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Nitration of 2′-Deoxyguanosine by a NO/O_2 Gas Mixture: Identification and Characterization of N^2-Nitro-2′-deoxyguanosine

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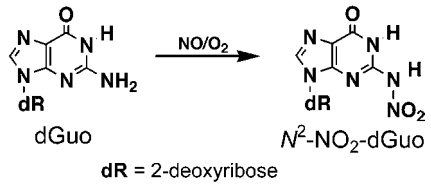
ABSTRACT



A gas mixture of NO and O_2 was bubbled into 2′-deoxyguanosine solution at neutral pH and 37 °C. A novel nitrated nucleoside was generated in the reaction mixture in addition to 8-nitroguanine, 8-nitroxanthine, 2′-deoxyxanthosine, xanthine, and guanine. The novel nucleoside was identified as N^2-nitro-2′-deoxyguanosine by spectrometric data.

Nitric oxide (NO) is a messenger molecule mediating various physiological functions.[1] Today inhaled NO is used as a vasodilator for newborns with persistent pulmonary hypertension, despite its clinical risk.[2] However, NO is also a major constituent of air pollutant.[3] NO can react with biomolecules such as protein and DNA to exert cytotoxicity and genotoxicity.[4] Nucleic acid bases are converted to their deaminated derivatives by incubation with NO under aerobic but not unaerobic conditions, suggesting that reactive nitrogen species generated from the reaction of NO and molecular oxygen (O_2) cause the deamination.[5−7] Reaction of 2′-deoxyguanosine (dGuo) with NO under aerobic conditions generates 2′-deoxyxanthosine (dXao) as a major deaminated product[5−7] and 2′-deoxyoxanosine (dOxo)[8] as a byproduct. Recently, our group has reported that 8-nitroguanine (8-NO_2-Gua), which is a product from dGuo by reaction with several reactive nitrogen species such as peroxynitrite ($ONOO^-$) and nitryl chloride (NO_2Cl),[9−11] is also formed from dGuo by reaction with a gas mixture of NO and O_2.[12] In the present

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study, we report the formation, identification, and characterization of a novel nitrated nucleoside in the reaction of dGuo with the gas mixture of NO and O_2 in addition to 8-NO_2-Gua, 8-nitroxanthine (8-NO_2-Xan), dXao, xanthine (Xan), and guanine (Gua).

An aqueous dGuo solution (100 μmol/10 mL) was treated by the gas mixture of NO (0.5 mL/s) and O_2 (2.0 mL/s) at pH 7.4 and 37 °C until 10 mmol of NO was consumed, and the solution was subsequently allowed to stand for 3 h at room temperature.[13] Figure 1 shows a reversed-phase HPLC

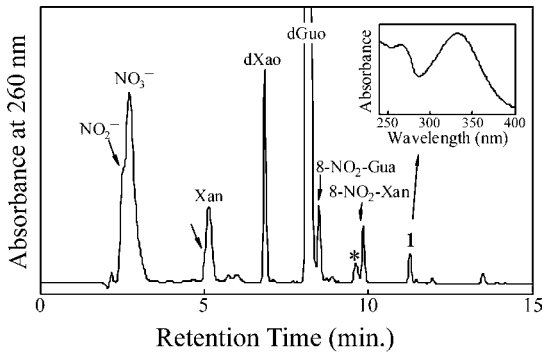

Figure 1. RP-HPLC chromatogram of the reaction mixture of dGuo with the gas mixture of NO and O_2. The inset is the on-line detected UV spectrum of **1**.

(RP-HPLC) chromatogram of the reaction mixture.[14] Several product peaks were detected in addition to void peaks including NO_2^- and NO_3^- and the starting dGuo peak. Among the product peaks, a peak with the retention time (t_R) of 5.1 min contained Xan with a small amount of Gua. The products of t_R = 6.8 and 8.5 min were identified as dXao and 8-NO_2-Gua, respectively. A peak of t_R = 9.6 min denoted by an asterisk in Figure 1 contained a trace amount of dOxo overlapped by another unknown product. The identification of these products was based on their identical retention times and UV spectra with the authentic samples.[15] The product of t_R = 9.8 min was identified as 8-NO_2-Xan on the basis of coincidence of their retention time, UV spectrum (λ_{max} = 382 nm), and APCI-MS spectrum (negative, m/z = 196) with those of the authentic sample.[15]

