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Near ultraviolet radiation-mediated reaction between *N*-nitrosoproline and DNA: Isolation and identification of two new adducts, (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine

Shuhei Aoyama[a], Kayoko Sano[a], Chiharu Asahi[a], Masaki Machida[a], Keinosuke Okamoto[a], Tomoe Negishi[a], Sachiko Kimura[b], Toshinori Suzuki[c], Tsutomu Hatano[a], Sakae Arimoto-Kobayashi[a,*]

[a] *Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Tsushima, Kita-ku, Okayama 700-8530, Japan*
[b] *School of Human Science and Environment, University of Hyogo, Shinzaike-hon-machi, Himeji, Hyogo 670-0097, Japan*
[c] *School of Pharmaceutical Sciences, Shujitsu University, Nishigawara, Naka-ku, Okayama 703-8516, Japan*

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ABSTRACT

To explore possible genotoxicity of *N*-nitrosoproline (NPRO), we investigated near-ultraviolet radiation (UVA)-mediated chemical reaction of NPRO with 2′-deoxyguanosine (dG). An acidic solution containing NPRO and dG was irradiated with UVA and products were analyzed by HPLC. In addition to the three known modified guanine nucleosides, *i.e.*, 2′-deoxyxanthosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine and 2′-deoxyoxanosine, we found two products. These new products were isolated and identified as (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine (G1 and G2, respectively). Experiments using monochromatic UVA in the range 300–400 nm were performed to determine the relation between products-yield and wavelength, and the highest yields of both G1 and G2 were found to occur at 340 nm. This wavelength coincided with the absorption maximum of NPRO. We propose that the NO radical produced from NPRO by photolysis triggers the formation of these products. Further investigation using calf thymus DNA showed that G1 and/or G2, probably both, could be produced by UVA irradiation of DNA + NPRO as well.

1. Introduction

Recently, the International Agency for Research on Cancer (IARC) announced that it has classified outdoor air pollution as carcinogenic to humans (Group 1) [1]. Occurrence of carcinogenic *N*-nitroso compounds in the human environment including air pollution and tobacco smoke has been extensively studied [2–4]. Secondary amines and amino acids can react with nitrite to form *N*-nitroso derivatives [4,5]. Endogenous formation of *N*-nitrosoproline (NPRO) from L-proline by nitrite in humans has been reported by Ohshima and Bartsch [6] and its excretion has been used as an index for endogenous nitrosation [7,8]. NPRO was not carcinogenic in a long-term study in rats [9]. In addition, NPRO was non-mutagenic in the Ames test [10]. Thus, NPRO is regarded as non-mutagenic and non-carcinogenic for humans [5–8].

However, we have detected the direct mutagenicity of NPRO plus natural sunlight toward *Salmonella typhimurium* [11]. Our previous studies have also shown that DNA exposed to NPRO plus UVA results in strand breaks [11,12]. We observed that UVA irradiation of NPRO in aqueous solution generates OH radical, singlet oxygen and nitric oxide (NO). By using inhibitors against these active components, we showed that DNA strand breaks were caused by these three species [11]. We also found that 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a mutagenic adduct, was formed in DNA on treatment with NPRO plus simulated sunlight [11]. In addition, Suzuki et al. have shown that treatment of dG with nitric oxide in acetate buffer (3.0 M, pH 3.7) produces 2′-deoxyoxanosine (dO), 2′-deoxyxanthosine (dX) and 2-nitro-deoxyinosine in addition to 8-oxodG [13]. Stefan and Bolton [14] reported that the reaction rate of the photolysis of *N*-nitrosodimethylamine (NDMA) at pH 3 is greater than at pH 7. We have suspected that other modified nucleosides may also be produced during the photoreaction of NPRO with dG and DNA. We therefore investigated the photoproducts from an UVA-irradiated mixture of NPRO and dG under acidic conditions. Here, the isolation and identification of two new photoproducts generated from NPRO and dG are described. Formation of these new adducts in

Abbreviations: NPRO, *N*-nitrosoproline; UVA, near-ultraviolet light; dG, 2′-deoxyguanosine; NO, nitric oxide; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dO, 2′-deoxyoxanosine; dX, 2′-deoxyxanthosine.

* Corresponding author. Tel.: +81 86 251 7947; fax: +81 86 251 7947.
E-mail address: arimoto@cc.okayama-u.ac.jp (S. Arimoto-Kobayashi).

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calf thymus DNA on exposure to UVA + NPRO at pH 7 is also reported.

