Journal of Photochemistry & Photobiology A: Chemistry 400 (2020) 112621

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Isolation of new photoadducts from UVA-irradiated *N*-nitrosoproline with 2'-deoxyadenosine and characterization of photoadducts from DNA irradiated with *N*-nitrosoproline

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ARTICLE INFO

Keywords:
Photoreaction
N-nitrosoproline
Adenine adduct
DNA adduct
Reactive oxygen species

ABSTRACT

N-nitrosoproline (NPRO) is formed from nitrosation of proline and has been reported to be non-carcinogenic and non-mutagenic. However, earlier studies in our laboratory showed that pre-irradiated NPRO can be converted to a mutagenic form. We previously investigated the reaction of NPRO with dA or dG under UVA irradiation and identified the formation of 2-pyrrolidyl-dA adducts (P1 & P2) and 8-pyrrolidyl-dG adducts (G1 & G2) as well as four known modified nucleosides, although several peaks found in the HPLC profiles of UVA-irradiated mixtures of dA and NPRO remain unidentified. In the present study we isolated new photoproducts from irradiated mixtures of dA and NPRO and identified (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyadenosine (A1 and A2) as products by MS and NMR. We also investigated the photoadducts formed in DNA treated with NPRO under UVA irradiation, and detected A1 and/or A2 (probably both), P1, P2, G1 and/or G2, and 8-oxodG as products. Under anaerobic conditions, formation of A1 and A2 was greater than that under aerobic conditions, suggesting that photo-reactions comprising pyrrolidyl radical with dA may increase under anaerobic conditions given reduced competition with oxidative photo-reactions which may decompose pyrrolidyl-dA adducts.

1. Introduction

Cancer is driven by genetic changes, and especially by the accumulation of driver mutations in the genome [1]. Pan-cancer analysis of whole genomes comprising cancer genomes had 4–5 driver mutations on average [2]. Investigation of natural chemicals that react with DNA to produce mutations are important in understanding the influence of mutagenesis and carcinogenesis. *N*-nitrosamines, most of which are known mutagens, are found in water, air pollution and tobacco smoke, as well as in internal body fluids [3,4]. *N*-nitroso compounds are thought to play a significant role in the development of cancer [5,6]. We investigated *N*-nitrosoproline (NPRO), a cyclic *N*-nitroso-α-amine acid, whose structure is indicated in Fig. 1. Proline is nitrosated to NPRO in the stomach of rat [7] and NPRO has also been found in nitrite-preserved cooked food [8]. NPRO was detected in human urine at an average value of 4.30 μg/g Cr of NPRO [5]. NPRO was reported to be non-carcinogenic [9] and non-mutagenic in an Ames test using *S. typhimurium* [10].

We noticed that NPRO can absorb UVA light. Solar ultraviolet radiation (UV) is a causal factor for skin cancer in humans, and ultraviolet-A (UVA, 315 − 400 nm) is the predominant source of radiant energy in sunlight [11]. UVA can reach the subcutaneous area of the skin containing blood vessels. Biological compounds that absorb solar photons, particularly in the UVA region, serve as photosensitizers involved in skin phototoxicity. Earlier studies in our laboratory showed that pre-irradiated NPRO can be converted to a mutagenic form [12]. We investigated the mutagenic spectrum of NPRO on M13mp2 DNA with UVA irradiation, and found that the most frequent mutation comprised GC to CG transversions. A hot spot of mutation was also found comprising AT to GC only following treatment of DNA with NPRO and UVA [13]. From UVA-irradiated solutions of NPRO and 2′-deoxyguanosine (dG), we found new guanine adducts, comprising (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine (hereafter referred to as G1 and G2, respectively), in addition to three previously known modified guanine nucleosides 2′-deoxyxanthosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), and 2′-deoxyoxanosine (Fig. 1) [14]. We also

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https://doi.org/10.1016/j.jphotochem.2020.112621
Received 23 March 2020; Received in revised form 27 April 2020; Accepted 2 May 2020
Available online 21 May 2020
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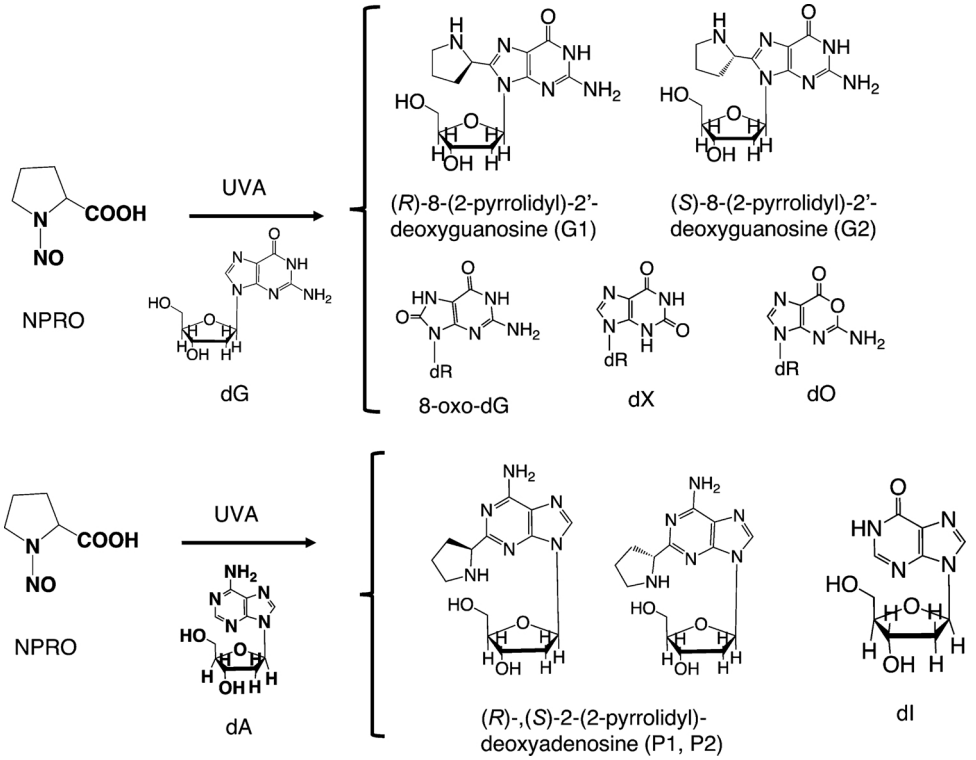


Fig. 1. Upper: Chemical structures of photoproducts from dG with NPRO, (*R*)-8-(2-pyrrolidyl)-2′-deoxyguanosine (G1), (*S*)-8-(2-pyrrolidyl)-2′-deoxyguanosine (G2), 8-oxodG, dX and dO. Lower: Those from irradiated dA with NPRO, (*R*)- and (*S*)-2-(2-pyrrolidyl)-deoxyadenosine (P1, P2) and dI.

found that reaction of NPRO with 2′-deoxyadenosine (dA) under UVA irradiation produced photoproducts including new adenine adducts identified as (*R*)- and (*S*)-2-(2-pyrrolidyl)-2′-deoxyadenosine (hereafter referred to as P1 and P2) and previously known nucleoside deoxyinosine (dI) (Fig. 1) [15], although several photoproduct peaks found in the HPLC profiles of UVA-irradiated mixtures of dA and NPRO remain unidentified. In the present study we isolated and identified new photoproducts from irradiated mixtures of dA and NPRO and investigated the formation of photoadducts in irradiated mixtures of DNA with NPRO.

