Uric Acid as a Photosensitizer in the Reaction of Deoxyribonucleosides with UV Light of Wavelength Longer than 300 nm: Identification of Products from 2’-Deoxycytidine

Toshinori Suzuki,* Atsuko Ozawa-Tamura, Miyu Takeuchi, and Yuriko Sasabe
School of Pharmacy, Shujitsu University; 1–6–1 Nishigawara, Okayama 703–8516, Japan.
Received June 14, 2021; accepted August 10, 2021

DNA reacts directly with UV light with a wavelength shorter than 300 nm. Although ground surface sunlight includes little of this short-wavelength UV light due to its almost complete absorption by the atmosphere, sunlight is the primary cause of skin cancer. Photosensitization by endogenous substances must therefore be involved in skin cancer development mechanisms. Uric acid is the final metabolic product of purines in humans, and is present at relatively high concentrations in cells and fluids. When a neutral mixed solution of 2’-deoxycytidine, 2’-deoxyguanosine, thymidine, and 2’-deoxyadenosine was irradiated with UV light with a wavelength longer than 300 nm in the presence of uric acid, all the nucleosides were consumed in a uric acid dose-dependent manner. These reactions were inhibited by the addition of radical scavengers, ethanol and sodium azide. Two products from 2’-deoxycytidine were isolated and identified as N5-hydroxy-2’-deoxycytidine and N7,5-cyclic amide-2’-deoxycytidine, formed by cycloaddition of an amide group from uric acid. A 15N-labeled uric acid, uric acid-1,3-15N2, having two 14N and two 15N atoms per molecule, produced N7,5-cyclic amide-2’-deoxycytidine containing both 14N and 15N atoms from uric acid-1,3-15N2. Singlet oxygen, hydroxyl radical, peroxynitrous acid, hypochlorous acid, and hypobromous acid generated neither N4-hydroxy-2’-deoxycytidine nor N7,5-cyclic amide-2’-deoxycytidine in the presence of uric acid. These results indicate that uric acid is a photosensitizer for the reaction of nucleosides by UV light with a wavelength longer than 300 nm, and that an unidentified radical derived from uric acid with a delocalized unpaired electron may be generated.

Key words uric acid; photosensitizer; UV light; deoxycytidine; cyclic amide

Introduction
Uric acid is the final metabolic product of purine catabolism in humans; the enzyme uricase that catalyzes the conversion of uric acid to a further oxidation product, allantoin, became inactivated during human evolution.1,2) Thus, humans have a relatively high concentration of uric acid in serum: 2.6–7.2 mg/dL (155–428 μM) is considered normal in many countries.3) Uric acid is an important antioxidant in humans, since it safely eliminates various reactive oxygen species.4,5) The effect of uric acid to scavenge reactive oxygen species may contribute to extending the human lifespan. However, uric acid can also act as a pro-oxidant. Exposure of cells to uric acid induces oxidative stress.6,7) Epidemiological cancer studies have reported both positive and negative associations between the serum uric acid concentration and cancer incidence.8) A study on this association in a large cohort (approx. 500000 persons) and with a long follow-up period (up to 25 years) was recently reported.9) Positive, negative, and neutral associations between the concentration of serum uric acid and cancer incidence were reported. The incidence of non-melanoma skin cancer in the highest quartile for the serum uric acid concentration compared with the lowest quartile showed hazard ratios of 1.42 for men and 1.67 for women, whereas that of melanoma skin cancer showed hazard ratios of 1.09 for men and 0.83 for women. Skin cancer in humans is closely associated with exposure to UV light from the sun.10,11) DNA and its components directly absorb UV light with a wavelength shorter than 300 nm to generate photoproducts, including thymine dimers.12,13) However, solar radiation shorter than 300 nm is almost completely absorbed by the atmosphere, primarily by molecular oxygen and ozone.10) In addition to direct UV reactions, DNA indirectly reacts with UV light at a wavelength longer than 300 nm and even to visible (Vis) light in the presence of photosensitizers, i.e., substances that cause photosensitization.13) Photosensitization comprises diverse processes including energy transfer, electron transfer, hydrogen atom abstraction, and formation of singlet oxygen (O2) and reactive free radicals including hydroxyl radical (·OH).14) Endogenous photosensitizers include riboflavin and bilirubin.15,16) We recently reported that salicylic acid is a photosensitizer of the reaction of thymidine (dThd) with UV light between 300 and 350 nm, resulting in cyclobutane dThd dimers via energy transfer.17) We conducted experiments to identify other compounds that enhance nucleoside consumption by UV irradiation. Among the biological substances we examined, uric acid decreased the concentration of nucleosides when a nucleoside mixture was irradiated with UV light at a wavelength longer than 300 nm. In the present study, we report the effects of uric acid on UV irradiation of nucleosides and identification of the products of 2’-deoxycytidine (dCyd). We discuss the reactive species in this reaction system based on the results using radical scavengers and 15N-labeled uric acid.

