ion scan mode. In the latter

[1] Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; ABAP, 2,2′-azobis(2-methylpropionamidine) hydrochloride; BrOHdT, 5-bromo-6-hydroxy-5,6-dihydrothymidine (thymidine bromohydrin); dF, formamido residue; dG, 2′-deoxyguanosine; dT, thymidine; dTGly, (5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol); dZ, 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone (oxazolone); IT-ES-MS, ion trap electrospray mass spectrometry.

Table 1. Parameters Used for the HPLC-Tandem MS Detection of the Modified Dinucleoside Monophosphates in the MRM Mode

compd	parent ion	daughter ions	retention time (min)
dZpdT	505	321, 195	14.4
dTpdZ	505	321, 263, 195	14.8
8-oxodGpdT	586	321, 166	26.2
dTp8-oxodG	586	460, 362, 195	23.1
dZpdF	424	240, 195	2.5
dFpdZ	424	240, 195	3.0
8-oxodGpdF	505	240, 166	18.9
dFp8-oxodG	505	362, 166	16.5
dZpdTGly	539	396, 212	6.8, 18.7
dTGlypdZ	539	396, 281	6.5, 14.4
8-oxodGpdTGly	620	477, 212, 166	20.3, 21.6, 24.3
dTGlyp8-oxodG	620	477, 362, 166	19.0, 21.7

experiments, the pseudo-molecular ion of the compound of interest was isolated and fragmented. The fragmentation mass spectrum was then recorded (mass range of 100−650). In quantitative studies, the mass spectrometer was used in the multiple reaction monitoring (MRM) mode. The molecular ion of the targeted compound was isolated on the first quadrupole, and specific daughter ions were collected and quantified. The specific monitored transitions and retention times of each of the studied compounds are listed in Table 1.

Synthesis of the Modified Dinucleoside Monophosphates. 1. 5-Bromo-6-hydroxy-5,6-dihydrothymidylyl-(3′-5′)-2′-deoxyguanosine (BrOHdTpdG) and 2′-Deoxyguanosylyl-(3′-5′)-5-bromo-6-hydroxy-5,6-dihydrothymidine (dGpBrOHdT). Bromine (0.8 equiv) suspended in 500 μL of water was added to a solution of dGpdT (5 mL, 1 mg/mL) cooled in an ice bath. After 30 min, air was bubbled to remove traces of bromine. The product of the bromination reaction was purified by HPLC. Peaks corresponding to the main reaction products (retention time (Rt), 31 and 33 min) were observed on the chromatogram in addition to unreacted dinucleoside monophosphate (Rt, 27 min). They were identified by ion trap electrospray mass spectrometry (IT-ES-MS) on an ion trap LCQ apparatus (Thermoquest, San Jose, CA) as the two trans diastereoisomers of dGpBrOHdT. The spectrum exhibited two pseudo-molecular ions (m/z = 666 and 668), corresponding to the presence of the two main stable bromine isotopes (M_w = 79 and 81). The fragmentation mass spectrum exhibited a base peak at m/z = 586, corresponding to the loss of HBr. A weaker signal was observed at m/z = 435 (relative intensity (r.i.): 15%, [M − H − 8-bromoguanine]$^-$). Similar results were obtained with dTpdG. The respective retention times of the unmodified dinucleoside monophosphate and the two diastereoisomers of BrOHdTpdG were 25, 29, and 30 min, respectively.

2. 5,6-Dihydroxy-5,6-dihydrothymidylyl-(3′-5′)-2′-deoxyguanosine (dTGlypdG) and 2′-Deoxyguanosylyl-(3′-5′)-5,6-dihydroxy-5,6-dihydrothymidine (dGpdTGly). Sodium hydrogen carbonate was added to the solution of either dGpBrOHdT or BrOHdTpdG to reach pH 8. The resulting solutions were left overnight at room temperature. The resulting mixture was purified by HPLC. Two main fractions that were found to contain each of the cis diastereoisomers of dGpdTGly were isolated (Rt, 24 and 26 min). They were analyzed by IT-ES-MS. The pseudo-molecular ion was observed at m/z = 604. MS^2 fragmentation spectra exhibited ions at m/z = 586 (r.i.: 20%, [M − H − H_2O]$^-$) and 461 (r.i.: 100%, [M − H− 143]$^-$, loss of O=C=N−CO−C(CH_3)(OH)−CH=O, $C_5NO_4H_5$). Similar results were obtained for the synthesis of dTGlypdG from BrOHdTpdG. Two fractions were isolated (Rt, 23 and 25 min). MS^1 and MS^2 IT-ES-MS spectra were similar to those of dGpdTGly. Differences between the thymine glycol derivatives arising from dGpdT and dTpdG were only observed on MS^3 spectra (fragmentation of the ion at m/z = 461 from the MS^2 spectrum). Indeed, dTGlypdG yielded a single intense fragment at m/z = 346 (r.i.: 100%, [2′-deoxyguanosine 5′-monophosphate]$^-$) while dGpdTGly gave rise to ions at m/z = 346 (r.i.: 55%, [2′-deoxyguanosine 3′-monophosphate]$^-$), 310 (r.i.: 50%, [M − H − $C_5NO_4H_5$ − guanine]$^-$), and 150 (r.i.: 100%, [guanine − H]$^-$).

3. Thymidylyl-(3′-5′)-2,2-diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5-(2*H*)-oxazolone (dTpdZ) and 2,2-Diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5-(2*H*)-oxazolone-(3′-5′)-thymidine (dZpdT). A saturated solution of riboflavin in water (200 μL) was added to aqueous solutions of either dGpdT or dTpdG (1 mg/mL, 2 mL). The resulting solutions were then exposed under air bubbling for 15 min to the UVA radiation delivered by a lamp consisting of two 15 W tubes. The irradiated mixtures were left overnight at 37 °C. The solutions were purified by HPLC, and the main peaks were collected. For both dGpdT and dTpdG, the major product (Rt, 18 and 19 min, respectively) was identified as dZpdT and dTpdZ, respectively. Indeed, the UV spectrum of each of these isolated compounds lacked the absorption bands corresponding to the guanine moiety. In addition, both compounds were found to release guanidine upon hot alkaline treatment. Final proof of structure was obtained from IT-ES-MS analysis. The MS^1 spectrum exhibited the expected pseudo-molecular ion at m/z = 549 (r.i.: 30%), together with a more intense signal at m/z = 505 (r.i.: 100%), corresponding to the loss of CO_2, a characteristic behavior of oxazolone derivatives. For both dTpdZ and dZpdT, fragmentation of the pseudo-molecular ion (m/z = 549) yielded fragments at m/z = 505 (r.i.: 25%) and 477 (r.i.: 100%). In contrast, fragmentation mass spectra of the ion at m/z = 505 were different from one compound to the other. Indeed, dTpdZ yielded fragments at m/z = 379 (r.i.: 35%, [M − H − thymine]$^-$) and 263 (r.i.: 33%, [M − H − thymidine]$^-$), whereas a single daughter ion was observed at m/z = 321 (r.i.: 45%, [thymidine 5′-monophosphate]$^-$) in the fragmentation mass spectrum of the m/z = 505 ion arising from the loss of CO_2 from dZpdT.

