Peroxynitrite reaction products of 3′,5′-di-*O*-acetyl-8-oxo-7,8-dihydro-2′-deoxyguanosine

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Of the DNA bases, peroxynitrite ($ONOO^-$) is most reactive toward 2′-deoxyguanosine (dGuo), but even more reactive with 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), requiring a 1,000-fold excess of dGuo to provide 50% protection against the reaction with 8-oxodGuo. Therefore, it seems reasonable that 8-oxodGuo is a potentially important target in DNA and that the structures of the reaction products with $ONOO^-$ should be characterized. Using 3′,5′-di-*O*-Ac-8-oxodGuo as a model compound, the reaction products with $ONOO^-$ have been isolated and identified under simulated physiological reaction conditions (phosphate/bicarbonate buffer at pH 7.2). The major reaction product, II, is unstable and undergoes base-mediated hydrolysis to 2,5-diaminoimidazol-4-one, IIa, and 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-5-iminoimidazolidine-2,4-dione, IIb. The latter compound further hydrolyzes to 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)oxaluric acid, IIc. Other products include 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-2,4,6-trioxo-[1,3,5]triazinane-1-carboxamidine, I, which further hydrolyzes to 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid, Ia. 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)parabanic acid, III, is a minor product that also may contribute to formation of IIc. The major products formed in these reactions are biologically uncharacterized but are similar to modified DNA bases that have been shown to be both premutagenic and blocks to DNA polymerization.

Peroxynitrite ($ONOO^-$) is formed by the diffusion-limited combination of nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) (1) and is a highly reactive molecule; it is stable in the anion form (pH = 12) but decays rapidly with a half-life of 1 s at physiologic pH in a process that involves protonation to form peroxynitrous acid (ONOOH, $pK_a = 6.8$). It is proposed that the protonation of $ONOO^-$ results in a high-energy, reactive intermediate, ONOOH*, which can either decay to give nitrate or react with substrate (2, 3). In the presence of bicarbonate, the peroxynitrosocarbonate anion, ($ONOOCO_2^-$) is formed from $ONOO^-$ in a carbon dioxide catalyzed reaction (4–7). Although $ONOOCO_2^-$ has a shorter lifetime than $ONOO^-$, the former is still a potent oxidizing agent, with a redox potential in excess of + 1 V (4, 8).

An important source of $ONOO^-$ *in vivo* is the activated macrophage. This cell type has been implicated in causing tissue damage during acute and chronic inflammatory conditions by mechanisms that might involve $ONOO^-$ (9). Indeed, 3-nitrotyrosine, a putative marker of $ONOO^-$ formation, has been detected in people with acute lung injury (10, 11) and rheumatoid arthritis (12). In addition to reacting with lipids (13), proteins (14), and cellular thiols (15–19), $ONOO^-$ can cause DNA damage. The spectrum of DNA damage includes strand breakage (20–22) and both oxidation (22) and nitration (23) of bases.

Of the DNA nucleosides, 2′-deoxyguanosine (dGuo) is the most reactive toward $ONOO^-$. Several reaction products are observed including the nitration product, 8-nitro-2′-deoxyguanosine (23); the addition product, 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine (24); and the oxidation products, 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone and 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine (25). Furthermore, 8-oxodGuo is more reactive toward $ONOO^-$ than is dGuo, requiring a 1,000-fold excess of dGuo to provide 50% protection of 8-oxodGuo from reaction with $ONOO^-$ (26). 8-oxodGuo can be present in cellular DNA at levels in the range of 4 to 11 in 10^7 bases (27). Further, given the preferential reactivity of this lesion toward $ONOO^-$, we have hypothesized that knowledge of the structure of the lesions produced by reaction of 8-oxodGuo with $ONOO^-$ could be essential in understanding how $ONOO^-$ induces DNA damage and plays a role in mutagenesis.

Given the propensity for 8-oxodGuo to undergo further oxidation, we reasoned that this would be the most likely mode of reaction of 8-oxodGuo with $ONOO^-$. There are several important models for the oxidation of 8-oxodGuo: (*i*) the alkaline potassium permanganate or iodine-mediated oxidation of uric acid; (*ii*) the electrochemical oxidation of 8-oxoGua; and (*iii*) the photooxidation of 8-oxodGuo. Alkaline oxidation of uric acid leads to the formation of allantoin and dehydroallantoin as major products (28). Electrochemical oxidation of 8-oxoGua results in formation of 2,5-diaminoimidazol-4-one (**IIa**) and 5-guanidinohydantoin (29). Photooxidation of 8-oxodGuo leads to the formation of 1,3,5-triazine-1(2*H*)-carboximidamide, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)tetrahydro-2,4,6-trioxo-(**I**) as the major product. This compound then undergoes base-mediated hydrolysis to yield 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid (**Ia**) (30).

Using these systems as models for the $ONOO^-$-mediated oxidation of 8-oxodGuo, the major immediate products of this reaction have been identified.

Materials and Methods

Materials. Cyanuric acid, parabanic acid, potassium oxonate, and 2-amino-6,8-dihydroxypurine (8-oxoGua) were obtained from Aldrich (Milwaukee, WI). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) was either obtained from Sigma or synthesized as described by Kasai and Nishimura (31). Potassium permanganate (Fisher Scientific), sodium borohydride ($NaBH_4$) (American Bioanalytical, Natick, MA), and acetic anhydride (Mallinckrodt) were analytical grade. Silylation grade pyridine was obtained from Pierce, and bis(trimethylsilyl)trifluoroacetamide was from Supelco. Peroxynitrite was synthesized by the ozonation of an aqueous alkaline solution of sodium azide (32). All solvents were HPLC grade.

