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The pH-Dependent Role of Superoxide in Riboflavin-Catalyzed Photooxidation of 8-Oxo-7,8-dihydroguanosine

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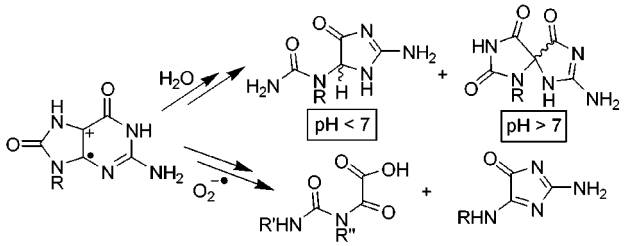
ABSTRACT



The riboflavin-catalyzed photooxidation of 2′,3′,5′-tri-*O*-acetyl-8-oxo-7,8-dihydroguanosine generates a radical intermediate that is competitively trapped by H_2O, $O_2^{-\bullet}$, or O_2. The products of H_2O trapping have been previously described as the spiroiminodihydantoin (pH ≥ 7) and iminoallantoin/guanidinohydantoin (pH < 7) nucleosides. Trapping by $O_2^{-\bullet}$ leads to the oxaluric acid (pH ≤ 7) and imidazolone (pH ≥ 8.6) pathways (R′′, R′′ = H or 2,3,5-tri-*O*-Ac-ribofuranosyl). The pH-dependent role of superoxide was probed using Mn–SOD and compared to guanosine and 8-methoxyguanosine photooxidation.

The oxidized DNA base lesion 8-oxo-7,8-dihydroguanosine (**OG**) is a major product of radiation damage in the cell[1,2] and is recognized as a site highly susceptible to further oxidation by one-electron oxidants,[3,4] singlet oxygen,[5−7] photochemical processes,[8] and peroxynitrite.[9] We previously showed that the oxidation of **OG** in the form of a nucleoside triacetate by Ir^{IV} and other one-electron oxidants was highly pH-dependent, leading to nearly exclusive formation of the spiroiminodihydantoin nucleoside (**Sp**) at neutral pH (Scheme 1).[10] At lower pH or in oligodeoxynucleotides, **OG** oxidation additionally leads to an equilibrating mixture of the decarboxylated products iminoallantoin (**Ia**) and guanidinohydantoin (**Gh**).[11−13] The various products were proposed to arise from a common intermediate, 5-hydroxy-8-oxoG (**5-OH-OG**), formed from trapping of a radical or radical cation intermediate by a solvent water molecule.

Photochemical oxidation of **OG** has been reported to yield different products depending upon the type of photosensitizer employed. Among these, riboflavin (**Rf**) is thought to follow primarily the "Type I" pathway involving one-electron transfer from the base to **Rf**.[14,15] Because the **Rf**-catalyzed photooxidation of both guanosine[16−19] and 8-methoxyG[20,21]

(1) Wang, D.; Kreutzer, D. A.; Essigmann, J. M. *Mutat. Res.* **1998**, *400*, 99−115.
(2) Cadet, J.; Delatour, T.; Douki, T.; Gasparutto, D.; Pouget, J.-P.; Ravanat, J.-L.; Sauvaigo, S. *Mutat. Res.* **1999**, *424*, 9−21.
(3) Muller, J. G.; Duarte, V.; Hickerson, R. P.; Burrows, C. J. *Nucleic Acids Res.* **1998**, *26*, 2247−2249.
(4) Hickerson, R. P.; Prat, F.; Muller, J. G.; Foote, C. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9423−9428.
(5) Sheu, C.; Foote, C. S. *J. Am. Chem. Soc.* **1995**, *117*, 474−477.
(6) Duarte, V.; Gasparutto, D.; Yamaguchi, L. F.; Ravanat, J.-L.; Martinez, G. R.; Medeiros, M. H. G.; Di Mascio, P.; Cadet, J. *J. Am. Chem. Soc.* **2000**, *122*, 12622−12628.
(7) Raoul, S.; Cadet, J. *J. Am. Chem. Soc.* **1996**, *118*, 1892−1898.
(8) Adam, W.; Saha-Möller, C. R.; Schönberger, A. *J. Am. Chem. Soc.* **1996**, *118*, 9233−9238.
(9) Niles, J. C.; Burney, S.; Singh, S. P.; Wishnok, J. S.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11729−11734.
(10) Luo, W.; Muller, J. G.; Rachlin, E.; Burrows, C. J. *Org. Lett.* **2000**, *2*, 613−616.
(11) Duarte, V.; Muller, J. G.; Burrows, C. J. *Nucleic Acids Res.* **1999**, *27*, 496−502.
(12) Leipold, M.; Muller, J. G.; Burrows, C. J.; David, S. S. *Biochemistry* **2000**, *39*, 14984−14992.
(13) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Chem. Res. Toxicol.* **2001**, *14*, 927−938.
(14) Foote, C. S. *Photochem. Photobiol.* **1991**, *54*, 659.
(15) Cadet, J.; Decarroz, C.; Wang, S. Y.; Midden, W. R. *Isr. J. Chem.* **1983**, *23*, 420−429.

