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

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SUPPORTING INFORMATION

Experimental Section

Materials. Guanosine hydrate, 4-dimethylaminopyridine and riboflavin were purchased from Acros, Br_2 from Fisher Scientific, benzyl alcohol and Pd (10% on activated carbon) from Aldrich, Na from Mallinckrodt and $H_2{}^{18}O$ (>93.5% purity) from Icon. Mn-SOD and catalase were from Sigma. The nucleoside 2',3',5'-tri-*O*-acetyl-8-oxo-7,8-dihydroguanosine and 2',3',5'-tri-*O*-acetylguanosine were synthesized by a published method.[1,2]

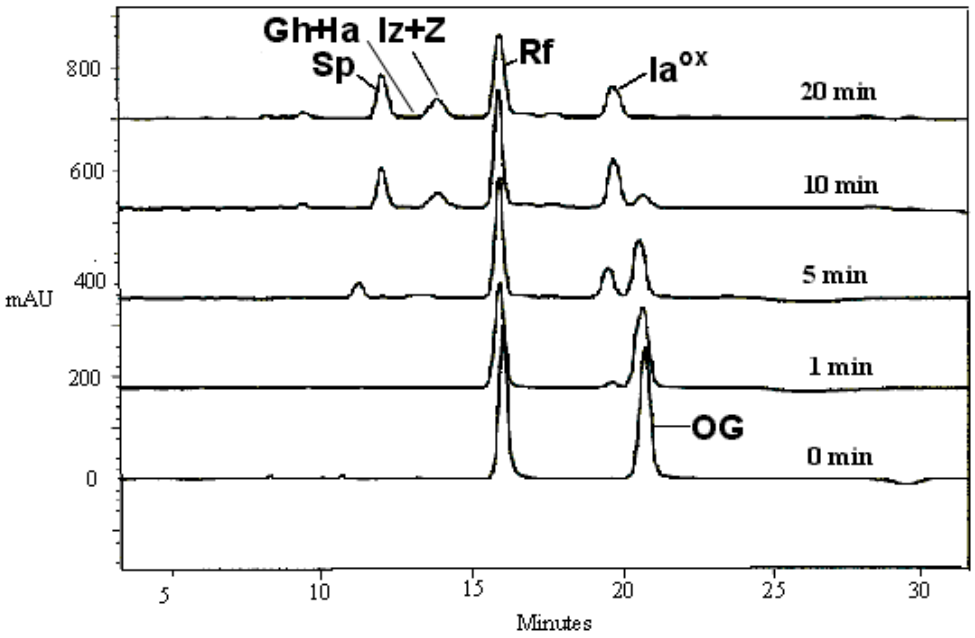
Instrumentation. Photooxidation reactions were carried out in a Rayonet UV irradiator (120 volts, 50/60 Hz, Southern New England, MA) equipped with 8 UVA (360 nm)-emitting lamps. ESI mass spectra were recorded on a Micromass Quatro II spectrometer. HPLC analyses were carried out using a Waters Alliance 2690 Sample Module attached to a Waters 2487 Dual Absorbance Detector and an Alltech (Deerfield, Illinois) Alltima C-18 Nuc analytical reverse phase column (5 μm, 250 mm x 4.6 mm). All solvents used for HPLC were HPLC grade and filtered and sonicated before use. All aqueous solutions utilized purified water (Nanopure, Sybron/Barnsted).

Synthesis of 2',3',5'-tri-O-acetyl-8-methoxyguanosine.[2-4] After 8-bromoguanosine (3 g, 8.3 mmol) was suspended in 24 mL methanol, a freshly prepared 3.5 N $NaOCH_3/CH_3OH$ solution (20.5 mL) was added dropwise. The reaction mixture was refluxed for 26 hours under nitrogen. The mixture was cooled in an ice bath while 2N HCl was added to neutralize the reaction solution. The solvent was evaporated to dryness under reduced pressure and after the residue was washed with water, the obtained solid (1.1 g, containing small amount of starting material 8-bromoguanosine and 8-methoxyguanosine) was dried under vacuum. To the solid (0.32 g, 1 mmol) and 4-dimethylaminopyridine (9 mg, 0.074 mmol) in a mixture of acetonitrile (12 mL) and triethylamine (0.56 mL, 4.0 mmol) was added acetic anhydride (0.34 mL, 3.6 mmol) at room temperature. After stirring the mixture for 30 min, methanol (0.5 mL) was added to the mixture and stirring was continued for another 5 min. The mixture was evaporated to dryness under reduced pressure and the resulting residue was purified by silica gel chromatography with $CHCl_3$:MeOH/10:1.5 (v:v) as mobile phase to obtain **8-OMeG**. Yield 0.21 g, 48 %. ^{1}H NMR (300 Hz, DMSO-d_6, δ): 6.44 (b, 2H, NH_2), 5.88 (dd, 1H, J=5.1 Hz, H-2'), 5.81 (d, 1H, J=5.1 Hz, H-1'), 5.48 (dd, 1H, J=5.1 Hz, H-3'), 4.13-4.38 (m, 3H, H-4', H-5', H-5''), 4.00 (s, 3H, OMe), 2.02 (s, 3H, Me), 2.06 (s, 3H, Me),

2.10 (s, 3H, Me). ESI-MS: m/z 440 ($M+H^+$).

