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Generation of Guanine – Thymidine Cross-links in DNA by Peroxynitrite/Carbon Dioxide

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Abstract

Nitrosoperoxycarbonate derived from the combination of carbon dioxide and peroxynitrite, is an important chemical mediator of inflammation. In aqueous solutions, it rapidly decomposes to the reactive species $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals that are known to initiate the selective oxidation and nitration of guanine in DNA. We have previously demonstrated that the reactions of carbonate radical anions with guanine in 2′-deoxyoligoribonucleotides generate a previously unknown intrastrand cross-linked guanine-thymine product G*-T* with a covalent bond between the C8 (G*) and thymine N3 (T*) atoms (Crean *et al*., *Nucleic Acids Res*., **2008**, *36*, 742–755). In this work we demonstrate that G*-T* cross-linked products are also formed when peroxynitrite (0.1 mM) reacts with native DNA in aqueous solutions (pH 7.5–7.7) containing 25 mM carbon dioxide/bicarbonate, in addition to the well known nitration/oxidation products of guanine such as 8-nitroguanine (8-nitroG), 5-guanidino-4-nitroimidazole (NIm), 8-oxo-7,8-dehydroguanine (8-oxoG) and spiroiminodihydantoin (Sp). The yields of these products, after enzymatic digestion with P1 nuclease and alkaline phosphatase to the nucleotide level, and reversed phase HPLC separation, were compared with those obtained with the uniformly, isotopically labeled ^{15}N,^{13}C-labeled 2′-deoxy oligoribonucleotides 5′-dGpT and 5′-dGpCpT. The d(G*pT*) and d(G*-T*) cross-linked products derived from the di- and tri-oligonucleotides, respectively, were used as standards for identifying the analogous lesions in calf thymus DNA by isotope dilution LC-MS/MS methods in the selected reaction-monitoring mode. The Nim and 8nitroG are the major products formed (~ 0.05% each), and lesser amounts of 8-oxoG (~ 0.02%), and d(G*pT*) and d(G*-T*) enzymatic digestion products (~ 0.002% each) were found. It is shown that the formation of d(G*pT*) enzyme digestion product can arise only from intrastrand cross-links, whereas d(G*-T*) can arise from both interstrand and intrastrand cross-linked products.

Introduction

In human cells, oxidative stress is associated with the inflammatory response that plays an important role in the progression of human cancers (1, 2). This response is triggered by infections and environmental factors such as tobacco smoke and ionizing radiation (3). Under inflammatory conditions, neutrophils and macrophages are activated and overproduce nitric oxide and superoxide radical anions, which rapidly combine to form the highly toxic peroxynitrite, $ONOO^-$ (4, 5). The NO molecules that are overproduced by inflammatory cells can freely diffuse across cellular membranes and develop oxidative stress inside cells, in part due to elevated levels of $O_2^{\bullet-}$ radicals (6–8). The toxic effects of peroxynitrite are most likely associated with free radicals derived from its decomposition (6). In neutral

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solutions, the decomposition of $ONOO^-$ occurs via intermediate formation of the conjugated peroxynitrous acid, ONOOH, that decomposes to form $^{\bullet}OH$ and $^{\bullet}NO_2$ radicals (9). However, in vivo, the formation of $^{\bullet}OH$ radicals plays a minor role only, because of the rapid reaction of $ONOO^-$ with CO_2 (10), which is ubiquitous in tissues due to the high levels of bicarbonate (10–30 mM) in biological fluids (6). This reaction results in the formation of a highly unstable nitrosoperoxycarbonate species, $ONOOCO_2^-$, that rapidly decomposes to form $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (11).

The primary target of oxidative damage in DNA is guanine, the most easily oxidizable DNA base (12). In vitro experiments have shown that guanine lesions generated by decomposition products of nitrosoperoxycarbonate include both nitration and oxidation products (13, 14). The nitration of guanine is known to generate two major products, 8-nitroguanine (8-nitroG)1 (15, 16) and 5-guanidino-4-nitroimidazole (NIm) (17, 18) lesions (Figure 1). Other oxidation products generated by peroxynitrite include 8-oxo-7,8-dehydroguanine (8-oxoG), the diastereomeric spiroiminodihydantoin (Sp), guanidinohydantoin (Gh), and imidazolone/oxazolone lesions (19, 20).

Among the radicals arising from the spontaneous homolysis of nitrosoperoxycarbonate, only $CO_3^{\bullet-}$ radicals (reduction potential of E^o = 1.59 V vs NHE (21)) can directly react with guanine bases in DNA (22). In contrast, the $^{\bullet}NO_2$ radical is a milder oxidant with E^o = 1.04 V vs NHE (23) that does not react with guanine (24). Our laser flash photolysis experiments have shown that $CO_3^{\bullet-}$ radicals selectively oxidize guanine in DNA by a one-electron abstraction mechanism that gives rise to the guanine neutral radicals (22, 25). We found that further reactions of guanine radicals are base sequence-dependent and in the 5′-..GT.. and 5′-..GCT.. sequence contexts produce novel intrastrand cross-links between guanine an thymine bases (5′-dG*pT*, and 5′-dG*pCpT*) together with the other, known guanine oxidation products (8-oxoG, Sp, Gh) shown in Figure 1 (26, 27).

In this work, we show that such lesions are also formed in native double-stranded DNA exposed to peroxynitrite in aqueous carbon dioxide/bicarbonate solutions, and we also provide estimates of the levels of G*-T* cross-linked products formed relative to other lesions such as 8-oxoG, Nim, and 8-nitroG. The distributions of the end-products, intrastrand G*-T* cross-links, 8-oxoG, Sp, 8-nitroG and NIm lesions, are markedly different in the oligonucleotides 5′-d(GpT) and 5′-d(GpCpT) and in calf thymus DNA. This difference in the distributions of the end-products in single-stranded di- and tri-nucleotides and calf thymus DNA is discussed in terms of the competition between (1) nucleophilic addition reactions of water to guanine radicals that result in the formation of 8-oxoG, and the addition of T(N3) to G(C8) radicals to form the G*-T* cross-links, and (2) competing radical-radical combination reactions (addition of $CO_3^{\bullet-}$ radicals to form 8-oxoG and $^{\bullet}NO_2$ radicals) that generate 8-nitroG and NIm lesions.

Experimental Procedures

Materials

All chemicals (analytical grade) were used as received. Snake venom phosphodiesterase was purchased from Pharmacia (Piscataway, NJ); nuclease P1 and alkaline phosphatase were from Sigma Chemical (St. Louis, MO). Sodium peroxynitrite solutions were obtained from Cayman Chemical (Ann Arbor, MI); the concentrations of $ONOO^-$ were determined in 0.01

[1]The abbreviations used are: 8-nitro-G, 8-nitroguanine; NIm, 5-guanidino-4-nitroimidazole; 8-oxo-G, 8-oxo-7,8-dihydroguanine; 5′-d(G*-T*), guanine-thymine cross-link with a covalent bond between the C8 (G*) and thymine N3 (T*) atoms; 5′-d(G*pT*), intrastrand guanine-thymine cross-link resistant to nuclease P1; 5′-d(G*pCpT*); cyclic intrastrand guanine-thymine cross-link; R, 2-deoxy-β-D-ribofuranosyl; Gh, guanidinohydantoin; Sp, spiroiminodihydantoin.

