Oxidation of single-stranded oligonucleotides by carbonate radical anions: generating intrastrand cross-links between guanine and thymine bases separated by cytosines

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ABSTRACT

The carbonate radical anion is a biologically important one-electron oxidant that can directly abstract an electron from guanine, the most easily oxidizable DNA base. Oxidation of the 5'-d(CCTAGCGTACC) sequence by photochemically generated CO$_3^{3-}$ radicals in low steady-state concentrations relevant to biological processes results in the formation of spiroiminodihydantoin diastereomers and a previously unknown lesion. The latter was excited from the oxidized oligonucleotides by enzymatic digestion with nuclease P1 and alkaline phosphatase and identified by LC-MS/MS as an unusual intrastrand cross-link between guanine and thymine. In order to further characterize the structure of this lesion, 5'-d(GpCpT) was exposed to CO$_3^{3-}$ radicals, and the cyclic nature of the 5'-d(G'pCpT') cross-link in which the guanine C8-atom is bound to the thymine N3-atom was confirmed by LC-MS/MS, 1D and 2D NMR studies. The effect of bridging C bases on the cross-link formation was studied in the series of 5'-d(GpCn,pT) and 5'-d(TpCn,pG) sequences with n = 0, 1, 2 and 3. Formation of the G'-T' cross-links is most efficient in the case of 5'-d(GpCpT). Cross-link formation (n = 0) was also observed in double-stranded DNA molecules derived from the self-complementary 5'-d(TTACGTACGTAA) sequence following exposure to CO$_3^{3-}$ radicals and enzymatic excision of the 5'-d(G'pT') product.

INTRODUCTION

Interest in oxidative chemistry of carbonate radical anion, CO$_3^{3-}$ has significantly grown within the last decade due to its important role in inflammatory processes (1,2). Persistent oxidative stress developed in response to inflammation in tissues is associated with overproduction of nitric oxide and superoxide radical anions that rapidly combine to form peroxynitrite (3). In cellular environments, the major mode of reaction of peroxynitrite (4–6) is the formation of a complex with CO$_2$ that decomposes rapidly to the oxidizing species, CO$_3^{3-}$ and *NO$_2$ radicals (7,8). Only CO$_3^{3-}$ radical can directly abstract an electron from guanine, the most easily oxidizable normal DNA base (9). This one-electron abstraction triggers a cascade of subsequent chemical reactions that yields stable oxidatively modified guanine (1,2). In previous work (10–12), we generated CO$_3^{3-}$ radicals by a 308-nm excimer laser pulse-induced photochemical dissociation of peroxodisulfate anions to sulfate radical anions that subsequently oxidize bicarbonate anions to yield CO$_3^{3-}$ radicals. The mechanisms of oxidation of 2'-deoxyoligonucleotides were studied by monitoring the transient absorbances of guanine and carbonate radicals, and by employing analytical and chemical methods to isolate and identify the chemical end products. Under these conditions, CO$_3^{3-}$ radicals were generated in high concentrations and it was found that a consecutive four-electron oxidation of guanine yields, predominantly, the diastereomeric spiroiminodihydantoin (Sp) lesions (Figure 1). In this work, we explored the oxidation of the 5'-d(CCTAGCGTACC) sequence by CO$_3^{3-}$ radicals generated by continuous illumination methods that generate low steady-state concentrations of carbonate radicals that are more relevant to biological conditions. The chemical end products of these oxidative processes were isolated by anion-exchange HPLC. In addition to the two diastereomeric Sp lesions found previously under conditions of laser pulse excitation that yields much higher transient concentrations of CO$_3^{3-}$ radicals, a novel oxidatively generated lesion was found that was not observed previously (13,14). Using enzymatic digestion of the irradiated oligonucleotide followed by LC-MS/MS
analysis of the digestion products, an excision fragment was found that has a mass smaller by 2 Da than the combined masses of dG and dT. The structure of this d(G*-T*) dimer is unusual because G and T are not adjacent to one another in the oligonucleotide. To gain further insights into the precursor of the enzymatic d(G*-T*) excision product, the trinucleotide 5'-d(GpCpT) was subjected to the same oxidation protocol as the oligonucleotide and the structure of the trinucleotide oxidation product was investigated by LC-MS/MS, 1D and 2D NMR methods. A cross-linked product, 5'-d(GpCpTpC*) that has a covalent bond between the C8-atom of guanine and the N3-atom of thymine was found that has the same characteristics as the corresponding product excised from the 11-mer oligonucleotide. The cross-linked product generated by oxidation with CO$_3^-$ radicals (Figure 1) differs from the cross-links between adjoining G and T bases produced by ionizing radiation in deoxygenated solutions that involve a covalent bond between the C8-atom of guanine and the C-atom of the methyl group in thymine (15–24). Here, we investigated the effects of bridging C bases between G and T on the efficiency of the cross-link formation in a series of the oligonucleotide sequences 5'-d(GpCpT) and 5'-d(TpCpC) with $n = 0, 1, 2$ and $3$, and cross-linking in a 12-mer duplex derived from the self-complementary 5'-d(TTACGTACGTAA) sequence.

**MATERIALS AND METHODS**

**Materials**

All chemicals (analytical grade) were used as received. Snake venom phosphodiesterase was purchased from Pharmacia (Piscataway, NJ, USA); calf spleen phosphodiesterase, nuclease P1 and alkaline phosphatase were from Sigma Chemical (St Louis, MO, USA). The oligonucleotides were purchased from Sigma Genosys (Woodlands, TX, USA), purified and desalted using reverse-phase HPLC. The integrity and composition of the oligonucleotides were confirmed by MALDI-TOF/MS and LC-MS/MS techniques.