General procedure for OG or G photooxidation by riboflavin. 300 μL of 75 mM potassium phosphate buffer solution (pH 6, 7 or pH 8.6) containing **OG** (0.2 mM), **8-OMeG** (0.2 mM) or **G** (0.2 mM) and riboflavin (16 μM) was irradiated for 10 min for the **OG** reaction, 20 min for **8-OMeG** and 20 min for **G** reaction at 22˚C. The mixture was analyzed by ESI-LC-MS immediately after the reaction. The HPLC method was a linear gradient of 10 % solvent B to 20 % solvent B in 20 min at 1 mL/min. where solvent A was 0.1 % aqueous CF_3CO_2H (TFA) and solvent B was 0.08 % TFA in CH_3CN. The chromatograms were recorded with monitoring at 220 nm. For the reaction with Mn-SOD and catalase, Mn-SOD (125 units/mL) and catalase (375 units/mL) were added into the reaction solution before irradiation.

Figure A. HPLC trace of OG photooxidation mediated by riboflavin at pH 7 as a function of time. HPLC analysis was carried out on an Alltech Alltima C-18 Nuc analytical reversed-phase column (5 μm, 250 mm x 4.6 mm) with a gradient of 10% B to 20% B in 20 min and flow rate=1 mL/min. Solvent A was 0.1% TFA/water and solvent B was 0.08% TFA/acetonitrile. Spectra were recorded at 230 nm.



$H_2{}^{18}O$ labeling experiments. After 100 μL of potassium phosphate buffer (pH 7) of **OG** (0.2 mM) or **G** (0.2 mM) and riboflavin (16 μM) was lyophilized to dryness in the dark, $H_2{}^{18}O$ (100 μL) was added. The mixture was irradiated for 10 min for **OG** reaction and 20 min for **G** reaction and analyzed by ESI-LC-MS.

Quantification of product yields by ESI-MS. The yield of the products and the recovered starting material, including **Sp**, **Gh**/**Ia**, **Ia^{ox}**, **G** and **OG**, was calculated from its signal intensity of ESI-MS spectra (positive ion mode) after the intensity was corrected for variations in ion current as a function of molecular structure. For example, if the original signal intensity of **Gh**/**Ia** was 58721 counts, this number was corrected to 24467 by a factor of 0.43. The reason for the correction is that different compounds at the same concentration would show different mass spectrometric signal intensity. Most of the oxidation

products of nucleosides are very polar and are easily protonated by 0.1% TFA and showed strong signals on positive ion ESI-MS. For each of the **OG** or **G** oxidation products of riboflavin, the specific signal intensity correction factor was determined as follows: (*i*) a mixture of **Gh**/**Ia**, **Sp**, **OG** and **G** with the same concentration (7.5 mM) was analyzed by LC-MS and the relative signal intensity was calculated by normalizing each peak to that of **Sp**. The experiments were repeated three times and the results are shown in Table 1 (Entry 1-4). (*ii*) **Ia^{ox}** was generated by further oxidizing **Gh**/**Ia** (7.5 mM in 75 mM potassium phosphate buffer at pH 7) with Na_2IrCl_6 (37.5 mM) at 22˚C for 1h. After the reaction, **Sp** and **G** were added as internal standards and the mixture was analyzed by LC-MS. The remaining **Gh**/**Ia** and the product **Ia^{ox}** were calculated and the correction factor of **Ia^{ox}** was calculated by using either **Sp** or **G** as an internal standard. The average correction factor of **Ia^{ox}** *vs.***Sp** was shown in Table1 (Entry 5). (*iii*) Since the half life of **Iz** was only 147 minutes (pH 7, 37˚C), a purified **Iz** standard was not obtainable. LC-MS showed that Gh/Ia eluted at 13.10/13.67 min and Iz and Z co-eluted at 13.82 min. Though the peak associated with **Iz**/**Z** overlapped with the tail of the peak of **Ia** to some extent, since the extinction coefficient ϵ of Iz (ϵ_{254nm} = 5000 $L \cdot M^{-1}cm^{-1}$)[5] was about 1.7 fold of the ϵ of Gh/Ia ($\epsilon_{230nm} \approx 3000$ $L \cdot M^{-1}cm^{-1}$)[6] and **Z** should have a much smaller ϵ than Iz due to the lack of efficient conjugation system, the peak at 13.82 min could be considered mainly contributed by **Iz**. Thus, in the reaction of **OG** or **G** photooxidation by riboflavin, we were able to use the HPLC peak area of **Sp** (retention time 12.27 min, $\epsilon_{230nm} \approx 4900$ $L \cdot M^{-1}cm^{-1}$) and that of **Iz** (retention time 13.82 min, $\epsilon_{230nm} \approx 5000$ $L \cdot M^{-1}cm^{-1}$) to calculate the concentration ratio of Sp/Iz. By combining this data with the ESI-MS signal intensity ratio of **Sp**/**Iz**, we were able to obtain the mass spectrometric correction factor for **Iz** *vs.* **Sp** as 0.93 *vs.* 1 (Table 1, Entry 6). **Z** was not corrected due to the lack of proper method. But by comparing all the correction factors, it was found that all these heterocycles behave similarly in the positive mode ESI-MS with the factor ranging from 0.43-1. The assumption that **Z** gave the same signal intensity as Sp should not dramatically change the total product distribution was reasonable, also because only a small amount (less than 25 %) of **Iz** was hydrolyzed to **Z** before the immediate LC-MS analysis.

Table 1. ESI-MS signal intensity correction factor for OG or G photooxidation products.

Entry	Compounds	Correction factor (normalized to Sp)
1	**Sp**	1 ± 0
2	**Gh**/**Ia**	0.43 ± 0.063
3	**OG**	0.86 ± 0.13
4	**G**	0.61 ± 0.12
5	**Ia^{ox}**	0.89 ± 0.30
6	**Iz**	0.93
7	**Z**	not corrected

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