Efficient Oxidation of 2′-Deoxyguanosine by Mn-TMPyP/$KHSO_5$ to Imidazolone dIz without Formation of 8-Oxo-dG

Corine Vialas, Geneviève Pratviel, Catherine Claparols, and Bernard Meunier*

Contribution from the Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex 4, France

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Abstract: Oxidation of 2′-deoxyguanosine dG with the chemical nuclease Mn-TMPyP/$KHSO_5$ leads within one minute to a nearly quantitative amount of 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one (dIz or imidazolone for short) (90%) in a non-oxygen-dependent pathway and without formation of 8-oxo-dG. This new mechanism of dIz formation involves, as a crucial first step, the abstraction of two electrons from guanine by a high-valent porphyrin−Mn(V)═O species, leading to the dG^+ cation instead of the classical $dG^{\bullet+}$ radical cation. We describe also the oxidation of imidazolone into the corresponding imidazolone *N*-oxide in the presence of $KHSO_5$.

Introduction

Oxidative damage to DNA is strongly involved in mutagenesis and carcinogenesis.[1−5] Among all of the different possible oxidation sites on DNA, guanine is one of the main target because of its low redox potential which is accessible for many different oxidizing systems[6−10] (the one-electron oxidation potential $E°$ of guanosine at pH 7 is equal to 1.29 V vs NHE[6c]). The structure of four main oxidation products of 2′-deoxyguanosine (dG) have been identified: 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 2,6-diamino-5-formamidopyrimidine derivative (FAPy), 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one (dIz or imidazolone for short) (Scheme 1), and 2,2-diamino-4-[2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-2,5-dihydrooxazol-5-one (dZ or oxazolone) (Scheme 1). However, 8-oxo-dG received a considerable attention in the literature, leading to consider it as the most biologically relevant G-damage in oxidized DNA. Here we report the nearly quantitative formation of imidazolone within 1 min by oxidation of 2′-deoxyguanosine dG with the chemical nuclease Mn-TMPyP/$KHSO_5$[11−13] *without* intermediate formation of 8-oxo-dG.

Imidazolone dIz and its hydrolysis product oxazolone dZ were first described by Cadet et al. as the major oxidation products of dG in the presence of hydroxyl radicals or type I photosensitizers.[2,14,15] The proposed mechanism (Scheme 1A) involves one-electron oxidation of guanine base, deprotonation of the resulting guanine radical cation to guanine radical ($dG^{\bullet}$), and reaction of this latter radical with molecular oxygen at the C5 position. This mechanism was supported by the incorporation of one atom of ^{18}O in both dIz and dZ under $^{18}O_2$ atmosphere.[14] After attack of a water molecule on the electrophilic C8 position of guanine, one-electron reduction, and release of CO_2 (from the former C6 of the base), intermediate formation of **4** and subsequent release of formamide were proposed to explain the formation of dIz. While imidazolone can arise directly from guanine oxidation, it was also reported that it can be generated via the oxidation of 8-oxo-dG in photooxidation reactions with both type I or II photosensitizers.[16] In a similar way, the one-electron oxidation of 8-oxo-dG would give the corresponding radical after losing a proton and would react with O_2 at the C5 position (Scheme 1B). After one-electron reduction and elimination of CO_2 (from the former C6), the formation of dIz would necessitate the release of CO_2 and NH_3 instead of formamide from **5** (an oxidized form of intermediate **4**). In both mechanisms of dIz formation, the one-electron oxidation of either dG or 8-oxo-dG into the corresponding radical which reacts with O_2 implied that the formation of imidazolone is oxygen-dependent. Formamide and intermediates **4** or **5** have not been identified up to now.

On oxidatively damaged DNA, most of the guanine lesions are revealed by cleavage at guanine residues with a piperidine

* Corresponding author. E-mail: bmeunier@lcc-toulouse.fr.

