Chemical Research in Toxicology

Article

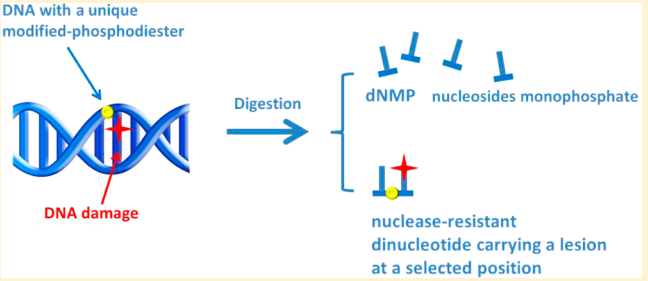
pubs.acs.org/crt

A Single Nuclease-Resistant Linkage in DNA as a Versatile Tool for the Characterization of DNA Lesions: Application to the Guanine Oxidative Lesion "G+34" Generated by Metalloporphyrin/$KHSO_5$ Reagent

Agnieszka Tomaszewska, Sophie Mourgues, Piotr Guga, Barbara Nawrot, and Geneviève Pratviel*

Supporting Information

ABSTRACT: The oxidation of an oligonucleotide containing a single nuclease-resistant phosphodiester link, a stereoisomerically pure methylphosphonate, by manganese (Mn-TMPyP) or iron (Fe-TMPyP) porphyrin associated to $KHSO_5$ allowed the isolation and characterization of a guanine lesion corresponding to an increase of mass of 34 amu as compared to guanine ("G+34"), namely, 5-carboxamido-5-formamido-2-iminohydantoin. Enzymatic digestion of the damaged oligonucleotide afforded, apart from the undamaged nucleotide monomer pool, a unique dinucleotide doubly modified with a methylphosphonate and an oxidized guanine base that is suitable for NMR analysis. The method can be applied to the study of any DNA lesion. More importantly, the method can be extended to the analysis of DNA damage in a sequence context. Any preselected residue in a DNA sequence may be individually analyzed by the easy introduction of a single nuclease-resistant link at the 3′- or 5′-position.



INTRODUCTION

Oxidative damage of DNA can be induced by ionizing radiation, photosensitization, or various chemical oxidants giving rise to a number of DNA lesions such as oxidized nucleic acid bases, oxidized sugar units, and DNA breaks.[1−3] DNA damage may also be caused by various electrophiles (alkylating agents and metal complexes) that lead to chemically modified bases.[3,4] DNA damage plays a key role in mutagenesis, carcinogenesis, and cellular aging and is also considered as the main mechanism of action of anticancer drugs. Consequently, it is important to identify at a molecular level the DNA lesions that may be responsible for biological effects. We report a strategy that will facilitate the analysis of modified DNA and the study of DNA repair.

The modifications of DNA bases can be characterized by nuclear magnetic resonance (NMR) of nucleosides either after hydrolysis of damaged high molecular weight DNA or by performing in vitro damage on individual nucleosides. However, some DNA lesions may escape detection at the nucleoside level because of low UV absorbance or inappropriate chromatographic properties. The characterization of such lesions may be easier on dinucleotides[5−7] or trinucleotides.[8] The UV absorbance of unmodified residues of di(tri)-nucleotides facilitates the detection on HPLC chromatograms, and different chromatographic properties may allow a better separation. On the other hand, di(tri)nucleotide models are still not considered as ideal substrates for the study of DNA damage. A double-stranded DNA oligonucleotide model is physiologically more relevant. Because structural analysis of damaged oligonucleotides by NMR is not trivial, we designed a versatile method to isolate dinucleotide units (instead of nucleosides) by enzymatic digestion of a damaged double-stranded oligonucleotide. It is based on the preparation of a self-complementary oligonucleotide carrying a single nuclease-resistant phosphodiester linkage at a precise site on the sequence. The nuclease-resistant link must be located at the 3′- or 5′-position of the nucleoside unit in the DNA sequence that is considered as the target of the DNA-damaging reaction. After DNA damage and enzymatic hydrolysis, the nuclease-resistant dinucleotide containing the damaged residue (and the vicinal nondamaged residue) can be isolated and characterized by NMR.

We applied this method to the structural characterization of a guanine oxidation product formed by the reaction of a DNA oxidizing reagent based on a metalloporphyrin/$KHSO_5$ system with an oligonucleotide modified with a single stereoisomerically pure methylphosphonate linkage. We found that the product corresponds to the previously identified 5-carboxamido-5-formamido-2-iminohydantoin (2-Ih) formed with other oxidizing reagents.[9,10] Additionally, labeling studies clarify the mechanism of its formation and support epoxidation as the oxidation event leading to this particular guanine lesion under our experimental conditions.

EXPERIMENTAL PROCEDURES

Chemicals. H_2O was of Milli-Q grade (Millipore). Oligodeoxyribonucleotide (ODN) 5′-d(CAGCTG) was from Eurogentec SA

Received: July 13, 2012
Published: October 1, 2012

(Belgium) and purified by HPLC if necessary. The concentration was determined by UV at 260 nm, ε = 55000 M^{-1} cm^{-1}. Manganese porphyrin was prepared as described.[11] Nuclease P1 from *Penicillium citrinum* was purchased from Sigma-Aldrich. $KHSO_5$ (triple salt $2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$) was from Aldrich. The ^{18}O-labeled water (97.2 atom %) was from Eurisotop, France.

Synthesis of P-Stereopure Methylphosphonate Oligonucleotide. Stereochemically pure methylphosphonate-modified oligonucleotide, 5′-d($CAGCT_{PMe}G$) ("isomer I", 400 OD, about 7 μmol and 12 mg), was prepared with an OligoPilot II synthesizer (Pharmacia Biotech, Sweden) using commercially available phosphoramidite monomers (Glen Research, Sterling, VA) and synthesis protocols provided by the manufacturer. Nine consecutive syntheses at the 40 μmol scale each furnished ca. 6000 OD units of crude product. One has to notice that the coupling of the methylphosphonate monomer was significantly less effective despite an extended time of condensation. Because of intrinsic instability of methylphosphonate linkages under alkaline conditions, the deprotection of nucleobases was performed in concentrated ammonia at 55 °C for 4 h only. The crude product was fractionated by means of preparative RP HPLC (a column Pursuit XR, C18, 10 μm, 250 mm × 21.4 mm) with a gradient of acetonitrile in TEAB (3%/min, rt ca. 10 min), and ca. 1000 OD units of desired oligonucleotide was collected as a mixture of two P-diastereoisomers (R_P and S_P). The mixture was subjected to preparative anion exchange chromatography (a column BioBasicAX, 5 μm, 150 mm × 21.2 mm) with a nonlinear gradient of 1 M KCl in 20 mM NaH_2PO_4 (0−50% over 10 min, then 50−100% over 4 min, then isocratic at 100% for 10 min, rt 14 and 15 min), which yielded almost pure P-diastereomers. They were further desalted using the aforementioned preparative C18 column eluted with a gradient of acetonitrile in TEAB (1%/min).

