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Characterization of Spiroiminodihydantoin as a Product of One-Electron Oxidation of 8-Oxo-7,8-dihydroguanosine

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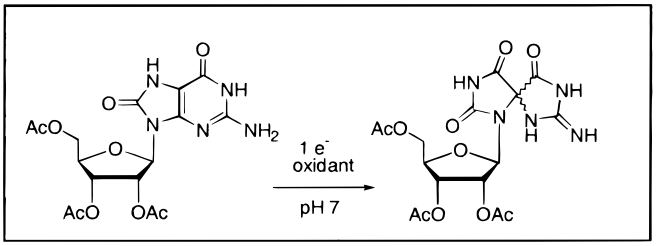
ABSTRACT



Further oxidation of the common DNA lesion 8-oxo-7,8-dihydroguanosine by one-electron oxidants such as $IrCl_6^{2-}$, $Fe(CN)_6^{3-}$, or $SO_4^{-\bullet}$ leads to two major products, depending upon reaction conditions. In nucleosides at pH 7, 22 °C, the principal product is shown herein to be a spiroiminodihydantoin nucleoside, as a diastereomeric mixture, that can be characterized by NMR, ESI-MS/MS, and independent synthesis.

8-Oxo-7,8-dihydroguanosine, hailed as the marker of oxidative damage in the cell,[1,2] is a problematic lesion to detect in DNA owing to its inability to inhibit polymerases and its lack of piperidine sensitivity.[3,4] 8-OxoG analysis is also complicated by its high sensitivity to further oxidation,[5−7] a feature that we have exploited in the development of Ir^{IV} complexes as selective oxidants of 8-oxopurines.[8] While the products of oxidation of 8-oxoG by $^1O_2$ have been extensively investigated,[9−13] the pathway via one-electron oxidants hitherto has not. We report here the major product of oxidation of the *nucleoside* 2′,3′,5′-triacetoxy-8-oxo-7,8-dihydroguanosine, **1**, in neutral aqueous solution mediated by the one-electron oxidants Na_2IrCl_6, $K_3Fe(CN)_6$, and $CoCl_2$/$KHSO_5$.

In previous mass spectral studies of *oligodeoxynucleotides*, two products of Na_2IrCl_6-mediated oxidation of 8-oxoG were observed at masses M − 10 and M + 16, with the M − 10 product predominating (Figure 1).[14] This product was proposed to be the guanidinohydantoin heterocycle **3**[15] by analogy with the well-characterized uric acid pathway that leads to allantoin as the major oxidation product.[16] The corresponding 5-hydroxy-8-oxopurines are proposed as intermediates. However, subsequent studies in our laboratory revealed that 5-OH-8-oxoG (**2**) could not be the stable M + 16 product observed by ESI-MS since prolonged heating did not convert it to the M − 10 product.

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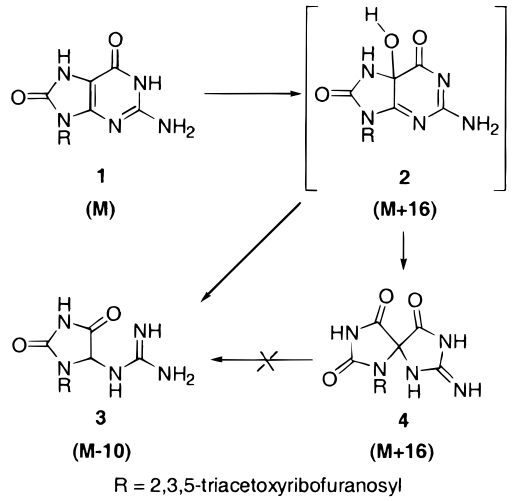


Figure 1. Oxidation of 8-oxoG (**1**) to **3** and **4**.

An alternative hypothesis was provided by Johnson[17] based on the studies of Poje and co-workers who found formation of spirodihydantoin, an isomer of 5-hydroxyurate, to be an alternative product not on the pathway to allantoin.[18] A related spirodihydantoin analogue was confirmed by Johnson et al. to be the oxidation product of arylamine adducts to C8 of guanine.[19] By analogy, it seemed plausible that the spiroiminodihydantoin structure **4** could account for the M + 16 product of 8-oxoG oxidation. To confirm this, we carried out studies with the triacetoxy derivative of the nucleoside whose higher hydrophobicity led to clean separations by reverse-phase HPLC.