Instrumentation. UV/Vis measurements were made by using an HP8452 Diode Array Spectrophotometer (Hewlett–Packard). ^{1}H-NMR spectra were recorded at 300 or 500 MHz and ^{13}C-NMR spectra (proton decoupled) at 75 or 125 MHz on Unity 300 and Inova 500 spectrometers, respectively. HPLC was performed

Abbreviations: ESI, electrospray ionization; MS/MS, tandem MS; HRMS, high-resolution MS.

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by using an HP1090 pump equipped with a 1090 Diode Array Detector (Hewlett–Packard). Electrospray ionization (ESI)-MS and tandem MS (ESI-MS/MS) experiments were carried out by using either an HP 5989B (Hewlett–Packard) or TSQ 7000 (Finnigan, San Jose, CA) mass spectrometer, respectively. High-resolution MS (HRMS) experiments were done in ESI mode on an APEX II Fourier transform MS (Bruker, Billerica, MA). All ESI-MS and ESI-MS/MS spectra were obtained in negative ion mode by using a spraying solution with the composition 49:40:10:1 water/methanol/acetonitrile/ammonium hydroxide or 78:20:2 water/2-propanol/ammonium hydroxide. In exchange experiments, D_2O was substituted for H_2O. GC/MS analyses were carried out on a HP 5989A mass spectrometer (Hewlett–Packard).

3′,5′-Di-*O*-Ac-8-oxodGuo Synthesis and Purification. 3′,5′-Di-*O*-Ac-8-oxodGuo was prepared by heating a suspension of 8-oxodGuo (1 mg) in pyridine (100 μl) and acetic anhydride (60 μl) at 70°C for 30 min. Unreacted acetic anhydride was quenched by addition of methanol (250 μl), and the mixture was dried *in vacuo*. The residue was dissolved in double-distilled water and purified by semipreparative HPLC on a 250 × 10 mm, 7 μm RP-300 column (Alltech Associates). Solvents A and B were 50 mM ammonium acetate, pH 5.5 and acetonitrile, respectively. Isocratic elution with 5% B for 5 min was followed by a gradient from 5% B to 22% B in 18 min. After holding at 22% B for 4 min, the column was ramped from 22% B to 5% B in 3 min. A flow rate of 2.5 ml/min was used, and products were monitored simultaneously at 225 nm and 260 nm. The desired 3′,5′-di-*O*-Ac-8-oxodGuo eluted at 19.8 min under these conditions. Fractions containing the desired product were collected, pooled, and lyophilized. Excess ammonium acetate was removed by taking up the residue in double-distilled water and lyophilizing several times. ^{1}H-NMR (DMSO-d_6, δ): 10.84 ppm (s, 1H, NH); 10.71 ppm (s, 1H, NH); 6.49 ppm (s, 2H, NH_2); 6.02 (m, 1H, H1′); 5.35 ppm (m, 1H, H3′); 4.32 ppm (m, 1H, H4′); 4.09 ppm (m, 2H, H5″); 2.23 ppm (m, 1H, H2′); 2.09 (m, 6H, 2 x CH_3); UV/Vis (0.1 M HCl): λ_{max} = 248 nm, 292 nm; ESI-MS (negative ions) *m/z* 366 $[M-H]^-$.

Synthesis of 5-Azauracil and (2-Imino-5-Oxo-Imidazolidin-4-yl)Urea. 5-Azauracil was synthesized by the decarboxylation of potassium oxonate as described by Pike (33). The pure compound was obtained by recrystallization of the crude product from methanol. ^{1}H-NMR (DMSO-d_6, δ): 11.32 ppm (s, 1H, NH), 8.14 ppm (s, 1H, CH); ^{13}C-NMR (DMSO-d_6, δ): 157.18 ppm (C2/C4), 66.71 (C6); ESI-MS (negative ions) *m/z* 112 $[M-H]^-$.

(2-Imino-5-oxo-imidazolidin-4-yl)urea was prepared by the alkaline potassium permanganate oxidation of 8-oxoGua by analogy with the preparation of allantoin from uric acid (34). The analytical sample was obtained after recrystallization of the crude product from water. ^{1}H-NMR (DMSO-d_6, δ): 7.92 ppm (s, 1H, 3-NH), 7.68 ppm (s, 1H, 2-NH), 6.93 ppm (s, 1H, 1-NH), 6.7 ppm (d, 1H, J = 7.8 Hz, 6-NH), 5.69 ppm (s, 2H, 8-NH_2), 5.08 ppm (d, 1H, J = 8.1 Hz, 4-CH); ^{13}C-NMR (DMSO- d_6, δ): 185.99 ppm (C2), 171.94 ppm (C5), 158.36 ppm (C7), 64.34 (C4); HRMS calculated for $C_4H_6N_5O_2$ $[M-H]^-$ 156.0522, found 156.0525.