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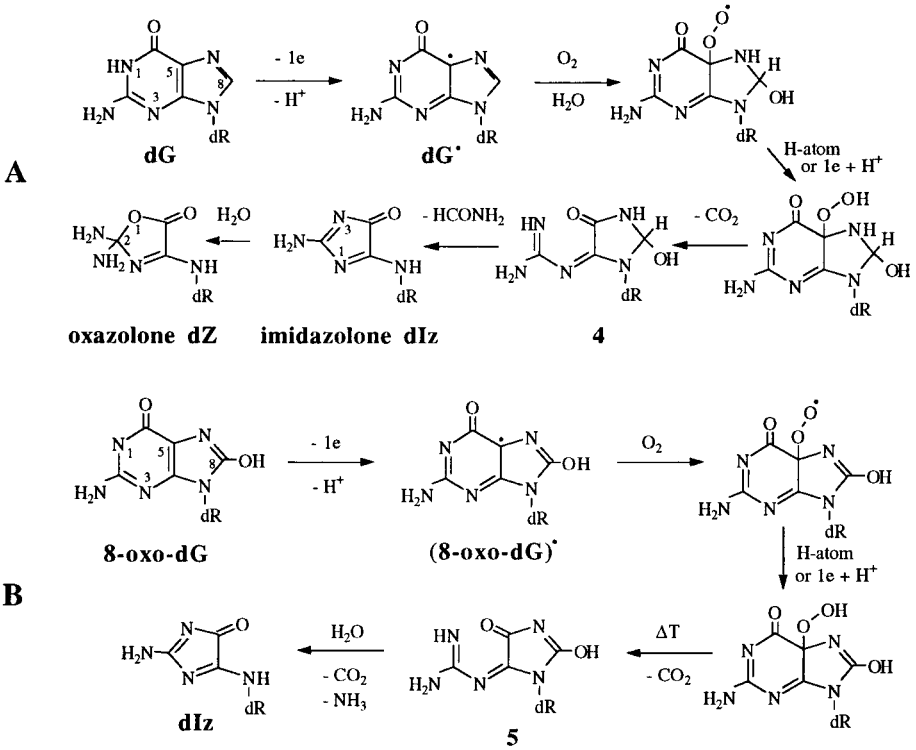
Scheme 1. (A) Proposed Mechanism of Formation of the Imidazolone dIz in Type I Photooxidation Reactions (i.e., Via an Electron Transfer) According to Cadet[14,a] and (B) Proposed Mechanism of Formation of dIz from 8-Oxo-dG According to Adam[16]



[a] dR stands for the deoxyribose unit. The addition of water at C8 might occur after the opening of the six-membered ring of dG and not at the level of the neutral radical cation of dG•.

treatment. Both dIz and dZ have been shown to be alkali-sensitive[15] and consequently might account for the facile piperidine lability of guanine lesions found in oxidized DNA. However, the formation of dIz and dZ from oxidized guanine in a double-stranded DNA structure may not be favored since they both depend on the evolution of the guanine radical cation $dG^{\bullet+}$ generated within DNA.[2,5] $dG^{\bullet+}$ might retain more cationic character because of the base pairing with cytosine in double-stranded DNA,[5] leading to a diminution of the formation of imidazolone (the proton shuttle between $dG^{\bullet+}$ and its complementary base reduces the deprotonation of $dG^{\bullet+}$). A longer lifetime of a guanine radical cation in a DNA structure would rather allow its hydration, leading to the formation of 8-oxo-7,8-dihydroguanine base damage.[2,5] However, the 8-oxo-7,8-dihydroguanine lesion was recently found not to be directly alkali-labile.[5,17−20] Since 8-oxo-dG is more oxidizable than dG,[5,16] its oxidation products (including imidazolone, oxazolone, and yet unknown derivatives) may be responsible of the alkali-labile lesions observed at guanine residues.[21] So, the understanding of guanine oxidation is still a matter of debate and is probably complicated by sequence effects.[22]

We became interested in the identification of alkali-sensitive guanine oxidation products during our work on sequence-directed oxidative cleavage of DNA with manganese cationic metalloporphyrins attached to oligonucleotides. Contrary to the 2-deoxyribose oxidation mainly observed with the nonvectorized Mn-TMPyP,[11,12] alkali-labile sites were observed at guanine residues of the DNA target.[23−25] This reactivity was tentatively attributed to a different mode of interaction of the metalloporphyrin moiety with respect to the DNA target. The free Mn-TMPyP interacts with the minor groove of B-DNA and thus the $KHSO_5$ activated metalloporphyrin, namely a high-valent (porphyrin)Mn(V)═O species,[11,26,27] can attack the C−H bonds of 2-deoxyribose units, while when attached to the 5′-end of an oligonucleotide vector, this interaction within the minor groove might be impeded, leading to electron abstractions from the easily oxidizable guanine residues. The yield of DNA cleavage through guanine oxidation after piperidine treatment was as high as 80% with 100 nM of metalloporphyrin−oligonucleotide conjugate reacting with 10 nM of target DNA,[24,25] and the oxidation of guanine residues was fast (less than 1 min at room temperature).[25] We eliminated the hypothesis of sugar oxidation at C1′ carbon because the lesions were only located at guanine residues and were not revealed by a simple 90 °C heating step previously shown to promote the release of the corresponding