**Photochemical oxidation of oligonucleotides**

Oligonucleotides (10 nmol) were dissolved in 1 ml of air-equilibrated solutions containing 300 mM NaHCO$_3$ and 10 mM Na$_2$S$_2$O$_8$ and the pH was adjusted to 7.5 with 1 M NaH$_2$PO$_4$. The solutions were irradiated with light from a 100-W high-pressure Xenon lamp that was reflected from a dichroic mirror with the reflectance in the 300–340-nm spectral range. In the experiments with riboflavin (RF) (~15 μM), the solutions were irradiated by 350–390 nm light from the same Xenon lamp and this wavelength range was isolated by means of an appropriate dichroic mirror. The energy incident on the sample was ~100 mW/cm$^2$ and the irradiation time was varied from 10 to 30 s depending on the sequence. After irradiation, the sample was immediately desalted by reversed-phase HPLC, concentrated and subjected to HPLC analysis.

**Separation by anion-exchange HPLC methods**

The photochemical reaction products were separated using an Agilent 1200 Series LC system (quaternary LC pump with degasser, thermostated column compartments, and diode array detector) equipped with an analytical (250 × 4 mm i.d) anion-exchange DNA-Pac PA-100 column (Dionex, Sunnyvale, CA, USA). Typically, the anion-exchange HPLC separations of the oxidized oligonucleotides were performed employing a 10–90% linear gradient of solvent B (10% acetonitrile and 90% 1.5 M ammonium acetate) in solvent A (10% acetonitrile and 90% water) for 30 min at a flow rate of 1 ml/min (25,26). The trinucleotide products were separated using an isocratic mobile phase (90% solvent A and 10% solvent B) at a flow rate of 1 ml/min. The purified adducts were desalted by reversed-phase HPLC using the following mobile phases: 5 mM sodium phosphate (pH 7.5) (10 min), deionized water (10 min) and 50% methanol in water (15 min). The desalted samples were concentrated under vacuum and subjected to LC-MS/MS and NMR analysis.

**Enzymatic and chemical digestion of oligonucleotides**

The cross-linked oligonucleotide adducts (10 nmol) were digested with nuclease P1 (2 U) in 35 μl of 30 mM acetate buffer (pH 5.2) overnight at 37°C; following digestion, the samples were treated with 2 U of alkaline phosphatase in a Tris–HCl (~100 mM) buffer solution (pH 8.2) for 1 h at 37°C. The incubation mixtures were passed through a Millipore centrifugal filter to remove the enzymes. The nucleosides were separated by reversed-phase HPLC using a 0–40% linear gradient of acetonitrile in 20 mM ammonium acetate for 60 min at a flow rate of 1 ml/min and subjected to LC-MS/MS analysis.

Chemical hydrolysis of the cross-linked products was performed using a commercially available solution of hydrogen fluoride (~70%) in pyridine (HF/Pyr) as previously described (27). The dry, cross-linked product (1–3 nmol) generated by the oxidation of 5'-d(GpCpT) was treated in a plastic vial with 50 μl of HF/Pyr at 37°C for 30 min. The reaction mixture was diluted with 1 ml of water and neutralized by vigorous agitation with 80 mg of CaCO$_3$. The insoluble inorganic salts were removed by centrifugation. The sample was evaporated to dryness and traces of pyridine were removed by repeated lyophilization. The final aqueous solution (~20 μl) was passed through a Millipore centrifugal filter and diluted by water up to ~100 μl prior to HPLC separation. The hydrolysis...
products were isolated on a (100 × 3 mm i.d.) Hypercarb column (Thermo-Hypersil-Keystone, Bellefonte, PA) employing 0–90% gradient of acetonitrile in 2-mM ammonium acetate for 40 min at a flow rate 1 ml/min, lyophilized and subjected to LC-MS/MS analysis.

LC-MS/MS experiments

Analysis of the photoproducts was performed with an Agilent 1100 Series capillary LC/MSD Ion Trap XCT mass spectrometer equipped with an electrospray ion source (ion trap conditions: ESI ionization, nebulizing gas 40 psi, drying gas 81/min, drying gas temperature 350°C, acquisition range m/z 80–2000, target mass set to molecular ion of interest, manual MS/MS, fragmentation amplitude 1.0 V, fragmentation time 10 ms, fragmentation width 2 Da). The end products derived from oxidation of 5′-d(GpCnpT) sequences (n = 0, 1) were analyzed using 1–8 μl injection of the sample solution in a narrowbore (50 × 1 mm i. d.) Zorbax SB-C8 column and elution with an isocratic mixture (50% acetonitrile in water with 0.1% formic acid) at a flow rate of 0.2 ml/min. The mass spectra were recorded in the positive mode. The spectrometer was tuned to maximum sensitivity using a direct infusion of the d(GpCnpT) standard solutions. The oxidatively modified 4–11mer oligonucleotides were analyzed using 1–8 μl loop injection elution with an isocratic mixture (50% acetonitrile in 15 mM imidazole) at a flow rate of 0.2 ml/min. The negative multiplex charged ion spectra were deconvoluted using Agilent Data Analysis 5.2 software.

NMR spectra of the oligonucleotide irradiation products

The 1D 1H and 13C spectra, and 2D DQF-COSY (double quantum filtered) and magnitude COSY (correlation spectroscopy), HSQC (heteronuclear single quantum correlation), HMBC (heteronuclear multiple bond correlation) and NOESY (nuclear Overhauser effect spectroscopy) spectra were recorded in D2O using 400 MHz and 500 MHz Bruker NMR instruments and a sample concentration of 1.3 mM. The chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

RESULTS

Generation and identification of the oligonucleotide reaction products

The CO3− radicals were generated by the photochemical method used in our previous experiments (10–12). This method involves the photodissociation of peroxodisulfate to sulfate radical anions, followed by the one-electron oxidation of HCO3− anions with SO4+ radicals. The reaction products derived from the reactions of the single-stranded oligonucleotide, 5′-d(CCCTCGCTACC) with the photochemically generated CO3− radicals were separated from the unmodified oligonucleotides by anion-exchange HPLC methods (Figure 2A). The products were collected, desalted and subjected to analysis by LC-MS/MS. The mass spectra were recorded in the negative mode and deconvoluted. The unmodified sequence eluted after 16.7 min (mass, M: 3237.2), the sequences with diastereomeric spiroiminodihydantoin lesions at 20.0 and 20.8 min (M + 32: 3259.2) and a novel product eluted at 19.0 min (M-2: 3235.2) as shown in Figure 2A. The retention times of the fractions 3 and 4 containing the oligonucleotides with single Sp lesions instead of guanine were identical to those of the authentic standards containing the diastereomeric Sp lesions with (+)-R and (−)-S absolute configurations (11,28). Therefore, in the experiments described here, we focused on the identification of the M-2 adducts that eluted at 19.0 min (fraction 2 in Figure 2A).

