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Formation of diazoate intermediate upon nitrous acid and nitric oxide treatment of 2′-deoxyadenosine

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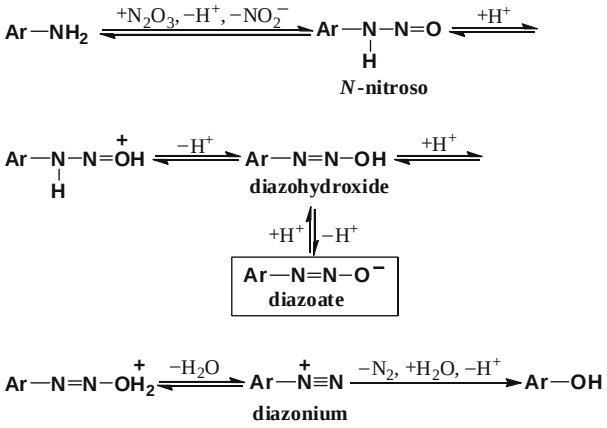
ABSTRACT

When 2′-deoxyadenosine was treated with HNO_2 or NO, a small amount of a previously unidentified product was formed. The product was also formed by the reaction of 2′-deoxyadenosine with isoamyl nitrite in tetrahydrofuran as a major product. The product was identified as a diazoate derivative of 2′-deoxyadenosine, a reaction intermediate. At the initial stage of the HNO_2 or NO reaction, the concentration of the diazoate was greater than or comparable to 2′-deoxyinosine, a deamination product of 2′-deoxyadenosine. The diazoate was fairly stable and decomposed with a half-life of 66 h at pH 7.4 and 37 °C. These results suggest that the diazoate can be formed in cellular nucleosides or DNA with biologically relevant dose of HNO_2 and NO.

Nitrous acid (HNO_2) generated by protonation of nitrite is known to be mutagenic to living cells.[1,2] Under mildly acidic conditions, the real active species of nitrous acid is thought to be dinitrogen trioxide (N_2O_3).[3] Nitric oxide (NO) is synthesized in various types of cells by the enzyme nitric oxide synthase and is involved in numerous biological functions, including vasodilation, neurotransmission, and inflammation.[4,5] Although NO is a radical, the self-reactivity of NO is relatively low.[6] However, in the presence of O_2, NO is converted to N_2O_3.[3] N_2O_3 can react with amino groups in various biological molecules resulting in corresponding *N*-nitroso compounds. Amino groups of nucleic acid components react with N_2O_3 resulting in deamination compounds[7,8] since the formed *N*-nitroso compounds are unstable and subsequent hydrolyses are performed. Deamination of nucleobases by N_2O_3 is thought to proceed via several intermediates including diazoahydroxide, diazoate, and diazonium derivatives based on the diazo chemistry of aromatic primary amines (Scheme 1).[9] The detection and isolation of an intermediate in the reaction of 2′-deoxycytidine with HNO_2 and NO was reported.[10] The intermediate was identified as a diazoate derivative of 2′-deoxycytidine. At pH 7.4 and 37 °C, the diazoate intermediate was stable and converted to 2′-deoxyuridine with a half-life of 330 h. The diazoate intermediate of guanosine was also reported, although it was unstable with a half-life of 5.6 min at pH 7.0 and 20 °C.[11] A diazoate derivative of 5-methyl-2′-deoxycytidine with a half-life of 21 h at pH 7.4 and 37 °C was also reported.[12] For adenine nucleobase, a diazoate derivative of 9-propyladenine was reported as a major product of the reaction of 9-propyladenine with isoamyl nitrite and $NaNH_2$ in tetrahydrofuran.[13] In this study, we examined the reaction of 2′-deoxyadenosine (dAdo) with HNO_2 or NO, and found the formation of a diazoate intermediate of dAdo (dAdo-diazoate). This paper reports the identification and characterization of dAdo-diazoate.

When 10 mM dAdo, which had been purified by RP-HPLC (reversed phase high performance liquid chromatography), was



Scheme 1. Proposed reaction pathways for the nitrosative deamination of aromatic amines (Ar–NH_2) by N_2O_3.