Results and Discussion
A mixed solution of nucleosides (dCyd, 2’-deoxyguanosine (dGuo), dThd, and 2’-deoxyadenosine (dAdo); 100 μM each) with uric acid in 100 mM potassium phosphate buffer at pH

© 2021 The Pharmaceutical Society of Japan
7.4 was irradiated with UV light from a high-pressure mercury lamp through a 300-nm longpass filter at a temperature of 37°C. The reaction mixture was analyzed by reversed phase (RP) HPLC. The nucleoside concentrations were determined from the absorbance area of HPLC detected at 260 nm. Figure 1A shows the uric acid dose-dependent changes in concentrations of nucleosides when a solution of nucleosides with 0–400 μM uric acid was irradiated by UV light for 10 min. At 0 μM uric acid, no nucleosides reacted. The concentrations of all nucleosides decreased with an increasing uric acid dose up to 100 μM. At 200–400 μM uric acid, the concentrations of dCyd, dThd, and dAdo were almost constant, whereas the consumption of dGuo diminished with an increasing uric acid dose. The most reacted nucleoside was dCyd in the presence of dCyd, dThd, and dAdo. Figure 1B shows the irradiation time-dependence of the concentrations of nucleosides when a solution of nucleosides with 400 μM uric acid was irradiated by UV light for 0–30 min. In this range of irradiation times, the concentration of uric acid in the solution was low due to the decomposition of uric acid (data not shown). This acceleration of the dGuo reaction under prolonged irradiation is consistent with the profile of uric acid dose-dependence shown in Fig. 1A, in which high consumption of dGuo occurs at low concentrations of uric acid, and indicates that at least two mechanisms, one of which involves all four nucleosides and the other involves only dGuo, are associated with the present reaction system. Urlic acid can act as a scavenger of various reactive species, and at high concentrations it should eliminate the reactive species specific to dGuo efficiently and safely.

To obtain information about the reaction mechanisms, the UV irradiation reactions of nucleosides were conducted in the presence of additives: ethanol (EtOH, a scavenger of radicals),18,19) sodium azide (NaN3, a scavenger of radicals and 1O2),20–22) or deuterium oxide (D2O, an enhancer of 1O2 reaction by prolongation of the lifetime of 1O2).23) Table 1 shows the effects of 1% EtOH, 10 mM NaN3, and 99.9% D2O on the UV irradiation reactions of nucleosides. The mixed nucleoside solution with 0, 100, or 400 μM uric acid in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV light through a 300-nm longpass filter for 0–30 min at a temperature of 37°C.

![Fig. 1](image)

**Fig. 1.** (A) Uric Acid Dose-Dependence of the Concentration Changes in Nucleosides When a Solution of 100 μM Each of dCyd, dGuo, dThd, and dAdo with 0–400 μM Uric Acid Was Irradiated with UV Light through a 300-nm Longpass Filter at pH 7.4 and 37°C; (B) Time-Course of the Concentration Changes in Nucleosides When a Solution of 100 μM Each of dCyd, dGuo, dThd, and dAdo with 400 μM Uric Acid in 100 mM Potassium Phosphate Buffer at pH 7.4 Was Irradiated with UV Light through a 300-nm Longpass Filter for 0–30 min at a Temperature of 37°C.

Table 1. Effects of Additives on the Reactions of Nucleosides and Uric Acid with UV Light (>300 nm)24)

| Additives | dCyd (μM) | dGuo (μM) | dThd (μM) | dAdo (μM) |
|-----------|-----------|-----------|-----------|-----------|
| —0 μM Uric acid— | | | | |
| None | 100.9 ± 0.8 | 99.0 ± 0.3 | 100.7 ± 0.2 | 100.4 ± 0.2 |
| 1% EtOH | 100.3 ± 0.6 | 99.1 ± 0.4 | 100.3 ± 0.3 | 100.4 ± 0.3 |
| 10 mM NaN3 | 99.6 ± 0.9 | 100.2 ± 0.4 | 100.4 ± 0.5 | 100.5 ± 0.4 |
| 99.9% D2O | 95.5 ± 0.3 | 91.7 ± 0.5 | 99.3 ± 0.0 | 99.7 ± 0.3 |
| —100 μM Uric acid— | | | | |
| None | 61.1 ± 2.0 | 58.1 ± 2.6 | 72.7 ± 0.8 | 88.7 ± 0.9 |
| 1% EtOH | 98.5 ± 0.4 | 100.3 ± 0.7 | 99.1 ± 0.3 | 100.4 ± 0.5 |
| 10 mM NaN3 | 98.4 ± 1.0 | 99.4 ± 1.2 | 99.2 ± 1.0 | 100.0 ± 1.2 |
| 99.9% D2O | 57.8 ± 0.2 | 49.4 ± 1.7 | 65.1 ± 0.3 | 83.0 ± 0.2 |
| —400 μM Uric acid— | | | | |
| None | 47.8 ± 1.2 | 81.2 ± 1.0 | 62.2 ± 0.7 | 85.0 ± 1.0 |
| 1% EtOH | 99.9 ± 0.6 | 100.8 ± 0.8 | 99.1 ± 0.8 | 100.4 ± 1.1 |
| 10 mM NaN3 | 99.9 ± 0.3 | 100.3 ± 0.2 | 100.3 ± 0.2 | 100.6 ± 0.2 |
| 99.9% D2O | 47.0 ± 1.6 | 78.2 ± 0.6 | 62.5 ± 1.3 | 82.2 ± 1.1 |