2. Methods

2.1. Materials

N-nitrosoproline (NPRO) shown in Fig. 1 was a gift from Dr. Mochizuki of Tokyo University of Science, who synthesized this compound as described by Lijinsky et al. [16], with purity > 99%, as determined by HPLC. 2′-Deoxyadenosine (dA) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were purchased from Wako Pure Chemicals (Osaka, Japan). Calf thymus DNA was purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other materials used were of reagent grade and obtained from commercial sources.

2.2. Identification and characterization of photoproducts formed from an irradiated mixture of dA and NPRO

dA (10 mM) and NPRO (100 mM) dissolved in sodium acetate buffer (3 M, pH 3.7) was irradiated using 20-W black light bulbs (Panasonic, Japan) emitting radiation in the range 300 − 400 nm in a chamber kept at 4 °C. Use of a 4-mm thick glass plate between the light source and the reaction solution excluded radiation of wavelengths < 320 nm. The UVA dose rate at 360 nm was measured using a black-ray UV intensity meter (Ultraviolet Products, San Gabriel CA). Light intensity was 8.30 ± 0.95 W/m^2 at the surface of the solution, namely 359 ± 41 kJ/m^2 for 12 h, if not stated otherwise. Irradiated samples were analyzed using HPLC apparatus equipped with a photodiode array detector SPP-M 10Avp (Shimadzu, Kyoto). The column used was Inertsil ODS-3 (4.6 mm × 250 mm) and the eluent comprised 20 mM triethylammonium acetate with 2–30% methanol (pH 7.0). Irradiated samples were fractionated by HPLC with a column comprising TSK-GEL ODS-80 Ts (21.5 mm × 300 mm) and eluent consisting of 40 mM triethylammonium acetate with 15% methanol (pH 7.0). Peak fractions were collected and re-chromatographed by HPLC with a column comprising TSK-GEL ODS-80 Ts (4.6 mm × 250 mm) and eluent consisting of 20 mM triethylammonium acetate (pH 7.0) with 20% methanol. ^{1}H NMR spectra of samples dissolved in dimethylsulfoxide-d6 were recorded on a Unity INOVA AS600 spectrometer (Varian Technologies Japan LTD., Tokyo) at 600 MHz. Chemical shifts were based on those of the solvent signals (δ_H 2.49; δ_C 39.8) and given in δ (ppm) from tetramethylsilane.

Monochromatic irradiation was performed under the auspices of the National Institute for Basic Biology which employs the Okazaki Large Spectrograph (Okazaki, Japan). By adjusting the time of irradiation, the UV dose at each wavelength was adjusted to 30 kJ/m^2. Solutions of 100 mM NPRO and 10 mM dA dissolved in Na-acetate buffer (3.0 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 produced was determined by HPLC analysis. The column comprised Inertsil ODS-3 (4.6 mm × 250 mm) and the eluent consisted of 20 mM triethylammonium acetate (pH7.0) with 2–30% methanol. Experiments were performed in triplicate.

2.3. Analysis of products formed from UVA-irradiated DNA with NPRO

Calf thymus DNA was dialyzed against 15 mM NaCl–1.5 mM sodium citrate (pH 7.0) for 12 h at 4 °C. A mixture of calf thymus DNA (0.75 mg/mL) and NPRO (100 mM) in 100 mM sodium phosphate buffer (pH 7.4) was irradiated with UVA for 0 − 12 h (0 − 359 kJ/m^2) at 4 °C. Irradiated DNA was dialyzed against 1 mM Tris–HCl–0.1 mM EDTA buffer (pH 8.0) for 12 h at 4 °C. The DNA (0.1 mg) was then digested