The M-2 oligonucleotide adducts obtained in a series of photochemical experiments were combined, desalted and enzymatically digested by nuclease P1 and alkaline phosphatase; the digestion products were separated by...
The ion at \( m/z \) 276.1 is assigned to the \( \text{G}^+\text{-T}^+ \) fragment and its mass is equal to the sum of the masses of G and T minus 2 Da. These results further suggest that the M-2 product derived from the oxidation of \( 5^-\text{dT}(\text{CCATCGCTACC}) \) with CO\(_3^+\) radicals contains an intrastrand cross-link between G and either \( 3^-\text{T} \) or \( 5^-\text{T} \). This cross-linked product is resistant to digestion by the nuclease P1, although the nucleotide pdCp between the bases is removed by the combined action of nuclease P1 and alkaline phosphatase.

The \( 5^-\text{dT}(\text{CCATCGCTACC}) \) sequence contains two isolated T bases, one positioned on the \( 5^-\) side and the other on the \( 3^-\)side of the central G that is initially oxidized by the carbonate radical (10). The site of the cross-link was identified by enzymatic digestion with phosphodiesterases followed by MALDI-TOF/MS analysis of the digestion products. The mass spectra recorded in the negative mode showed that digestion of the M-2 oligonucleotide adduct from the \( 3^-\)end by snake venom phosphodiesterase I stalls at base T2 thus yielding the modified fragment \( 5^-\text{dT}(\text{CCAT}_1\text{CGCT}_2)\)\(^+\), which shows a signal at \( m/z \) 2342.2 after a prolonged digestion. In contrast, the fragment \( 5^-\text{dT}(\text{CGCT}_2\text{ACC})\)\(^+\) was detected at \( m/z \) 2038.2 when the digestion of the full modified 11-mer oligonucleotide with a mass of M-2 was digested from the \( 5^-\)end by bovine spleen phosphodiesterase II. The successful removal of the base T1 by phosphodiesterase II indicates that the intramolecular cross-link is involved in reaction at T2. The involvement of T2 in cross-link formation was confirmed by treating \( 5^-\text{dT}(\text{CCAT}_1\text{CGCT}_2) \) sequences with hot piperidine at 90°C for 30 min (32) and resolving the cleavage products using high-resolution denaturing polyacrylamide gel electrophoresis (data not shown). Cleavage was observed mostly at T2 (~70%), although lesser extents of cleavage at the G (~7%) and T1 (~9%) sites were also detected. These observations indicate that the base T2 is damaged and that hot piperidine treatment generates the fragments \( 5^-\text{dT}(\text{CCAT}_1\text{CGCT}_0) \) and \( 5^-\text{dT}(\text{CGCT}_0\text{ACC}) \) in which G\(^+\) is only partially alkali-labile. The minor extent of cleavage at T1 indicates that cross-linking at T1 does not exceed ~9%.

To obtain insights into the structure of this unusual product, we analyzed the fragmentation patterns of the parent molecular ion, \([M + H]^+\) at \( m/z \) 508.3 (Figure 3). Fragmentation of \([M + H]^+\) occurs via the cleavage of the N-glycosidic bond with the concomitant transfer of a hydrogen atom from the sugar residue to the base (29–31). The signals detected at \( m/z \) 392.2 and \( m/z \) 276.1 correspond to the ions, \([M + H - 116]^+\) and \([M + H - 232]^+\), respectively, derived from the consecutive detachment of two sugar residues from \([M + H]^+\).

Reversed-phase HPLC (Figure 2B). The digestion product eluting at 11.4 min is characterized by a strong UV absorption band at 270 nm (inset in Figure 2B) indicating that this product contains aromatic nucleobases. The 11.4 min product was desalted, concentrated and subjected to LC-MS/MS analysis. The positive ion spectra (MS/MS) of this digestion product are shown in Figure 3. The molecular ion, \([M + H]^+\) is detected at \( m/z \) 508.3. Interestingly, the mass of the digestion product (507.3 Da) is equal to the combined masses of the dG and dT nucleosides minus 2 Da. A plausible structure of the digestion product is shown in Figure 3, and involves a covalent interstrand cross-link between the C8 position of dG and the N3-position of dT that requires the removal of two H atoms. This product lacks the nucleotide pdCp that normally separates the dG and dT in the pdCpG sequence.

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The fragmentation of \([M + H]^+\) occurs via typical pathways for protonated oligonucleotides involving cleavage of the N-glycosidic bond and the phosphodiester C3-O3 bond with the concomitant transfer of two hydrogens from the sugar residue to the base and phosphate (29–31). The ion, \([M + H/C0]^98\]+ arising from the fragmentation of \([M + H]^+\) associated with the release of a furan-type residue is detected at \(m/z\) 761.1. Further cleavage of \([M + H/C0]^98\]+ at \(m/z\) 761.1. Further cleavage of \([M + H/C0]^98\]+ that releases cytosine, generates the ion \([M + H = 98]^+\) observed at \(m/z\) 650. The ions, \([M + H = 387]^+\) at \(m/z\) 472.0 and \([M + H = 583]^+\) at \(m/z\) 276.0 are derived from cleavage of the C3-O3 bond in \([M + H = 98]^+\) and the N-glycosidic bond in \([M + H = 387]^+\). The mass of \([M + H = 583]^+\) is identical to that of the ion at \(m/z\) 276.0 observed in the spectra of the d(GpCpT) and d(TpCpG) sequences by CO3/C15/C0 radicals yield the cyclic products with the identical masses in which G and T are linked to one another and are separated by a single C residue (Figures 5 and S1).