4. 5,6-Dihydroxy-5,6-dihydrothymidylyl-(3′-5′)-2,2-diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5-(2*H*)-oxazolone (dTGlypdZ) and 2,2-Diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5-(2*H*)-oxazolone-(3′-5′)-5,6-dihydroxy-5,6-dihydrothymidine (dZpdTGly). Solutions of the isolated cis diastereoisomers of dTGlypdG and dGpdTGly were photosensitized to UVA in the presence of riboflavin as described for dTpdZ and dZpdT. Irradiated solutions were purified by HPLC. In both cases, the major photooxidation product was shown to be either dTGlypdZ or dZpdTGly, on the basis of UV spectroscopic features, guanidine release, and mass spectrometry analyses. For the latter experiments, the pseudo-molecular ion was observed for all compounds at m/z = 583. A fragment, corresponding to the loss of CO_2, was also detected at m/z = 539. Fragmentation of the latter species yielded for all compounds an ion at m/z = 396 (r.i.: 100%, [M − H − $C_5NO_4H_5$]$^-$). An additional signal was observed at m/z = 212 (r.i.: 15%, [5,6-dihydroxy-5,6-dihydrothymidine 5′-monophosphate − $C_5NO_4H_5$]$^-$) and 281 (r.i.: 20%, [dZ 5′-monophosphate − CO_2]$^-$) for the cis diastereoisomers of dZpdTGly and dTGlypdZ, respectively.

Preparation of Calibrated Solutions of 8-Oxo-7,8-dihydro-2′-deoxyguanosylyl-(3′-5′)-thymidine (8-oxodGpdT), Thymidylyl-(3′-5′)-8-oxo-7,8-dihydro-2′-deoxyguanosine (dTp8-oxodG), 8-Oxo-7,8-dihydro-2′-deoxyguanosylyl-(3′-5′)-5,6-dihydroxy-5,6-dihydrothymidine (8-oxodGpdTGly), and 5,6-Dihydroxy-5,6-dihydrothymidylyl-(3′-5′)-8-oxo-7,8-dihydro-2′-deoxyguanosine (dTGlyp8-oxodG). Methylene blue at a final optical density at 550 nm of **1** and $FeSO_4$ (200 μL, 1 mM) were added to solutions of either dTpdG, dGpdT, dTGlypdG, or dGpdTGly (2 mL). The resulting mixtures were placed in a glass tube under a 500 W halogen lamp (Phillips). Air was continuously bubbled during the 10 min irradiation period. An aliquot fraction (5 μL) of each of the irradiated solutions was diluted to 50 μL by addition of water. The modified dinucleoside monophosphates were digested by sequential incubation at pH 5 in the presence of nuclease P1 and phosphodiesterase II and pH 8 following addition of alkaline phosphatase and phosphodiesterase I. The content in 8-oxodG of the resulting samples was determined by HPLC-EC using a previ-

ously described method (*18*). Fragmentation mass spectra of the compounds were recorded by HPLC coupled to tandem mass spectrometry. Three peaks, corresponding to diastereoisomers exhibiting the same mass spectra, were observed on the chromatogram of dTGlyp8-oxodG and 8-oxodGpdTGly. In both cases, the first eluting peak was broad, suggesting the coelution of two diastereoisomers. Only one peak exhibiting the expected mass was observed within oxidized solutions of dTpdG and dGpdT. The pseudo-molecular and the main daughter ions were the following:

dTp8-oxodG ([M − H]$^-$= 586): 460 (r.i.: 70%, [M − H − thymine]$^-$); 362 (r.i.: 20%, [8-oxodG 5′-monophosphate]$^-$); 195 (r.i.: 100%, [2-deoxyribose monophosphate]$^-$); 166 (r.i.: 15%, [8-oxoGua − H]$^-$).

8-oxodGpdT ([M − H]$^-$= 586): 419 (r.i.: 15%, [M − H − 8-oxoGua]$^-$); 321 (r.i.: 15%, [thymidine 5′-monophosphate]$^-$); 166 (r.i.: 100%, [8-oxoGua − H]$^-$).

8-oxodGpdTGly ([M − H]$^-$= 620): 477 (r.i.: 80%, [M − H − $C_5NO_4H_5$]$^-$); 212 (r.i.: 20%, [5,6-dihydroxy-5,6-dihydrothymidine 5′-monophospate − $C_5NO_4H_5$]$^-$); 166 (100%, [8-oxoGua − H]$^-$).

dTGlyp8-oxodG ([M − H]$^-$= 620): 477 (100%, [M − H − $C_5NO_4H_5$]$^-$); 362 (40%, [8-oxodG 5′-monophosphate]$^-$); 166 (10%, [8-oxoGua − H]$^-$).

ABAP Treatment of dTpdG and dGpdT. Two sets of conditions were used for the ABAP-mediated oxidation of dGpdT and dTpdG. In a first series of experiments, ABAP was used at concentrations of 0, 2.5, 5, 10, and 20 mM by addition of a freshly prepared 1 M solution into tubes containing 2 mL of a 1 mM aqueous solution of dinucleoside monophosphate. The samples were left under constant air bubbling for 1 h at 37 °C. Then, air bubbling was stopped and the samples were left at room temperature overnight. ABAP was also used under much milder conditions. The solutions of either dTpdG or dGpdT were placed at 0 °C in an ice bath. Air was bubbled in the solution for 15 min. Then, a 1 mM solution of ABAP was added in order to reach final concentrations of 0, 10, 20, 50, and 100 μM. The samples were left under air bubbling in the ice bath for 1 h. Air bubbling was stopped, and the samples were left at 4 °C for 2 h. They were then left at room temperature overnight. In both cases, samples were analyzed by HPLC-MS/MS.