*The nucleoside concentrations were determined by RP-HPLC analysis. Means ± S.D. (n = 3) are presented. A solution containing a mixture of nucleosides (dCyd, dGuo, dThd, and dAdo, 100 μM each) and 0, 100, or 400 μM uric acid containing the additives in 100 mM potassium phosphate buffer (pH 7.4) was irradiated with UV light (300-nm longpass filter) at 37°C for 10 min.*
the effects of EtOH, NaN₃, and D₂O on the reaction by methylene blue with Vis light, a well-established ¹O₂-producing system, as a comparison.²⁵ A mixed solution of nucleosides with 0, 100, or 400 µM uric acid was irradiated with Vis light from a tungsten lamp through a 520-nm longpass filter in the presence of 25 µM methylene blue at pH 7.4 and 37°C for 10 min. With 0 µM uric acid, only dGuo was consumed. EtOH showed no effect, whereas NaN₃ suppressed and D₂O strongly enhanced the reaction of dGuo. With 100 µM uric acid, the effects of the additives were similar to those with 0 µM uric acid. With 400 µM uric acid, the reaction of dGuo was suppressed. Table 3 shows the effects of EtOH, NaN₃, and D₂O on the reaction by nitrite with UV light, a ·OH-producing system, as another comparison. It has been reported that nitrite with UV light generates ·OH and nitric oxide (·NO), and the formed ·OH reacts with dCyd, dGuo, dThd, and dAdo with comparable reactivities.²⁶,²⁷ A mixed solution of nucleosides with 0, 100, or 400 µM uric acid was irradiated by UV light from a high-pressure mercury lamp through a 350-nm longpass filter in the presence of 1 mM NaN,O₂ at pH 7.4 and 37°C for 20 min. With 0 µM uric acid, all the nucleosides were consumed. EtOH and NaN₃ suppressed the reactions, whereas D₂O enhanced the reaction of dGuo. It has been reported that with 100 µM uric acid, the reactions of all the nucleosides were slightly suppressed. The reaction profiles with 100 and 400 µM uric acid by the ·OH system (Table 3) were similar to those for the uric acid/UV system (Table 1). The results shown in Tables 1–3 suggest that the

### Table 2. Effects of Additives on the Reactions of Nucleosides and Methylene Blue with Vis Light (>520 nm)³⁰

| Additives | dCyd (µM) | dGuo (µM) | dThd (µM) | dAdo (µM) |
|-----------|-----------|-----------|-----------|-----------|
| —0µM Uric acid— |           |           |           |           |
| None      | 99.9 ± 0.2 | 60.9 ± 0.7 | 99.8 ± 0.3 | 99.8 ± 0.3 |
| 1% EtOH   | 100.8 ± 0.2 | 57.4 ± 0.9 | 100.4 ± 0.4 | 100.2 ± 0.2 |
| 10mM NaN₃ | 100.5 ± 0.4 | 98.7 ± 0.8 | 100.8 ± 0.3 | 100.5 ± 0.6 |
| 99.9% D₂O | 99.0 ± 0.4 | 0.3 ± 0.1  | 93.7 ± 0.3  | 98.3 ± 0.5  |
| —100µM Uric acid— |           |           |           |           |
| None      | 99.9 ± 0.9 | 61.9 ± 0.6 | 99.6 ± 0.7 | 99.8 ± 1.0 |
| 1% EtOH   | 100.5 ± 0.4 | 62.4 ± 0.5 | 100.2 ± 0.3 | 100.0 ± 0.4 |
| 10mM NaN₃ | 99.8 ± 0.4 | 98.3 ± 0.3 | 99.9 ± 0.2 | 100.0 ± 0.2 |
| 99.9% D₂O | 99.7 ± 0.2 | 0.6 ± 0.2  | 94.9 ± 0.2  | 99.0 ± 0.1  |
| —400µM Uric acid— |           |           |           |           |
| None      | 99.8 ± 0.5 | 90.2 ± 0.2 | 100.1 ± 0.4 | 99.7 ± 0.4 |
| 1% EtOH   | 100.0 ± 0.2 | 90.0 ± 0.8 | 100.4 ± 0.3 | 99.9 ± 0.1 |
| 10mM NaN₃ | 99.9 ± 0.1 | 99.4 ± 0.2 | 99.8 ± 0.1 | 99.5 ± 0.1 |
| 99.9% D₂O | 99.8 ± 0.8 | 58.8 ± 1.6 | 99.0 ± 0.6 | 99.6 ± 0.7 |

³⁰ The nucleoside concentrations were determined by RP-HPLC analysis. Means ± S.D. (n = 3) are presented. A solution containing a mixture of nucleosides (dCyd, dGuo, dThd, and dAdo, 100µM each), 25 µM methylene blue, and 0, 100, or 400 µM uric acid containing the additives in 100mM potassium phosphate buffer (pH 7.4) was irradiated with Vis light (520-nm longpass filter) at 37°C for 10 min.

