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PAPER

Characterization of 2′-deoxyguanosine oxidation products observed in the Fenton-like system Cu(II)/H_2O_2/reductant in nucleoside and oligodeoxynucleotide contexts†‡

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Reactive oxygen species attack both base and sugar moieties in DNA with a preference among the bases for reaction at guanine. In the present study, 2′-deoxyguanosine (**dG**) was oxidized by a copper-mediated Fenton reaction with the reductants ascorbate or *N*-acetyl-cysteine, yielding oxidation on both the base and the sugar. The primary oxidized lesions observed in these studies include the 2′-deoxyribonucleosides of 8-oxo-7,8-dihydroguanosine (**dOG**), spiroiminodihydantoin (**dSp**), guanidinohydantoin (**dGh**), oxazolone (**dZ**), and 5-carboxamido-5-formamido-2-iminohydantoin (**d2Ih**), as well as the free base guanine. **d2Ih** was the major product observed in the nucleoside, single- and double-stranded oligodeoxynucleotide contexts and is proposed to arise from oxidation at C5 of guanine. Product distribution studies provide insight into the role of the reductant in partitioning of **dG** base oxidation along the C5 and C8 pathways.

Introduction

Cellular damage resulting from oxidative stress has been implicated in many disorders including aging, cancer, and neurological diseases.[1,2] Formation of reactive oxygen species (ROS) *in vivo* results from incomplete reduction of O_2 to H_2O in mitochondria as well as from inflammation or environmental factors.[3,4] These diffusible oxidants can impose damage to cellular macromolecules including DNA.[2] H_2O_2 is a relatively stable ROS produced within mitochondria that is capable of diffusing throughout the cell promoting free radical oxidation reactions *via* the Fenton reaction.[5,6] The Fenton reaction occurs when H_2O_2 is activated by Fe(II) yielding hydroxyl radical (eqn (1)), or by Cu(I) yielding either a free or metal bound hydroxyl radical (eqn (2), (3)). In the presence of DNA, a DNA bound Cu(I)–OOH complex has been proposed.[5,7] All of these species are strong one-electron oxidants. Isolated cellular DNA often contains copper,[8] and studies suggest that Cu-mediated oxidation of DNA is associated with aging and cancer disease states.[9]

$$\mathrm{Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO^{\bullet}} \quad (1)$$

$$\mathrm{Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^- + HO^{\bullet}} \quad (2)$$

$$\mathrm{Cu(I)\text{-}OOH + H^+ \rightarrow Cu(III)\text{-}OH + HO^-} \quad (3)$$

DNA oxidation by Cu-mediated Fenton chemistry has been shown to induce oxidation of 2-deoxyribose leading to base release and strand scission when Cu is not bound to DNA;[6,10] when Cu is bound to DNA, base-centered oxidations have been proposed.[6] Binding of both Cu(I) and Cu(II) ions with DNA occurs primarily at N7 of 2′-deoxyguanosine (**dG**, mass = M)[11] in which the coordination of Cu(I) ($K_a \approx 10^9$ M^{-1})[12] is ~10^5 times stronger than Cu(II) ($K_a \approx 10^4$ M^{-1}).[13] Furthermore, copper-mediated Fenton oxidation of DNA with bound Cu(I) yields base oxidation at **dG** sites,[6] and nucleosome oxidation studies using Cu(II)/H_2O_2 indicated formation of more base lesions than single-strand breaks.[8] Further studies have suggested that Cu-mediated DNA damage is sequence selective with **dG** repeats being more prone to base modification than other sequences.[14] It has been suggested that hydroxyl radical is not the primary reactive oxidant responsible for base damage.[7,15]

Guanine is the most electron-rich site in DNA; therefore, oxidation occurs preferentially at **dG** to yield electrophilic intermediates and a wide spectrum of end products that are typically identified by mass spectrometry (Fig. 1).[5] These products can be categorized as resulting from initial chemistry at either the C5 or C8 carbons. Alternatively, base release and/or strand scission occur from oxidation of the 2-deoxyribose moiety.[5] The oxidation of **dG** (mass = M) at C8 by $HO^{\bullet}$ (produced by Fenton chemistry or radiolytically),[3,16,17] $CO_3^{\bullet-}$,[18] chromate,[19] and

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† Dedicated to the memory of Professor Athel Beckwith and his distinguished career in free-radical chemistry.

‡ Electronic supplementary information (ESI) available: Experimental details of HPLC separation and mass spectrometric characterization of products, time and pH-dependent studies, and compilations of product yield data. See DOI: 10.1039/c1ob05112a

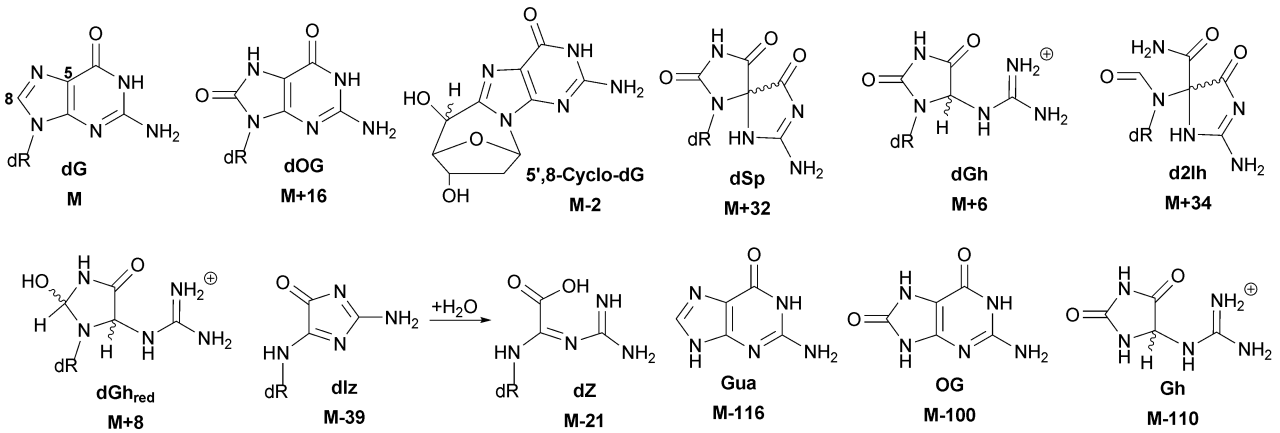


Fig. 1 Structures of dG oxidation products and their relative mass differences (dR = 2′-deoxyribose).

the photoexcited state of riboflavin[20] yields 8-oxo-7,8-dihydro-2′-deoxyguanosine (**dOG**, M + 16) as a key product. When **dG** is oxidized within the DNA context by HO$^{\bullet}$ (produced by Fenton chemistry or radiolytically), the products observed were **dOG** and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (**Fapy-dG**, M + 18) with **dOG** observed under oxidizing conditions and **Fapy-dG** under reducing conditions.[21,22] The *in vivo* concentrations of **dOG** can be monitored to provide levels of cellular oxidative stress. Basal concentrations of **dOG** are ~1 in 10^6 bases,[23] but increase under certain disease states.[24] **dOG** lesions in DNA are moderately mutagenic causing G→T transversion mutations *in vivo*[25] when **dOG** is not removed from the genome by repair processes.[26]

