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for

Oxidation of Guanine by Carbonate Radicals Derived from Photolysis of Carbonatotetramminecobalt(III) Complexes and the pH Dependence of Intrastrand DNA Cross-links Mediated by Guanine Radical Reactions

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Kinetics of Oligonucleotide Oxidation by $CO_3^{\bullet-}$ Radicals: In the case of 5'-d(CC-ATC[X]CTACC) sequences (X = G or 8-oxoGua), the solution of the differential equations describing this reaction scheme at $[CO_3^{\bullet-}]_0 < [\text{oligo}]_0$ yield the following equation for the decay of the transient absorbance of $CO_3^{\bullet-}$ radicals at 600 nm:

$$A_{600}(t) = k_n' A_{600}(t_0)/\{[k_n' + 2\,k_5 A_{600}(t_0)/\varepsilon_{600}] \exp(k_n' t) - k_5 A_{600}(t_0)/\varepsilon_{600}\} + A_{600}(t_\infty) \qquad (1a)$$

where $A_{600}(t_0)$ and $A_{600}(t_\infty)$ are the initial and final absorbances at 600 nm (data not shown), $k_n' = k_n[\text{oligo}]_0$ is the pseudo first order rate constant of the $CO_3^{\bullet-}$ decay in reactions 1 – 4, and k_5 is the rate constant of the recombination of $CO_3^{\bullet-}$ radicals measured in the absence of oligonucleotides. Figure S1 shows that the values of k_n' do not depend on the method of $CO_3^{\bullet-}$ generation and linearly increase with the rise of oligonucleotide concentrations. From the slopes of the straight lines, the values of k_n are obtained that are identical to those obtained in our previous experiments using $CO_3^{\bullet-}$ radicals derived from oxidation of HCO_3^- anions by $SO_4^{\bullet-}$ radicals.[1, 2]

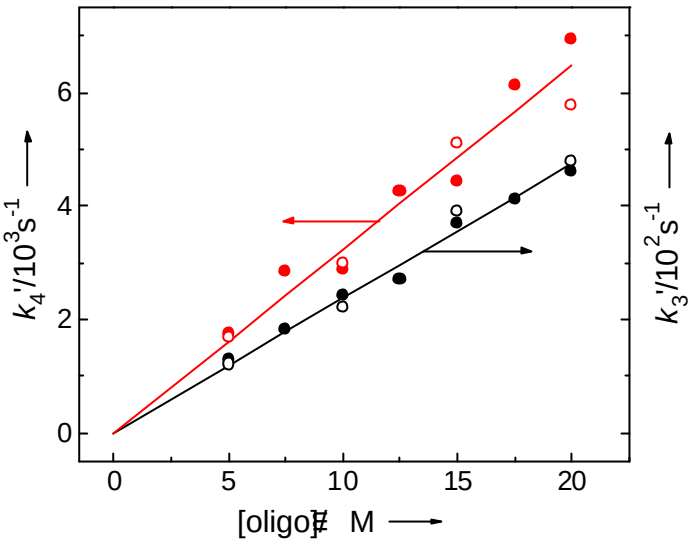

Figure S1. Rate constants for the decay of $CO_3^{\bullet-}$ radicals in the presence of 5'-d(CCATC-GCTACC) (black circles) and 5'-d(CCATC[8-oxodGua]CTACC) (red circles) sequences as a function of the oligonucleotide concentration. The values of k_3' and k_4' obtained fitting equation 1a to the experimental transient absorption profiles recorded at 600 nm do not depend on the method of $CO_3^{\bullet-}$ generation (open circles – by the photolysis of $[Co(NH_3)_4CO_3]^+$, closed circles – by the oxidation of HCO_3^- anions by $SO_4^{\bullet-}$ radicals).

Identification of 5'-d(G*CT*) by LC-MS/MS: The mass of the cross-linked product with molecular ion, $[M + H]^+$ detected at *m/z* 859.1 (Figure S2) is smaller by 2 Da than the mass of the unmodified 5'-d(GpCpT) observed at *m/z* 861.2. The ion, $[M + H - 98]^+$ 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 $[M + H - 98]^+$ that releases cytosine, generates the ion $[M + H - 209]^+$ observed at *m/z* 650.1. 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 $[M + H - 209]^+$ and the *N*-glycosidic bond in $[M + H - 387]^+$, respectively. The $[M + H - 583]^+$ fragment detected at *m/z* 276.0 is a direct evidence for cross-linking the G and T bases.