HPLC and HPLC Coupled to Electrospray Mass Analysis (LC/ESI-MS). The HPLC apparatus was an Agilent 1200 system. The oligonucleotide reactions were analyzed on a reverse-phase Uptishere column (C18, 5 μm, 250 mm × 4.6 mm from Interchrom, France) eluted in the gradient mode: linear gradient from 4 to 15% of acetonitrile in 10 mM triethylammonium acetate buffer (TEAA), pH 6.5, for 60 min. The flow rate was 0.5 mL min^{-1}, and detection was at λ = 260 nm. LC/ESI-MS analyses were carried out under the same chromatographic conditions with an Agilent 1640 or a Qtrap AB Sciex spectrometer (electrospray ionization in the negative mode).

Analytical Oxidation of 5′-d(CAGCTG) and 5′-d($CAGCT_{PMe}G$). Self-complementary oligonucleotide 5′-d(CAGCTG) or 5′-d($CAGCT_{PMe}G$) (20 μM strand concentration, 10 μM duplex concentration) was allowed to anneal in 50 mM phosphate buffer, pH 8, and 100 mM NaCl at 0 °C for 15 min and was then reacted with Mn-TMPyP (or Fe-TMPyP) (10 μM) and $KHSO_5$ (100 μM). The concentrations are final concentrations in a reaction volume of 100 μL. The reaction lasted for 10 min at 0 °C. The addition of excess HEPES buffer with respect to $KHSO_5$ stopped the reaction. When the reaction was carried out in labeled water in the case of 5′-d(CAGCTG), the reaction medium was lyophilized before the addition of the minimum volume of a solution of $KHSO_5$ prepared in nonlabeled water. The final content of ^{18}O-labeled water in the reaction medium was 96.7 atom %. LC/ESI-MS analyses were carried out on a small range of m/z values for better resolution of isotopic patterns (905−920 amu).

Oxidation of 5′-d($CAGCT_{PMe}G$) on a Preparative Scale for Isolation of 5′-d[$CAGCT_{PMe}$(G+34)]. Stereochemically pure 5′-d($CAGCT_{PMe}G$) (isomer I, 50 OD, ∼10 μmol, 20 μM strand concentration) was reacted with Fe-TMPyP (20 μM) and $KHSO_5$ (200 μM) in 50 mM phosphate buffer, pH 8, and 100 mM NaCl at 0 °C. The concentrations are final concentrations. The reaction lasted 20 min and was performed in a final volume of 50 mL. Individual reactions were pooled together and desalted on a SepPak C18 (5 g cartridge from Waters), and the concentrated material eluted with acetonitrile/H_2O (30:70, v/v) was lyophilized.

Isolation of 5′-d[$CAGCT_{PMe}$(G+34)]. A first chromatographic separation was carried out on a C18eq 400 mg cartridge from Macherey-Nagel eluted with 50 mM TEAA buffer, pH 6.5, and acetonitrile (90:10, v/v). The collected fraction (oxidized strands) was concentrated by lyophilization and subjected to a second chromatography on a Hichrom Inert (C18 ODS2, 5 μm, 250 mm × 10 mm) eluted in the gradient mode with 50 mM TEAA buffer, pH 6.5, and acetonitrile: 6−10% acetonitrile in 40 min followed by 10−15% acetonitrile in 5 min. The flow rate was 4.7 mL/min, and detection was at λ = 260 nm. The collected fractions (separated oxidized strands) were desalted one by one on SepPak cartridges (elution with H_2O/acetonitrile) and lyophilized. The oxidized oligonucleotide 5′-d[$CAGCT_{PMe}$(G+34)] eluted at Rt = 4 and 4.5 min for the fast- and slow-eluting isomers, respectively. The fast-eluting, minor isomer fraction contained 7.5 OD (∼150 nmol), and the slow-eluting, major isomer contained 18 OD (∼360 nmol). The purity of the products was checked by analytical LC/ESI-MS.

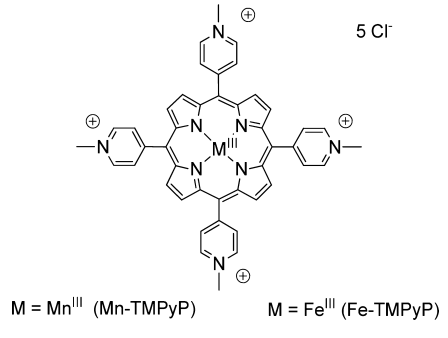
Enzymatic Digestion. Typically, the purified oligonucleotide carrying the damaged residue at G6, 5′-d[$CAGCT_{PMe}$(G+34)] (3 OD, ∼6 nmol), was incubated with 1 unit of nuclease P1 in 30 mM sodium acetate buffer, pH 5.4, for 5 min at 37 °C. The total reaction volume was 100 μL. The enzyme was extracted by dichloromethane, and the aqueous phase was injected on HPLC column for the isolation of the modified dinucleotide 5′-d[pT_{PMe}(G+34)] by chromatography. The collected fractions from several hydrolysis reactions were pooled and lyophilized. The purified dinucleotide of interest 5′-d[pT_{PMe}(G+34)] was stored dry at −20 °C before NMR analysis.

NMR. NMR spectra were collected using a Bruker Avance 600 spectrometer equipped with a 5 mm triple resonance inverse *Z*-gradient cryoprobe. All chemicals shifts for ^1H are relative to the residual HOD resonance (relative to tetramethylsilane). ^1H NMR spectra were collected at 298 K in D_2O. Presaturation was used to suppress the residual water signal. All of the ^1H and ^{13}C signals were assigned on the basis of chemical shifts, spin−spin coupling constants, splitting patterns, and signal intensities and by using ^1H−^1H total correlation spectroscopy (TOCSY), ^1H−^1H transverse rotating-frame Overhauser enhancement spectroscopy (T-ROESY), ^1H−^{13}C heteronuclear multiple-quantum correlation (HMQC), and ^1H−^{13}C heteronuclear multiple bond correlation (HMBC) experiments. The 2D T-ROESY spectra were acquired with a mixing time of 300 ms, and TOCSY spectra were recorded with a spin-lock time of 60 ms. Typically, 2048 t_2 data points were collected for 256 t_1 increments. Spectra processing was performed using Bruker Topspin software.

