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# Spiroiminodihydantoin Is the Major Product of the 8-Oxo-7,8-dihydroguanosine Reaction with Peroxynitrite in the Presence of Thiols and Guanosine Photooxidation by Methylene Blue

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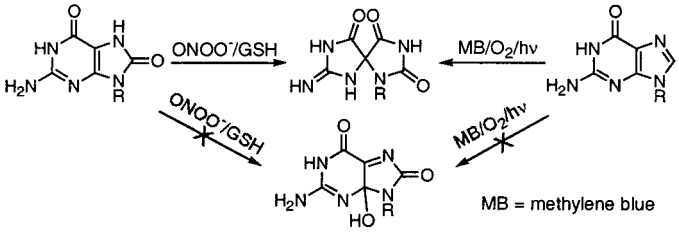
## ABSTRACT




**The potent oxidant, peroxynitrite, will oxidize 8-oxo-7,8-dihydroguanosine to give several products. In the presence of a thiol agent, the major final product has been determined to be a spiroiminodihydantoin compound. Additionally, we have found that the spiroiminodihydantoin, and not the previously reported 4-hydroxy-8-oxo-4,8-dihydroguanosine, is the major final product formed during the methylene blue-mediated photooxidation of guanosine.**

The biologically relevant lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)[1] can arise when $ONOO^-$ interacts with DNA[2] and is itself more reactive than its parent dG toward this oxidant.[3] Consequently, we have been exploring the possibility that 8-oxodGuo might be a target of $ONOO^-$-induced damage in vivo and have been characterizing the various products that arise during this reaction[4,5] in order to elucidate their role in mutagenesis and cellular toxicity. Additionally, we have also been investigating the mechanism by which the various chemical transformations occur and have performed studies using thiols in an attempt to trap reaction intermediates and $^{18}O$-isotope labeling studies to trace the origin of O atoms incorporated from an exogenous source. Hitherto, no attempts have been made to consolidate the chemistry, including the product identity, of the various 8-oxoGuo oxidizing systems. Here, we report on the identification and mechanism of formation of the major product of the 8-oxoGuo/thiol/$ONOO^-$ system and a com-

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(1) Abbreviations used: 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxo-dGuo; 8-oxo-7,8-dihydroguanosine, 8-oxoGuo; methylene blue, MB; peroxynitrite, $ONOO^-$; singlet oxygen, $^1O_2$.

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parison with the major products of the 8-oxoGuo/$CoCl_2$/$KHSO_5$ and Guo/MB/$^1O_2$ systems.

Reactions of 2′,3′,5′-tri-*O*-acetyl-8-oxoGuo (200 $\mu$M) with $ONOO^-$ (2 mM) in 150 mM potassium phosphate, 25 mM sodium carbonate, pH 7.2−7.4 buffer yielded the previously reported products, **I**−**IV** shown in Figure 1, with **II** as the

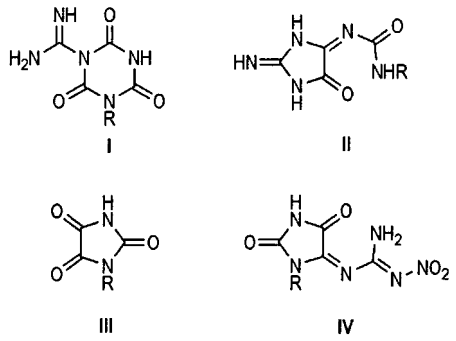


**Figure 1.** Summary of the previously elucidated products of the reaction between 2′,3′,5′-tri-*O*-acetyl-8-oxoGuo and peroxynitrite.

major product.[4,5] When a thiol, such as glutathione or cysteine (0.5−2 mM), was included in the reaction mixture, the oxidation of 8-oxoGuo was suppressed (17 mol % based upon added $ONOO^-$ in the absence of thiol vs 4 mol % in the presence of 2 mM thiol). A decrease was expected since thiols will react with $ONOO^-$ ($k \approx 0.58-6 \times 10^3$ $M^{-1}$ $s^{-1}$)[6,7] and, therefore, compete with 8-oxoGuo for available oxidant. Consistent with the overall decrease in the oxidation of 8-oxoGuo, there was a corresponding decrease in the yield of **I**−**IV**. Simultaneously, however, the yield of another product, **V**, dramatically increased in the presence of thiol, and at a thiol concentration above 200 $\mu$M, **V** became the major reaction product (Figure 2). Since the relative yields of **I**−**IV** remained constant in the presence of thiol despite