2. Materials and methods

2.1. Materials

N-Nitrosoproline (NPRO) was a gift from Dr. M. Mochizuki of Tokyo University of Science, who synthesized this compound as described by Lijinsky et al. [15]. 2′-Deoxyguanosine (dG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were purchased from Wako Pure Chemicals (Osaka, Japan). 2′-Deoxyxanthosine (dX) and 2′-deoxyoxanosine (dO) were synthesized as described [13] both with purities >99%. All other materials used were of reagent grade obtained from commercial sources.

2.2. Characterization of photoproducts formed in the irradiation of dG with NPRO

dG (10 mM) was irradiated in sodium acetate buffer (3 M, pH 3.7) in the presence of NPRO (100 mM) in a chamber kept at 4 °C. Continuous mixing was done during the reaction. UVA irradiation was performed for 12 h with 20-W black-light bulbs emitting radiation in the range 300–400 nm (Panasonic, Japan). Use of a 4-mm thick glass plate between the light source and the reaction solution excluded radiation of wavelength <320 nm. The UVA dose rate at 360 nm on the surface of the solution, measured using a black-ray UV intensity meter (Ultraviolet Products, San Gabriel, CA), was 0.98 ± 0.060 mW/cm^2. Irradiated samples were fractionated using HPLC (TSK-GEL ODS-80Ts, 21.5 mm × 300 mm) equipped with a photodiode array detector SPP-M 10Avp (Shimadzu, Kyoto). These fractions were analyzed by LC–MSMS (API3000 and API4000, AB SCIEX, MA, USA) with positive mode ESI. The column used was Inertsil ODS-3 (1.0 mm × 150 mm), and the eluent was 5 mM ammonium acetate with 0–40% methanol. Co-chromatography with authentic specimen was performed with 10 μL of irradiated sample mixed with 1 nmol of dX, 1 nmol of dO or 2 nmol of 8-oxodG. ^{1}H NMR spectra of samples dissolved in dimethylsulfoxide-d_6 were recorded on a Unity INOVA AS600 spectrometer (Agilent Technologies, Tokyo) at 600 MHz.

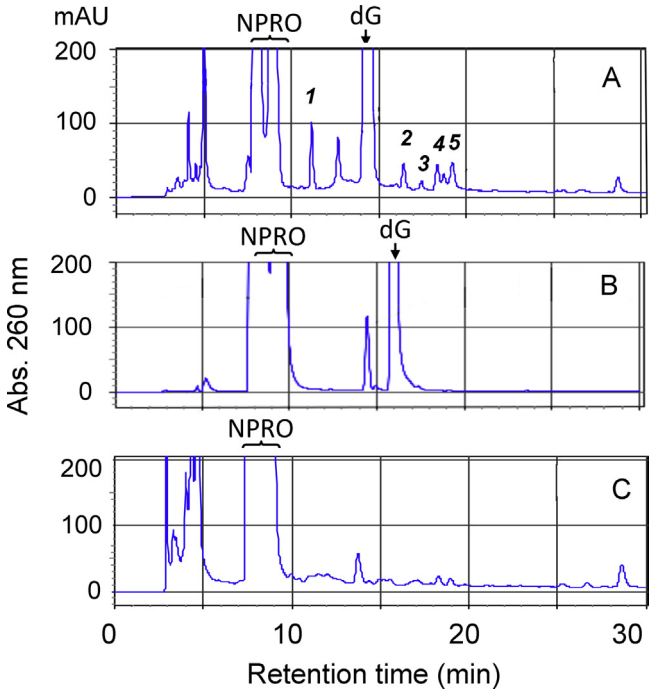


Fig. 1. HPLC profiles of UVA-dG-NPRO reaction. (A) A solution containing 10 mM dG and 100 mM NPRO at pH 3.7 was incubated at 4 °C for 12 h under UVA (320-400 nm) irradiation. (B) 10 mM dG-100 mM NPRO, pH 3.7, incubated at 4 °C for 12 h without UVA. (C) 100 mM NPRO, pH 3.7, incubated at 4 °C for 12 h under UVA without dG. Peaks 1–5 indicate products to be identified.