The oxidation of **dOG** is much more facile than that of **dG** as reflected in its lower redox potential (E^{o} = 0.7 V and 1.3 V *vs.* NHE, pH 7.0, respectively);[27,28] thus, a further two-electron oxidation of **dOG** occurs readily in the presence of such oxidants as Ir(IV),[29,30] chromate,[31] $CO_3^{\bullet-}$,[18] $^{\bullet}NO_2$,[32] or $ONOO^-$[33] to yield the hydantoin products, spiroiminodihydantoin (**dSp**, M+32) and guanidinohydantoin (**dGh**, M+6). **dSp** has also been observed by the iron and copper-mediated Fenton oxidation of **dOG**.[34] Furthermore, **dSp** and **dGh** can be formed directly from **dG** by four-electron oxidation mediated by singlet oxygen; a mechanistic proposal for this pathway has been reported.[35] Yields of **dSp** and **dGh** are affected by the reaction pH and the structural context with **dSp** formation favored at high pH (>6.0) and in nucleoside contexts and **dGh** favored at low pH (<6.0) and in oligonucleotide contexts.[36–38] **dSp** has been observed in chromate-stressed *E. coli*,[39] and it has been shown that **dSp** and **dGh** are highly mutagenic causing G→T and G→C transversion mutations.[40]

When **dG** was oxidized by either HO$^{\bullet}$ (formed radiolytically),[21] $SO_4^{\bullet-}$, or photoexcited riboflavin, many additional products were observed. Those characterized as resulting from chemistry at C5 of **dG** were imidazolone (**dIz**, M − 39) and its hydrolysis product oxazolone (**dZ**, M − 21). **dZ** has been observed *in vivo*,[41] and predominantly causes G→T transversion mutations.[42] In the case of **dG** oxidation by NiCR/$KHSO_5$,[43] Mn-TMPyP/$KHSO_5$,[44,45] or the epoxidizing agent dimethyldioxirane,[46] oxidation at C5 is also observed yielding the product 5-carboxamido-5-formamido-2-iminohydantion (**d2Ih**, M+34). In work by Karlin and Rokita, a compound with the same mass as **d2Ih** was observed upon oxidation of an oligodeoxynucleotide (ODN) with a dinuclear copper(II) complex.[47] Other coordination compounds of copper formed with phenanthroline and related ligands have been shown to mediate primarily ribose oxidation.[10] The large distribution of base and ribose-derived products observed from **dG** oxidation highlight the complexity in understanding **dG** oxidation pathways with free radical-generating oxidants.

Fenton oxidation (Fe(II)/H_2O_2/reductant) of **dG** in the nucleoside context yields **dOG** and 5′,8-cyclo-2′-deoxyguanosine (**5′,8-cyclo-dG**, M-2) in nearly equal amounts.[16,48] In the absence of the reductant, **5′,8-cyclo-dG** was observed in a 16-fold greater yield.[16] In addition, significant amounts (~50%) of sugar chemistry occurred leading to base release that was characterized by the presence of guanine (**Gua**, M-116); the remaining mass balance was comprised of many sugar oxidation products in low yield.[16] These studies suggest that the reductant plays a critical role in determining product distributions from Fenton-mediated oxidation of **dG**. Radiation-induced oxidation of **dG** in DNA was shown to yield both **5′,8-cyclo-dG** and **dOG**.[49] Copper-mediated Fenton oxidation of **dG** in DNA was reported to yield both sugar- and base-oxidation products in which the sugar-oxidation products were determined by monitoring strand breaks, and the base-oxidation products **dOG** and **Fapy-dG** were quantified using GC-MS.[22,50,51] These studies consistently found **dOG** to be the major oxidation product, with yields at least 10-fold greater than that of **Fapy-dG**. When ODNs or DNA were exposed to Cu(II)/H_2O_2/ascorbate, intrastrand cross-links between **dG** and thymidine (**T**), or **dG** and 5-methyl-2′-deoxycytidine were observed, pointing to the fact that the DNA context yields new products that were not observed within nucleoside models.[52,53] In this study, we have analyzed **dG** oxidation products within the nucleoside, single- and double-stranded **ODN** contexts resulting from exposure to Cu(II) and H_2O_2 in the presence of either ascorbate (Asc) or *N*-acetyl-cysteine (NAC) as reductant. In these studies a recently identified compound, **d2Ih**, was observed as the major product of **dG** oxidation in all contexts.

Results

Product characterization

The nucleoside oxidation studies were conducted with **dG** (2.0 mM) in buffer (75 mM NaP_i, pH 7.0), followed by stepwise addition of Cu(II) acetate (1.0 mM, final concentration), Asc or NAC (2.0 mM), and then H_2O_2 (10.0 mM). The reaction was held at 22 °C for 60 min under aerobic conditions, and then terminated by addition of Na_2EDTA (10.0 mM). The reaction was analyzed initially by reversed-phase HPLC (RP-HPLC) with a MS detector that allowed the identification of the free base products **Gua**, 8-oxo-7,8-dihydroguanine (**OG**) and the nucleoside products **dOG** and **5′,8-cyclo-dG**. The void volume from the previous HPLC run was collected and then reinjected on a Hypercarb HPLC column. This run was monitored by MS to provide initial identification for the free base **Gh**, and the nucleosides **d2Ih**, **dGh**, **dSp**, and **dZ**. Under these conditions, **dIz** elutes past the void volume on the RP-HPLC run and was collected with the void volume. During the process to prepare a sample for the Hypercarb column, **dIz** hydrolyzed to **dZ**, therefore only **dZ** was observed and quantified. Chromatography utilizing the Hypercarb column allowed the separation of diastereomers, and two peaks were observed for both **d2Ih** and **dSp**, although the diastereomers of **dGh** were not separable (see electronic supplementary information‡). Further support for the products **d2Ih**, **dSp** and **dZ** was obtained through ESI^+-MS/MS fragmentation of the free bases derived from in-source fragmentation of the glycosidic bond followed by comparison of the daughter fragments to previously published data (see ESI‡).[30,41,46] Because **dGh** could not be fragmented, an HRMS was obtained for further characterization. Reactions were also conducted using cysteine as the reductant, and in order to avoid amine adducts to electrophilic **dG** oxidation intermediates, *N*-acetyl-cysteine (NAC) was used.[54,55]

To evaluate the context effect on the oxidation products, the complementary single-stranded ODNs (**ODN-1** and **ODN-2**) and the double-stranded ODN (**ODN-12**) were allowed to react with the same oxidant systems described above. The ODN and nucleoside oxidation studies were conducted under slightly different conditions; the ODN oxidation utilized 100 μM ODN, 20.0 mM NaP_i, pH 7.0, 100 mM NaCl at 37 °C for 8 h reaction with the oxidant system comprising Cu(II) acetate (10.0 μM) reductant (1.0 mM) and H_2O_2 (1.0 mM). To study the product distributions from **dG** oxidation in the ODN contexts, the ODNs had to be digested to nucleoside mixtures that could be analyzed by the method outlined for the previous studies. The ODN samples were exhaustively digested with DNaseI, nuclease P1, and snake venom phosphodiesterase, followed by phosphate removal with calf intestinal phosphatase to yield an analyzable nucleoside sample. The digestion method used was shown previously to liberate both diastereomers of **dSp** and **dGh** in high yields from ODNs containing synthetic standards of the hydantoins.[56] The ODN oxidation studies yielded the same products observed in the nucleoside studies, but in different ratios that will be discussed below. Oxidation of duplex **ODN-12** yielded a new product, $\mathbf{dGh_{red}}$. It is assumed that these conditions would have the same efficiency for digesting **d2Ih** and $\mathbf{dGh_{red}}$ from ODN samples allowing a direct comparison of the base oxidation product yields. The sequences of the ODNs used are as follows:

ODN-1: 5′-TCA TGG GTC GTC GGT ATA

ODN-2: 3′-AGT ACC CAG CAG CCA TAT

Product quantification

Comparison of UV-vis signatures for **5′,8-cyclo-dG**, **dOG**, **dSp**, **dGh**, **d2Ih**, **dZ**, **Gh**, **Gua**, and **OG** to literature data allowed fast identification and quantification of each compound utilizing an HPLC with a photodiode array (PDA) detector (see ESI‡).[16,30,37,41,46] Quantification of the reactions to determine the yields and product distributions was achieved through integration of reactant and product peak areas while monitoring absorbance at 240 nm, followed by normalization of peak areas using each compound's unique extinction coefficent ε_{240}. Because no literature data exists for $\mathbf{dGh_{red}}$, the ε_{240} was assumed to be the same as that of **dGh**. The individual product yields will be discussed in the following sections.