**Acid hydrolysis of the cross-linked trinucleotide and LC-MS/MS analysis of the hydrolysis products**

The positive ion spectra (MS/MS) show that the cross-linked products can be easily fragmented in our ion trap mass spectrometer to the level of a G-T dimer detected at \(m/z\) 276.0 setting (Figure S1). Thus, the LC-MS/MS results indicate that the oxidation of S-d(GpCpT) and S-d(TpCpG) sequences by CO3/C15/C0 radicals yield the cyclic products with the identical masses in which G and T are linked to one another and are separated by a single C residue (Figures 5 and S1).
hydrogen fluoride stabilized by pyridine (HF/Pyr). Cadet and co-workers showed that HF/Pyr treatment allows for the fast and quantitative release of oxidatively modified guanine (27) and thymine (33) bases from DNA at 37°C. After hydrolysis of 5'-d(G'pCpT') with HF/Pyr, the products were isolated by reversed-phase HPLC and subjected to LC-MS/MS analysis. The positive ion spectra of the main hydrolysis product are shown in Figure 6. The molecular ion, [M + H]⁺ detected at m/z 392.0 corresponds to the G'-dT' dimer containing a single sugar residue. Since, the N-glycosidic bonds in pyrimidines are more stable to acid hydrolysis than the N-glycosidic bonds of purines (34,35), we propose that the sugar residue is linked to the T base rather than to the G base (Figure 6). Chemical hydrolysis of 5'-d(G'pCpT') was also performed in 1 N HCl at 85°C for 1 h. The hydrolysis products were separated by reversed-phase HPLC and identified by LC-MS/MS analysis. The positive ion spectra of these products exhibit molecular ions, [M + H]⁺ detected at m/z 392.0 and at m/z 276.0, which were assigned to the dimers with either a single sugar residue (G'-dT') or without any sugar residue (G'+-T'), respectively. However, the yields of these dimers produced by HCl hydrolysis were lower than in the case of HF/Pyr hydrolysis, most likely due to the formation of side products associated with the isomerization and anomerization of the thymine 2-deoxyribose residue that compete with hydrolysis of the N-glycosidic bond (36).

The fragmentation of the molecular ion, [M + H]⁺ at m/z 392.0 (Figure 6) generates the ion, [M + H - 116]⁺ at m/z 276.0 derived from the cleavage of the N-glycosidic bond with release of a sugar residue (29–31). Further fragmentation of [M + H - 116]⁺ yields the daughter ions, [M + H - 199]⁺ at m/z 192.9 and [M + H - 208]⁺ at m/z 84.2 (Figure 6). We note that further fragmentation of these ions in our XCT ion trap produces only negligible amounts of daughter ions that could not be identified. The ions at m/z 192.9 and m/z 84.2 are derived from the cleavage of the N3–C4 and C2–N1 bonds of thymine (Figure 6); these are typical of the fragmentation of thymine derivatives (37). According to this mode of fragmentation, only the N3 or O2 atoms of thymine can be involved in the formation of a bond with the G base.

However, Singer and co-workers (38) have shown that alkylation of the O2 atom of thymine greatly destabilizes the N-glycosidic bond, and in acid solutions (pH 1.5) the O2-alkylated thymines undergo a rapid deproimidination, followed by dealkylation. The O4-alkylated thymidine residues are also dealkylated under these conditions; however, dealkylation does not occur because of the more stable N-glycosidic bonds in these thymidine derivatives. We expect that any cross-linked products containing the C8–O2 (or C8–O4) linkage would not have survived acid hydrolysis under our conditions. Therefore, we conclude that the cross-link occurs via a C8/G–N3/T bond rather than a C8/G–O/T linkage.

**NMR characteristics of the cross-linked product derived from the oxidation of 5'-d(GpCpT)**

The participation of the C8 atom of guanine in the cross-linked product is further supported by NMR data. The formation of a G'pT' cross-link (tandem lesion) in the tetranucleotide, d(CGTA) subjected to ionizing radiation in aqueous solution under anoxic conditions was first reported by Box et al. (15). Utilizing NMR methods they showed that the protons at the C8 position of G and a proton at the methyl group of T were absent, in contrast to the unirradiated controls, and concluded that the irradiation produced a cross-link involving a covalent bond between the C8 carbon atom of guanine and the carbon atom of the thymine methyl group. Unlike the cross-link in our 11-mer and trimer oligonucleotides that involve a covalent bond between G and T separated by C, the cross-linked product observed by Box et al. (15–19), Cadet et al. (20–22) and by Hong et al. (23,24), involved the adjacent G and T residues.

In order to further characterize the sites at the G and T residues that are involved in the formation of the covalent bond in the cross-linked product generated by the oxidation of the 5'-d(GpCpT) sequences with CO₃⁻ radicals, we carried out extensive 1D and 2D NMR analysis. Initially, we compared the 1D proton NMR
spectra of $5'$-$d(G^\prime pCpT^\prime)$ (product 2, Figure 4A) and the unirradiated, intact $5'$-$d(GpCpT)$ trinucleotide. These spectra were recorded in D$_2$O solutions where the resonances of the exchangeable protons are not detectable (Figure 7). Comparison of the 5.5–8.5 p.p.m. portions of the spectra clearly show that the guanine H8 proton is present in the spectrum of the intact sequence (Figure 7A), but is absent in the spectrum of the cross-linked product (Figure 7B). This is straightforward evidence that guanine in $5'$-$d(G^\prime pCpT^\prime)$ is linked to the T base via its C8 carbon atom.

The thymine H6 singlet is evident at 7.6 p.p.m. (Figure 7A and B), and the characteristic –CH$_3$ singlet due to the thymine methyl group is evident below 1.9 p.p.m. in the control as well as in the cross-linked trinucleotide (Figure 7C and D). This clearly suggests that the covalent bond in our cross-linked G-T fragment does not involve the thymine methyl group as in the G-T tandem lesions reported previously (15–19). It is noteworthy that the 2-deoxyribose H1$'$ protons appear as three distinct multiplets in $5'$-$d(G^\prime pCpT^\prime)$ as opposed to the overlapping multiplet in $5'$-$d(GpCpT)$. Also there is a distinct shift in the 2-deoxyribose H2$'$/H2$''$ signals in $5'$-$d(G^\prime pCpT^\prime)$ compared to the control. These differences allowed for the assignment of the majority of the proton (Table 1) and $^{13}$C NMR resonances (Table 2) for the cross-linked $5'$-$d(G^\prime pCpT^\prime)$ oxidation product.