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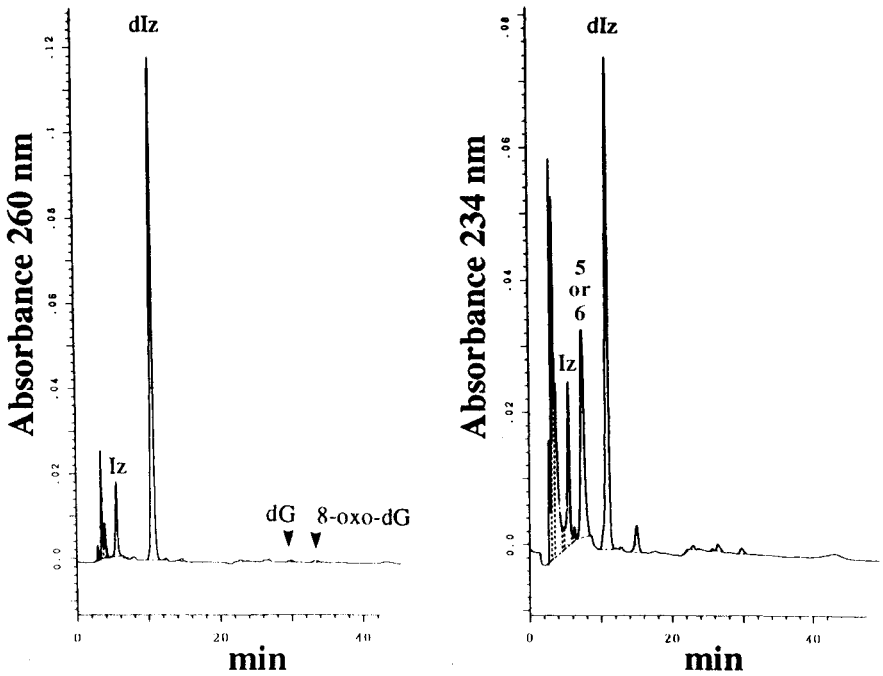

Figure 1. Formation of imidazolone dIz resulting of dG oxidation by Mn-TMPyP/$KHSO_5$. HPLC profiles after 1 min of reaction at 260 nm (left) and at 234 nm (right). Iz stands for the base moiety of dIz without the sugar. **5** (or **6**) is a transient species. The arrows indicate the positions of dG and 8-oxo-dG.

sugar oxidation product.[28] To get some molecular insight on the guanine lesions generated by metalloporphyrin−oligonucleotide conjugates, we first investigated the oxidation of 2′-deoxyguanosine as a model compound. Here we report the mechanism of its very efficient catalytic oxidation by the Mn-TMPyP/$KHSO_5$ system.

Oxidation of dG into Imidazolone

The model nucleoside dG was converted to dIz in a nearly quantitative amount by the Mn-TMPyP/$KHSO_5$ system at room temperature. In a final volume of 250 μL of 20 mM triethylammonium acetate buffer (pH 6.5), dG (1 mM) was incubated with Mn-TMPyP (8 μM) and $KHSO_5$ (10 mM). After 1 min of reaction at ambient temperature, the reaction medium was either stopped by the addition of Hepes buffer (pH 8) (100 mM) for regular HPLC analysis or directly injected (20-μL aliquot) for LC-ESI-MS analyses (LC-ESI-MS: liquid chromathography coupled to an electrospray spectrometer). This soft method was preferred to a derivatization of dIz followed by a GC-MS analysis. The starting material dG was catalytically oxidized within 1 min into dIz in a very high yield (90%) (Figure 1, left panel). This oxidation product had the same chromatographic behavior (and the same UV−vis spectrum obtained with a diode-array detector) as a dIz sample prepared by photosensitized oxidation of dG in the presence of benzophenone as previously described by Cadet et al.[15] The ESI-MS spectra of dIz showed *m*/*z* peaks corresponding to the molecular peak $[M + H]^+$ at 229, the sodium salt adduct $[M + Na]^+$ at 251, the potassium adduct $[M + K]^+$ at 267, and the loss of the 2-deoxyribose moiety (dR) $[M - dR + 2H]^+$ at 113 (Figure 2).