Oxidation of a 7.5 mM solution of **1** in aqueous phosphate buffer (75 mM, pH 7) at 22 °C by 7.5 mM Na_2IrCl_6 led to the formation of only one stable new product observable by HPLC (Figure 2). Depending upon the separation conditions used, the new HPLC peak eluting at 12.5 min was sometimes observable as two closely spaced peaks of nearly equal

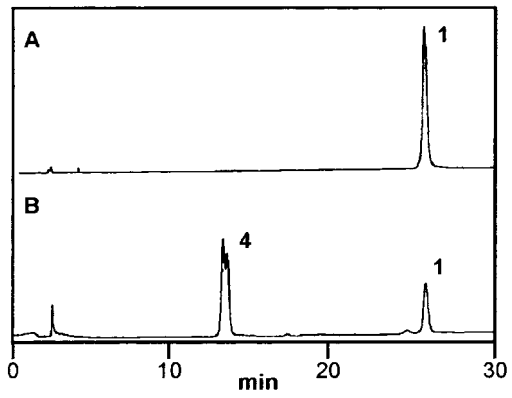


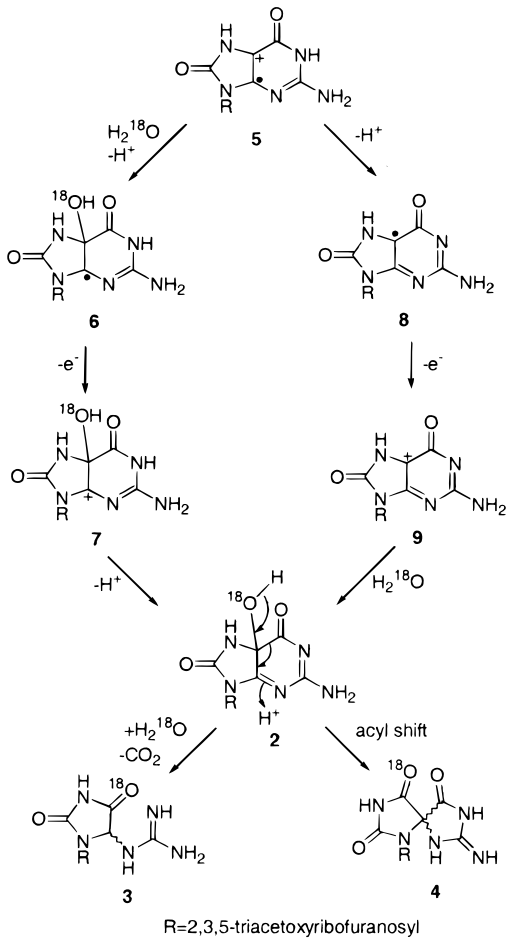
Figure 2. HPLC traces of (**A**) 8-oxoG starting material **1** (R = 2,3,5-triacetoxyribofuranosyl) and (**B**) the product mixture after oxidation with Na_2IrCl_6 at pH 7, 22 °C.[20]

intensity (as in Figure 2B), consistent with the product being a ~1:1 mixture of diastereomers due to the generation of a new stereogenic center during oxidation. ESI-LC-MS analysis[20] of the product indicated that its mass is 16 amu higher than that of 8-oxoG (**1**). Essentially identical results were obtained with other oxidants such as $K_3Fe(CN)_6$ (7.5 mM) or $KHSO_5$ (7.5 mM) in the presence of catalytic $CoCl_2$ (0.13 mM) which is thought to form $SO_4^{-\bullet}$.[21,22]

The product could be purified by silica gel chromatography to remove all starting material, although it remained a diastereomeric mixture. The high-resolution FAB mass spectrum was consistent with a molecular formula of $C_{16}H_{19}N_5O_{10}$ as expected for spiroiminodihydantoin **4**.[20] The NMR spectra were complicated by the doubling of nearly all the peaks due to the mixture of diastereomers; however, 2D-COSY NMR analysis allowed assignment of all of the CH protons of **4**.[20] Importantly, the ^{13}C NMR spectrum in CD_3OD indicated a new resonance at 85.1 ppm that we assign as the spiro carbon of **4** because of its similar position to quaternary carbon resonances in other spirodihydantoins (80.0 ppm[18] and 82.2 ppm,[19] for example, both in d_6-DMSO).