M NaOH solution using $\varepsilon_{302} = 1.67\times10^3\ M^{-1}cm^{-1}$ (28). Calf thymus DNA was from Sigma Chemical; the concentrations of nucleobases in DNA solutions were determined using $\varepsilon_{260} = 6.6\times10^3\ M^{-1}cm^{-1}$ (29). The di- and trinucleotides were purchased from Sigma Genosys (Woodlands, TX) and were purified and desalted using reversed-phase HPLC; their integrity was confirmed by LC/MS methods. The uniformly labeled 2′-deoxyguanosine, d($^{15}N_5$-G) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Oxidation of DNA by Flow-Mixing with Peroxynitrite

Equal volumes (0.5 mL) of DNA and peroxynitrite solutions were flow-mixed using two manually driven syringes connected to a mixer (30). The first syringe contained the oligonucleotide (200 nmol) or calf thymus DNA (200 nmol nucleobases) in 40 mM phosphate solution (pH 7.5) containing 50 mM $NaHCO_3$; the second syringe contained a peroxynitrite solution prepared by dilution of the ~35 mM peroxynitrite stock solution in 300 mM NaOH. After mixing, the changes in the pH of the solutions were determined and did not vary from the original solutions by more than 0.1 – 0.2 pH units. Rapid flow-mixing methods were employed because the lifetime of peroxynitrite in 25 mM $CO_2/NaHCO_3$ solution (pH 7.5 – 7.7) is only 50 – 80 ms (10). Samples of the oxidatively damaged di- and trinucleotides were subjected to reversed-phase HPLC analysis. The oxidatively damaged calf thymus DNA samples were desalted using Amicon Ultra-0.5 (MWCO = 10 kDa) centrifugal filters (Millipore, Billerica, MA), evaporated to dryness and subjected to enzymatic digestion to the nucleotide level.

HPLC Isolation of Oxidation Products

An Agilent 1200 Series LC system (quaternary LC pump with degasser, thermostated column compartments, and diode array detector) was used to separate the oxidatively modified di- and trinucleotides using an analytical (250 mm × 4.6 mm i.d.) Microsorb-MV C18 column (Varian, Walnut Creek, CA) employing a 1 – 45% linear gradient of methanol in 20 mM ammonium acetate in water (pH 7) for 60 min at a flow rate of 1 mL/min. The fractions collected were evaporated under vacuum, dissolved in water and subjected to LC-MS/MS analysis.

Synthesis of the Isotope-Labeled Standards

The ^{15}N-labeled 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxod($^{15}N_5$-G) was synthesized by oxidation of d($^{15}N_5$-G) using Fe^{2+}/H_2O_2 Fenton reagent (31); the 8-nitro-2′-deoxyguanosine (8-nitrod($^{15}N_5$-G)), and 5-guanidino-1-[2-deoxy-*β*-D-*erythro*-pentofuranoyl]-4-nitroimidazole d($^{15}N_5$-NIm), were prepared by photochemical nitration of d($^{15}N_5$-G) (32). The 5′-d($^{15}N_5$,$^{13}C_{10}$-G)pT and 5′-d($^{15}N_5$,$^{13}C_{10}$-G)pCpT sequences were synthesized by standard automated phosphoramidite chemistry technique using uniformly labeled 2′-deoxyguanosine phosporamidites (Cambridge Isotope Laboratories), and then oxidized by photochemically generated $CO_3^{\bullet-}$ radicals to form the 5′-d($^{15}N_5$,$^{13}C_{10}$-G)*pT* and 5′-d($^{15}N_5$,$^{13}C_{10}$-G)*pCpT* cross-linked products (26). All isotope-labeled standards were isolated, purified, and desalted by HPLC methods and their identities were confirmed by LC/MS/MS methods.

Isotope Dilution LC-MS/MS Detection of Guanine Lesions in DNA

The 200 nmol samples of oxidized calf thymus DNA were spiked with 2 – 4 pmol of the selected isotope labeled standard and digested with nuclease P1 (2 units) in 100 μL of 30 mM sodium acetate buffer (pH 5.2) containing 0.1 mM $ZnCl_2$ overnight at 37 °C; following digestion, the samples were treated with 2 units of alkaline phosphatase and 4 milliunits of snake venom phosphodiesterase in a Tris-HCl (~ 100 mM) buffer solution (pH 8.2) containing 10 mM $MgCl_2$ for 2 h at 37 °C. The incubation mixtures were passed through

Amicon Ultra-0.5 (MWCO = 10 kDa) centrifugal filters to remove the enzymes. The nucleosides were separated by reversed-phase HPLC using a 1 – 45 % linear gradient of methanol in 20 mM ammonium acetate for 60 min at a flow rate of 1 mL/min and subjected to LC-MS/MS analysis.

Analysis of 8-nitrodG poses a special problem since it undergoes depurination with a half-life ranging from 1 – 10 h depending on the temperature (15, 33, 34). To overcome this problem, we used acid hydrolysis to release 8-nitroG from DNA in the free base form (15). Samples (0.5 mL) of solutions of oxidized calf thymus DNA (100 nmol) were desalted using Amicon Ultra-0.5 (MWCO = 10 kDa) centrifugal filters, and 50 μL aliquots of 1 M HCl were added until the pH reached a value of 0.1 M. The samples were heated at 60 °C for 30 min, neutralized by the addition of 50 μL aliquots of 1 M NaOH and passed through Amicon Ultra-0.5 (MWCO = 10 kDa) centrifugal filters. After addition of 2 pmol of ^{15}N-labeled 8-nitroG, the samples were subjected to LC-MS/MS analysis.

The LC-MS/MS analysis of the digestion products was performed with the Agilent 1100 Series capillary LC/MSD Ion Trap XCT mass spectrometer equipped with an electrospray ion source as described elsewhere (26).

Results

Decomposition Products of Nitrosoperoxycarbonate Generate G*-T* Intrastrand Cross-links in Di- and Trinucleotides

In these experiments 5′-dGpCpT (or 5′-dGpT) sequences in buffer solutions (pH 7.5) containing CO_2/$NaHCO_3$ were flow-mixed with alkaline solutions (pH~11.3) of peroxynitrite. Under these conditions (pH 7.5–7.7) carbon dioxide, which is in equilibrium with HCO_3^- anions, dramatically reduces the lifetime of $ONOO^-$ anions from 10 – 15 s in the absence of bicarbonate to 50 – 80 ms at [HCO_3^-] = 25 mM (10). The highly unstable nitrosoperoxycarbonate formed in this reaction readily decomposes to form the strong oxidants $CO_3^{\bullet -}$ and $^{\bullet}NO_2$, which can selectively oxidize and subsequently nitrate guanine bases. We found that the end products of these reactions include the previously unknown G*-T* instrastrand cross-links (26, 27), as well as the more familiar guanine nitration/oxidation products such as 5-guanidino-4-nitroimidazole, 8-nitroguanine, 8-oxoguanine and the diastereomeric spiroiminodihydantoin lesions (15, 17, 19, 20).