Imidazolone was quantified on HPLC chromatograms by comparison of the peak area with a calibration curve obtained from standard solutions of dIz prepared by photooxidation[15] (the ϵ value of dIz at λ_{max} 254 nm was determined as being 5 ± 0.5 mM^{-1} cm^{-1}). Under the experimental conditions used with Mn-TMPyP/$KHSO_5$, the hydrolysis of dIz into dZ was not significant because of the slow conversion of dIz into dZ (half-life of dIz is 10 h at 20 °C at neutral pH).[2]

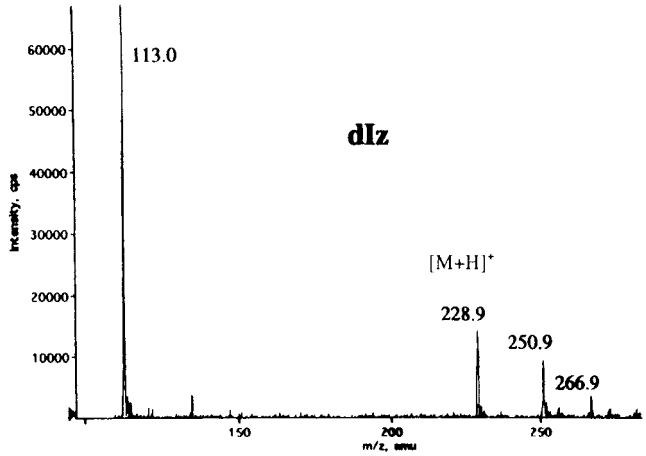

Figure 2. LC-ESI-MS spectra of the peak of dIz.

We addressed the question of the possible formation of 8-oxo-dG as an intermediate in the reaction pathway. A reference sample of 8-oxo-dG was prepared as previously described.[29a] This compound eluted after dG under the HPLC conditions employed (retention time = 33 min) and was not observed during the oxidation of dG by Mn-TMPyP/$KHSO_5$. It should be noted that 8-oxo-dG is as easily monitored as dG, by UV−vis during HPLC analysis, since its absorbance coefficients are at pH 1 248 nm, 13 500 M^{-1} cm^{-1} and 295 nm, 11 300 M^{-1} cm^{-1} or at pH 12 248 nm, 13 400 M^{-1} cm^{-1} and 282 nm, 11 140 M^{-1} cm^{-1}.[29b] In fact, 8-oxo-dG was degraded either by $KHSO_5$ alone or by Mn-TMPyP/$KHSO_5$ within 5 min to nondetected species. No dIz was observed. Under the same experimental conditions, dG was proved to be stable in the presence of

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$KHSO_5$ alone during 5 min. Consequently, we can conclude that 8-oxo-dG is not an intermediate in the formation of dIz during dG oxidation by Mn-TMPyP/$KHSO_5$.

The formation of dIz was not affected when the oxidation of dG was performed in anaerobic conditions, strongly suggesting that this metalloporphyrin-catalyzed production of dIz is not oxygen-dependent. To obtain confirmation that molecular oxygen was not involved in dIz formation, we carried out the dG oxidation under an $^{18}O_2$ atmosphere. The LC-ESI-MS analysis of the imidazolone peak confirmed that the molecular mass of dIz was unchanged ($[M + H]^+$ at 229), indicating that no ^{18}O-incorporation occurred. As a control, the photooxidation of dG in the presence of benzophenone under $^{18}O_2$ produced imidazolone with the expected increase of two units on the molecular peak of dIz ($[M + H]^+$ at 231 and the glycosidic bond cleavage product $[M - dR + 2H]^+$ at 115) when analyzed by the same method. Thus, clearly, molecular oxygen does not participate in the mechanism of formation of dIz mediated by Mn-TMPyP/$KHSO_5$.

This result led us to postulate a fast second electron abstraction from the neutral dG$^\bullet$ radical (resulting from the fast deprotonation of the radical cation dG$^{\bullet+}$) by the high-valent metal−oxo species of the metalloporphyrin, that would lead to the dG^+ cation. The relatively slow reaction of dG$^\bullet$ with O_2 (millisecond scale)[6b] allows the oxidation of this neutral radical by a manganese−oxo species. The high-valent Mn(V)═O being equivalent to compound I of a peroxidase, its reduction by one electron generates a Mn(IV)═O species (comparable to compound II) which is still able to abstract one electron from a neutral radical. The reaction was then performed in $H_2{}^{18}O$ to detect a possible addition of a molecule of water at C5 of dG^+ in a fashion reminiscent to the mechanism of degradation of uric acid by urate oxidase.[30] No ^{18}O atom from $H_2{}^{18}O$ was incorporated in the imidazolone compound. So we concluded that the dG^+ cation would be trapped by the inorganic peroxide in excess which has a stronger nucleophilic character than water itself.