A mechanism for the formation of the spirocycle **4** from **1** is proposed in Scheme 1. 8-Oxoguanosine (**1**) has a redox potential of about 0.6 V vs NHE[23] compared to $IrCl_6^{2-}$ (~0.9 V)and should be readily oxidized by Ir^{IV} to the radical cation

Scheme 1



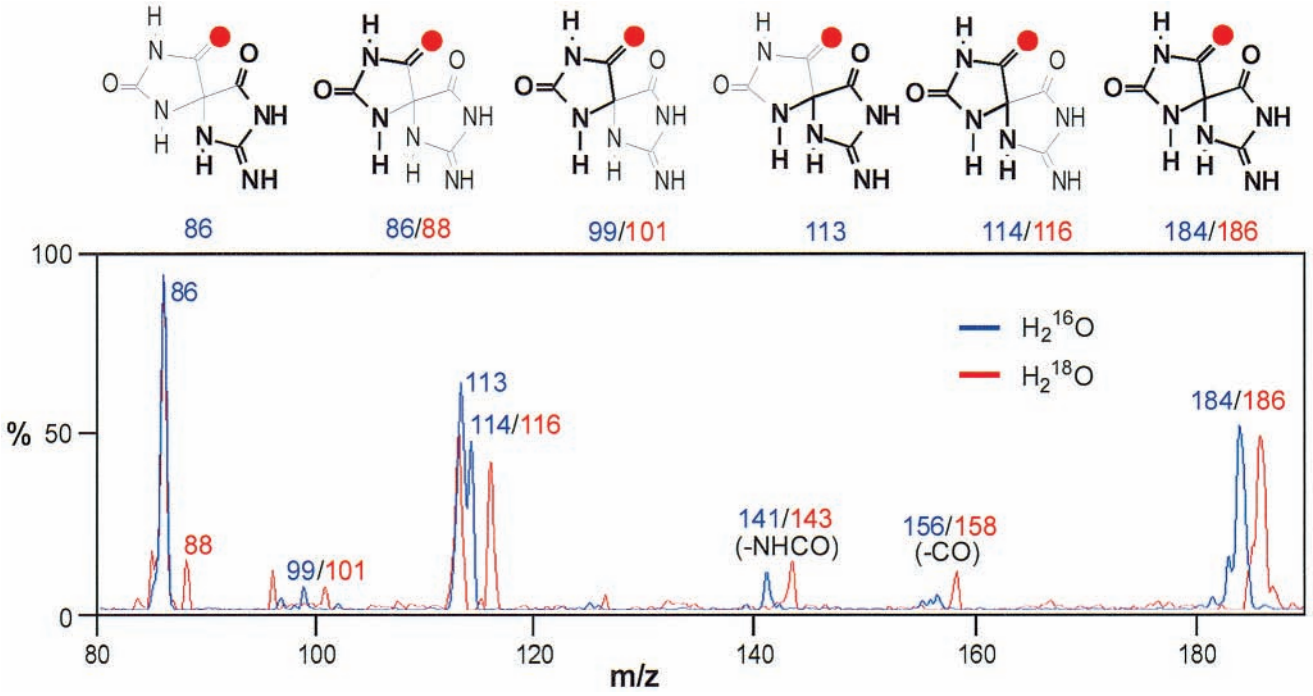


Figure 3. ESI-MS/MS analysis of fragment ions of **4** following in-source ionization to break the N-glycosidic bond. Nucleoside **4** was formed from Ir^{IV} oxidation of **1** in either $H_2{}^{16}O$ (blue) or $H_2{}^{18}O$ (red). Structural assignments are suggested above as indicated in the bolded portions of the heterocycles. The red oxygen is proposed to be the one labeled in $H_2{}^{18}O$ according to Scheme 1.

5. Althought the exact sequence of events that follows cannot be determined (i.e. pathways shown on left vs right), we propose that 55 M H_2O traps the cation at C5 before a second, facile one-electron oxidation by another Ir^{IV} that ultimately gives the intermediate 5-hydroxy-8-oxoG, **2**.[24] This species is apparently not stable, but is the branchpoint for formation of two stable products, **3** and **4**. Under the conditions used here for nucleoside studies, **2** apparently undergoes deprotonation and concomitant acyl migration to yield the spiroiminodihydantoin **4**.[25] The mechanism in Scheme 1 predicts that the spirocycle should be labeled with ^{18}O at the C4 carbonyl if the oxidation is carried out in $H_2{}^{18}O$, and this hypothesis could be tested using tandem MS techniques.