UV Photolysis of dGpBrOHdT. The two trans diastereoisomers of dGpBrOHdT were prepared from 5 mL of dGpdT (1 mM) and isolated by HPLC as described above. The mixture of both diastereoisomers was solubilized in water and transferred into a 3 mL quartz cell (1 cm optical path, 1 cm width). The sample was then exposed to the UVC light emitted by a 2 × 15 W germicidal lamp (fluence rate: 3.5 kJ m^{-2} min^{-1}) for 2 h under constant stirring. The irradiated solution was then concentrated under vacuum to 100 μL and analyzed by HPLC coupled to mass spectrometry in the product ion scan mode. The selected precursor ion was 505, corresponding to the pseudo-molecular ion of 8-oxodGpdF. A peak was observed at the expected retention time (18.9 min). The corresponding fragmentation mass spectrum was the following: 505 (r.i.: 20%, [M − H]$^-$); 338 (r.i.: 10%, [M − H − 8-oxoGua]$^-$); 240 (r.i.: 15%, [dF 5′-monophosphate]$^-$); 166 (r.i.: 100%, [8-oxoGua − H]$^-$).

γ-Irradiation of dGpdT and dTpdG. Solutions of either dGpdT or dTpdG (1 mM, 1 mL) that contained 10 mM potassium phosphate (pH 7) were exposed to the γ-rays emitted by a ^{60}Co source (dose rate 20 Gy/min) in a glass tube under constant air bubbling. The overall dose applied was either 0, 20, 40, 100, 200, or 400 Gy. Each sample was then analyzed in triplicate for its content in lesions by HPLC-MS/MS. Irradiation of dGpdT was also carried out under ^{18}O-labeled molecular oxygen atmosphere (96.7%, Eurisotop, St. Aubin, France). For this purpose, the sample was degassed by bubbling argon followed by a series of freezing/thawing steps under vacuum. Labeled oxygen was then added into the tube. The latter was sealed and exposed to γ-radiation for 30 min (overall dose, 600 Gy). The content of the irradiated solution was then analyzed by HPLC-MS in the single mass spectrometry, product ion scan, and MRM modes.

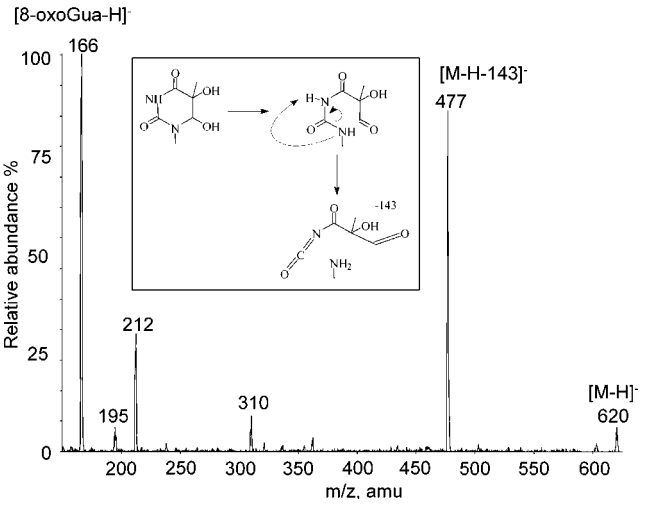


Figure 1. Fragmentation spectrum in the negative mode of 8-oxodGpdTGly obtained by triple quadrupole mass spectrometry analysis. The parent ion was set at m/z = 620.

Results

Synthetic and Analytical Aspects. A series of modified dinucleoside monophosphates were prepared for this work using established synthetic pathways. They were isolated by HPLC and characterized, mostly on the basis of mass spectrometry analyses. Indeed, the latter technique allowed the assignment of the chemical structure of the two base moieties of the modified dinucleoside monophosphates through the study of the fragmentation pattern. As a general trend, a first fragmentation involved the loss of the 5′-end base. In addition, the nucleoside 5′-monophosphate containing the 3′-end base was obtained as a daughter ion. Additional fragmentation pathways were observed for some lesions, as discussed below. A key step in the present work was the synthesis of the trans thymine bromohydrins (5-bromo-6-hydroxy-5,6-dihydrothymine) of dTpdG and dGpdT. The corresponding derivatives have been used as precursors in the synthesis of thymidine and 2′-deoxycytidine hydroperoxides (*19*, *20*). In the present work, reaction conditions were optimized in order to prevent the addition of bromine to the C8 position of the guanine residue of the starting dinucleoside monophosphates. This was achieved by lowering the reaction temperature and limiting the conversion of thymine to ca. 60%. For each of the dinucleoside monophosphates, the two trans diastereoisomers were isolated and unambiguously characterized by mass spectrometry. The hydrolytic conversion into the corresponding cis thymine glycol derivatives under slightly alkaline conditions (*21*) was quantitative. The resulting modified dinucleoside monophosphates (dTGlypdG and dGpdTGly) exhibited similar fragmentation spectra, with a major daughter ion at [M − H − 143]$^-$, which could not be accounted for by the loss of the 5′-end base. The favored loss of 143 amu from dTGlypdG and dGpdTGly, which was also observed for dTGlypdZ, dTGlyp8-oxodG, dZpdTGly, and 8-oxodGpdTGly, can be rationalized in terms of a rearrangement of the ring-opened form of the thymine glycol moiety (Figure 1).

Another series of modified dinucleoside monophosphates involved degradation of guanine into its oxazolone derivative. All corresponding compounds, dTpdZ, dZpdT, dTGlypdZ, and dZpdTGly, were found to release guanidine upon alkaline treatment. In addition, these mol-

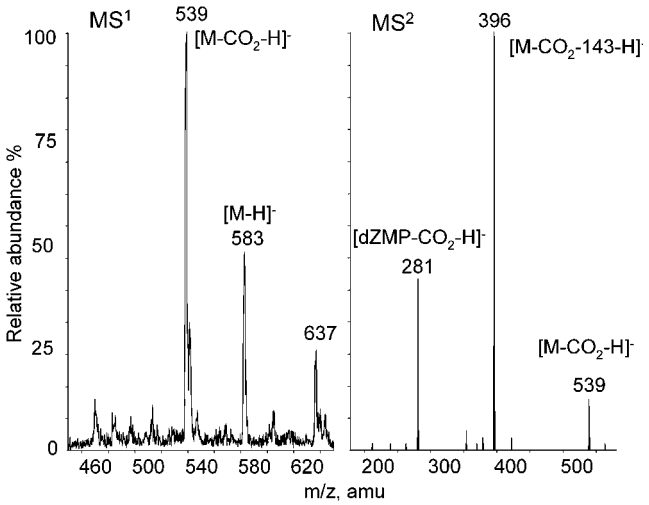


Figure 2. MS^1 and MS^2 mass spectra of dTGlypdZ obtained by IT-MS analysis. The fragmentation spectrum was recorded with the parent ion set at $m/z = 539$ ($[M - CO_2 - H]^-$).