In the ODN studies, only products derived from oxidation of **dG**'s base and sugar in which H_2O or $O_2^{\bullet-}$ trapped the reactive intermediates leading to product formation were quantified, and reactions with another base (*i.e.*, base cross-links) were not analyzed. Because HPLC with a PDA detector was used to quantify products from the ODN-oxidation studies, the levels of **5′,8-cyclo-dG** could not be accurately quantified due to its overlap with the large excesses of the other bases in the HPLC chromatogram; therefore, we conclude that this product may exist, but was not quantified. Thus, only relative distributions between the contexts were compared. In the ODN contexts, the mass balance was not quantitative, which appears to result from additional **dG** oxidation products forming that were not accounted for (*e.g.*, cross-links and **5′,8-cyclo-dG**).[57,58] With this experimental limitation in mind, the following data should only be interpreted with respect to context effects on **dG** base oxidation products.

C5 oxidation products

In the nucleoside reactions studied with Cu(II)/H_2O_2/Asc or NAC, **d2Ih** was the major oxidation product observed when reductant concentrations were near physiological (2 mM), as shown in Table 1. Oxazolone (**dZ**), also a product of the C5 oxidation pathway, was a minor nucleoside product under both reaction conditions (Table 1). Inspection of the relative product distributions in all nucleoside and ODN contexts yielded **d2Ih** as the major oxidation product (Fig. 2). **dZ** was a minor product observed in the single-stranded ODN contexts (**ODN-1** and **ODN-2**, Fig. 2) and not observed in the double-stranded ODN context (**ODN-12**, Fig. 2). Within the double-stranded ODN context, $\mathbf{dGh_{red}}$ was observed in low yields for copper-mediated oxidation in the presence of either Asc or NAC (Fig. 2).

C8 oxidation products

The products derived from oxidation at C8 of **dG** include the initial product **dOG**, and its further oxidation products **dSp** and **dGh** that were all observed with each oxidant system (Table 1) in all

Table 1 Absolute product yields from dG nucleoside oxidations with Cu(II)/H_2O_2/Asc or NAC at pH 7.0

Absolute % product yields[a,b]						
	Reaction conditions					
	Reductant	Asc	Asc	NAC (H_2O)	NAC (D_2O)	NAC
Product	None	2.0 mM	20.0 mM	2.0 mM	2.0 mM	20.0 mM
d2Ih	3.0	11.9	28.2	18.0	2.7	28.9
dZ	1.3	1.1	5.5	0.4	1.8	1.5
Ghred	0.0	0.0	0	0.0	0.0	0.0
dOG	0.0	5.4	36.2	2.6	9.6	34.2
dSp	2.0	4.7	6.3	2.9	6.3	4.7
dGh	0.2	0.7	1.1	0.8	1.4	1.6
5′,8-Cyclo-dG	1.4	1.1	1.9	0.3	0.6	2.3
Gua	1.6	8.5	5.8	6.3	5.8	8.8
OG	0.0	2.6	0.7	0.0	0.0	1.2
Gh	0.0	0.3	0	0.0	0.0	0.0
% Conversion	9.5[c]	36.3[c]	85.7[c]	31.3[c]	28.2[c]	83.2[c]

[a] The average error on each value was ~4% of the value, determined by three independent studies (error = 1 standard deviation). Individual error bars are provided in the ESI. [b] The reactions were conducted with **dG** (2.0 mM), Cu(II) acetate (1.0 mM), Asc or NAC (2.0 or 20 mM), and H_2O_2 (10.0 mM). [c] After accounting for unreacted dG, the mass balance for each nucleoside reaction was >90%.

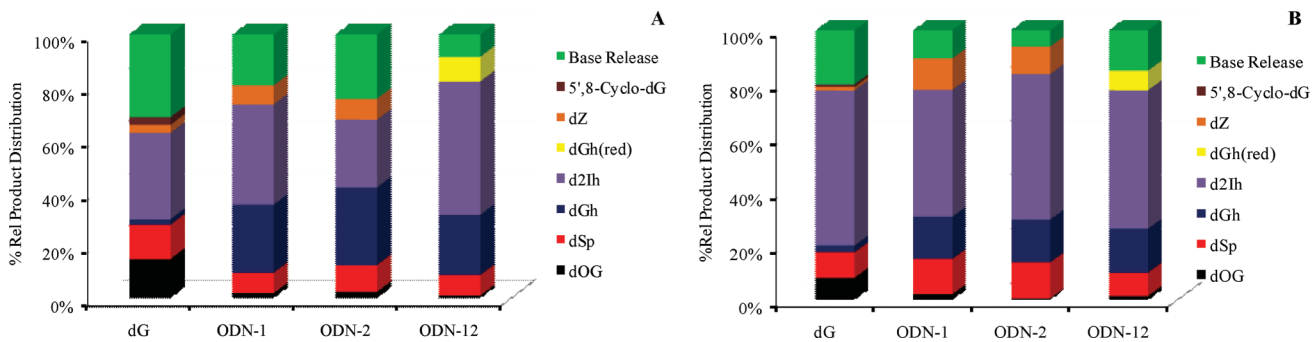


Fig. 2 Context effects on relative product distributions of **dG** when oxidized with (**A**) Cu(II)/H_2O_2/Asc or (**B**) Cu(II)/H_2O_2/NAC Fenton-like oxidant systems. The calculated mass balance for each context study was: **dG** nucleoside >90%, **ODN-1** and **-2** ~70%, and **ODN-12** ~60%.

contexts studied (Fig. 2). Combined yields of **dOG**, **dSp**, and **dGh** provide the amount of initial **dG** oxidation chemistry that occurs at C8; thus, these combined amounts can be compared to the C5 oxidation product totals to gain insight into the partitioning of products along the C5 and C8 base oxidation pathways. In the nucleoside studies, the combined absolute yields of **dOG**, **dSp**, and **dGh** provided the second largest pathway with Cu(II)/H_2O_2/Asc and NAC (Table 1, combined yields for Asc (2 mM) = 10.8%, and NAC (2 mM) = 6.3%). In terms of relative yields for which all structural contexts can be compared, the combined yields of **dOG**, **dSp**, and **dGh** were ~30–40% of the total products with Asc as reductant, and ~20–30% with NAC (Fig. 2). In other words, more C8 oxidation occurs when Asc is the reductant. The absolute yield of **dOG** was greatest in the nucleoside studies, and decreased in the ODN studies; whereas, the hydantoin yields increased in the ODN studies. This suggests that **dOG** was more susceptible to further oxidation in the ODN contexts than in the nucleoside, which likely resulted from the longer reaction time for the ODN studies (8 h *vs.* 1 h). In addition, the yield of **dSp** was greater in the nucleoside, and the yield of **dGh** was greater in the ODN contexts, in accordance with previous studies (Fig. 2).

