Acetylation of the Amino Group on Guanosine Induced by Nitric Oxide in Acetonitrile under Aerobic Conditions

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Received August 25, 2008; accepted October 24, 2008; published online October 31, 2008

When nitric oxide was bubbled into acetonitrile under aerobic conditions, the solution showed a cobalt-blue color. Addition of guanosine into the solution generated N^2-acetylguanosine as a major product. The result of the reaction using ^{15}N labeled acetonitrile indicated that the nitrogen atom of the acetylated exocyclic amino group on N^2-acetylguanosine originated from acetonitrile. We discuss the reaction mechanism for the acylation.

Key words nitric oxide; acetonitrile; acetylation; guanosine

Results and Discussion

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.[2,3)] Although NO is a radical, the reactivity of NO *per se* is relatively low.[4)] However, in the presence of O_2, NO is converted to a reactive nitrosating reagent, dinitrogen trioxide (N_2O_3).[5)] 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,[6,7)] since the formed *N*-nitroso compounds are unstable and subsequent hydrolyses are performed. Suzuki *et al.* reported that N_2O_3 converted guanosine (Guo) into the novel compound oxanosine (Oxo) in addition to the well-known deamination compound xanthosine (Xao).[8)] A possible overall reaction pathway of Guo with N_2O_3 generating Xao and Oxo was proposed as follows.[9,10)] N_2O_3 reacts with the amino group on C2 of Guo, resulting in the N^2-nitroso intermediate. The nitroso intermediate is converted immediately to the diazoate intermediate (–N=N–O$^-$), which appears to be the most stable intermediate throughout the reaction pathway. Then the diazoate intermediate is converted to the diazonium intermediate (–N_2^+) and the ring-opened cation is formed synchronously by the release of a nitrogen molecule from the diazonium. Reaction by H_2O on the carbodiimide (–N=C=N–) of the ring-opened cation and subsequent ring closure and tautomerization generates Xao. In contrast, reaction by H_2O on the carbonyl group of the ring-opened cation and subsequent ring closure generates Oxo. Lucas *et al.* reported that *N*-nitrosoindoles, which are NO donors, also induced generation of Xao and Oxo from Guo in neutral solution including 50% acetonitrile (CH_3CN).[11)] In addition, N^2-acetylguanosine (N^2-Ac-Guo) was generated as a co-product in the reaction mixture (Chart 1). However, the reaction mechanism of the acetylation of the amino group induced by NO in CH_3CN is unclear. In the present study, we studied the acetylation of the exocyclic amino group on Guo by NO and CH_3CN under aerobic conditions. From the results of the reactions using N^2-methylguanosine (N^2-Me-Guo) and ^{15}N labeled acetonitrile ($CH_3C^{15}N$), the reaction mechanism for the acylation is proposed.

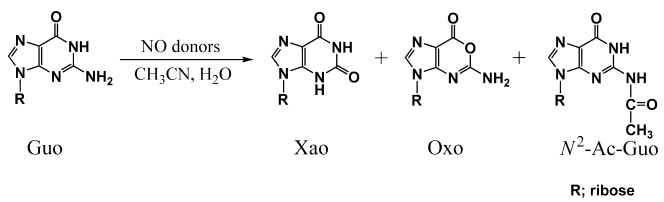

Chart 1. Reaction Scheme and Structures of the Products in the Reaction of Guo with NO Donors in CH_3CN/H_2O=1/1 (v/v)

R in the structure denotes a ribose moiety.

NO gas (4 ml/min) was bubbled into 2 ml CH_3CN in an open vessel at ambient temperature in open air. By 10 min NO bubbling, the solution showed a cobalt-blue color. The visible light spectrum of the solution with the absorbance peak maximum of 661 nm is shown in Fig. 1. It was similar to the reported spectrum of N_2O_3 in organic solvents.[12,13)] The cobalt-blue color disappeared within 30 min when the solution was left without a cap at ambient temperature. However, the color was maintained at least for one month in the vessel with a cap. Under Ar atmosphere, NO gas bubbling into CH_3CN did not change the color. These results suggest that NO reacts with O_2 in the air generating N_2O_3 in CH_3CN, and that N_2O_3 can exist stably in CH_3CN. When 10.0 mg (35.3 μmol) Guo was added into the N_2O_3/CH_3CN solution, Guo dissolved generating a gas. The solution in the open vessel was left for 10 min at ambient temperature. Figure 2 shows the reversed phase HPLC (RP-HPLC) chromatogram of the reaction mixture. Guo eluted at an HPLC retention time of 13.7 min with λ_{max}=253 nm in the on-line detected UV spectrum disappeared completely: The peak around 13.7 min, actually at 13.6 min, in the chromatogram of the reaction mixture showed a different UV spectrum with λ_{max}=261 and 284 nm. A product (compound **1**) eluted at an