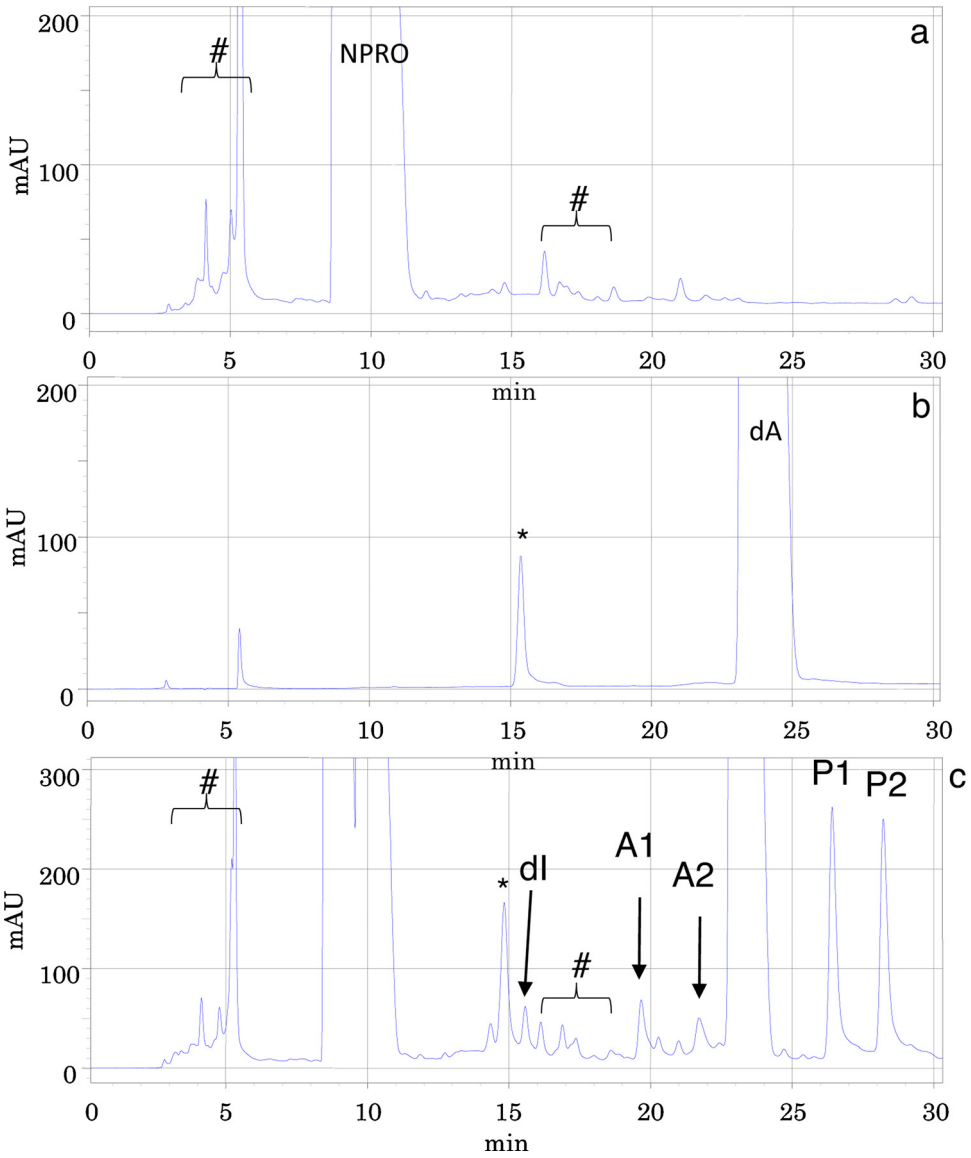


Fig. 2. HPLC profiles of (a) an irradiated solution of 100 mM NPRO with UVA for 12 h (253 ± 41 kJ/m^2); (b) an irradiated solution of 10 mM dA with UVA for 12 h; (c) an irradiated solution of 10 mM dA and 100 mM NPRO with UVA for 12 h. The UVA intensity was 586 ± 94.6 μW/cm^2. Peaks marked as A1 and A2 indicate products to be identified. Peaks marked as dI, P1 and P2 indicate products already identified as dI, P1 and P2. Peaks marked as # were observed in both profiles a and c. Peak marked as * were observed in both profiles b and c.

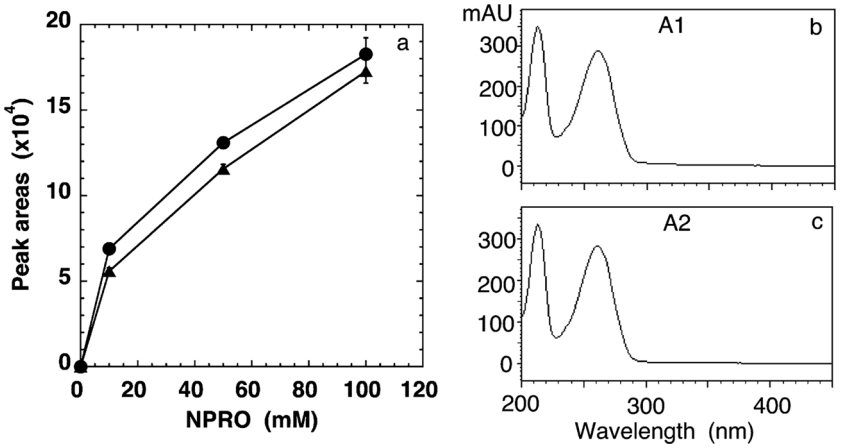


Fig. 3. (a) NPRO-dependent formation of A1 (circle) and A2 (triangle). (b, c) UV absorption spectra of peak fractions A1 and A2, respectively. The concentration of dA was 10 mM and UVA irradiation was performed for 50 kJ/m^2.

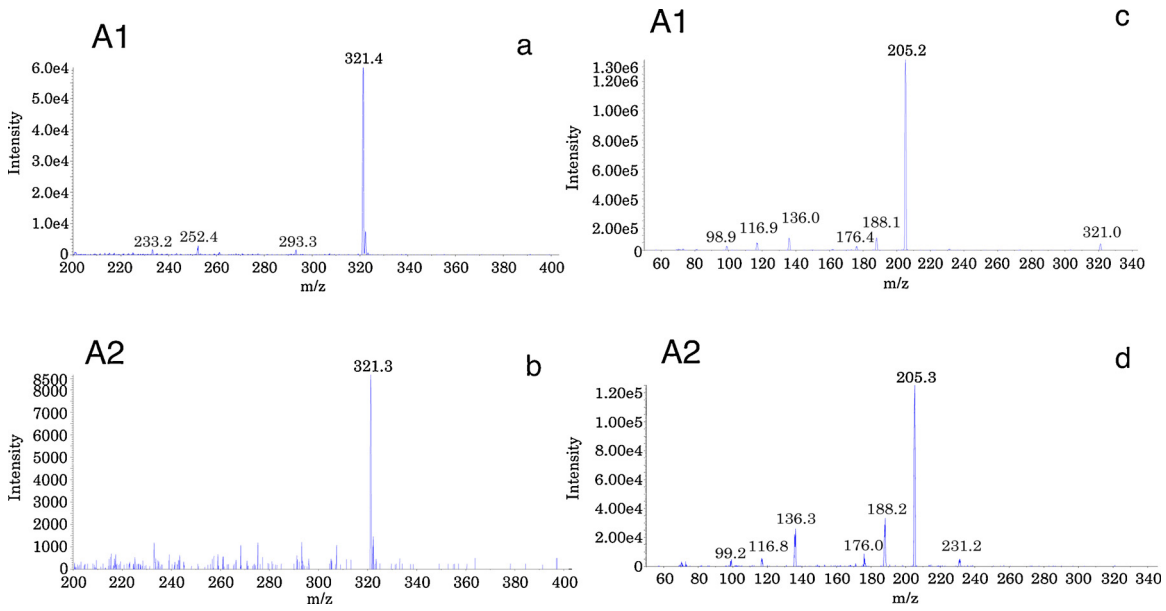


Fig. 4. Positive mode LC–MS/MS profiles under a constant neutral scan that should release a fragment of 116 amu from (a) A1 and (b) A2, and under a product ion scan from the precursor ion (*m/z* 321) of (c) A1 and (d) A2.