2.3. Relation between photoproducts-yield and UVA wavelength

Monochromatic irradiation was performed at the Spectrography and Bioimaging Facility, National Institute of Basic Biology (Okazaki, Aichi Prefecture) using the Large Spectrograph. The intensity of radiation at 300, 320, 340, 360, 380 and 400 nm employed was 5.5, 13.5, 19.4, 24.0, 22.5 and 21.3 W/m^2, respectively. By adjusting the time of irradiation, the UV-dose at each wavelength was made equal. Mixtures containing 10 mM NPRO and 10 mM dG in sodium acetate buffer (3 M, pH 3.7) were exposed to these monochromatic radiations at 25 °C. Immediately after irradiation, samples were taken and stored at −80 °C. The amount of each photoproduct formed was determined by LC–MSMS analysis. The experiments were performed in triplicate.

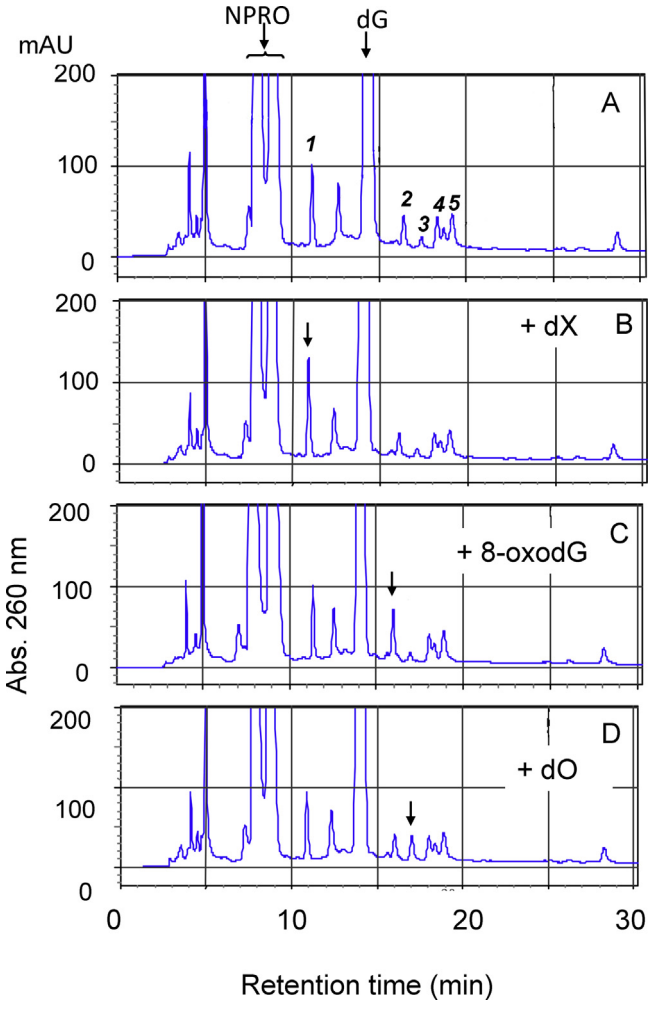


Fig. 2. Co-chromatographic profiles. NPRO (100 mM) and dG (10 mM) were used. (A) Chromatographic profile of the sample of NPRO and dG after UVA irradiation for 12 h as shown in Fig. 1A. (B) Co-chromatographic profile of irradiated sample (10 μL) with dX (1 nmol). The arrow indicates the retention time of authentic dX. (C) Co-chromatographic profile of irradiated sample (10 μL) with 8-oxodG (2 nmol). The arrow indicates the retention time of authentic 8-oxodG. (D) Co-chromatographic profile of irradiated sample (10 μL) with dO (1 nmol). The arrow indicates the retention time of authentic dO.

2.4. Analysis of products formed in the UVA irradiation of DNA plus NPRO

A mixture of calf thymus DNA (0.5 mg/mL) and NPRO (50 mM) in 15 mM sodium chloride–1.5 mM sodium citrate buffer (pH 7.0) was irradiated with UVA for 4 h at 4 °C. UVA irradiation was performed with 20-W black-light bulbs, with an inserted glass plate (see above). Irradiated DNA was dialyzed against 1 mM Tris–HCl – 0.1 mM EDTA buffer (pH 8.0). The DNA (0.1 mg) was then digested with nucleaseP1 (Sigma, St. Louis, MO) followed by alkaline phosphatase (Sigma) into nucleosides. The mixture was centrifuged with a filter (Amicon Ultra free-MC, Milipore, Billerica, MA) and the filtrate was subjected to HPLC, with 20 mM triethylammonium acetate (pH 7.0) and 2–30% methanol as elution media. The column used was Inertsil ODS-3 (4.6 mm × 250 mm). Eluent from 19 to 22 min, which corresponded to that of G1 and G2, was collected and evaporated to dryness. The residue was dissolved in water and analyzed by positive mode LC–MSMS with a constant neutral scan, monitoring the precursor/product ion transitions *m*/*z* 337/221.