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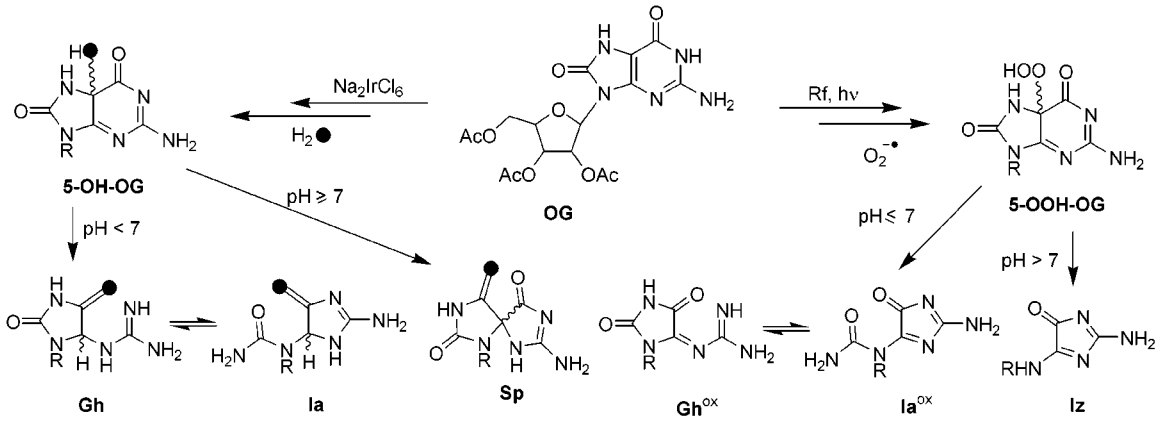
Scheme 1. Oxidation of OG by Ir^{IV} vs Type I Photooxidation (● = ^{18}O Label)



were reported to give the imidazolone **Iz** as a major product, we sought to understand the difference between chemical (i.e., metal-mediated) and photochemical one-electron oxidation of **G**, **OG**, and **8-MeOG**.

For these studies, we selected the nucleoside triacetate in which the three sugar hydroxyls are prevented from acting as nucleophiles with any base-centered intermediates.[22] In addition, *O*-acylated nucleosides are more highly retained on reversed-phase HPLC columns, facilitating identification of hydrophilic oxidation products. Products were identified and quantified by HPLC using both UV−vis and ESI-MS detection. Because authentic pure samples of **Sp**, **Gh/Ia**, **OG**, and **G** were available[13] and the ϵ value of **Iz** was known,[23] we were able to determine relative ionizabilities of the products, and ESI-MS could be used for quantification. In the case of **Ia^{ox}**, a relatively pure sample could be prepared as a standard from Na_2IrCl_6 oxidation of **Gh/Ia**,[13,24] although its hydrolytic instability necessitated rapid analysis.

A pH 7 (75 mM KP_i) solution of **OG** (**1**, 0.2 mM) containing 16 μM **Rf** was irradiated with a 360-nm Rayonet apparatus for 10 min at 22 °C. Samples were immediately analyzed by LC-ESI-MS according to the procedures detailed in Supporting Information. The quantitative values shown for the products obtained are displayed in Figure 1 and represent the averages of three or more independent experiments. The reactions were repeated in the presence of manganese superoxide dismutase (125 units/mL) plus catalase (375 units/mL).[25] Since the mass balance was ≥90−95% for all reactions, we believe that all major products have been identified.