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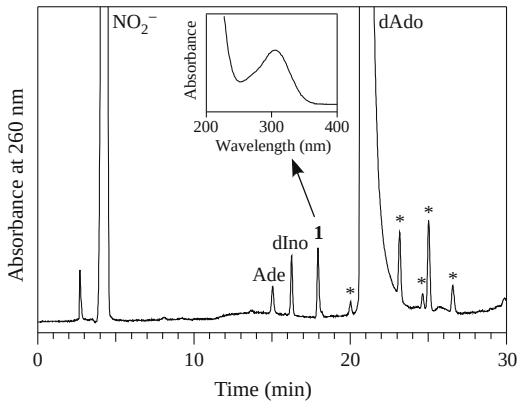


Figure 1. RP-HPLC chromatogram for HNO_2-treated dAdo detected at 260 nm. The inset is the on-line UV spectrum of **1**. A solution of 10 mM dAdo was incubated with 100 mM $NaNO_2$ in 100 mM potassium phosphate buffer at pH 3.7 and 37 °C for 5 min. The sample was separated by an ODS column (4.6 × 250 mm). The eluent was 20 mM triethylammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0% to 50% for 30 min with a linear gradient mode. The column temperature was 40 °C and the flow rate 1 mL/min. The peaks indicated by asterisks are impurities in the starting dAdo.

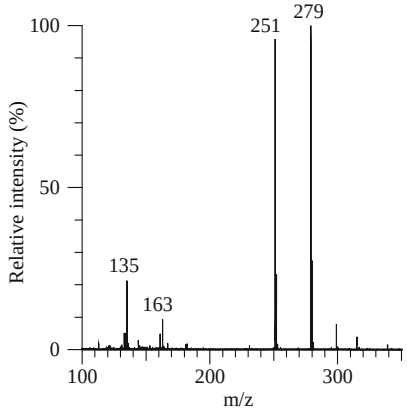


Figure 2. Negative ion electrospray ionization time of flight mass spectrometry (ESI-TOF/MS, MicroTOF, Bruker) spectrum of **1**, a diazoate derivative of dAdo (dAdo-diazoate).

incubated with 100 mM $NaNO_2$ in 100 mM potassium phosphate buffer at pH 3.7 and 37 °C for 5 min, several product peaks appeared in the RP-HPLC chromatogram (Fig. 1). In addition to peaks of 2′-deoxyinosine (dIno), a deamination product, and adenine (Ade), a depurination product, a small amount of a previously unidentified product peak (termed **1**) appeared at the retention time of 17.9 min. The product showed a UV spectrum with λ_{max} = 306 nm (inset of Fig. 1). A product with the same retention time and UV spectrum was also formed by the reaction of dAdo with isoamyl nitrite in tetrahydrofuran as a major product.[14] The product formed by isoamyl nitrite was collected and subjected to spectrometric measurements.[15] The ^{1}H NMR spectrum of the product showed two aromatic protons, H2 and H8, slightly shifted to the downfield relative to dAdo. In addition, a set of signals of intact 2-deoxyribose moiety was observed. However, no exchangeable proton signal indicative of an amino group was observed. The ^{13}C NMR spectrum of the product showed five aromatic protons and five aliphatic protons. Negative ion ESI-TOF/MS showed a signal *m*/*z* 279 attributable to the molecular ion (Fig. 2). In addition, a signal *m*/*z* 251 attributable to a fragment ion produced by loss of molecular nitrogen through a four-center skeletal rearrangement specific to aromatic diazoate compounds was observed.[16,17] High-resolution ESI-TOF/MS (negative) of the molecular ion showed *m*/*z* 279.084971, which agreed with the theoretical molecular mass for $C_{10}H_{11}N_6O_4$ composition within 0.3 mmu (1 ppm). Combining these data, we concluded that the product was a diazoate derivative of dAdo (dAdo-diazoate), 9-(β-D-2′-deoxyribofuranosyl)-purine-4-diazoate.

The time courses of concentration change in dAdo-diazoate, dIno, and Ade are shown in Figure 3A when 10 mM dAdo was incubated with 100 mM $NaNO_2$ in 100 mM potassium phosphate buffer at pH 3.7 and 37 °C. The concentration of dAdo-diazoate increased rapidly in the initial stage of the reaction and reached a plateau, showing the characteristic profile of a reaction intermediate. The concentration of dAdo-diazoate was greater than that of dIno up to 5 min. In addition, a small amount of Ade was generated, probably due to acidic depurination, which includes protonation at the N7 atom of dAdo and subsequent cleavage of the *N*-glycosidic bond.

The time courses of concentration change in dAdo-diazoate, dIno, and Ade are shown in Figure 3B when NO was bubbled into a 10 mM dAdo solution with 100 mM potassium phosphate buffer