Examples of some of the assignments of $^{13}$C resonances based on HSQC and HMBC correlation spectra are depicted in Figure 8A and B, respectively. The $^1$H resonances corresponding to the 2-deoxyribose H4$'$, H5$'$ and H5$''$ and their respective $^{13}$C signals could not be unambiguously assigned. The process by which all the NMR signals were assigned (Tables 1 and 2) is described, and more complete 1D $^{13}$C NMR, 2D COSY, HSQC and HMBC spectra are provided in Supplementary Data.

**Effect of number of C residues separating G and T on the efficiency of cross-link formation**

In the sequences, $5'$-$d(CCATCGCTACC)$ and $5'$-$d(GpCpT)$ that we studied, the G and T bases are separated by a single C residue. We explored the effects of the number of bridging C bases between G and T on the efficiencies of intrastrand cross-link formation in the series of oligonucleotides $5'$-$d(GpCnpT)$ and $5'$-$d(TpCnpG)$ with $n = 0, 1, 2$ and 3. The intrastrand cross-links generated by the oxidation of these sequences by CO$_3^{2-}$ were isolated by HPLC and identified by LC-MS/MS methods. The positive ion spectra of the cross-linked product, $5'$-$d(G^\prime pC^\prime pT^\prime)$ derived from the oxidation of the dinucleotide, $5'$-$d(GpT)$ exhibits a molecular ion, [M + H]$^+$ at $m/z$ 570.3, as well as the characteristic ion at $m/z$ 276.2 arising from the fragmentation of [M + H]$^+$ (Figure S2, Supplementary Data). The cross-linked product, $5'$-$d(G^\prime C^\prime C^\prime T^\prime)$ generated by the oxidation of $5'$-$d(GCCCT)$ was identified using the negative ion spectrum that shows molecular ions, [M – H]$^-$ at $m/z$ 1146.4 and [M – 2H]$^{2-}$ at $m/z$ 572.9 (Figure S3, Supplementary Data).

The highest efficiency of intrastrand $5'$-$d(G^\prime pCpT^\prime)$ cross-link formation is found in the $5'$-$d(GpCpT)$ sequence; in the case of $5'$-$d(GpT)$ without intervening C

**Table 1. $^1$H NMR chemical shifts of $5'$-$d(G^\prime pCpT^\prime)$**

| Thymine   | Cytosine | Guanine   |
|-----------|----------|-----------|
| T-CH$_3$  | C-H$_5$  | G-H$_1'$  |
| T-H$_6$   | C-H$_6$  | G-H$_2'$  |
| T-H$_1'$  | C-H$_1'$ | G-H$_2''$ |
| T-H$_2'$  | C-H$_2'$ | G-H$_3'$  |
| T-H$_2''$ | C-H$_2''$| G-H$_4'$  |
| T-H$_3'$  | C-H$_3'$ | G-H$_5'$  |
| T-H$_4'$  | C-H$_4'$ |          |
| T-H$_5'$  | C-H$_5'$ |          |
| T-H$_5''$ | C-H$_5''$|          |

*Overlapping peaks.

**Table 2. $^{13}$C NMR chemical shifts of $5'$-$d(G^\prime pCpT^\prime)$**

| Thymine | Cytosine | Guanine      |
|---------|----------|--------------|
| T-C$_2$ | C-C$_2$  | G-C$_2''$    |
| T-C$_4$ | C-C$_4$  | G-C$_4$      |
| T-C$_5$ | C-C$_5$  | G-C$_4''$    |
| T-C$_6$ | C-C$_6$  | G-C$_6''$    |
| T-CH$_3$ | C-C'$_1'$ | G-C$_8$     |
| T-C'$_1'$ | C-C'$_2''$|          |
| T-C'$_2$ | C-C'$_3$ | G-C'$_2''$   |
| T-C'$_3$ | C-C'$_4'$| G-C'$_3'$    |
| T-C'$_4'$ | C-C'$_5'$| G-C'$_4'$    |
| T-C'$_5'$ | C-C'$_5'$| G-C'$_5'$    |

*Assignments based on their expected positions in 2'-deoxyguanosine.
bases the efficiency is almost three times lower (Figure 9). Increasing the number of the C bases also resulted in a decrease in the efficiency of cross-link formation when there are two C bridging residues as in the case of 5'-d(GCCCT). In the case of 5'-d(GCCCT), sequence with three bridging C residues the cross-linked products were not detected. Furthermore, the intrastrand 5'-d(TpG) cross-links were not detected in 5'-d(TpG), although there is a substantial yield in the isomeric 5'-d(GpT) (Figure 6).

This latter result supports the conclusion, reached on the basis of experiments with the isomeric 5'-d(GpCpT) and 5'-d(TpCpG), that the cross-link formation is more efficient when the T residue is on the 3'-side rather than on the 5'-side of the guanine.