Reaction of 3′,5′-Di-*O*-Ac-8-oxodGuo with ONOO$^-$. Reactions of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$ were carried out in 150 mM KH_2PO_4, 25 mM Na_2CO_3, pH 7.2 buffer. The desired concentrations of 3′,5′-di-*O*-Ac-8-oxodGuo and $ONOO^-$ were determined spectrophotometrically by using the extinction coefficients 13.5 $mM^{-1}\cdot cm^{-1}$ (λ = 248 nm, 0.1 M HCl) (35) and 1,670 $M^{-1}\cdot cm^{-1}$ (λ = 302 nm, 0.1 M NaOH) (36), respectively. Reactions were performed by adding the desired amount of 3′,5′-di-*O*-Ac-8-oxodGuo to a buffered solution in an Eppendorf tube (1.5 ml). $ONOO^-$ was placed as a droplet on the underside of the lid. After carefully closing, the tube was vigorously vortexed for 1 min to effect complete reaction.

Separation of 3′,5′-Di-*O*-Ac-8-oxodGuo/ONOO$^-$ Reaction Products. After reacting 100 μM 3′,5′-di-*O*-Ac-8-oxodGuo with 500 μM $ONOO^-$, products were separated by HPLC using a 250 × 4.6 mm, 5 μm LC-18-DB column (Supelco). The mobile phases used were 50 mM ammonium acetate and acetonitrile. HPLC conditions used were: isocratic 5% B for 10 min; 5% B to 27.4% B in 20 min; 27.4% B to 5% B in 5 min. The flow rate was 1 ml/min, and products were monitored simultaneously at 230 nm and 260 nm.

Reduction of Compound II. For analytical studies, a 100 μM 3′,5′-di-*O*-Ac-8-oxodGuo solution was treated with 500 μM $ONOO^-$ (final total volume = 1 ml). Treating either the HPLC-purified product or the crude 3′,5′-di-*O*-Ac-8-oxodGuo/$ONOO^-$ reaction mixture with $NaBH_4$ effected reduction of **II**. The fraction containing purified **II** (volume = 350–400 μl) or the crude reaction mixture (volume = 1 ml) was treated with 5 μl of an aqueous 1 M $NaBH_4$ stock solution. The mixtures were incubated at room temperature for 5–10 min and then analyzed by HPLC using the same conditions for purifying the 3′,5′-di-*O*-Ac-8-oxodGuo/$ONOO^-$ reaction products. Reduction of **II** was complete and yielded two products, $\mathbf{II}_{red}$ (λ_{max} = 229 nm, M_r = 357) and $\mathbf{II'}_{red}$ (λ_{max} = 227 nm, M_r = 357), which had retention times of 18.1 and 18.9 min, respectively. In preparative studies, 0.084 mmol (30 mg) of 3′,5′-di-*O*-Ac-8-oxodGuo was treated with 0.50 mmol $ONOO^-$ in 150 mM KH_2PO_4, 25 mM Na_2CO_3, pH 7.2 (80 ml), and 0.80 mmol $NaBH_4$ added to reduce the products formed. The reaction mixture was concentrated by rotary evaporation and purified by semipreparative HPLC on a Nucleosil C18 250 × 10 mm, 5 μm column (Alltech Associates) with the same gradient and solvents used in the analytical studies and a flow rate = 3.0 ml/min. Under these conditions, $\mathbf{II}_{red}/\mathbf{II'}_{red}$ eluted as a single peak. The product was desalted on a C18 Mega Bond Elut column (Varian), eluted with methanol and dried *in vacuo*. ^{1}H-NMR (DMSO-d_6, δ): (diastereomer 1) 7.44 ppm (s, br, 2H); 5.81 ppm (dd, J = 5.5 Hz, 10 Hz, 1H, H1′); 5.30 ppm (s, 1H, H4); 5.06 ppm (m, 1H, H3′); 4.15–3.90 ppm (m, 3H, H4′, H5′, H5″); 2.30 ppm* (m, 1H, H2′); 2.18 ppm* (m, 1H, H2″); 2.07 ppm (m, 6H, 2 x CH_3); (diastereomer 2) 7.44 ppm (s, br, 2H); 5.66 ppm (dd, J = 6 Hz, 9 Hz, 1H, H1′); 5.19 ppm (s, 1H, H4); 5.00 ppm (m, 1H, H3′); 4.15–3.90 ppm (m, 3H, H4′, H5′, H5″); 2.30 ppm* (m, 1H, H2′); 2.18 ppm* (m, 1H, H2″); 2.05 ppm (m, 6H, 2 x CH_3); HRMS calculated for $C_{13}H_{18}N_5O_7$ $[M-H]^-$ 356.1205, found 356.1207. * indicates recorded in D_2O. Considerable overlap of the H2′ and H2′′ resonances for the respective diastereomers is observed.

Hydrolysis of II. A 100-μM 3′,5′-di-*O*-Ac-8-oxodGuo solution was treated with 750 μM $ONOO^-$ and **II** isolated by analytical HPLC as described before, except that 60 mM KH_2PO_4, pH 7.4 was used as the aqueous mobile phase. Ten microliters of concentrated ammonium hydroxide was added to the fraction containing **II** (520 μl), and the mixture was incubated at room temperature for 1.5 hr. This hydrolysis mixture was analyzed by HPLC using the same conditions used to isolate **II**. Hydrolysis of **II** yielded **IIa** (λ_{max} = 249 nm and 313 nm; M_r = 112), **IIb** (λ_{max} = 225 nm, shoulder 260–310 nm; M_r = 313), and **IIc** (λ_{max} = 213 nm; M_r = 332), which eluted at 3.3, 26.2, and 21 min, respectively.