An unknown product (termed **1**) was eluted at t_R = 11.3 min showing a UV spectrum with a λ_{max} = 330 nm (Figure 1, inset). Compound **1** was isolated by preparative RP-HPLC and subjected to structural assignment.[16] ^{1}H NMR spectrum of **1** in DMSO-d_6 showed signals for an aromatic proton and an exchangeable proton with a set of signals derived from 2′-deoxyribose moiety. The ^{13}C NMR spectrum showed 10 signals, of which five were in the aromatic region. APCI-MS (negative) showed a signal at m/z = 311 as the deprotonated molecular ion $[M - H]^-$, which was 45 mass units greater than that of the starting dGuo. Additionally, signals at m/z = 195, 166, and 150 assignable to base fragments $[M_{base}]^-$, $[M_{base} + H - NO]^-$, and $[M_{base} + H - NO_2]^-$, respectively, were observed. High-resolution FAB-MS (negative) of **1** showed m/z = 311.0743 for the deprotonated molecular ion, which agreed with the theoretical molecular mass for composition $C_{10}H_{11}O_6N_6$ within 0.3 mmu. IR exhibited absorption bands at 1561 and 1304 cm^{-1} attributable to asymmetric and symmetric vibrations of a nitro group in a nitramine (−NH−NO_2), respectively.[17] Combining these spectrometric data, we identified **1** as a nitrated derivative of dGuo on the exocyclic amino group, N^2-nitro-2′-deoxyguanosine (N^2-NO_2-dGuo) (Figure 2). We detected

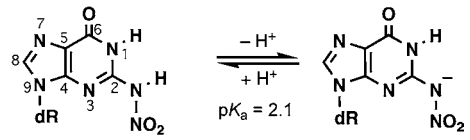

Figure 2. Structure of N^2-NO_2-dGuo and its possible acid−base equilibrium. dR stands for 2-deoxyribose. The numbers designate the atomic position.

only one exchangeable proton signal derived from N^2-NO_2-dGuo, although two exchangeable proton signals were expected for this structure. The pH titration of isolated N^2-NO_2-dGuo solution using absorbance at 260 nm showed the existence of two acid−base equilibria of pK_a = 2.1 and 9.2.

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(13) **Reaction Conditions.** dGuo (100 μmol) was dissolved in 10 mL of 100 mM sodium phosphate buffer (pH 7.4) in an open vessel. An NO/O_2 gas mixture with flow rates of 0.5 mL/s for NO and 2.0 mL/s for O_2 was bubbled into well-stirring dGuo solution at 37 °C. The pH of the solution was maintained at 7.4 ± 0.4 by the titration of 1 M NaOH throughout the reaction. NO dose was measured as the moles of NaOH required to neutralize the solution. The reaction mixture was allowed to stand for 3 h at room temperature before RP-HPLC analysis. The NO/O_2 gas mixture was prepared as follows. NO (99.8%, Kyoto Teisan, Kyoto, Japan) was passed through soda lime and then mixed with O_2 (99.5%, Kyoto Teisan) at a Y-type connector. The mixed gas was run through a Teflon-lined tube (5.0 × 200 mm) and bubbled into the dGuo solution by a glass frit (4.0 × 200 mm, pore size 20−30 μm, Kinoshita Rika, Tokyo, Japan).

(14) **HPLC Conditions.** RP-HPLC analyses were performed using an octadecylsilane column (ULTORON VX-ODS, 6.0 × 150 mm, particle size 5 μm, Shinwa Chemical Industry, Kyoto, Japan) eluted with 100 mM triethylammonium acetate buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 20% for 20 min in a linear gradient mode. The flow rate was 1.5 mL/min.

(15) **Authentic Samples.** Gua and Xan were purchased. dXao and dOxo were prepared from dGuo by reaction with nitrous acid (see ref 8). 8-NO_2-Gua was synthesized from Gua by reaction with peroxynitrite (see ref 9). 8-NO_2-Xan was synthesized from Xan by the treatment with nitric acid (see ref 10). The samples synthesized were purified by RP-HPLC.

