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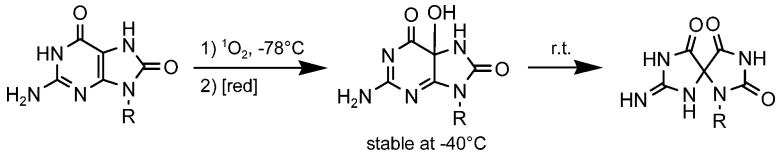

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Characterization of 5-Hydroxy-8-oxo-7,8-dihydroguanosine in the Photosensitized Oxidation of 8-Oxo-7,8-dihydroguanosine and Its Rearrangement to Spiroiminodihydantoin

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Abstract: The photosensitized oxidation of 2′,3′,5′-tris-(O-*tert*-butyldimethylsilyl)-8-oxo-7,8-dihydroguanosine (8-oxoG) with singlet oxygen was studied by low-temperature NMR. A stable intermediate was characterized at −60 °C by ^{13}C, 2D NMR HMBC spectra, and chemical shifts calculated by hybrid Hartree−Fock density functional theory which agreed with the structure 5-hydroperoxy-8-oxo-7,8-dihydroguanosine. Reduction of this intermediate at low temperature afforded the corresponding alcohol, the long-postulated 5-hydroxy-8-oxo-7,8-dihydroguanosine, the last intermediate in the formation of spiroiminodihydantoin. Upon warming to room temperature, this alcohol rearranges to form the spiroiminodihydantoin in good yield within 2 h.

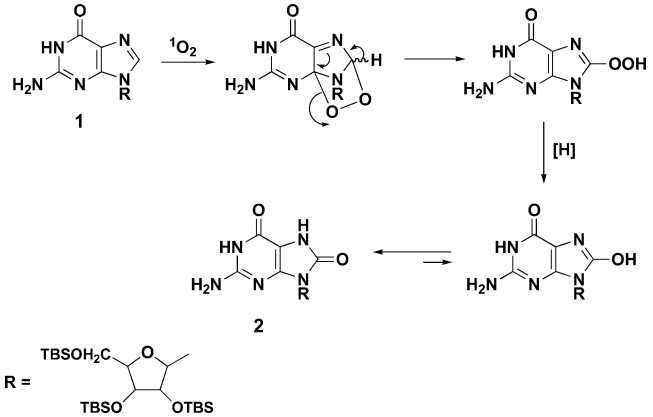
Introduction

DNA bases are prime targets for oxidative attack by reactive oxygen species or other oxidants. Oxidation of electron-rich nucleobases may lead to alkali-labile sites that can result in strand cleavage. Examination of the mechanism of oxidative damage has helped in understanding oxidative stress, which has been linked to many diseases.[1−4] A key to this understanding is the identification of short-lived intermediates.

Guanine (**1**) is the most easily oxidized base, and a great deal of research has been devoted to studying products from the oxidation of guanosine and its derivatives.[1−3] Of these guanine base modifications, 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG (**2**), is the most common lesion observed in duplex DNA and is routinely used as an indicator of oxidative stress.[1−8] Interestingly, 8-oxoG is also susceptible to further oxidation and is much more reactive than guanosine.[9−14] In an attempt to better understand the mechanisms involved in oxidative damage, our group investigated the $^1O_2$-mediated oxidation of guanosine and various guanosine derivatives.[11−13,15−17]

The proposed mechanism of the formation of 8-oxoG (**2**) in the photooxidation of guanosine by singlet oxygen is shown in Scheme 1. This mechanism is consistent with experimental evidence that an unstable endoperoxide forms as the result of a singlet oxygen-mediated cycloaddition with guanosine.[13,17] Opening of the endoperoxide and subsequent reduction of the hydroperoxide results in the enol tautomer of 8-oxoG. Structural and NMR studies show the 6,8-diketo isomer is the predominant species in solution.[18,19]

Scheme 1. Mechanism of 8-oxoG Formation from Photooxidation of Guanosine by Singlet Oxygen



The main decomposition products identified from the oxidation of guanosine and guanosine derivatives are shown in Figure