Preparation of 2,3,5-Tri-*O*-Ac-β-D-*erythro*-Pentofuranosyl Derivative of IIc (IIc′). The starting material, 2′,3′,5′-tri-*O*-Ac-8-oxoGuo, was prepared according to the procedure by Ikehara *et al.* (37), except that the 8-BrGuo was acetylated by using standard procedures (38). **IIc′** then was prepared by reacting 2′,3′,5′-tri-

O-Ac-8-oxoGuo (0.047 mmol, 20 mg) dissolved in 150 mM KH_2PO_4, 25 mM Na_2CO_3, pH 7.2 buffer (10 ml) with $ONOO^-$ (0.141 mmol, 1.68 ml). Reactions were initiated by forcefully pipetting the $ONOO^-$ into the vigorously stirred nucleoside solution. The product corresponding to **II**, (**II′**), was purified by semipreparative HPLC on a Nucleosil C18 250 × 10 mm, 5-μm column (Alltech Associates) by using 10 mM KH_2PO_4, pH 7.4 (solvent A) and acetonitrile (solvent B) as mobile phases. HPLC elution conditions used were: isocratic, 5% B for 10 min; 5% to 40% B in 35 min; 40% to 5% B in 5 min. The flow rate was 3.5 ml/min, and products were monitored simultaneously at 225 nm and 260 nm. **II′** eluted at 32 min. Fractions containing **II′** were pooled, dried, and taken up in 1.5 ml of double-distilled water and left at room temperature for 30 hr. Complete hydrolysis to **IIc′** was confirmed by HPLC before the mixture was desalted on a C18 Mega Bond Elut column (Varian), eluted with methanol, and dried *in vacuo*. ^{1}H-NMR (DMSO-d_6, δ): 9.81 ppm (s, 1H, COOH); 8.87 ppm (d, J = 9.3 Hz, 1H, NH); 7.04 (s, 1H, NH); 5.51 ppm (m, 1H, J = 5.1 Hz, 9.3 Hz, H1′); 5.22 ppm (m, 2H, H2′ and H3′); 4.23 ppm (m, 1H, H4′), 4.11 ppm (m, 2H, H5′ and H5″); 2.07 ppm (m, 9H, 3 x CH_3); UV (H_2O): λ_{max} = 213 nm; ESI-MS (negative ions): *m/z* 390 $[M-H]^-$.

Preparation of Reaction Products for GC/MS. Ten 1-ml reactions of 100 μM 3′,5′-di-*O*-Ac-8-oxodGuo with 750 μM $ONOO^-$ were combined and dried *in vacuo*. The residue was taken up in double-distilled water (1 ml), and **I**, **II,** and **III** were purified by analytical HPLC using 50 mM ammonium acetate and acetonitrile as mobile phases. Isolated **II** was base-hydrolyzed to give **IIa** and **IIb** as described above. $\mathbf{II}_{red}$ and $\mathbf{II'}_{red}$ were HPLC-purified from 10 separate reactions of 100 μM 3′,5′-di-*O*-Ac-8-oxodGuo with 750 μM $ONOO^-$, with $NaBH_4$ (5 μl of a 1 M stock) added to effect reduction of **II**. The HPLC fractions containing **I**, **IIb**, **III**, $\mathbf{II}_{red}$, and $\mathbf{II'}_{red}$ were dried *in vacuo*, and the residues were taken up in 6 M HCl (75 μl), transferred to silinized reacti-vials (0.5 ml), and heated at 100°C for 5 min to remove the 3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl moiety. These solutions then were cooled to room temperature and dried *in vacuo*. **IIa**, which lacks the 3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl moiety, was not subjected to acid hydrolysis. Hence, the HPLC fraction containing **IIa** was dried *in vacuo* and subsequently derivatized as described below. Samples were derivatized for GC/MS analysis using a 1:1 mixture of bis(trimethylsilyl)trifluoro-acetamide in acetonitrile (100 μl). The aglycons of **IIb** and **III** were derivatized by heating at 130°C for 10 min. The aglycons of **I**, **IIa**, $\mathbf{II}_{red}$, and $\mathbf{II'}_{red}$ were derivatized similarly, except that these samples were heated for 20 min. All samples were cooled to room temperature, and 5–10 μl was used per GC/MS analysis.

Preparation of Authentic Standards for GC/MS. One hundred micrograms each of 5-azauracil, cyanuric acid, (2-imino-5-oxo-imidazolidin-4-yl)urea, and parabanic acid were transferred in aqueous solution to silinized reacti-vials. The solutions were dried *in vacuo*, and the residues were taken up in 100 μl of a 1:1 bis(trimethylsilyl)trifluoro-acetamide/acetonitrile mixture. Heating the respective solutions at 130°C for 10 min effected derivatization of 5-azauracil and parabanic acid. Cyanuric acid and (2-imino-5-oxo-imidazolidin-4-yl)urea were derivatized similarly, except that these solutions were heated for 20 min. Derivatized samples were diluted 10-fold with hexane and 1–5 μl was injected per analysis.