(16) **Spectrometric Data of N^2-Nitro-2′-deoxyguanosine (1).** ^{1}H NMR (600 MHz, in DMSO-d_6 at 30 °C): δ (ppm/TMS) 12.3 (br, 1H, NH), 8.01 (s, 1H, H8), 6.13 (dd, $J_{1'2'}$ = 7.1 Hz, $J_{1'2''}$ = 6.7 Hz, 1H, H1′), 5.28 (br, 1H, 3′-OH), 5.02 (br, 1H, 5′-OH), 4.31 (ddd, $J_{3'4'}$ = 2.8 Hz, 1H, H3′), 3.78 (ddd, $J_{4'5'}$ or $J_{4'5''}$ = 2.6 or 4.3 Hz, 1H, H4′), 3.48 (ABX, $J_{5'5''}$ = 40.1 Hz, 2H, H5′, 5″), 2.51 (ddd, 1H, H2′ or H2″), 2.16 (ddd, 1H, H2′ or H2″). ^{13}C NMR (125 MHz, in DMSO-d_6 at 30 °C): δ (ppm/TMS) 155.8, 154.0, 149.3, 137.2 (C8), 118.4, 87.9 (C4′), 83.4 (C1′), 70.7 (C3′), 61.6 (C5′), 39.8 (C2′). The signal assignments for ^{1}H and ^{13}C resonances were performed by TOCSY, ^{1}H−^{13}C HMQC, and ^{1}H−^{13}C HMBC. IR (KBr): 3418, 1688, 1561, 1520, 1468, 1396, 1304, 1219, 1094, 1063, 943, 781, 725, 642 cm^{-1}. UV: λ_{max} 272 and 319 nm (pH 1), 266 and 330 nm (pH 7), 244 nm (pH 11). APCI-MS (negative, CH_3OH): m/z 311, 200, 195, 186, 166, and 150; HR-MS (FAB, negative, CH_3OH): m/z = 311.0743 $[M - H]^-$ (calcd for $C_{10}H_{11}N_6O_6$, 311.0740).

At neutral pH, an anion-exchange resin trapped N^2-NO_2-dGuo completely, whereas a cation-exchange resin did not. Generally, primary nitramines are weak acids.[17−19] Thus, the acid−base equilibrium of $pK_a = 2.1$ is attributable to protonation-deprotonation of the nitramino group of N^2-NO_2-dGuo, indicating that N^2-NO_2-dGuo exists as the anionic form in neutral solution (Figure 2). In the NMR solvent DMSO-d_6, N^2-NO_2-dGuo may exist as the anion form due to a trace of H_2O present in the solvent or the sample, thus obscuring the nitramine proton signal. Under the above reaction conditions, the yields for each product relative to the starting dGuo were 0.35% Gua, 0.86% Xan, 1.60% dXao, 0.31% 8-NO_2-Gua, <0.10% dOxo, 1.33% 8-NO_2-Xan, and 0.14% N^2-NO_2-dGuo.[20]

Figure 3A shows NO dose-dependent changes of the yields of the nitrated products, N^2-NO_2-dGuo, 8-NO_2-Gua, and

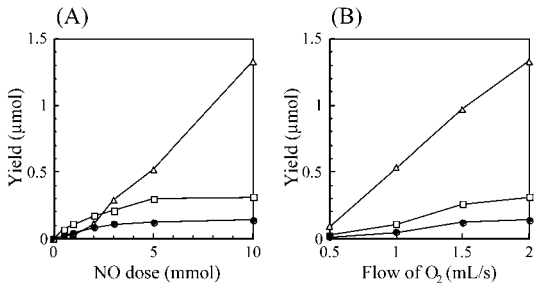

Figure 3. (A) NO dose- and (B) O_2 flow rate-dependence of the yields for N^2-NO_2-dGuo (●), 8-NO_2-Gua (□), and 8-NO_2-Xan (△).

8-NO_2-Xan, generated from dGuo by the NO/O_2 (0.5/2.0 mL/s) gas mixture. The yields of all of the nitrated products increased with increasing NO dose, while the yield of 8-NO_2-Xan increased significantly at high doses of NO. Figure 3B shows changes of the yields at 10 mmol NO dose when the O_2 flow rate decreased from 2.0 to 0.5 mL/s with the fixed flow rate of NO (0.5 mL/s). The yields of all the nitrated products decreased as the O_2 flow decreased. Figure 4 shows the yields at various pHs, when dGuo was reacted with the NO/O_2 (0.5/2.0 mL/s) gas mixture of 10 mmol NO dose.

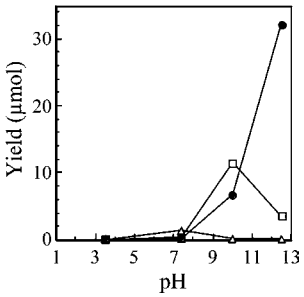

Figure 4. pH-dependence of the yields of N^2-NO_2-dGuo (●), 8-NO_2-Gua (□), and 8-NO_2-Xan (△). The pHs were maintained within the indicated values ± 0.5.