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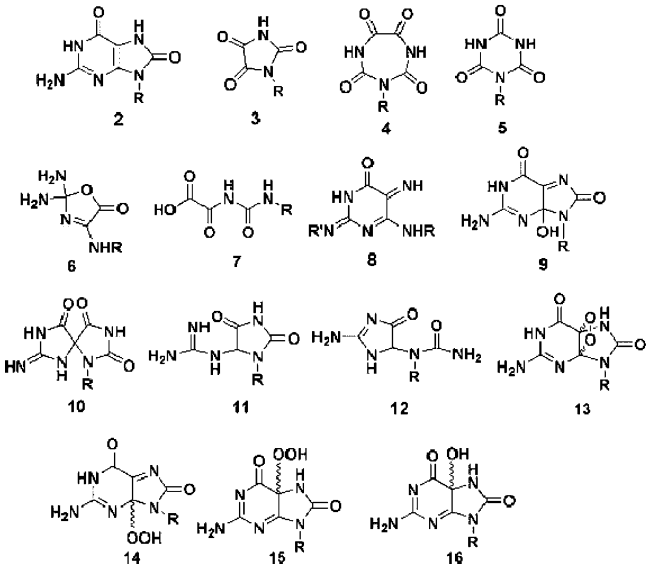

Figure 1. Oxidation products of guanosine and 8-oxo-7,8-dihydroguanosine.

1. 8-oxodG (**2**) was the major product when DNA was photooxidized in the presence of methylene blue or treated with a chemical source of singlet oxygen.[7,20] Other products of guanine oxidation have also been characterized.

Using type I photooxidation conditions with 2′-deoxyguanosine, Cadet et al. identified oxazolone **6** as the major product.[21] Upon photosensitized oxidation of 2′-deoxyguanosine with methylene blue, Cadet et al. identified 8-oxodG along with a compound to which he originally assigned the structure of 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (4-OH, **9**) as the major products formed.[5] More recently, Tannenbaum et al. showed that the main product from the oxidation of the acetyl-protected guanosine nucleoside is actually spiroiminodihydantoin (spiro) **10** rather than the 4-hydroxy compound (**9**).[22] Burrows et al. identified spiro **10** as the major product at pH > 7 and guanidinohydantoin **11** at pH < 7 from guanosine oxidation with singlet oxygen.[23] Spiro **10** was also found as the major decomposition product in the oxidation of 2′-deoxyguanosine by the triplet state generated from hydroxyacetophenone photolysis.[24] This spiro compound also has been identified in single- and double-stranded oligonucleotides. When dG- and 8-oxodG-containing oligonucleotides were oxidized by carbonate radical anion, a spiro-containing oligonucleotide was identified as the major product.[25] When a hydrophobic guanosine derivative was photooxidized with TPP in organic solvent, Foote et al. identified compound **8** as the major product formed with minor amounts of parabanic acid **3**, the seven-membered ring **4**, and 8-oxoG (**2**).[16]

8-OxoG is itself susceptible to further oxidation and is far more reactive than guanosine.[9−14,26] For this reason, research has also been focused on the characterization of 8-oxoG oxidation products. When Cadet first investigated the photosensitized reaction of 8-oxodG with methylene blue in aqueous solution, he found three main products: the compound assigned 4-OH **9** in 10% yield, oxazolone **6** in 35% yield, and cyanuric acid **5** at 50%.[9,26] Cadet et al. also studied the oxidation of 8-oxodG-derivatized oligonucleotides by chemically generated singlet oxygen (from di-(2,3-dihydroxypropyl)-1,4-naphthalene-dipropanoate endoperoxide) in aqueous solution. In this study, oxaluric acid (**7**) was the main decomposition product identified.[10] When Burrows et al. treated 8-oxodG **2** with a one-electron oxidant ($IrCl_6^{2-}$), spiro **10** was the major decomposition product at pH ≥ 7. Guanidinohydantoin **11** was the major decomposition product from the one-electron oxidation at pH < 7.[27−29] Additionally, Tannenbaum et al. identified spiro **10** as the main oxidation product from the oxidation of the acetyl-protected 8-oxodG-nucleoside derivative with peroxynitrite.[22]