### Table 3. Effects of Additives on the Reactions of Nucleosides and Nitrite with UV Light (>350 nm)³⁰

| Additives | dCyd (µM) | dGuo (µM) | dThd (µM) | dAdo (µM) |
|-----------|-----------|-----------|-----------|-----------|
| —0µM Uric acid— |           |           |           |           |
| None      | 64.1 ± 0.1 | 79.1 ± 0.7 | 64.1 ± 0.5 | 81.4 ± 0.2 |
| 1% EtOH   | 99.2 ± 0.2 | 98.4 ± 0.2 | 99.0 ± 0.5 | 99.8 ± 0.2 |
| 10mM NaN₃ | 96.4 ± 0.2 | 98.4 ± 0.1 | 96.1 ± 0.2 | 97.9 ± 0.2 |
| 99.9% D₂O | 62.1 ± 0.6 | 60.2 ± 0.9 | 62.2 ± 1.2 | 77.7 ± 0.6 |
| —100µM Uric acid— |           |           |           |           |
| None      | 65.4 ± 0.4 | 80.3 ± 0.3 | 64.7 ± 0.2 | 81.8 ± 0.2 |
| 1% EtOH   | 99.5 ± 0.1 | 99.7 ± 0.2 | 99.3 ± 0.1 | 100.2 ± 0.1 |
| 10mM NaN₃ | 97.5 ± 0.2 | 99.1 ± 0.3 | 96.5 ± 0.1 | 99.1 ± 0.2 |
| 99.9% D₂O | 61.4 ± 0.7 | 66.2 ± 0.3 | 62.0 ± 0.9 | 77.6 ± 0.7 |
| —400µM Uric acid— |           |           |           |           |
| None      | 70.5 ± 1.1 | 85.9 ± 0.8 | 76.2 ± 1.2 | 84.4 ± 0.8 |
| 1% EtOH   | 99.9 ± 0.6 | 101.1 ± 0.2 | 99.6 ± 0.5 | 100.6 ± 0.5 |
| 10mM NaN₃ | 97.7 ± 0.2 | 99.7 ± 0.2 | 97.4 ± 0.3 | 99.3 ± 0.2 |
| 99.9% D₂O | 64.7 ± 0.7 | 75.7 ± 0.5 | 67.9 ± 0.8 | 80.0 ± 0.5 |

³⁰ The nucleoside concentrations were determined by RP-HPLC analysis. Means ± S.D. (n = 3) are presented. A solution containing a mixture of nucleosides (dCyd, dGuo, dThd, and dAdo, 100µM each), 1 mM nitrite, and 0, 100, or 400 µM uric acid containing the additives in 100mM potassium phosphate buffer (pH 7.4) was irradiated with UV light (350-nm longpass filter) at 37°C for 20 min.
contribution of $^{1}$O$_2$ is small and that -OH and other radicals are the major reactive species in the uric acid/UV system.

We then investigated the reaction of dCyd in the presence of uric acid to obtain information on the products of this UV irradiation system. A solution of 100 $\mu$M dCyd with 400 $\mu$M uric acid in 100mM potassium phosphate buffer at pH 7.4 was irradiated with UV light through a 300-nm longpass filter at a temperature of 37°C for 10 min. Figure 2 shows the RP-HPLC chromatogram of the reaction mixture. Two product peaks with retention times of 14.2 and 14.9 min, referred to as Product 1 and Product 2, respectively, were detected in addition to the peaks of dCyd, uric acid, and decomposition products of uric acid denoted by asterisks. UV spectra of the products are shown in the Fig. 2 insets. The products were isolated by RP-HPLC and subjected to MS and NMR.

For Product 1, UV spectra of the products agreed with the theoretical molecular mass for C$_9$H$_{12}$N$_3$O$_5$ and the TOF-MS value of the molecular ion (M$^+$) of Product 1 showed a signal at $m/z$ 242 within 3 ppm. Product 1 showed a $^{1}$H-NMR spectrum similar to that reported for N$^4$-hydroxy-2'-deoxycytidine (N$^4$-OH-dCyd). The $\lambda_{max}$ values and their relative intensities of Product 1 were similar to those reported for N$^4$-OH-dCyd. Thus, Product 1 was identified as N$^4$-OH-dCyd. It has been reported that N$^4$-OH-dCyd is synthesized from dCyd by the reaction with hydroxylamine (NH$_2$OH). That reaction mechanism was proposed via addition of hydroxylamine to the 5,6-double bond of the cytosine moiety and replacement by hydroxylamine in the exocyclic amino group of cytosine. We could not find any report on the formation of N$^4$-OH-dCyd by oxidation of dCyd. Regarding biological activities, it has been reported that 5'-monophosphate of N$^4$-OH-dCyd is a strong inhibitor of thymidylate synthase.

For Product 2, ESI-TOF-MS showed a signal at $m/z$ 267 in negative mode. HR-ESI-TOF/MS values of the molecular ion (M$^+$) of Product 2 were similar to those reported for N$^4$-OH-dCyd. Thus, Product 2 was identified as N$^4$-OH-dCyd. It has been reported that N$^4$-OH-dCyd is synthesized from dCyd by the reaction with hydroxylamine (NH$_2$OH). That reaction mechanism was proposed via addition of hydroxylamine to the 5,6-double bond of the cytosine moiety and replacement by hydroxylamine in the exocyclic amino group of cytosine. The 13C spectrum of Product 2 showed the disappearance of an aromatic proton attributable to the 5-position of the cytosine base. The $^{13}$C spectrum of Product 2 showed the appearance of an aromatic carbon signal at 155.2 ppm. In conjunction with the results of two-dimensional NMR measurements ($^{1}$H–$^{1}$H correlation spectroscopy, $^{1}$H–$^{13}$C heteronuclear multiple quantum coherence, $^{1}$H–$^{13}$C heteronuclear multiple bond connectivity), Product 2 was identified as N$^4$-5-cyclocytosine (N$^4$,5-cAmd-dCyd). The cyclocaddition of the amide group between N4 and C5 of the pyrimidine ring of dCyd generated a bicyclo structure, a purine. We found only two reports of nucleoside synthesis of the same base moiety as N$^4$,5-cAmd-dCyd. A compound with the base moiety of N$^4$,5-cAmd-dCyd was synthesized from a 5-aminocytosine derivative treated by chloroethyl formate to insert a carbonyl group between the 4-amino and 5-amino groups of the 5-aminocytosine moiety. The structures of N$^4$-OH-dCyd and N$^4$,5-cAmd-dCyd are shown in Fig. 3. Uric acid dose-dependent changes in the reaction of dCyd with UV light were examined. A solution of 100 $\mu$M dCyd with 0–400 $\mu$M uric acid in 100mM potassium phosphate buffer at pH 7.4 was irradiated with UV light through a 300-nm longpass filter at a temperature of 37°C for 10 min. The concentrations were determined by RP-HPLC. Figure 4A shows the changes in concentrations of N$^4$-OH-dCyd and N$^4$,5-cAmd-dCyd. With 25–100 $\mu$M uric acid, N$^4$-OH-dCyd was greater than N$^4$,5-cAmd-dCyd, whereas with 200–400 $\mu$M uric acid, N$^4$,5-cAmd-dCyd was greater than N$^4$-OH-dCyd. Figure 4B shows the dCyd concentration.

