Peroxynitrite Reacts with 8-Nitropurines to Yield 8-Oxopurines

Joseph M. Lee,[†] Jacquin C. Niles,[†] John S. Wishnok,[†] and Steven R. Tannenbaum*,[†,‡]

Division of Bioengineering and Environmental Health and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

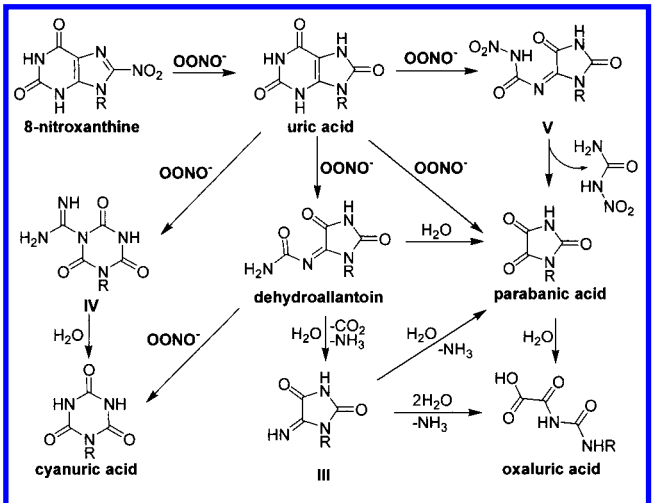
Received May 15, 2001

Peroxynitrite reacts with 2′-deoxyguanosine to yield several major products, including 8-oxo-2′-deoxyguanosine (8-oxodG) and 8-nitroguanine (8-nitroGua). While the terminal products formed during the reaction of 8-oxodG with peroxynitrite have been previously characterized, those formed from 8-nitroGua have not. To identify these products, 9-ethyl-8-nitroxanthine was used as a model for 8-nitroGua, since the former could be easily synthesized in high yield, and facilitated reversed-phase HPLC separation of the resulting products. Using this model substrate, the products formed during the peroxynitrite reaction were identified as the ethyl derivatives of oxaluric acid, 5-iminoimidazolidin-2,4-dione, **III**, [*N*-nitro-*N*′-[2,4-dioxo-imidazolidine-5-ylidene]-urea, **V**, dehydroallantoin, parabanic acid, cyanuric acid, and uric acid. Upon the basis of the previous studies with 8-oxodG, these products were recognized as those expected to arise from peroxynitrite-mediated uric acid oxidation. Furthermore, the presence of uric acid in the reaction mixture led us to propose a model in which the 8-nitropurine is first converted to the 8-oxopurine which is further oxidized by peroxynitrite to give the observed final products. We have also provided evidence suggesting that the peroxynitrite anion, acting as a nucleophile, might be responsible for the initial conversion of the 8-nitropurine to the 8-oxopurine and that a hydroxyl radical or oxidative process is less likely to explain this conversion.

Introduction

During chronic inflammation and infection, activated immune cells release increased amounts of reactive oxygen and nitrogen species, which can lead to tissue damage (*1*−*3*). Peroxynitrite, a one- and two-electron oxidizing and nitrating agent, is one such toxic agent that can directly modify a wide range of biological targets, including lipids (*4*), proteins (*5*−*9*), and DNA bases/sugars (*10*, *11*). Peroxynitrite is formed from the diffusion-limited reaction between nitric oxide and superoxide (*12*, *13*), and at the nucleoside and DNA levels, it reacts almost exclusively with Gua and dG, respectively (*14*, *15*). Several oxidation and nitration products, illustrated in Figure 1, are formed including 8-nitroguanine (8-nitroGua) (*11*), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (*16*), 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-guanine (nox-dG) (*17*), and 2,2-diamino-4-[(2′-deoxy-β-D-*erythro*-pentofuranosyl)amino-5-(2H)-oxazolone, dX (*18*−*20*).

It was subsequently shown that 8-nitroGua (*14*) and 8-oxodG (*14*, *16*) were not only major peroxynitrite products but also several orders of magnitude more reactive than dG toward peroxynitrite. The reaction of peroxynitrite with 8-oxodG has been studied, and the final products have been characterized as the β-D-*erythro*-pentofuranosyl deriviatives of oxaluric acid, parabanic acid, and cyanuric acid (*21*, *22*). Three key intermediates, **I**, **II**, and **IV**, shown in Figure 1, were also identified. **I** and **II** were shown to undergo hydrolysis to yield parabanic and oxaluric acid, via **III**, while **IV** underwent hydrolysis to cyanuric acid. The results at the nucleoside level were reproduced in 8-oxoG-containing double stranded oligonucleotides treated with peroxynitrite, with the exception that no parabanic acid was detectable (*23*).



Summary of Peroxynitrite Products of 8-Nitropurines (R = ethyl)

8-NitroGua is formed in a dose-dependent fashion in vitro when Gua-containing oligonucleotides have been treated with peroxynitrite (*24*). In studies by Tretyakova

* To whom correspondence should be addressed. Phone (617) 253-3729. Fax: (617) 252-1787. E-mail: srt@mit.edu.
[†] Division of Bioengineering and Environmental Health.
[‡] Department of Chemistry.

10.1021/tx010093d CCC: $22.00
Published on Web 12/22/2001

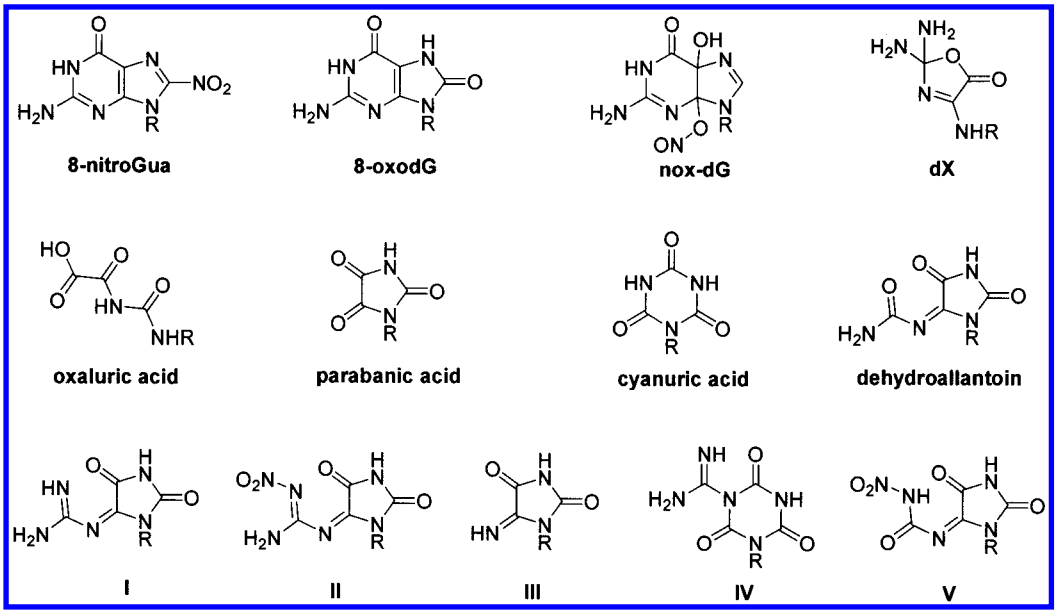


Figure 1. Structures of 8-oxodG, 8-nitroGua, nox-dG, dX, and the former two products peroxynitrite products (R = ethyl except for 8-oxodG, dX, and nox-dG where R′ = 3′,5′-di-*O*-Ac-beta-D-erythropentafuranosyl).