Sheu and Foote identified parabanic acid (**3**), cyanuric acid (**5**), and the seven-membered ring product **4** as additional decomposition products from the rose bengal photosensitized oxygenation of 8-oxoG (**2**) in organic solvent.[15]

Low-temperature studies by Sheu and Foote characterized intermediates assigned at the time as the diastereomeric 4-hydroperoxy-8-oxo-4,8-dihydroguanosines (4-OOH, **14**) solely by low-temperature NMR. Reduction of these hydroperoxides afforded the corresponding alcohols, which were assigned as the 4-hydroxy-8-oxo-4,8-dihydroguanosine (4-OH, **9**), on the basis of similarities in the spectra with Cadet's earlier assignment of the 4-OH structure.[15]

Interestingly, the other possible regioisomer of this alcohol, 5-hydroxy-8-oxo-7,8-dihydroguanosine, 5-OH (**16**), is the putative precursor of spiro **10**. This long postulated intermediate has been suggested to be the last intermediate in the formation of spiroiminodihydantoin.[22,23,27−29]

We now report NMR and computational studies that require us to reassign the structure of intermediates **9** and **14**.

Experimental Section

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker ARX-400, ARX-500, and Avance 500 and 600 spectrometers. Low-temperature NMR spectra were taken in a precooled probe maintained at the desired temperature. Electrospray ionization mass spectra were collected on a PE SCIEX API III Biomolecular Mass Analyzer. Column chromatography was performed on silica gel 60, 230−400 mesh, from E. Merck. The photosensitized oxidation of 8-oxoG (**2**) was carried out at −78 °C using 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine (TPP) as a sensitizer in acetone-d_6 or methylene chloride-d_2 using a chromium glass filter to cut off wavelengths below 540 nm, with a Cermax 300-W xenon lamp as the light source. Samples were irradiated for 30 min and kept at −78 °C until placed in the precooled NMR probe for analysis. Reduction of hydroperoxide (**15**) was achieved with 10 equiv of dimethyl sulfide (DMS) at −40 °C. Spiro **10** was formed when 5-OH (**16**) was allowed to warm to room temperature in acetone-d_6. Rate constants were determined by following ^{1}H NMR.

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Table 1. Relative Energies for $C_6H_7N_5O_4$ Isomers

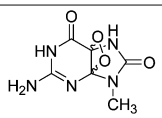
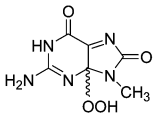
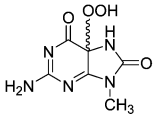
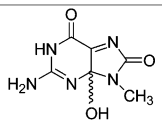
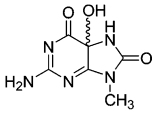
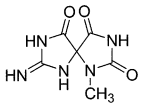
$C_6H_7N_5O_4$ isomer		Relative Energies (kcal/mol)
9-methyl-8-oxoG-4,5-dioxetane		12.3
4-hydroperoxy-9-methyl-8-oxoGua		16.3
5-hydroperoxy-9-methyl-8-oxoGua		0.0

Table 2. Relative Energies for $C_6H_7N_5O_3$ Isomers

$C_6H_7N_5O_3$ isomer		Relative Energies (kcal/mol)
4-hydroxy-9-methyl-8-oxoGua		31.4
5-hydroxy-9-methyl-8-oxoGua		18.2
9-methylspiroiminodihydantoin		0.0

NMR spectra were calculated using Gaussian 98.[30,31] All structures were optimized at the B3LYP/6-31G(d) level of theory and characterized by frequency analysis, and ^{13}C NMR spectra were predicted at the GIAO/B3LYP/6-311+G(2p,d) level.[32−42] Relative energies reported in Tables 1 and 2 are based on B3LYP/6-311+G(2p,d) single-point calculations on the geometries optimized at the B3LYP/6-31G(d) level and include zero-point energy corrections from frequency calculations at the B3LYP/6-31G(d) level scaled by 0.9806.[43] As a first step, a series of calibrations was run to determine that this level of theory was appropriate for predicting the NMR spectra for 8-oxoGua derivatives. Calculated spectra for *N*-9 substituted 8-oxo-7,8-dihydroguanines were compared to the experimental spectrum of TBS protected 8-oxoG. Hydrogen, methyl, hydroxymethyl, and methoxymethyl were chosen as models for the ribose at *N*-9. The predicted spectrum of *N*-methyl-8-oxo-7,8-dihydroguanine closely matched the experimental spectrum of the nucleoside (with an average absolute deviation of 1.4 ppm from the actual chemical shifts), so all subsequent calculations incorporated a methyl group as a surrogate for the ribose.