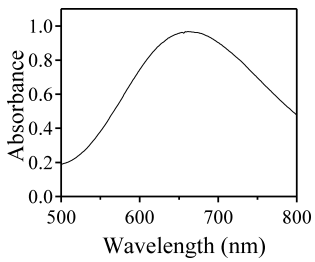

Fig. 1. UV Spectrum of the NO Bubbled Solution of CH_3CN

NO gas (4 ml/min) was bubbled into 2 ml CH_3CN in an open vessel at ambient temperature for 10 min under aerobic conditions. The flow rate of NO gas (99.7%, Sumitomo Seika, Tokyo) was controlled by a Model 3660 mass flow controller (KOFLOC, Kyoto, Japan).

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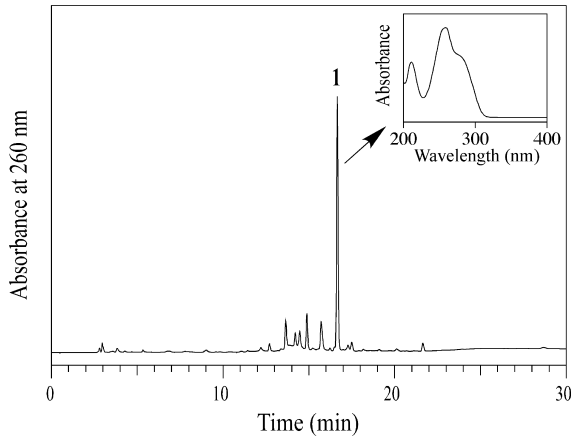

Fig. 2. RP-HPLC Chromatogram of a Reaction Solution of Guo with N_2O_3/CH_3CN

The insets are the on-line UV spectrum of **1**. Guo (10 mg) was added to the N_2O_3/CH_3CN solution. The reaction mixture in the open vessel was left to stand for 10 min at ambient temperature. The RP-HPLC chromatogram was detected at 260 nm. Guo eluted at an HPLC retention time of 13.7 min with λ_{max}=253 nm in the on-line detected UV spectrum was disappeared completely: The peak around 13.7 min, actually at 13.6 min, in this chromatogram showed a different UV spectrum with λ_{max}=261 and 284 nm.

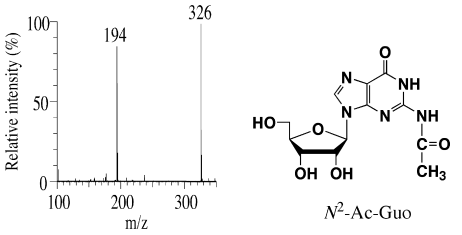

Fig. 3. Positive-Ion Electrospray Ionization Time of Flight Mass Spectrometry Spectrum (ESI-TOF/MS) of **1** and the Structure of N^2-Ac-Guo

The sample isolated by RP-HPLC was directly infused into the MS system (MicroTOF, Bruker, Bremen, Germany) by a syringe pump without a column.

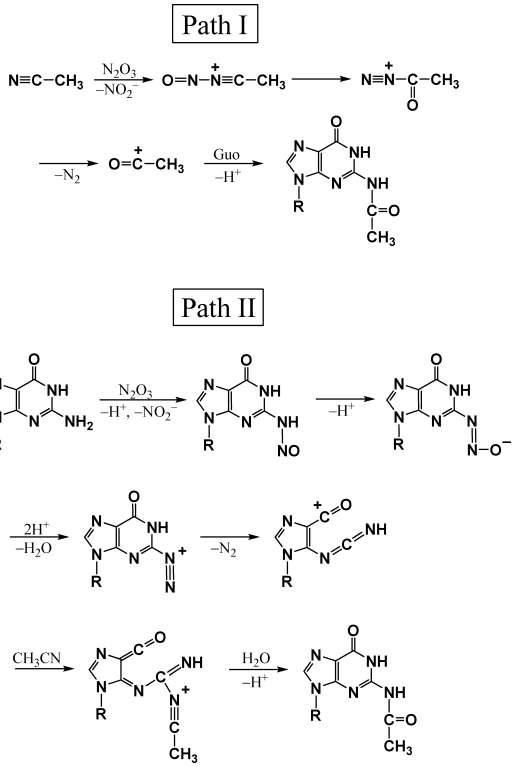

Chart 2. Two Conceivable Reaction Pathways for the Formation of N^2-Ac-Guo from Guo by the Reaction of NO and CH_3CN under Aerobic Conditions

R in the structure denotes a ribose moiety.