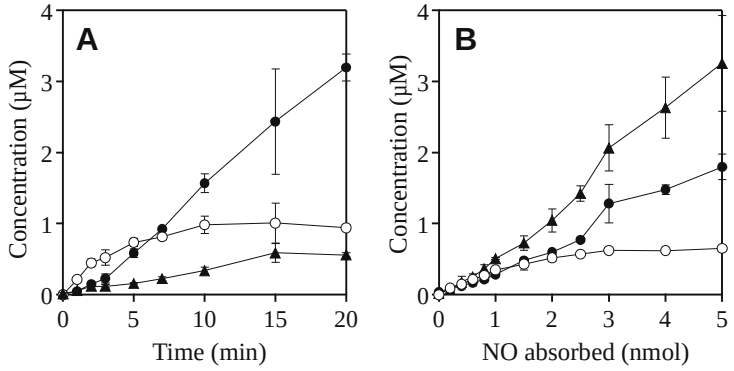


Figure 3. (A) The time course of the concentration changes in dAdo-diazoate (open circle), dIno (closed circle), and Ade (closed triangle) when 10 mM dAdo was incubated with 100 mM $NaNO_2$ in 100 mM potassium phosphate buffer (1 mL) at pH 3.7 and 37 °C in a microtube. Means ± SD (n = 3) are presented. (B) Correlation of the amounts of dAdo-diazoate (open circle), dIno (closed circle), and Ade (closed triangle) with that of absorbed NO. 10 mM dAdo was dissolved in 10 mL of 100 mM potassium phosphate buffer (pH 7.4) at 37 °C in a 20 mL beaker, and NO was bubbled at a flow rate of 1.0 mL/s into the stirring solutions under aerobic conditions. The pH of the solutions was maintained between 7.2 and 7.6 by the titration of 1 M NaOH throughout the reaction. The amount of NO absorbed means the amount of NaOH added. Means ± SD (n = 2) are presented. The concentration was determined by RP-HPLC. The concentrations of dIno and Ade in the reaction mixtures were evaluated from integrated peak areas on HPLC chromatograms detected at 260 nm, compared with those of authentic standard solutions. The concentration of the diazoate was estimated from the integrated peak areas of HPLC chromatograms and molar extinction coefficients (ε) at 300 nm. The ε_{300nm} of the diazoate was estimated as 1.77 × 10^4 M^1 cm^1 from the UV spectrum and the ε_{260nm} of the diazoate which was determined by integration of the H2 and H8 proton signals and RP-HPLC peak area detected at 260 nm relative to those of dAdo.

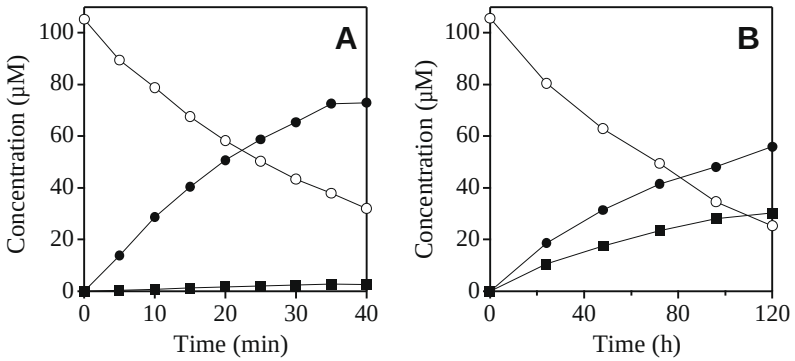
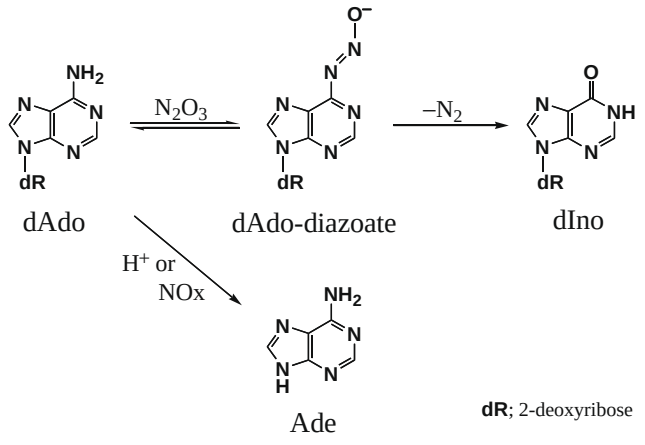


Figure 4. The time course of the concentration changes in dAdo-diazoate (open circle), dIno (closed circle), and dAdo (closed square) when isolated 105 μM dAdo-diazoate was incubated in 100 mM potassium phosphate buffer at (A) pH 3.7 and (B) pH 7.4 at 37 °C in a microtube. The concentration was determined by RP-HPLC.

at neutral pH and 37 °C. The concentration of dAdo-diazoate was comparative to those of dIno and Ade at the initial stage of the reaction (<1 nmol NO). In addition, a greater amount of Ade was generated. The release of Ade from dAdo is probably catalyzed by addition of NO^+, generated from nitrogen oxides (NO_X), at the N7 atom of dAdo.[18]