GC/MS Analyses. All analyses were performed by using a 12.5 m × 0.22 mm × 0.33 μm film thickness HP-1 column (Hewlett–Packard). Either electron ionization or positive ion chemical ionization (PICI) was used. In PICI studies, methane was used as the bath gas. The injector and inlet temperatures were set at 250°C and the source temperature at 200°C. The quadrupole temperature was kept at 100°C. An electron ionization energy of 70 eV was used in all studies. Helium was used as the carrier gas, and the column head pressure was set at 6 psi. In all studies of the derivatized aglycons and authentic standards, the column temperature was ramped from 60°C at 0 min to 250°C at a rate of 20°C/min and then held constant at 250°C for 5 min. All injections were made in splitless mode.

Results and Discussion

The treatment of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$ followed by immediate HPLC analysis reveals the formation of four

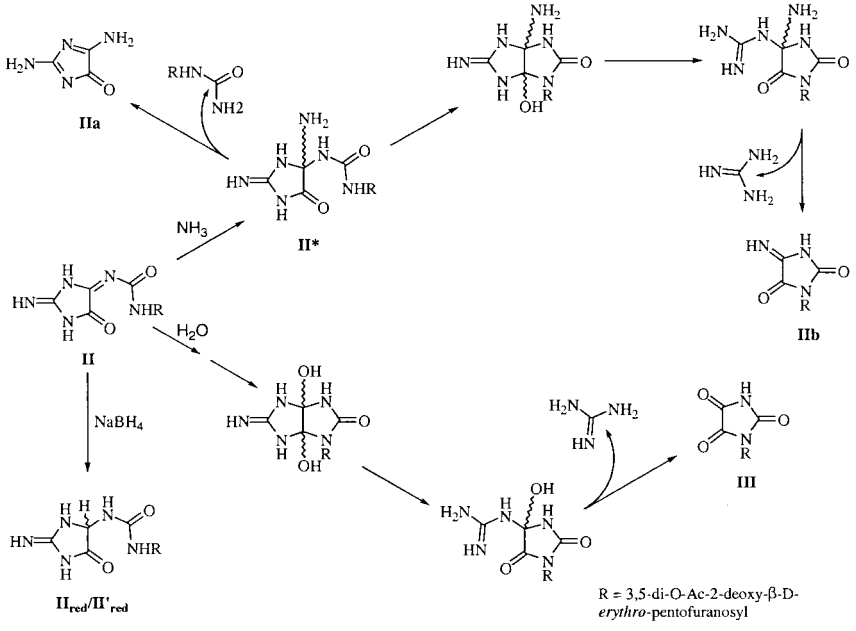

Fig. 1. Structure proposed for **II**, along with its reduction and hydrolytic products.

Table 1. GC/MS data for authentic standards

Compound	Retention time, min	Ions
5-Azauracil, **IIb**	5.3	257 (M^+, 59%); 242 (54%); 143 (68%); 127 (31%); 100 (base peak)
Cyanuric acid, **Ia**	6.8	345 (base peak, M^+); 330 (36%); 147 (44%); 100 (40%)
(2-Imino-5-oxo-imidazolidin-4-y)urea, $\mathbf{II}_{red}$	9.0	517 (base peak, M^+); 502 (22%); 428 (78%); 356 (33%)
Parabanic acid, **III**	5.8	258 (M^+, 25%); 243 (76%); 215 (15%); 188 (5%); 100 (base peak)

The aglycons of the $OONO^-$-reaction products of 3′,5′-di-*O*-Ac-8-oxodGuo are shown in bold type next to the authentic standard that gave an identical retention time and fragmentation pattern.

additional compounds. The first compound, **I** (λ_{max} = 216 nm, M_r = 371), eluted at 23 min. The second compound, **II** (λ_{max} = 236 nm; shoulder 275–350 nm; M_r = 355), eluted at 24.5 min, and the third compound, **III** (λ_{max} = 220 nm and 280 nm; M_r = 314), eluted at 24.9 min. The fourth compound is not presently characterized and will not be discussed further. Over a range of $ONOO^-$ concentrations, **II** is the major product of the reaction of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$ whereas **I** and **III** are formed in lower but similar amounts. These three compounds have been studied in detail and structurally characterized.

Characterization of II. Direct structural studies of **II** were impaired because of its inherent instability. However, HRMS revealed that the molecular formula for this compound is $C_{13}H_{17}N_5O_7$ (calculated exact mass: 355.1128; found: 355.1125). **II** can be reduced by $NaBH_4$ in 100% yield to give equal amounts of two stable compounds, $\mathbf{II}_{red}$ and $\mathbf{II}'_{red}$, both with molecular formulae $C_{13}H_{19}N_5O_7$ (calculated exact mass: 357.1284; found: 357.1286). By characterizing the stable reduction products along with the hydrolysis products, it was possible to propose that the intermediate **II** is most likely 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-imino-5-oxo-imidazolidin-4-ylidene)urea (Fig. 1).