RESULTS AND DISCUSSION

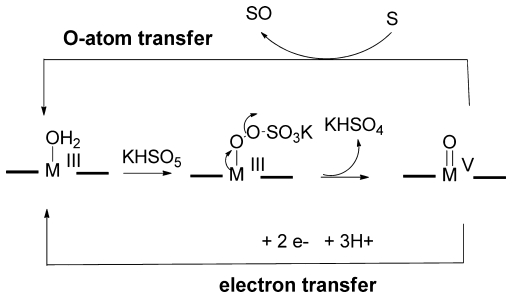
DNA Oxidative Damage by Manganese Porphyrin Associated to $KHSO_5$. The manganese porphyrin *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrinatomanganese(III) (Mn-TMPyP)/$KHSO_5$ is a powerful oxidation reagent (Scheme 1).[12] It has been used as a tool to identify DNA lesions under oxidative stress.[5−7,13−18] The mechanism of action of this oxidation catalyst is reminiscent of cytochrome P450 enzymes since it involves as an active species a high-valent metal-oxo entity in the form of a Mn^V=O entity (Scheme 2). On DNA, the metal-oxo porphyrin is able to perform oxidation

Scheme 1. Structure of the Metalloporphyrins Mn-TMPyP and Fe-TMPyP



dx.doi.org/10.1021/tx300319y | Chem. Res. Toxicol. 2012, 25, 2505−2512

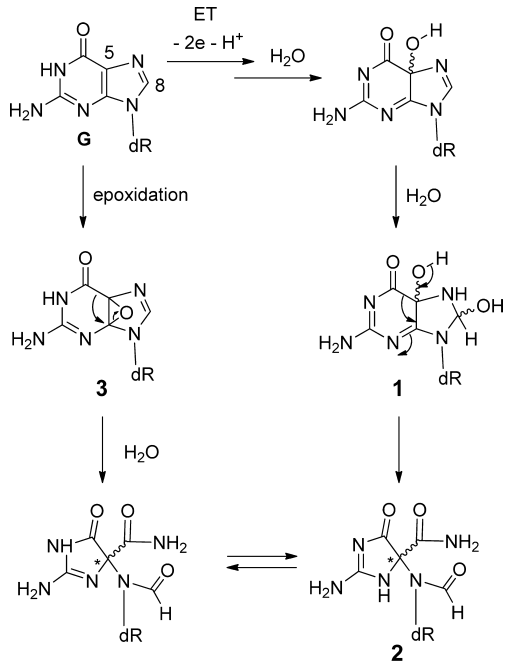
Scheme 2. Formation of an Active Metal-Oxo Porphyrin by Reaction of $KHSO_5$ with Metalloporphyrins Mn-TMPyP or Fe-TMPyP (M = Mn or Fe)[a]



[a]The metal-oxo entity (M^V=O) is a strong oxidant and is capable of oxygen atom transfer and/or electron transfer reactions like the active intermediate of heme enzymes. The porphyrin macrocycle is simplified. S refers to a substrate.

by electron transfer from guanine bases as well as oxygen atom transfer at 2-deoxyribose units.[16,18,19] Previously, among the products of guanine oxidation, we observed a product with a molecular mass corresponding to an increase of 34 amu as compared to the mass of the nondamaged DNA.[14,16] This compound, referred to as "G+34", was only identified by mass spectrometry, and a structure was proposed (**1** in Scheme 3) on

Scheme 3. Two Possible Mechanisms of Guanine Oxidation Leading to the G+34 Lesion[a]



[a]C5 and C8 of G are indicated. dR stands for 2′-deoxyribose unit. ET, electron transfer.

the basis of labeling studies implying guanine oxidation by electron transfer followed by the attack of a water molecule at carbon C5 of a guanine cationic intermediate. The aim of the present study was to further characterize this guanine lesion.

The G+34 lesion has also been observed by others, using different DNA-oxidizing agents, copper complexes,[20,21] nickel complexes,[22] epoxidizing agents,[9,10] and one-electron oxidants.[23] The 2-Ih structure (**2** in Scheme 3) was determined by NMR on the nucleobase[9] or nucleos(t)ides.[10] It was obtained with epoxidizing agents such as dimethyldioxirane[9] and *m*-chloroperbenzoic acid.[10] Resolution of diastereomers in the oxidized nucleos(t)ides and 5-mer oligonucleotide indicates that the oxidized base contains a chiral carbon.[10] The compound may be formed by two different mechanisms depending on the oxidizing reagent used: epoxidation of the C4−C5 bond of guanine[9,10] or guanine oxidation by electron transfer.[23] In both cases, it arises from a rearrangement of an initial intermediate, **1** in the case of electron transfer and **3** in the case of epoxidizing reagents (Scheme 3). The rearrangement is reminiscent of the acyl shift previously reported in the mechanism of formation of another oxidative guanine lesion, spiroiminodihydantoin (Sp),[24−28] which corresponds to a four-electron oxidation product of guanine instead of a two-electron oxidation product in the case of G+34. Alternatively, compound **2** was also recently described to form through the coordination of a nickel and a copper complex at N7 of guanine in the presence of $KHSO_5$ and H_2O_2, respectively.[21,22]

We decided to isolate the lesion of interest from the oxidation of small DNA duplex using a modified oligonucleotide with a single nuclease-resistant internucleotide link 5′ to the guanine residue targeted for damage. In the case of short oligonucleotide duplexes subjected to oxidation in vitro, the most accessible residues are located at external base pairs. The natural self-complementary oligonucleotide 5′-d(CAGCTG) affords an external CG base pair, which was the site of major oxidation by Mn-TMPyP/$KHSO_5$. From liquid chromatography coupled to negative electrospray mass spectrometry analysis (LC/ESI-MS) of the oxidation reaction, the oxidized strands contained either an oxidized guanine residue (guanine lesions) or an oxidized sugar (ribonolactone) (Figure S1 and Table S1 in the Supporting Information). The control experiment in the presence of $KHSO_5$ alone does not show any degradation. Oligonucleotide 5′-d(CAGCTG) contains two guanines (G3 and G6). The determination of the site of oxidation is possible by piperidine treatment of oxidized DNA. Most oxidized guanine residues are alkali-labile sites, and a DNA break is generated at the site of the oxidized guanine. Clearly, the 5-mer fragment 5′-d(CAGCT) sequence with a 3′-phosphate group, 5′-d(CAGCTp), was the major species from piperidine treatment of oxidized 5′-d(CAGCTG) after Mn-TMPyP/$KHSO_5$ oxidation (not shown). Consequently, we decided to position a nuclease-resistant link at the junction between T5 and terminal guanine residues to isolate oxidative damage products at G6.