ecules lost CO_2 upon ionization in the mass spectrometer (Figure 2). These two features have been already observed upon characterization of the 2′-deoxyribonucleoside of oxazolone (*22*, *23*). It should be added that the guanidine release was used to calibrate the solutions, using a previously described HPLC/fluorescence assay (*24*). Finally, solutions containing 8-oxodG derivatives were prepared. The synthesis in large amount was not attempted from these dinucleoside monophosphates because 8-oxodG is a minor oxidation product within small model systems. Therefore, dTpdG, dGpdT, dTGlypdG, and dGpdTGly were exposed to singlet oxygen, a specific oxidant of guanine residues, in order to keep unmodified the adjacent pyrimidine moiety. Ferrous ions were added to the solution in order to increase the yield of 8-oxodG derivatives by reduction of the transient guanine endoperoxide (*25*). The isolation of pure dinucleoside monophosphates bearing both an oxazolone and a formamido residue was not possible because of the lack of hydrophobicity of the compounds, which are likely to be almost not retained on reverse phase HPLC columns. However, γ-irradiated solutions of dGpdT and dTpdG contained a degradation product exhibiting the expected molecular weight and undergoing the loss of CO_2 upon ionization. The fragmentation mass spectra of the latter ion were in agreement with the presence of a formamido moiety since the main observed fragment corresponded to the phosphorylated derivative of the latter nucleoside. Therefore, these lesions were tentatively identified as dZpdF and dFpdZ.

The modified dinucleoside monophosphates prepared as reported above were then used to optimize the response of the mass spectrometer operating in the sensitive and specific MRM mode. The latter approach is receiving major attention in the field of DNA damage with a growing number of applications. Among them, assays involving the detection of modified dinucleoside monophosphates have been developed for tandem lesions (*15*) and UV-induced pyrimidine dimeric photoproducts (*26*, *27*). In the MRM mode, the pseudo-molecular ion of the targeted molecule ($[M - H]^-$) is isolated in the first quadrupole of the apparatus. It is then fragmented, and specific fragments are quantified in the last quadrupole. The ions used in the quantitative analyses of radiation-induced degradation products of dGpdT and dTpdG are

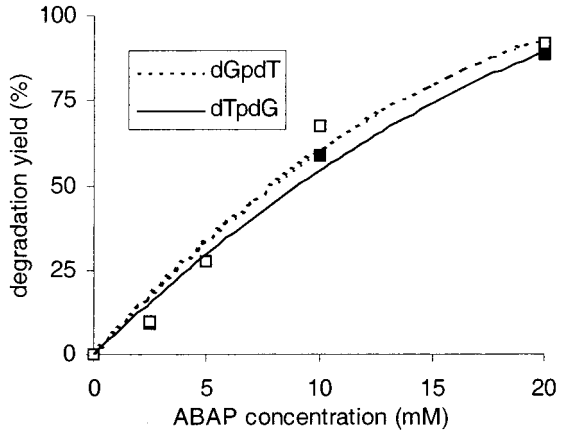


Figure 3. Degradation of dGpdT and dTpdG upon incubation with large concentrations of ABAP in aerated aqueous solution at 37 °C. The amount of dinucleoside monophosphate was inferred from HPLC-UV analysis. Dinucleoside monophosphates were solubilized at 1 mM concentration in 10 mM, pH 7, phosphate buffer. The reported values represent the average of two independent determinations.

listed in Table 1. It should be noted that the precursor ion of the transition used for dZ-containing lesions corresponded to $[M - CO_2 - H]^-$.

Peroxyl Radical-Induced Oxidation of dGpdT and dTpdG. To further investigate the reaction of a 6(5)-hydroxy-5(6)-peroxy-5,6-dihydrothymine moiety with an adjacent guanine base, as previously proposed in the mechanism of formation of 8-oxodG/dF tandem lesions, peroxyl radicals produced by thermal decomposition of ABAP were used as model systems together with dTpdG and dGpdT as substrates. The former chemical generates 2-methyl-3-yl-priopionamidine radicals, which further react with oxygen to yield the related peroxyl radicals. Each molecule of ABAP gives rise to two identical radicals, with release of nitrogen. Therefore, in contrast to other systems, only one class of radical species is involved. In addition, ABAP is a water soluble radical precursor, a feature required in order to react with dinucleoside monophosphates. Peroxyl radicals were found to be very efficient damaging species of the guanine moieties of dGpdT and dTpdG. Indeed, when used at high concentrations (up to 20 mM), an almost complete degradation of both dinucleoside monophosphates was observed (Figure 3). The major product obtained under these conditions was identified as a modified dinucleoside monophosphate bearing an oxazolone and a thymine moiety. The rate of formation of the latter lesion and of the corresponding 8-oxodG analogue was determined within solutions of dGpdT and dTpdG treated by ABAP under milder conditions (ABAP concentration lower than 100 μM). The level of 8-oxodG was found to slightly decrease while that of oxazolone increased in a quadratic relationship with respect to the ABAP concentration (Figure 4). These features are very similar to recent observations made upon type I photosensitization of 2′-deoxyguanosine (Ravanat and Cadet, manuscript in preparation). Altogether, the present results show that peroxyl radicals may undergo electron abstraction from the guanine moiety of dinucleoside monophosphates.

Formation of dFp8-oxodG and 8-oxodGpdF upon Photolysis of the Bromohydrins of dTpdG and dGpdT. Previous results have led to the conclusion that formation of the 8-oxodG/d*F* tandem lesions upon exposure to γ-radiation arose from the initial formation of the

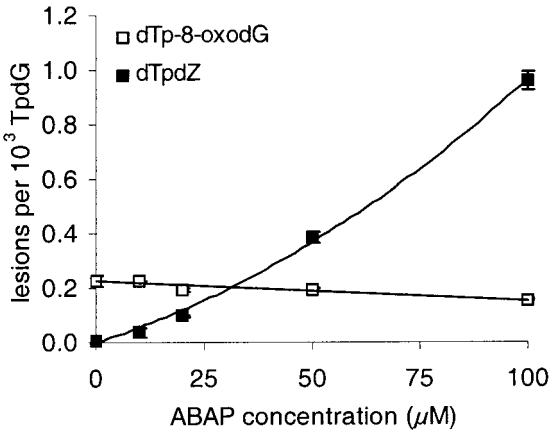


Figure 4. Formation of guanine oxidation products of dTpdG upon incubation at 0 °C with a low amount of ABAP. Dinucleoside monophosphates were solubilized at 1 mM concentration in 10 mM, pH 7, phosphate buffer. The reported values, expressed as number of lesions per 10^3 molecules of dinucleoside monophosphate, represent the average ± standard deviation of three independent determinations.