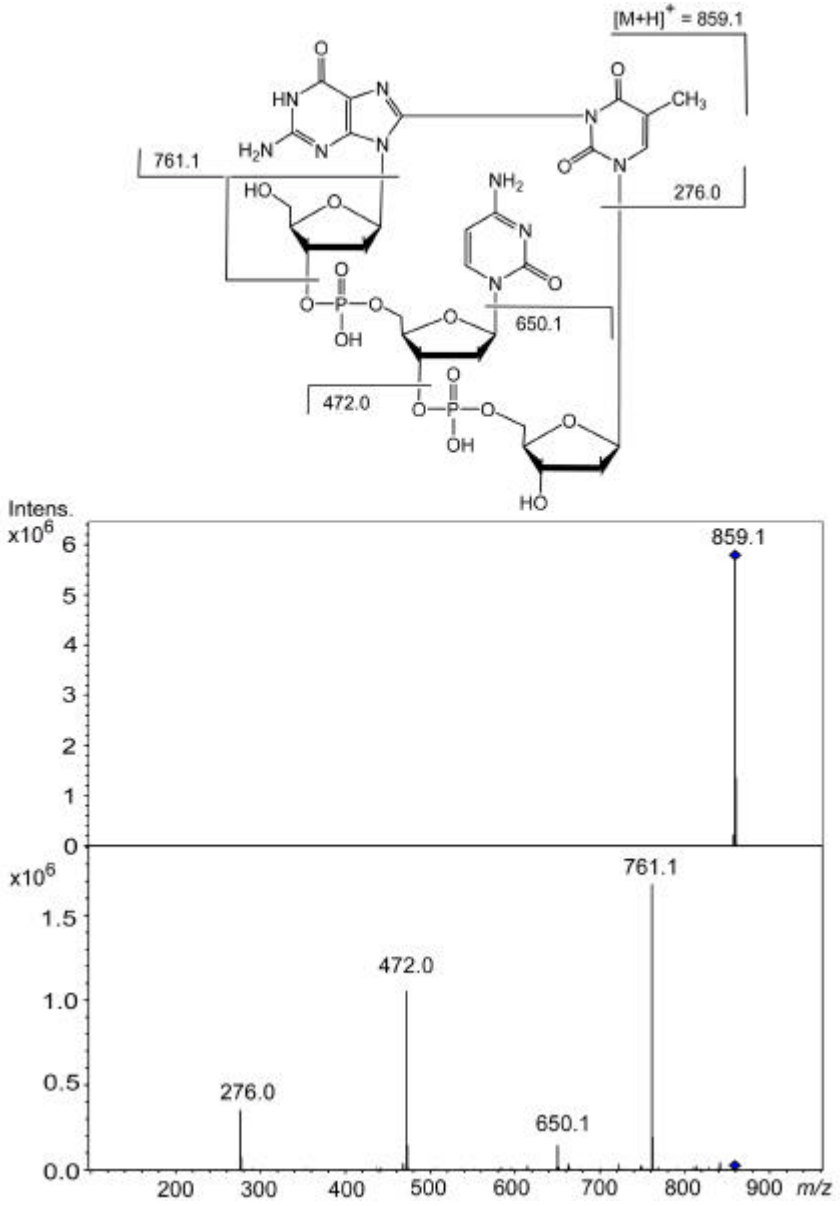

Figure S2. Positive ion spectra (MS/MS) of 5'-d(G*pCpT*). MS spectrum of the molecular ion, $[M + H]^+$ at *m/z* 859.1. MS^1 product ion spectrum obtained by fragmentation of the molecular ion, $[M + H]^+$ at *m/z* 859.1.