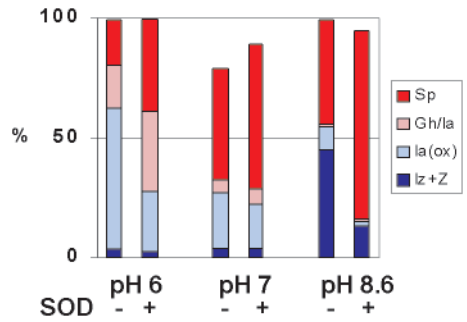


Figure 1. Products of **Rf**-catalyzed photooxidation of **OG** as a function of pH and added Mn-SOD: red, **Sp**; pink, **Gh** + **Ia**; light blue, **Ia^{ox}**; dark blue, **Iz** + **Z**. Errors are estimated at ±5%.

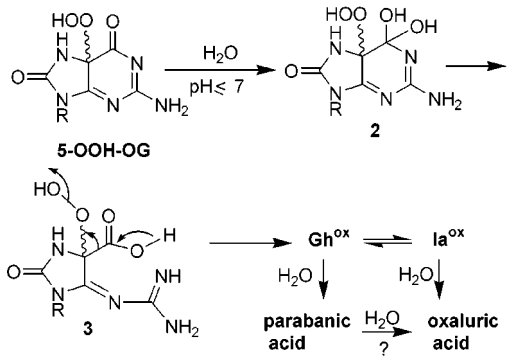
Surprisingly, **Iz** was found to be only a trace product at pH 7 for **Rf**-catalyzed oxidation of **OG**. Instead, water quenching of the presumed **OG**$^{+\bullet}$ intermediate to yield **Sp** predominated, giving the same result as for metal-catalyzed oxidation.[10] Use of $H_2{}^{18}O$ as solvent showed quantitative incorporation of one O atom from H_2O. A substantial amount of a two-electron further oxidized product, tentatively identified as **Ia^{ox}**, was also formed by photooxidation. Such a product represents a 4e$^-$ oxidation of **OG** suggesting the involvement of O_2 as oxidant. Indeed, no photooxidation of **OG** was observed in degassed solutions, and no isotopic incorporation from $H_2{}^{18}O$ was found, confirming a role for O_2 in either the **OG** chemistry, the regeneration of the **Rf** catalyst, or both. The excited state **Rf*** is known to generate $O_2{}^{-\bullet}$ by electron transfer from **Rf**$^{-\bullet}$ to O_2,[26,27] which would suggest the pathway shown in eq 1.

$$\mathbf{OG} + \mathbf{Rf}^* \rightarrow \mathbf{OG}^{+\bullet} + \mathbf{Rf}^{-\bullet} \xrightarrow[O_2]{} O_2{}^{-\bullet} + \mathbf{Rf} \qquad (1)$$

(16) Kasai, H.; Yamaizumi, Z.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1992**, *114*, 9692−9694.
(17) Ito, K.; Inoue, S.; Yamamoto, K.; Kawanishi, S. *J. Biol. Chem.* **1993**, *298*, 13221−13227.
(18) Cadet, J.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Raoul, S.; Ravanat, J.-L. *J. Am. Chem. Soc.* **1994**, *116*, 7403−7404.
(19) Adam, W.; Saha-Moeller, C. R.; Schoenberger, A. *J. Am. Chem. Soc.* **1997**, *119*, 719−723.
(20) Ikeda, H.; Saito, I. *J. Am. Chem. Soc.* **1999**, *121*, 10836−10837.
(21) Sugiyama, H.; Kino, K. *Chem. Biol.* **2001**, *8*, 369−378.
(22) Buchko, G. W.; Wagner, J. R.; Cadet, J.; Raoul, S.; Weinfeld, M. *Biochim. Biophys. Acta* **1995**, *1263*, 17−24.
(23) Vialas, C.; Pratviel, G.; Claporols, C.; Meunier, B. *J. Am. Chem. Soc.* **1998**, *120*, 11548−11553.
(24) No cyanuric acid derivatives were detected in this study. While cyanuric acid shows low ionizability and a low ϵ by UV−vis detection, its presence is still unlikely since the mass balance of the reaction was always >95%.
(25) Subsequent experiments showed that catalase had no effect on the reaction.
(26) Ballou, D.; Palmer, G.; Massey, V. *Biochem. Biophys. Res. Commun.* **1969**, *36*, 898−904.
(27) Armitage, B. *Chem. Rev.* **1998**, *98*, 1171−1200.