Several lines of evidence support this conclusion. The reduction products $\mathbf{II}_{red}$ and $\mathbf{II}'_{red}$ were acid-hydrolyzed to yield the aglycons, and the trimethylsilyl derivatives of the aglycons were prepared and analyzed by GC/MS. Both $\mathbf{II}_{red}$ and $\mathbf{II}'_{red}$ released a single aglycon that had the same retention time and fragmentation pattern as that of authentic (2-imino-5-oxo-imidazolidin-4-yl)urea (Table 1). This finding suggested that $\mathbf{II}_{red}$ and $\mathbf{II}'_{red}$ constituted a diastereomeric pair. The ^{1}H-NMR spectra of $\mathbf{II}_{red}$ and $\mathbf{II}'_{red}$ were essentially identical, a finding consistent with these compounds being diastereomers. Additionally, ESI-MS/MS experiments revealed that these compounds fragmented identically, yielding a major fragment in both cases with m/z = 113, which corresponds to the 5-amino-2-imino-imidazolidin-4-one portion of $\mathbf{II}_{red}/\mathbf{II}'_{red}$ (Fig. 2). Taken together, these data indicate that $\mathbf{II}_{red}/\mathbf{II}'_{red}$ are diastereomers of 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-imino-5-oxo-imidazolidin-4-yl)urea.

The H1′ proton of the 3,5-di-*O*-Ac-β-D-*erythro*-pentofuranosyl diastereomers of $\mathbf{II}_{red}$ appear as a doublet of doublets in DMSO-d_6 (J = 5.8 Hz and 9.5 Hz) as expected from coupling between the H1′ and the nonidentical H2′ and H2′′ protons. The expected coupling between the H1′ and the 1-NH protons was not observed. This finding likely arises because of rapid exchange

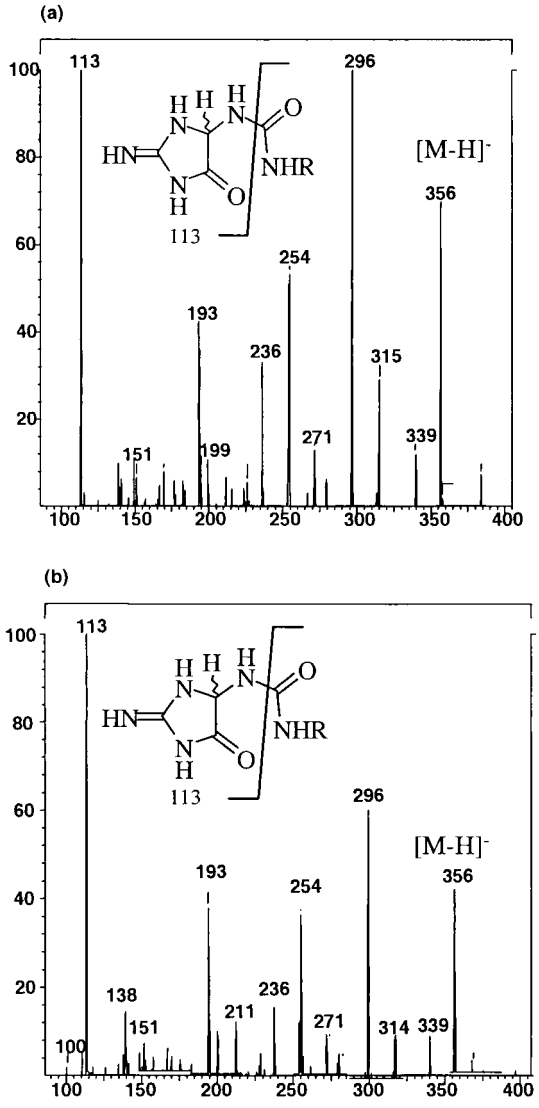

Fig. 2. Negative ion ESI-MS/MS spector for (*a*) $\mathbf{II}_{red}$ and (*b*) $\mathbf{II}'_{red}$. Both compounds fragment identically, a finding consistent with their assignment as the diastereomers of 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-imino-5-oxo-imidazolidin-4-yl)urea.

of the 1-NH proton with trace amounts of water present in the solution. However, in ^{1}H-NMR experiments carried out on the 2,3,5-tri-*O*-Ac-β-D-*erythro*-pentofuranosyl diastereomers of $\mathbf{II}_{red}$ in DMSO-d_6, the H1′ appeared as a doublet of doublets, indicating coupling of the H1′ to both the H2′ (J = 6.3 Hz) and the 1-NH proton (J = 12 Hz). On the other hand, when ^{1}H-NMR spectra were obtained in D_2O, the H1′ appeared as a doublet (J = 6.5 Hz). This indicates exchange of the 1-NH proton for deuterium with concomitant loss of coupling between the H1′ and 1-NH protons. These findings further support the structural assignment of $\mathbf{II}_{red}$ as 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-imino-5-oxo-imidazolidin-4-yl)urea.

The hydrolysis products of **II** also were characterized to gain further insight into its structure. Ammonium hydroxide-mediated hydrolysis yielded three compounds, **IIa**, **IIb**, and **IIc**. IIa (M_r = 112) has UV maxima at 249 nm and 313 nm and an HPLC retention time of 3.3 min. The dramatically shortened retention time relative to all of the primary reaction products along with the molecular weight of **IIa** indicated that the 3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl moiety, M_r = 201) was not attached to **IIa**. The UV