et al., 8-NitroGua formation reached a maximum at a peroxynitrite concentration of 400 μM and subsequently declined with increasing peroxynitrite concentration (*25*). When peroxynitrite reacts with DNA, the 8-nitrodG formed rapidly depurinates [$t_{1/2}$ = 1−4 h at neutral pH and room temperature (*25*)] to yield the measurable 8-nitroGua. However, since complete depurination of 8-nitrodG was ensured prior to measurement of 8-nitroGua, the decline in 8-nitroGua formation could not be attributed to incomplete depurination. Instead, this result suggested that both at the nucleobase and DNA level, 8-nitroGua can undergo further reaction with peroxynitrite, leading us to hypothesize that similar to the 8-oxodG/peroxynitrite reaction, peroxynitrite can induce formation of 8-nitroGua oxidation and nitration products.

In this paper, we have studied the reactions of 8-nitroGua, 8-nitroGuo and 9-ethyl-8-nitroxanthine with peroxynitrite in an effort to elucidate the final products and the mechanism by which this chemistry occurs. We found 9-ethyl-8-nitroxanthine to be a useful model compound in these studies since it is readily synthesized in high yield, recapitulates 8-nitroGua chemistry almost identically, and along with its oxidation products, is amenable to reversed-phase chromatography. In addition, we have provided in this paper a reproducible method for synthesizing 8-nitroGua, and have characterized this compound directly by several spectroscopic methods, instead of by the indirect methods that have been previously employed (*11*, *24*, *26*−*28*).

Experimental Procedures

General. Guanosine, 4-amino-5-imidazole-carboxyamide (AICA), benzoyl isothiocyanate, copper (II) acetate, bromoethane, sodium nitrite, and ^{15}N nitric acid (70 wt %, 98 atom % ^{15}N) were obtained from Aldrich (Milwaukee, WI). Uric acid, 9-methyluric acid, 8-aminoguanosine, 8-bromoguanosine, and 2′-deoxyguanosine were obtained from Sigma (St. Louis, MO). Acetic anhydride, pyridine, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and hydrazine, were purchased from either Mallinckrodt (Paris, KY) or Aldrich. NMR solvents, DMSO-d_6 and D_2O, were obtained from Cambridge Isotope Laboratories (Andover, MA). Peroxynitrite was prepared as described by Pryor et al. (*29*), and the concentration was determined by measuring its UV absorbance (ϵ_{302nm} = 1670 M^{-1} cm^{-1}) (*30*). Partisil ODS-40 (C18) packing material and C18 Sep-packs were acquired from Whatman (Springfield Mill, U.K.) and Varian (Harbor City, CA), respectively. All solvents were HPLC-grade.

Instrumentation. UV−vis spectra were obtained using an HP8452 Diode Array Spectrophotometer (Hewlett-Packard). ^{1}H and ^{13}C NMR (proton decoupled) spectra were obtained using a Unity 300 spectrometer at 300 and 75 MHz, respectively. HPLC analyses were performed using an HP 1100 pump equipped with an 1090 Diode Array Detector (Hewlett-Packard, Palo Alto, CA). All HPLC and LC-MS runs were carried out using either a 250 × 4.6 mm, 5 μm LC18-DB column (Supelco, Bellefonte, PA) or a 30 cm × 1 mm, 5 μm LC18-DB column (Supelco). Electrospray ionization mass spectrometry (ESI-MS), tandem mass spectrometry (ESI-MS/MS), and ion trap mass spectrometry experiments were performed using an HP 5989B (Hewlett-Packard), a TSQ 7000 (Finnigan, San Jose, CA), and an 1100 LC/MSD Ion Trap (Agilent, Palo Alto, CA), respectively. FT-MS spectra were obtained using ESI in negative ions mode on an APEX II FT-MS (Bruker, Billerica, MA). Spectra were obtained in either positive or negative ions mode using a spraying solution consisting of a 50:50 water-methanol solution spiked with 0.02% glacial acetic acid. Hydroxyl radical was generated in situ by irradiating N_2O-flushed aqueous samples with γ-rays emitted at 2.36 Gy/min by a Gammacell-220 machine equipped with a ^{60}Co source.

For the 250 × 4.6 mm, 5 μm LC18-DB column, two HPLC methods were employed. Method A: isocratic elution at 1.0 mL/min, using a mixture consisting of 90% 100 mM triethylammonium acetate (TEAA), pH 7 and 10% methanol. Method B: 100% 20 mM ammonium acetate, pH 7 for 10 min, followed by an acetonitrile gradient from 0 to 25% over the next 15 min with a flow rate of 1.0 mL/min. Similarly, for the 30 cm × 1 mm, 5 μm LC18-DB column, two HPLC methods, C and D were used. In Methods C and D, respectively, the column was isocratically eluted with water and 20 mM ammonium acetate, at a flow rate of 0.06 mL/min.

Synthesis of 8-Nitrogua. 8-nitroGua was synthesized by stirring 8-bromoguanosine (1 g, 2.7 mmol) with five equivalents of sodium nitrite (0.95 g, 14 mmol) under reflux in DMF at 60 °C overnight to produce both 8-nitroGua and 8-nitroxanthine. The DMF reaction mixture was next concentrated in vacuo. The dried orange solid was dissolved in 100 mM TEAA (pH 7) and loaded onto a 2 cm × 8.5 cm MegaBond Elut C18 sep pack

(Varian) that was preequilibrated with 100 mM TEAA. The desired products were eluted with 100 mM TEAA buffer, while the unreacted starting material was later eluted with methanol. The fractions containing the products were concentrated and further purified on a 3 cm × 18 cm ODS-40 C18 column that was preequilibrated and eluted under gravity with 100 mM TEAA. The second eluting yellow band was collected, concentrated, and rechromatographed on a 2 cm × 8.5 cm C18 Sep Pak with ddH_2O as the eluent to remove excess salts in and obtain pure 8-nitroGua. Yield: 0.027 g, (0.14 mmol, 5.2%). ^1H NMR (DMSO-d_6, δ) 10.25 ppm (s, 1H, NH), 5.89 ppm (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6, δ) 159.42, 158.76, 157.60, 151.77, and 121.27 ppm; HRMS calculated for $C_5H_3N_6O_3$ $[M-H]^-$ 195.0267, observed 195.0269; UV−vis (H_2O) λ_{max} = 400 nm; HPLC Method A, single peak eluting at 4 min.