**Cross-link formation in DNA duplexes**

The formation of cross-links between adjacent G and T bases in oligonucleotides by ionizing radiation has

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**Figure 8.** (A) Portion of the 2-D HSQC NMR spectrum of 5'-d(GpCpT) showing the correlations that allow assignment of C-C5, C-C6, T-C6 and the C1' carbons of each guanine, cytosine and thymine residue. (B) Portion of the 2-D HMBC NMR spectrum of 5'-d(GpCpT) showing some of the multiple bond correlations that allow assignment of the aromatic base carbons with no attached protons.
been extensively studied by Box et al. (15-19), Cadet et al. (20–22) and by Hong et al. (23,24). Here, we investigated whether oxidation of oligonucleotides by CO$_3^{+}$ radicals can also induce cross-linking reactions in double-stranded DNA and whether the linkage formed involves the T5-CH$_3$ methyl as in the experiments initiated by ionizing radiation. To test this hypothesis, we explored the oxidation of the duplex derived from the self-association of the self-complementary sequence, 5'-d(TTACG TACGTAA) with adjacent G and T nucleotides by CO$_3^{+}$ radicals. After the reactions, the oxidized duplexes were desalted, enzymatically digested by nuclease P1 and alkaline phosphatase, and the products were separated by reversed-phase HPLC methods. The digestion product eluting at 4.1 min after dC at 3.1 min (Figure S8A in Supplementary Data), was desalted, concentrated and subjected to LC-MS/MS analysis. The positive ion spectra (MS/MS) of this digestion product (Figure S8B, Supplementary Data) are identical to the spectra of a 5'-d(GpCpT) cross-link standard (Figure S2) prepared by the oxidation of the dinucleotide 5'-d(GpT) by CO$_3^{+}$ radicals. We also found that the 5'-d(GpCpT) sequence excised from the self-complementary duplex exposed to CO$_3^{+}$ radicals co-elutes with the 5'-d(GpCpT) standard and that the phosphate group between G and T bases is thus not removed by the treatment of 5'-d(GpCpT) with nuclease P1 and alkaline phosphatase. In contrast, such a treatment in the case of the 5'-d(GpCpT) sequence removes the pCp moiety thus leaving a residual G$^\ast$T$^\ast$ cross-linked product (Figures 2 and 3). The yields of the G$^\ast$T$^\ast$ cross-links in the double-stranded DNA are lower than in the case of 5'-d(GpT) dinucleosides and do not exceed 2–3%.

**Role of different one-electron oxidants in the cross-linking reaction**

Our experiments have shown that the highest yields of the G$^\ast$T$^\ast$ cross-links are observed in the case of 5'-d(GpCpT). Using this trinucleotide we found that the yields of the two major products, 5'-d(GpCpT$^\ast$) and 5'-d([Sp)pCpT] increase monotonically with increasing irradiation time as shown in Figure 10. These observations can be considered as an indication that 5'-d(GpCpT$^\ast$) and 5'-d([Sp)pCpT] are formed via two parallel processes and that the cross-linked product 5'-d(GpCpT$^\ast$) is generated by a two-electron oxidation of 5'-d(GpCpT) and is not a precursor of the 5'-d([Sp)pCpT] product derived from a four-electron oxidation of guanine in 5'-d(GpCpT). Removal of O$_2$ by purging the sample solutions with argon during the irradiation significantly decreases the yield of the cross-linked 5'-d(GpCpT$^\ast$) product by a factor of 16; in contrast, the yield of 5'-d([Sp)pCpT] decreases by factor of only 1.4 (Figure 11). Since, the cross-linked product does not contain any additional oxygen atoms in comparison with the parent 5'-d(GpCpT), we propose that oxygen is required for the oxidation of the intermediates. In agreement with our previous $^{18}$O-isotope labeling experiments, which have shown that the major
pathway of 5′-d([Sp]pCpT) formation involves a consecutive four-electron oxidation of 5′-d(GCT) mediated by carbonate radicals (12), oxygen is not critical for the formation of the Sp lesions.

Further experiments performed in air-equilibrated solutions have shown that carbonate radicals are not uniquely necessary for the generation of the cross-linked 5′-d(GpCpT) products. To explore these issues further, we used a series of one-electron oxidants with different reduction potentials (39,40) as well as riboflavin (RF), a typical type I photosensitizer that reacts with DNA via electron abstraction mechanisms (41), to oxidize the trinucleotide 5′-d(GpCpT). The results are summarized in Table 3. After irradiation (30 s, ~100 mW/cm²), the end products were separated by reversed-phase HPLC methods and analyzed by LC-MS/MS that allowed us to identify three major end products: 5′-d(GpCpT)*, 5′-d([Sp]pCpT) and 5′-d([Iz]pCpT), where Iz is 2,5-diamino-4H-imidazolone. The results of these experiments summarized in Table 3 show that the generation of the end products from 5′-d(GpCpT) correlate with the reduction potential of the oxidant.

The reduction potential of carbonate radical of 1.59 V versus NHE (39) is greater than that of guanine neutral radical, 1.29 V versus NHE (9) and the 5′-d(GpCpT)* and 5′-d([Sp]pCpT) are the major end products, but minor quantities of 5′-d([Iz]pCpT) are also observed. The SO₄* radical, a stronger one-electron oxidant with the reduction potential of 2.43 V versus NHE (39), oxidizes 5′-d(GpCpT) thus forming 5′-d(GpCpT)* and 5′-d([Iz]pCpT) as the major oxidation products, but 5′-d([Sp]pCpT) products are not generated. The azide radical, which is a mild one-electron oxidant with a reduction potential of 1.33 V versus NHE (40), was generated by the oxidation of the azide anion by photochemically generated SO₄* radicals. In order to minimize the reactions with nucleotides, the SO₄* radicals were trapped by high concentrations of N₃⁻ anions (0.1 M). Under these conditions, the N₃* radicals do not produce well-defined products such as 5′-d(GpCpT)*, 5′-d([Sp]pCpT) and 5′-d([Iz]pCpT). The value of $E = 1.33$ V versus NHE for N₃* radicals is very close to that of 1.29 V versus NHE for dG(=H)* (9), and under our experimental conditions ([N₃⁻] = 0.1 M $\gg [d(GpCpT)] = 0.1$ mM, Table 1) the driving force of one-electron oxidation of guanine by N₃* radicals is not sufficient for the generation of oxidation products of 5′-d(GpCpT) with observable rates. In contrast, utilizing RF as the photosensitizer which, in the triplet excited state, has a reduction potential of ~1.7 V versus NHE (42), all three products, 5′-d(GpCpT)*, 5′-d([Sp]pCpT) and 5′-d([Iz]pCpT) are generated, with d([Iz]pCpT) being the major product (Table 3). In the case of free nucleosides, photosensitization of the oxidation of dG by RF generates dIz, dSp and dCdG [2,5-anhydro-l-(2′-deoxy-β-d-erythro-pentofuranosyl)-5-guanidinylidene-2-hydroxy-4-oximidazolidine] cyclonucleoside products (41).