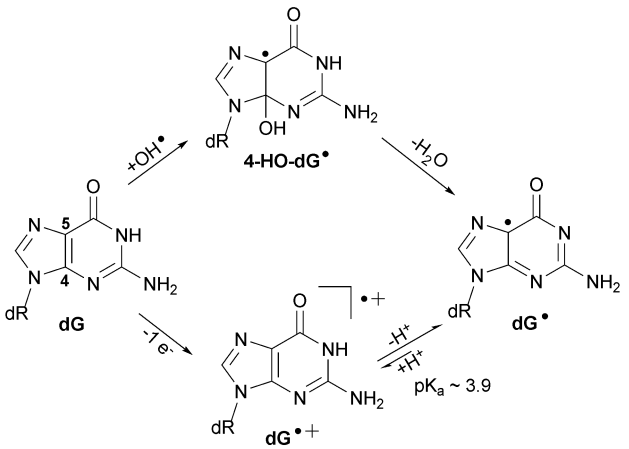
Attempts were made to identify **Fapy-dG** as a product of nucleoside or ODN oxidation, both at 2.0 and 20 mM concentrations of Asc and NAC. None was detected by reversed-phase LC-MS.

Ribose oxidation products

The 2-deoxyribose moiety of **dG** is also prone to oxidation by Cu-mediated Fenton chemistry.[10] In the studies with 2 mM reductant present, two products were identified from sugar oxidation, **5′,8-cyclo-dG** (Asc = 1.1%; NAC = 0.3%, Table 1) and **Gua** (Asc = 8.5%, and NAC = 6.3%, Table 1). In the Fe-mediated Fenton oxidation of **dG**, both **5′,8-cyclo-dG** and **Gua** were observed along with some minor sugar oxidation products that were not detected in these studies.[10,16] In the nucleoside studies using Cu(II)/H_2O_2/Asc, **Gua** oxidation leading to **OG** and **Gh** free bases was detected in low yields (Asc, OG = 2.6%, and Gh = 0.3%, Table 1). In the ODN oxidation studies, **Gua** was observed but in lower relative yields than characterized in the nucleoside studies (Fig. 2). This effect is most likely a result of the ODN context providing other sugar oxidation sites besides **dG**, leading to release of the other bases as observed in the HPLC analysis (see ESI‡).

Partitioning between pathways

When the reductant concentration was increased 10-fold, the total conversion to products increased dramatically in the nucleoside experiment (Table 1). Interestingly, the increase in the C8 oxidation products was more dramatic than the C5 pathway (four to seven-fold compared to two to three-fold). As expected for the higher reductant concentration, pathways dependent on one-electron chemistry or hydroxyl radical (**dOG** oxidation to **dSp** and sugar oxidation to release free bases) were relatively small. To test an earlier hypothesis that singlet oxygen may be involved in the reaction, we also conducted a nucleoside experiment in D_2O in which $^1O_2$ has a longer lifetime. Consistent with an earlier study,[15] the yield of C8 products (**dOG**, **dSp**, **dGh**) increased by a factor of 3.7 (Table 1). However, the yield of C5 products (**d2Ih**, **dZ**) decreased in a compensatory fashion by a factor of 4.1.



Scheme 1 Proposed pathways for the initial one-electron oxidation of **dG** to **dG**•.

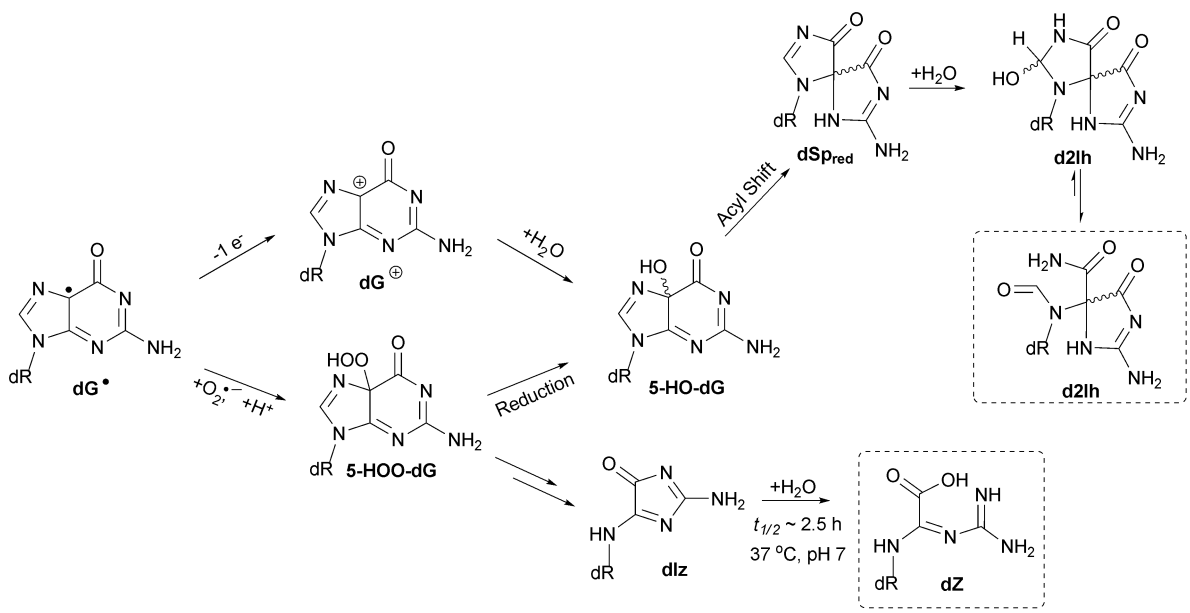
Discussion

Mechanisms of base oxidation

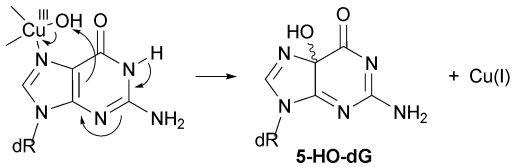
The C5 oxidation pathway of guanosine leads to two principal products **dZ** and **d2Ih** that were observed in all nucleoside reactions. The proposed mechanisms for formation of these products are the topic of many reviews, where the full details of these reactions can be found.[5,45,59] Both **dZ** and **d2Ih** may originate from the guanine radical intermediate **dG**• (Scheme 1) that then bifurcates along two proposed coordinates (Scheme 2): (1) Quenching by $O_2^{\bullet-}$ leads to **5-HOO-dG**, or (2) one-electron oxidation to **dG**$^+$, followed by addition of H_2O at C5 leads to **5-HO-dG**.[45] The fate of **5-HOO-dG** is either reduction to **5-HO-dG** or decomposition to **dIz** and hydrolysis to **dZ** (Scheme 2).[45,60] **5-HO-dG** is the proposed key intermediate leading to **d2Ih** *via* acyl migration giving an intermediate spirocycle that adds H_2O at C8, finally yielding **d2Ih** as a mixture of diastereomers and structural isomers. The ring-opened form of **d2Ih** shown in Scheme 2 was identified by Ye, *et al.*[46]

An additional pathway can be considered for formation of the key intermediate **5-HO-dG** that is particularly relevant to metal-mediated oxidation. Coordination of a high-valent copper species (eqn (3)) to N7 of **dG** as shown in Scheme 3 could lead to the direct C5 hydroxylation of guanine and commitment to the **d2Ih** pathway shown in Scheme 2. This mechanism is attractive because it helps explain why the product profiles for copper and nickel-mediated oxidations[43] are quite different from those of G radical chemistry initiated by radiation, riboflavin photochemistry, and related processes.[3] The former produces **d2Ih** as a major product *via* **5-HO-dG** while the latter forms **dIz**/**dZ** *via* **5-HOO-dG**.

Oxidation at C8 of guanosine leads to the products **dOG** and its further oxidation products **dSp** and **dGh**. The formation of **5′,8-cyclo-dG** is likely initiated by sugar oxidation that will be discussed below. Observation of **dOG** in the nucleoside reactions



Scheme 2 Proposed pathways to form **dZ** and **d2Ih**. Compounds characterized in this work are labeled with a dashed box.