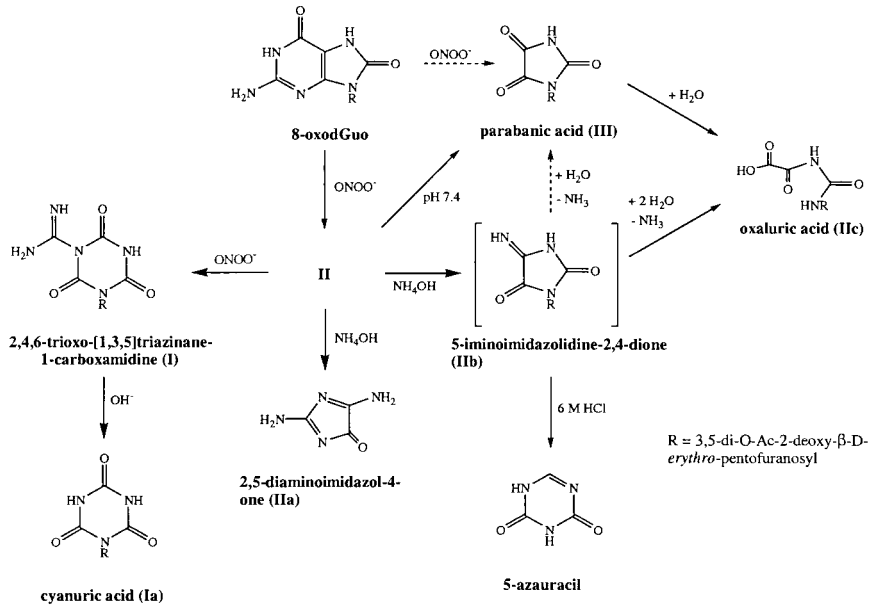

Fig. 3. Summary of the peroxynitrite reaction products of 3′,5′-di-*O*-Ac-8-oxodGuo.

spectrum of **IIa** was found to be identical to that reported for 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-*4H*-imidazol-4-one (39). Furthermore, the mass spectrum of **IIa** using GC/MS in electron ionization mode revealed ions at m/z = 328, 313, 285, and 198, a spectrum identical to that reported for 2,5-diaminoimidazol-4-one (29). Hence, **IIa** is identified as 2,5-diaminoimidazol-4-one.

The second hydrolysis product, **IIb** (M_r = 313), has λ_{max} = 225 nm and a shoulder from 260 to 310 nm and an HPLC retention time of 26.2 min. HRMS indicated that this compound has a molecular formula $C_{12}H_{15}N_3O_7$ (calculated exact mass: 313.0910; found: 313.0908) and hence, a base fragment with molecular formula, $C_3H_2N_3O_2$. Negative ion ESI-MS analysis of **IIb** performed by using H_2O and D_2O in the spray solvent resulted in $[M\text{-}H]^-$ ions at m/z = 312 and 313, respectively. This result indicated that **IIb** has two exchangeable protons, because in addition to having one deuteron in the observed ion, another deuteron was lost to form the observed negative ion. These exchangeable protons must be present in the base fragment because the 3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl moiety has no exchangeable protons. Therefore, **IIb** is identified as 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-5-iminoimidazolidine-2,4-dione. Further, as has been reported previously for substituted 5-iminoimidazolidine-2,4-dione compounds, strong acid or base treatment will cause ring expansion to yield s-triazine structures, including oxonic acid and 5-azauracil, by mechanisms that are not clearly understood (28). Indeed, acid hydrolysis of **IIb** led to release of an aglycon that was identified as 5-azauracil based on an identical GC/MS retention time and fragmentation pattern as authentic 5-azauracil (Table 1), thus further indirectly supporting the proposed structure for **IIb**.

Importantly, **IIb** is unstable and undergoes hydrolysis to yield **IIc** as a stable end product. **IIc**, as determined by negative ion ESI-MS, has a M_r = 332 and three exchangeable protons. To facilitate ^{1}H-NMR studies aimed at identifying the aglycon of **IIc**, the 2,3,5-tri-*O*-Ac-β-D-*erythro*-pentofuranosyl analog (**IIc′**), was used. **IIc′** was used because it could be prepared from the parent compound, 2′,3′,5′-tri-*O*-Ac-8-oxoGuo, which is easily accessible synthetically and in high yield. We verified that **IIc** and **IIc′** had identical UV/Vis spectra and that the molecular weight of **IIc′** (M_r = 390) was the expected 58 atomic mass units greater than that of **IIc** (M_r = 332).

The aglycon of **IIc′** (and hence that of **IIc**) was identified as oxaluric acid. Three nonsugar protons were observed at δ = 9.81 ppm (s, 1H, COOH), 8.87 ppm (d, 1H, J = 9.3 Hz, 3-NH), and 7.04 ppm (s, 1H, 1-NH). All three protons are D_2O exchangeable, in agreement with results determined by ESI-MS. The δ = 8.87 ppm peak is coupled to the sugar H1′ (δ = 5.51 ppm, 1H, J = 5.1 Hz, 9.3 Hz). Exchange of the 3-NH proton when D_2O is added results in H1′ appearing as a doublet with J = 5.1 Hz, which confirms that the sugar is attached at the N3 position. Therefore, it is concluded that **IIc** is 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)oxaluric acid. Indeed, if substitution were at the N1 position, both amide protons would be identical ($3\text{-}NH_2$) and no coupling between the $3\text{-}NH_2$ and H1′ would be expected.

Any structure proposed for **II** must account for the fact that reduction will yield the diastereomeric pair, $\mathbf{II}_{red}$ and $\mathbf{II'}_{red}$ and the observed hydrolysis products, **IIa**, **IIb,** and **IIc**. As shown in Fig. 1, reduction of **II** will directly yield the diastereomers $\mathbf{II}_{red}$ and $\mathbf{II'}_{red}$. Ammonium hydroxide-mediated ammonolysis or hydrolysis of **II** likely proceeds via nucleophilic attack at C4. Ammonolysis with elimination of (3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)urea leads to formation of **IIa**. Alternatively, ring closure to yield intermediate **II*** followed by elimination of guanidine yields **IIb** (Fig. 1), which ultimately hydrolyses to **IIc**.