Synthesis of 8-nitroGuo. Using a similar strategy as above, 8-nitroGuo was prepared by stirring 8-bromoguanosine (*31*) (1 g, 2.8 mmol) with 1.5 equiv potassium nitrite (0.35 g, 4.2 mmol) in DMF at room temperature for 2 days. 8-NitroGua and 8-nitroxanthine were also present as side products. Using the same purification steps outlined above, 8-nitroGuo was purified and desalted. Yield: 0.045 g, (0.14 mmol, 3.3%). ESI-MS 327, $[M - H]^-$; UV−vis (H_2O): λ_{max} = 394 nm; HPLC Method A, single peak eluting at 12.3 min.

Synthesis of 9-Ethylguanine. 9-Ethylguanine was synthesized according to a previously published report (*32*). ESI-MS 180, $[M + H]^+$; UV−vis (H_2O): λ_{max} = 272, 252 nm; HPLC Method B, single peak eluting at 19 min.

Synthesis of 9-Ethyl-8-nitroxanthine. 9-Ethyl-8-nitroxanthine was prepared by refluxing 9-ethylguanine (0.501 g, 2.8 mmol) in glacial acetic acid (10 mL) and nitric acid solutions (1 mL, 70%) at 132 °C for 50 min, followed by careful rotary evaporation of the greenish solution to apparent dryness on an ice bath. The product was taken up in ddH_2O and desalted on an ODS-40 (C18) 3 cm × 18 cm column that was preequilibrated, washed, and eluted with water. Yield: 0.6 g, (2.8 mmol, 100%). ESI-MS: 224, $[M - H]^-$; UV−vis (H_2O): λ_{max} = 410 nm; HPLC Method B, single peak eluting at 23.3 min. Likewise, 9-ethyl-8-$^{15}NO_2$-xanthine was synthesized using 1 mL of a 40 wt % nitric acid (98% atom ^{15}N) solution. Yield: 0.6 g, (2.8 mol, 100%). ESI-MS: 225, $[M - H]^-$; UV−vis (H_2O): λ_{max} = 412 nm; HPLC Method B, single peak eluting at 23.3 min.

Reaction of 8-Nitro and 8-Oxopurines with Peroxynitrite. The reaction of peroxynitrite with several modified DNA bases was analyzed by measuring either the loss of starting material or by the detection of new products using HPLC and LC-MS. These reactions were carried out in a 1 mL eppendorf tube containing 0.5−5 mM peroxynitrite and 100 μM 8-nitroGua, 8-nitroGuo, or 9-ethyl-8-nitroxanthine ($^{14}NO_2$ and $^{15}NO_2$ labeled), in 120 mM potassium phosphate, 25 mM sodium bicarbonate, pH 7.2 buffer.

Reaction of 9-Ethyl-8-nitroxanthine with Hydroxyl Radical. Samples containing 9-ethyl-8-nitroxanthine (100 μM) in 100 mM phosphate buffer (pH 7.4), were prepared in 1.5 mL eppendorf tubes and sealed with rubber septae. The tubes were flushed with a 9:1 nitrous oxide (N_2O):oxygen gas (O_2) mixture for five minutes, prior to irradiation with up to 1000 Gy. HPLC and off-line ESI-MS were used to analyze the reaction mixture.

Reaction of 9-Ethyl-8-Nitroxanthine with Hydroxylamine. The 9-ethyl-8-nitroxanthine standard (100 μM) was incubated with hydroxylamine (15 μM to 1.5 M) in 0.1 N KOH (pH 13) for up to 4 h, to determine whether nucleophilic displacement of the nitro group occurred. The reaction was monitored by UV−vis spectroscopy at 412 nm (λ_{max} for 9-ethyl-8-nitroxanthine).

Results and Discussion

Characterization of 8-NitroGua and 8-NitroGuo. The synthesis of 8-nitroGua via the reaction of 8-diazoguanine with sodium nitrite has previously been reported (*33*−*35*). However, we were unable to reproduce these results and devised another synthetic route involving the nitrite-mediated Br^- displacement from 8-bromoguanosine to give 8-nitroGuo and 8-nitroGua by depurination of the former. UV−vis spectroscopy, FT-MS, ^1H NMR, and ^{13}C NMR were used to characterize the 8-nitroGua. In agreement with previous reports, 8-nitroGua has a maximum UV−vis absorbance at 400 nm and is extremely stable even at pH 1−14 and elevated temperatures (up to 100 °C). An exact mass of 195.0267 calculated for the formula $C_5H_3N_6O_3$ $[M - H]^-$ ion was consistent with the observed 195.0269. ^1H NMR carried out in DMSO-d_6 revealed two resonances at 10.25 ppm (1H) and 5.89 ppm (2H), corresponding to the N1 proton and the exocyclic N2 amino protons, respectively. Noticeably, the resonance at 8 ppm corresponding to the C8−H in the parent Guo is absent, as would be expected for 8-nitroGua.

Reactions of Peroxynitrite with 8-NitroGua and 8-NitroGuo. When 8-nitroGua (100 μM) was treated with peroxynitrite (0.5−5 mM), there was a dose-dependent decrease in the amount of 8-nitroGua remaining at the end of the reaction. At 5 mM peroxynitrite, all the 8-nitroGua had been destroyed. The increased destruction of 8-nitroGua was accompanied by the appearance of at least four new peaks during HPLC analysis. Unfortunately, these products were extremely polar and very poorly resolved by reversed-phase chromatography. In an effort to improve our chromatography, we synthesized 8-nitroGuo, which itself is well retained on a reversed-phase column (retention time = 12.3 min using HPLC Method A). However, we encountered several problems with this strategy. First, 8-nitroGuo will undergo depurination in aqueous solution, albeit slowly at room temperature, at a rate of 0.6%/h. Second, addition of peroxynitrite leads to rapid and near complete depurination despite there being no change in the pH of the reaction mixture. Consequently, the peroxynitrite products observed using 8-nitroGuo were the same as those between 8-nitroGua with peroxynitrite. Hence, 8-nitroGuo did not offer any advantage over using 8-nitroGua. As a result, we turned our attention toward finding a suitable substrate that did not undergo depurination, but was amenable to reversed-phase chromatography, readily synthesized and expected to undergo chemistry similar to that of 8-nitroGua.

