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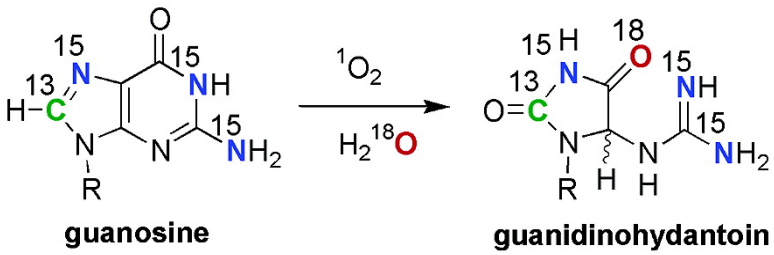

guanosine **guanidinohydantoin**

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Formation of ^{13}C-, ^{15}N-, and ^{18}O-Labeled Guanidinohydantoin from Guanosine Oxidation with Singlet Oxygen. Implications for Structure and Mechanism

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The action of reactive oxygen species upon DNA bases damages genomic and mitochondrial DNA as well as the cellular nucleotide pool and is therefore implicated in cancer, aging, and neurological disorders.[1−3] Oxidation of guanosine by singlet oxygen has been the focus of considerable interest because of its importance in both cancer etiology and its cure via photodynamic therapy.[4,5] Additionally, $^1O_2$ reaction with the guanine heterocycle presents a plethora of pathways and potential products depending upon the reaction conditions and the structural context − nucleoside versus double-stranded DNA.[6,7] It is now well established that the principal product of G nucleoside oxidation by $^1O_2$ above pH 7 is spiroiminodihydantoin (Sp),[8−10] although 8-oxo-7,8-dihydroguanosine (OG) predominates in cellular DNA.[11] We characterized Sp as the main product of OG oxidation by one-electron oxidants in nucleoside studies, while oxidation in ds-DNA led to a cationic base, guanidinohydantoin (Gh).[12] We report here that Gh is also the major product of $^1O_2$ reaction with G at pH < 7. Furthermore, isotopic labeling resolves controversial issues concerning its structure and points to a mechanistic explanation for products observed in our laboratory and others.

Previously, we proposed that 5-hydroxy-OG is an unstable common intermediate in the pH and structure-dependent formation of both Sp and Gh (Scheme 1),[12] by analogy to uric acid oxidation to allantoin via hydration and decarboxylation of 5-hydroxyurate.[13] While an acyl shift to generate Sp is preferred at pH 7 and above, formation of the tetrahedral carbon of Sp is inhibited in ds-DNA. In addition, lower pH facilitates hydration of C6. In the present study, oxidation of guanosine using Rose Bengal photosensitization in buffered aqueous solution led to the pH-dependent product distribution shown in Table 1, as determined by quantitative LC-MS analysis (see ref 14 and Supporting Information). The product distribution alters dramatically in favor of Gh below pH 7, and overall Sp and Gh account for the bulk of the products. Evidence for further oxidation to Gh^{ox} [12] is also seen because of the high level of conversion. 2,5-Diaminoimidazol-4-one[15] accounts for a small amount of the total products but is more pronounced at higher pH.

When the oxidation was conducted in 95% enriched $H_2{}^{18}O$ at pH 4.5, MS analysis indicated that 84% of Gh and 83% of Sp contained one atom of ^{18}O. Because both products each gain two oxygen atoms upon singlet oxygen oxidation, one oxygen atom must be derived from O_2 while the other is derived from H_2O. That both Sp and Gh show identical isotopic incorporation lends further support to 5-OH-OG as a common intermediate.