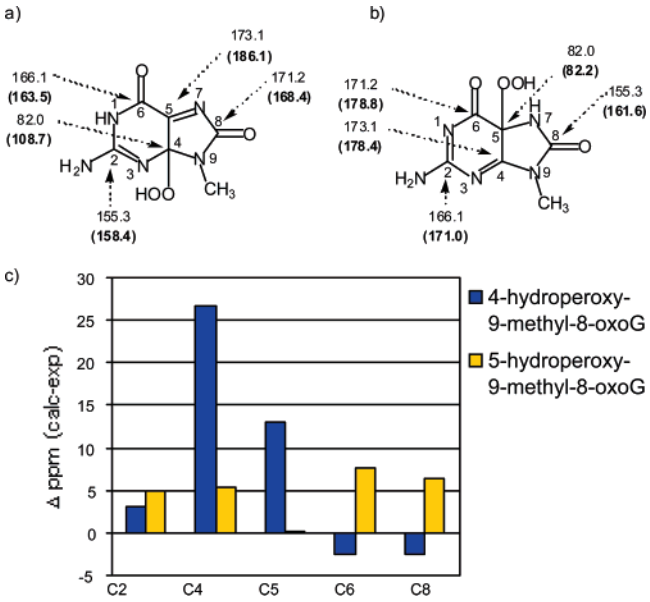


Figure 2. (a) Observed (regular) and computed (bold, parentheses) chemical shifts (ppm) for 4-OOH-8-oxoG (**14**). (b) Observed (regular) and computed (bold, parentheses) chemical shifts (ppm) for 5-OOH-8-oxoG (**15**). (c) Difference between observed and computed chemical shifts calculated for 4-OOH-8-oxoG (blue) and 5-OOH-8-oxoG (gold).

Materials. Unless otherwise noted, all chemicals were from Aldrich. All common solvents were from Fisher. Deuterated solvents were from Cambridge Isotope Laboratory and used as received. 2′,3′,5′-Tris-(O-*tert*-butyldimethylsilyl)-8-oxo-7,8-dihydroguanosine was synthesized as previously described.[13]

Results

Low-Temperature Photooxidation. Low-temperature photooxygenation of 2′,3′,5′-tris-(O-*tert*-butyldimethylsilyl)-8-oxo-7,8-dihydroguanosine was carried out as previously described.[15] The ^{13}C NMR spectrum at −60 °C showed five guanine peaks at 82.0, 155.3, 166.1, 171.2, and 173.1 ppm, which agree with the chemical shifts of the intermediate previously assigned the structure 4-hydroxy-8-oxo-4,8-dihydroguanosine by Sheu and Foote.[15]

To help interpret this spectrum, chemical shifts were calculated for 9-methyl-substituted guanine derivatives (Figure 2) with the B3LYP/6-311+G(2p,d)//B3LYP/6-31G(d) method. Comparison with the experimental observations (Figure 2c) suggests that the observed intermediate is not the 4-OOH (**14**) regioisomer but rather the 5-OOH (**15**) regioisomer. The computed shifts for the 4-OOH isomer deviate from the observed spectrum by values between +26.7 and −2.6 ppm, with an average absolute deviation of 9.6 ppm (Figure 2c). On the other hand, the computed shifts for the 5-OOH isomer deviate by values between +7.6 and +0.2 ppm, with an average absolute