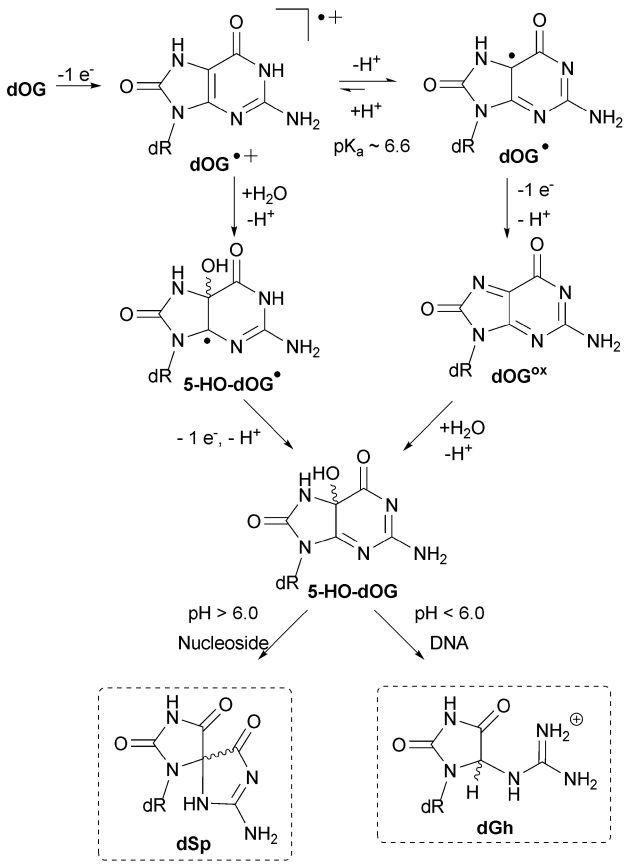


Scheme 3 Proposed mechanism for oxidation by a high-valent copper species yielding **5-HO-dG**.

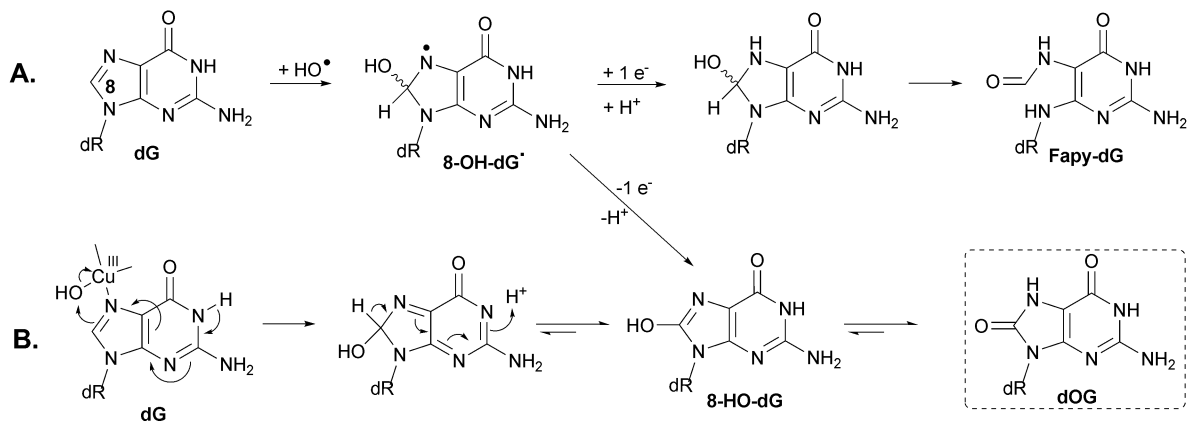
is consistent with previous studies in which **dG** was oxidized in double-stranded DNA under similar reaction conditions to yield **dOG**.[16,22,50,51] A key difference is that most of the prior work was done in the absence of added reductants. In those studies, the role of copper was proposed to be as a vehicle to deliver hydroxyl radical in the vicinity of C8 of **dG** *via* Cu binding to N7.[3,5,45,59] When HO$^{\bullet}$ adds to C8 of **dG**, the intermediate radical **8-HO-dG$^{\bullet}$** is formed; this species can yield either **dOG** under oxidizing conditions or **Fapy-dG** under reducing conditions (Scheme 4A). This latter point is relevant because all of our studies reported here had reductant present in 2–20 mM concentrations.

In the present work, **dOG** was observed from the oxidation of **dG** by Cu(II)/H_2O_2/Asc or NAC in yields of 5.4% or 2.6%, respectively (Table 1). We could not detect **Fapy-dG** as a product of any copper-mediated reaction of **dG**, either as a nucleoside substrate or in an ODN. Other laboratories report that Fe- and Cu-mediated Fenton reagents produce more **dOG** (>10-fold) than **Fapy-dG**.[15,22,50] There are two possible explanations for the complete absence of **Fapy-dG** in our work; either the presence of more oxidant (10.0 mM H_2O_2) than reductant (2.0 mM Asc or NAC) favors the oxidation pathway of **8-HO-dG$^{\bullet}$** leading to **dOG**, or Cu facilitates a different mechanism than that proposed in Scheme 4A in which the formation of **dOG** bypasses the radical intermediate. We present such a possibility in Scheme 4B. In this mechanism, a Cu(III)-OH species mediates 2-electron oxidation of **dG** directly to a tautomer of **dOG** without any opportunity for reduction of an intermediate radical. If the mechanism in Scheme 4B predominates, no **Fapy-dG** would be formed, consistent with our observations. It should be pointed out the role of copper in C8 oxidation as shown in Scheme 4B is completely analogous to that proposed in Scheme 3 for C5 oxidation.

The products **dGh** and **dSp** result from subsequent oxidation events on **dOG** due to its 600 mV lower redox potential.[27,28] Oxidation pathways for **dOG** have been reviewed and are outlined in Scheme 5.[3,45,59,61,62] Formation of **dSp** and **dGh** is proposed from the common intermediate **5-HO-dOG** that then partitions to the hydantoins in a pH and context dependent manner. Rearrangement to **dSp** is observed at high pH (>6.0) and in the less sterically encumbered nucleoside context,[30,36] while low pH (<6.0)



Scheme 5 Proposed pathways for the formation of hydantoin products from **dOG**. Compounds characterized in this work are labeled with a dashed box.



Scheme 4 Proposed pathway for HO$^{\bullet}$ addition to C8 of **dG** leading to **dOG** and **Fapy-dG**. Compounds characterized in this work are labeled with a dashed box.

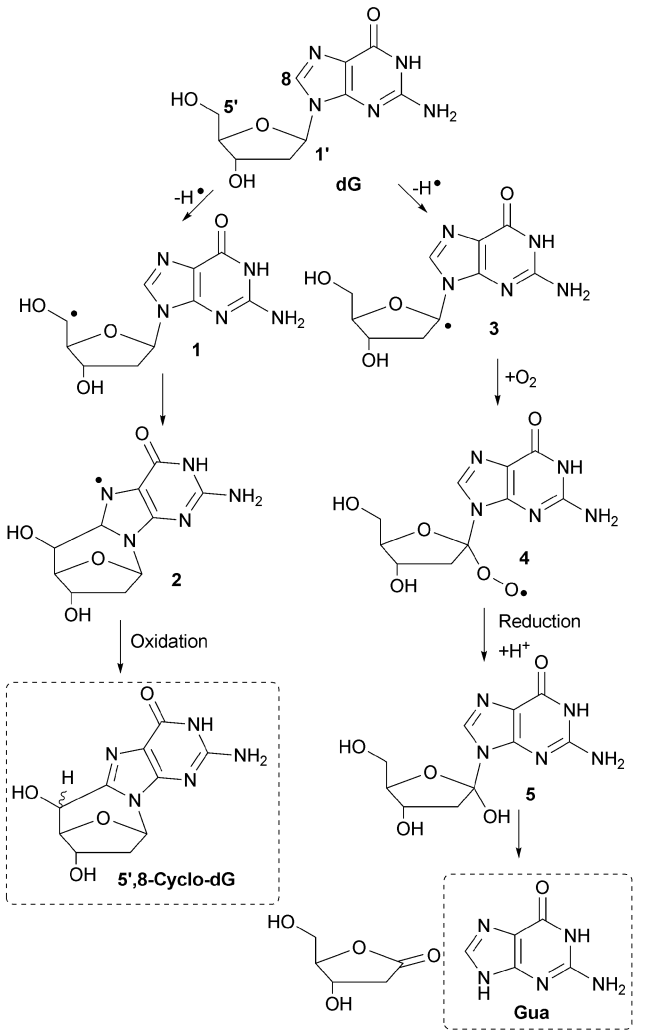
and a duplex DNA context favor hydration and decarboxylation to yield **dGh** (Scheme 5).[36,37]