The end products generated by the reactions of peroxynitrite/carbon dioxide with the di- and trinucleotides were separated by reversed-phase HPLC methods (Figure 2).

The fractions collected after multiple HPLC injections were combined, desalted and subjected to LC-MS/MS analysis. The negative ion spectra of the end products are identical to the spectra of the authentic standards synthesized by photochemical methods developed in our group for the selective nitration (32) and oxidation (26) of the parent di- and trinucleotides. The molecular ions, $[M-H]^-$ of the unmodified di- and trinucleotides eluted at 27 min (5′-dGpCpT) and 24 min (5′-dGpT) as shown in Figure 2, and were detected at *m/z* 859.2 and *m/z* 570.1 (Table 1).

The intrastrand cross-links eluted at 16 min (5′-dG*pCpT*) and 13 min (5′-dG*pT*) exhibit molecular ions at *m/z* 857.2 and *m/z* 568.1. Increasing the pH from 7.5–7.7 to 8.0–8.2 enhances the yields of 5′-d(G*pCpT*) and 5′-d(G*pT*) cross-linked product yie;ds by factors of ~ 3.4 and 2.5, respectively (Figure 3).

The guanine nitration products (NIm and 8-nitroG adducts) were monitored at 385 nm (blue curves, Figure 2) due to the characteristic absorption band at 380 – 400 nm (15–17, 32). The

molecular ions of the 5-guanidino-4-nitroimidazole adducts eluted at 22 min (5′-d[NIm]pCpT) and 19 min (5′-d[NIm]pT) were recorded at *m/z* 878.2 and *m/z* 589.2. The adducts containing the 8-nitroG lesions eluted at 36 min (5′-d[8-nitroG]pCpT) and 34 min (5′-d[8-nitroG]pT) were identified by the corresponding $[M-H]^-$ ions detected at *m/z* 904.2 and *m/z* 615.1.

The diastereomeric spiroiminodihydantoin (Sp) adducts eluted at 11 – 12 min (Figure 2A) and 11.5 – 13 min (Figure 2B) were identical to 5′-d[Sp]pCpT (*m/z* 891.2) and 5′-d[Sp]pT (*m/z* 602.1) derived from the oxidation of the tri- and dinucleotides by photochemically generated $CO_3^{\bullet-}$ radicals (26). The 8-oxoguanine (8-oxoG) lesions are present in smaller quantities. The 8-oxoG adducts eluted at 29.5 min (5′-d[8-oxoG]pCpT) and at 26.1 min (5′-d[8-oxoG]pT) were detected at *m/z* 875.1 and *m/z* 586.1. Distributions of the nitration/oxidation products generated by treatment of di- and trinucleotides by peroxynitrite/carbon dioxide are shown in Figure 4.

The major products generated by peroxynitrite/carbon dioxide are the NIm adducts. The yields of these adducts are by factor of 2.8 greater than the yields of 8-nitroG adducts. The ratios of the NIm/8-nitroG product yields are typical for free radical nitration of single-stranded oligonucleotides initiated by the combined action of photochemically generated $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (32). The yields of the intrastrand cross-linked products, 5′-dG*pCpT* and 5′-dG*pT* are by a factor of 7 – 9 smaller than the yields of the NIm adducts. The efficiency of intrastrand cross-link formation in trinucleotides is higher than in dinucleotides. Indeed, in the case of 5′-dGpT without an intervening C base, the yield of 5′-dG*pT* is by a factor of 2 lower than the yield of 5′-dG*pCpT*. In the case of reactions of these oligonucleotides with photochemically generated $CO_3^{\bullet-}$ radicals, the ratio of the 5′-dG*pCpT*/5′-dG*pT* yield is ~3, thus somewhat higher (26) than in the case of oxidation by peroxynitrite/carbon dioxide (Figure 4). On the other hand, the yields of the diastereomeric Sp adducts lesions are close to the yields of the G*-T* intrastrand cross-links in both types of experiments (26). The 8-oxoG adducts attained levels of 0.07 – 0.1% (Figure 4). Although these levels are small compared to the yields of the other adducts, they are still larger by a factor of ~10 than the background levels of 8-oxoG lesions in di- and trinucleotide control samples. These results clearly indicate that the 8-oxoG adducts are also formed, but in minor quantities.

Distribution of guanine nitration/oxidation products in calf thymus DNA generated by peroxynitrite/carbon dioxide

In these experiments we focused on the detection of the G*-T* cross-links and the guanine lesions 8-oxoG, NIm and 8-nitroG. It was shown previously that G*-T* cross-links can be excised from the oligonucleotides by the combined action of nuclease P1 and alkaline phosphatase yielding either, or both enzyme digestion-resistant products 5′-d(G*pT*) and 5′-d(G*-T*) depicted in Figure 5. When the dinucleotide d(GpT) is modified, the resulting d(G*pT*) lesion is completely resistant to P1 nuclease. We thus conclude that the product d(G*pT*) must entirely originate from intrastrand cross-linked lesions. However, the same enzyme digestion treatment of 5′-dG*pCpT* leads to a loss of pdCp, thus yielding the 5′-d(G*-T*) digestion product (26). While such an intrastrand G*-T* lesion can arise from any 5′-d(GpN_npT) sequence with N = C or A, and n = 1,2,3 (26), the possibility cannot be excluded that such a cross-linked lesion can also arise from an interstrand cross-linked product in native DNA.

The 8-oxoG lesion is a classical guanine oxidation product that can serve as an indicator of oxidative damage to DNA (35). A number of guanine nitration products have been detected in calf thymus DNA treated with peroxynitrite (16, 18). However, the yields of the nitro products were reported in separate experiments and the ratio of the NIm/8-nitroG yields

remain unknown, but have been evaluated (relative to 8-oxoG and the G*-T* lesions) in this work. The quantitation of the Sp diastereomers relative to 8-oxoG resulting from the treatment of native DNA with peroxynitrite/carbon dioxide were thoroughly investigated by Yu et al. (20) and the [Sp]/[8-oxoG] ratio was found to be in the range of 2 – 3 at [$ONOO^-$] = 50 – 200 μM. We therefore did not find it necessary to quantitate the Sp oxidation products in this work.

The samples of calf thymus DNA in buffer solutions (pH 7.5) containing 25 mM CO_2/$NaHCO_3$ were flow-mixed with alkaline solutions (pH~11.3) of peroxynitrite. After desalting the oxidized DNA, the solutions were spiked with the isotope labeled nucleoside standards and enzymatically digested to the nucleoside level. The fractions containing the lesions and the isotope-labeled standard were isolated by reversed-phase HPLC and subjected to LC-MS/MS analysis.

The lesions excised from the oxidized calf thymus DNA by enzymatic digestion were detected using multiple reaction monitoring (MRM) mode. In the case of 5′-d(G*pT*) cross-linked product, we found that the most intensive transition is associated with the detachment of two sugar residues from the molecular ion, $[M+H]^+$ (Figure 5). The most intense transition associated with the detachment of two sugar residues from the sodium adduct, $[M+Na]^+$, was found for the 5′-d(G*-T*) cross-linked product.