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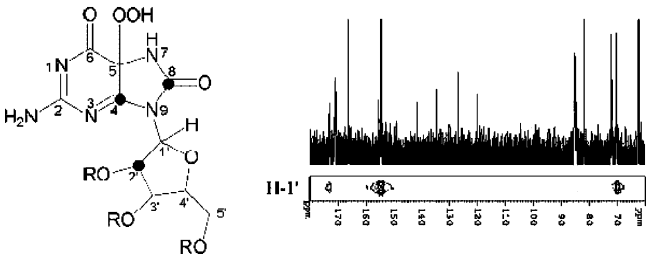
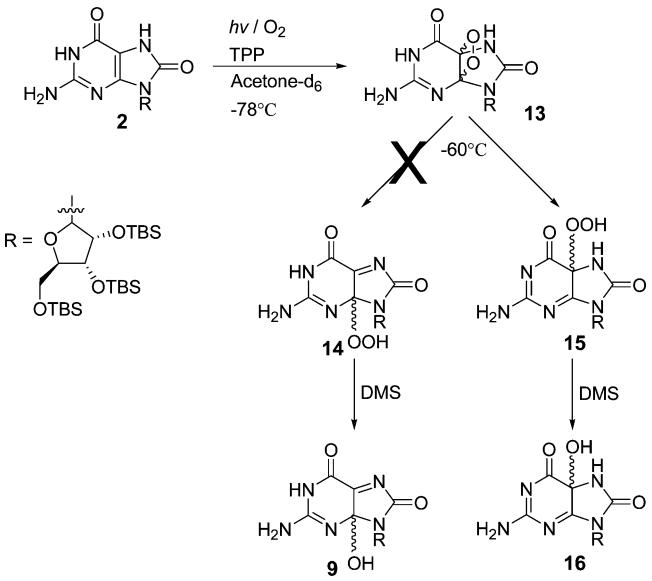


Figure 3. HMBC of 5-hydroperoxy-8-oxo-7,8-dihydroguanosine (**15**).

Scheme 2. Singlet Oxygen Oxidation of 8-oxoG (**2**)



deviation of 4.9 ppm. The observed chemical shift at C-5 of 82.0 is similar to that observed for analogous hydroperoxy derivatives of thymidine.[44,45]

2-D HMBC experiments confirmed the assignment as the 5-regioisomer (**15**). A clear three-bond coupling from the anomeric proton (H-1′) to C-8 at 155 ppm and C-4 at 173.1 ppm is evident in Figure 3. As C-5 is an sp^3-hybridized carbon and C-4 is sp^2-hybridized, the 2-D HMBC correlation to C-4 at 173.1 ppm clearly indicates correlation to an sp^2-hybridized carbon. The coupling between H-1′ and C-2′ at 70 ppm is also present. Upon warming, the hydroperoxide decomposed to several products, as previously reported.[15]

When **2** was oxidized at −78 °C (Scheme 2), warmed to −60 °C, then treated with DMS at −40 °C, a new product was formed. The low-temperature ^{13}C NMR spectra at −40 °C showed five guanine peaks at 72.5, 154.6, 165.7, 175.6, and 175.7 ppm. These peaks also corresponded to the peaks reported by Sheu and Foote, previously identified as the 4-OH alcohol (**9**).[15] The reduction step was necessary for formation of 5-OH (**16**) under these conditions. Again, relative chemical shifts were calculated for the possible alcohol regioisomers (Figure 4), and calculated shifts for the 5-substituted hydroxyl (**16**) correspond more closely to the experimental observations (Figure 4c). Deviations for the 4-OH isomer ranged from +29.6 to −7.8 ppm with an average absolute deviation of 10.0 ppm, while