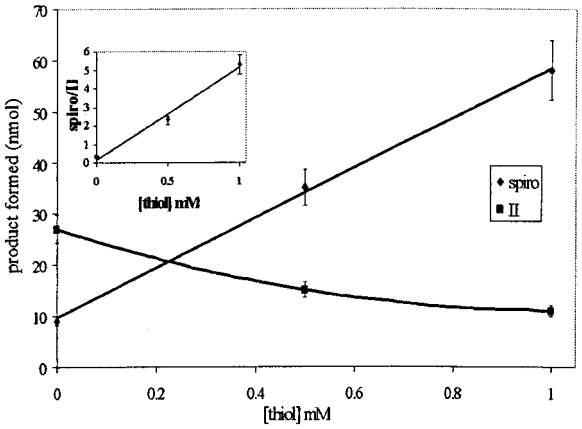


**Figure 2.** The yield of **V** increases with the concentration of thiol used, while that of **II** decreases. The ratio of **V**/**II** increases linearly with thiol concentration.

a decrease in their absolute levels, we reasoned that the product **V** must arise via trapping of an early reaction intermediate.

To identify **V**, we first conducted ESI-MS experiments in both negative and positive ion modes, which indicated a molecular weight of 441. Consultation with the literature on the oxidation of Guo and 8-oxoGuo revealed that the 2,3,5-tri-*O*-acetyl-$\beta$-D-*erythro*-pentofuranosyl derivative of two previously reported compounds would have exactly this molecular weight. These were 4-hydroxy-8-oxo-4,8-dihydroguanine reported as the major product of the $^1O_2$-mediated oxidation of Guo[8,9] and spiroiminodihydantoin reported as the major product formed during the $CoCl_2$/$KHSO_5$-mediated oxidation of 8-oxoGuo.[10] Hence, we wanted to determine whether **V** was identical to either of these products.

To do so, authentic standards from both reactions were prepared by (i) irradiating an aerated $D_2O$ solution of 2′,3′,5′-tri-*O*-acetyl-Guo (200 $\mu$M) and MB (100 $\mu$M) with visible light for 2 h and (ii) treating 2′,3′,5′-tri-*O*-acetyl-8-oxoGuo (200 $\mu$M) with $CoCl_2$ (3.3 $\mu$M) and $KHSO_5$ (290 $\mu$M) in 150 mM potassium phosphate, pH 7.0 buffer for 2 h. The respective products were then purified using analytical HPLC.[11] We then compared these standards with **V** using HPLC,[12] UV/vis spectroscopy, and ESI-MS/MS. First, we observed that during HPLC all three compounds eluted as a double peak, consistent with the previous reports,[8,10] and that they all had identical retention times as shown in Figure 3.

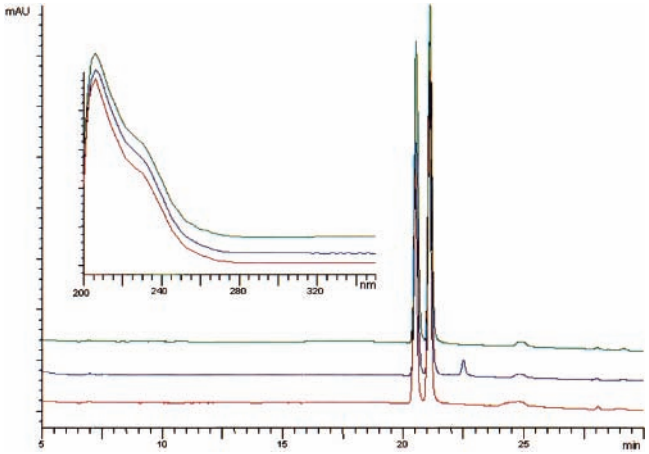


**Figure 3.** HPLC traces of the major product isolated from the 8-oxoGuo/$CoCl_2$/$KHSO_5$ (red line), 8-oxoGuo/$ONOO^-$/GSH (blue line), and Guo/methylene blue/$h\nu$ (green line) reactions. All three products have identical retention times and UV spectra.