Analysis of product evolution *vs.* reaction time shows that **dOG** is formed before **dSp** or **dGh**, supporting previous data that suggest **dOG** to be the intermediate leading to the hydantoin products (see ESI‡).[3,45,59,61] The reactions investigated in these studies, at 2 mM reductant concentration, consistently gave **dSp** yields (Asc = 4.7%, and NAC = 2.9%, Table 1) greater than **dGh** (Asc = 0.7%, and NAC = 0.8%, Table 1) as anticipated because the reactions were conducted under conditions with pH > 6.0 [33,37] Also, as expected, the yield of **dGh** increased in the single- and double-stranded ODN contexts (Fig. 2). To further confirm that **dSp** and **dGh** result from the intermediate **dOG** in the Cu(II)/H_2O_2/Asc reaction, authentic **dOG** nucleoside was allowed to react with Cu(II)/H_2O_2/Asc providing the two hydantoins, **dSp** and **dGh** (see ESI). Another possible pathway for formation of **dSp** is through the two-electron oxidation of **d2Ih**. Purified **d2Ih** was allowed to react with the Cu-ascorbate oxidant system, and the only product observed was **2Ih** free base, suggesting sugar oxidation as the major oxidation product from **d2Ih**. To further understand if the base of **d2Ih** could be oxidized, the one-electron oxidant Na_2IrCl_6 was allowed to react with **d2Ih**. Reinjection of the oxidation reaction provided no change in **d2Ih** by HPLC (see ESI). These observations suggest that **d2Ih** cannot be oxidized to **dSp**, and that the hydantoins both result from initial oxidation of **dG** at C8 yielding the intermediate **dOG**; subsequently, a two-electron oxidation of **dOG** occurs at C5 yielding the hydantoins, **dSp** or **dGh**.

Mechanisms of 2-deoxyribose oxidation

Formation of **5′,8-cyclo-dG** is thought to be initiated by oxidation of **dG** at C5′ yielding a carbon-centered radical **1** that adds to C8 of the heterocyclic ring of **dG** (Scheme 6); subsequent addition of this radical to C8 of guanine leads to the intermediate radical **2**. Radical **2** can then be further oxidized by one electron along with loss of a proton to yield **5′,8-cyclo-dG**. Sugar oxidation also causes **Gua** base release from the nucleoside sugar. **Gua** base release *via* the Fenton reaction is proposed to be initiated by the one-electron oxidation of **dG** at C1′ yielding an intermediate carbon-centered radical **3** that provides peroxyl radical **4** after reaction with dioxygen. Finally, one-electron reduction and proton transfer occur to yield the alcohol **5**. Compound **5** decomposes to **Gua** free base and a sugar lactone (Scheme 6).[10,16] **OG** (M − 100) and **Gh** (M − 110) free bases result from the two- and four-electron oxidation of **Gua**, respectively, following the previously proposed mechanisms (Schemes 4 and 5).[5,37,63]

The free base **Gua** resulting from sugar oxidation was observed in all Cu-mediated Fenton reactions. This observation parallels previous studies that characterized **Gua** base release from oxidation of **dG** nucleoside with Fe-based Fenton reagents.[16] Additionally, DNA oxidation with Cu-mediated Fenton reagents led to free base oxidation rather than deglycosylation of **dOG** or **dGh**, respectively (see ESI†). Only **Gh** free base was observed and not **Sp** free base; in previous studies, **OG** free base oxidation at pH 7.0 yielded **Gh**, not **Sp**, consistent with our findings and the fact that uric acid oxidation leads to allantoin in preference to spirodihydantoin.[63]



Scheme 6 Proposed pathways for the sugar oxidation products **5′,8-cyclo-dG** and **Gua**. Compounds characterized in this work are labeled with a dashed box.

Partitioning between pathways

The overall yields of **dG** oxidation products (Table 1) were similar in the Cu(II)/H_2O_2/Asc and Cu(II)/H_2O_2/NAC reactions (36.3 ± 1.5 and 31.3 ± 1.3%, respectively, 2 mM reductant). However, closer inspection of the product yields grouped by reaction pathway indicates a bigger difference between the two reductants studied than is first apparent. The products can be grouped into three clusters: the C5 pathway (**d2Ih** and **dZ**), the C8 pathway (**dOG** and its two-electron oxidation products **dSp** and **dGh**), and the sugar oxidation pathway (**Gua**, **OG** and **Gh**). The ratio of C5 : C8 : sugar reactivity for Cu(II)/H_2O_2/Asc was 1.00 : 0.84 : 0.96, whereas for Cu(II)/H_2O_2/NAC it was 1.00 : 0.34 : 0.36, based on the nucleoside values in Table 1. Within error, the Asc-based system gave similar product yields along each reaction channel. In previous studies that quantified product distributions from **dG** oxidation by Fe(III)-EDTA/H_2O_2/NADH, similar yields of C8 products (**dOG**) and sugar oxidation products (**5′,8-cyclo-dG**, **Gua**, and **OG**) were observed,[16,48] but C5 oxidation products were not identified because of the HPLC conditions used.[16] The Cu(II)/H_2O_2/Asc and NAC systems both gave equal reactivity at C8 and the ribose

of **dG**, suggesting a common feature to both Fe- and Cu-mediated Fenton oxidation of the nucleoside **dG**. The present work also permitted the characterization and quantification of C5 oxidation products, and notably, **d2Ih** yields increased with the stronger reductant, NAC. With Asc, the relative reactivities at C5 and C8 were similar, but NAC provided roughly three-fold more reaction at C5 than C8 or the sugar (Table 1). The reducing agent effect on product distribution was evaluated by Cadet and coworkers when **dG** was oxidized with HO$^{\bullet}$ (generated by γ irradiation) in the presence of Asc or cysteine to yield the C5 product **dZ** and the C8 products **dOG** and **Fapy-dG**.[64] In their studies, the absolute product yields differed from ours, but the trends were the same. With the stronger reductant cysteine, C8 oxidation product yields decreased, and they concluded that cysteine, the stronger reductant, quenched the radicals leading to C8 oxidation products better than Asc.[64] This observation was mirrored by our results in which the NAC reactions gave lower yields of C8 oxidation products when compared to the Asc reaction. The base-oxidation pathway dependence on reductant suggests two possibilities: (1) Radical chemistry plays a larger role in **dOG** formation than in the pathway to **d2Ih**, and/or (2) the coordination environment for Cu in the Asc reaction is different than the NAC reaction causing a shift in the base oxidation pathway. The latter point is expected based on the propensity for cysteine thiols to coordinate directly to Cu(I) and Cu(II). Of course, the precise structure of the copper complex that carries out the key oxidation step is unknown.