Exonuclease ladders of 5'-d(CCATCG*CT*ACC): Dried G*CT* 11-mer oligonucleotides (150 pmol) were dissolved in 6 µL of water. For digestion from the 3'-end, 6 µL of 100 mM ammonium citrate (the pH was adjusted to 9.4 by NH_4OH) and 0.5 µL snake venom phosphodiesterase (0.005 units/µL) were added to the sample solution. The solution was incubated at 37 °C. For digestion from the 5'-end, 7 µL of water and 1 µL bovine spleen phosphodiesterase (0.005 units/µL) were added to the sample

solution. Aliquots (1 µL) of the digested solutions were removed at fixed periods of time and placed on dry ice for 20 s to interrupt the digestion reaction. Aliquots (1 – 2 µL) of the samples and the trihydroxyacetophenone matrix solution were mixed and immediately spotted on a MALDI target and air-dried before analysis. The mass spectra were acquired using a Bruker OmniFLEX instrument. The mass spectrometer was operated in the negative linear mode (accelerating voltage 19 kV, extraction voltage 92.7% of the accelerating voltage, ion focus 9 kV, and delay time 250 ns). Each spectrum was obtained with an average of 50–100 laser shots. The mass spectra were internally calibrated by using synthetic oligonucleotides of known molecular weights.

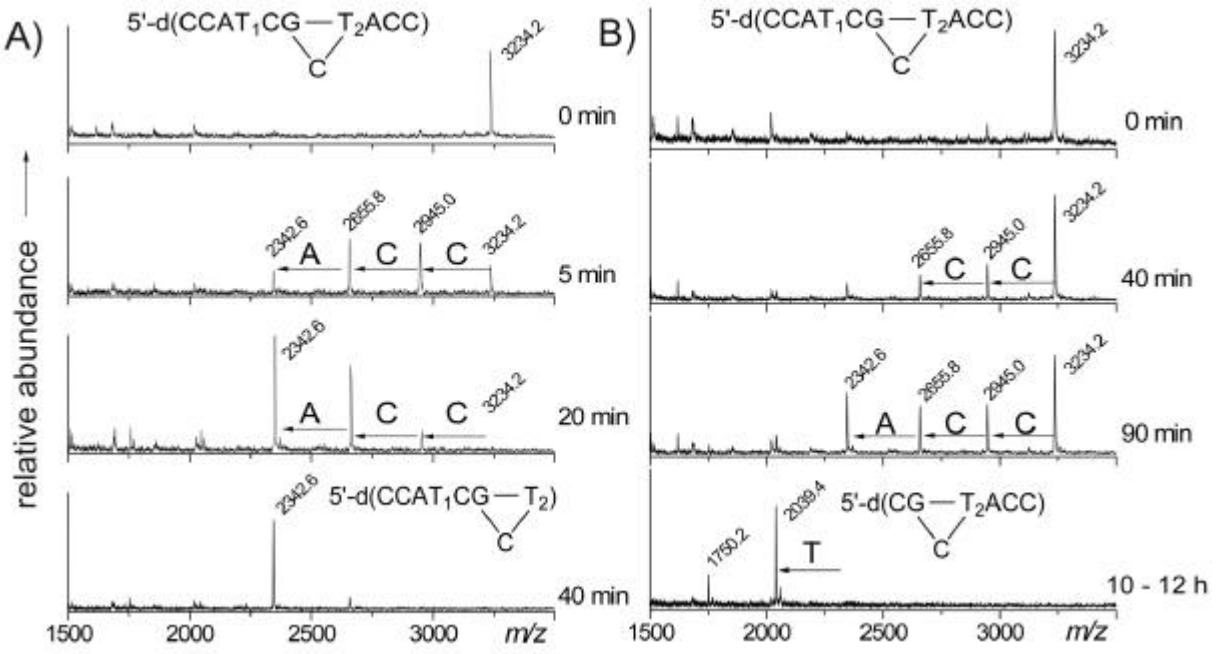

Figure S3. Negative MALDI-TOF mass spectra of the M-2 adduct containing the G*CT* intrastrand cross-linked 11-mer oligonucleotide product digested for the indicated periods of time either by snake venom phosphodiasterase I (Panel A), or by bovine spleen phosphodiesterase II (Panel B). Panel A - a prolonged digestion of the M-2 adduct from the 3'-end by snake venom phosphodiesterase I stops at base T_2 yielding the 8-mer fragment 5'-d(CCA-T_1CGCT_2)* detected at *m/z* 2342. Panel B - in contrast, digestion of this adduct from the 5'-end by bovine spleen phosphodiesterase II generates the shorter 7-mer fragment 5'-d(CGC-T_2ACC)* detected at *m/z* 2039.4. The successful removal of the base T_1 by phosphodiesterase II indicates that the intrastrand cross-link mostly involves reaction at T_2.