The ion-current chromatograms recorded in the positive mode using selective reaction monitoring of the transitions: *m/z* 570.1 → *m/z* 273.6 for the unlabeled 5′-d(G*pT*) and *m/z* 530.0 → *m/z* 297.7 for the unlabeled 5′-d(G*-T)* are indeed formed in calf thymus DNA treated with peroxynitrite/carbon dioxide because these nucleoside excision products co-elute with the $^{15}N_5$,$^{13}C_{10}$-labeled 5′-d(G*pT*) and 5′-dG*-dT* internal standards (Figure 6). In these experiments we observed a peroxynitrite/carbon dioxide concentration-dependent increase in the quantities of 5′-d(G*pT*) and 5′-d(G*-T*) reaching a maximum value at a concentration of 0.1 mM, followed by a small decline of the 5′-d(G*T*) product at a 0.2 mM concentration (Figure 6).

The authentic, isotope-labeled 8-oxodG standard was synthesized from $^{15}N_5$-dG using Fe^{2+}/H_2O_2 Fenton reagent (31). The negative ion spectra of this standard showed the molecular ion $[M–H]^-$ at *m/z* 287, and the daughter ion $[M–H–C_3H_6O_3]^-$ at *m/z* 197 that arises from the cleavage of C′2 – C′3 and C′4 – O bonds in the sugar residue (36). The authentic standards of the isotope labeled dNIm and 8-nitrodG were prepared by the nitration of $^{15}N_5$-dG initiated by photochemically generated $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals as described elsewhere (32). The ion spectra of the $^{15}N_5$-dNIm standard were recorded in the positive mode because in the negative mode the signals were weaker than in the positive mode; the $^{15}N_5$-dNIm products show the molecular ion $[M+H]^+$ at *m/z* 293, and the daughter ion $[M–H–C_5H_6O_2]^-$ at *m/z* 177 that arises from detachment of the sugar residue. The $^{15}N_5$-8-nitrodG was depurinated by soft acid hydrolysis at 60 °C (15); the 8-nitroG thus formed showed a molecular ion $[M–H]^-$ at *m/z* 200, and a daughter ion $[M–H–NH_2]^-$ at *m/z* 183 that arises from the loss of ammonia.

The analysis 8-oxodG, dNIm, and 8-nitroG excised by enzymatic digestion (or acid hydrolysis in the case of 8-nitroG lesions) from calf thymus DNA oxidized peroxynitrite/carbon dioxide, was based on selective reaction monitoring of the relevant transitions for the unlabeled products and the labeled internal standards. The ion-current chromatograms show that the 8-oxodG, dNIm, and 8-nitroG are indeed formed in calf thymus DNA treated with peroxynitrite/carbon dioxide and the nucleoside excision products co-elute with the corresponding $^{15}N_5$-labeled internal standards (Figure 7).

In calf thymus DNA the nitration/oxidation products of guanine are formed in a dose-dependent manner. Using the relevant concentration profiles for NIm, 8-nitroG and 8-oxoG (data not shown) and 5′-d(G*pT*) and 5′-d(G*-T*) (Figure 6) we calculated the distribution of these lesions generated by treatment of calf thymus DNA with peroxynitrite/carbon dioxide (Figure 8).

The major lesions generated by peroxynitrite/carbon dioxide in calf thymus DNA are guanine nitration products. In calf thymus DNA the yield of the NIm adducts is greater by a factor of only ~1.2 than the yield of 8-nitroG adducts. This [NIm]/[8-nitroG] ratio is close to those (0.8 – 1.1) detected in the free radical nitration of double-stranded oligonucleotides initiated by the combined action of photochemically generated $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (32). The [NIm]/[8-nitroG] ratios of 2.1 – 2.6 in the single-stranded oligonucleotides are greater than in DNA duplexes. The relative efficiency of 8-oxoG formation in calf thymus DNA is smaller by a factor of 2 – 2.5 than the yield of NIm and 8-nitroG lesions. The yields of the intrastrand cross-links, 5′-d(G*pT*) and 5′-dG*pN_npT* and/or interstrand cross-linked products (all enzymatically excised as 5′-d(G*-T*) are smaller by a factor of 10 – 12 than the yield of 8-oxoG.

Discussion

Fates of guanine radicals

Nitrosopeoxycarbonate is a unique generator of $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (37). The selective one-electron oxidation of guanine bases in DNA by $CO_3^{\bullet-}$ radicals monitored by direct spectroscopic methods (22, 25) supports the hypothesis that the $CO_3^{\bullet-}$ radicals are the actual one-electron oxidants in the reactions induced by $ONOOCO_2^-$. The guanine radicals are key intermediates of the one-electron oxidation of guanine in DNA. The radical intermediates decay by competitive pathways, which include the addition of free radicals or nucleophiles to the C5 or C8 position of guanine.

We have shown that in the case of guanosine (38), or guanine embedded in an oligonucleotide duplex (27), oxidation by $CO_3^{\bullet-}$ radicals in $H_2{}^{18}O$ solutions gives rise to the insertion of one ^{16}O atom in 8-oxoG and two ^{16}O atoms (shown in red in Figure 9) in Sp; these findings indicate that the additional oxygen atoms in 8-oxoG and Sp originate from $HC^{16}O_3^-$ and not from $H_2{}^{18}O$. These results clearly indicate that the formation of 8-oxoG occurs via the addition of $C^{16}O_3^{\bullet-}$ radicals to the C8 position of G(-H)$^{\bullet}$ radicals followed by hydrolysis of the esters of the carbonic acids formed (Figure 9).

The addition of $CO_3^{\bullet-}$ to G(−H)$^{\bullet}$ radicals leading to 8-oxoG, as well as the further oxidation steps resulting in the formation of the Sp diastereomers, compete with the combination of $^{\bullet}NO_2$ with G(−H)$^{\bullet}$ radicals that results in the formation of nitro products (Figure 9). The addition of $^{\bullet}NO_2$ to the C8 position of G(−H)$^{\bullet}$ generates 8-nitroG, whereas addition to C5 produces unstable adducts, which rapidly decompose to form NIm lesions (32). The ratio of NIm to 8-nitro-G lesions decreases from 2.8 in 5′-d(GpCpT) and 5′-d(GpT) (Figure 4) to 1.2 in calf thymus DNA (Figure 8). This value is consistent with earlier studies of the effects of secondary DNA structure on NIm/8-oxoG ratios since values of 3.4 (free nucleoside), 2.6 (single-stranded oligonucleotides), and 0.8 - 1.0 (double-stranded oligonucleotides) were found (32). The probabilities of addition of the $^{\bullet}NO_2$ radical to the C5 or the C8 positions of G(−H)$^{\bullet}$ radicals are dissimilar because, in contrast to the single-stranded di- and tri-nucleotides, the accessibilities of these positions to reactive species from the major groove of double-stranded DNA are likely to be different.