The location of the oxygen atom derived from water was determined by positive ion ESI-MS/MS studies of Gh formed with $H_2{}^{16}O$ versus $H_2{}^{18}O$. In-source fragmentation of the glycosidic bond followed by mass selection of the Gh heterocycle (m/z = 158 or 160) led to a major fragmentation of the C5−N6 bond, forming guanidinium ion at m/z = 60. The second most intense ion at m/z = 98 (shifted to 100 in $H_2{}^{18}O$) is thought to be due to a two-bond fragmentation whose product ion would include C4 and C5 of Gh along with an ^{18}O as a carbonyl group at C4 (originally C5 in G). To gain further support for this assignment, guanosine isotopically labeled at C8, N1, amino, and N7[16] was oxidized with Rose Bengal photosensitization again in $H_2{}^{16}O$ versus $H_2{}^{18}O$. As expected, the guanidinium ion shifted to m/z = 62 due to the presence of two ^{15}N atoms (Figure 1). Significantly, the m/z = 100 fragment, now with two ^{15}N atoms in the guanidinium group, was shifted to m/z = 102 in the $H_2{}^{18}O$ experiment. The only pattern consistent with such a labeling scheme involves introduction of an oxygen atom from water at C5 of guanine (C4 of Gh) along with an oxygen atom from O_2 at C8 of G (C2 of Gh).[17]

Scheme 1. Oxidation Pathways of Guanosine

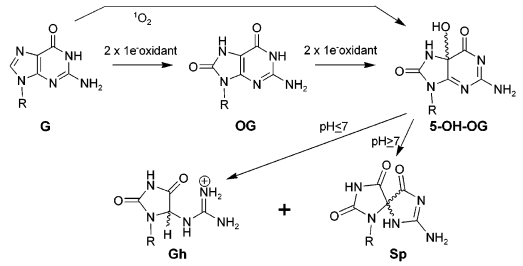

Table 1. Product Distribution (%) as a Function of pH

	pH 4.5	pH 6	pH 7	pH 8.6
Gh	38	34	3	1
Sp	1	16	52	70
Gh^{ox} [a]	7	2	1	1
Iz[b]	5	4	4	15

[a] Gh^{ox} = 5,6-dehydroGh. [b] Iz = 2,5-diaminoimidazol-4-one.

The use of isotopes also permitted resolution of a controversy surrounding the structure of Gh, a species formed as a mixture of two slowly equilibrating isomers. Subject to debate was whether the two HPLC peaks observed represent equilibrating diastereomers via enolization of the C4 carbonyl[18,19] or an additional set of iminoallantoin isomers formed by ring closure of a guanidine nitrogen attacking C4 followed by opposite ring opening.[12] We previously proposed the iminoallantoin structure on the basis of an analogous isomerization observed in the urate pathway to allantoin,[20] as well as a related proposal from products of arylamine adducts to C8 of G.[21] To resolve this issue, Gh was formed by $^1O_2$ oxidation of a G labeled at C2, N1, amino, and N7[22] at pH 4.5, and the relevant NMR spectra in 10% D_2O buffered to pH 7 are shown in Figure 2.

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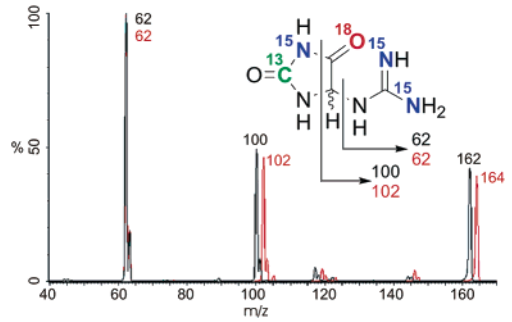


Figure 1. Positive ion ESI-MS/MS studies of labeled Gh in $H_2{}^{16}O$ (black) versus $H_2{}^{18}O$ (red).