Hot piperidine cleavage of 5'-d(CCATCG*CT*ACC): The conclusion that the cross-linked product indeed involves G* and T* was confirmed by treating the 5'-^{32}P-end-labeled oligonucleotides in the G*CT* fraction with hot piperidine at 90 ^{o}C, and resolving the cleavage products using high resolution denaturing polyacrylamide gel electrophoresis. Figure S4 shows that the standard hot piperidine treatment (30 min) of the G*CT* 11-mer oligonucleotide (Lane 3) induces strong cleavage of the modified oligonucleotide at T_2 (~70%) resulting in the formation of two partially resolved cleavage products (T_2', and T_2"). Lesser extents of cleavage are also observed at the central guanine G (~7%) and T_1 (~9%) sites.

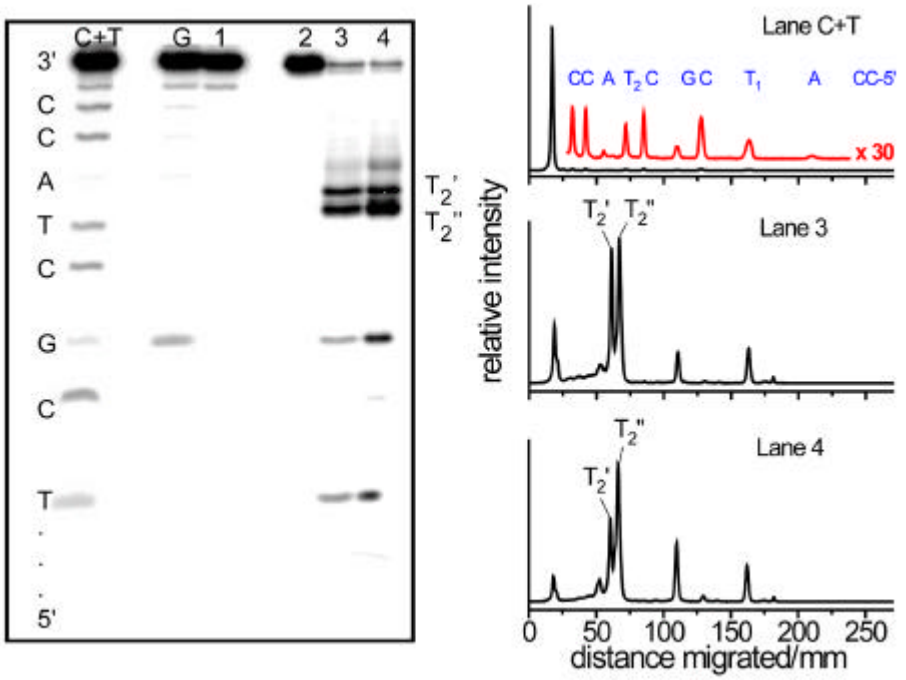

Figure S4. Autoradiograph and histograms of the denaturating gel (7 M urea, 20% polyacrylamide gel) showing the cleavage patterns induced by hot piperidine treatment of the adduct containing the G*CT* intrastrand cross-link labeled at its 5'-termini. Lanes C+T and G: Maxam-Gilbert C+T and G sequencing of the 5'-d(CCATCGCTACC) control sequence; Lane 1: Control sequence (with piperidine treatment); Lane 2: Intact M-2 adduct (without piperidine treatment); Lanes 3 and 4: M-2 adduct treated with hot piperidine at 90 ^{o}C for 30 and 60 min, respectively.

Increasing the hot piperidine incubation time up to 60 min (Lane 4, and histogram in Panel C) results in an almost complete cleavage of the starting M-2 adduct (~93%). These observations indicate that the base T_2 is damaged and that hot piperidine

treatment generates the fragments 5'-^{32}P-d(CCAT$_1$CG*C) in which G* is only partially alkali-labile. The minor extent of cleavage at T_1 indicates that cross-linking at T_1 does not exceed ~9%. This fragmentation mode was confirmed by the MALDI-TOF/MS analysis. The negative MALDI-TOF mass spectrum of the non-labeled M-2 adduct treated by hot piperidine (Figure S5) shows the expected fragments including 5'-pACC (*m/z* 908.2) and 5'-CCATCp (*m/z* 1501.2) that arise from the cleavage at T_2 and G. A group of unidentified products (*m/z* 2199, 2257.4) are also observed, which can be tentatively assigned to the 5'-CCAT$_1$CG*Cp fragments (T_2', and T_2" in Figure S4) derived from cleavage at T_2.