Synthesis of 9-Ethyl-8-nitroxanthine as a Model Compound. We chose to use 9-ethyl-8-nitroxanthine to model the reaction of 8-nitroGua with peroxynitrite. First, since this compound is alkyl substituted at the N9, it will not undergo depurination, as would the N9-β-D-*erythro*-pentofuranosyl-substituted compound. Second, the precursor 9-ethylguanine is readily afforded in good yield by a previously reported synthesis (*32*). Third, C8-nitration of 9-ethylguanine using concentrated nitric and glacial acetic acid gives quantitatively, and in one step, the 9-ethyl-8-nitroxanthine, in contrast with the low yields (∼5%) expected for the route to the corresponding 9-ethyl-8-nitroGua via the nitrite-mediated displacement of Br^- from 9-ethyl-8-bromoguanine. Reversible protection of the N2-exocyclic amino group of 9-ethylguanine during the reaction with concentrated nitric and glacial acetic acid was not a viable option, since most of these protecting moieties contain acyl groups that are readily hydrolyzed under these harsh nitrating conditions. Finally, 9-ethyl-8-nitroxanthine is more water-soluble than

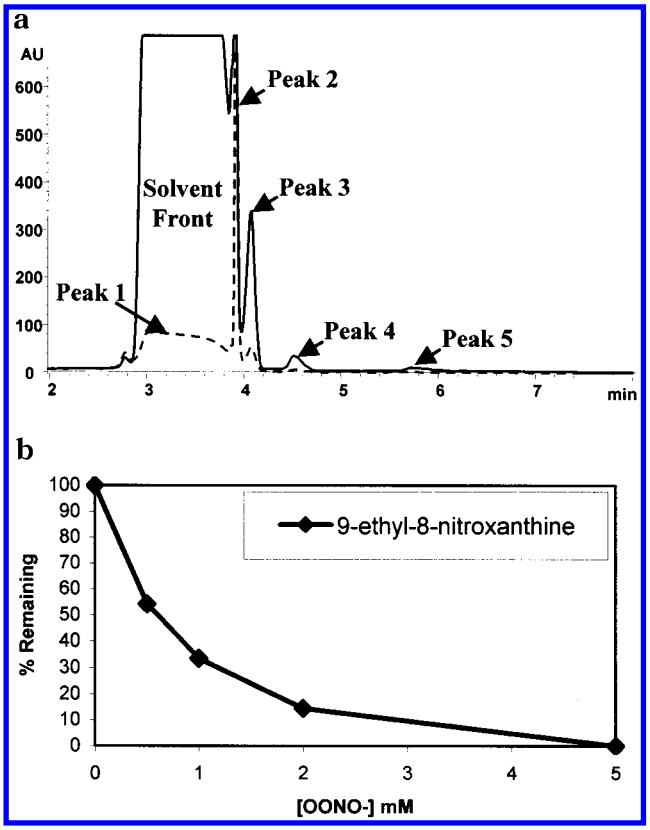


Figure 2. (a) Five new reaction products arising from the reaction between 9-ethyl-8-nitroxanthine and 5 mM peroxynitrite. Employed Method C and analyzed reaction with $\lambda = 238$ nm (solid line) and $\lambda = 282$ nm (dashed line). (b) Dose-dependent depletion of 9-ethyl-8-nitroxanthine in the presence of increasing peroxynitrite concentration (pH 7.2).

8-nitroGua, and is well retained during reversed-phase chromatography, eluting at 23.3 min using HPLC Method B.

Reaction of 9-Ethyl-8-nitroxanthine with Peroxynitrite. When we reacted 9-ethyl-8-nitroxanthine (100 μM) with peroxynitrite (0.5−5 mM), there was a dose-dependent decline in the amount of starting material present at the end of the reaction (Figure 2b). Similar to the reaction with 8-nitroGua, all of the 9-ethyl-8-nitroxanthine had reacted at a peroxynitrite concentration of 5 mM. Correspondingly, five new peaks appeared when the reaction mixture was analyzed by reversed-phase HPLC (Figure 2a).

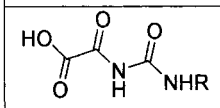
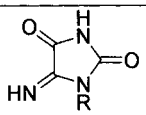
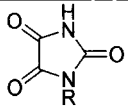
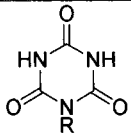
Upon the basis of our initial studies of the reaction products formed during the reaction of 8-nitroGua and 8-nitroGuo with peroxynitrite, we suspected that the 8-nitropurines were giving rise to terminal products corresponding to those known to arise from the reaction of the 8-oxopurine with peroxynitrite. Therefore, we hypothesized that the 8-nitropurine products might be identical or analogous to the 8-oxopurine products and set out to compare the identity of the products arising from these two 8-substituted purines using UV−vis and ESI-MS. Since the 3′,5′-di-*O*-Ac-8-oxodG products had previously been characterized (*21*, *22*), we could calculate the expected molecular weight of the various products by (i) subtracting 201 amu for the 3′,5′-di-*O*-Ac-β-D-*erythro*-pentofuranosyl moiety and adding 29 amu to account for the ethyl substituted derivative used in this study, and (ii) adding 1 amu to the expected molecular weight of any product retaining the C2-oxo group, to account for the presence of an amino group in the 3′,5′-di-*O*-Ac-8-oxodG versus a hydroxyl group in 9-ethyl-8-nitroxanthine.

Characterization of the Products Eluting in Peaks 1−5. Table 1, parts a and b, summarizes the results of the comparison between the products arising from the reaction of 3′,5′-di-*O*-Ac-8-oxodG and 9-ethyl-8-nitroxanthine with peroxynitrite. Using HPLC Method B, peak 1 was not sufficiently resolved from the solvent front to facilitate ESI-MS analysis. However, when the peak was collected, dried in vacuo and reanalyzed by LC-MS using Method D, two new peaks arose and were identified as 1-ethyl-5-iminoimidazolidin-2,4-dione, **III** and 1-ethyloxaluric acid. For peak 2, we were also unable to ascertain its molecular ion weight since it eluted at the end of the solvent front. However, this compound has a characteristic UV spectrum shown in Figure 3, with λ_{max} at 228 and 264 nm, that is essentially identical to that reported for **II** (*22*) as illustrated in Figure 3. Thus, we conclude that peak 2 corresponds to [*N*-nitro-*N*′-[1-ethyl-2,4-dioxoimidazolidin-5-ylidene]-urea, **V**. Using Method D in LC-MS experiments, the product eluting in peak 3 was determined to be 1-ethyldehydroallantoin, which is analogous to **I** formed in the reaction of 3′,5′-di-*O*-Ac-8-oxodG with peroxynitrite. Peak 4 was analyzed by LC-MS (using HPLC Method D) and determined to be 1-ethylparabanic acid, and peak 5 as 1-ethylcyanuric acid. During our LC-MS experiments using a narrow bore column and lower flow rates (60 μL/min), we were able to detect an additional compound with $\lambda_{max} = 245$ and 290 nm, and that gave rise to an $[M - H]^-$ ion at *m*/*z* 195. The UV spectrum was identical to that of authentic 9-methyluric acid, so based upon the UV−vis spectrum and the molecular weight, this compound was identified as 9-ethyluric acid. Furthermore, we ascertained that the C8-nitro group was not retained in any of these products by carrying out the same experiments using 9-ethyl-8-[^{15}N]nitroxanthine, and determining that the molecular weights of the products arising from the ^{14}N- and ^{15}N-labeled substrates were identical.