HPLC retention time of 16.7 min showed a λ_{max}=259 nm in the on-line detected UV spectrum (Fig. 2, inset). The product was collected by RP-HPLC and subjected to MS and NMR measurements. Figure 3 shows an ESI-TOF/MS spectrum of the product. Two major peaks, *m/z* 326 and 194, which are attributable to the molecular ion and the base fragment ion, respectively, were observed. ^{1}H-NMR showed a set of signals of intact ribose protons and H8 aromatic proton. In addition, two imino protons and three protons on a methyl group were observed. Combining these spectrometric data, the product was identified as N^2-acetylguanosine (N^2-Ac-Guo). These spectrometric data accorded well with the reported data of N^2-Ac-Guo.[14] The N^2-Ac-Guo purified by RP-HPLC was 3.1 mg (9.5 μmol, yield: 27%).

Concerning the mechanism of N^2-Ac-Guo formation, two conceivable pathways exist (Chart 2). In Path I, N_2O_3 initially reacts with the tertiary nitrogen atom of CH_3CN. The following rearrangement produces the acetyldiazonium ion. It is converted to the carbonium ion by the subsequent release of the nitrogen molecule. The carbocation reacts with the amino group on Guo, resulting in N^2-Ac-Guo. An alternative pathway in line with the pathway proposed for the reaction of Guo with N_2O_3 generating Xao and Oxo is shown in Path II.[9,10] N_2O_3 reacts with the amino group on C2 of Guo, resulting in the N^2-nitroso intermediate. The nitroso intermediate is converted to the diazonium intermediate ($-N_2^+$) *via* the diazoate intermediate ($-N=N-O^-$). Then the ring-opened cation is formed synchronously by the release of a nitrogen molecule from the diazonium. The carbodiimide ($-N=C=N-$) of the ring-opened cation reacts with the lone pair on the tertiary nitrogen atom of CH_3CN, resulting in the adduct cation. It is converted to N^2-Ac-Guo by subsequent ring closure and addition of water. If path II is involved, the reaction is initiated by nitrosation of the exocyclic amino group of Guo. The N_2O_3 reaction of N^2-Me-Guo in lieu of Guo should generate the relatively stable nitroso derivative, since N^2-Me-Guo carries a secondary amine and, in general, the nitroso derivative of a secondary amine is stable. When N^2-Me-Guo (1 mg) was added into the N_2O_3/CH_3CN solution, a product (compound **2**) showing λ_{max}=240 and 301 nm was detected on the RP-HPLC chromatogram (data not shown). The product was collected by RP-HPLC and subjected to an MS measurement. Figure 4 shows the ESI-TOF/MS spectrum on the negative mode of compound **2**. Two peaks, *m/z* 325 and 295, attributable to the molecular ion and the fragment ion produced by a loss of NO, respectively, were observed. The spectrometric data accorded well with the reported data of N^2-methyl-N^2-nitrosoguanosine (N^2-Me-N^2-NO-Guo).[10] This result suggests that the reaction of Guo with N_2O_3/CH_3CN initiates by nitrosation of the amino group on Guo. If Path II is involved, the acetylated nitrogen atom of 2 position on N^2-Ac-Guo should originate from CH_3CN. To clarify the origin of the nitrogen atom of the exocyclic amino group on N^2-Ac-Guo, the reaction of Guo with N_2O_3/CH_3CN was performed using ^{15}N labeled CH_3CN ($CH_3C^{15}N$, ^{15}N