The product labeled **Ia^{ox}** represents a species of mass **OG** − 12 that has been observed in singlet oxygen[6] and peroxynitrite oxidation of **OG** in nucleosides and oligomers,[9,28] from Mn-porphyrin/$KHSO_5$-mediated oxidation of **G**[29] and from further oxidation of the **Gh/Ia** mixture by 2 equiv of Ir^{IV}.[13] Unfortunately, the species is hydrolytically unstable, yielding oxaluric acid, so its exact identity remains in question. NMR data plus the fact that oxaluric acid rather than parabanic acid is obtained after hydrolysis better support the **Ia^{ox}** structure (Scheme 2).[13,30]

Scheme 2. Decomposition of **5-OOH-OG** to **Ia^{ox}** and Related Products



Curiously, the photooxidation of **OG** at pH 7 was only slightly inhibited in the presence of SOD, although the mechanism proposed in eq 1 suggests that formation of **Ia^{ox}** should be dependent on [$O_2^{-\bullet}$]. In addition, recent studies imply that $O_2^{-\bullet}$ should be much more reactive than O_2 with radical intermediates formed from **G** and likely **OG** as well.[31,32] Thus, one would expect that SOD would greatly diminish the formation of **Ia^{ox}**.

A complicating feature in the mechanistic analysis is the pK_a of **OG**$^{+\bullet}$, measured to be 6.6.[33] This means that about 80% of the radical cation should be deprotonated and exist as **OG**$^{\bullet}$ at pH 7. To better understand the reaction of **OG**$^{+\bullet}$ with $O_2^{-\bullet}$, we repeated the **Rf**-mediated photooxidation of **OG** at pH 6 (75 mM KP_i). At this pH, most of the radical intermediate remains as **OG**$^{+\bullet}$, and $O_2^{-\bullet}$ (pK_a 4.8)[34] is anionic. These data (Figure 1) show several interesting features. First, the overall yield of products was consistently higher (∼100%) at pH 6 compared to pH 7. Second, the distribution of products arising from H_2O quenching of **OG**$^{+\bullet}$ shifted as expected toward a higher proportion of **Gh/Ia** (pink) compared to **Sp** (red). Also, the overall proportion of **Ia^{ox}** (light blue) was greater than the H_2O-derived products (pink + red). Finally, the addition of SOD had a substantial effect on the product distribution, such that formation of **Ia^{ox}** was suppressed while **Gh/Ia** + **Sp** increased.

Since Mn−SOD reacts with $O_2^{-\bullet}$ with a second-order rate constant of about 10^9 M^{-1} s^{-1} over the pH range 6−9,[34] the lack of SOD quenching at pH 7 cannot be explained as an ineffectiveness of the enzyme. Rather, there must be competing processes that either react with the available $O_2^{-\bullet}$ at pH 7, or alternatively less $O_2^{-\bullet}$ is formed at this pH. A possible explanation is the competing reaction of **HRf**$^{\bullet}$ with $O_2^{-\bullet}$. This occurs with a large rate constant for the protonated form of **Rf**$^{-\bullet}$ as shown in eqs 2−5:[35]

$$\mathbf{Rf}^{-\bullet} + H^+ \rightleftarrows \mathbf{HRf}^{\bullet} \qquad (pK_a = 8.5) \qquad (2)$$

$$\mathbf{HRf}^{\bullet} + O_2^{-\bullet} \rightarrow \mathbf{HRfOO}^- \qquad (k = 10^9\ M^{-1}\ s^{-1}) \qquad (3)$$

$$\mathbf{HRfOO}^- + H^+ \rightleftarrows \mathbf{HRfOOH} \qquad (pK_a \approx 12) \qquad (4)$$

$$\mathbf{HRfOOH} \rightarrow \mathbf{Rf} + H_2O_2 \qquad (k \approx 10^9\ M^{-1}\ s^{-1}) \qquad (5)$$

Taken together, these data suggest that **OG**$^{+\bullet}$, the predominant species at pH 6, must react with $O_2^{-\bullet}$ with a rate constant very similar to that of SOD with $O_2^{-\bullet}$ since substantial quenching is observed. At pH 7, the [**OG**$^{+\bullet}$] is diminished as a result of conversion to the neutral deprotonated form, **OG**$^{\bullet}$, which must have a much slower rate constant for reaction with $O_2^{-\bullet}$, allowing the fast reaction (eq 3) to compete. The overall amount of O_2-derived products is lower at pH 7, and the **Ia^{ox}** formed may also arise from a slow but competitive reaction of **OG**$^{\bullet}$ with O_2.