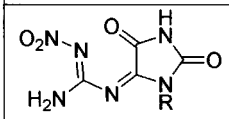
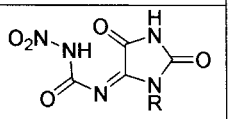
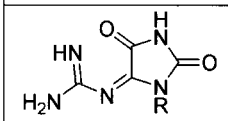
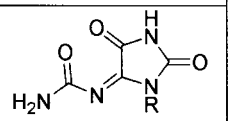
Proposed Mechanism for the Reaction of 9-Ethyl-8-nitroxanthine with Peroxynitrite. The above data confirm the hypothesis that the terminal products arising from the 8-nitropurine are identical to those arising from the 8-oxopurine. The presence of 9-ethyluric acid in the reaction mixture also suggests that displacement of the C8-nitro group occurs during the reaction of the 8-nitropurine with peroxynitrite. Upon the basis of the data above, we therefore propose that early in the reaction, peroxynitrite mediates conversion of the 8-nitropurine to the 8-oxopurine, and that subsequently, the 8-oxopurine reacts with peroxynitrite to give the observed terminal products. In exploring the mechanism, we hypothesized that either oxidative or nucleophilic processes might be involved in the initial displacement of the C8-nitro group and the subsequent oxidation of the 8-oxopurine, since peroxynitrite is a powerful oxidant (*36*, *37*) and a good nucleophile (*30*, *38*). It has been shown previously that peroxynitrite decomposes into potent one-electron oxidants, either by a proton or CO_2-catalyzed process. The former pathway yields the hydroxyl radical ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) (*39*−*41*), while the latter results in formation of the carbonate radical ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ radicals, via homolytic cleavage of peroxynitrosocarbonate ($ONOOCO_2^-$) (*30*, *36*, *38*, *42*, *43*).

Table 1. (a) Comparison between Products Arising from the Reaction of 3′,5′-Di-O-Ac-8-oxodG and 9-Ethyl-8-nitroxanthine with Peroxynitrite Where the Products of the Former Contain a C2-oxo Group; (b) Comparison between Products Arising from the Reaction of 3′,5′-Di-O-Ac-8-oxodG and 9-Ethyl-8-nitroxanthine with Peroxynitrite Where the Products of the Former Retain the C2-Amino Group

a

Compound	R = 3′,5′-di-*O*-Ac-β-D-*erythro*-pentofuranosyl		R = ethyl	
	M_r (amu)	λ_{max} (nm)	M_r (amu)	λ_{max} (nm)
 **oxaluric acid**	332	213	160	213
 **3**	313	225, 260-310 (s)	141	230, 284 (s)
 **parabanic acid**	314	220, 280	142	225, 288
 **cyanuric acid**	329	216	157	216

b

3′,5′-di-*O*-Ac-8-oxodG	9-ethyl-8-nitroxanthine
 **2**	 **5**
M_r (amu): 400	NA
λ_{max} (nm): 238, 272	λ_{max} (nm): 228, 264
 **1**	 **dehydroallantoin**
M_r (amu): 355	M_r (amu): 175
λ_{max} (nm): 236, 275-350 (s)	λ_{max} (nm): 236, 270-340 (s)

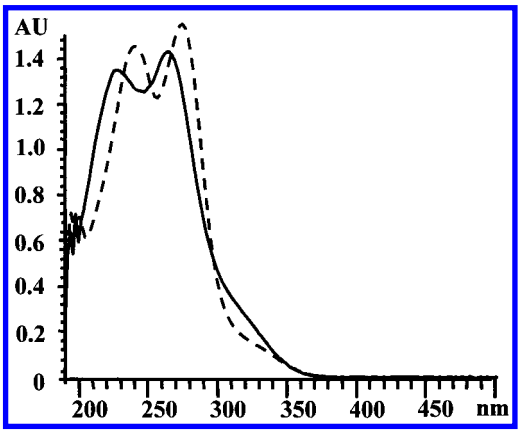
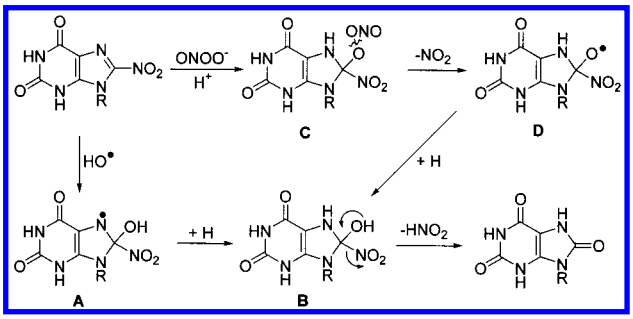


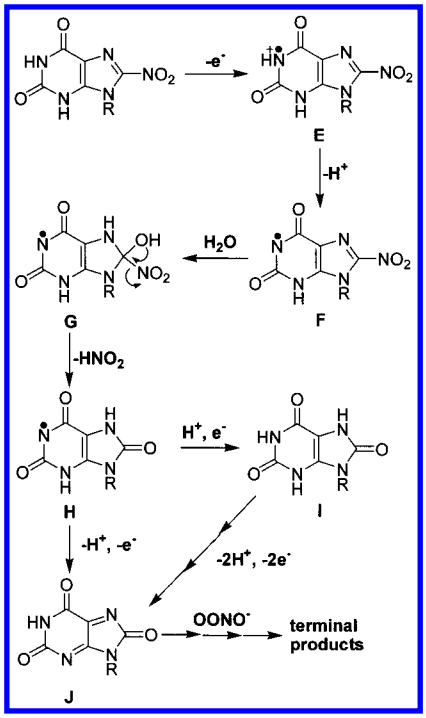
Figure 3. Overlaid UV−vis spectra of peak 2 (—) and II (---). Absorbance maximum measured at λ = 228, 264 nm and λ = 238, 272 nm, respectively, using ddH_{2O}.