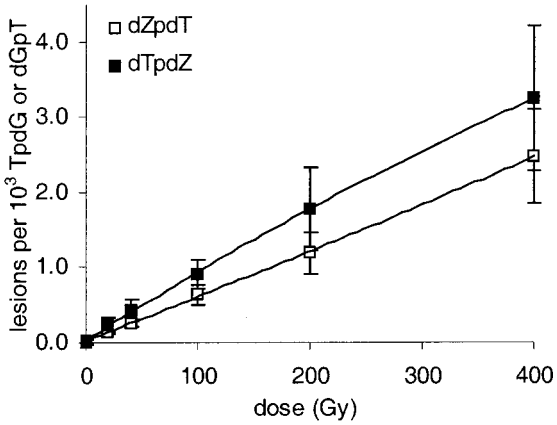


Figure 5. Formation of oxazolone-containing single lesions upon exposure of dGpdT and dTpdG to increasing doses of γ-radiation. Dinucleoside monophosphates were solubilized at 1 mM concentration in 10 mM, pH 7, phosphate buffer. The reported values, expressed as number of lesions per 10^3 molecules of dinucleoside monophosphate, represent the average ± standard deviation of three independent determinations.

Table 2. Radiolytic Yield of Formation of Oxidation Products of dGpdT and dTpdG upon Exposure to γ-Radiation in Aerated Aqueous Solution[a]

dinucleoside monophosphate	dGpdT	dTpdG
8-oxodG/dT	3.25 ± 0.32	1.38 ± 0.07
dZ/dT	5.90 ± 0.12	8.73 ± 0.24
8-oxodG/dF	8.17 ± 0.87	0.63 ± 0.14
8-oxodG/dTGly	0.10 ± 0.01	0.09 ± 0.01

[a] Results are expressed as nmol/J.

5-hydroxy-6-yl and/or the 6-hydroxy-5-yl radicals of thymine (and cytosine) (*15*). To confirm this hypothesis, the bromohydrins of dGpdT were used as precursors of the latter transient species. Indeed, UVC irradiation of dGpBrOHdT leads to the homolytic cleavage of the C−Br bond, yielding the oxidizing pyrimidine radical that is also obtained by addition of °OH to the C6 position of the thymine moiety. Analysis of the resulting sample by HPLC-MS/MS unambiguously showed the presence of 8-oxodGpdF among the photolysis products (yield, 5%). Indeed, the fragmentation mass spectrum of the compound eluting at the expected retention time was identical to that of synthetically prepared 8-oxodGpdF. The formation of other products was also observed. However, they were found to be only modified on the thymine moiety, as shown by the loss of unmodified guanine upon fragmentation during HPLC-MS/MS analysis.

Formation of Degraded Guanine Single Lesions upon Exposure of dTpdG and dGpdT to γ-Radiation. The formation of a wide set of modified dinucleoside monophosphates was monitored within aerated aqueous solutions of dGpdT and dTpdG exposed to γ-radiation. First, the formation of oxidation products exhibiting either a 8-oxodG or an oxazolone moiety on one hand and thymine on the other hand was quantified. In both cases, oxazolone was found to be the main hydroxyl radical-induced product when compared to 8-oxodG (Table 2). The ratio between the yields of the two former lesions was 1.8 ± 0.4 and 6.3 ± 0.8 for dGpdT and dTpdG, respectively. A similar trend has been observed upon exposure of aerated aqueous solution of 2′-deoxyguanosine to γ-radiation (*28*). However, a ratio of 62 was then found between the yield of oxazolone (measured as guanidine releasing compound) and the yield of 8-oxodG. The low yield of 8-oxodG upon γ-irradiation of dG was proposed to be accounted for by a reaction between the oxidizing and the reducing radicals of guanine (*28*). Ongoing studies indicate that secondary oxidation of 8-oxodGuo by the guanine oxidizing radical is also involved (Ravanat and Cadet, manuscript in preparation). The present observation of the formation of 8-oxodG-containing dinucleoside monophosphates in significant yield suggests that these reactions are much less efficient with dGpdT and dTpdG. The high yield of 8-oxodG/dF tandem lesions also indicates that the reaction between the 8-oxodG moieties and the guanine oxidizing radical is much less efficient that with isolated nucleosides. These observations may be accounted for by the electrostatic repulsion of the negative charge of phosphodiester linkage of dinucleoside monophosphates. Altogether, both oxazolone and 8-oxodG were found to be primary °OH radical-induced degradation products of guanine moieties in dinucleoside monophosphates, as confirmed by the linearity of their dose−course formation (Figure 5 for dZpdT and dTpdZ).

Formation of 8-oxodG- and Oxazolone-Containing Tandem Lesions upon Exposure of dTpdG and dGpdT to γ-Radiation. The formation of 8-oxodGpdF and dFp8-oxodG was first monitored in aerated aqueous solutions of dinucleoside monophosphates exposed to γ-radiation. They were both found to be produced in a linear relationship with respect to the dose (Figure 6), in agreement with previous data (*5*). A drastic sequence effect was observed since the radiolytic yield of 8-oxodGpdF (G = 8.2 ± 0.9 nmol J^{-1}) was higher than that of dFp8-oxodG (G = 0.6 ± 0.1 nmol J^{-1}). The predominant formation of 8-oxodG/dF tandem lesion at GT with respect to TG sequence has already been observed within γ-irradiated isolated DNA (*15*). Interestingly, a similar sequence effect has been reported for oligonucleotides and DNA concerning the hydrogen abstraction from a 2-deoxyribose moiety by a vicinal uracil-5-yl radical produced by photochemical decomposition of 5-bromouracil (*29*, *30*). Similar conformational effects are thus likely to be involved in both reactions, in agreement with the production of a thymine radical located at the C5/C6 positions as the initial event in the formation of 8-oxodGpF. The tandem lesions exhibiting both formamido and oxazolone