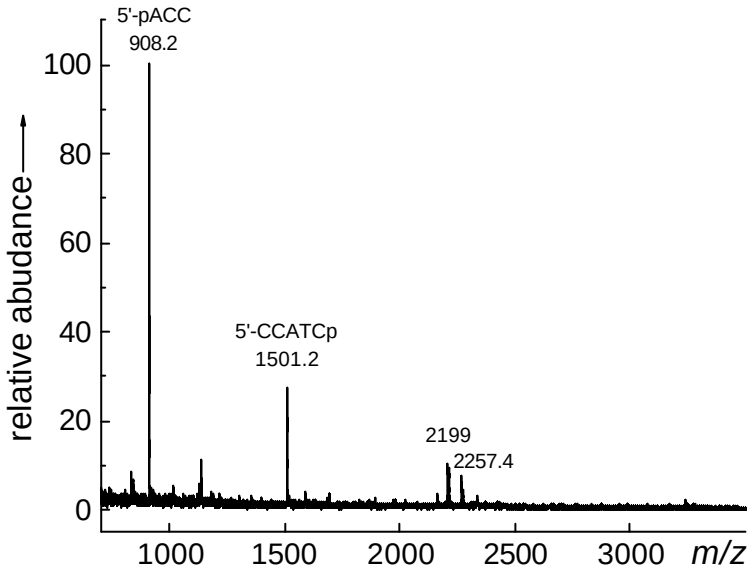

Figure S5. Negative MALDI-TOF mass spectrum of the M-2 adduct treated with hot piperidine at 90 ^{o}C for 30 min.

Hot piperidine cleavage of the diastereomeric 5'-d(CCATC[Sp]CTACC) adducts: The 5'-^{32}P-endlabeled spiroiminodihydantoin adducts with (+)-*R*-Sp and (–)-*S*-Sp were treated with hot piperidine at 90 ^{o}C, and the cleavage products formed were detected by a high resolution denaturing polyacrylamide gel electrophoresis. Figure S6 shows that the hot piperidine treatment of the both diastereomeric Sp adducts induces cleavage mostly at Sp-sites.

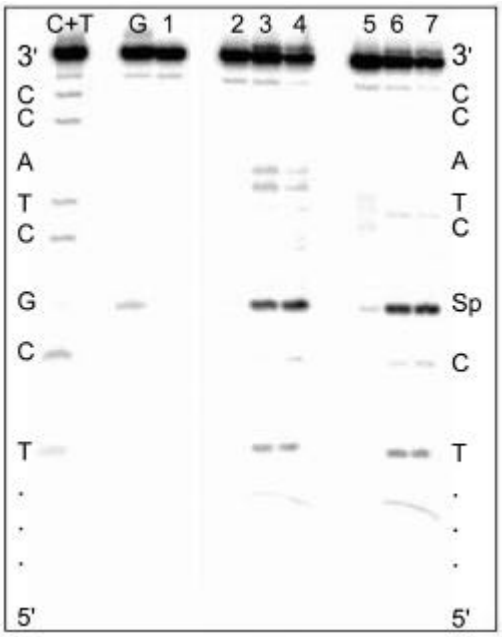

Figure S6. Autoradiograph of the denaturating gel (7 M urea, 20% polyacrylamide gel) showing the cleavage patterns induced by hot piperidine treatment of the 5'-d(CCATC[Sp]CTACC adducts with (+)-*R*-Sp and (–)-*S*-Sp configurations labeled at their 5'-termini. Lanes C+T and G: Maxam-Gilbert C+T and G sequencing of the 5'-d(CCATCGCTACC) control sequence; Lane 1: Control sequence (with piperidine treatment); Lane 2: Intact (+)-*R*-Sp adduct (without piperidine treatment); Lanes 3 and 4: (+)-*R*-Sp adduct treated by hot piperidine at 90 °C for 30 min (~14% cleavage) and 60 min (~31%); Lane 5: Intact (–)-*S*-Sp adduct (without piperidine treatment); Lanes 6 and 7: (–)-*S*-Sp adduct treated by hot piperidine at 90 °C for 30 min (~19% cleavage) and 60 min (~28%).