3. Results

3.1. UVA-mediated reaction of 2′-deoxyguanosine (dG) and N-nitrosoproline (NPRO)

Fig. 1A shows HPLC fractionation pattern of the UVA-dG-NPRO reaction, Fig. 1B that of dG-NPRO incubation without UVA, and Fig. 1C that of UVA-NPRO incubation without dG. As can be seen,

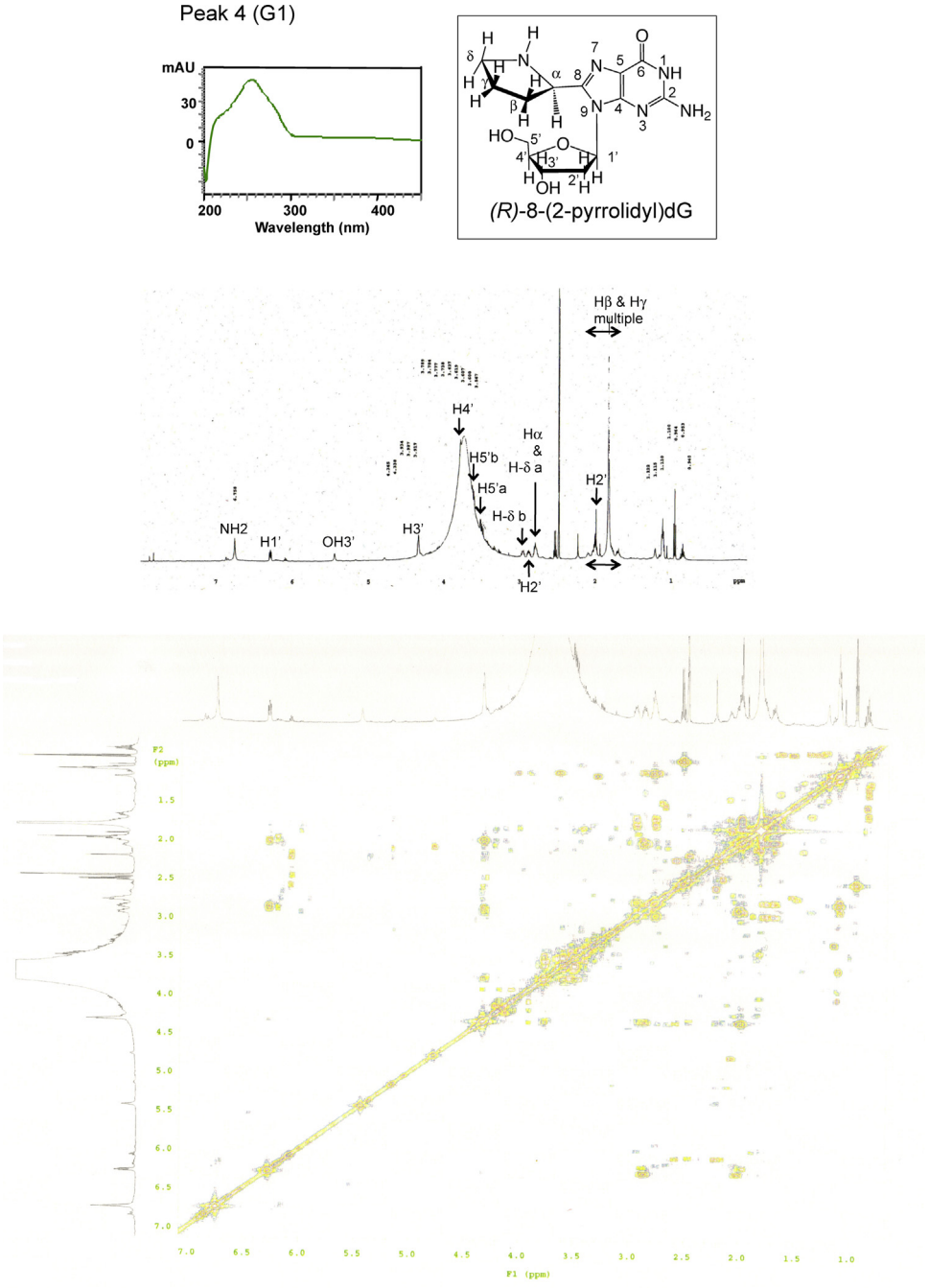


Fig. 3. Identification of peak 4 as (*R*)-8-(2-pyrroldyl)dG (G1). UV and NMR spectra, and the chemical structure.