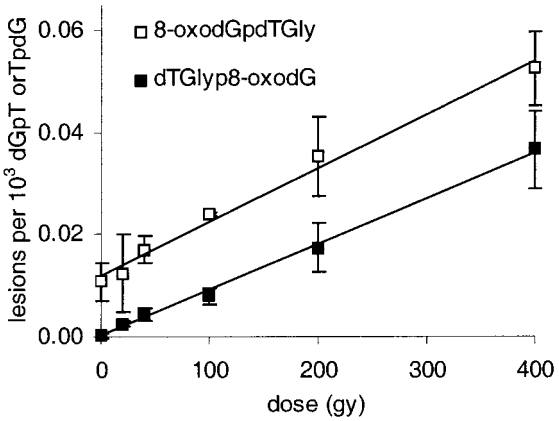


Figure 6. Formation of dTGly/8-oxodG tandem lesions upon exposure of dTpdG and dGpdT to increasing doses of γ-radiation. Dinucleoside monophosphates were solubilized at 1 mM concentration in 10 mM, pH 7, phosphate buffer. The reported values, expressed as number of lesions per 10^3 molecules of dinucleoside monophosphate, represent the average ± standard deviation of three independent determinations.

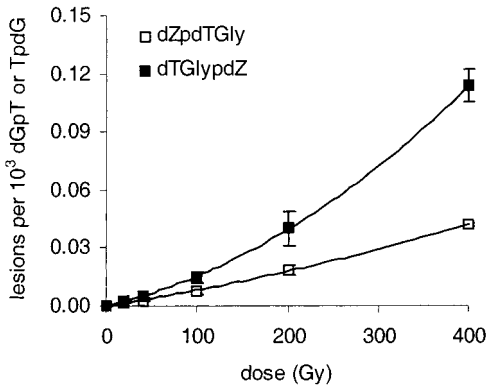


Figure 7. Formation of dTGly/dZ tandem lesions upon exposure of dTpdG and dGpdT to increasing doses of γ-radiation. Dinucleoside monophosphates were at 1 mM concentration and solubilized in 10 mM, pH 7, phosphate buffer. The reported values, expressed as number of lesions per 10^3 molecules of dinucleoside monophosphate, represent the average ± standard deviation of three independent determinations.

moieties were found to be produced upon exposure of aqueous solutions of dinucleoside monophosphates to γ-radiation. However, the dose−course formation of dZpdF and dFpdZ within irradiated solutions of dGpdT and dTpdG, respectively, was found to be quadratic.

The formation of tandem lesions containing both a 8-oxodG and a thymine glycol residue was then quantified (Figure 6). It should be pointed out that this class of damage consists of a mixture of diastereoisomers, well-resolved by HPLC, due to the presence of two asymmetric carbon atoms on the thymine glycol moiety. Both dTGlyp8-oxodG and 8-oxodGpdTGly were detected in γ-irradiated samples of dTpdG and dGpdT, respectively. However, their yield of formation was low, when compared to dFp8-oxodG and 8-oxodGpdF. Tandem lesions bearing both an oxazolone and a thymine glycol moiety were also quantified. They were easily detected, even though produced in significantly lower yield than the corresponding thymine-containing modified dinucleoside monophosphates. Their dose−course formation was found to be quadratic (Figure 7), as already observed for dFpdZ and dZpdF, strongly suggesting that these lesions resulted from two separate radical events.

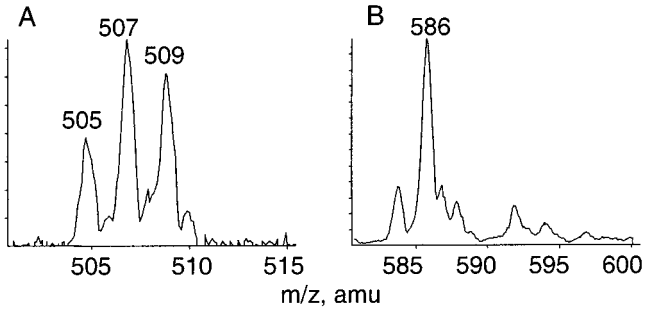


Figure 8. Mass spectrum of 8-oxodGpdF and 8-oxodGpdT obtained upon γ-irradiation of dGpdT in the presence of ^{18}O-labeled molecular oxygen. The irradiated solution was injected onto the HPLC-MS system with the spectrometer operating in the MS^1 mode. The chromatogram was simultaneously recorded between two mass ranges, namely, 500−515 and 580−600 amu. Identification of the products was inferred from both their mass spectra and retention times.

Labeling of 8-oxodGpdF and 8-oxodGpdT upon γ-Irradiation of dGpdT in $^{18}O_2$-Saturated Aqueous Solution. To gain further insight into the mechanism of formation of 8-oxodGpdF, γ-irradiation of dGpdT was then carried out in aqueous solution saturated with $^{18}O_2$. The irradiated samples were analyzed by HPLC-MS/MS for their content in 8-oxodG-containing dinucleoside monophosphates. First, single mass spectrometry detection allowed to determine the level of ^{18}O incorporation into 8-oxodGpdF and 8-oxodGpdT. The pseudo-molecular ion ($[M - H]^-$) of the former molecule was found at m/z = 505, 507, and 509 in a 20/43/37 ratio (Figure 8). This can be accounted for by the involvement of two oxygen atoms in the mechanism of formation of 8-oxodGpdF. In contrast, no significant labeling of 8-oxodGpdT was observed. The lack of oxygen incorporation into single 8-oxodG lesions is in agreement with the fact that the oxygen atom at the C8 position arises from the initial addition of an hydroxyl radical, the latter produced from water. To identify the labeled position in 8-oxodGpdF, the mass spectrometer was then used in the product ion scan mode, which allowed the recording of the fragmentation mass spectra of pseudo-molecular ions at m/z = 505 ($[M - H]^-$), 507 ($[M - H + 2]^-$), and 509 ($[M - H + 4]^-$). Interestingly, fragmentation of 8-oxodGpdF yields ions containing either the formamido (m/z = 240, [dF 5′-monophosphate]$^-$) or the 8-oxoGua moiety (m/z = 166, [8-oxoGua − H]$^-$). Therefore, it was shown that 8-oxodGpdF molecules containing only one ^{18}O atom were all labeled on their 8-oxodG residue. Lesions containing two ^{18}O atoms were labeled on both the 8-oxodG and the dF moieties. These results were confirmed by more quantitative MRM analyses (Figure 9). In control experiments, similar HPLC-MS/MS measurements were performed on dGpdT samples exposed to γ-radiation in $^{16}O_2$-saturated solution. It was found that the abundance of the M + 2 and M + 4 pseudo-molecular ions for both 8-oxodGpdT and 8-oxodGpdF was negligible.