Effects of pH on cross-link formation

The formation of G*-T* cross-links initiated by the one-electron oxidation of guanine, suggests that the C8-centered G(−H)$^{\bullet}$ radical can react with the N3-site of thymine (26, 27). Thymine is a weak nucleophile and the deprotonation of the N3-H hydrogen atom greatly enhances its nucleophilicity. Consistent with a nucleophilic mechanism of crosslink formation, the product yields in the case of 5′-d(GpCpT) trinucleotides are greater at pH 10 than at neutral pH (27). At this pH value the thymine exists mostly in the deprotonated form since its pK_a is 9.67 (39). Here we show that the mere increase of the solution pH from 7.5 to only 8.0 can lead to a sizable increase in the yields of cross-linked products in the case of 5′-d(GpT) and 5′-d(GpCpT) oxidized by nitrosperoxycarbonate in solution (Figure 3). This observation is consistent with a nucleophilic mechanism involving thymine N3-H. The alternative mechanism in which thymine attacks the C8 position of the G$^{\bullet+}$ radical cation (pKa 4.7) would be favored when the pH is decreased since this would favor the protonation of the G(−H)$^{\bullet}$ radical to the cation G$^{\bullet+}$. However, this mechanism is not supported by experiments that show that the yields of cross-linked G*-T* products are negligible when the pH is decreased from 7.5 to < 5.0 (27).

Effects of sequence context

The effects of sequence context on the formation of cross-links between guanine and thymine bases suggest that the covalently linked (G-C8)-(N3-T) products arise via non-canonical conformational intermediates in double-stranded DNA. The yields of cross-links in 5′-GT and 5′-GCT sequences are ~one order of magnitude higher than in 5′-TG and 5′-TCG sequences (26). Furthermore, the yields in 5′-GCT are higher than those in 5′-GT, regardless of the method of generation of $CO_3^{\bullet-}$ radicals by either photochemical methods or the spontaneous decomposition of nitrosoperoxycarbonate (26) (Figure 4). Since the nuclease P1 does not cleave the phosphodiester bond in 5′-G*pT*, it can be concluded that such lesions arise from an intrastrand rather than an interstrand cross-linking mechanism (Figure 5). However, nuclease P1 efficiently excises the nucleotide C from 5′-G*CT* (26, 27) or A from 5′-G*AT* (data not shown), thus yielding G*-T* P1 nuclease digestion products. These results suggest that 5′-dG*-dT* cross-links excised from calf thymus DNA (Figures 5) could have originated from either intrastrand (5′-..G*NT*..) or interstrand interactions in double-stranded DNA.

An essential step in the formation either G*pT* or G*-T* cross-links is the removal of an electron by O_2 after the addition of N3-T to the G(−H)$^{\bullet}$ radical (Figure 9). Indeed, O_2 is essential since the yields of these cross-linked products are negligible in the absence of O_2 (26). In contrast, the formation of other known intrastrand lesions is observed only in the absence of molecular oxygen. These include cross-linked lesions between adjacent G and T, or G and C bases (the so-called tandem lesions) that are produced when DNA is exposed to ionizing radiation (40–42) or Fenton reagents (43–45). These cross-linked products involve a covalent bond between C8-guanine and either the methyl groups of thymine or C5-cytosine, or the C5 position of C. These products arise from the addition to C8-guanine of pyrimidine C-centered radicals generated by H-atom abstraction from the CH_3-groups of T, or C5H of cytosine. The formation of these products is suppressed by O_2 because the latter efficiently traps the C-centered radical intermediates and thus prevents the formation of the cross-linked products (46, 47). A similar mechanism is responsible for the formation of adenine and guanine 5′,8-cyclo-2′-deoxyribonucleoside products that occurs via the addition of C-centered 5′-hydroxylmethyl radicals produced by H-atom abstraction from 2-deoxyribose moieties (48, 49). In the presence of molecular oxygen the formation of such products is also suppressed because O_2 reacts readily with the C-centered radicals (50, 51).

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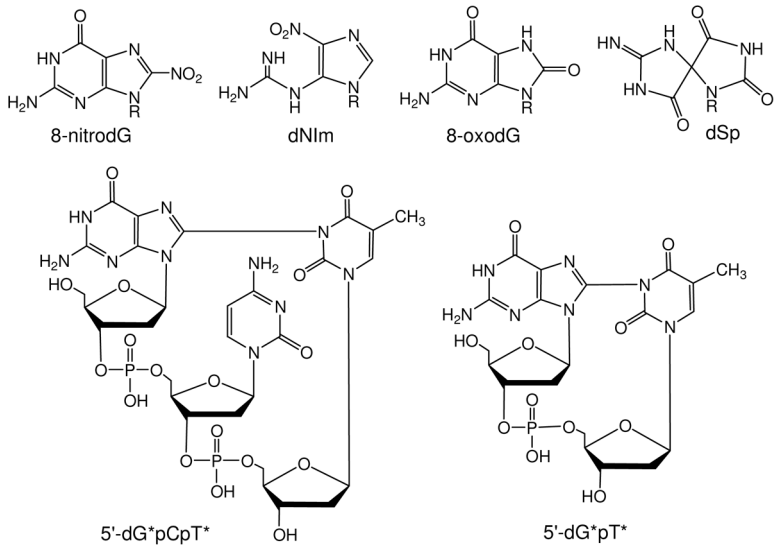

Figure 1.
Major nitration and oxidation products of guanine generated by the decomposition products of nitrosoperoxycarbonate.

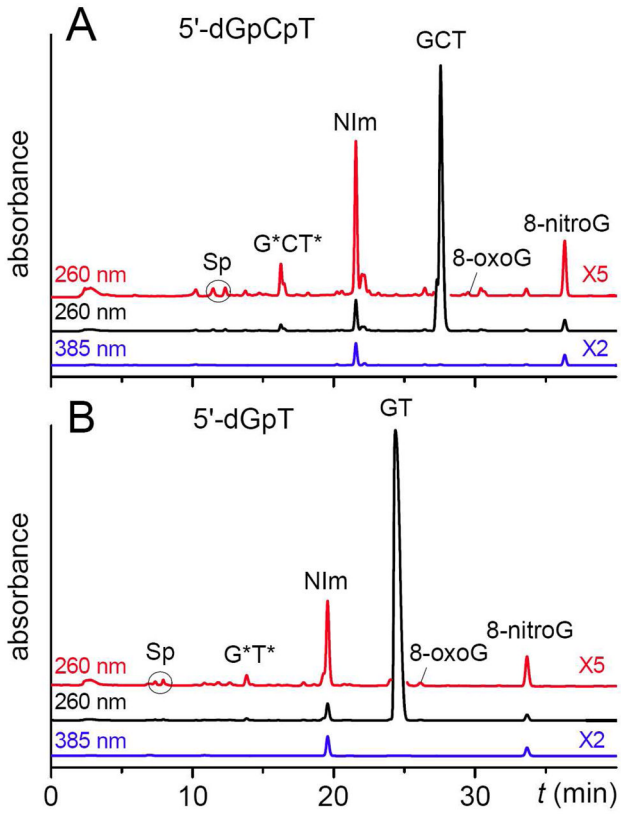

Figure 2.