If the hypothesis that **HRf**$^{\bullet}$ can compete with SOD for trapping of $O_2^{-\bullet}$ is correct, one would predict that SOD would have more effect at pH > 8.5 where **Rf**$^{-\bullet}$ remains as a radical anion. Accordingly, **Rf**-mediated photooxidation of **OG** was repeated at pH 8.6; the results are shown in Figure 1.

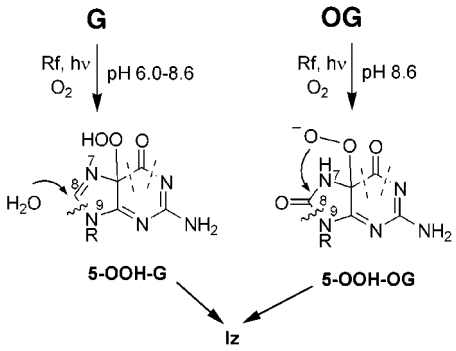
The data obtained at pH 8.6 include a higher amount of O_2-derived (blue) as opposed to H_2O-derived (red) products compared to results at pH 7, in agreement with the notion that **Rf**$^{-\bullet}$ cannot effectively diminish the [$O_2^{-\bullet}$]. In the presence of SOD, the amount of $O_2^{-\bullet}$ is greatly reduced, such that the fate of **OG**$^{\bullet}$ is principally to react with H_2O as well as an approximately constant (∼15−20%) reaction with O_2 seen at all pHs studied.

The other striking observation at pH 8.6 is a major shift in the identity of the O_2-derived product. Under more alkaline conditions, the imidazolone pathway (**Iz**, dark blue) predominates at the expense of **Ia^{ox}** (light blue). This behavior is highly reminiscent of the situation with H_2O-derived products in which **Gh** + **Ia** are formed at low pH from **5-OH-OG**, while **Sp** is formed at pH ≥ 7 (Scheme 1).[13]

A partial mechanism to explain the pH-dependent bifurcation in the further chemistry of **5-OOH-OG** is shown in

(28) Tretyakova, N. Y.; Niles, J. C.; Burney, S.; Wisnok, J. S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1999**, *12*, 459−466.
(29) Vialas, C.; Claparols, C.; Pratviel, G.; Meunier, B. *J. Am. Chem. Soc.* **2000**, *122*, 2157−2167.
(30) The hydrolysis product of **Gh^{ox}** would be expected to be parabanic acid, a species that is relatively stable at pH 7 but not detected in these reactions. Further characterization is underway.
(31) Candeias, L. P.; Steenken, S. *Chem. Eur. J.* **2000**, *6*, 475−484.
(32) Shafirovich, V.; Cadet, J.; Gasparutto, D.; Dourandin, A.; Huang, W.; Geacintov, N. *J. Phys. Chem. B* **2001**, *105*, 586−592.
(33) Steenken, S.; Jovanovic, S. V.; Bietti, M.; Bernhard, K. *J. Am. Chem. Soc.* **2000**, *122*, 2372−2374.
(34) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041−1100.
(35) Anderson, R. F. In *Flavins and Flavoproteins*; Massey, V., Williams, C. H., Eds.; Elsevier: New York, 1982; pp 278−283.

Scheme 3



Scheme 3. The formation of **Iz** requires at some stage the hydrolytic cleavage of the C8−N9 bond. This is relatively easy to accomplish when the precursor is **G** containing essentially an amidine functional group at C8. However, the urea moiety of **OG**-derived products should be more resilient to formation and breakdown of the required tetrahedral intermediate. A possible explanation for the fact that this happens at all is intramolecular attack of a peroxy anion at C8, although this would have to occur before the decarboxylative ring contraction of the six-membered ring (Scheme 3).