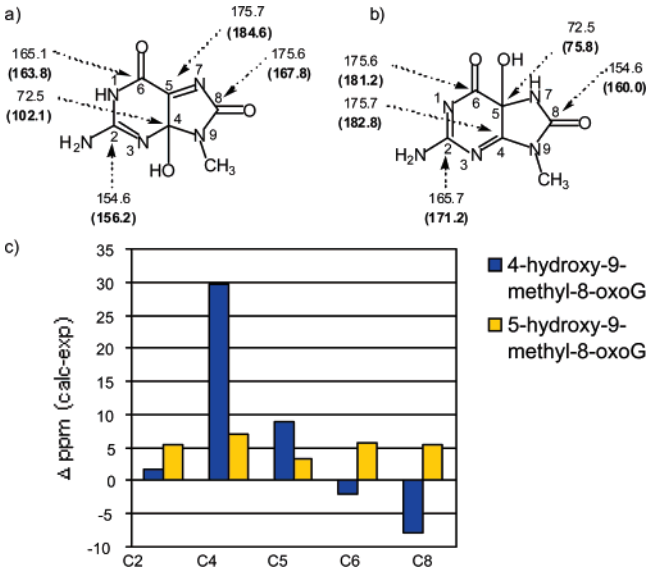


Figure 4. (a) Observed (regular) and computed (bold, parentheses) chemical shifts (ppm) for 4-OH-8-oxoG (**9**). (b) Observed (regular) and computed (bold, parentheses) chemical shifts (ppm) for 5-OH-8-oxoG (**16**). (c) Difference between observed and computed chemical shifts calculated for 4-OH-8-oxoG (blue) and 5-OH-8-oxoG (gold).

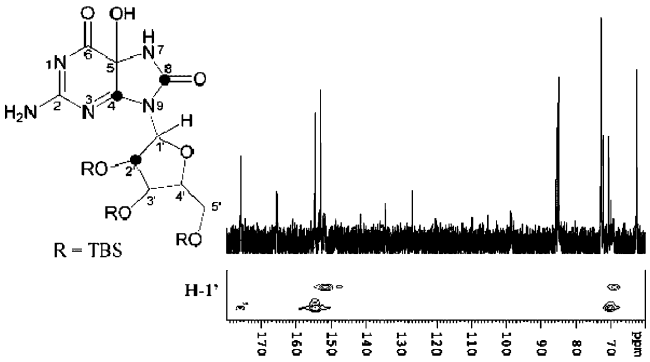


Figure 5. HMBC of 5-hydroxy-8-oxo-7,8-dihydroguanosine (**16**).

deviations for the 5-OH isomer ranged from +7.1 to +3.3 ppm with an average absolute deviation of 5.4 ppm.

The structure characterization of alcohol **16** is further confirmation of the structure of hydroperoxide **15**. In addition, the changes observed when 5-OOH (**15**) is reduced to 5-OH (**16**) are similar to changes between analogous hydroperoxy and hydroxy thymidine derivatives.[44,45] In particular, C5 of **15** shifts upfield by 9.5 ppm occurred upon reduction, similar to ca. 10 ppm shifts of the thymidine derivatives.[44] Also, C6 and C4 of **15** shift downfield by 4.4 and 2.6 ppm, respectively, again similar to analogous carbons in thymidine derivatives.

The 2-D HMBC NMR spectra confirmed that the compound is the regioisomer with the hydroxyl at the 5-position (**16**), derived from the hydroperoxide (**15**) (Figure 5). The double peaks in Figure 5 correspond to the two diastereomeric alcohols.

Rearrangement of 5-OH (16). Upon warming to room temperature, alcohol **16** rearranges to spiroiminodihydantoin **10**. This species was characterized via NMR and MS/MS and matched literature values. 2-D HMBC experiments further confirmed the assignment as spiro **10**. A clear three-bond coupling from the anomeric proton (H-1′) to the quaternary spiro carbon is evident at 80 ppm in Figure 6. Additional couplings are seen at 154 and 73 ppm, corresponding to the urea carbonyl and C-2′, respectively.

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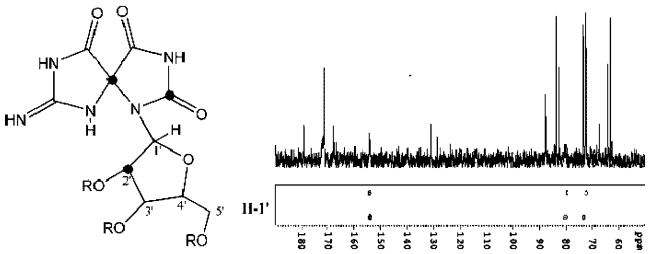


Figure 6. HMBC of spirominodihydantoin (**10**).