Oxidation of the Natural 5′-d(CAGCTG) Oligonucleotide by Iron Porphyrin Associated to $KHSO_5$. The iron porphyrin *meso*-tetrakis(4-*N*-methylpyridiniumyl)-porphyrinatoiron(III) (Fe-TMPyP)/$KHSO_5$ is also a powerful oxidation reagent with similar oxidation properties as compared to its manganese analogue.[12] However, the two catalytic systems do not show exactly the same reactivity.[12] In the present work, oxidation of 5′-d(CAGCTG) with Fe-TMPyP/$KHSO_5$ system afforded an oxidation profile including a higher proportion of the so-called G+34 lesion (Figure S2 and Table S2 in the Supporting Information). The G+34 lesions obtained with the two different porphyrin catalysts were identical according to their retention times on HPLC trace (coinjection not shown). Therefore, in the present work, we decided to use the Fe-TMPyP/$KHSO_5$ system instead of the Mn-TMPyP/$KHSO_5$ system for the isolation of G+34.

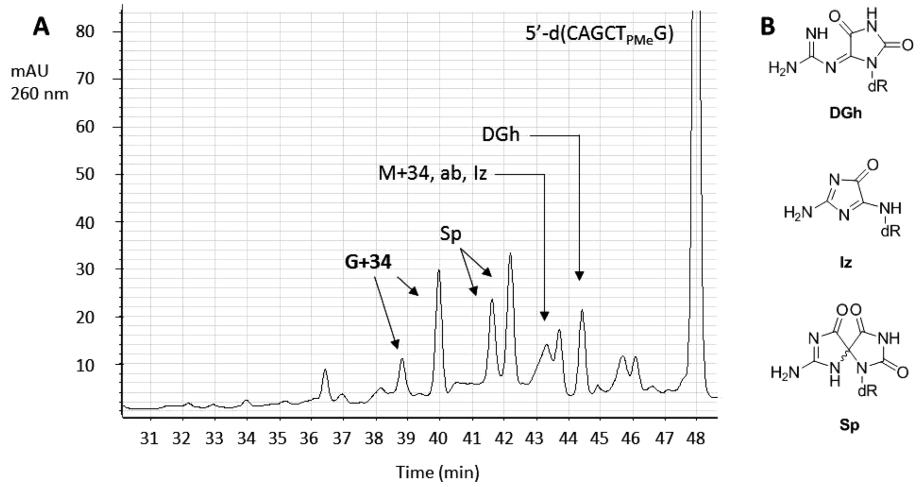


Figure 1. (A) HPLC trace of the oxidation of 5′d(CAGCT$_{PMe}$G) by Fe-TMPyP/KHSO$_5$ (C18 reverse phase). The single-stranded 6-mer elutes at 48 min. Oxidized oligonucleotides elute at different retention times. They carry different lesions, namely, G+34, spiroiminohydantoin (Sp), abasic site (ab), imidazolone (Iz), and oxidized guanidinohydantoin (DGh). (B) Structure of the observed DNA lesions resulting from guanine oxidation. "M+34" refers to a nonidentified lesion corresponding to an increase of 34 amu with respect to the nondamaged oligonucleotide.

Stereoisomerically Pure Single Methylphosphonate-Modified Oligonucleotides. In the context of seeking guanine oxidative lesions, care must be taken in the choice of the nuclease-resistant phosphodiester modification introduced in DNA. Phosphorothioates are transformed to phosphates under oxidative conditions[29] and were discarded. Methylphosphonate linkage was selected because it proved resistant under oxidation conditions, and single-methylphosphonate modified oligonucleotides can often be separated into P-diastereomerically pure forms. The synthesis of 5′-d(CAGCTG) oligonucleotide with a methylphosphonate link between T5 and G6, 5′-d(CAGCT$_{PMe}$G) was performed using the phosphoramidite approach, followed by chromatographic separation of P-diastereomers on an anion-exchange column. That procedure furnished fast-eluting (400 OD) and slow-eluting (338 OD) P-diastereomeric compounds, further referred to as the isomers I and II, respectively. The stereochemistry of the methylphosphonate link was not determined. According to T_m values of 22 and 29 °C for isomer I duplex and isomer II duplex, respectively, one may propose that isomer I is the R_P diastereomer and isomer II is the S_P diastereomer of methylphosphonate.[30]

Both duplexes composed of two strands of either isomer I or isomer II allowed us to observe the typical oxidation products of guanine at the external base pair position. Isomer I was used in the present study. It was analyzed by LC/ESI-MS. The doubly charged ion observed at m/z = 894.0 amu was consistent with the calculated m/z = 894.1 amu (Figure S3 in the Supporting Information).

Oxidation of Methylphosphonate-Modified 5′-d(CAGCT$_{PMe}$G) with Fe-TMPyP/KHSO$_5$ and Mn-TMPyP/KHSO$_5$ Systems. The duplex oligonucleotide composed of two strands of the self-complementary 5′-d(CAGCT$_{PMe}$G) (10 μM duplex DNA) was incubated in 50 mM phosphate buffer, pH 8, and NaCl 100 mM, with Fe-TMPyP (10 μM) and KHSO$_5$ (100 μM) at 0 °C for 10 min. The reaction was stopped by the addition of excess HEPES buffer[31] when 30% of the oligonucleotide was damaged. HPLC/ESI-MS analysis of the reaction showed several types of oxidized oligonucleotide strands eluting between 38 and 46 min before the residual nondamaged oligonucleotide at 48 min (Figure 1A). The lesions were attributed to known modified guanine residues, including spiroiminohydantoin (Sp), oxidized guanidinohydantoin (DGh), imidazolone (Iz), and abasic site (Figure 1B and Table 1). Most of them have been previously characterized from DNA oxidation by the Mn-TMPyP/KHSO$_5$ system[5−7,13,14] except the guanine lesion with a mass corresponding to an increase of 34 amu with respect to the molecular mass of G. It is referred to as the G+34 lesion. Among the oxidized oligonucleotide strands, two peaks appeared with a mass corresponding to G+34 (m/z = 911.1) at 38.8 and 40 min. They were referred to as fast- and slow-eluting G+34 isomers. The fast-eluting compound was in a lower amount as compared to the slow-eluting one (about 1/3 ratio) (Figure 1A). Another product with a m/z signal also corresponding to an increase of 34 amu was observed at 43−44 min, "M+34" in Figure 1A. However, the peak contained several nonseparated products and was not studied in the present work (Table 1). The two oxidized oligonucleotide strands (fast- and slow-eluting G+34 isomers) at 38.8 and 40 min were collected separately by preparative HPLC from a larger scale preparation.