Figure 3 shows the fragmentation pattern that results from oxidation of **1** by Na_2IrCl_6 in $H_2{}^{16}O$ (blue) vs $H_2{}^{18}O$ (red). Use of $H_2{}^{18}O$ (>95% puritiy) led to 98% incorporation of ^{18}O into the parent molecular ion $(M + H)^+$ of **4**. The oxidized mixture of **4** plus starting material **1** was separated by HPLC and then subjected to in-source ESI fragmentation in order to break the N-glycosidic bond.[20] The protonated base with a mass of 184 (or 186 in the $H_2{}^{18}O$ experiment), corresponding to 16 Da higher than 8-oxoguanine, was selected in MS-1, ionized for further fragmentation, and analyzed in MS-2. Essentially all of the fragmentation products can be explained by the partial structures shown in bold at the top of Figure 3. Only those fragments that should contain the C4 carbonyl group were found to shift by 2 amu in the $H_2{}^{18}O$ experiment.[26] For example, there are two fragments with M^+ or $(M + H)^+ = 86$; one contains the ^{18}O carbonyl and shifts to mass 88 while the other does not. There are also multiple structures for mass 141 and 156 (M − CHNO and M − CO, respectively). The most telling evidence of the spirocyclic structure is the pattern obtained at masses 113 and 114. These ions correspond to the two five-membered rings, one with an imino group rather than an oxo group. The ^{18}O label is incorporated specifically on the oxo-containing heterocycle and therefore shifts from 114 to 116 Da.

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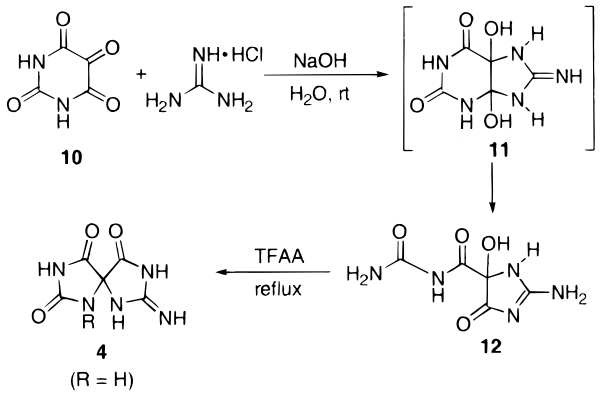
(24) Although **6** or **8** might be expected to be O_2 sensitive, a reaction carried out under Ar was identical to that in air.
(25) At pH 4 where deprotonation is disfavored, guanidinohydantoin **3** is also observed.
(26) Incubation of **4** with $H_2{}^{18}O$ confirmed that the label is not exchangeable with solvent.

To further support the assignment of the spiroiminodihydantoin structure, the heterocycle was independently synthesized by the route shown in Scheme 2. The procedure

Scheme 2. Independent Synthesis of **4** (R = H)



followed closely that reported by Poje[27] for the synthesis of the parent spirodihydantoin, with the modification that guanidinium hydrochloride in the presence of NaOH was used in place of urea in the first condensation with alloxan (**10**). The presumed intermediate **11** underwent spontaneous ring opening to generate the stable product **12** in low yield. Nevertheless **12** was crystallized and an X-ray crystallographic analysis[20] confirmed that the six-membered ring was the one that had opened. Subsequent treatment of **12** with trifluoroacetic anhydride generated a mixture of two products with $(M + H)^+$ ions at 184 and 269 Da, respectively. Analysis of the 184 ion using the same MS/MS analysis described above was identical to that shown in Figure 3, thereby confirming the structure of **4** as the spiroiminodihydantoin.[20]

The spiro product **4** appears to represent an alkaline-labile lesion. Treatment of the nucleoside at pH 12, 22 °C, showed nearly complete hydrolysis in 1 min; however, the rate of deglycosylation in oligomers is substantially slower. Complete studies of the rate and conditions of base lability are underway.

Mounting evidence suggests that all purines substituted at C8 with an electron-donating group are readily subject to further oxidation leading to products akin to the uric acid oxidation pathway.[28−30] For 8-oxoG, spiroiminodihydantoin is the predominant product in the nucleoside at pH 7, although oxidation in the context of a DNA oligomer leads, especially in duplex DNA, to guanindinohydantoin via ring opening and decarboxylation. This dichotomy highlights the point that subtle differences between the environment of 8-oxoG in a monomer vs a polymer can lead to entirely different chemistries. Given the sensitivity of 8-oxoG to further oxidation,[6] and the ability of 8-oxoG to act as a hole sink over long sections of DNA,[31] the processing of such lesions as guanidinohydantoin and spiroiminodihydantoin by polymerases and repair enzymes will be of immediate interest.

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Supporting Information Available: Experimental procedures, NMR, UV and mass spectra for **4** and crystallographic data for **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) One exception is 8-methoxyguanosine whose photochemical oxidation leads to an imidazolone product from a pathway that is two electrons more oxidized than **3** or **4**.[30] There appears to be an additional bifurcation in Scheme 1 involving O_2 under photochemical conditions.

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