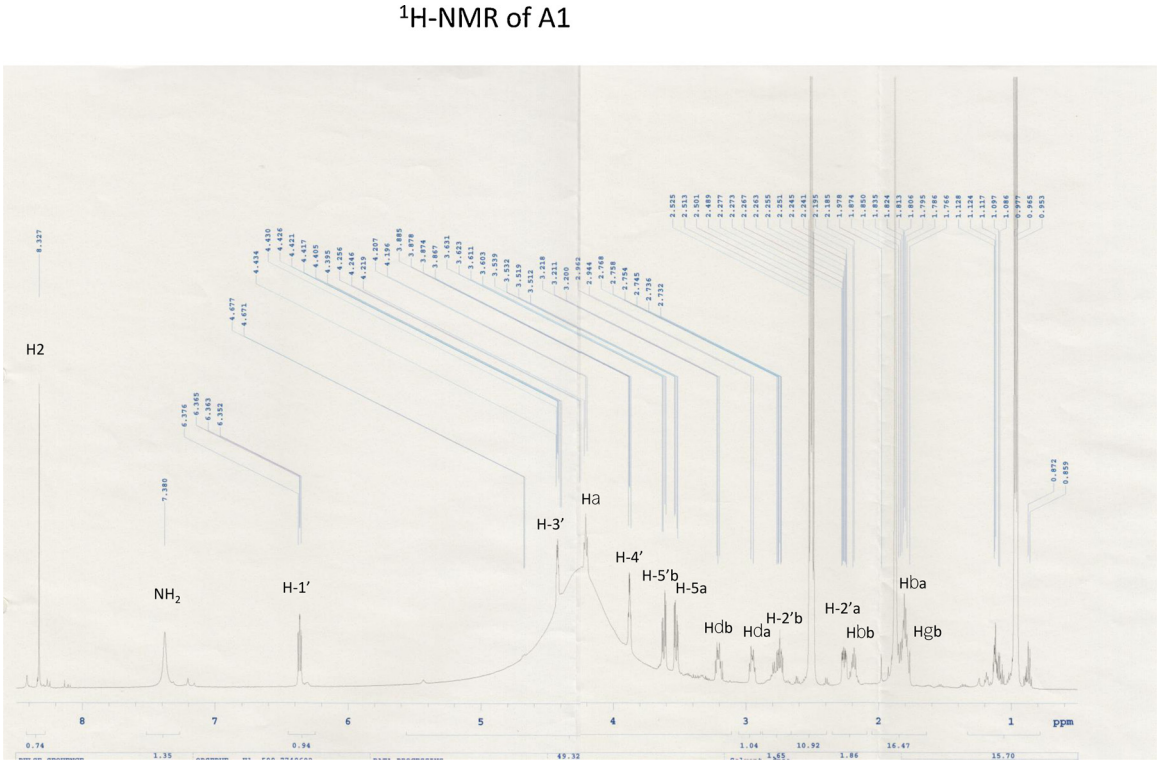


Fig. 5. ^{1}H NMR spectra of photoproduct A1.

with nuclease P1 (Sigma, St. Louis, MO) followed by alkaline phosphatase (Sigma) into nucleosides. The obtained samples were analyzed by positive ion mode LC-MSMS with MRM mode, with monitoring of the precursor/product ion transitions at *m/z* 284.142/167.900 for 8-oxodG, *m/z* 321.300/205.200 for P1, P2, A1 & A2, and 337.300/221.200 for G1 & G2. The column comprised Inertsil ODS-3 (1.0 mm × 150 mm) and the eluent consisted of 5 mM ammonium acetate with 4–35% methanol. Isolated and structure-determined samples from irradiated mixtures of dA and NPRO were used as authentic specimens of P1, P2, A1, A2, G1 and G2.

Investigation of the effects of aerobic and anaerobic conditions on the photoreaction of NPRO with DNA was performed as follows. A sample tray was placed into an airtight bag. N_2 gas or air was blown into the sample solution at a flow rate of approximately 300 mL/min for 20 s to replace dissolved gasses with N_2 or air. Sample solution was then irradiated. Products were analyzed as described above. Experiments were performed in triplicate.

2.4. Statistical analyses

Data are expressed as means ± standard deviation for each data point as indicated. *P* values < 0.01 were considered to be statistically

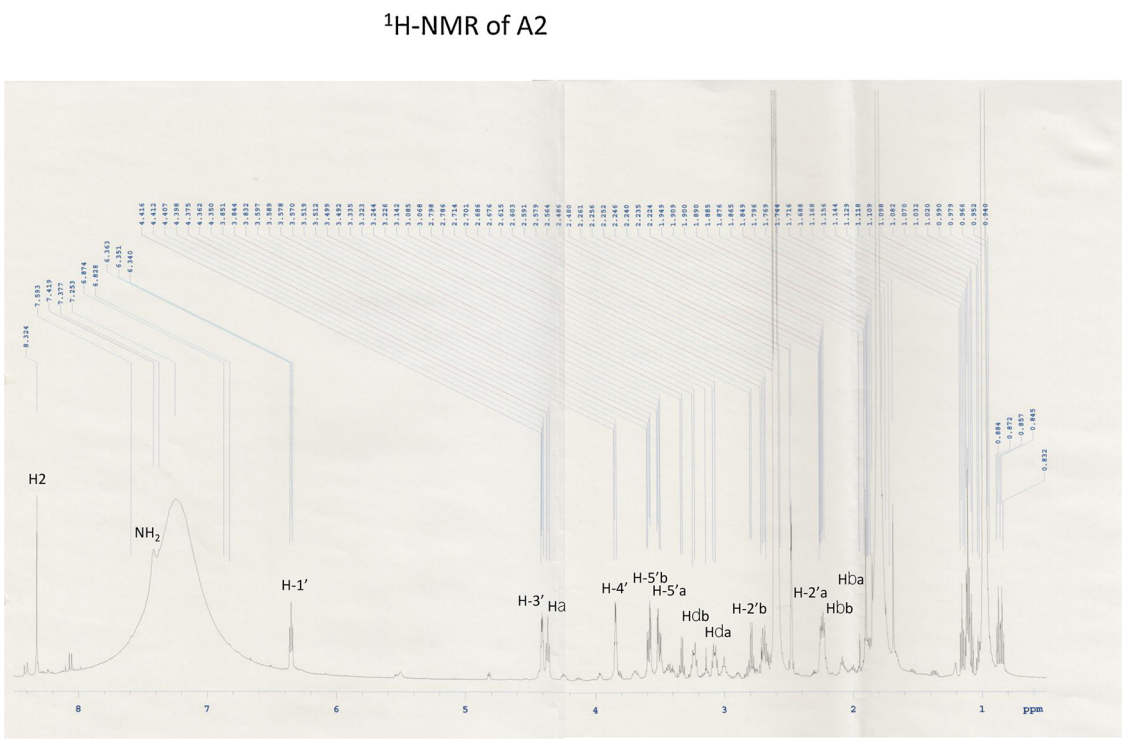


Fig. 6. ^{1}H NMR spectra of photoproduct A2.

Table 1
^{1}H data of dA, A1 and A2 in DMSO-d6.

dA position	δ	A1 position	δ	COSY	A2 position	δ	COSY
H2	8.34 (1H, s)	H2	8.33(1H, s)		H2	8.32 (1H, s)	
H8	8.14(1H, s)	H8	**		H8	**	
NH2	7.29 (2H, s)	NH2	7.38 (2H, s)		NH2	7.42 (2H, s)	
H1′	6.36 (1H, dd, 7.8, 6.0)	H1′	6.36 (1H, dd, 7.8, 6.6)	H2′a,b	H1′	6.35 (1H, t, 6.9)	H2′a,b
3′-OH	**	3′-OH	**		3′-OH	**	
5′-OH	**	5′-OH	**		5′-OH	**	
		Hα	4.21 (1H, t, 6.9)	Hβa,b	Hα	4.36 (1H, t, 7.5)	Hβa,b
H3′	4.12 (1H, m)	H3′	4.41 (1H, m)	H2′a,b	H3′	4.41 (1H, m)	H2′a,b
H4′	3.89 (1H, m)	H4′	388 (1H, dd, 6.6, 4.4)	H5′a,b	H4′	3.84 (1H, dd, 7.2, 4.2)	H5′a,b
H5′b	3.63 (1H, dd, 12.0, 4.2)	H5′b	362 (1H, dd, 12.0, 4.8)	H4′ ; H5′a	H5′b	3.58 (1H, dd, 11.4, 4.8)	H4′ ; H5′a
H5′a	3.53 (1H, dd, 11.7, 4.2)	H5′a	353 (1H, dd, 12.0, 4.2)	H4′ ; H5′b	H5′a	3.51 (1H, dd, 12.0, 4.2)	H4′ ; H5′b
		Hδb	3.21 (1H, m)	Hδa ; Hγa,b	Hδb	3.24 (1H, m)	Hδa ; Hγa,b
		Hδa	2.95 (1H, m)	Hδb ; Hγa,b	Hδa	3.08 (1H, m)	Hδb ; Hγa,b
H2′b	2.73 (1H, ddd, 13.4, 7.8, 5.4)	H2′b	2.75 (1H, m)	H2′a ; H1 ; H3′	H2′b	2.79 (1H, m)	H2′a ; H1 ; H3′
H2′a	2.27 (1H, ddd, 13.4, 6.0, 3.0)	H2′a	2.26 (1H, ddd, 13.2, 6.0, 2.4)	H2′b ; H1 ; H3′			
					H2′a,Hβb	2.24 (2H, m)	H2′b ; H1 ; H3′ ; Hα ; Hβa
		Hβb	2.19 (1H, m)	Hβa : Hγa,b			
		Hβa	1.83 (m)	Hβb : Hγa,b	Hβa	1.86 (m)	Hα ; Hβb
		Hγb	*	Hγa ; Hδa,b	Hγb	*	Hδa,b
		Hγa	*	Hγb ; Hδa,b	Hγa	*	Hδa,b