We decided also to address the question of the release of formamide in the final steps of the formation of dIz as initially suggested by Cadet et al.[14] We found by two different methods (GC and GC-MS with *N*-methylformamide as internal standard in both cases) that 30% of formamide was generated in the catalytic oxidation of dG to dIz. This value represents the real amount of released formamide since we checked separately that this product was stable in a control reaction performed with Mn-TMPyP/$KHSO_5$. In the case of a reaction carried out in $H_2{}^{18}O$, the released formamide was 85% ^{18}O-labeled (molecular mass increased by two units in GC-MS analysis), confirming the attack of a water molecule on C8.

Furthermore, the analysis of the reaction HPLC profiles at 234 nm allowed us to identify a transient species in the formation of imidazolone (peak **5** or **6**, Rt = 6 min) (Figure 1, right panel). This compound was only observed after 1 min of reaction and disappeared after 5 min (Figure 4). Its mass spectrum obtained by LC-ESI-MS analysis exhibited a peak at $m/z = 272$ attributed to $[M + H]^+$ and a fragmentation peak $[M - dR + 2H]^+$ at $m/z = 156$ corresponding to the loss of the intact sugar ring (Figure 3). The mass spectrum of this intermediate compound is compatible with the structure labeled as **5** (or **6**) in Scheme 2. This compound has been postulated before as being an intermediate in the reaction pathway proposed for the oxidation of 8-oxo-dG to dIz.[16] These results suggest

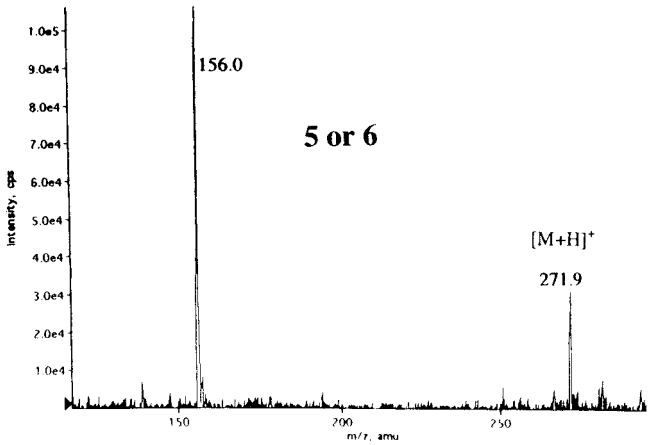

Figure 3. LC-ESI-MS spectra of the transient product **5** or **6**.

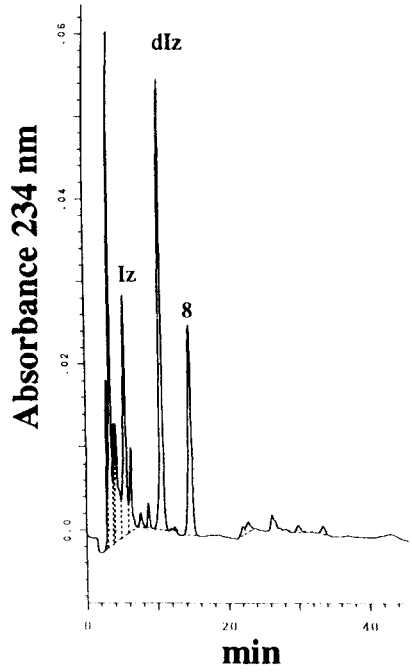

Figure 4. Degradation of imidazolone during dG oxidation by Mn-TMPyP/$KHSO_5$. HPLC profile at 234 nm after 5 min of reaction. Iz stands for the base moiety of dIz due to the release of the sugar. The putative imidazole *N*-oxide is indicated as compound **8**.

that at least two independent routes lead to the imidazolone, the major route involving the evolution of carbon dioxide and ammonia and the minor one involving the elimination of formamide (Scheme 2).