five peaks numbered 1–5 (Fig. 1A) were the products to be identified. Without dG, recovery of NPRO after 12 h irradiation was 12% (Fig. 1C). Without NPRO, the recovery of dG after 12 h irradiation was 99% and no new peaks were observed (data not shown). The retention times (Fig. 1A) and UV-absorption profiles (data not shown) of the product in peaks 1–3 (hereafter referred to as P1, P2 and P3, respectively) were identical with those of authentic dX, 8-oxodG and dO, respectively. The yields of dX, 8-oxodG and dO from dG (1 mM) with NPRO (10 mM) irradiated in the acidic solution were 1.3–2.0%, 2.0–5.0% and 0.5–2.5% of original dG, respectively,

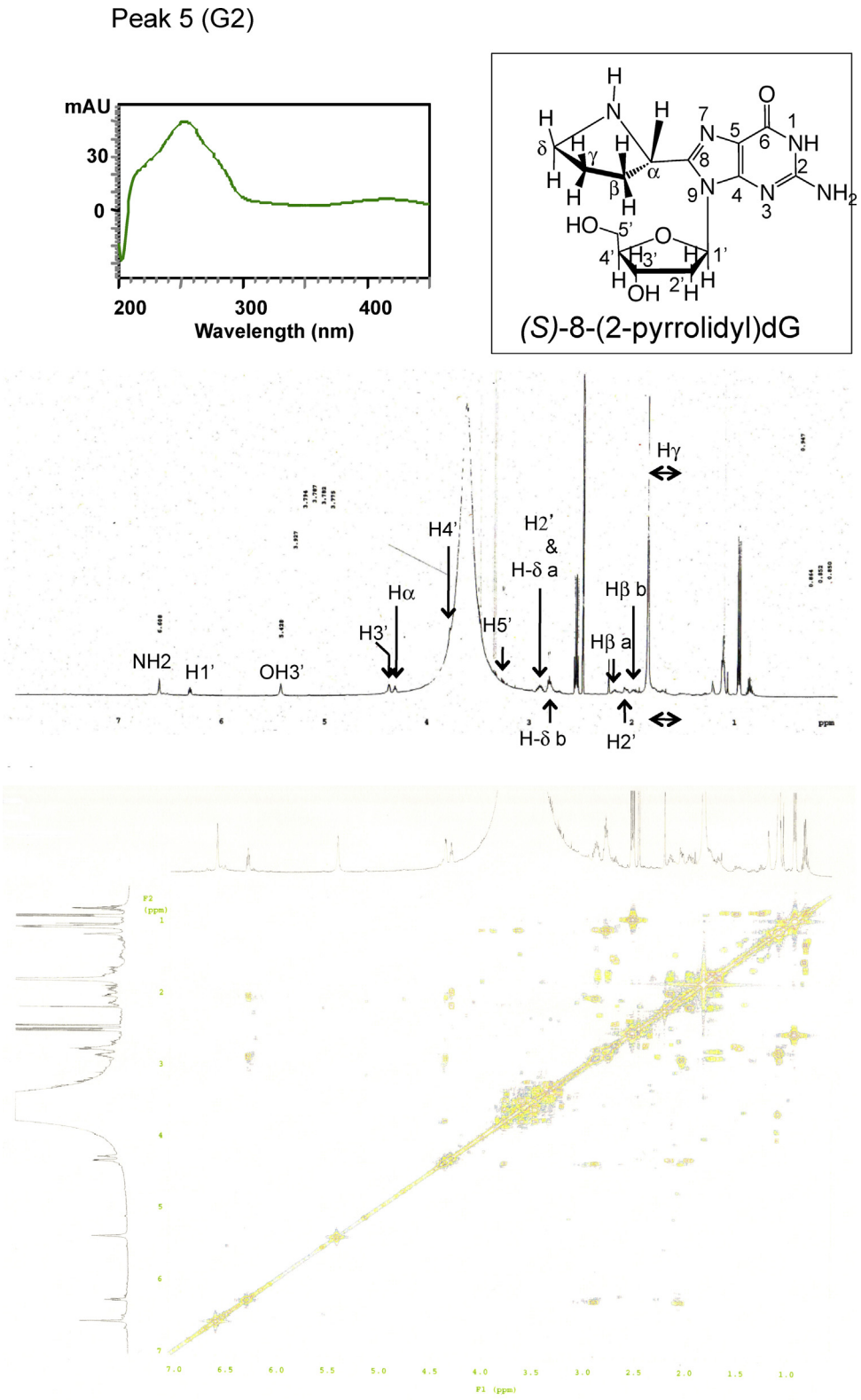


Fig. 4. Identification of peak 5 as (*S*)-8-(2-pyrrolidyl)dG (G2). UV and NMR spectra, and the chemical structure.