The conclusions above are presented for the copper-mediated Fenton reaction conducted in the presence of physiological concentrations of reductant, either Asc or NAC, of 2 mM. Further mechanistic insight could be gained by increasing the reductant concentration ten-fold. Under these conditions, the total conversion to products increased, indicating that the formation of the reactive oxidant is dependent upon reductant. This step is presumably the reduction of Cu(II) to Cu(I). Consistent with this hypothesis is the observation of very low levels of product formation when the Cu(II) concentration remains the same, but no reductant is added (Table 1, column 1). Curiously, the effect of increasing the reductant concentration is more dramatic for the C8 pathway than for the C5 pathway. These data would argue that the reactive species responsible for oxidation at C5 *vs.* C8 of **dG** is not the same. Also pertinent to this point is the fact that the C8 pathway was preferred when the solvent was D_2O compared to the C5 pathway dominating in H_2O (Table 1). One possibility is that $^1O_2$ contributes to the C8 pathway as suggested by Frelon *et al.*,[15] but not to the formation of C5 products. The formation of singlet oxygen in this system could result from dimerization of a Cu(I) peroxyl species that decomposes to generate $^1O_2$ and 2 Cu(III)-OH complexes, as shown in eqn (4)–(6).

$$\text{Cu(I)} + O_2 \rightarrow \text{Cu(II)-OO}^{\bullet} \quad (4)$$

$$2\ \text{Cu(II)-OO}^{\bullet} \rightarrow \text{Cu(II)-O-OO-O-Cu(II)} \quad (5)$$

$$\text{Cu(II)-O-OO-O-Cu(II)} + 2\ H^+ \rightarrow 2\ \text{Cu(III)-OH} + {}^1O_2 \quad (6)$$

With respect to ribose chemistry, cysteine has been shown to reduce sugar radicals more efficiently than Asc,[65,66] consistent with our observation that NAC yields less sugar oxidation chemistry. In the Cu(II)/H_2O_2/Asc system, yields of base oxidation products (**d2Ih** + **dZ** + **dOG** + **dSp** + **dGh**) were approximately two-fold greater than sugar oxidation products. This supports the proposal that Cu coordination to N7 of **dG** favors base oxidation products, as well as the observation that DNA oxidation with Cu(II)/H_2O_2 gives more base oxidation than strand breaks.[8,11,13]

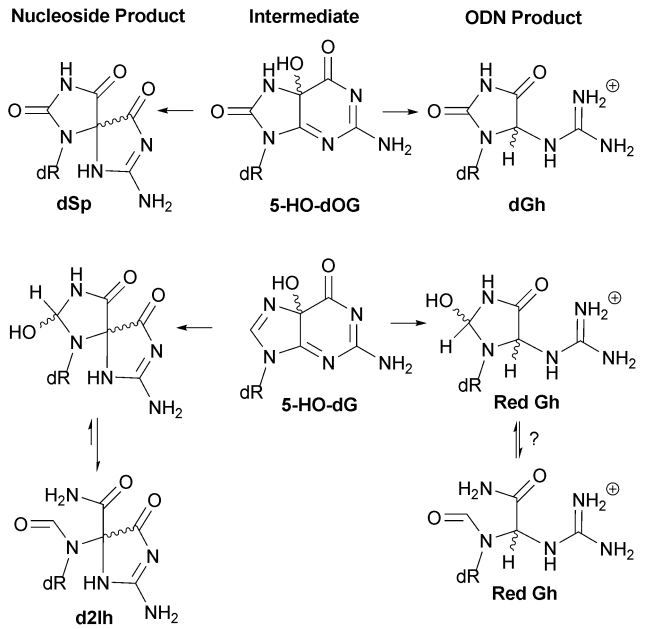
Context effects on dG base oxidation products

Previous studies have alluded to the observation that reaction context effects the oxidation product distributions; for example, **dSp** is a major nucleoside product, while **dGh** is a major DNA product under the same reaction conditions.[36] With this thought in mind, the distribution of base oxidation products from **dG** were compared in the nucleoside, single- and double-stranded ODN contexts in which the products formed were the same, but their relative yields differed. From the data it appears that oxidation of ODNs with Cu(II)/H_2O_2/reductant, Asc or NAC, is affected most by the double-stranded **ODN-12** context (Fig. 2). Three differences were observed in these context studies: (1) **dZ** was not observed in the double-stranded **ODN-12** context, but appears in small amounts in the nucleoside and single-stranded **ODN-1** and **ODN-2** contexts. (2) The ratios for the hydantoin products, **dSp** and **dGh**, are context dependent, as expected, favoring more **dGh** in single- and double-stranded ODN contexts studied. It has been proposed that the ODN context favors **dGh** because it is less sterically demanding, and the acid/base chemistry of the intermediate **5-HO-dOG** in the ODN context favors acid-catalyzed decarboxylation leading to **dGh**.[33,36,62] (3) Importantly, a new product of double-stranded DNA oxidation, **dGh$_{red}$** (M + 8), was observed by analysis with HPLC-ESI$^+$-MS. This structure was previously proposed by Pratviel and Meunier,[45] and in the present studies a mass consistent with **dGh$_{red}$** was observed. Because the ODN context affects the reactivity of **5-HO-dOG** leading to higher yields of **dGh**, it was proposed that the analogous intermediate **5-HO-dG** would be affected in the same way (Scheme 7).[45] Our results are consistent with this idea, and the hydrolytic chemistry of **5-HO-dG** appears to parallel that of **5-HO-dOG** to yield **dGh$_{red}$** and **dGh**, respectively. It is not known if the five-membered ring of **dGh$_{red}$** is opened or closed, but the ring-opened form is similar to that of **d2Ih**,which has been more extensively characterized.[46]

Lastly, we note that the diastereomers of both **dSp** and **d2Ih** were separable on the Hypercarb HPLC column.[67,68] Cu-mediated oxidation of **dG** yielded **dSp** diastereomer ratios that are approximately 1 : 1 in all contexts studied. In contrast, it is interesting to note that the Cu-mediated formation of **d2Ih**, in all reaction contexts, favored the later eluting **d2Ih** diastereomer by a factor of 2 : 1. Without further structural analysis, the absolute stereochemistry of each **d2Ih** diastereomer remains unknown.

Conclusions

DNA isolated from cellular extracts contains high concentrations of associated Cu, and it has been proposed that Cu-mediated oxidation of DNA underlies certain disease states.[9] In this study, the major oxidation product of the copper-mediated Fenton reaction observed in all contexts was the recently characterized hydantoin **d2Ih**. The reductant appears to play a role in defining the overall product distribution between oxidation of guanosine



Scheme 7 Product comparison for the intermediates **5-HO-dOG** and **5-HO-dG**.

at C5 *vs.* C8 *vs.* the ribose group. In these studies, the overall two-electron oxidation of **dG** at C5 was a major reaction channel leading initially to **5-OH-dG**, an isomer of the well-studied **dOG**. Subsequent rearrangement and hydration of **5-OH-dG** led to the characterized product **d2Ih**. Secondarily, oxidation at C8 of the purine using physiologically-relevant concentrations of reductant yielded **dOG** and no detectable quantities of **Fapy-dG**, highlighting a key difference in the mechanisms between radiation *vs.* metal-mediated oxidation of the guanine base. In radiation-induced damage, a free hydroxyl radical is formed that adds to C8 of **dG**, yielding an intermediate radical that can partition to **dOG** under oxidizing conditions or yield **Fapy-dG** under reducing conditions. Whereas in Cu-mediated oxidation of **dG**, the intermediate radical leading to **dOG** is bypassed because Cu is coordinated to N7 of **dG** for which direct transfer of the hydroxyl group to C8 of **dG** occurs following Scheme 4B. In the double-stranded ODN context a new product, $\mathbf{dGh_{red}}$, was observed supporting the hypothesis of Pratviel and Meunier for formation of this structure.[45] Questions remain about the exact identity of the copper species responsible for guanine oxidation at C5 *vs.* C8 and the possible role of singlet oxygen in the C8 pathway. Nevertheless, the observation of significant amounts of **d2Ih** from both nucleoside and oligonucleotide studies underscores the importance of future chemical and biological studies of this interesting heterocycle.