Scheme 1. Peroxynitrite Addition and Hydroxyl Radical Insertion with 9-Ethyl-8-nitroxanthine to Yield 9-Ethyluric Acid (R = ethyl)



The •OH may play a role in the reaction of 9-ethyl-8-nitroxanthine by initial insertion into the purine ring. Such a mechanism has been previously been proposed to explain the formation of dX by C4 addition, and 8-oxodG and formamidopyrimidine (Fapy G) by C8 addition, during the •OH-mediated dG oxidation (*44*). Indeed, as shown in Scheme 1, •OH addition at C8 can give rise to the N7-centered radical **A**, followed by hydrogen abstraction to form **B**, which then eliminates nitrous acid to yield the 9-ethyluric acid. However,

Scheme 2. One Electron Oxidation of 9-Ethyl-8-nitroxanthine to Yield the Observed Intermediates and Terminal Products (R = ethyl)



certain facts argue against a primary role for •OH in this mechanism. First, all our reactions with peroxynitrite were carried out in 25 mM sodium bicarbonate-containing buffers (~1 mM CO_2). Under these conditions, peroxynitrite is expected to decay predominantly into the $CO_3^{\bullet-}$/•NO_2 radical pair, without significant •OH formation (*42*). Second, we treated 9-ethyl-8-nitroxanthine with •OH generated in situ by γ-irradiation of N_2O/O_2 purged aqueous solutions. At the 1000 Gy dose, over 95% of the starting material had been consumed, and analysis of the reaction mixture demonstrated formation of 1-ethylparabanic acid as the major product along with a minor unknown compound (λ_{max} = 240 nm). Since 1-ethylparabanic acid is the major product with •OH radical, and only a minor product with peroxynitrite, it is unlikely that the •OH plays a significant role in the overall chemistry with peroxynitrite. However, we cannot fully exclude the possibility that •OH plays a minor role in the initial conversion of the 8-nitropurine to the 8-oxopurine, and that the differences in product profiles between the •OH and peroxynitrite reactions arise due to additional chemistry when the latter is present.

Alternatively, since the •OH and $CO_3^{\bullet-}$ radicals are potent oxidants [$E°$ = 1.9 V (*45*) and 1.5 V (*46*), respectively], while •NO_2 is a milder oxidant ($E°$ = 1.04 V) (*45*), these species may mediate one-electron oxidation of 9-ethyl-8-nitroxanthine to initiate the reaction. Unfortunately, redox potentials have not been determined for any of the 8-nitropurines, so meaningful predictions about the ability of the peroxynitrite-derived species to effect one-electron oxidation of these substrates cannot be made. 1-Electron oxidation of 8-nitroxanthine, in a manner similar to that previously reported for dGuo (*47*−*50*) and 8-oxodG (*51*, *52*) will lead to formation of the 8-nitroxanthine radical cation, **E**, as shown in Scheme 2. Because of the strong electron-withdrawing C8-nitro group, this radical is expected to be even more acidic than the dG and 8-oxodG radical cations [pK_a = 3.9 (*49*) and 6.6 (*50*), respectively], and, therefore, will rapidly deprotonate to the neutral 8-nitroxanthine radical, **F**. In fact, given that the overall oxidation of 8-nitroxanthine by the •OH and $CO_3^{\bullet-}$ radicals is likely close to thermoneutral, proton transfer is likely to accompany electron transfer, as the former provides additional energy to make the oxidation step more favorable (*53*). Hydration of the neutral radical at the C8 to give **G**, followed by elimination of nitrous acid would then yield the neutral uric acid radical, **H**. This species could either abstract a H-atom to give 9-ethyluric acid, or undergo further oxidation to the quinone diimine species, **J**, analogous to that previously reported (*54*). The latter species may also be an important intermediate in the reaction of 9-ethyluric acid with peroxynitrite to give the observed final products.

Another initiating reaction involves the peroxynitrite anion, $ONOO^-$, acting as a nucleophile, leading to loss of the C8-nitro group. $ONOO^-$ is considered a good nucleophile, and is the species that reacts with CO_2 to give peroxynitrosocarbonate, $ONOOCO_2^-$ (*42*). To assess whether a nucleophile could displace the C8-nitro group in 9-ethyl-8-nitroxanthine, we decided to use hydroxylamine, which was expected to give the stable 9-ethyl-8-hydroxylaminoxanthine for isolation and characterization. Therefore, we incubated 9-ethyl-8-nitroxanthine (100 μM) with hydroxylamine (15 μM to 1.5 M) in 0.1 M potassium hydroxide solution and monitored the decline in absorbance at 412 nm with time. At a hydroxylamine concentration ≥10 mM, there was a progressive decline in the absorbance, which indicated that the C8-nitro group was being displaced. Indeed, when the reaction mixture was neutralized, and analyzed by HPLC and off-line ESI-MS, the major product gave rise to an $[M - H]^-$ ion at *m*/*z* 210, consistent with that expected for the 9-ethyl-8-hydroxylaminoxanthine. Furthermore, the λ_{max} = 240 and 285 nm at pH 9 are in close agreement with those reported for 8-hydroxylaminoxanthosine (λ_{max} = 246 and 291 nm at pH 11) (*55*). Additionally, 9-ethyluric acid was a very minor product, likely arising as a result of hydroxide-mediated nitro group displacement. In a control experiment, incubation of 9-ethyl-8-nitroxanthine (100 μM) in 0.1 M potassium hydroxide resulted in no significant degradation of this substrate, demonstrating its stability under these conditions. This finding supports the hypothesis that nucleophilic displacement of the C8-nitro group can occur. Since peroxynitrite has a pK_a of ~6.8 (*29*), at pH 7.2−7.4, between 70 and 80% is present as $ONOO^-$ and thus is available to react as a nucleophile.

The displacement of the C8-nitro group is expected to occur via an addition−elimination route as shown in Scheme 1. Under the experimental conditions used (pH 7.2−7.4), 9-ethyl-8-nitroxanthine exists as the neutral molecule, since pK_a values of 4.1 and 13.2 have previously been reported for the analogous 9-methyl-8-nitroxanthine (*56*). Addition of $ONOO^-$ at the C8 of 9-ethyl-8-nitroxanthine thus leads to formation of **C**, which, like $ONOOCO_2^-$, contains a weak peroxo bond and undergoes homolysis to give **D**. H-atom abstraction by this species then leads to **B**, which then eliminates either nitrous acid or water to yield, respectively, 9-ethyluric acid, or the starting 9-ethyl-8-nitroxanthine.