Discussion

Identification of the radiation-induced products of DNA components has been carried out for decades on simple monomeric compounds such as nucleosides and bases. Recently, more relevant model systems such as dinucleoside monophosphates have been used. These allowed the discovery, by Box and co-workers (*5*, *12*−*14*), of oxidative pathways leading to the degradation of two adjacent

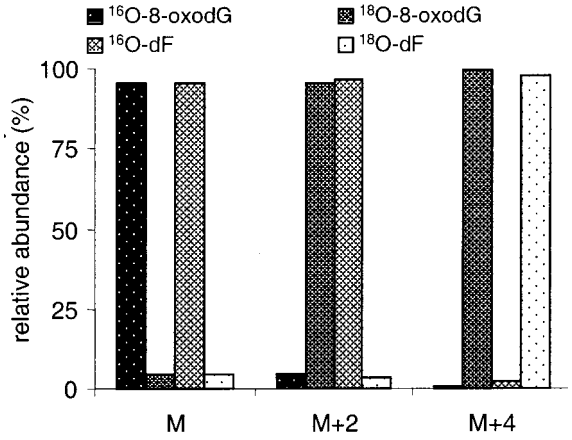


Figure 9. Repartition of the ^{16}O and ^{18}O atoms in 8-oxodGpdF produced upon irradiation of dGpdT in the presence of ^{18}O. The reported values represent the proportion (expressed as %) of each of the ^{16}O- and ^{18}O-labeled (M + 2) 8-oxoGua and dF moieties in the 8-oxodGpdF molecules exhibiting pseudo-molecular ions at m/z = 505 (M), 507 (M + 2), and 509 (M + 4).

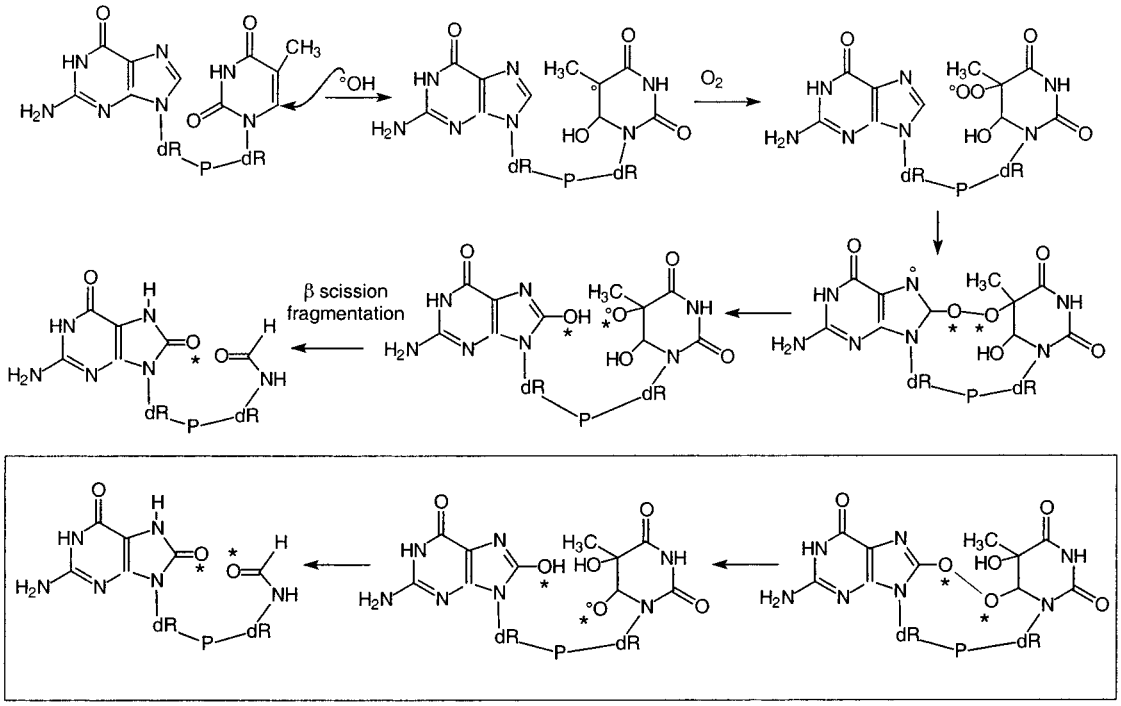
bases after one initial radical event. Indeed, tandem lesions exhibiting both a 8-oxodG and a formamido residue arising from guanine on one hand and thymine or cytosine on the other hand have been identified within short oligonucleotides and DNA exposed to low LET radiation in aerated aqueous solutions. Until now, the accepted mechanism (*5*, *15*) for the formation of 8-oxodG/dF tandem lesions involved the initial addition of a single °OH to the C5−C6 double bond of a thymine residue, followed by oxygen fixation on the resulting radical. This was mainly inferred from experiments involving oxidation of DNA under various conditions (*15*). In the present work, a more direct evidence was provided by the study of the photolysis of thymine bromohydrins. Indeed, homolytic cleavage of the C−Br bond of the latter compounds leads to the formation of the radical arising from the addition of °OH at the C6 position of thymine. Under these conditions, 8-oxodGpdF was actually characterized as a UV-induced degradation product of dGpBrOHdT. The second key step in the proposed mechanism of formation of 8-oxodG/dF tandem lesion is the intramolecular electron abstraction from guanine by a thymine peroxyl radical. The possibility for such a reaction to occur has been already strongly suggested by the study of methylperoxyl radicals arising from the radiolysis of aqueous solutions containing dimethyl sulfoxide (*31*). This has received additional support from experiments using ABAP as a source of alkylperoxyl radical. The latter oxidizing compound has previously been used in mutagenesis experiments involving transfected plasmids (*32*), and guanine was found to be a major target. This has also been visualized by sequencing techniques (*33*, *34*). In addition, ABAP-treated DNA was found to contain a much higher level of 8-oxodG than of 5-hydroxy-2′-deoxycytidine (*35*). The overwhelming degradation of guanine has also been observed upon treatment of DNA with *tert*-butoxyl radicals (*36*). These results demonstrate that even though peroxyl radicals are weaker oxidants than reactive species such as hydroxyl radicals, they are able to undergo one electron oxidation of guanine. This selectivity might be accounted for by the low oxidation potential of guanine with respect to other DNA bases. This was confirmed in the present study since ABAP was found to induce a very efficient degradation of dGpdT and dTpdG through a one electron oxidation process, as inferred from the formation of oxazolone as the major product. Altogether, evidence has been obtained for the possible involvement of a thymine peroxyl radical-induced oxidation of guanine in the formation of 8-oxodG/dF tandem lesions.