Experimental section

Nucleoside studies

All reagents were obtained from commercially available sources and used without further purification unless otherwise stated. A 200-μL solution of **dG** (3.0 mM, 0.60 μmol, 0.16 mg) in NaP_i buffer (75.0 mM, pH 7.0) was oxidized by addition of copper(II) acetate (1.0 mM, 0.20 μmol, 0.04 mg), Asc or NAC (*N*-acetyl-cysteine, 2.0 mM, 0.40 μmol, 0.03 mg), followed by addition of H_2O_2 (10.0 mM, 2.0 μmol, 0.07 mg). The solution was kept at 22 °C for 60 min and then quenched with Na_2EDTA (10.0 mM, 2.0 μmol, 0.50 mg). The reaction mixture was first analyzed by RP-HPLC that retained **Gua**, **OG**, **dOG**, and **5′,8-cyclo-dG**. The void volume from the RP-HPLC run was collected, dried down, resuspended in the Hypercarb column mobile phase (0.1% acetic acid), and then injected on a Hypercarb HPLC column to analyze **Gh**, **d2Ih**, **dGh**, **dSp**, and **dZ**. All HPLC separation parameters are presented in the supporting information. HPLC-ESI^+-MS results for each identified compound are as follows: *m/z* $(M+H)^+$ 152.1 (**Gua**), 168.1 (**OG**), 268.1 (**dG**) 284.1 (**dOG**), 266.3 (**5′,8-cyclo-dG**), 158.1 (**Gh**), 302.1 (**d2Ih** diastereomers), 274.1 (**dGh**), 300.1 (**dSp** diastereomers), and 247.1 (**dZ**); all found values matched their calculated values. Collected samples provided the following ESI^+-MS/MS data for the free bases of the following compounds: **d2Ih** diastereomers (186, 158, and 141; lit.,[46] 186, 158, 141) **dSp** diastereomers (184, 156, 141, 113, 99, and 86; lit.,[30] 184, 156, 141, 113, 99, and 86) and **dZ** (247, 203, 131; lit.,[41] 247, 203, 131). HRMS-ESI^+ (*m/z*) for **dSP**, $C_{10}H_{13}N_5O_6Na$ $(M)^+$ calcd 322.0764, found 322.0761; **dGh**, $C_9H_{15}N_5O_5Na$ $(M)^+$ calcd 296.0971, found 296.0980; **dZ**, $C_8H_{14}N_4O_5Na$ $(M)^+$ calcd 269.0862, found 269.0870. UV-vis profiles for each compound are shown in the ESI.‡ Integrated peak areas obtained from absorbance at 240 nm on each HPLC run, were used to quantify the reaction yields through normalization of each area by its unique $\varepsilon_{240\,nm}$ (ddH_2O): **dG** (lit.,[16] 14 080 $dm^3\ mol^{-1}\ cm^{-1}$), **dOG** (lit.,[16] 14 300 $dm^3\ mol^{-1}\ cm^{-1}$), **5′,8-cyclo-dG** (lit.,[16] 14 080 $dm^3\ mol^{-1}\ cm^{-1}$), **d2Ih** (lit.,[43] 3275 $dm^3\ mol^{-1}\ cm^{-1}$), **dGh** (lit.,[37] 2412 $dm^3\ mol^{-1}\ cm^{-1}$), **dSp** (lit.,[30] 3275 $dm^3\ mol^{-1}\ cm^{-1}$), **dZ** (lit.,[41] 1778 $dm^3\ mol^{-1}\ cm^{-1}$), **Gua** (lit.,[16] 14 080 $dm^3\ mol^{-1}\ cm^{-1}$), **OG** (lit.,[16] 14 300 $dm^3\ mol^{-1}\ cm^{-1}$), and **Gh** (lit.,[37] 2412 $dm^3\ mol^{-1}\ cm^{-1}$).

Oligodeoxynucleotide studies

The ODNs were synthesized following standard procedures by the DNA/Peptide Core facility at the University of Utah were used after HPLC purification (ESI‡). Formation of double-stranded **ODN-12** was achieved by heating equimolar ratios of **ODN-1** and **ODN-2** in NaP_i buffer (20.0 mM, pH 7.0) with NaCl (100.0 mM), at 90 °C for 5 min, followed by slowly cooling the samples to room temperature over 3 h. Cu-mediated oxidations were conducted in a 100-μL solution of **ODN-1**, **ODN-2**, or **ODN-12** (100 μM, 10.0 nmol) in NaP_i buffer (20.0 mM, pH 7.0) and NaCl (100 mM) at 37 °C. First, the ODN samples were incubated with Cu(II) acetate (10.0 μM, 1.0 nmole) for 5 min. Next, the reaction was initiated by addition of Asc or NAC (1.0 mM, 100.0 nmol) then H_2O_2 (1.0 mM, 100.0 nmol), and the reaction was allowed to proceed for 8 h. The ODN samples were then digested with a suite of nucleases to liberate the oxidized nucleotides as follows: (1) The ODN samples were lyophilized to dryness and then resuspended in DNase I reaction buffer (20.0 mM Tris (pH 8.4), 2.0 mM $MgCl_2$, 50.0 mM KCl) followed by addition of DNase I (2.0 U), and incubated at 37 °C for 3 h. The antioxidant butylated hydroxytoluene (2.0 mM) along with the deaminase inhibitors pentostatin (100 μM) and tetrahydrouridine (100 μM) were initially added before commencement of the digestion. (2) 10 μL of a NaOAc buffer solution (100.0 mM, pH 5.3) containing zinc acetate (10.0 mM) was added to the digestion solution, followed by nuclease P1 (2.0

U). The reaction was incubated at 45 °C for 9 h, followed by addition of more nuclease P1 (2.0 U) and incubation for another 9 h. (3) 11 μL of tris buffer (100.0 mM, pH 7.8) with $MgCl_2$ (10.0 mM) and snake venom phosphodiesterase (2.0 U) was added to the digestion mixture. The reaction was incubated at 45 °C for 9 h after which snake venom phosphodiesterase (2.0 U) and calf intestinal phosphatase (16.0 U) were added and allowed to react for 9 h to liberate the damaged and undamaged nucleosides from the reacted ODNs. The digestion proteins were removed before HPLC analysis by passing the sample through a 10 000 molecular weight cutoff filter (Millipore), and then analyzed by HPLC following the previous method. The HPLC areas for the canonical DNA bases were normalized by their extinction coeffiencts $\varepsilon_{240\,nm}$ (ddH_2O): 2′-deoxyadenosine (lit.,[69] 9500 $dm^3\ mol^{-1}\ cm^{-1}$), 2′-deoxycytidine (lit.,[69] 7400 $dm^3\ mol^{-1}\ cm^{-1}$), and thymidine (lit.,[69] 9900 $dm^3\ mol^{-1}\ cm^{-1}$). An **ODN-12** reaction with Cu(II)/H_2O_2/Asc was analyzed by HPLC-ESI$^+$-MS running the Hypercarb HPLC column to provide the additional MS data m/z $(M+H)^+$ 276.1 (**dGh$_{red}$**).

Acknowledgements

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