Taking in consideration all the different data obtained on this oxidation of dG by Mn-TMPyP/$KHSO_5$, we can summarize the different steps of Scheme 2 as follows: (i) two electrons and one proton are abstracted from dG by the high-valent Mn(V)═O[11,26,27] species leading to dG^+, then (ii) the excess of monopersulfate acting as a nucleophile attacks this cation to generate the intermediate peroxide **1**, (iii) the addition of a water molecule at C6 will give **2**, a suitable intermediate for a Grob fragmentation,[31] then (iv) addition of a water molecule at the former C8 position will produce after decarboxylation the compound **4**, which can be oxidized (pathway **a**) by the Mn(V)═O species (or by monopersulfate itself) into **5** (or **6**, the rearranged compound resulting from the nucleophilic attack of the guanidine NH_2), and (v) the decomposition of **6** will

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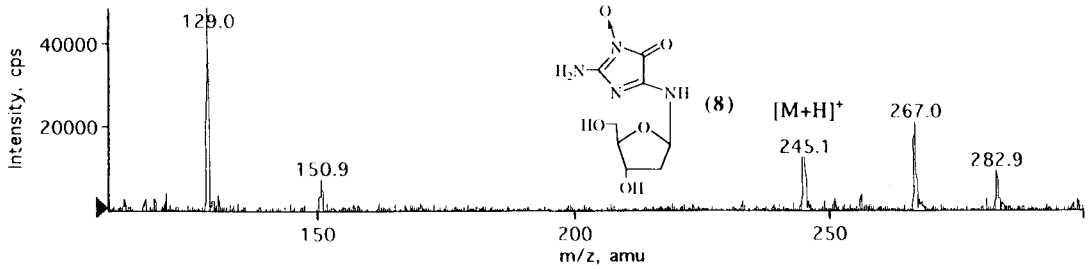
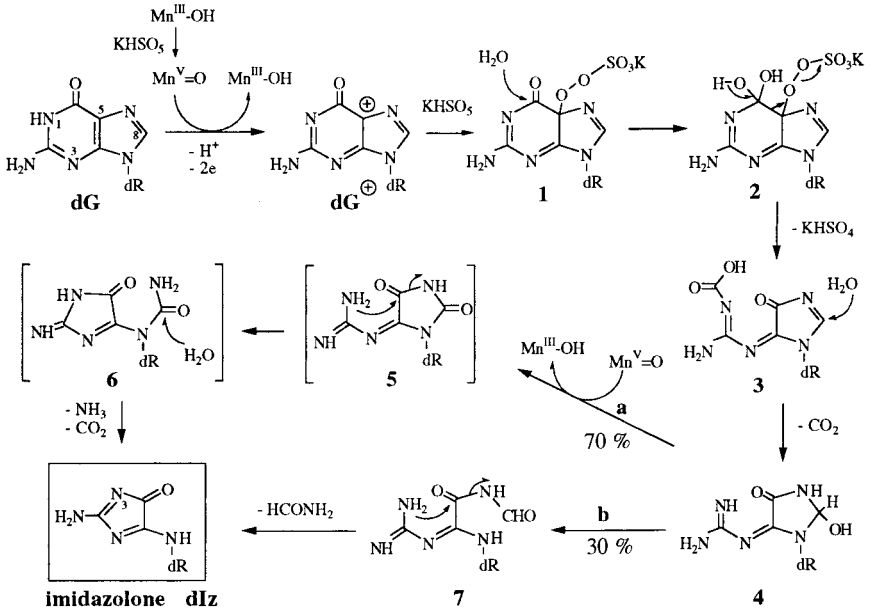


Figure 5. LC-ESI-MS mass spectrum corresponding to the peak of the putative *N*-oxide **8**.

Scheme 2. Proposed Mechanism of Imidazolone dIz Formation by Oxidation of dG by the Chemical Nuclease Mn-TMPyP/$KHSO_5$ (a = Oxidative Pathway, b = Formamide Elimination)



generate dIz, by elimination of ammonia and carbon dioxide. The alternative competing minor route (pathway **b**) corresponds to the ring opening of **4** leading to intermediate **7** followed by the intramolecular nucleophilic attack of the guanidine NH_2 on compound **7** to produce dIz and formamide.