*Signal was overlapped with solvent signal.
**not detected.

significant. Statistical analyses were performed using KaleidaGraph (Synergy Software, Reading, PA) and an Excel add-in (SSRI Co. Ltd, Tokyo, Japan).

3. Results

3.1. Isolation and identification of new photoadducts in UVA-irradiated mixtures of dA and NPRO

Fig. 2 shows the peaks observed in the HPLC profile of irradiated NPRO and/or dA samples. Since peaks from 2.5 min to 6 min and from 16 min to 19 min in Fig. 2c were also found in the irradiated solution of NPRO (100 mM) without dA (Fig. 2a), we deduced that compounds represented by these peaks (marked #) are degradation products from NPRO. Similarly, since the peak at 14.8 min (Fig. 2c) was also found in the irradiated solution of dA (10 mM) without NPRO (Fig. 2b), we deduced that the compound represented by this peak (marked *) is a degradation product from dA formed during the treatment for 12 h at pH3.7, or an impurity of the sample. Compounds represented by the three peaks indicated as dI, P1 and P2 (Fig. 2c) were previously identified and reported as deoxyinosine (dI), and (*R*)- and (*S*)-2-(2-pyrrolidyl)-2′-dA (P1, P2), respectively [15]. In addition to dI, P1 and P2, two peaks at 19.5 and 22 min (peaks A1 & A2) were also observed (Fig. 2c), and since these were absent in profiles of irradiated samples of

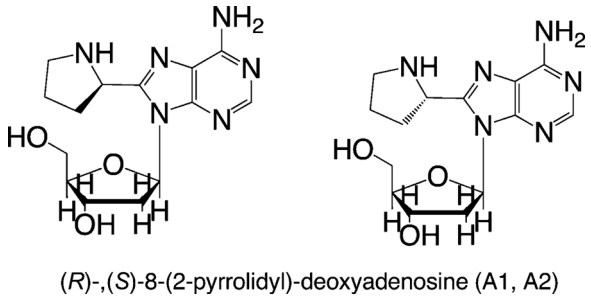


Fig. 7. Chemical structures of A1 and A2 identified as (*R*)- and (*S*)-8-(2-pyrrolidyl)-deoxyadenosine.

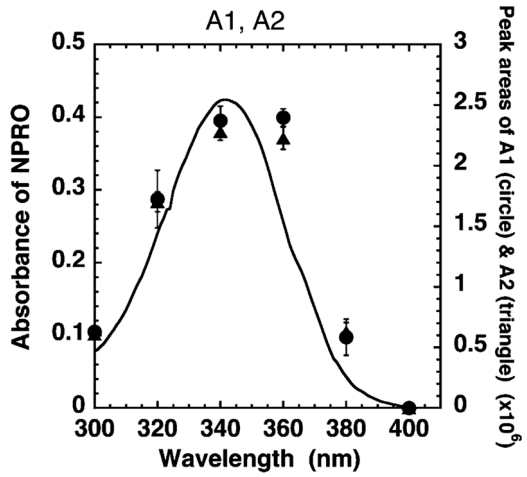


Fig. 8. UV absorption spectrum of 5 mM NPRO at pH 3.7 (line) and amount of product formed from dA and NPRO following irradiation at individual wavelengths at 30 kJ/m^2. A1 is represented by circles and A2 by triangles. Peak area values are those of the integrator reading.

NPRO without dA and dA without NPRO (Fig. 2a and b), we decided that these two peaks represented new photoproducts. The results shown in Fig. 3a show that the peak areas of A1 & A2 increased in a dose-dependent manner with respect to NPRO. We then attempted to determine the structures of compounds in peaks A1 and A2 (hereafter referred to as A1 and A2, respectively). UV spectra of peak fractions A1 and A2 (Fig. 3b and c, respectively) have similar curves and absorption maxima at 260 nm, leading us to suspect that A1 and A2 might be adenine derivatives. Fractions pertaining to peaks A1 and A2 were collected and freeze-dried. The obtained products (A1 and A2) were subjected to LC-MSMS and ^{1}H NMR analysis. With a constant neutral loss scan, we found a major peak (*m/z* 321) releasing a fragment at *m/z* 116 from both of A1 and A2 (Fig. 4a and b). We deduced that the released fragment (*m/z* 116) might be deoxyribose from dA adducts. With a product ion scan from the precursor ion (*m/z* 321) of A1 and A2, we found a major peak MS/MS product ion (*m/z* 205) that could be formed by loss of deoxyribose (116 amu) from the protonated molecular ion ([MH]+) (*m/z* 321), and a minor product ion (*m/z* 136) that seems to represent the protonated adenine ion formed by loss of the pyrrolidyl moiety (69 amu) from the product ion (*m/z* 205) (Fig. 4c and d). Results were very similar to those of P1 & P2 [15]. The ^{1}H NMR spectral data are shown in Figs. 5 and 6, and Table 1. Couplings were confirmed by COSY measurements (Table 1). The proton signal of the H8 position in the dA residue at 8.14 ppm was absent in both A1 and A2 profiles (Fig. 5 and 6, and Table 1), indicating that substitution had occurred at the C8 position of dA. Several proton signals (Hα-δ) coupled together are also apparent (Figs. 5 and 6, and Table 1). The chemical shifts and coupling constants indicated that products A1 and A2 are enantiomers of 8-(2-pyrrolidyl)-2′-deoxyadenosine, i.e., (*R*)- and (*S*)-8-(2-pyrrolidyl)-2′-deoxyadenosine (Fig. 7).

3.2. Photodynamic analysis of photoproducts formed in the irradiated mixture of dA and NPRO

Experiments using monochromatic UVA light at 300, 320, 340, 360, 380 and 400 nm were performed to determine the relation between product yield and wavelength. As shown in Fig. 8, the highest yields of both A1 and A2 were found to occur with monochromatic irradiation at 340 nm or 360 nm. NPRO has no absorption at 400 nm, and neither A1 nor A2 was detected from the sample of NPRO with dA irradiated at 400 nm.