**DISCUSSION**

**Structural characterization of the 5′-d(GpCpT) lesion**

The 1D proton NMR data clearly indicate that the G-C8 proton is missing in the cross-linked product, thus suggesting that guanine is covalently linked to T via its C8 carbon atom. The T-CH₃ methyl group protons are characterized by the usual single resonance at 1.85 p.p.m. (Table 1), indicating that all three protons are present and the covalent linkage to guanine does not involve the thymine methyl group. The ¹³C chemical shift at 14.28 p.p.m. (Table 2) is consistent with an sp² hybridization of the methyl group carbon atom, and is in agreement with the conclusions derived from the 1D NMR spectrum. The T-H6 proton resonates at 7.61 p.p.m. in the ¹H spectrum, and the HSQC correlation diagram (Figure 8A) identifies the T-C₆ (¹³C) resonance at 140.49 p.p.m.; the T-C₁ resonance can also be identified via its cross-peak in the HMBC spectrum (Figure 8B). The downfield ¹³C chemical shift of T-C₆ in the 5′-d(GpCpT) product is close to the 139.73 p.p.m. value for T-C₆ in the 5′-d(CpGpT) control indicating that sp² hybridization is intact (Figure S4B and S4D in Supplementary Data).

Similarly, the chemical shifts of the T-C₅ carbon atom at 113.85 p.p.m. in the 5′-d(GpCpT) and 113.85 p.p.m. in the 5′-d(GpCpT) control, indicate that the T-C₅/T-C₆ double bond is intact. These observations demonstrate that neither T-C₅ nor T-C₆ are involved in the covalent bond formation between G-C₈ and thymidine in the 5′-d(GpCpT) product. The T-C₂ and T-C₄ ¹³C resonances at 153.69 and 167.16 p.p.m., respectively, are close to the analogous values at 151.91 and 168.65 p.p.m. in the 5′-d(GpCpT) control; these downfield chemical shifts are consistent with carbon atoms in carbonyl groups and suggest that the covalent linkage between G-C₈ and thymine does not involve the T-C₂ or T-C₄ oxygen atoms. Thus, none of the chemical shifts in the carbon atoms in thymidine appear to be significantly different in the cross-linked product and the control 5′-d(GpCpT) sequence. This is in agreement with ¹³C NMR data for 3-methyl thymidine, where N-functionalization results in minimal changes in the chemical shifts of the other carbon atoms in the modified base (44).
The G-H1’ multiplet at 5.82–5.86 p.p.m. (see, Supplementary Data for the assignment) shows HMBC correlations to two 13C signals at 137.5 p.p.m. and 153.8 p.p.m. (Figure 8B), which can be expected to represent G-C4 and G-C8. Literature values for the G-C4 and G-C8 resonances (44) suggest that G-C4 is the signal at 153.8 p.p.m. and G-C8 is the signal at 137.5 p.p.m. The 13C signal of the G-C8 atom in guanine derivatives functionalized in the C8-position might be expected to undergo a downfield shift relative to the unmodified nucleoside, at least based on the substitution of H by N at the G-C8 position. However, the chemical shift of this G-C8 resonance has not changed significantly in 5’-d(GpCpT) (137.50 p.p.m.) relative to the unmodified 5’-d(GpCpT) (140.13 p.p.m.) (Figure S4B and S4D in Supplementary Data). As in our case, no significant downfield shifts have been observed in the case of N-(2’-deoxyguanosin-8-yl)-2,4-dimethylaniline where G-C8 resonates at 138.0 p.p.m. (45), although a downfield shift from 136.2 to 143.2 p.p.m. was reported in the case of 8-(N-fluoren-2-ylacetamido)-2’-deoxyguanosine 5’-monophosphate (46).

Overall, an analysis of the 13C NMR spectra suggests that the covalent link between G and T in the 5’-d(GpCpT) product occurs via the G-C8 and T-N3 atoms. The G-C8/T-N3 bond may account for the pronounced shift of the H1’ and H2’/H2” protons because the constrained relative orientations of the G-T bases may influence the syn/anti configuration of the ribose units thus affecting the magnetic environment of these H1’, H2’, H2” protons. However, more extensive studies that were beyond the scope of this work would be needed to confirm this hypothesis.

The conclusions based on the NMR data are supported by the experiments involving acid hydrolysis of the cross-linked 5’-d(GpCpT) sequence, followed by LC-MS/MS analysis (Figure 6) that clearly indicates formation of a G+-dT hydrolysis product. Fragmentation of the corresponding molecular ion, [M + H]+ at m/z 392.0 ion leads to the loss of the sugar residue to yield the base-base heterodimer product G”-T” (m/z 276.0). Further cleavage of the N3–C4 and C2–N1 bonds of thymine yields the ions m/z 192.9 and m/z 84.2 (Figure 6). These patterns are typical of the fragmentation of thymine derivatives (37) and suggest that only the N3 or O2 atoms of thymine can be covalently linked to guanine in the G”-T” cross-linked product. Since any C8–O2 (or C8–O4) bonds should be unstable under conditions of acid hydrolysis, we conclude that the cross-link in the G”-T8 product occurs via a G-C8/T-N3 bond rather than a G-C8/T-O linkage, in agreement with the NMR results.