Oxidation of Imidazolone

Two products arose from dIz oxidation; one corresponds to the base moiety of dIz, liberated after oxidative glycosidic bond cleavage and is referred to as Iz in Figures 1 and 4, and the other one (**8**) is a further oxidized product of dIz (Figure 4). It should be noted that the formation of these two compounds is diminished when the catalytic oxidation of dG is performed in a more diluted reaction mixture. Both products have been identified by LC-ESI-MS. The Iz peak eluted at a retention time of 5 min and the corresponding mass spectrum exhibited a peak $[M + H]^+$ at *m*/*z* 113. This product is present after 1 min of reaction and represents about 10% of the amount of the formed dIz in these reaction conditions (Figure 1). Iz may arise from C1′ sugar carbon hydroxylation of dIz by the activated Mn(V)═O species of the metalloporphyrin. This type of reactivity has been well documented on DNA deoxyriboses.[11] The cleavage of the glycosidic bond of dIz is not a simple hydrolysis here, since we could note the absence of Iz formation in the control reaction when standard dIz was incubated with $KHSO_5$ alone (see Figure 6). The second product (**8**) eluted with a retention time of 15 min. Its corresponding mass spectrum (Figure 5) presents $[M + H]^+$ *m*/*z* peak at 245, $[M + Na]^+$ and $[M + K]^+$ adducts respectively at *m*/*z* 267 and 283, and the usual glycosidic fragmentation peak at $[M - dR + H]^+$ at *m*/*z* 129 accompanied by its sodium adduct at *m*/*z* 151. One oxygen atom was incorporated on the imidazolone ring and, thus, implies a further oxidation of dIz in the oxidative reaction

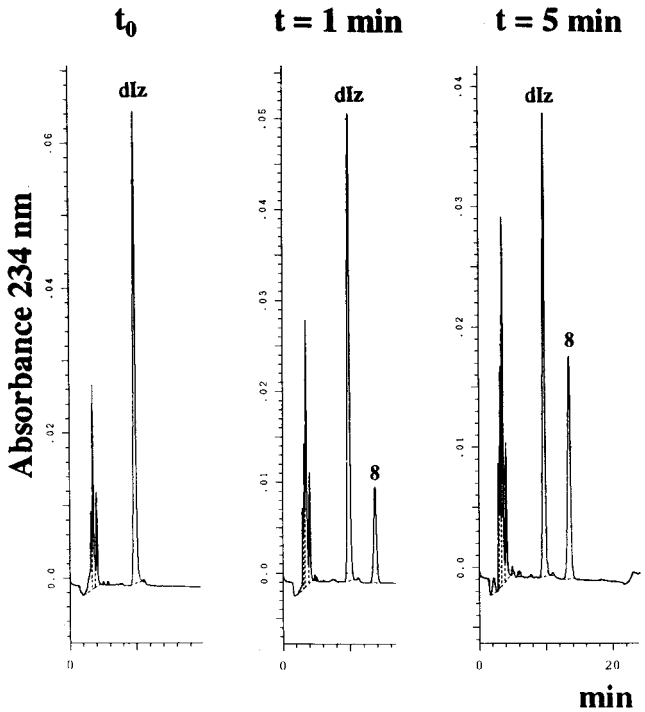


Figure 6. Oxidation of dIz by $KHSO_5$. HPLC profiles at 234 nm.

mixture. We propose that this product corresponds to a *N*-oxide derivative of dIz with the structure represented in Figure 5. The amount of product **8** was negligible at a reaction time of 1 min but increased with time (compare Figures 1 and 4). It corresponds to about 30% of degradation of dIz after 5 min of incubation in the catalytic oxidation conditions. Formation of *N*-oxide of nucleoside bases in the presence of $KHSO_5$ has been reported before.[32,33] On the basis of Hückel calculations, the nitrogen atom N_3 of dIz corresponds to the highest coefficient of the HOMO, and therefore, this position is favored in the *N*-oxide formation with $KHSO_5$. To support this hypothesis, we tested the reactivity of $KHSO_5$ alone with dIz in the same conditions as for the oxidation of dG with Mn-TMPyP/$KHSO_5$. Product **8** represented 40% of the initial dIz within 5 min of incubation (Figure 6). The UV−vis spectrum of compound **8**, obtained with a diode array detector during the HPLC analysis, presented an absorbance maximum at 238 nm, while the absorbance maximum of dIz was at 254 nm. This hypsochromic shift is consistent with a substitution of the imidazolone ring. The half-life of the *N*-oxide derivative of dIz at pH 7 is about 2 h at room temperature.

Conclusion

In conclusion, we found a very efficient formation of dIz by oxidation of dG with the artificial nuclease Mn-TMPyP/$KHSO_5$ without formation of 8-oxo-dG. In this two-electron oxidation of 2′-deoxyguanosine, the incorporation of molecular oxygen is not observed, contrarily to one-electron oxidants able to produce the neutral radical dG• which is trapped by the triplet state of dioxygen.

We are currently investigating the mechanism of oxidation of guanine residues (in particular 5′-GG motifs) in short oligonucleotides to generalize this remarkably efficient two-electron process on DNA which can be related to base damage observed in the presence of adventitious hydrogen peroxide reacting with redox active metal ions interacting with nuclear DNA.