3.3. Analysis of photoadduct (A1, A2, P1, P2, G1, G2 and 8-oxodG) formation in DNA irradiated with NPRO under neutral conditions

Fig. 9 shows an LC-MSMS profile of a sample prepared from DNA irradiated for 12 h with 100 mM NPRO at pH 7.0 under air conditions. Retention times of dC, dG, dT and dA with LC-MSMS were 4.94, 9.58, 12.48 and 17.90 min, respectively (Fig. 9, lane a). Retention times of authentic G1, G2, P1, P2, A1, A2 and 8-oxodG with LC-MSMS were 8.7, 9.2 14.1, 16.7, 15.9, 16.0 and 12.0 min, respectively (data not shown). As shown in Fig. 9, lane b, a peak representing 8-oxodG produced in a sample prepared from irradiated DNA with NPRO appeared at 13.83 min. A combined peak of A1 and A2 appeared at the retention time of 17.45 min. Peaks representing P1 and P2 are found at 15.52 and 19.10 min, respectively (Fig. 9, lane c). A combined peak of G1 and G2 appeared at 8.43 min, as described in our previous report [14] (Fig. 9, lane d).

Amounts representing the sum of A1 and A2 (hereafter referred to as A1 + A2), P2 and 8-oxodG that had formed in DNA irradiated with 100 mM NPRO were dependent on irradiation time (Fig. 10a, b and d). Although no peaks of P1 were found in DNA irradiated with 100 mM NPRO, formation of P1 was observed in DNA irradiated with 200 mM and 300 mM NPRO (Fig. 11b). A combined peak representing G1 and G2 was found in DNA irradiated for 6 h and 12 h in the presence of 100 mM NPRO, although a smaller amount representing the sum of G1 and G2 (hereafter referred to as G1 + G2) was detected with 12 h irradiation compared with 6 h irradiation. (Fig. 10c). Adduct formation from DNA irradiated for 12 h with 100 mM NPRO at pH 7.0 under aerobic conditions was determined to be 2.29 ± 0.17 for A1 + A2 per 10^5 dA, 3.84 ± 0.027 for P2 per 10^5 dA, 285.4 ± 7.63 for G1 + G2 per 10^5 dG and 230.4 ± 3.02 for 8-oxodG per 10^5 dG. P1 formed from DNA irradiated for 12 h with 300 mM NPRO at pH 7.0 under aerobic conditions was calculated to be 12.7 ± 0.041 per 10^5 dA.

The amount of A1 + A2, P1 and P2 formed in DNA was also dependent on NPRO concentration under aerobic and anaerobic conditions with 12 h UVA (Fig. 11a and b). Under anaerobic conditions, the amount of A1 + A2 and P1 photoproducts formed with 200 and 300 mM NPRO was greater compared with that formed under aerobic conditions (Fig. 11ab). There was no change in P2 formation between aerobic and anaerobic conditions (Fig. 11c). Photochemical formation of G1 + G2 was found in the presence of 50 and 100 mM NPRO, while G1 and G2 peaks were absent with 200 and 300 mM NPRO under UVA (Fig. 11d). Formation of 8-oxodG was observed in irradiated DNA with NPRO under aerobic conditions (Fig. 11e). Smaller amounts of 8-oxodG were found with 200 and 300 mM NPRO compared with 100 mM NPRO.

4. Discussion

Photoactivated NPRO reacted with dA to produce C8-modified adducts (A1 and A2) as well as C2-modified adducts (P1 and P2), as previously described [15]. With metabolic activation of nitrosamines, alkyl diazonium ions are considered to be the ultimate alkylating species formed, and nucleophilic centers in DNA are the most highly

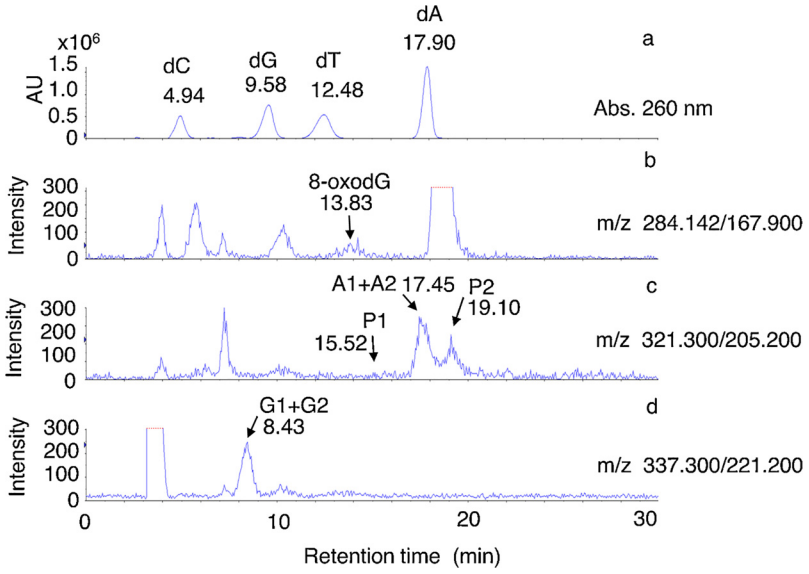


Fig. 9. LC-MSMS profiles of a sample prepared from DNA irradiated by UVA for 12 h with 100 mM NPRO at pH 7.0 under air conditions. Eluent was monitored (lane a) for dC, dG, dT and dA by measuring the absorbance at 260 nm, and (lanes b–d) in the MRM mode by monitoring the precursor/product ion transitions ($[MH]^+/[MH\text{-}116]^+$, loss of deoxyribose) of adducts, with (lane b) *m/z* 284.142/167.900 for 8-oxodG, (lane c) *m/z* 321.300/205.200 for A1, A2, P1 & P2, and (lane d) *m/z* 337.300/221.200 for G1 & G2. Retention times of adduct peaks are indicated.