In addition, the UV spectra of these compounds were identical, and when all three compounds were mixed and analyzed by HPLC, they coeluted. Furthermore, in ESI-MS/

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MS studies all three compounds gave identical fragmentation patterns, as shown in Figure 4. Taken together, these data

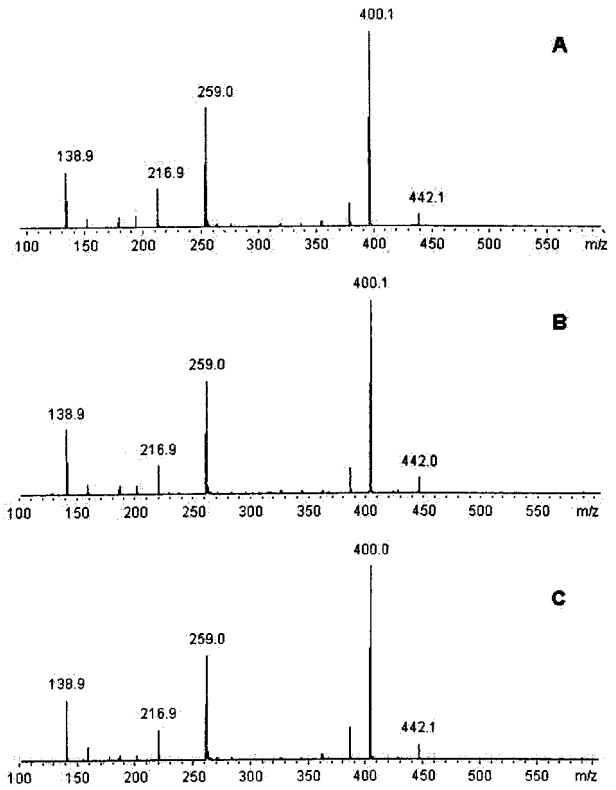


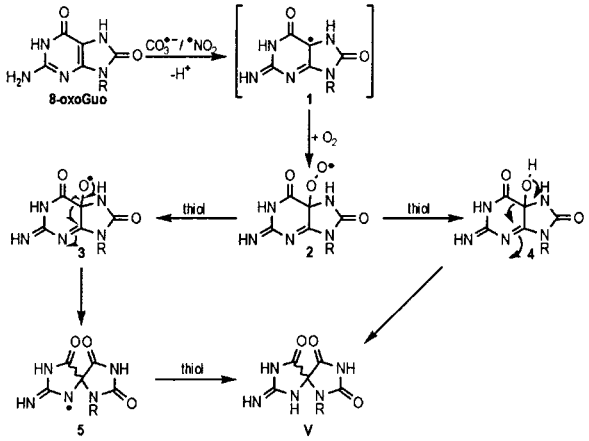

**Figure 4.** The major products from the Guo/methylene blue/$h\nu$ (**A**), 8-oxoGuo/$CoCl_2$/$KHSO_5$ (**B**), and 8-oxoGuo/$ONOO^-$/GSH (**C**) reactions fragment identically in MS/MS experiments.

demonstrate that (i) the Guo/MB/$^1O_2$, 8-oxoGuo/$CoCl_2$/$KHSO_5$, and 8-oxoGuo/GSH/$ONOO^-$ systems all yield the same productand (ii) the product of the Guo/MB/$^1O_2$ reaction had previously been misidentified. The 4-hydroxy-8-oxo-4,8-dihydroguanosine previously identified by Sheu and Foote during low temperature photooxidation of 8-oxoGuo was shown to be unstable at room temperature, ultimately undergoing hydrolysis to yield parabanic acid.[9] Thus, the unstable lesion characterized by Sheu and Foote cannot be identical to the stable lesion characterized by Raoul and Cadet.[8] Thus, on the basis of the recent report by Luo et al. and on our presently reported results, it is reasonable to conclude that the true major product of the Guo/MB/$^1O_2$ reaction is the spiroiminodihydantoin. Indeed, the 1-D $^{13}C$ spectrum of the product obtained from the Guo/MB/$^1O_2$ reaction (see Supporting Information) is similar to that reported by Luo et al. for the 8-oxoGuo/$CoCl_2$/$KHSO_5$ product.

Additionally, we wanted to examine the mechanism by which **V** was formed and to specifically identify the source of the O atom incorporated into this product. Therefore, the 8-oxoGuo/GSH/$ONOO^-$ reaction was carried out in the presence of $^{18}O_2$, $H_2{}^{18}O$, and $^{18}O$-labeled $ONOO^-$ and **V** was analyzed by either on-line LC-ESI-MS or off-line ESI-MS. Only in the presence of $^{18}O_2$ did the molecular weight of **V** increase by two atomic mass units, indicating that molecular oxygen was the sole source of the incorporated O atom. Further supporting a role for molecular oxygen was the finding that carrying out the reaction in deoxygenated buffer led to a 5-fold decrease in the yield of **V** relative to that obtained in oxygenated buffer.