Experimental Section

Materials. Potassium monopersulfate, $KHSO_5$ (triple salt $2KHSO_5 \cdot K_2SO_4 \cdot KHSO_4$, Curox), was from Interox, 2′-deoxyguanosine from Sigma, benzophenone from Aldrich, formamide from Gibco BRL, and methylformamide from Fluka. Mn-TMPyP was prepared as previously described.[34] Labeled molecular oxygen (96.8 atom % ^{18}O) and water (96.5 atom % ^{18}O) were from Leman (France).

HPLC Analyses. The reaction medium was analyzed by HPLC, on an analytical reverse phase column (nucleosil C18, 10 μm, 250 × 4.6 mm) eluted isocratically with MeOH/H_2O (2/98) for 13 min then MeOH/H_2O (10/90) at 1 mL/min. The column was coupled to a diode array detector (Kontron Instrument) that allowed detection of the products at 260 and 234 nm and monitoring of UV−vis spectra for routine analysis.

LC-ESI-MS Analyses. In the case of LC-ESI-MS analysis, the same column was used with a quaternary pump, in the same conditions as above, but only 4% of the flow eluted from the column was introduced into the electrospray source after automated mixing with a methanol solution containing 0.1% of formic acid using a Harvard Apparatus syringe pump. The ESI-MS spectrometer was a Perkin-Elmer SCIEX API 100. The ionization voltage was 5 kV, and the orifice voltage was 50 V. The analyses were performed in the positive mode.

Oxidation of dG by MnTMPyP/$KHSO_5$. In a final volume of 250 μL of 20 mM triethylammonium acetate buffer (pH 6.5), dG (1 mM) was incubated with Mn-TMPyP (8 μM, 0,8 mol % with respect to dG) and $KHSO_5$ (10 mM). After 1 or 5 min of reaction at ambient temperature, the reaction medium was either directly injected for LC-ESI-MS analysis (20 μL) or stopped by the addition of Hepes buffer pH 8 (100 mM) for classical HPLC analysis.

Oxidation of 8-Oxo-dG by MnTMPyP/$KHSO_5$. In a final volume of 100 μL of 20 mM triethylammonium acetate buffer (pH 6.5), 8-oxo-dG (50 μM) was incubated with Mn-TMPyP (2 μM) and $KHSO_5$ (1 mM). After 5 min of reaction at ambient temperature, the reaction medium was stopped by the addition of Hepes buffer (pH 8) (10 mM) and directly injected for classical HPLC analysis.

Labeling Experiments. Samples of a 4× scale of reaction medium as described above were first deoxygenated in Schlenck tubes. After four freeze−pump−thaw cycles, these tubes were pressurized under $^{18}O_2$ (3 bar) at the end of the last pumping step. The samples were incubated overnight under $^{18}O_2$ before irradiation or addition of $KHSO_5$ that started the oxidation reactions.

In the case of reaction in $H_2{}^{18}O$, 100 μL of the reaction medium, prealably prepared in water without $KHSO_5$, was lyophilized and dissolved in 99 μL of labeled water. A minimal volume (1 μL) of a 1 M solution of $KHSO_5$ in $H_2{}^{16}O$ water was then added to the reaction.

Formamide Analysis. GC/MS analysis were carried out with a HP6870 gas chromatograph interfaced with a HP5973 mass spectrometer. Separations were obtained on a polar capillary column carbowax (20 m × 0.2 mm) with a constant temperature (120 °C). Injector and GC-MS interfaces were respectively at 250 and 230 °C. Helium (ultrahigh purity) was used as the carrier gas at an inlet pressure of 1.6 bar. Mass spectra are recorded on the electron-impact mode at 70 eV. Quantification of formamide was performed using methylformamide as an internal standard and was done by classical GC equipped with a ionization flame detector. Methylformamide was present in the reaction medium. We also checked that it was not degradated in the oxidation medium. Gas chromatography analyses were performed on a HP5890 chromatograph equipped with a ionization flame detector (260 °C) and a polar capillary column AT-WAX (30 m × 0.25 mm) from Alltech (oven temperature at 130 °C and injector at 260 °C).

Oxidation of Imidazolone by $KHSO_5$. In a final volume of 250 μL of 20 mM triethylammonium acetate buffer (pH 6.5), dIz (1 mM) was incubated with $KHSO_5$ (10 mM). After 5 min of reaction at ambient temperature, the reaction medium was either directly injected for LC-ESI-MS analysis (20 μL) or stopped by the addition of Hepes buffer (pH 8) (100 mM) for classical HPLC analysis.

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