![Fig. 2. RP-HPLC Chromatogram of a Reaction Mixture of dCyd with Uric Acid Detected at 230 nm](image)

A solution of 100 $\mu$M dCyd and 400 $\mu$M uric acid was irradiated with UV through a 300-nm longpass filter in 100mM potassium phosphate buffer at pH 7.4 and 37°C for 10 min. The HPLC system consisted of LC-10A (Shimadzu, Kyoto, Japan). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6 × 250 mm and particle size of 5 $\mu$m (GL Sciences, Tokyo, Japan) was used. The eluent was 20 mM ammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 50% over 30 min in linear gradient mode. The column temperature was 40°C and flow rate was 1 mL/min. The insets are the UV spectra of Products 1 and 2.

![Fig. 3. Structures of the Products N$^4$-OH-dCyd and N$^4$,5-cAmd-dCyd](image)

![Fig. 4. (A) Uric Acid Dose-Dependence of Concentration Changes in N$^4$-OH-dCyd (Open Triangles) and N$^4$,5-cAmd-dCyd (Open Squares); (B) Uric Acid Dose-Dependence of Concentration Changes in dCyd (Closed Circles) and the Total of N$^4$-OH-dCyd and N$^4$,5-cAmd-dCyd (Open Circles)](image)

A solution of 100 $\mu$M dCyd and 0–400 $\mu$M uric acid in 100mM potassium phosphate buffer at pH 7.4 was irradiated with UV light from a high-pressure mercury lamp through a 300-nm longpass filter for 10 min at a temperature of 37°C. The whole reaction mixture was analyzed by RP-HPLC. Means ± S.D. (n = 3) are presented.
and total concentration of $N^4$-OH-dCyd plus $N^4$-5-cAmd-dCyd. The consumption of dCyd was maximal with 100$\mu$M uric acid. Irradiation time-dependent changes of the dCyd reaction with UV light were examined. A solution of 100$\mu$M dCyd with 400$\mu$M uric acid was irradiated with UV light at pH 7.4 and 37°C for 0–30 min. Figure 5A shows the changes in concentrations of $N^4$-OH-dCyd and $N^4$-5-cAmd-dCyd. The concentrations of both products increased up to 10 min then decreased, indicating that both decompose quickly under UV irradiation. Figure 5B shows the dCyd concentration and total concentration of $N^4$-OH-dCyd plus $N^4$-5-cAmd-dCyd. As shown in Figs. 4B and 5B, the total concentration of $N^4$-OH-dCyd plus $N^4$-5-cAmd-dCyd was one-tenth or smaller than that of the consumed dCyd over the range of uric acid concentrations and irradiation times examined. This indicates that undetected products remained in the reaction solution.

In order to obtain information about the origin of the amide group added to dCyd generating $N^4$-5-cAmd-dCyd, a similar UV irradiation experiment was conducted using $^{15}$N-labeled uric acid (uric acid-1,3-15N2; 98 atom % $^{15}$N) with two $^{14}$N atoms and two $^{15}$N atoms per molecule. A solution of 100$\mu$M dCyd with 400$\mu$M uric acid-1,3-15N2 in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV light through a 300-nm longpass filter at a temperature of 37°C for 10 min. The products of $N^4$-OH-dCyd and $N^4$-5-cAmd-dCyd were isolated by RP-HPLC and subjected to MS. For $N^4$-OH-dCyd, the spectrum of ESI-TOF-MS obtained using uric acid-1,3-15N2 was similar to that using unlabeled uric acid (data not shown). For $N^4$-5-cAmd-dCyd, the spectrum of ESI-TOF-MS obtained using uric acid-1,3-15N2 was different from that using unlabeled uric acid. Figure 6A shows the MS of $N^4$-5-cAmd-dCyd formed using unlabeled uric acid. The molecular ion peak [M − H$^+$]$^-$ (m/z 267) and another peak [M − H$^+$ + 1]$^-$ (m/z 268) were observed with a naturally-occurring isotope ratio. Figure 6B shows the MS of $N^4$-5-cAmd-dCyd formed using uric acid-1,3-15N2. The relative peak area of m/z 268 increased, but the peak of m/z 267 was still present with a larger peak area than that of m/z 268. These results indicate that both the $^{14}$N atom and $^{15}$N atom of uric acid-1,3-15N2 were incorporated into $N^4$-5-cAmd-dCyd. In order to estimate the ratio of 14N:15N introduced into $N^4$-5-cAmd-dCyd from uric acid-1,3-15N2, the peak area of m/z 267 in Fig. 6B was compared with the peak area of m/z 268 originating from 15N of uric acid-1,3-15N2, which was obtained by subtraction of the contribution of the naturally-occurring isotopes shown in Fig. 6A. The ratio of 14N:15N introduced into $N^4$-5-cAmd-dCyd from uric acid-1,3-15N2 was calculated as 8:5. This result indicates that the amide group added to dCyd originates from both the five-membered imidazole ring and six-membered pyrimidine ring of uric acid. The reaction scheme of dCyd with uric acid-1,3-15N2 is shown in Fig. 7.