Conclusion

The reaction of peroxynitrite with both 8-oxodG and 9-ethyl-8-nitroxanthine (a model for 8-nitroGua) results

in the formation of four identical and two analogous products, the latter arising only because of the presence of a C2-oxo group in the model compound versus a C2−NH_2 group in 8-oxodG. These findings suggest that the two reactions converge, likely due to formation of the 8-oxopurine from the 8-nitropurine and subsequent reaction of the 8-oxopurine with peroxynitrite. A primary role for the hydroxyl radical in the peroxynitrite-mediated chemistry has been ruled out, since a very distinct product profile results when this species reacts with 9-ethyl-8-nitroxanthine. Absence of an ^{15}N-label in the products arising from the reaction of 9-ethyl-8-[$^{15}NO_2$]-nitroxanthine with peroxynitrite, along with the hydroxylamine-mediated displacement of the C8-nitro group, argue in favor of a mechanism in which $ONOO^-$ initially attacks the C8 of 9-ethyl-8-nitroxanthine, leading to loss of the C8-nitro group and ultimately to 9-ethyluric acid formation. This work, therefore, illustrates an interesting and novel aspect of 8-nitropurine oxidation by peroxynitrite as well as suggests an additional mechanism via which 8-oxodG might be produced in DNA under circumstances where peroxynitrite levels are insufficient to facilitate further reaction of the 8-oxodG produced.

Acknowledgment. We would like to thank Gary Kruppa from Brüker for allowing us to run our 8-nitroGua sample on the APEX II Fourier Transform Mass Sspectrometer. We would also like to acknowledge Mohamad Awada and Professor R. P. Marini for their support in using the Gammacell-220 machine and acquiring N_2O, respectively. This work was supported by NIH Grants T32ES07020 and 5P01CA26731. J.C.N. was supported by 5F31HG00144.

References

(1) Szabo, C., Zingarelli, B., O'Connor, M., and Salzman, A. L. (1996) DNA strand breakage, activation of poly (ADP-ribose)-synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and small muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1753−1758.

(2) Rachmilewitz, D., Stamler, J., Karmeli, F., Mullins, M. E., Singel, D., Loscalzo, J., Xavier, R. J., and Podolsky, D. K. (1993) Peroxynitrite-induced rat colitis: A new model of colonic inflammation. *Gastroenterology* **105**, 1681−1688.

(3) Hussain, S. P., and Harris, C. C. (1998) Molecular epidemiology of human cancer: Contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res.* **58**, 4023−4037.

(4) Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481−487.

(5) Mohr, S., Stamler, J. S., and Brune, B. (1994) Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite, and related nitrosating agents. *FEBS Lett.* **348**, 223−227.

(6) Ischiropoulos, H., and Al-Mehdi, A. B. (1995) Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett.* **364**, 279−282.

(7) Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244−4250.

(8) Moreno, J. J., and Pryor, W. A. (1992) Inactivation of alpha-1 proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.* **5**, 425−431.

(9) Gotti, R. M., Radi, R., and Augusto, O. (1994) Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Lett.* **348**, 287−290.

(10) Kennedy, L. J., Moore, K., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997) Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. *Chem. Res. Toxicol.* **10**, 386−392.

(11) Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Oshima, H. (1995) Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis* **16**, 2045−2050.

(12) Huie, R. E., and Padmaja, S. (1993) The reaction of NO with superoxide. *Free Radical Res. Commun.* **18**, 195−199.

(13) Koppenol, W. H., Pryor, W. A., Moreno, J. J., Ischiropoulos, H., and Beckman, J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5**, 834−842.

(14) Burney, S., Niles, J. C., Dedon, P. C., and Tannenbaum, S. R. (1999) DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite. *Chem. Res. Toxicol.* **12**, 513−520.

(15) Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S., and Tannenbaum, S. R. (1999) The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat. Res.* **424**, 37−49.

(16) Uppu, R. M., Cueto, R., Squadrito, G. L., Salgo, M. G., and Pryor, W. A. (1996) Competitive reactions of peroxynitrite with 8-oxodG: Relevance to the formation of 8-oxodG in DNA exposed to peroxynitrite. *Free Radical Biol. Med.* **21**, 407−411.

(17) Douki, T., Cadet, J., and Ames, B. N. (1996) An adduct between peroxynitrite and 2′-deoxyguanosine: 4,5-Dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. *Chem. Res. Toxicol.* **9**, 3−7.

(18) Gasparutto, D., Ravanat, J. L., Gerot, O., and Cadet, J. (1998) Characterization and chemical stability of photooxidized oligonucleotides that contain 2,2-diamino-4-[(2-deoxy-beta-D-erythro-pentofuranosyl)amino]-5-(2H)-oxazolone. *J. Am. Chem. Soc.* **120**, 10283−10286.

(19) Douki, T., and Cadet, J. (1996) Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Res.* **24**, 369−380.

(20) Cadet, J., Berger, M., Buchko, G. W., Joshi, P. C., Raoul, S., and Ravanat, J. L. (1994) 2,2-Diamino-4-[(3,5-di-O-acetyl-2-deoxy-beta-D-erythro-pentofuranosyl)amino]-5-(2H)-oxazolone: A novel and predominant radical oxidation product of 3′,5′-di-O-acetyl-2′deoxyguanosine. *J. Am. Chem. Soc.* **116**, 7403−7404.

(21) Niles, J. C., Burney, S., Singh, S. P., Wishnok, J. S., and Tannenbaum, S. R. (1999) Peroxynitrite reaction products of 3′,5′-di-O-acetyl-8-oxo-7,8-dihydro-2′-deoxyguanosine. *Proc. Natl. Acad. Sci.* **96**, 11729−11734.

(22) Niles, J. C., Wishnok, J. S., and Tannenbaum, S. R. (2000) A novel nitration product formed during the reaction of peroxynitrite with 2′,3′,5′-tri-O-acetyl-7,8-dihydro-8-oxo-guanosine: N-nitro,N′-[1-(2,3,5-tri-O-acetyl-beta-D-erythro-pentofuranosyl)-2,4-dioxo-imidazolidin-5-ylidene]guanidine. *Chem. Res. Toxicol.* **13**, 390−396.

(23) Tretyakova, N. Y., Niles, J. C., Burney, S., Wishnok, J. S., and Tannenbaum, S. R. (1999) Peroxynitrite-induced reactions of synthetic oligonucleotides containing 8-oxoguanine. *Chem. Res. Toxicol.* **12**, 459−466.

(24) Yermilov, V., Rubio, J., and Oshima, H. (1995) Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett.* **376**, 207−210.

(25) Tretyakova, N. Y., Burney, S., Pamir, B., Wishnok, J. S., Dedon, P. C., Wogan, G. N., and Tannenbaum, S. R. (2000) Peroxynitrite-induced DNA damage in the supF gene: Correlation with the mutational spectrum. *Mutat. Res.* **447**, 287−303.

(26) Tuo, J., Liu, L., Poulsen, H. E., Weimann, A., Svendsen, O., and Loft, S. (2000) Importance of guanine nitration and hydroxylation in DNA in vitro and in vivo. *Free Radical Biol. Med.* **29**, 147−155.