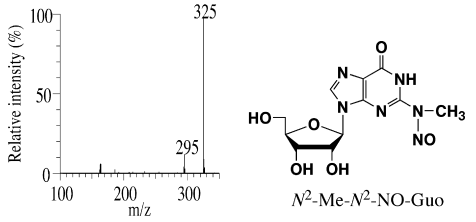

Fig. 4. Negative-Ion ESI-TOF/MS of **2** and the Structure of N^2-Me-N^2-NO-Guo

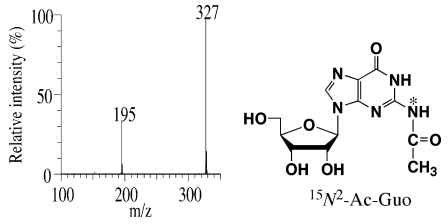

Fig. 5. Positive-Ion ESI-TOF/MS of N^2-Ac-Guo Generated from the Reaction in $CH_3C^{15}N$ and Its Structure

98%+, CIL, MA, U.S.A.). NO gas (4 ml/min) was bubbled into 0.25 ml $CH_3C^{15}N$ in an open vessel at ambient temperature for 20 min under aerobic conditions. Guo (2 mg) was added into the solution and left for 10 min. The formed N^2-Ac-Guo was isolated by RP-HPLC. Its ESI-TOF/MS spectrum showed two major peaks, *m*/*z* 327 and 195, attributable to the molecular ion and the base fragment ion, respectively, (Fig. 5) which were one mass unit higher than those of the product in CH_3CN (Fig. 3). This result indicates that the acetylated nitrogen atom on 2 position of N^2-Ac-Guo originated from CH_3CN and that Path II is involved as a major path.

In conclusion, we propose the reaction mechanism for the formation of N^2-Ac-Guo in the reaction of Guo with NO in CH_3CN under aerobic conditions as follows: NO reacts with O_2 in the air resulting in N_2O_3 in CH_3CN. N_2O_3 reacts with the exocyclic amino group on Guo resulting in the N^2-nitroso derivative. The nitroso derivative is converted to the ring-opened cation *via* the diazonium and diazoate intermediates. The carbodiimide of the ring-opened cation reacts with CH_3CN, resulting in the adduct cation. It is converted to N^2-Ac-Guo by subsequent ring closure and addition of water.

Experimental

HPLC and MS Conditions The HPLC system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. An Inertsil ODS-3 octadecylsilane column of 4.6×250 mm and particle size of 5 μm (GL Science, Tokyo) was used. The eluent was 20 mM ammonium acetate buffer (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 50% over 15 min in linear gradient mode and kept at 50% until 30 min. The column temperature was 40 °C and the flow rate was 1.0 ml/min. The ESI-TOF/MS measurements were performed on a MicroTOF spectrometer (Bruker, Bremen, Germany) in the positive mode. The sample isolated by RP-HPLC using 20 mM ammonium acetate buffer (pH 7.0) containing methanol as the eluent was directly infused into the MS systems by a syringe pump without a column at a flow rate of 2 μl/min.

Spectrometric Data of N^2-Acetylguanosine (N^2-Ac-Guo) ^{1}H-NMR (500 MHz, in DMSO-d_6 at 25 °C) δ (ppm/TMS): 12.04 (s, NH, 1H), 11.71 (s, NH, 1H), 8.24 (s, H8, 1H), 5.78 (d, H1′, 1H), 5.47 (br, OH, 1H), 5.17 (br, OH, 1H), 5.02 (br, OH, 1H), 4.42 (dd, H2′, 1H), 4.11 (dd, H3′, 1H), 3.89 (m, H4′, 1H), 3.58 (m, H5′,5″, 2H), 2.17 (s, CH_3, 3H); ^{13}C-NMR (125 MHz, in DMSO-d_6 at 25 °C) δ (ppm/TMS): 173.4 ($CH_3\underline{C}O$), 154.7, 148.7, 147.9, 137.5 (C8), 120.1, 86.5 (C1′), 85.2 (C4′), 73.9 (C2′), 70.1 (C3′), 61.0 (C5′), 23.7 ($\underline{C}H_3CO$); UV: λ_{max}=259 nm (pH 7.0); ESI-TOF/MS (positive) *m*/*z* 326 $[M+H]^+$, 194 $[M_{base\ fragment}+H]^+$.

Spectrometric Data of N^2-Methyl-N^2-nitrosoguanosine (N^2-Me-N^2-NO-Guo) UV: λ_{max}=240, 301 nm (pH 7.0); ESI-TOF/MS (negative) *m*/*z* 325 $[M-H]^-$, 295 $[M-NO-H]^-$.

References and Notes

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