Uric acid can reportedly react with various reactive species including O$_2$, ·OH, peroxynitrous acid (ONOOH), hypochlorous acid (HOCl), and hypobromous acid (HOBr), since the redox potential of uric acid is low. In reactions with these reactive species, uric acid generates various intermediates including radicals and products. We examined the possibility that these intermediates or products of uric acid react with dCyd to generate $N^4$-OH-dCyd and $N^4$-5-cAmd-dCyd. The reaction solution was analyzed by RP-HPLC. Table 4 shows the
concentrations of \( N^{4}\text{-OH-dCyd} \), \( N^{4},5\text{-cAmd-dCyd} \), dCyd, and uric acid in the reaction solutions. The detection limits were 0.05 \( \mu \text{M} \) for both \( N^{4}\text{-OH-dCyd} \) and \( N^{4},5\text{-cAmd-dCyd} \). Neither \( N^{4}\text{-OH-dCyd} \) nor \( N^{4},5\text{-cAmd-dCyd} \) was detected in any of the reaction solutions except for the present uric acid/UV system. It has been reported that the reactivity of \( \text{O}_2^\cdot \) to dCyd is low.\(^{3}\) \( \text{O}_2^\cdot \) readily reacts with uric acid to form parabanic acid and its hydrolysate, and oxaluric acid via several intermediates.\(^{34,35}\)

The present methylene blue/Vis \( \text{O}_2^\cdot \)-generating system did not decrease dCyd in the presence or absence of uric acid, whereas uric acid readily reacted and decreased. For \( \cdot \text{OH} \), although a detailed study of the Fenton reaction of dCyd reported the formation of 26 products, neither \( N^{4}\text{-OH-dCyd} \) nor \( N^{4},5\text{-cAmd-dCyd} \) was formed.\(^{36}\) The reactivity of \( \cdot \text{OH} \) to uric acid is low, and the Fenton system generates small amounts of allantoin, oxonic/oxaluric acid, and parabanic acid.\(^{37}\) A urate radical, in which the unpaired electron is highly delocalized, resulting in resonance stabilization, is generated by pulse radiolysis of uric acid solution with one-electron oxidation.\(^{38,39}\)

The present two \( \cdot \text{OH} \)-producing systems, \( \text{NaNO}_2/\text{UV} \) and \( \text{Methylene blue/Vis} \), reportedly generates \( \cdot \text{NO} \) in addition to \( \cdot \text{OH} \).\(^{26} \) \( \cdot \text{NO} \) can react with ONOO\(^-\) to generate several radicals including urate radical, triuretcarbonyl radical, and aminocarbonyl radical (\( \text{H}_2\text{N}^-\text{C}=\text{O} \)), plus several products including allantoin and triuret.\(^{43-45}\) In the present experiment, ONOO\(^-\) did not react with dCyd in the presence or absence of uric acid, although uric acid reacted slightly. HOCl has been reported to react with dCyd, generating \( N^3\text{-chloro-dCyd} \) and \( 5\text{-chloro-dCyd} \).\(^{46}\) HOCl rapidly reacts with uric acid, forming allantoin, oxonic/oxaluric acid, and parabanic acid.\(^{37}\) For HOBr, 5-bromo-dCyd is generated from dCyd as the major product.\(^{47}\) Uric acid reacts with HOBr with a reactivity similar to that of HOCl.\(^{48}\) In the present experiments, although HOCl and HOBr reactions were eliminated effectively by uric acid, neither \( N^{4}\text{-OH-dCyd} \) nor \( N^{4},5\text{-cAmd-dCyd} \) was generated. Table 4 shows that in all the reactions examined, except for UV irradiation in the presence of uric acid, \( N^{3}\text{-OH-dCyd} \) and \( N^{4},5\text{-cAmd-dCyd} \) were not generated. These results indicate that reactive species other than those in the reaction systems examined are generated in the uric acid/UV system.

In conclusion, the present study showed that uric acid is a

\[ \text{Table 4. Concentrations of } N^{4}\text{-OH-dCyd, } N^{4},5\text{-cAmd-dCyd, dCyd, and Uric Acid in the Reaction Solution of dCyd in the Presence or Absence of Uric Acid for Various Reaction Systems}^{a} \]