(27) Chen, H. C., Chen, Y., Wang, T., Wang, K., and Shiea, J. (2001) 8-nitroxanthine, an adduct derived from 2′-deoxyguanosine or DNA reactions with nitryl chloride. *Chem. Res. Toxicol.* **14**, 536−546.

(28) Yermilov, V., Yoshie, Y., Rubio, J., and Oshima, H. (1996) Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett.* **399**, 67−70.

(29) Pryor, W. A., Cueto, R., Jin, X., Koppenol, W. H., Ngu-Schwemlein, M., Squadrito, G. L., Uppu, P. L., and Uppu, R. M. (1995) A practical method for preparing peroxynitrite solutions of low ionic strength and free of hydrogen peroxide. *Free Radical Biol. Med.* **18**, 75−83.

(30) Lymar, S. V., and Hurst, J. K. (1995) Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* **117**, 8867−8868.

(31) Long, R. A., Robins, R. K., and Townsend, L. B. (1968) 8-Bromoguanosine. Bromination of the purine ring of a purine nucleoside. In *Synthetic Procedures in Nucleic Acid Chemistry* (Zorbach, W. W., and Tipson, R. S., Eds.) pp 228−229, Wiley-Interscience, New York.

(32) Alhede, B., Clausen, F. P., Juhl-Christensen, J., McCluskey, K. K., and Preikschat, H. F. (1991) A simple and efficient synthesis

of 9-substituted guanines. Cyclodesulfurization of 1-substituted 5-[(thiocarbamoyl)amino]imidazole-4-carboxamides under aqueous base conditions. *J. Am. Chem. Soc.* **56**, 2139−2143.
(33) Sodum, R. S., and Fiala, E. S. (2001) Analysis of peroxynitrite reactions with guanine, xanthine, and adenine nucleosides by high-pressure liquid chromatography with electrochemical detection: C8-nitration and -oxidation. *Chem. Res. Toxicol.* **14**, 438−450.
(34) Jones, J. W., and Robins, R. K. (1960) Potential purine antagonists. XXIV. The preparation and reaction of some 8-diazopurines. *J. Am. Chem. Soc.* **82**, 3773−3779.
(35) Ratsep, P. C., Robins, R. K., and Vaghefi, M. M. (1990) 8-Diazoguanosine, 2,8-diaminoadenosine and other purine nucleosides derived from guanosine. *Nucleosides Nucleotides* **9**, 1001−1013.
(36) Lymar, S. V., and Hurst, J. K. (1998) CO2-catalyzed one-electron oxidations by peroxynitrite: Properties of the reactive intermediate. *Inorg. Chem.* **37**, 294−301.
(37) Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35**, 7855−7861.
(38) Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333**, 49−58.
(39) Coddington, J. W., Hurst, J. K., and Lymar, S. V. (1999) Hydroxyl radical formation during peroxynitrous acid decomposition. *J. Am. Chem. Soc.* **121**, 2438−2443.
(40) Merenyi, G., and Lind, J. (1998) Free radical formation in the peroxynitrous acid (ONOOH)/peroxynitrite (OONO-) system. *Chem. Res. Toxicol.* **11**, 243−246.
(41) Richeson, C. E., Mulder, P., Bowry, V. W., and Ingold, K. U. (1998) The complex chemistry of peroxynitrite decomposition: New insights. *J. Am. Chem. Soc.* **120**, 7211−7219.
(42) Goldstein, S., and Czapski, G. (1998) Formation of peroxynitrate from the reaction of peroxynitrite with CO2: Evidence for carbonate radical production. *J. Am. Chem. Soc.* **120**, 3458−3463.
(43) Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996) Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327**, 335−343.
(44) Steenken, S. (1989) Purine-bases, nucleosides and nucleotides-aqueous-solution redox chemistry and transformation reactions of their radical cations and e- and OH radical adducts. *Chem. Rev.* **89**, 503−520.
(45) Stanbury, D. M. (1989) Reduction potentials involving inorganic free radicals in aqueous solution. *Adv. Inorg. Chem.* **33**, 69−138.
(46) Huie, R. E., Clifton, C. L., and Neta, P. (1991) Electron-transfer reaction rates and equilibria of the carbonate and sulfate radical anions. *Radiat. Phys. Chem.* **38**, 477−481.
(47) Ravanat, J., and Cadet, J. (1995) Reaction of singlet oxygen with 2′deoxyguanosine and DNA. Isolation and characterization of the main oxidation products. *Chem. Res. Toxicol.* **8**, 379−388.
(48) Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J., Ravanat, J., and Sauvaigo, S. (1999) Hydroxyl radicals and DNA base damage. *Mutat. Res.* **424**, 9−21.
(49) Candeias, L. P., Steenken, S. (1989) Structure and acid−base properties of one electron-oxidized deoxyguanosine, guanosine, and 1-methylguanosine. *J. Am. Chem. Soc.* **111**, 1094−1099.
(50) Steenken, S., Jovanovic, S. V., Bietti, M., and Bernhard, K. (2000) The trap depth (in DNA) of 8-oxo-7,8-dihydro-2′deoxyguanosine as derived from electron-transfer equilibria in aqueous solution. *J. Am. Chem. Soc.* **122**, 2373−2374.
(51) Goyal, R. N., Jain, N., and Garg, D. K. (1997) Electrochemical and enzymatic oxidation of guanosine and 8-hydroxyguanosine and the effects of oxidation products in mice. *Bioelectrochem. Bioenerg.* **43**, 105−114.
(52) Goyal, R. N., and Dryhurst, G. (1982) Redox chemistry of guanine and 8-oxyguanine and a comparison of the peroxidase-catalyzed and electrochemical oxidation of 8-oxyguanine. *J. Electroanal. Chem.* **135**, 75−91.
(53) Weatherly, S. C., Yang, I. V., and Thorp, H. H. (2001) Proton-coupled electron transfer in duplex DNA: Driving force dependence and isotope effects on electrocatalytic oxidation of guanine. *J. Am. Chem. Soc.* **123**, 1236−1237.
(54) Goyal, R. N., Brajter-Toth, A., Besca, J. S., and Dryhurst, G. (1983) The electrochemical and peroxidase-catalyzed redox chemistry of 9-beta-D-ribofuranosyluric acid. *J. Electroanal. Chem.* **144**, 163−190.
(55) Long, R. A., Robins, R. K., and Townsend, L. B. (1967) Purine nucleosides XV. The synthesis of 8-amino- and 8-substituted aminopurine nucleosides. *J. Org. Chem.* **32**, 2751−2756.
(56) Mosselhi, M. A., and Pfleiderer, W. (1993) Purines. XIV Synthesis and properties of 8-nitroxanthine and its *N*-methyl derivatives. *J. Heterocycl. Chem.* **30**, 1221−1228.

TX010093D