As a consequence, the formation of a large series of tandem lesions arising from the decomposition of thymine hydroperoxides on one hand and the fate of the guanine radical cation on the other hand might be predicted. Thymine glycols (dTGly) were chosen as target lesions of thymine since they have been reported to be major final stable products arising from the decomposition of thymine hydroperoxides (*19*). Tandem lesions carrying dTGly on one hand and either 8-oxodG or oxazolone on the other hand were thus studied. dTGly/8-oxodG tandem lesions were actually found to be produced in very low yield upon exposure of solutions of dTpdG and dGpdT to γ-radiations. They were shown to be generated as primary products, on the basis of their linear dose−course formation. In contrast, the related oxazolone-containing tandem lesions studied (dTGly/dZ) were found to be "two hits" degradation products as inferred from the quadratic relationship of their formation with increasing doses. Similar observations were made for the tandem lesions bearing both an oxazolone and a formamido residue.

It can be concluded from the results presented above that 8-oxodGpdF and dFp8-oxodG are the major tandem lesions produced upon exposure of dGpdT and dTpdG, respectively, to γ-rays in aqueous aerated solution. Therefore, the diversity of radiation-induced tandem lesions is not as large as predicted from the mechanism involving intramolecular electron transfer between a thymine peroxyl radical and a guanine residue. To investigate a possible alternative mechanism, experiments were carried out in order to establish the origin of the oxygen atom at position 8 of the 8-oxodG moiety in 8-oxodG/dF. Indeed, the overwhelming formation of 8-oxodG/dF may be accounted for by the addition of the 5(6)-hydroxyl-6(5)-peroxyl-5,6-dihydrothymine radical to the guanine moiety, followed by the rearrangement of the resulting peroxide (Scheme 3). This alternative mechanism would lead to the direct formation of 8-oxodG, without formation of oxazolone. Similarly, thymine peroxyl radical would be transiently converted into an alkoxy radical, without formation of hydroperoxides. This might therefore account for the high yield of formamido remnant. Indeed, 5(6)-hydroxy-6(5)-oxyl-5,6-dihydroythymine radicals have been shown to be precursors of formamido residue via a β-scission (*19*, *37*). In contrast, 8-oxoGua/dTGly are produced in low yield by the reduction of the alkoxy radical.

Evidence was provided for this alternative mechanism of formation of 8-oxodG/dF tandem lesions by an experiment involving irradiation of dGpdT in the presence of $^{18}O_2$. A very efficient labeling of 8-oxodGpdF was observed (80%), in agreement with a major role played by molecular oxygen in the formation of the tandem lesion. HPLC-MS/MS analysis allowed to show that either one or two ^{18}O atoms were incorporated. However, the 8-oxoGua residue was labeled in both cases. This definitively shows that the oxygen atom of the keto group of 8-oxodG in 8-oxodG/dF is provided by molecular oxygen, likely through the addition of the thymine peroxyl radical. Indeed, conversion of the guanine radical cation into 8-oxodG requires hydration and would thus yield unla-

Scheme 3. Presently Proposed Mechanism for the Formation of 8-oxodG/dF Tandem Lesions[a]



[a] The described pathway only details the reaction occurring upon addition of °OH to the C6 position of thymine. The inset shows the final steps of the reaction upon initial addition of °OH to the C5 position of thymine. The key step is the addition of the peroxyl radical to the C8 position of guanine. The asterisks represent the position of the oxygen atoms arising from the molecular oxygen.

beled 8-oxodG moiety. Involvement of hydroxyl radicals can be ruled out for similar reasons. The latter point was emphasized by the observation that the 8-oxodGpdT single lesion, produced by addition of °OH to dGpdT, was not labeled in the presence of $^{18}O_2$. The second ^{18}O atom present in the M + 4 derivative of 8-oxoGpdF was found to be located on the formamido residue. However, less than half of the ^{18}O-labeled 8-oxodGpdF molecules carried this second stable isotope. This may be accounted for by the site of the initial addition of the hydroxyl radical to the C5−C6 thymine double bond. Indeed, formation of the formamido residue involves the fragmentation of the pyrimidine ring, with loss of the C5, C4, N3, and C2 atoms, together with their respective substituents. Therefore, formation of 8-oxodGpdF from a C5-peroxyl radical would lead to the loss of one of the two ^{18}O atoms arising from the addition of oxygen. In contrast, involvement of C6-peroxyl derivative would yield 8-oxodGpdF bearing two ^{18}O atoms. Interestingly, the abundance of the M + 2 pseudo-molecular ion was slightly higher than that of the M + 4. This suggests that the amount of tandem lesions arising from the initial addition of °OH to the C6 position of thymine is higher than that produced by the occurrence of the latter reaction at C5. This contrasts with the respective reactivity of the C5 and C6 positions of thymine to which °OH adds in a 60/35 ratio (*3*). It might therefore be proposed that the 6-hydroxy-5-peroxyl-5,6-dihydrothymine radical leads to a higher yield of 8-oxodGpdF tandem lesions than its 5-hydroxy-6-peroxyl analogue.

Altogether, these data strongly support the occurrence of an intramolecular addition of a thymine peroxyl radical to vicinal guanine in the formation of 8-oxodGpdF tandem lesions. Interestingly, alkylperoxyl radicals produced in the bulk of the solution upon ABAP thermal decomposition were found to undergo one electron oxidation of guanine. It might therefore be proposed that the addition of a thymine peroxyl radical to a vicinal guanine residue is favored by entropic factors. The identification of the latter mechanism for the formation of 8-oxodG/dF allows us to restrict the unidentified candidates for the tandem lesions likely to be found within irradiated DNA. Indeed, only degradation products arising from the thymine alkoxy radicals, such as the diastereoisomers of 5-hydroxy-5-methylhydantoin nucleosides, might be produced in significant yield. This would account for the results obtained within DNA, showing a likely formation of 8-oxodG-containing exonucleases-resistant tandem lesions other than 8-oxodG/dF (*15*). Indeed, 5-hydroxy-5-methylhydantoin was found to be resistant to the action of snake venom and calf spleen phosphodiesterases (*38*).

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