Formation of the Sp Lesions: Insights from Oxygen-18 Isotope Labeling. A deeper understanding of the mechanistic aspects of the Sp lesion formation in double-stranded DNA was gained by performing photooxidation experiments in $H_2^{18}O$ buffer solutions. In these experiments the duplexes formed by the self-complementary sequence 5'-d(AAC**G**C**G**AATTC**G**C**G**TT**)** were oxidized by $CO_3^{\bullet-}$ radicals derived from HCO_3^- oxidation by photochemically generated $SO_4^{\bullet-}$ radicals in air-equlibrated buffer solutions (pH 7.5) containing 40% $H_2^{18}O$. The photooxidized samples were enzymatically digested using the following protocol. The irradiated DNA samples (6 nmol) were dissolved in 17 µL of sodium acetate (30 mM, pH 5.2) and incubated with 1 unit of nuclease P1 for 30 min at 37°C. Then 3 µL of Tris-HCl (1M, pH 8) and 1 unit of calf intestinal phosphatase were added and the sample was then incubated for 60 min at 37°C. The enzymes were removed by centrifugal filtration and the sample was subjected to LC-MS/MS analysis. Under these conditions the major product is the 5'-

d(C[Sp]) dinucleoside because the C-[Sp] phosphodiester bond is strongly resistant to enzymatic digestion.[2] The 5'-d(C[Sp]) dinucleoside was identified by LC-MS using the authentic 5'-d(C[Sp]) standard synthesized as described earlier.[3] The complete digestion to the level of dSp mononucleoside requires a combined action of nuclease P1 and snake venom phosphodiesterase and overnight incubation;[4] here, we prefered a short incubation time in order to minimize potential isotope exchange reactions. The positive ion spectra of the d(C[Sp]) dinucleoside excised from oxidized DNA duplexes are shown in Figure S7.