Reversed-phase HPLC elution profiles of the end-products derived from the oxidation of 0.2 mM 5′-dGpCpT (A) and 5′-dGpT (B) by 0.1 mM peroxynitrite in buffer solutions (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$. HPLC elution conditions (detection at 260 nm): 3 – 35% (A), or 1 – 45% (B) linear gradient of methanol in 20 mM ammonium acetate in water (pH 7) for 60 min at a flow rate of 1 mL/min. Panel A – The diastereomeric spiroiminodihydantoin adducts (Sp) elute at 11 – 12 min, the cross-linked products (G*CT*) at 16 min, the 5-guanidino-4-nitroimidazole adduct (NIm) at 22 min, the unmodified trinucleotide (GCT) at 27 min, the 8-oxoguanine adduct (8-oxoG) at 29.5 min, and the 8-nitroguanine adduct (8-nitroG) adduct at 36 min. Panel B – The Sp adducts elute at 7 – 7.5 min, the intrastrand cross-link (G*T*) at 13 min, the NIm adduct at 19 min, the unmodified dinucleotide (GT) at 24 min, the 8-oxoG adduct at 26 min, and the 8-nitroG adduct at 34 min. The adducts were identified by LC-MS/MS as described in the text.

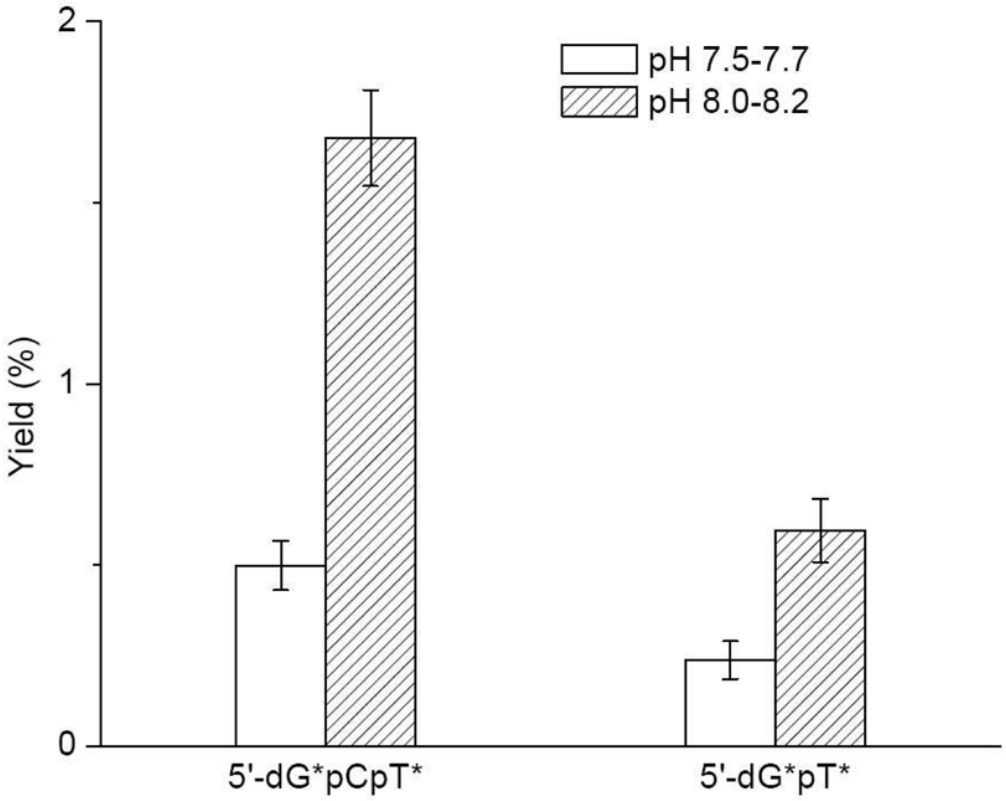

Figure 3.

Yields of the 5′-d(G*pT*) and 5′-d(G*-T*) cross-links generated by the oxidation of 0.2 mM 5′-dGpCpT and 5′-dGpT by 0.1 mM peroxynitrite in buffer solutions (pH 7.5–7.7 and 8.0–8.2) containing 25 mM CO_2/$NaHCO_3$. The yields calculated from the integrated peak areas in the HPLC elution profiles and the molecular absorbtivities at 260 nm represent the mean of at least four independent experiments.

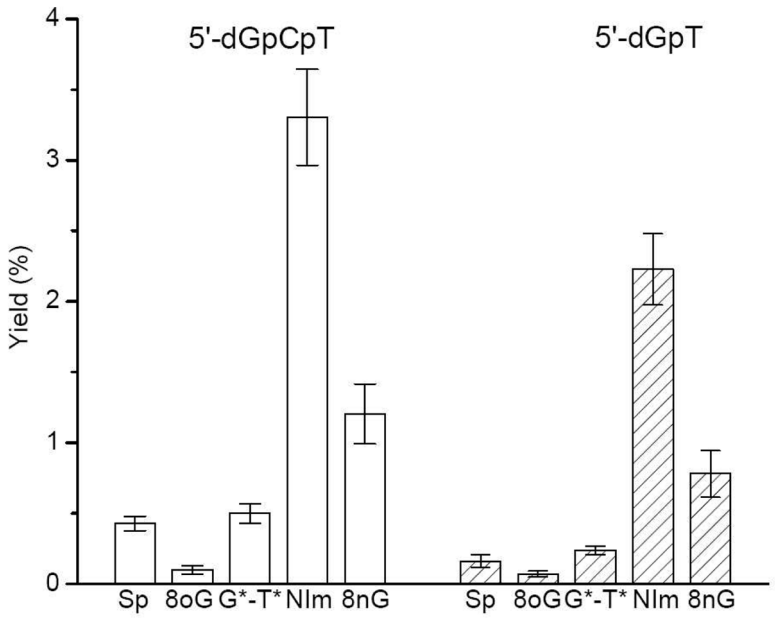

Figure 4.

Distributions of the nitration/oxidation products generated by the oxidation of 0.2 mM 5′-dGpCpT and 5′-dGpT by 0.1 mM peroxynitrite in buffer solutions (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$. The yields calculated from the integrated peak areas in the HPLC elution profiles and the molecular absorbtivities at 260 nm represent the mean of at least four independent experiments.

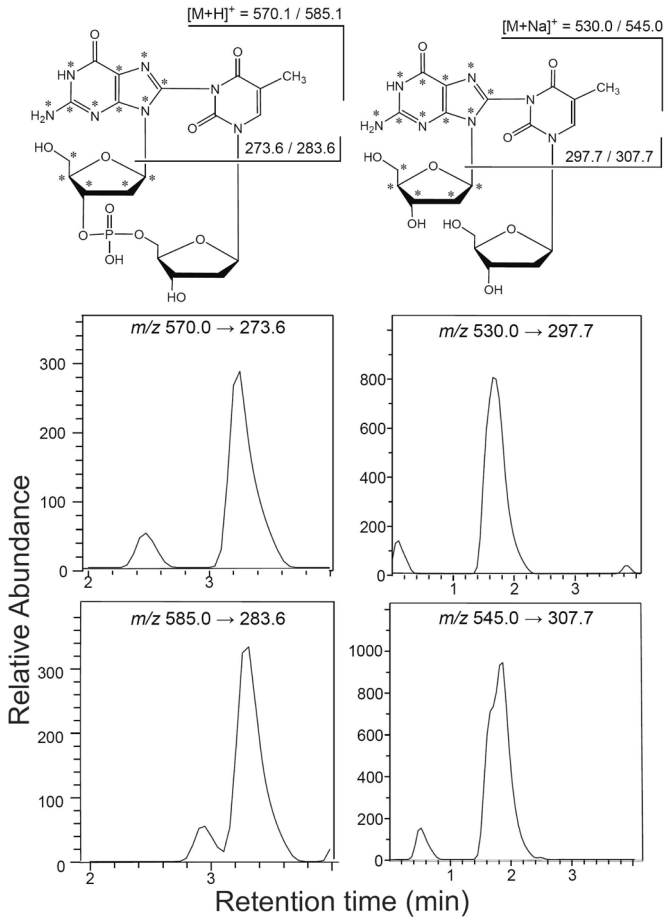

Figure 5.