For purposes of comparison, the **Rf**-mediated photooxidation of **G** was also studied with and without SOD at various pHs, and the data are shown graphically in Figure 2. The pK_a of $\mathbf{G}^{+\bullet}$ has been measured as 3.9,[36] and thus the

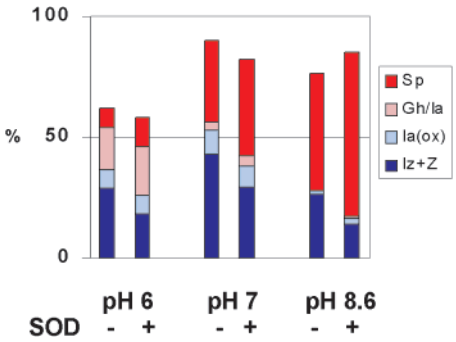


Figure 2. Products of **Rf**-catalyzed photooxidation of **G** as a function of pH and added Mn-SOD: red, **Sp**; pink, **Gh** + **Ia**; light blue, **Ia^{ox}**; dark blue, **Iz** + **Z**. Errors are estimated at ±5%.

principle species over the pH range studied is always the neutral radical **G$^{\bullet}$**. No dramatic change is observed between pH 6 and 7 other than the expected shift in product distribution between **Sp** and **Gh**/**Ia**. The reactions show some influence of quenching of $O_2^{-\bullet}$ by SOD, but mostly the quantity of **Iz** is reduced in the presence of SOD with little effect on other products. Interestingly, no **OG** was ever detected as a product of **Rf**-mediated **G** photooxidation at any pH.[37] The most notable difference between data obtained for **G** vs **OG** photooxidation is the predominant formation of **Iz**, even at pH 6. This must be due to the ease of hydration at C8 followed by cleavage of the C8−N9 bond and elimination of the N7−C8 fragment (Scheme 3).

Despite the fact that **Iz** is a major product pathway of **Rf**-catalyzed **G** oxidation at pH 7,[18] it is by no means the only product formed. **Sp** is also formed in substantial amounts; furthermore, **Sp** must be derived from $O_2^{-\bullet}$ and not $^1O_2$ because D_2O had no effect on the product distribution. This product was previously identified in the **Rf**-catalyzed oxidation of **G** as 4-hydroxy-8-oxo-7,8-dihydroguanosine,[19] but that assignment has been called into question.[38] The formation of **Sp** (and **Gh** + **Ia**) in this reaction must involve an intramolecular redox reaction in which the peroxyl group introduced from O_2 or $O_2^{-\bullet}$ at C5 participates in oxidation of C8 to generate **5-OH-OG** as the immediate precursor to these products. That **Sp** **Gh**/**Ia** and **Ia^{ox}** are unaffected by the presence of SOD, while **Iz** is, may suggest that these two sets of products are derived from different initial intermediates in the case of **G** oxidation.

In contrast, **Iz** was found as only a trace product at pH 7 when the substrate was **OG** because of a preference for decarboxylation and cleavage of the O−O bond, leading to six-membered ring contraction before hydrolytic decomposition of the five-membered ring was possible. For **G**, these two processes must be reversed in order. For **8-OMeG**, nucleophilic attack at C8 and subsequent cleavage of the C8−N9 bond might be intermediate between the two. In our hands, the pH 7 **Rf**-catalyzed oxidation of the **8-OMeG** nucleoside did form **Iz** as the major product, but it was accompanied by formation of three other products as well (**Iz**:**Ia^{ox}**:**Sp**:**OMe-Sp** in the ratio 1.0:0.2:0.3:0.3), and the overall reactivity of **8-OMeG** was substantially less than that of **OG**.

Overall, the large number of competing pathways in **G**, **OG**, and **8-OMeG** oxidation make the interpretation of reaction mechanisms extremely complex. For **OG**, the pK_a of the radical cation intermediate contributes to the pH dependence of $O_2^{-\bullet}$ reactivity, but it is not the only factor. Competing reactions of other species in the reaction mixture, including $\mathbf{Rf}^{-\bullet}$ (with $O_2^{-\bullet}$) and perhaps O_2 (with $\mathbf{OG}^{+\bullet}$), further complicate the nucleoside model studies. The chemistry of **OG** in duplex DNA in the cellular environment will likely include additional factors and should be expected to differ to some extent from nucleoside oxidation.

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Supporting Information Available: Experimental procedures including details of MS analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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(36) Steenken, S.; Jovanovic, S. *J. Am. Chem. Soc.* **1997**, *119*, 617−618.

(37) **OG** cannot be an intermediate in the formation of **Sp** from **G** because the **Sp** formed from **Rf** photooxidation does not incorporate an isotope from $H_2{}^{18}O$.[10]

(38) Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Org. Lett.* **2001**, *3*, 763−766.