Comparison with other oxidatively generated DNA cross-linked products

The pioneering work of Box and co-workers (15–19) has provided experimental evidence that ionizing radiation produces intrastrand cross-links between the adjacent G and T bases, thus generating the so-called tandem lesions in deoxygenated solutions. A similar G’pT* intrastrand cross-link was also observed when native DNA was subjected to oxidation by a Fenton reaction pathway (24). In these tandem lesions, the C8-atom of guanine is bound to the C-atom of the methyl group in thymine and the formation of these lesions involves intermediate formation of the C-centered 5’-(2’-deoxyuridinyl)methyl radicals. Cadet and co-workers (20–22) have reported that molecular oxygen completely suppresses the formation of such tandem lesions, probably due to the fast reaction of 5’-(2’-deoxyuridinyl)methyl radicals with oxygen, and that tandem lesions formed in the presence of O2 are composed from the oxidogenated G and T bases in DNA. In contrast, Greenberg and co-workers (14,47,48) have proposed that reaction of 5’-(2’-deoxyuridinyl)methyl radicals with O2 is reversible [k(O2) = 2 × 109 M−1 s−1, and k−(O2) = 3.4 s−1] and that molecular oxygen does not prevent the addition of these radicals to adenine. Our experiments demonstrate that oxidation of the single-stranded oligonucleotides by CO3• radicals generate a new type of intrastrand cross-link between G and T bases separated by 0, 1 and 2 cytosine bases and the presence of oxygen is required for formation of these cross-links (Figure 11). High-resolution NMR studies show that in these lesions the H-atom at the C6 of thymine, the CH2-group of thymine and the H-atoms at the C5 and C6 positions of cytosine remain intact. These observations, coupled to the loss of 2 Da in the cross-linked products are consistent with a covalent bond formation between the C8-atom of guanine and the N3 atom of thymine (Figure 1). These new lesions do not require the formation of 5’-(2’-deoxyuridinyl)methyl radicals that typically react rapidly with molecular oxygen (48) and CO3• radicals generate these cross-links in air-equilibrated solutions.

Mechanistic considerations

The cross-linking reaction in the GCT sequence is triggered by the one-electron abstraction from guanine bases by CO3• radicals (Figure 12). This step can be initiated by other one-electron oxidants with appropriate reduction potentials (Table 3). The guanine neutral radicals, G(-H)* generated by CO3• radicals in single- and double-stranded DNA were detected by a direct spectroscopic approach in our previous experiments (10,11,49). The G(-H)* radicals do not react with observable rates with oxygen (10,11) and their fates are determined by further reactions with free radicals or nucleophiles (2,50). Perrier et al. (51) have shown that the RF-mediated photosensitization of d(TpGpT) in aerated solutions containing the KKK tripeptide gives rise to an efficient cross-linking reaction between the guanine and lysine residues. These authors suggested that the cross-linking reaction occurs via a nucleophile addition of the ε-amino group of the lysine residue to the C8 atom of the G”• or G(-H)* radicals followed by oxidation of the radical adduct formed to the Ne-(guanin-8-yl)-lysine cross-link. Here, we propose a similar mechanism for the formation of the G”-T” cross-link that includes the nucleophilic addition of the N3 atom of the thymine residue to the C8 atom of the guanine radical (Figure 12). The radical adduct formed can be oxidized by O2 to abstract the second electron that is required for the
cross-linking reaction. An alternative mechanism might involve the oxidation of the thymidine base (‘second hit’), followed by the combination reaction of the guanine and thymine radicals. Since CO$_3^{3-}$ radicals are not capable of oxidizing thymine bases with observable rates (10), only the SO$_4^{2-}$ radicals can oxidize T in our experiments. However, this mechanism seems unlikely since ESR studies have shown that the yields of N3-centered thymine radicals generated by SO$_4^{2-}$ radicals are negligible (52). Isolation and structural analysis of T-T dimer formation produced by ionizing radiation and subsequent radical–radical interactions provides further evidence that the yields of N3-centered T radicals are small in comparison to the yields of radicals centered at T-CH$_3$, C5 and C6 (53). Therefore, it is unlikely that a bimolecular radical–radical reaction mechanism could have produced G-C8/8-oxo-dG and peroxyl radicals can result in the formation of the Sp diastereomers (10–12), cannot compete with the reactions of SO$_4^{2-}$ radicals with G that is in excess of G(-H)$^*$ under our experimental conditions and, therefore such end products are not observable (Table 3).

The formation of the Iz lesions is associated with the combination of G(-H)$^*$ radicals and superoxide radicals (2,26,50). This reaction dominates in the case of the RF-mediated photosensitization of 5'-d(GpCpT) because these radicals rapidly react with oxygen to form peroxyl radicals (54,58,59) to mostly form radicals of 8-oxo-dG monophosphate is rapidly oxidized to mostly form radicals of 8-oxo-dG (59). The formation of these radicals can also be triggered by the oxidation of 5'-d(GpCpT) by SO$_4^{2-}$ radicals. The SO$_4^{2-}$ radicals can oxidize all three bases in 5'-d(GpCpT) because these radicals rapidly react with dG [4.3 M$^{-1}$s$^{-1}$ (54)], dT [2.1 M$^{-1}$s$^{-1}$ (58)] and dC [1.6 M$^{-1}$s$^{-1}$ (59)] to mostly form radicals of the nucleobases. The G(-H)$^*$ radicals are oxidizing and do not react with oxygen (10,11). In contrast, the oxidation of the pyrimidine bases generates C-centered radicals that rapidly react with oxygen to form peroxyl radicals (58,59). Experiments performed in our laboratory have shown that the combination of G(-H)$^*$ and peroxyl radicals can result in the formation of Iz lesions.

**Biological implications**

The intrastrand crosslinking reaction reported here that covalently couples G and T in the 5'...GC$_2$T...3' sequence context is triggered by the carbonate radical.
anion. The CO$_3^{2-}$ radical is a ubiquitous byproduct of the inflammatory response (1,2) and can easily oxidize guanine in single- and double-stranded DNA by a one-electron transfer mechanism (10–12,49). In the absence of other radicals and further oxidative reactions, neutral guanine radical, G(-H)$^\bullet$ derived from the deprotonation of the guanine radical cation, G-H$^+$, has a lifetime that approaches seconds (10,11), and reaction of G-H$^+$ with T can occur by the nucleophilic N3 addition mechanism described (Figure 12). Oxygen is abundant and can oxidize the G-T radical adduct thus forming the superoxide anion in the process. This O$_2$-mediated reaction thus should add to the oxidative stress associated with CO$_3^{2-}$ radicals. The formation of G-T$^\bullet$ crosslinks requires a single hit by a one-electron oxidant, and is of course sequence dependent. The reparability of these novel cross-linked lesions by different DNA repair systems is not yet known, but is under study in our laboratory.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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