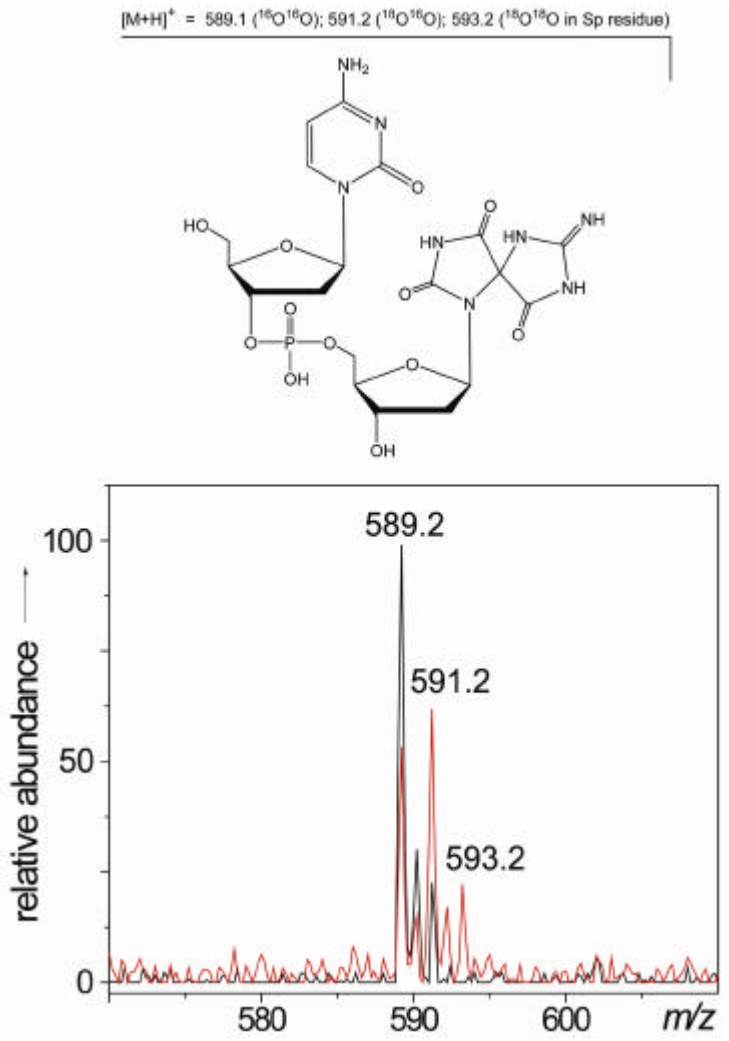

Figure S7. Postive ion spectra of 5'-d(C[dSp]) dinucleotides obtained from the partial digestion of duplexes formed by the self-complementary sequence 5'-d(AAC**G**C**G**AATTC**G**C-**G**TT**),** after exposure to and oxidation by $CO_3^{\bullet-}$ radicals derived from the oxidation of HCO_3^- by photochemically generated $SO_4^{\bullet-}$ radicals in air-equlibrated buffer solutions (pH 7.5) containing $H_2{}^{18}O$ (fraction of ^{18}O atoms $\alpha = 0.40 \pm 0.04$). The reaction mixtures did not contain

carbonic anhydrase (black), or contained 10 nM carbonic anhydrase to provide for a complete exchange of ^{18}O between $HC^{16}O_3^-$ and $H_2^{18}O$ (red). In the presence of 10 nM carbonic anhydrase, the experimentally observed fractions of isotopically labeled 5'-d(C[dSp]) dinucleotides are 0.39±0.04, 0.45±0.04, and 0.16±0.02 for the ions detected at *m/z* 589.2, 591.2 and 593.2. These fractions are close to the expected values of $(1-\alpha)^2 = 0.36\pm0.04$ ($^{16}O^{16}O$), $2(1-\alpha)\alpha = 0.5\pm0.01$ ($^{16}O^{18}O$), and $\alpha^2 = 0.16\pm0.03$ ($^{18}O^{18}O$) for a random transfer of two oxygen atoms from the carbonate radical anions to the Sp residues in 5'-d(C[dSp]).

Mechanisms of incorporation of oxygen atoms into Sp. We first consider the formation of Sp in the absence of carbonic anhydrase, under the reaction conditions described in Materials and Methods. Normally, isotope exchange between $HC^{16}O_3^-$ and $H_2^{18}O$ is slow because it occurs only through the hydration of carbon dioxide with the resulting H_2CO_3 being in equilibrium with bicarbonate.[5] Therefore, even if the reactions are carried out in heavy water, the isotopic composition of the $CO_3^{\bullet-}$ radicals will be close to that of the parent $HC^{16}O_3^-$ anions. Hence, the additional O atoms incorporated into the Sp dinucleosides in the absence of carbonic anhydrase could have been, in principle, transferred either from molecular oxygen ($^{16}O_2$) or from $C^{16}O_3^{\bullet-}$ radicals, but not from $H_2^{18}O$. Since Sp can be formed in the absence of oxygen,[2] O_2 can be ruled out as the source of the additional ^{16}O-atoms in Sp, and it can be concluded that the $C^{16}O_3^{\bullet-}$ radical is the source of the additional ^{16}O-atoms.

The experiment was also carried out in the presence of carbonic anhydrase, an enzyme which catalyzes oxygen isotope exchange between $HC^{16}O_3^-$ and $H_2^{18}O$.[6] The resulting mass spectrum (Figure S7, red line) shows molecular ions at 589.2, 591.2 and 593.2 indicating incorporation of one and two atoms of ^{18}O in the spiroiminodihydantoin produced. Thus, the mechanism of the Sp formation in double-stranded DNA is identical to that reported in our previous work for the oxidation of 2',3',5'-tri-O-acetylguanosine (tri-O-Ac-Guo) or 2',3',5'-tri-O-acetyl-8-oxo-7,8-dihydroguanosine (tri-O-Ac-8-oxoGuo) by $CO_3^{\bullet-}$ radicals.[7] In this work we showed that the oxygen atoms incorporated into the Sp end-products formed by the oxidation of either tri-O-Ac-Guo or tri-O-Ac-8-oxoGuo originate from $CO_3^{\bullet-}$ radicals and not from H_2O. These observations indicate that the intermediate steps occur by the radical-radical combination of $CO_3^{\bullet-}$ with Gua(-H)$^\bullet$ and 8-oxoGua(-H)$^\bullet$, and that these reactions can be formally viewed as O^--transfer mechanisms.

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