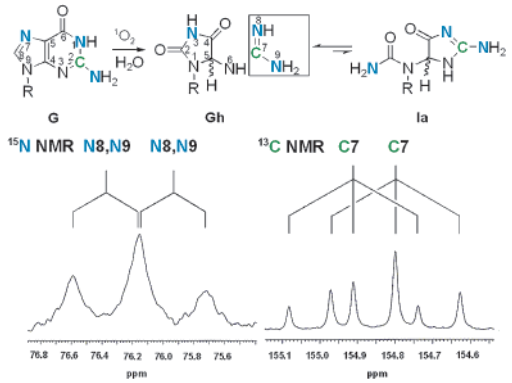


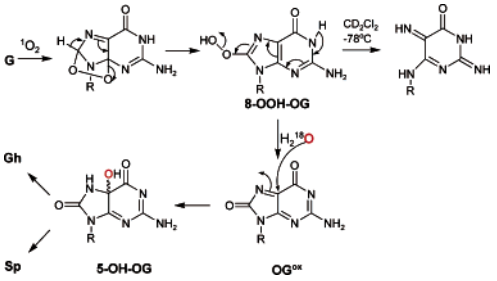
Figure 2. ^{15}N and ^{13}C NMR spectra of isotopically labeled Gh in 10% D_2O at pH 7. See Supporting Information for complete spectra and spectra in other solvents.

The ^{15}N NMR spectrum of labeled guanosine starting material displayed three resonances assigned as N7 (235.3 ppm), N1 (147.4 ppm, d, J = 14.7 Hz), and amino (72.5, d, J = 23.4 Hz). After oxidation, the signal corresponding to the original N7 (now N3 of Gh) was not seen at pH 7, although a weak resonance at 149.4 ppm was seen in the pH 4.5 spectrum.[17] The only peaks observed in the pH 7 ^{15}N spectrum were two overlapping doublets near 76 ppm, which became clearly resolved at pH 4.5.[17] These data are consistent with the formation of two diastereomers of Gh in which the two ^{15}N-labeled nitrogen atoms of the guanidinium group are equivalent and coupled to the adjacent ^{13}C. Even more convincing is the observation of two triplets (one for each diastereomer) in the ^{13}C NMR spectrum of the same material, showing the equivalency of the two nitrogen atoms attached to the ^{13}C. While it is still possible that iminoallantoin isomers exist, we could not detect them by NMR, and the upper limit of the equilibrium constant must therefore be about 0.03.

Taken together, the results point to Gh as a significant product of singlet oxygen damage to guanosine at pH < 7, while Sp predominates at pH ≥ 7, and the labeling studies along with prior observations are consistent with 5-OH-OG as their common intermediate. A mechanism consistent with the introduction of an oxygen atom from $H_2{}^{18}O$ at C5 of G while C8 of G reacts with O_2 is shown in Scheme 2.

Foote and co-workers have conducted detailed studies of $^1O_2$ oxidation of a ^{13}C, ^{15}N-labeled guanosine derivative in nonpolar organic solvents at −78 to −100 °C.[23−25] NMR evidence supports a [4+2] cycloaddition pathway with the imidazole ring of G that undergoes ring opening to 8-OOH-G. In a nonaqueous solvent, loss of CO_2 was observed, likely due to dioxirane formation from 8-OOH-G. Our studies suggest that the fate of 8-OOH-G in water is loss of H_2O to form a reactive oxidized form of OG (OG^{ox}) that is trapped by H_2O at C5, generating 5-OH-OG, the common

Scheme 2. Proposed Mechanism of the Formation of Gh and Sp in H_2O as Compared to Products Formed in Organic Solvents[23]



precursor to Sp and Gh. The mechanism in Scheme 2 explains all of the major G nucleoside products observed from $^1O_2$ in both aqueous and nonaqueous solvents. That OG is the most common product of singlet oxygen oxidation of cellular DNA is likely due to interception of the hydroperoxide intermediate by cellular reductants. While Gh may be less prevalent than OG in cellular DNA, its biological effects are more dramatic. An in vivo mutagenesis assay showed this lesion to cause 99% transversion mutations (G to C) as compared to <5% (G to T) for OG.[26]

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Note Added after ASAP: Scheme 2 contained errors in the version published on the Web 10/28/2003. The version published 11/3/2003 and the print version are correct.

Supporting Information Available: Experimental procedures, ^{13}C and ^{15}N NMR spectra in D_2O (pH 4.5 and 7.0) and d_6-DMSO, and ESI-MS/MS data for labeled and unlabeled Gh (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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