LC-MS/MS analysis of the 5′-d(G*pT*) and 5′-d(G*-T*) cross-linked products excised from calf thymus DNA (200 nmol) by enzymatic digestion in the presence of the $^{15}N,^{13}C$-labeled (isotopes are marked with an asterisk) internal standards (3 pmol). The 5′-dG*pT* cross-linked product is resistant to nuclease P1 digestion, whereas 5′-d(G*-T*) can be excised from 5′-..G*pN_npT*.. intrastrand or. G*.-.T*.. interstrand cross-links. The samples of DNA were oxidized by 0.1 mM peroxynitrite in 1 mL buffer solution (pH 7.5) containing 25 mM CO_2/$NaHCO_3$. The ion-current chromatograms were recorded in the positive mode using selective reaction monitoring of the transitions: 570.1 → *m/z* 273.6 (unlabeled) and *m/z* 585.1 → *m/z* 283.6 ($^{15}N,^{13}C$-labeled) 5′-d(G*pT*), 530.0 → *m/z* 297.7 (unlabeled) and *m/z* 545.0 → *m/z* 307.7 ($^{15}N,^{13}C$-labeled) 5′-d(G*-T*).

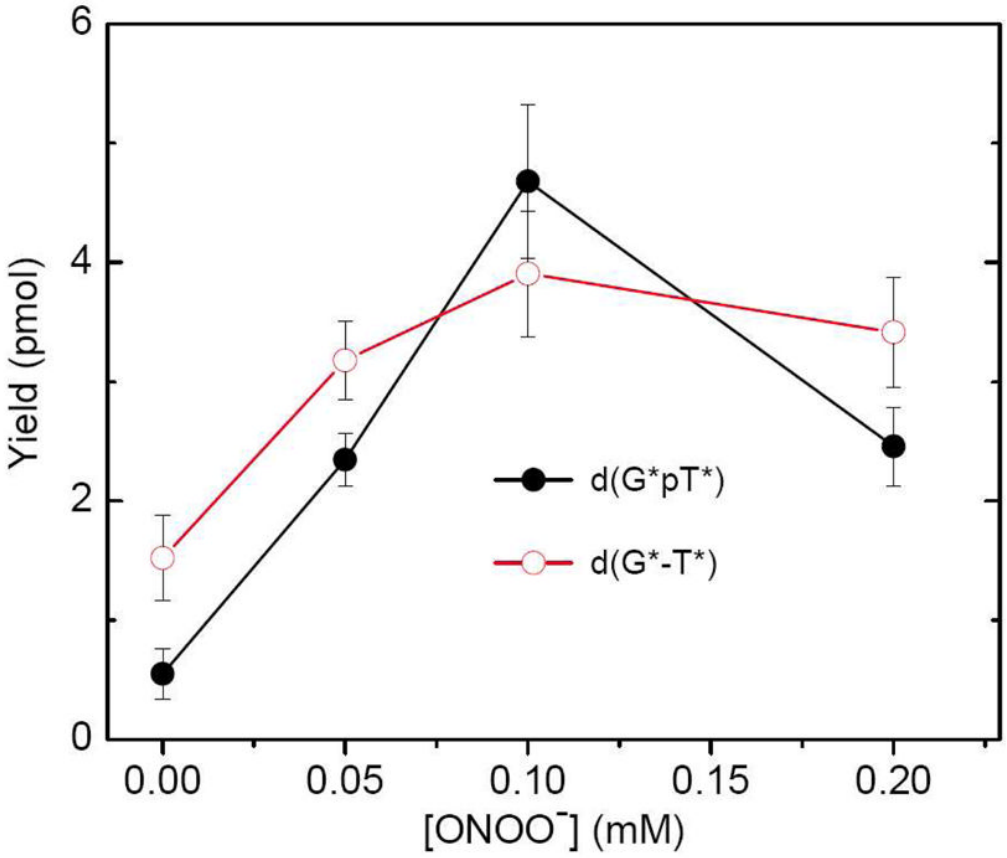

Figure 6.

Yields of the 5′-d(G*pT*) and 5′-d(G*-T*) cross-links excised by the enzymatic digestion of calf thymus DNA (200 nmol) after treatment with varying amounts of peroxynitrite in 1 mL buffer solution (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$. The 5′-d(G*pT*) and 5′-d(G*-T*) digestion products were quantitated by the isotope dilution LC-MS/MS method using $^{15}N_5$,$^{13}C_{10}$-labeled 5′-d(G*pT*) and 5′-d(G*-T*) internal standards as shown in Figure 5. The data points represent the mean values of at least four independent experiments.

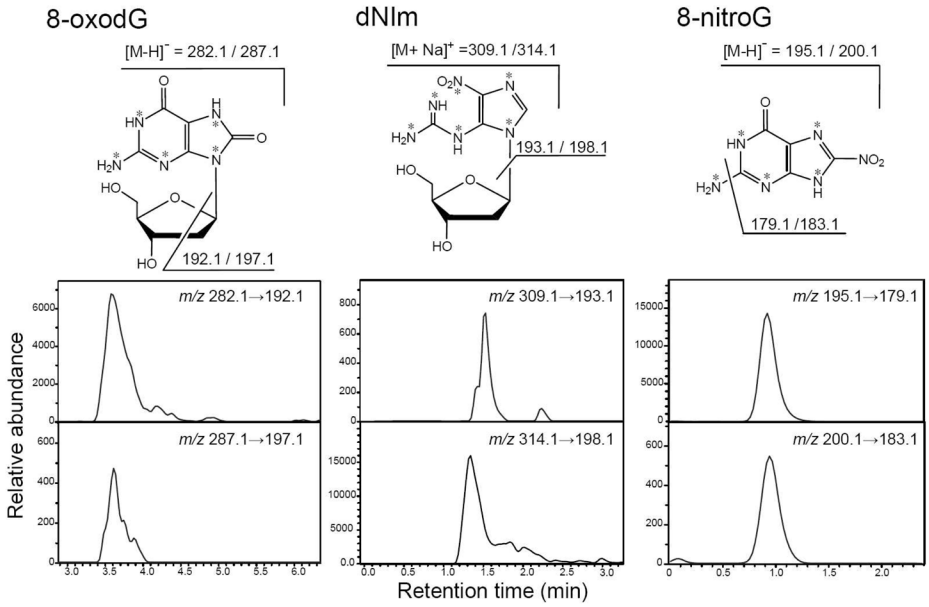

Figure 7.