Table 1. LC/ESI-MS Analysis of the Oxidation of 5′-d(CAGCT$_{PMe}$G) by Fe-TMPyP/KHSO$_5$

Rt (min)	m/z (z = 2) obsd	lesion	m/z (z = 2) calcd
38.8	911.2	G+34	911.1
40	911.0	G+34	911.1
41.5	909.9	Sp	910.1
42.2	909.9	Sp	910.1
43−44	911.1	M+34	911.1
	874.5	Iz	874.6
	827.4	abasic site	827.6
44.4	895.8	G+4	896.1
48	894.0	5′-d(CAGCT$_{PMe}$G) (isomer I)	894.1

For comparison, the reaction of Mn-TMPyP/KHSO$_5$ system with 5′-d(CAGCT$_{PMe}$G) under the same experimental conditions led to the same products but in different proportions (Figure S4 and Table S3 in the Supporting Information). As observed on natural oligonucleotides, DGh was the major

product of guanine oxidation, while G+34 was a minor compound. Besides, only the slow-eluting G+34 oligonucleotide could be detected at 40 min.

Hydrolysis of the Oxidized Methylphophonate Oligonucleotides, 5′-d[CAGCT$_{PMe}$(G+34)]. First, the nondamaged methylphosphonate oligonucleotide, 5′-d(CAGCT$_{PMe}$G) (isomer I), was reacted with nuclease P1 at 37 °C in 30 mM acetate buffer, pH 5.3, for 5 min. The rapid and complete hydrolysis led to the formation of nucleoside dC, nucleotides dGMP and dAMP with a 5′-phosphate group, as well as the dinucleotide carrying the methylphosphonate link with a 5′-phosphate group, 5′-d(pT$_{PMe}$G) (Rt = 34 min, Figure 2A). Hydrolysis of

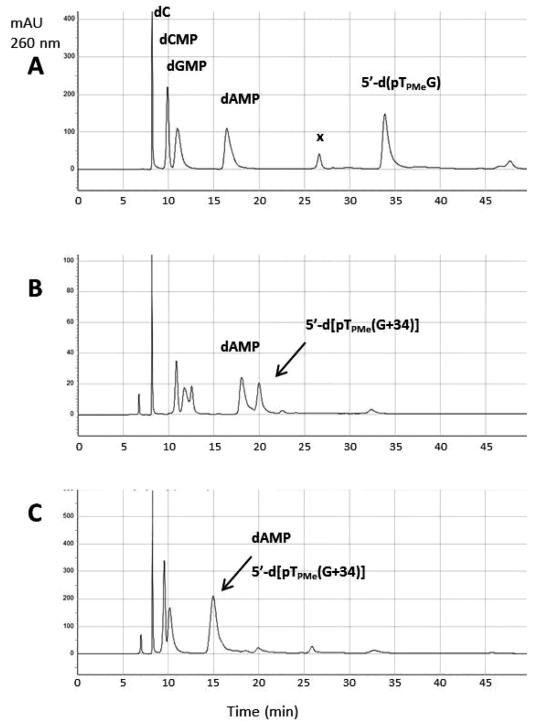


Figure 2. HPLC trace (λ = 260 nm, C18 reverse phase) of the nuclease P1 hydrolysis of 5′-d(CAGCT$_{PMe}$G) (A) and slow-eluting and fast-eluting G+34 lesion carrying oligonucleotide strands (B and C), respectively. The hydrolysis is complete, and the full-length ODN is absent from the chromatogram. The nucleoside dimer carrying the methylphosphonate bridge is observed at 35 (A) and 20 min (B) and coelutes with dAMP at 15 min (C). X = impurity.

each of the fast- and slow-eluting G+34 lesion carrying oligonucleotides led to the corresponding nuclease-resistant dinucleotides but carrying the oxidized guanine lesion, namely, d[T$_{PMe}$(G+34)] with a 5′-phosphate group, 5′-d[pT$_{PMe}$(G+34)] (Figure 2B,C). The elution of the modified dinucleotides followed the same order as the full-length oligonucleotides from which they originated. The dinucleotide obtained from the fast-eluting modified oligonucleotide eluted faster as compared to that originating from the slow-eluting oligonucleotide on the reverse-phase column.

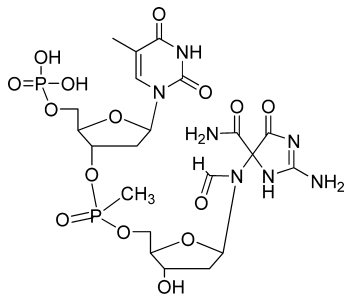
The dinucleotide 5′-d[pT$_{PMe}$(G+34)] carrying the methylphosphonate nuclease-resistant link and the oxidized guanine G+34 lesion was isolated in the case of the slow-eluting major isomer oligonucleotide (Figure 2B) and analyzed by ^{1}H and ^{13}C NMR (Tables 2 and 3). Importantly, a ^{1}H,^{13}C HMBC

Table 3. ^{13}C Resonances (δ, ppm) Extracted from Correlations in ^{1}H/^{13}C HSQC and ^{1}H/^{13}C HMBC Experiments for 5′-d[pT$_{PMe}$(G+34)] Dinucleotide in D$_2$O

	2-deoxyribose					thymine	G+34
	C1′	C2′	C3′	C4′	C5′	CH$_3$	C5
T	85.0	37.0	77.1	84.2	64.2	11.7	
G+34	88.2	38.4	70.9	84.2	65.7		79.2

cross-peak was observed between a carbon resonance at 79.2 ppm (attributed to the quaternary carbon) and a proton resonance at 8.76 ppm (attributed to the aldehyde group) on the modified base. Furthermore, a nuclear Overhauser effect (NOE) between the proton at 8.76 ppm from the modified base and the H2′H2″ protons of the 3′-sugar at 2.57−2.64 ppm confirms that the proton at 8.76 ppm belongs to the modified nucleoside. The proposed structure of the lesion is shown in Scheme 4. A minor form of this compound can be

Scheme 4. Structure of 5′-d[pT$_{PMe}$(G+34)] Dinucleotide Modified with a Nuclease-Resistant Methylphosphonate and Carrying the G+34 Lesion, Namely, 2-Ih



detected by an exchange correlation between the H-signal at 8.76 ppm with a signal at 8.22 ppm (accounting for 7−8%). As previously proposed, this minor form might correspond to a rotational isomer of the formyl group.[10]