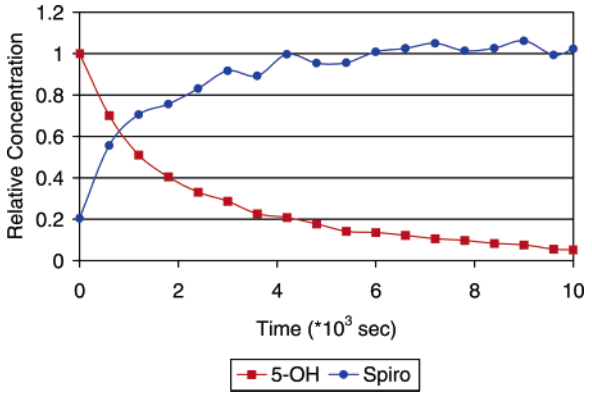


Figure 7. Kinetics of the rearrangement of 5-OH to spiro **10** in acetone at room temperature.

This rearrangement proceeds smoothly within 2 h. A rate constant of 2.24×10^{-4} s^{-1} was calculated for the decay of 5-OH as followed by ^{1}H NMR (Figure 7) at room temperature.

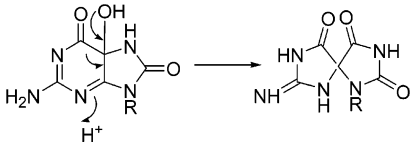
Relative Energies. Relative energies of the derivatives of *N*-methyl-8-oxoGua were calculated at the B3LYP/6-31G(d) level (Tables 1 and 2). Both regioisomers were calculated, along with the proposed dioxetane initial intermediate and rearranged spiro compound.

Discussion

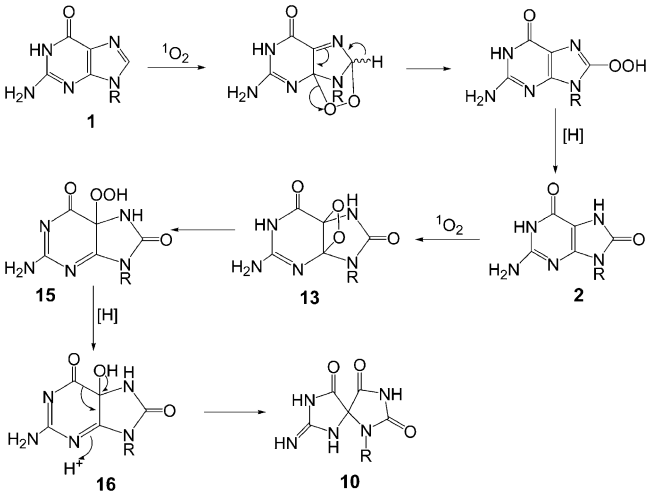
Photooxidation of guanosine derivatives with singlet oxygen results in formation of the dioxetane (**13**), the [2+2] adduct.[15] Warming to room temperature results in the opening of this dioxetane derivative to form a hydroperoxide (5-OOH, **15**). Reduction of the hydroperoxide resulted in the formation of alcohol **16** (5-OH). When we first attempted to assign these intermediates, Cadet et al. had assigned the structure of the compound they had isolated from the photooxygenation of 2′-deoxyguanosine as the 4-hydroxy compound (**9**).[5,26] Because our spectra appeared similar, we assigned the intermediates we had detected (under very different conditions from those of Cadet et al.) as the 4-isomers. While Cadet reported his compound to be stable at room temperature, our compound was not and decomposed to several compounds when warmed to room temperature.[5,15] When Tannenbaum, Burrows, and Adam reported that the correct structure of the Cadet product was the spiro compound (**10**), we reinvestigated our assignments.[22,29] Indeed, careful examination of the shifts we had observed with those reported for the spiro compound showed that they were significantly different.