On the basis of our findings, we have proposed the mechanism in Scheme 1 to account for the formation of **V**.

**Scheme 1.** Proposed Mechanism for the Formation of **V**




It has been determined that in the presence of $CO_2$ $ONOO^-$ will rapidly decay to ultimately produce the $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals,[13−17] which have redox potentials of 1.5[18] and 1.04 V,[19] respectively, and thus are capable of effecting the one-electron oxidation of 8-oxoGuo ($E^o$ = 0.59 V)[20] to yield the neutral 8-oxoGuo radical **1**. The latter species has recently been determined to have a p$K_{a1}$ = 6.6, and, thus, at physiologic pH, exists predominantly (>85%) as the neutral

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(11) For analytical HPLC, a 250 × 4.6 mm, 5 $\mu$m Columbus C18 column (Phenomenex) was used along with 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) as mobile phases. The elution program consisted of an isocratic phase (5%B for 10 min) followed by a gradient (5−40% B over 20 min). After another isocratic wash (40%B for 2 min), the initial mobile phase composition was restored (40% B to 5% in 3 min). The flow rate was 1.0 mL/min.

(12) In these studies, a 150 × 2.0 mm, 5 $\mu$m Columbus C18 column (Phenomenex) was used. The same solvents and elution program mentioned above were used, except with a flow rate = 0.2 mL/min.

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radical.[21] By analogy to the Guo radical, excess unpaired electron density would be expected at the O6 and C5 positions of the neutral radical. We propose that reaction of $O_2$ with the neutral radical at the C5 occurs to yield the hydroperoxyl radical species **2**, which in a thiol-dependent reaction is reduced to the alkoxide radical, **3**.

The latter species then undergoes rearrangement involving C5 oxo-bond formation and migration of the C5−C6 bond to form a new C4−C6 bond, thereby producing the *N*-centered spiro radical **5**, which is then reduced to **V**. Alternatively, **2** may be reduced to the 5-hydroxy-8-oxoGuo intermediate, **4**, in a thiol-dependent reaction. Subsequent rearrangement of **4** could then lead to formation of **V**. An additional mechanism, which cannot easily be eliminated, explaining the incorporation of an $O_2$-derived atom into **V** involves thiol-dependent superoxide production, followed by combination of the latter with the neutral 8-oxoGuo radical, to produce the C5-hydroperoxide which can subsequently be reduced to **3** or **4**.

Our observation that an $O_2$-derived O atom is incorporated into **V** contrasts with that previously reported for spiroiminodihydantoin isolated from the 8-oxoGuo/$Na_2IrCl_6$ system, in which $H_2O$ was shown to be the O atom source.[10] We believe that this difference arises at the level of the neutral 8-oxoGuo radical. Thus, in the presence of a thiol reductant, further oxidation of the 8-oxoGuo radical to the cation is inhibited, thereby facilitating competition for the neutral 8-oxoGuo radical by $O_2$. However, in the absence of a reductant, the neutral 8-oxoGuo radical is oxidized to the 8-oxoGuo cation, and this species rapidly reacts with nucleophiles such as $H_2O$.

In conclusion, these studies clarify the issue of the true identity of the major Guo/MB/$^1O_2$ product. This is an important correction that will aid in the accurate assignment of biological properties to the specific oxidation products of Guo and its derivatives. Indeed, this lesion has recently been incorporated into oligonucleotides,[22] thereby making it possible to proceed with an assessment of its biological consequences. Furthermore, this work illustrates the impact of biologically important reductants on the major products isolated from the oxidation of 8-oxoGuo. We have found that this is true not only for the 8-oxoGuo/thiol/$ONOO^-$ system but also for the 8-oxoGuo/± thiol/MB/$^1O_2$ system. In the latter case, the major product is the spiroiminodihydantoin, instead of cyanuric acid,[23] when a thiol is included. From a chemical perspective, this work demonstrates the intermediacy of the neutral 8-oxoGuo radical in the $ONOO^-$-mediated oxidation of 8-oxoGuo. This radical has only recently been characterized spectroscopically and kinetically,[21] and its chemical properties still remain to be fully elucidated. Finally, this study highlights the overlap in the product spectrum observed during the oxidation of 8-oxoGuo by various oxidizing agents, despite distinct differences in the underlying oxidation mechanism. Undoubtedly, this reflects the formation of common intermediates, including the 8-oxoGuo radical, that ultimately lead to formation of the same product(s).

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**Supporting Information Available:** 1-D $^{13}C$ spectrum of the product obtained from the Guo/MB/$^1O_2$ reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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