The yield of N^2-NO_2-dGuo increased extremely at basic pH. At pH 12.5, N^2-NO_2-dGuo was produced in 32% yield relative to the starting dGuo. The stability of N^2-NO_2-dGuo was evaluated under physiological and mildly acidic conditions. No decomposition was observed by RP-HPLC when the isolated N^2-NO_2-dGuo was incubated at pH 7.4 and 37 °C for 1 week. At pH 4.0 and 70 °C, N^2-NO_2-dGuo disappeared with a first-order rate constant of 1.2×10^{-4} s^{-1}, which was greater than that for dGuo (4.8×10^{-5} s^{-1}) but smaller than that for dXao (1.0×10^{-3} s^{-1}).

Possible pathways for the formation of the products are summarized in Scheme 1. Nitration, deamination, and depurination occur simultaneously in the reaction of dGuo with the NO/O_2 gas mixture. dGuo is converted via 8-nitro-2′-deoxyguanosine (8-NO_2-dGuo) to 8-NO_2-Gua by nitration and subsequent depurination. Reportedly, 8-NO_2-dGuo is unstable and undergoes depurination, readily releasing 8-NO_2-Gua with a half-life of less than 3 min at pH 7.4 and 37 °C.[21,22] Under the present conditions, almost all 8-NO_2-dGuo formed would be converted to 8-NO_2-Gua since a typical bubbling period of the NO/O_2 gas mixture of 10 mmol NO dose is 11 min and a reaction mixture is allowed to stand for 3 h at room temperature before RP-HPLC analysis. dGuo is also converted via Gua to 8-NO_2-Gua by depurination and subsequent nitration. We confirmed that Gua was nitrated readily by the NO/O_2 gas mixture, resulting in 8-NO_2-Gua as a major product (data not shown). On the other hand, dGuo gives rise to dXao by deamination. Analogous to dGuo, dXao is converted to 8-NO_2-Xan via 8-nitro-2′-deoxyxanthosine (8-NO_2-dXao) or via Xan since 8-NO_2-dXao is unstable to release 8-NO_2-Xan[10] and since Xan was nitrated readily to form 8-NO_2-Xan exclusively by the NO/O_2 gas mixture (data not shown). Independently of the formation of these products, N^2-NO_2-dGuo is generated by nitration on the exocyclic amino group of dGuo and stays as the nucleoside form in the reaction mixture since N^2-NO_2-dGuo is stable.

Several intermediates such as ONOO, ONOONO, NO_2, and N_2O_3 have been proposed in autoxidation of NO until NO_2^- is generated finally.[23−26] None of these compounds, however, can be candidates of the reactive intermediates to