The fast- and slow-eluting oligonucleotides at retention times of 38.8 and 40 min, respectively (Figure 1A), and/or dinucleotides carrying a G+34 lesion (Figure 2B,C) correspond probably to two diastereoisomers due to the presence of a new chiral carbon at the modified guanine residue. The stereochemistry of the new chiral center on the oxidized guanine residue could not be determined. This G+34-modified base

Table 2. ^{1}H NMR Data (δ, ppm) for 5′-p-d[pT$_{PMe}$(G+34)] Dinucleotide in D$_2$O

	2-deoxyribose					thymine		G+34	
	H1′	H2′, H2″	H3′	H4′	H5′, H5″	CH$_3$	H6	CHO	P-CH$_3$
T	6.36 (dd), $^{3}J_{HH}$ = 5.8 Hz, $^{3}J_{HH}$ = 8.3 Hz	2.56−2.45	5.14	4.38	4.05	1.90	7.73		1.68 (d), $^{3}J_{HP}$ = 17.7 Hz
G+34	5.66 (dd), $^{3}J_{HH}$ = 3.2 Hz, $^{3}J_{HH}$ = 7.1 Hz	2.64−2.57	4.44	4.11	4.11−4.18			8.76	

dx.doi.org/10.1021/tx300319y | Chem. Res. Toxicol. 2012, 25, 2505−2512

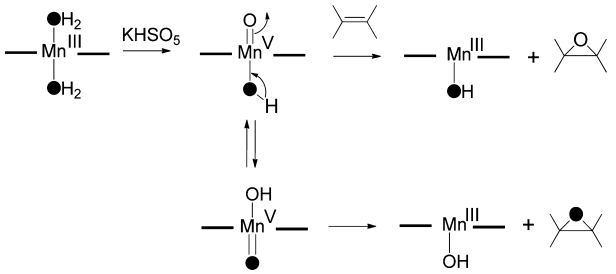
corresponds to the previously characterized 2-Ih.[9,10] As in the present work, the two isomers did not form in equal amounts.[10]

Mechanism of Formation of G+34 Lesion. The mechanism of formation of the G+34 lesion with Mn-TMPyP/$KHSO_5$ and Fe-TMPyP/$KHSO_5$ as oxidizing agent was investigated by labeling studies. According to Scheme 3, two new O-atoms are incorporated in the oxidized guanine residue.

It is possible to discriminate between mechanisms involving electron transfer and oxygen atom transfer by the analysis of the origin of the oxygen atoms incorporated in G+34 when the reaction is carried out in labeled water. Electron transfer should lead to the incorporation of two O-atoms from H_2O. O-atom transfer should lead to incorporation of the O-atom from the high-valent metal-oxo species of the activated metal-oxo porphyrin at the former C5 of guanine.

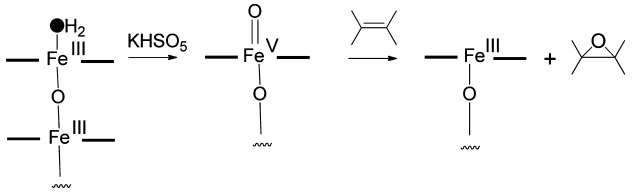
Manganese porphyrin undergoes oxo-hydroxo tautomerism at the Mn^V=O in water.[19,32] Thus, O-atom transfer in labeled water should lead to the incorporation of an O-atom at the former C5 of guanine in the form of $^{16}O/^{18}O$ in a 50/50 ratio (Scheme 5). In the case of iron porphyrin, the formation of μ-oxo species in pH 8-buffered solution precludes the oxo-hydroxo tautomerism. Thus, O-atom transfer mediated by Fe^V=O species should lead to the incorporation of one ^{16}O-atom at the former C5 of guanine (Scheme 6). On the other hand, the oxygen atom incorporated at the former C8 of guanine should always originate from the attack of a water molecule after modification of the guanine heterocycle by the oxidation event, whatever the porphyrin used.

Scheme 5. Oxygen Atom Transfer Mediated by Manganese-Oxo Porphyrin in $H_2{}^{18}O$ and Oxo-Hydroxo Tautomerism at the Mn^V=O Species[a]



[a]The porphyrin macrocycle is simplified. Black and white O-atom corresponds to ^{18}O- and ^{16}O-atom, respectively.

Scheme 6. Oxygen Atom Transfer Mediated by Iron-Oxo Porphyrin in $H_2{}^{18}O$[a]



[a]The porphyrin macrocycle is simplified. Black and white O-atom corresponds to ^{18}O- and ^{16}O-atom, respectively.

Thus, the nonlabeled mass signal for the G+34-modified natural oligonucleotide at m/z = 911.4 amu (z = 2) observed in nonlabeled water should appear, in the case of Mn-TMPyP/$KHSO_5$ oxidant, as a signal at m/z = 913.4 amu in labeled water for electron transfer mechanism (incorporation of two ^{18}O-atoms at former C5 and C8 carbons of guanine), while the mass signal should be composed of two species of equal intensity with m/z = 912.4 (one ^{18}O-atom incorporated at former C8) and 913.4 amu (for 2 ^{18}O-atoms incorporated at former C5 and C8) in labeled water. On the other hand, for Fe-TMPyP/$KHSO_5$ oxidant, an electron transfer mechanism should lead to the incorporation of two ^{18}O-atoms in G+34 as for Mn-TMPyP/$KHSO_5$, giving a mass signal at m/z = 913.4 amu in labeled water. An oxygen atom transfer mechanism should lead to a single mass signal at m/z = 912.4 amu (incorporation of one ^{16}O-atom at former C5 and one ^{18}O-atom at former C8).

Oxidation of the natural 5′-d(CAGCTG) oligonucleotide was carried out in normal and in labeled water with Mn-TMPyP/$KHSO_5$ and with Fe-TMPyP/$KHSO_5$. LC/ESI-MS analysis of the reaction with Mn-TMPyP showed that the G+34 carrying oligonucleotide with an m/z signal at 911.4 amu (z = 2) in $H_2{}^{16}O$ (Figure 3A) appeared as a mixture of two species of equal abundance in $H_2{}^{18}O$ (Figure 3B). The lesion incorporated one O-atom from water and one O-atom from $KHSO_5$ (m/z = 912.4 amu) or two O-atoms from labeled water (m/z = 913.4 amu). For Fe-TMPyP, the G+34-carrying oligonucleotide showed an in-line mass spectrum with a single mass signal at m/z = 912.4 amu when the reaction was performed in $H_2{}^{18}O$ (Figure 3C). These results are compatible with a mechanism involving an epoxide intermediate (**3** in Scheme 3) for both porphyrins. It was previously reported that the G+34 lesion did not undergo exchange of O-atoms with solvent,[10] and we also carried out control experiments consisting of incubation of the labeled lesion under different conditions for several hours (water, chromatography solvents), and no change in labeling occurred (not shown).