with 12 h UVA irradiation (triplicate experiments). Co-injection of P1 with dX, P2 with 8-oxodG, and P3 with dO gave respectively a single peak having areas coincident to the sum of the peak areas of P1 and dX, P2 and 8-oxodG, and P3 and dO, respectively (Fig. 2). The ESI-MS spectra of P1 and P3 showed protonated molecular ion ($[MH]^+$) m/z 269, and their major MS/MS product ion was m/z 153 that could be formed by a loss of deoxyribose (116 amu) from dX and dO (mw = 268). The ESI-MS spectra of P2 showed $[MH]^+$ m/z 284, and the major product ion was m/z 168 that could be formed by a loss of deoxyribose from 8-oxodG (mw = 283). ^{1}H NMR analysis of P1, P2, P3, and authentic specimens of 8-oxodG and dO dissolved in dimethylsulfoxide-d_6 were performed (summarized in Supporting information), and the ^{1}H NMR profiles of P1, P2 and P3 were identical with those of authentic specimens of dX, 8-oxodG and dO, respectively [13,16]. Thus, P1, P2 and P3 were identified as dX, 8-oxodG and dO, respectively.

We attempted to determine the structures of compounds in peaks 4 and 5 (hereafter referred to as G1 and G2, respectively). UV spectra of G1 and G2 seemed highest at about 260 nm (Figs. 2 and 3), making us to suspect that G1 and G2 might be guanine derivatives. With positive mode LC–MS/MS analysis under a constant neutral scan that should release a fragment of 116 amu, we observed an ion (m/z 337) from both G1 and G2. With the product ion scan from the precursor ion (m/z 337) of G1 and G2, we found two peaks; one major MS/MS product ion (m/z 221) that could be formed by loss of deoxyribose (116 amu) from the protonated molecule ion ($[MH]^+$) (m/z 337), and one minor product ion (m/z 152) that seems to represent the protonated guanine ion formed by a loss of the pyrrolidyl moiety (69 amu) from the product ion (m/z 221).

The ^{1}H NMR spectrum of G1 showed signals at δ 1.68–2.10 (2H, multiple, Hβa,b; 2H, m, Hγa,b), 2.01 (1H, m, H2′a), 2.79 (2H, m, Hα and H-δa), 2.87 (1H, ddd, H2′b), 2.95 (1H, ddd, H-δb), 3.50 (5H, m, H5′a,b), 3.78 (1H, dd, H4′), 4.34 (1H, br, H3′), 5.45 (1H, s, 3′-OH), 6.29 (1H, dd, H1′) and 6.76 (2H, s, NH2) (Fig. 3). The couplings were confirmed by COSY measurements (Fig. 3). The ^{1}H NMR spectrum of G2 showed signals at δ 1.57–1.79 (2H, m, Hγa,b), 1.96 (1H, m, Hβa), 2.06 (1H, m, H2′), 2.17 (1H, m, Hβb), 2.81 (1H, m, H-δa), 2.90 (2H, m, H2′ and H-δb), 3.26 (1H, dd, H5′a), 3.34 (1H, dd, H5′b), 3.78 (1H, dd, H4′), 4.31 (1H, t, Hα), 4.37 (1H, br, H3′), 5.43 (1H, s, 3′-OH), 6.31 (1H, t, H1′) and 6.76 (2H, s, NH2) (Fig. 4). The couplings were confirmed by COSY measurements (Fig. 4). The proton signal of the H8 position in the dG residue at 7.90 ppm was absent in either profiles of G1 or G2, indicating that substitution had occurred at the C8 position of dG. The ^{1}H NMR signal of the Hα proton of G1 at 2.79 ppm was upfield to that of G2 at 4.31 ppm. In the R-conformation of 8-(2-pyrrolidyl)dG, the Hα proton must have become close to protons of the deoxyribose moiety, thereby being shielded by steric compression; hence the signal of the Hα proton in the R-configuration would be shifted upfield. We concluded that G1 must be (*R*)-8-(2-pyrrolidyl)-2′-deoxyguanosine, and G2 (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine.