To clarify the stability of the diazoate, the isolated dAdo-diazoate was incubated in potassium phosphate buffer (pH 3.7 or 7.4) at 37 °C and the concentrations of dAdo-diazoate, dIno, and dAdo were monitored by RP-HPLC. dAdo-diazoate was decomposed quickly under acidic conditions with a half-life of 24 min at pH 3.7 and 37 °C, forming dIno almost exclusively (Figure 4A). Under the neutral conditions, dAdo-diazoate was stable at pH 7.4 and 37 °C and decomposed slowly with a half-life of 66 h (Fig. 4B). In addition to dIno, dAdo was generated with a half amount of dIno. All steps of the nitrosative deamination were reversible except for a step of N_2 release (Scheme 1). Under acidic conditions, dAdo-diazoate should be protonated resulting in N_2 release and subsequent formation of the deaminated product. Under neutral conditions, the forward reactions become slow and the backward reactions to form dAdo would increase relatively, resulting in an increase of dAdo production (Scheme 2).

Hypoxanthine (Hyp), a base moiety of dIno, can form a relatively stable base pair with cytosine in DNA.[19] When a DNA containing Hyp is replicated, 2′-deoxycytidine monophosphate is incorporated opposite Hyp. Thus, Hyp formation at the Ade site induces AT to GC transition mutation almost exclusively.[20,21] In cells, Hyp generated in DNA is repaired by a specific enzyme, hypoxanthine-DNA glycosylase, that catalyzes the release of free Hyp from DNA containing Hyp. A hypoxanthine-DNA glycosylase has been found in extracts of *E. coli* and HeLa cells.[22,23] It has been reported that HNO_2 and NO induce AT to GC transition mutations in addition to GC to AT transition mutations.[24,25] DNA damage for Ade moiety caused by HNO_2 and NO is elucidated only in the formation of Hyp, since studies for byproducts and intermediates in the reaction are few. In the present study, we found a previously unidentified product in the reaction of dAdo with HNO_2 and NO. The product was also generated in the reaction of dAdo with isoamyl nitrite as a major product. From the spectrometric date, the product was identified as a diazoate intermediate of dAdo. In the initial stage of HNO_2 reaction with dAdo, the diazoate derivative was the major product. In the initial stage of NO reaction, the concentration of diazoate derivative was comparative to dIno and Ade. dAdo-diazoate was stable under physiological conditions. The present results imply that dAdo-diazoate may have importance in elucidating the genotoxic effects of HNO_2 and NO.



Scheme 2. Proposed reaction pathways for the reaction of dAdo with HNO_2 or NO under aerobic conditions.

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14. Reaction conditions for isoamyl nitrite reaction. dAdo 1 mmol was treated with isoamyl nitrite 1.1 mmol and $NaNH_2$ 1 mmol in dry tetrahydrofuran (40 mL) under N_2 at room temperature for 14 days. Compound **1** was collected by RP-HPLC, using the same system for analysis, with an eluent of 5% MeOH in an isocratic mode and vacuum dried.
15. Spectrometric data of **1** [9-(β-D-2′-deoxyribofuranosyl)-purine-4-diazoate]. ^1H NMR (500 MHz, DMSO-d_6 at 22 °C): δ (ppm/TMS) 8.39 (s, 1H, H-2), 8.35 (s, 1H, H-8), 6.41 (dd, $J_{1'2'}$ = 7.8 Hz, $J_{1'2''}$ = 5.7 Hz, 1H, H-1′), 5.31 (br, 1H, 3′- or 5′-OH),

5.22 (br, 1H, 3′- or 5′-OH), 4.43 (ddd, $J_{3'4'}$ = 3.6 Hz, 1H, H-3′), 3.89 (ddd, $J_{4'5'}$ = 3.3 Hz, $J_{4'5''}$ = 3.3 Hz, 1H, H-4′), 3.59 (ABX, $J_{5'5''}$ = 11.1 Hz, 2H, H-5′,5″), 2.74 (ddd, $J_{2'2''}$ = 13.5 Hz, $J_{2'3'}$ = 5.5 Hz, 1H, H-2′), 2.28 (ddd, $J_{2''3'}$ = 2.7 Hz, 1H, H-2″). ^{13}C NMR (125 MHz, DMSO-d_6 at 22 °C): δ (ppm/TMS) 163.2 (C-6), 151.7 (C-2), 150.5 (C-4), 140.8 (C-8), 124.3 (C-5), 87.8 (C-4′), 83.6 (C-1′), 70.9 (C-3′), 61.8 (C-5′), 38.9 (C-2′). UV: λ_{max} 306 nm (pH 7). ESI-TOF/MS (negative ion): m/z 279 (M^-), 251 ($M^- - N_2$), 135 ($M^- - N_2$ – deoxyribose). HR-ESI-TOF/MS (negative ion): m/z 279.084971 (M^-) (calculated for $C_{10}H_{11}N_6O_4$, 279.084726).

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