| Reaction System | \( N^{4}\text{-OH-dCyd (µM)} \) | \( N^{4},5\text{-cAmd-dCyd (µM)} \) | dCyd (µM) | Uric Acid (µM) |
|-----------------|------------------|------------------|--------|------------|
| \( \text{UV} \) | <0.05 | <0.05 | 98.6 ± 1.0 | — |
| \( \text{Methylene blue/Vis} \) | <0.05 | <0.05 | 100.8 ± 0.7 | — |
| \( \text{NaNO}_2/\text{UV} \) | <0.05 | <0.05 | 57.6 ± 0.6 | — |
| \( \text{Fe}^{2+}/\text{H}_2\text{O}_2 \) | <0.05 | <0.05 | 85.3 ± 0.5 | — |
| \( \text{ONOOH} \) | <0.05 | <0.05 | 100.1 ± 0.5 | — |
| \( \text{HOCI} \) | <0.05 | <0.05 | 18.5 ± 2.0 | — |
| \( \text{HOBr} \) | <0.05 | <0.05 | 44.0 ± 1.9 | — |
| \( \text{HOCl} \) | <0.05 | <0.05 | 15.1 ± 0.7 | <1 |
| \( \text{Methylene blue/Vis} \) | <0.05 | <0.05 | 100.9 ± 0.3 | <1 |
| \( \text{NaNO}_2/\text{UV} \) | <0.05 | <0.05 | 63.7 ± 1.0 | <1 |
| \( \text{Fe}^{2+}/\text{H}_2\text{O}_2 \) | <0.05 | <0.05 | 91.9 ± 0.2 | 97 ± 0 |
| \( \text{ONOOH} \) | <0.05 | <0.05 | 100.5 ± 0.4 | 93 ± 3 |
| \( \text{HOCI} \) | <0.05 | <0.05 | 99.8 ± 0.2 | 33 ± 3 |
| \( \text{HOBr} \) | <0.05 | <0.05 | 97.1 ± 0.5 | 15 ± 3 |
| \( \text{HOCl} \) | <0.05 | <0.05 | 32.1 ± 2.8 | 79 ± 8 |
| \( \text{Methylene blue/Vis} \) | <0.05 | <0.05 | 100.5 ± 0.5 | 10 ± 3 |
| \( \text{NaNO}_2/\text{UV} \) | <0.05 | <0.05 | 63.6 ± 0.7 | 40 ± 5 |
| \( \text{Fe}^{2+}/\text{H}_2\text{O}_2 \) | <0.05 | <0.05 | 98.2 ± 0.3 | 396 ± 0 |
| \( \text{ONOOH} \) | <0.05 | <0.05 | 100.3 ± 0.3 | 388 ± 7 |
| \( \text{HOCI} \) | <0.05 | <0.05 | 99.9 ± 0.5 | 341 ± 4 |
| \( \text{HOBr} \) | <0.05 | <0.05 | 97.7 ± 0.3 | 313 ± 7 |

\( a \) The concentrations were determined by RP-HPLC analysis. Means ± S.D. (n = 3) are presented. b) A solution containing 100 µM dCyd and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was irradiated with UV light (590 nm longpass filter) at 37 °C for 10 min. c) A solution containing 100 µM dCyd, 25 µM methylene blue and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was irradiated with Vis light (520 nm longpass filter) at 37 °C for 10 min. d) A solution containing 100 µM dCyd, 1.1 mM NaNO\(_2\), and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was irradiated with UV light (350 nm longpass filter) at 37 °C for 20 min. e) A solution containing 100 µM dCyd, 100 mM FeSO\(_4\), 200 µM H\(_2\)O\(_2\), and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 30 min. f) A solution containing 100 µM dCyd, 100 µM peroxynitrite, and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 30 min. g) A solution containing 100 µM dCyd, 100 µM HOCI, and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 30 min. h) A solution containing 100 µM dCyd, 100 µM HOBr, and 0, 100, or 400 µM uric acid in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 30 min.
photosensitizer for nucleoside reactions by UV light with a wavelength longer than 300 nm. The reactive species of this reaction involve an unidentified radical from uric acid with a delocalized unpaired electron. Since uric acid exists ubiquitously at high concentrations in human cells and fluids, we should pay attention to the genotoxicity of uric acid in terms of DNA damage by sunlight. This is a fundamental study using nucleosides and artificial UV light. Further studies are needed to clarify the reliable structure of transient reactive species from uric acid using spin trap ESR and pulse photolysis, and to confirm the generation of specific products by uric acid in DNA of human skin cells exposed to sunlight.

Experimental

Materials dCyd, dGuo, dThd, dAdo, uric acid, and uric acid-1,3,15N2 (98 atom % 15N) were purchased from Sigma-Aldrich (MO, U.S.A.). Other chemicals were obtained from Sigma-Aldrich, Nacalai Tesque (Kyoto, Japan), Tokyo Chemical Industry (Tokyo, Japan), or Santoku Chemical Industries (Tokyo, Japan). Water was purified with a Millipore Milli-Q deionizer (MA, U.S.A.). Peroxynitrite (ONOO−) was synthesized by the reaction between acidified H2O2 and NaN3O2 followed by rapid mixing with excess NaOH in a quenched-flow reactor, as previously described. Unreacted H2O2 was removed by MnO2. The concentration of ONOO− was determined spectrophotometrically from its absorbance at 302 nm in 0.1 M NaOH using a molar extinction coefficient of 1670 M−1 cm−1. Chloride-free sodium hypochlorite (NaOCl) was prepared from bromine water (254 nm and 1 cm) for 254 nm and 1 cm.

Irradiation Conditions For UV light irradiations, UV light originating from a 250 W high-pressure mercury lamp (SP9-250UB, Ushio, Tokyo, Japan) with an optical filter through a light guide was used to directly irradiate the surface of a solution (1 mL) in a glass vial (12 mm i.d.) without a cap at 37°C. Longpass filters L10300 (cut-on 300nm) or LU0350 (cut-on 350nm) (Asahi Spectra, Tokyo, Japan) were used as the optical filters. The intensity of radiation on the surface of the sample solution was measured with a photometer (UVT-150, Ushio, Tokyo, Japan) equipped with the sensor UVD-S254 or UVD-S365. The intensities of the UV light were 0 mW/cm² for 254 nm and 263 mW/cm² for 365 nm with the 300 nm longpass filter, and 0 mW/cm² for 254 nm and 187 mW/cm² for 365 nm with the 350 nm longpass filter. For visible light irradiation, visible (Vis) light originating from a 100-W halogen lamp (MHAA-100W, Moritex, Tokyo, Japan) through a light guide with an orange cellulose sheet (cut-on 520 nm) was used to directly irradiate the surface of a solution (1 mL) in a glass vial (12 mm i.d.) without a cap at 37°C.