Experiments using monochromatic UVA in the range 300–400 nm were performed to determine the relation between products-yield and wavelength. As Fig. 5 shows, the highest yields of both G1 and G2 were found to occur at 340 nm. This wavelength coincided with the absorption maximum of NPRO, suggesting that sensitization of NPRO by UVA triggers the formation of both G1 and G2.

3.2. Formation of G1 and G2 in DNA-NPRO-UVA

DNA was treated with NPRO plus UVA under neutral conditions. The DNA was then digested into nucleosides and the mixture fractionated by HPLC. As the retention times of authentic G1 and G2 were 19.5 and 21.5 min, the eluent from 19 min to 22 min was collected (Fig. 6A). This fraction was analyzed with LC-MSMS in the MRM mode by monitoring the precursor/product ion transitions m/z 337/221 ($[MH]^+/[MH-116]^+$, the loss of deoxyribose) a signal for detecting G1 and G2. Indeed a peak was observed at retention time 25.7 min, which corresponded to the authentic G1 and G2 (Fig. 6B). We concluded that G1 and/or G2, probably both, were formed in DNA by the action of UVA-NPRO.

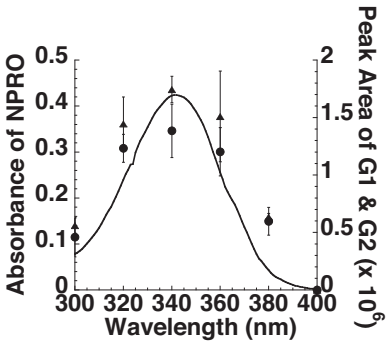


Fig. 5. UV absorption spectrum of 5 mM NPRO at pH 3.7 (line) and amounts of products (G1 by circles and G2 by triangles) formed on irradiation at individual wavelength. Peak area values are those of the integrator reading.

4. Discussion

Although *N*-nitroso compounds are thermally stable in aqueous solutions, Chow et al. described that nitroso-α-amino acid including NPRO are photolyzed to give CO_2 and piperidonoxime with UVC (Scheme, Reaction 1) [17]. In 1967, Chow observed that the formation of piperidinium radical from an acidic aqueous solution (pH 2) of *N*-nitrosopiperidine on photolysis, and proposed that a radical disproportion occurs to form HNO and tetrahydropyridine (Scheme, Reaction 2) [18].

The present findings show that new modified nucleosides, (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine can be formed by UVA-irradiation of NPRO-dG and of NPRO-DNA in aqueous solutions. Our finding that the plots of G1 and G2 formation match the absorption curve of NPRO, both being highest at 340 nm (Fig. 5), suggests that the rate limiting step of G1 and G2 formation is photo-cleavage of the N—N bond in NPRO. Thus, a possible mechanism of the G1 and G2 formation may involve release of NO from NPRO resulting in the formation of an aminium radical, and the latter reacting with NO radical to create an aminium cation. Subsequent nucleophilic attack of the C8 moiety of dG by the cation would give CO_2