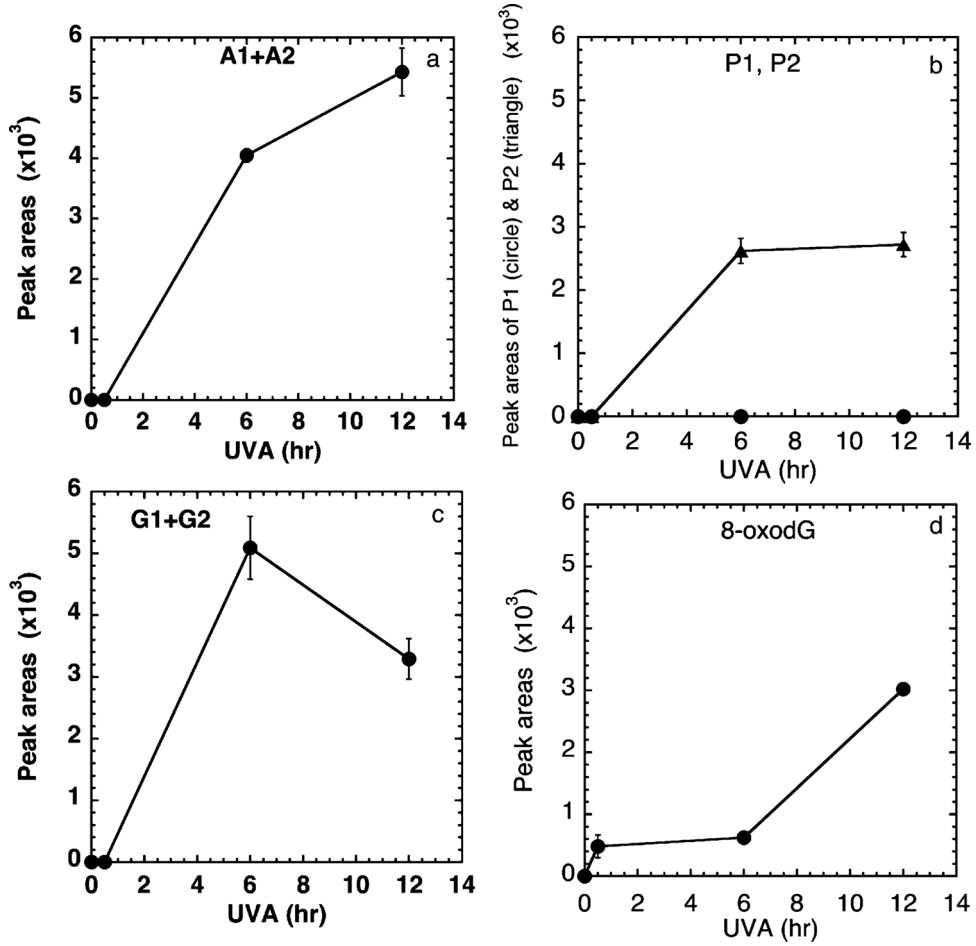


Fig. 10. UVA dose-dependent adduct formation of irradiated DNA with 100 mM of NPRO under air conditions. Formation of (a) A1 + A2, (b) P1 (circle) and P2 (triangle), (c) G1 + G2, and (d) 8-oxodG are shown.

reactive with alkylation agents. In particular, alkyl adducts are frequently formed at N1, N3, N6 & N7 of the adenine residue, and at N1, N^2, N3, N7 & O^6 of the guanine residue [17]. However, no adducts were formed at N1, N3, N6 or N7 of adenine with UVA-activated NPRO. Therefore, the photoactivation process is unlikely to be a mimic of metabolism. It is known that breakage of the N—N bond of N-nitrosamines is a very facile process [18]. Photochemical cleavage of nitrosamine produces the aminyl radical and nitric oxide, followed by oxidation of nitric oxide to nitrogen dioxide [18]. Stefan and Bolton [19] also suggested degradation pathways in the UV photolysis of N-nitrosodimethylamine (NDMA), where the primary step comprised photocleavage of the N—N bond, followed by dissociation to

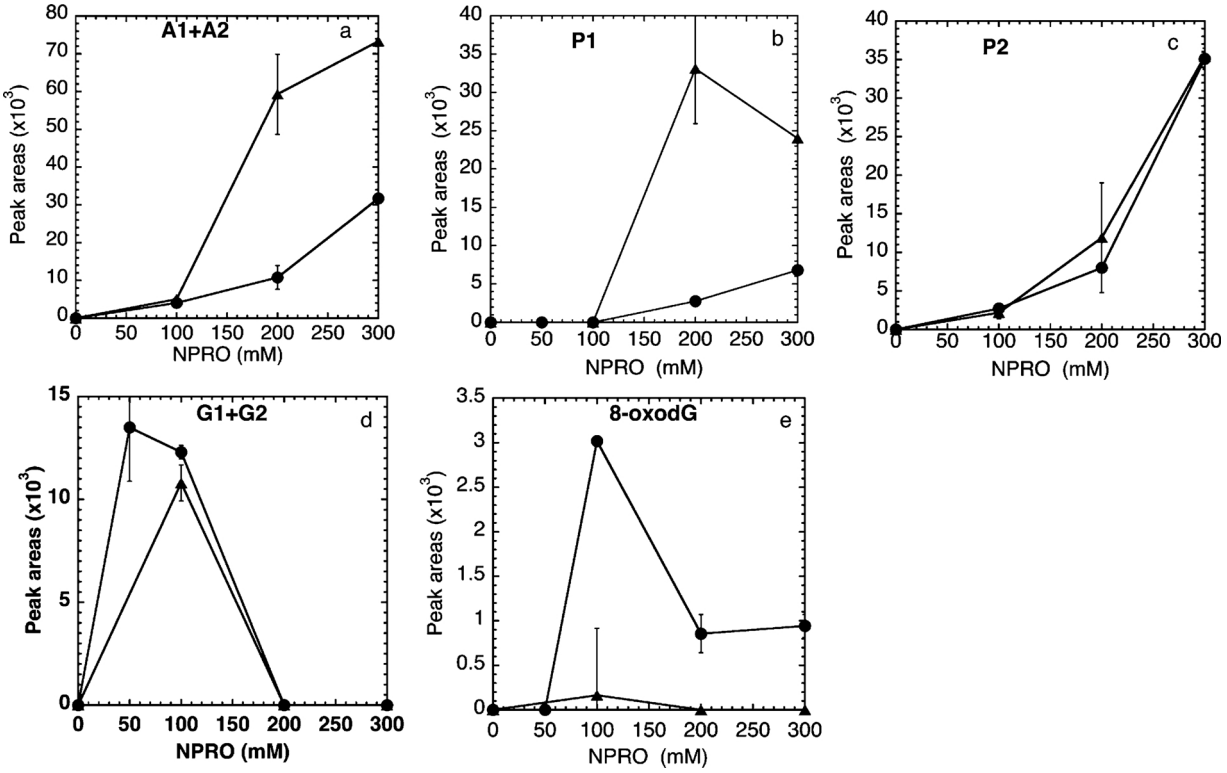
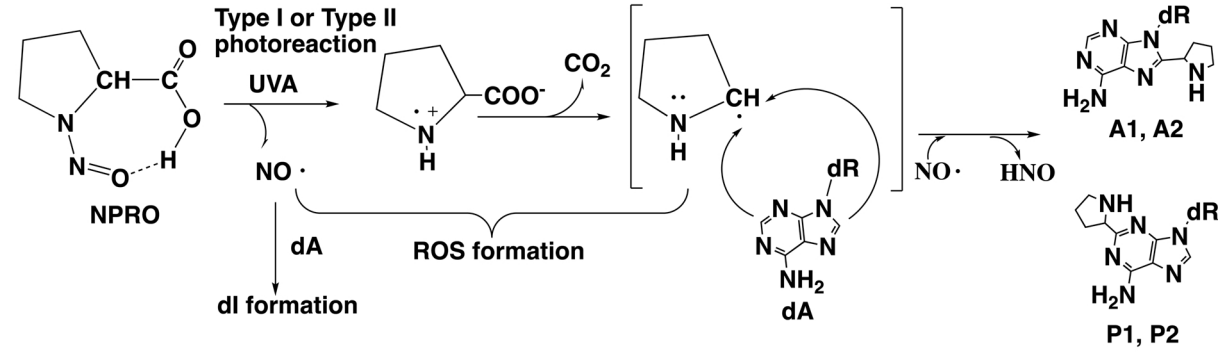


Fig. 11. NPRO dose-dependent adduct formation of irradiated DNA with NPRO under air conditions (circle) or N_2 replacement conditions (triangle). UVA irradiation was performed for 12 h (359 kJ/m^2). The amount of (a) A1 + A2, (b) P1, (c)P2, (d) G1 + G2, and (e) 8-oxodG photoadducts produced is shown.