Characterization of I and III. **I** and **III** were acid-hydrolyzed to obtain the aglycons. The trimethylsilyl derivative of the released aglycons was prepared and analyzed by GC/MS. The derivatized aglycons from **I** and **III** were found to have retention times and fragmentation patterns identical to those of authentic cyanuric acid and parabanic acid, respectively (Table 1). The molecular weight calculated for the 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)parabanic acid matched that observed by ESI-MS. Further, the UV spectrum of **III** (λ_{max} = 220 nm and 280 nm) matched that of the authentic parabanic acid. Therefore, **III** was identified as 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)parabanic acid. This compound also is formed as a hydrolysis product of **II** upon incubation in 150 mM KH_2PO_4, pH 7.4 buffer (Fig. 1) and also will yield **IIc** under the same conditions (Fig. 3).

In the case of **I**, the calculated molecular weight of the 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid (**Ia**) derivative (M_r = 329) was 42 atomic mass units lower than the molecular weight determined for **I** (M_r = 371) by ESI-MS. However, ESI-MS/MS of **I** revealed ions with m/z = 370, 328, and 285. The m/z = 370 ion corresponds to the $[M\text{-}H]^-$ ion of **I**. The m/z = 328 ion is the $[M\text{-}CH_3N_2\text{+}H]^-$ ion and corresponds to the ion expected for **Ia**. The m/z = 285 ion arises because of neutral loss of an [HN=C=O] fragment from the parent ion of **Ia**. These data indicate that **Ia** can arise from **I** either by hydrolysis or under ESI-MS/MS conditions and taken together led us to identify **I** and **Ia** as 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-2,4,6-trioxo-[1,3,5]triazinane-1-carboxamidine and 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid, respectively, both of which also are formed during the reaction of 8-oxodGuo with singlet oxygen (30).

Conclusion

Fig. 3 summarizes both the immediate and subsequent hydrolysis products formed by the reaction of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$. **II** is the major product, whereas **I** and **III** are formed in lower amounts. **I**, **II**, and **III** are present in the reaction mixture immediately after $ONOO^-$ treatment. Interestingly, formation of **II** plateaus in the presence of unreacted 3′,5′-di-*O*-Ac-8-oxodGuo, suggesting that it might be reacting further with $ONOO^-$. Indeed, treatment of isolated **II** with $ONOO^-$ led to formation of **I**, indicating that **I** can arise as a further oxidation product of **II**. Both **I** and **III** do not react further with $ONOO^-$ and hence are terminal oxidation products of the $ONOO^-$-mediated oxidation of 3′,5′-di-*O*-Ac-8-oxodGuo.

All three products are hydrolytically labile. In 150 mM KH_2PO_4, 25 mM Na_2CO_3, pH 7.2 and at ambient temperature, **II** has a half-life of 5 hr. This compound is therefore relatively more labile than **I** whose half-life previously was determined to be days (30). However, **IIc** is quite stable and little degradation occurs even after days of incubation at room temperature in pH 7.2 buffer. We have shown that **I** can arise from the further reaction of **II** with $ONOO^-$. At present, however, it is not possible to definitively say whether **III** arises solely as a hydrolysis product of **II** or whether it is also a direct product of the reaction of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$.

The instability of **II** has impaired our efforts to rigorously characterize this compound. However, the reduction products, in particular, are highly informative about its structure. Reduction leads to formation of the diastereomers, $\mathbf{II}_{red}$ and $\mathbf{II'}_{red}$, which is most consistent with the assignment of **II** as 1-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-imino-5-oxo-imidazolidin-4-ylidene)urea. Presently, we cannot completely exclude the possibility that **II** is the ring-closed structure, 3-(3,5-di-*O*-Ac-2-deoxy-β-D-*erythro*-pentofuranosyl)-3a-hydroxy-5-imino-3,3a,4,5-tetrahydro-1H-imidazo[4,5-d]imidazol-2-one. The bicyclic and the ring-opened structures are not mutually exclusive possibilities, however, as the former structure is highly strained and may be in equilibrium with the ring-opened structure.

In conclusion, we have identified the major immediate and hydrolysis products of the reaction of 3′,5′-di-*O*-Ac-8-oxodGuo with $ONOO^-$. Under our experimental conditions, **II** is the major product at the low $ONOO^-$ treatment levels. This is important when considering the reaction of 8-oxoGua lesions in DNA with $ONOO^-$. Our experiments indicate that these lesions also are formed in oligonucleotides containing an 8-oxoGua residue (40). Understanding the impact of the reaction of 8-oxoGua in DNA with $ONOO^-$ will require studying specifically the formation and fate of **II** in DNA. The half-life of **II** at physiologic pH (5 hr) indicates that within 2 days of formation, it is completely hydrolyzed to its stable hydrolysis product, **IIc**. Lesions bearing remarkable structural resemblance to **IIc**, for example the glycoside of β-ureidoisobutyric acid, have been shown to be premutagenic and to function as potent blocks to DNA replication (41).

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