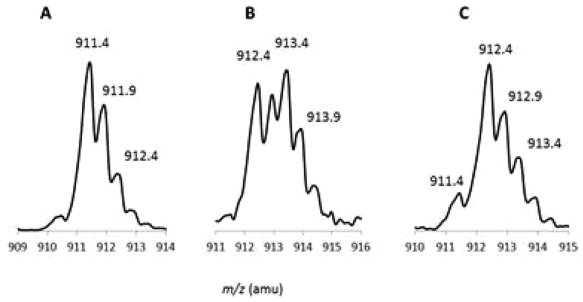


Figure 3. In-line mass spectrum of oxidized oligonucleotide strands carrying the G+34 lesion in the oxidation of 5′-d(CAGCTG) with Fe- and/or Mn-TMPyP/$KHSO_5$ in $H_2{}^{16}O$ (A), Mn-TMPyP/$KHSO_5$ in $H_2{}^{18}O$ (B), and Fe-TMPyP/$KHSO_5$ in $H_2{}^{18}O$ (C). Doubly charged ions are observed (z = 2). Corresponding HPLC traces are shown in Figure S1 (Mn) and Figure S2 (Fe) in the Supporting Information.

The last guanine base (G6 residue) of the short duplex formed with 5′-d(CAGCTG) is perfectly accessible for the high-valent metal-oxo form of the porphyrins. In the chemistry of oxygen atom transfer by metal-oxo porphyrins, a close contact between the metal-oxo and the double-bond that is the site of epoxidation is required. The accessibility of the external guanine base on the chosen oligonucleotide may explain the epoxidation mechanism.

dx.doi.org/10.1021/tx300319y | Chem. Res. Toxicol. 2012, 25, 2505−2512

CONCLUSION

The oxidation of an oligonucleotide containing a single nuclease-resistant internucleotide link, a stereoisomerically pure methylphosphonate, by Mn-TMPyP/$kHSO_5$ and Fe-TMPyP/$KHSO_5$ allowed the isolation and characterization of a guanine lesion corresponding to an increase of mass of 34 amu as compared to guanine (G+34). The lesion was attributed to 2-Ih. Enzymatic digestion of the damaged oligonucleotide afforded, apart from the nucleotide monomer pool, a unique dinucleotide doubly modified with a methylphosphonate and an oxidized guanine base that was suitable for NMR analysis. The method can be applied to the study of any DNA lesion. More importantly, the method can be extended to the analysis of DNA damage in a sequence context. Any preselected residue in a DNA sequence may be individually analyzed for damage at a molecular level by the easy introduction of a single nuclease-resistant link adjacent to the target nucleoside.

ASSOCIATED CONTENT

Supporting Information

HPLC traces, structures, and tables of LC/ESI-MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +33(0)561333146. Fax: +33(0)561553003. E-mail: genevieve.pratviel@lcc-toulouse.fr.

Funding

This work was partly supported by a joint program between CNRS, France, and the Polish Academy of Sciences (Polonium 20110NE) and by ANR 06SEST11.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Gauthier Foucras and Elsa Moreau for technical help; Dr. Yannick Coppel, Laboratoire de Chimie de Coordination, CNRS, for NMR analyses; and Dr. Chantal Zedde, service commun de chromatographie, Institut de Chimie, Toulouse, for the purification of the oligonucleotides. Dr. Milena Sobczak provided excellent technical assistance in synthesizing methylphosphonate oligonucleotides. We thank Prof. Wojciech J. Stec for invaluable discussions and permanent interest in the project.

REFERENCES

(1) Cadet, J., Douki, T., and Ravanat, J. L. (2010) Oxidatively generated base damage to cellular DNA. *Free Radical Biol. Med. 49*, 9–21.

(2) Dedon, P. C. (2008) The chemical toxicology of 2-deoxyribose oxidation in DNA. *Chem. Res. Toxicol. 21*, 206–219.

(3) Delaney, J. C., and Essigmann, J. M. (2008) Biological properties of single chemical-DNA adducts: A twenty year perspective. *Chem. Res. Toxicol. 21*, 232–252.

(4) Jamieson, E. R., and Lippard, S. J. (1999) Structure, recognition, and processing of cisplatin-DNA adducts. *Chem. Rev. 99*, 2467–2498.

(5) Chworos, A., Coppel, Y., Dubey, I., Pratviel, G., and Meunier, B. (2001) Guanine oxidation: NMR characterization of a dehydro-guanidinohydantoin residue generated by a 2e-oxidation of d(GpT). *J. Am. Chem. Soc. 123*, 5867–5877.

(6) Seguy, C., Pratviel, G., and Meunier, B. (2001) Characterization of an oxaluric acid derivative as a guanine oxidation product. *Chem. Commun.*, 2116–2117.

(7) Chworos, A., Seguy, C., Pratviel, G., and Meunier, B. (2002) Characterization of the dehydro-guanidinohydantoin oxidation product of guanine in a dinucleotide. *Chem. Res. Toxicol. 15*, 1643–1651.

(8) Perrier, S., Hau, J., Gasparutto, D., Cadet, J., Favier, A., and Ravanat, J. L. (2006) Characterization of lysine-guanine cross-links upon one-electron oxidation of a guanine-containing oligonucleotide in the presence of a trilysine peptide. *J. Am. Chem. Soc. 128*, 5703–5710.

(9) Ye, W., Sangaiah, R., Degen, D. E., Gold, A., Jayaraj, K., Koshlap, K. M., Boysen, G., Williams, J., Tomer, K. B., and Ball, L. M. (2006) A 2-iminohydantoin from the oxidation of guanine. *Chem. Res. Toxicol. 19*, 506–510.

(10) Ye, W., Sangaiah, R., Degen, D. E., Gold, A., Jayaraj, K., Koshlap, K. M., Boysen, G., Williams, J., Tomer, K. B., Mocanu, V., Dicheva, N., Parker, C. E., Schaaper, R. M., and Ball, L. M. (2009) Iminohydantoin lesion induced in DNA by peracids and other epoxidizing oxidants. *J. Am. Chem. Soc. 131*, 6114–6123.