HPLC and MS Conditions The HPLC system consisted of LC-10ADvp pumps and an SPD-M10Avp UV/Vis photodiode-array detector (Shimadzu, Kyoto, Japan). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6 × 250mm and particle size of 5µm (GL Sciences, Tokyo, Japan) was used. The eluent was 20mM ammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 50% over 15 min in linear gradient mode, and maintained at 50% from 15 to 30 min. The column temperature was 40°C and flow rate was 1 mL/min. The RP-HPLC chromatogram was detected at 200–500 nm. ESI-TOF/MS measurements were performed on a MicrOTOF spectrometer (Bruker, Bremen, Germany) in negative mode. The sample isolated by RP-HPLC was directly infused into the MS system by a syringe pump without a column.

Spectrometric Data Product 1. N4-Hydroxy-2’-deoxycytidine (N4-OH-dCyd), 1-(2-deoxy-b-D-ribofuranosyl)-4-hydroxylamin-2(1H)-pyrimidinone. ESI-TOF/MS (negative mode): m/z 242. HR-ESI-TOF/MS (negative mode): m/z 242.07891 obsd. (calcd for C12H12N4O5, 242.078244). UV (pH 7.0): λmax = 236, 266 nm, ε236nm = 9540 M−1 cm−1, ε260nm = 5250 M−1 cm−1. 1H-NMR (500 MHz, in dimethyl sulfoxide (DMSO))−: δ (ppm/tetramethylsilane (TMS)) 7.03 (d, J = 8.6 Hz, 1H, H-5), 6.15 (dd, J = 6.0, 8.3 Hz, 1H, H-1’), 5.56 (d, J = 8.1 Hz, 1H, H-6), 4.19 (m, 1H, H-3’), 3.71 (m, 1H, H-4’), 3.51 (m, 2H, H-5,5’), 2.01 (m, 1H, H-2’ or 2”), 1.95 (m, 1H, H-2’ or 2”).

Product 2. N5,5-Cyclic amide-2’-deoxycytidine (N5,5-cAmd-dCyd), 1-(2-deoxy-b-D-ribofuranosyl)-1H-purine-2,8(7H,9H)-dione. ESI-TOF/MS (negative mode): m/z 267. HR-ESI-TOF/MS (negative mode): m/z 267.0361 obsd. (calcd for C10H11N3O5, 267.03493). UV (pH 7.0): λmax = 219, 314 nm, ε238nm = 13280 M−1 cm−1, ε260nm = 2660 M−1 cm−1. 1H-NMR (500 MHz, in dimethyl sulfoxide (DMSO))−: δ (ppm/TMS) 7.79 (s, 1H, H-6), 6.22 (dd, J = 6.3, 6.3 Hz, 1H, H-1’), 4.23 (m, 1H, H-3’), 3.83 (m, 1H, H-4’), 3.60 (m, 2H, H-5,5’), 2.21 (m, 1H, H-2’ or 2”), 1.98 (m, 1H, H-2’ or 2”). 13C-NMR (125 MHz, in DMSO)−: δ (ppm/TMS) 158.0 (C-4), 155.2 (C-8), 153.7 (C-2), 118.4 (C-6), 112.3 (C-5), 87.5 (C-4’), 85.9 (C-1’), 70.1 (C-3’), 60.9 (C-5’), 40.8 (C-2’). The numbering of atoms in the base moiety of N5,5-cAmd-dCyd was based on that of a purine ring.

Quantitative Procedures For the reactions of nucleoside mixtures, the concentrations of the nucleosides were evaluated according to the integrated peak areas on RP-HPLC chromatograms detected at 260 nm compared with the peak areas of the starting nucleoside mixture. For the reactions of dCyd, the concentrations of the products were evaluated according to the integrated peak areas on RP-HPLC chromatograms detected at 230 nm and by the molecular extinction coefficients at 230 nm (ε230nm). The ε230nm value of 9540 M−1 cm−1 for N4-OH-dCyd was calculated using the UV spectrum from this study and the reported ε270nm value of 5200 M−1 cm−1 at pH 7.30 The ε230nm value of N5,5-cAm-dCyd was determined as 13280 M−1 cm−1 from the integration of proton signals of NMR and the HPLC peak area related to that of dCyd (ε230nm = 8160 M−1 cm−1) in the mixed solution.

Conflict of Interest The authors declare no conflict of interest.

References

1) Wu X. W., Lee C. C., Muzny D. M., Caskey C. T., Proc. Natl. Acad. Sci. U.S.A., 86, 9412–9416 (1989).
2) Oda M., Satta Y., Takenaka O., Takahata N., Proc. Natl. Acad. Sci. U.S.A., 106, 460–463 (2002).
3) Desideri G., Castaldo G., Lombardi A., Mussap M., Testa A., Ponzemoli R., Punzi L., Borghi C., Eur. Rev. Med. Pharmacol. Sci., 18, 1295–1306 (2014).