LC-MS/MS analysis of 8-oxodG, dNIm, and 8-nitroG lesions excised by enzymatic digestion (or acid hydrolysis in the case of 8-nitroG lesions) of calf thymus DNA (200 nmol) in the presence of ^{15}N-labeled (isotopes are marked with an asterisk) internal standards (3 pmol). The samples of DNA were oxidized with 0.2 mM peroxynitrite in 1 mL buffer solution (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$. The ion-current chromatograms were recorded in the negative mode (except for dNim that was recorded in the positive mode) using selective reaction monitoring of the transitions: *m/z* 282.1 → *m/z* 192.1 (unlabeled) and *m/z* 287.1 → *m/z* 197.1 ($^{15}N_5$-8-oxodG), *m/z* 309.1 → *m/z* 193.1 (unlabeled) and *m/z* 314.1 → *m/z* 198.1 ($^{15}N_5$-dNIm), *m/z* 195.1 → *m/z* 179.1 (unlabeled) and *m/z* 200.1 → *m/z* 183.1 ($^{15}N_5$-8-nitroG).

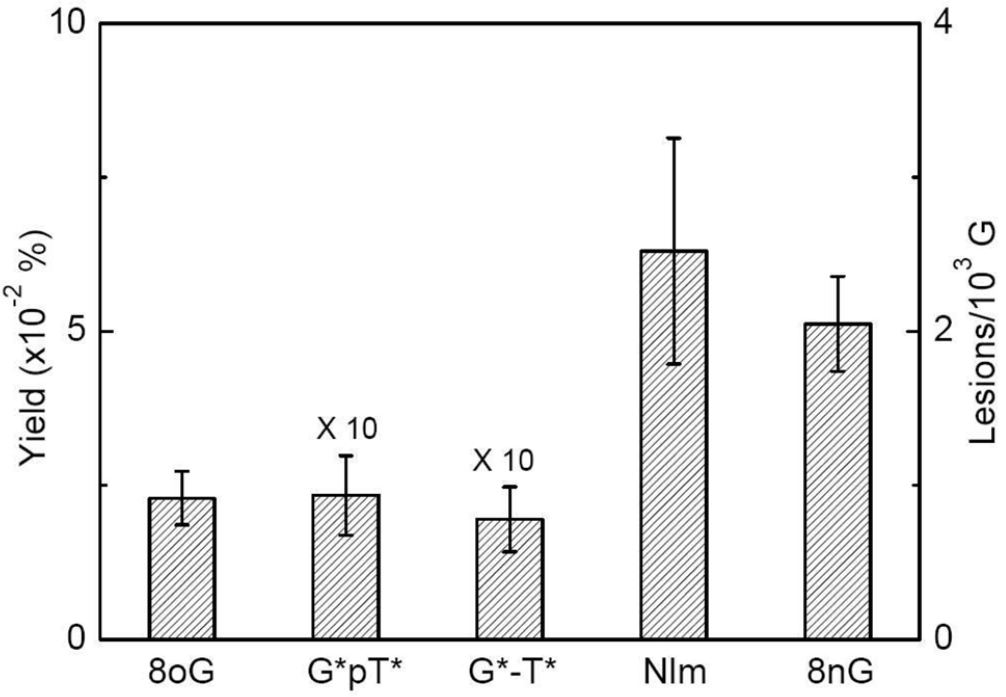

Figure 8.

Distributions of the nitration/oxidation products generated by the oxidation of calf thymus DNA by 0.1 mM peroxynitrite in buffer solutions (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$. The yields were calculated from the integrated peak areas in the HPLC elution profiles and the molecular absorbtivities at 260 nm.

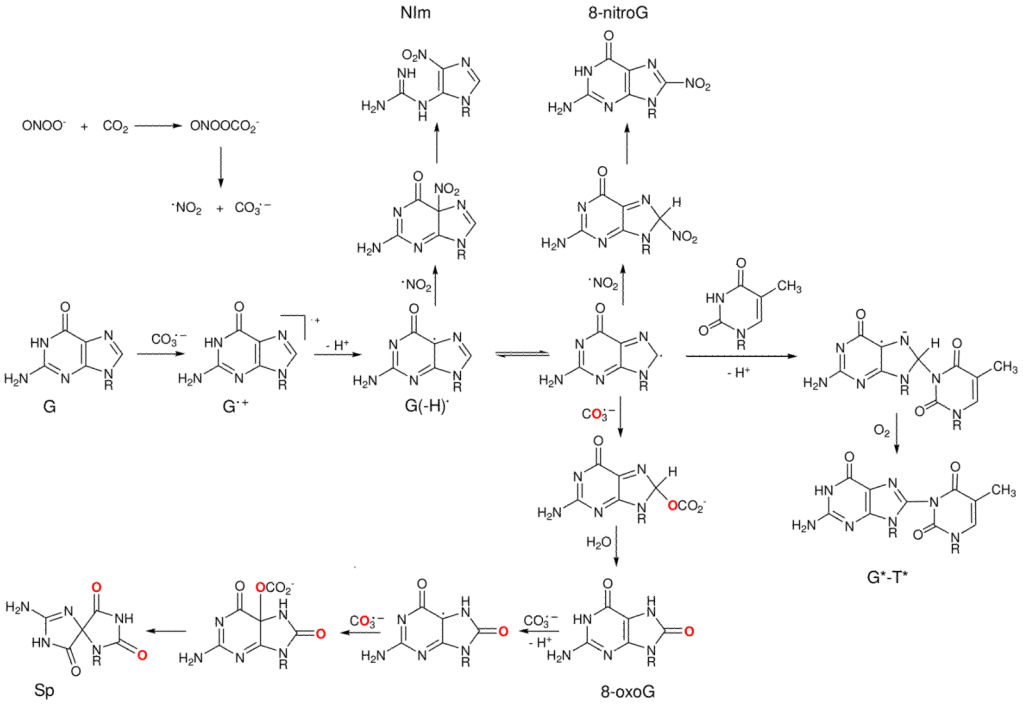

Figure 9.

Lesions derived from the oxidation of guanine in DNA by decomposition products of nitrosoperoxycarbonate ($CO_3^{\bullet -}$ and $^{\bullet}NO_2$ radicals). The O-atoms added to guanine as a result of the oxidative processes depicted are shown in red.

Table 1

LC-MS analysis (negative mode) of the nitration/oxidation products derived from the oxidation of di- and trinucleotides by peroxynitrite in buffer solutions (pH 7.5–7.7) containing 25 mM CO_2/$NaHCO_3$

Lesion	$[M–H]^-$ (*m*/*z*)		ΔM[b)]
	5′-dGpT	5′-dGpCpT	
GT/GCT[a)]	570.1	859.2	0
G*T*/G*CT*	568.1	857.2	− 2
NIm	589.2	878.2	+19
8-nitroG	615.1	904.2	+45
8-oxoG	586.1	875.1	+16
Sp	602.1	891.2	+32

[a)] Parent di- and trinucleotides.

[b)] Mass of the adduct minus the mass of di- or trinucleotide.