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(20) **Quanitative Procedures.** Yields of the products were estimated from integrated peak areas of the HPLC chromatogram detected at 260, 382, or 400 nm and the molar extinction coefficients (ϵ). The ϵ_{260} values of 7.5×10^3 M^{-1} cm^{-1} for Gua, 8.5×10^3 M^{-1} cm^{-1} for Xan, 7.8×10^3 M^{-1} cm^{-1} for dXao, and 5.1×10^3 M^{-1} cm^{-1} for dOxo were used (see ref 8 and Henle, E. S.; Luo, Y.; Gassmann, W.; Linn, S. *J. Biol. Chem.* **1996**, *271*, 21177−21186). The ϵ_{400} of 9.1×10^3 M^{-1} cm^{-1} for 8-NO_2-Gua and the ϵ_{382} of 7.7×10^3 M^{-1} cm^{-1} for 8-NO_2-Xan were used (see ref 10). The ϵ_{260} of N^2-NO_2-dGuo was estimated as 9.5×10^3 M^{-1} cm^{-1} by the comparison of the integration of H1′ proton signal and the RP-HPLC peak area detected at 260 nm with those for dGuo. Gua, Xan, and dOxo were separated and quantified using another HPLC eluent of 100 mM ammonium formate (pH 6.3) with acetonitrile, while 100 mM triethylammonium acetate (pH 7.0) was used as the eluent buffer for the nitrated products and dXao.
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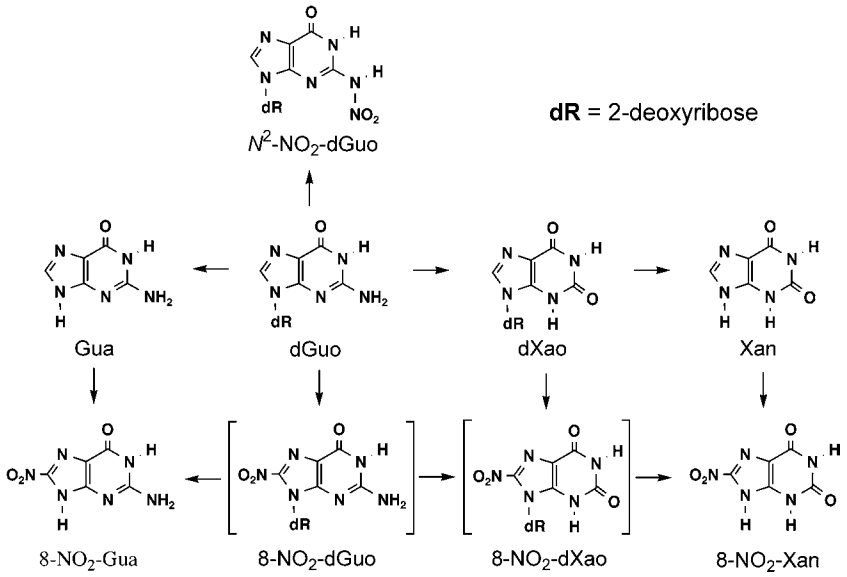
Scheme 1. Proposed Pathway for Reaction of dGuo with Gas Mixture of NO and O_2



nitrate dGuo directly, because of low reactivity of these compounds with dGuo.[25−28] Recently, Malhotra and Ross[29] have reported that aromatic compounds in liquid nitrogen tetroxide (N_2O_4), a liquid phase of NO_2, are nitrated by simultaneous passage of NO and O_2. They proposed the nitration mechanism as follows. The initial reaction of NO and O_2 produces ONOO. In the presence of excess NO_2, ONOO leads to nitrate radical (NO_3), a strong one-electron oxidant:

$$\text{ONOO} + (\text{excess } NO_2) \rightarrow NO_3 + (\text{excess } NO_2)$$

NO_3 oxidizes the aromatics to give their cation radicals. The cation radicals react with NO_2, forming nitrated aromatics. In the present study, NO_3 can be generated because a relatively large amount of NO_2 would exist in the NO/O_2 gas mixture. The formation of the nitrated products from dGuo may be explained by this nitration mechanism as follows. The NO_3 formed in the NO/O_2 gas mixture reacts with dGuo, resulting in a cation radical of dGuo. The cation radical of dGuo then deprotonates to become the neutral radical of dGuo in neutral solution since the pK_a of the equiribilium between the cation radical and the neutral radical is 3.9.[30] The cation or neutral radical of dGuo (or Gua) reacts with NO_2 at C8 to form 8-NO_2-dGuo (or 8-NO_2-Gua). Analogously, dXao (or Xan) is nitrated at the C8 position. If NO_2 reacts with the cation or neutral radical of dGuo on its exocyclic amino group, N^2-NO_2-dGuo can be generated. Another candidate of the reactive intermediate for the nitration is dinitrogen pentoxide (N_2O_5), since NO_3 can react with NO_2 to form N_2O_5:[31]

$$NO_3 + NO_2 \rightarrow N_2O_5$$

N_2O_5, a strong nitrating agent, can nitrate various compounds including aromatics and amines.[32] The nitration reactions are believed to be via the nitronium ion (NO_2^+).[32] NO_2^+ may react directly with C8 of dGuo, Gua, dXao, and Xan, resulting in their 8-nitro derivatives. When NO_2^+ attacks the exocyclic amino group of dGuo, N^2-NO_2-dGuo can be formed.

In conclusion, we found a novel nitrated nucleoside, N^2-NO_2-dGuo, in the reaction of dGuo with a gas mixture of NO and O_2 at neutral pH and 37 °C in addition to 8-NO_2-Gua, 8-NO_2-Xan, dXao, Xan, and Gua. N^2-NO_2-dGuo was a major product under basic conditions. Involvement of NO_3 and N_2O_5 was suggested as the reactive intermediate for the nitration. The isolated N^2-NO_2-dGuo was stable under physiological conditions. Studies would be required for N^2-NO_2-dGuo on formation and significance in biological systems.

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