(11) Romera, C., Sabater, L., Garofalo, A., Dixon, I. M., and Pratviel, G. (2010) Interaction of cationic nickel and manganese porphyrins with the minor groove of DNA. *Inorg. Chem. 49*, 8558–8567.

(12) Meunier, B., Robert, A., Pratviel, G., and Bernadou, J., Eds. (2000) *Metalloporphyrins in Catalytic Oxidation and Oxidative DNA Damage*, Vol. 4, Academic Press, San Diego, CA.

(13) Vialas, C., Pratviel, G., Claparols, C., and Meunier, B. (1998) Efficient oxidation of 2′-deoxyguanosine by Mn-TMPyP/$KHSO_5$ to imidazolone dIz without formation of 8-oxo-dG. *J. Am. Chem. Soc. 120*, 11548–11553.

(14) Vialas, C., Claparols, C., Pratviel, G., and Meunier, B. (2000) Guanine oxidation in double-stranded DNA by Mn-TMPyP/$KHSO_5$: 5,8-Dihydroxy-7,8-dihydroguanine residue as a key precursor of imidazolone and parabanic acid derivatives. *J. Am. Chem. Soc. 122*, 2157–2167.

(15) Angeloff, A., Dubey, I., Pratviel, G., Bernadou, J., and Meunier, B. (2001) Characterization of a 5′-aldehyde terminus resulting from the oxidative attack at C5′ of a 2-deoxyribose on DNA. *Chem. Res. Toxicol. 14*, 1413–1420.

(16) Mourgues, S., Kupan, A., Pratviel, G., and Meunier, B. (2005) Use of short duplexes for the analysis of the sequence-dependent cleavage of DNA by a chemical nuclease, a manganese porphyrin. *ChemBioChem 6*, 2326–2335.

(17) Kupan, A., Sauliere, A., Broussy, S., Seguy, C., Pratviel, G., and Meunier, B. (2006) Guanine oxidation by electron transfer: one- versus two-electron oxidation mechanism. *ChemBioChem 7*, 125–133.

(18) Pitié, M., and Pratviel, G. (2010) Activation of DNA carbon-hydrogen bonds by metal complexes. *Chem. Rev. 110*, 1018–1059.

(19) Pitié, M., Bernadou, J., and Meunier, B. (1995) Oxidation at carbon-1′ of DNA deoxyriboses by the Mn-TMPyP/$KHSO_5$ system results from a cytochrome P-450-type hydroxylation reaction. *J. Am. Chem. Soc. 117*, 2935–2936.

(20) Li, L., Murthy, N. N., Telser, J., Zakharov, L. N., Yap, G. P., Rheingold, A. L., Karlin, K. D., and Rokita, S. E. (2006) Targeted guanine oxidation by a dinuclear copper(II) complex at single stranded/double stranded DNA junctions. *Inorg. Chem. 45*, 7144–7159.

(21) Fleming, A. M., Muller, J. G., Ji, I., and Burrows, C. J. (2011) Characterization of 2′-deoxyguanosine oxidation products observed in the Fenton-like system Cu(II)/H_2O_2/reductant in nucleoside and oligodeoxynucleotide contexts. *Org. Biomol. Chem. 9*, 3338–3348.

(22) Ghude, P., Schallenberger, M. A., Fleming, A. M., Muller, J. G., and Burrows, C. J. (2011) Comparison of transition metal-mediated oxidation reactions of guanine in nucleoside and single-stranded oligodeoxynucleotide contexts. *Inorg. Chim. Acta 369*, 240–246.

(23) Rokhlenko, Y., Geacintov, N. E., and Shafirovich, V. (2012) Lifetimes and reaction pathways of guanine radical cations and neutral guanine radicals in an oligonucleotide in aqueous solutions. *J. Am. Chem. Soc. 134*, 4955–4962.

(24) Luo, W., Muller, J. G., Rachlin, E. M., and Burrows, C. J. (2000) Characterization of spiroiminodihydantoin as a product of one-electron oxidation of 8-oxo-7,8-dihydroguanosine. *Org. Lett. 2*, 613–616.
(25) Niles, J. C., Wishnok, J. S., and Tannenbaum, S. R. (2001) Spiroiminodihydantoin is the major product of the 8-oxo-7,8-dihydroguanosine reaction with peroxynitrite in the presence of thiols and guanosine photooxidation by methylene blue. *Org. Lett. 3*, 963–966.
(26) Adam, W., Arnold, M. A., Grune, M., Nau, W. M., Pischel, U., and Saha-Moller, C. R. (2002) Spiroiminodihydantoin is a major product in the photooxidation of 2′-deoxyguanosine by the triplet states and oxyl radicals generated from hydroxyacetophenone photolysis and dioxetane thermolysis. *Org. Lett. 4*, 537–540.
(27) Slade, P. G., Priestley, N. D., and Sugden, K. D. (2007) Spiroiminodihydantoin as an oxo-atom transfer product of 8-oxo-2′-deoxyguanosine oxidation by chromium(V). *Org. Lett. 9*, 4411–4414.
(28) Hailer, M. K., Slade, P. G., Martin, B. D., and Sugden, K. D. (2005) Nei deficient *Escherichia coli* are sensitive to chromate and accumulate the oxidized guanine lesion spiroiminodihydantoin. *Chem. Res. Toxicol. 18*, 1378–1383.
(29) Chworos, A., Arnaud, P., Zakrzewska, K., Guga, P., Pratviel, G., Stec, W., and Meunier, B. (2004) Comparison of the cleavage profiles of oligonucleotide duplexes with or without phosphorothioate linkages by using a chemical nuclease probe. *J. Biol. Inorg. Chem. 9*, 374–384.
(30) Reynolds, M. A., Hogrefe, R. I., Jaeger, J. A., Schwartz, D. A., Riley, T. A., Marvin, W. B., Daily, W. J., Vaghefi, M. M., Beck, T. A., Knowles, S. K., Klem, R. E., and Arnold, L. J., Jr. (1996) Synthesis and thermodynamics of oligonucleotides containing chirally pure R(P) methylphosphonate linkages. *Nucleic Acids Res. 24*, 4584–4591.
(31) Bernadou, J., Pratviel, G., Bennis, F., Girardet, M., and Meunier, B. (1989) Potassium monopersulfate and a water-soluble manganese porphyrin complex, [Mn(TMPyP)](OAc)5, as an efficient reagent for the oxidative cleavage of DNA. *Biochemistry 28*, 7268–7275.
(32) Bernadou, J., and Meunier, B. (1998) Oxo-hydroxo tautomerism as useful mechanistic tool in oxygenation reactions catalysed by water-soluble metalloporphyrins. *Chem. Commun.*, 2167–2173.