Chemical shift calculations were our first indication that our previous assignment of the 4-OOH (**14**) and 4-OH (**9**) was erroneous. As evident from Figures 2 and 4, it is apparent that

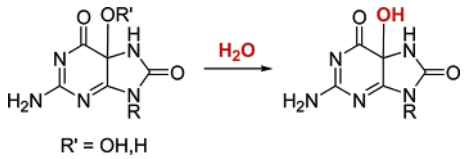
Scheme 3. Rearrangement of 5-OH to Spiro Compound



Scheme 4. Proposed Mechanism for the Formation of Spiro **10** from the $^1O_2$-Mediated Oxidation of Guanosine



Scheme 5. Possible Water Exchange of 5-OOH (**15**) or 5-OH (**16**)



the calculated and experimental chemical shifts are in much better agreement for the 5-substituted moieties.

Indeed, the 2-D HMBC NMR experiments prove that the hydroperoxide (**15**) and alcohol (**16**) formed are the 5-OOH and the 5-OH, respectively. The cross-peaks from the anomeric proton show a clear correlation to two sp^2-hybridized carbons (C8 and C4) in both compounds.

Alcohol **16** has been proposed as an intermediate in the formation of spiroiminodihydantoin (Scheme 3).[22,29] When alcohol **16** was warmed to room temperature, it rearranged smoothly to form spiroiminodihydantoin **10** as the major product. This result confirms the proposed intermediacy of the 5-hydroxy compound as the key intermediate in the formation of the spiroiminodihydantoin.

Calculations of the relative energies of the derivatives of *N*-methyl-8-oxoGua further support this scheme. 5-Hydroperoxy-9-methyl-8-oxoGua (**15**) is approximately 16 kcal/mol more stable than the 4-OOH derivative (**14**). Indeed, rearrangement for the dioxetane to **14** is calculated to be endothermic. Additionally, the 5-OH (**16**) is more stable than the 4-OH derivative (**9**) by approximately 13 kcal/mol. Interestingly, the spiro derivative (**10**) is approximately 18 kcal/mol more stable than alcohol **16**.

Two mechanisms have been postulated for the singlet oxygen-mediated oxidation of guanosine to spiro **10**. Burrows et al. have suggested this mechanism requires 1 equiv of singlet oxygen and subsequent hydration of the oxidized 8-oxoG species to form 5-hydroxy-8-oxo-7,8-dihydroguanosine (5-OH, **16**), the

precursor to spiro **10**.[23] Our proposed mechanism, shown in Scheme 4, requires 2 equiv of singlet oxygen and two reduction steps with 8-oxoG serving as an intermediate.[13,15] As mentioned in previous papers, the 8-hydroperoxide should have reactivity similar to that of a peroxyacid and therefore be relatively easily reduced.[13,17] The reduction of hydroperoxide **15** to alcohol **16** by the addition of dimethyl sulfide may be a model for in vivo reactivity. Unpublished studies in our laboratory indicate that 5-hydroperoxide **15** has mono-oxygen donor capabilities similar to those of substituted 4α-hydroperoxyflavins, which are easily reduced.[46−50]

Burrows et al. used isotope labeling studies to demonstrate that the oxygens of the amide carbonyl of the spiro and guanidinohydantoin compounds are derived from water.[23] Our mechanism does not involve the incorporation of an oxygen atom from water. It would be consistent with Burrow's results, however, if the exchange of water occurs readily with the hydroperoxy- or hydroxy-groups at the 5-position, which would be expected to be quite labile to hydrolysis, as depicted in Scheme 5. Of course, the formation of photooxidation products of 8-oxoG may be affected by the solvent, so these mechanisms may in fact coexist.

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Conclusions

Theoretical calculations of chemical shifts and HMBC experiments provide conclusive evidence that the photooxygenation of 8-oxoG provides 5-hydroperoxy-8-oxo-7,8-dihydroguanosine (5-OOH, **15**) at low temperature. Reduction of this species cleanly provides the corresponding alcohol, 5-hydroxy-8-oxo-7,8-dihydroguanosine (5-OH, **16**), which rearranges smoothly to the spiroiminodihydantoin species (**10**). This is the first direct evidence for this novel rearrangement. Combined with previous work on the formation of 8-oxoG, this study provides a comprehensive picture of the reaction of $^1O_2$ with guanosine.[11−13,15−17]

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Supporting Information Available: Experimental spectra, and geometries and energies for calculated structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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