Scheme 1



Scheme 2

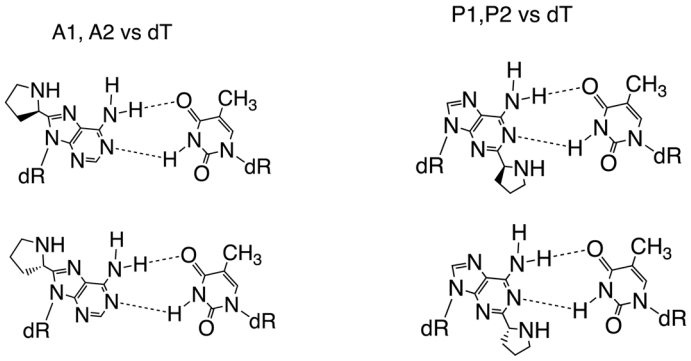


Fig. 12. Photoreaction Scheme.

dimethylaminiun and NO radicals. UVA-triggered NO release was reported from a N-nitrosamine in mitochondria in live cells [20]. We observed NO release from irradiated NPRO in a neutral solution [21]. Therefore, photo-excited NPRO is considered to release NO radical with photocleavage of the N—N bond, followed by subsequent production of a pyrrolidyl radical resulting in formation of pyrrolidyl-dA adducts (Fig. 12). Formation of A1 and A2 was dependent on NPRO dose (Fig. 3a), and this was reflected in the absorption curve of NPRO (Fig. 8). Wavelength-dependent formation of A1 and A2 suggests that sensitization of NPRO by UVA triggers the formation of both A1 and A2 (Fig. 8). Kwon et al. suggested that NDMA photo-decomposition produced hydroxyl radical-like reactive species besides NO radical [22]. Similar oxidative photo-reactions might also occur during NPRO photo-decomposition, leading to the formation of dI from dA, and 8-oxodG, deoxyoxanosine & deoxyxanthosine from dG, as previously described [14,15]. UVA-excited NPRO also reacted with DNA producing residues of A1, A2, P1, P2, G1, G2 and 8-oxodG (Fig. 9–11). Formation of A1 + A2, P1 and P2 were dependent on NPRO dose and irradiation (Fig. 10 and 11). Larger amounts of A1 + A2 and P1 adducts were detected under anaerobic conditions compared with aerobic conditions (Fig. 11). We observed that UVA irradiation of NPRO in aqueous solution generates oxidative reactive species, NO radical, OH radical, and singlet oxygen [12]. In the presence of O_2, photo-exited *N*-nitrosodimethylamine was reported to decompose to produce NO and superoxide anion radical, which probably generate peroxynitrite [22]. Reactive oxygen species (ROS) generated during photo-reaction in the presence of O_2 may act to destroy the activated form of irradiated NPRO. Under air condition, ROS production during the photoreaction with NPRO and dA might be occurred, and generated ROS might react with A1 + A2 and P1 as a secondary reaction to reduce the yields. Oxidative photo-reactions might decompose pyrrolidyl-dA adducts, thereby leading to lower yields of A1 + A2 and P1 under aerobic conditions. Alternatively, photo-reactions of pyrrolidyl radical with dA may increase under anaerobic conditions due to reduced competition with oxygen-related photo-processes. There was little difference in P2 formation observed between aerobic and anaerobic conditions. The difference observed in the formation of P1 and P2 might be due to restrictions imposed by steric hindrance on the reaction of pyrrolidyl radical with dA in the DNA strand (Fig. 12). The formation of G1 + G2 was independent of NPRO dose but dependent on the irradiation, with little difference being observed between aerobic and anaerobic conditions (Figs. 10c and 11c). As the amount of G1 + G2 formed from DNA irradiated for 12 h with 100 mM NPRO was quite high, adduct formation might quickly have reached a plateau, with no apparent difference being observed between aerobic and anaerobic conditions. The formation of 8-oxodG from dG in DNA observed under aerobic conditions probably resulted from oxidative photo-reactions during NPRO photo-decomposition (Figs. 10d, 11d, 12). Amount of 8-oxodG decreased with 200 or 300 mM NPRO. Since the molar absorption coefficient of NPRO at 340 nm in 10 mM sodium phosphate buffer at pH 7.4 was calculated to be 81.0, these decrease might be due to an filter effect of NPRO. In our earlier studies, we have shown that the activity of irradiated NPRO with DNA involves alkylation and oxidation of DNA [12]. Botta et al. [23] reported that reactive oxygen species in CHO cells formed by UVA radiation of benzo(a)pyrene causes increased level of micronuclei and 8-oxodG legions, and Warner et al. showed photo-oxidation of guanine base in DNA and RNA treated with UVA-sensitized furocoumarins with a mechanism involving singlet oxygen is known [24]. Reactive oxygen species including singlet oxygen might produce oxidation of DNA involving 8-oxodG formation. The formation ratio of dG adduct was one hundred times higher than that of the dA adduct, which may explain the most frequent mutations on M13mp2 DNA treated with NPRO and UVA being GC mutations [13].

Nitrosation of proline into NPRO may occur in the environment as well as with in vivo endogenous formation, and may be present on the skin with sunlight irradiation. Certain components of sunscreen have been reported to absorb and enter systemic circulation [25]. Since NPRO concentration in urine reported was an average value of 4.30 μg/g Cr [5] and molecular weight of NPRO is 144.130, NPRO concentration in urine is estimated an average value of about 0.30–0.25 nM. NPRO concentration in our research, 100 mM, is higher than those in body fluid. Because of the detection limits, photoreaction of NPRO at nM scale could not be examined in this research. Endogenous and exogenous NPRO might be photoactivated in vivo or in the environment thereby playing a role in the genotoxicity of sunlight. Further studies are required to delineate the mechanism pertaining to the phototoxicity of NPRO in vivo.

Author statement

Chiharu Asahi: Investigation on characterization of photoadducts from DNA, Shuhei Aoyama: Investigation on isolation of new photo-adducts, Sachiko Kimura: Supervision on LCMSMS analysis, Toshinori Suzuki: Supervision on MS analysis, Tsutomu Hatano: Investigation on NMR analysis, Sakae Arimoto-Kobayashi: Conceptualization, Supervision and wrighting

Funding

This work was supported by JSPS KAKENHI [Grant Number JP15K00556]; the NIBB Cooperative Research Program for the Okazaki Large Spectrograph [Grant Number 19-701&18-707]; and funding from The Okayama Medical Foundation.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgments

The authors wish to thank Dr. M. Mochizuki (Tokyo University of Science) for the gift of NPRO.

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