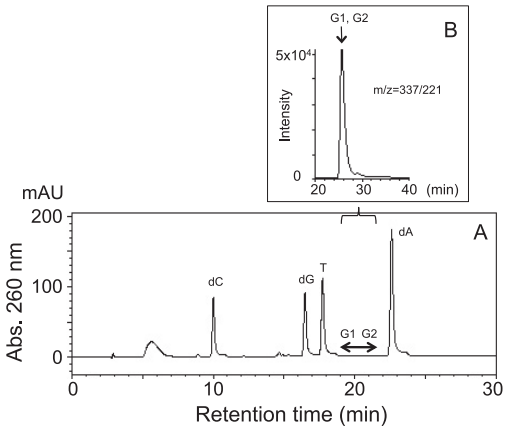
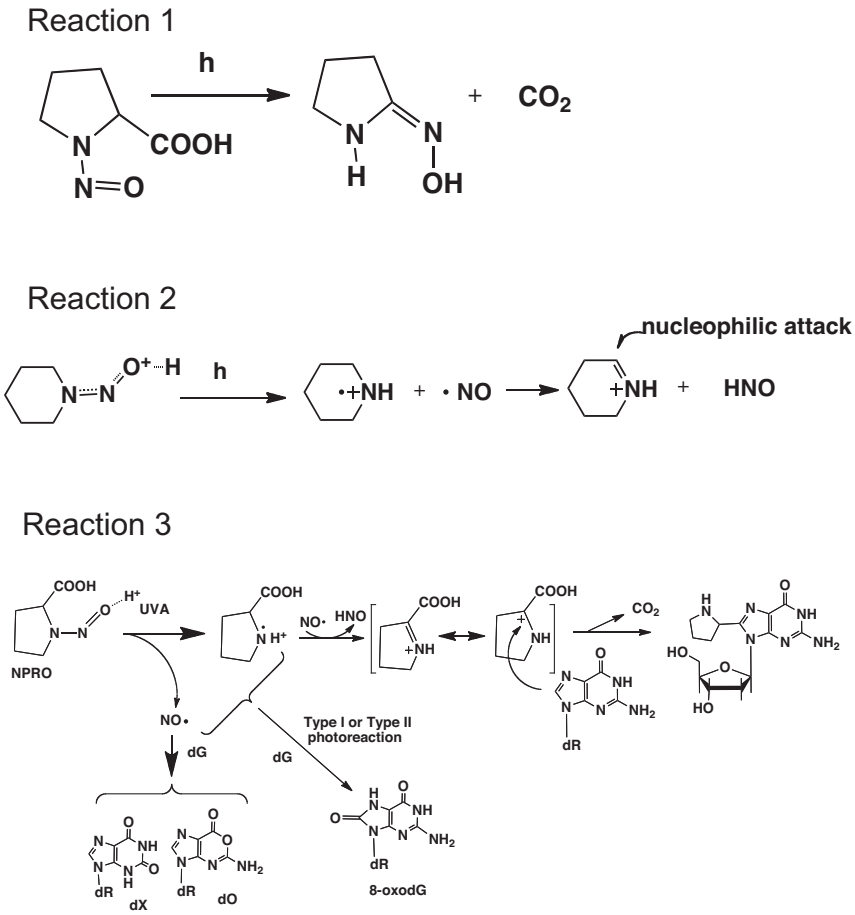


Fig. 6. Identification of G1 and G2 in DNA treated with NPRO plus UVA. (A) HPLC profile of nucleosides produced by enzymatic digestion of DNA that had been treated with NPRO and UVA. (B) LC–MSMS profile of the fraction pooled from 19 to 22 min in (A).



Scheme 1. Mechanisms of the reactions.

and 8-(2-pyrrolidyl)-dG adducts (Scheme, Reaction 3). Whether the sequence of intermediates, *i.e.*, the decarboxylation, is as shown or at an earlier step is not clear. Chow et al. [17] demonstrated that a carboxylic acid located at an α-position of nitrosamines could act as a proton donor causing the nitrosamino group to become photosensitive and decompose in neutral or acidic solutions without much difference in quantum efficiency.

In our earlier studies, we have shown that irradiation of NPRO with UVA or sunlight can produce mutagenic activity and that the activity of irradiated NPRO with DNA involves alkylation and oxidation of DNA [11]. The present results show that photoreaction of NPRO with dG produces 8-oxodG as well as dX and dO. We also observed nitric oxide formation in the solution of UVA-irradiated NPRO [12]. dX and dO were isolated from a reaction of dG with nitric oxide [13]. Therefore, photoproduction of dX and dO from dG-NPRO could occur by the attack of the released NO. Our present work shows that NPRO can facilitate UVA-induced NO release to create an aminium ion that can give 8-(2-pyrrolidyl)-dG adducts.

The role of UVA in the phototoxicity and photogenotoxicity of carcinogens and chemicals has been investigated by many research groups. For example, UVA-induced reaction of benzo(a)pyrene with DNA *in vitro* results in single-strand breaks and benzo(a)pyrene-nucleoside adducts *in vitro* [19]. Reactive oxygen species in CHO cells formed by UVA radiation of benzo(a)pyrene causes increased level of micronuclei and 8-oxodG legions [20]. UVA-sensitized riboflavin degrades purine and pyrimidine bases in DNA [21]. Wierckx et al. reported that UVA-irradiated 2-nitrofluorene becomes covalently bound to RNA to form a guanine C8-adduct [22]. Photooxidation of guanine base in DNA and RNA treated with UVA-sensitized furocoumarins with a mechanism involving singlet oxygen is known [23].

We propose that endogenous NPRO might have a role in the photogenotoxicity of sunlight. Further studies are required to gain a deeper understanding of the phototoxicity of NPRO.

Conflicts of interest

The authors declare that no financial or personal conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotochem.2013.11.009.

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