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Formation of a Diimino-Imidazole Nucleoside from 2′-Deoxyguanosine by Singlet Oxygen Generated by Methylene Blue Photooxidation

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Abstract—Singlet oxygen ($^1O_2$) is capable of inducing genotoxic, carcinogenic and mutagenic effects. It has previously been reported that the reaction of $^1O_2$ with 2′-deoxyguanosine, which is a major target of $^1O_2$ among the DNA constituents, leads to formation of various oxidized products including 8-oxo-7,8-dihydro-2′-deoxyguanosine and spiroiminodihydantoin, amino-imidazolone and diamino-oxazolone nucleosides. In addition to these products, we report that a novel diimino-imidazole nucleoside, 2,5-diimino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-2*H*,5*H*-imidazole (dD), is formed by reaction of 2′-deoxyguanosine with $^1O_2$ generated by irradiation with visible light in the presence of methylene blue under aerobic conditions. Its identification is based on identical chromatographic and spectroscopic data with an authentic compound, which we recently isolated and characterised from the reaction mixture of 2′-deoxyguanosine with reagent HOCl and a myeloperoxidase–H_2O_2–Cl^- system. The yield of dD was increased by D_2O and decreased by azide. dD was not generated from 8-oxo-7,8-dihydro-2′-deoxyguanosine. These results indicate that dD is generated by $^1O_2$ directly from 2′-deoxyguanosine, but not via 8-oxo-7,8-dihydro-2′-deoxyguanosine. dD may play a role in the genotoxicity of singlet oxygen in cells.

Introduction

Singlet oxygen ($^1O_2$) is an excited state of molecular oxygen. $^1O_2$ can be produced in biological systems by photoexcitation through energy transfer (type II reaction) upon exposure of endogenous photosensitizers.[1,2] $^1O_2$ is capable of inducing genotoxic, carcinogenic and mutagenic effects.[1,2] Among the constituents of DNA, 2′-deoxyguanosine (dG) is a major target of $^1O_2$.[3] The reaction of $^1O_2$ with dG leads to predominant formation of a spiroiminodihydantoin nucleoside (dS) together with 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an amino-imidazolone nucleoside (dIz) and its hydrolyzed nucleoside, a diamino-oxazolone nucleoside (dZ).[4,5] 8-OxodG is a much better substrate than dG for the reaction with $^1O_2$.[6] dS, dIz, dZ and other compounds including cyanuric acid and an oxidized guanidinohydantoin nucleoside have been reported as products of the reaction of 8-oxodG with $^1O_2$.[7,8] However, dS was a minor product in this system.

It has been proposed that $^1O_2$ may be involved in the human host defense system.[9] Steinbeck et al.[10] reported that about 20% of the oxygen consumed by neutrophils was converted to singlet oxygen within the phagosomes. However, the general consensus is that if singlet oxygen is produced in neutrophils, it is only in very small amounts.[11] On the other hand, there is no doubt that appreciable amounts of hypochlorous acid (HOCl) are discharged into the extracellular space surrounding neutrophils.[11] HOCl generated by myeloperoxidase is of central importance in the host defense mechanism. The HOCl formed also has the potential to harm normal tissue and contribute to inflammatory injury. HOCl has been reported to react with nucleic acid bases to form various compounds.[12–17] Recently, we showed that dS was generated from 8-oxodG and dG by HOCl as a major product.[18,19] We also found that a novel diimino-imidazole nucleoside (dD) was generated from dG by the reaction with reagent HOCl and a myeloperoxidase–H_2O_2–Cl^- system in addition to dIz and 8-chloro-2′-deoxyguanosine.[20,21] The involvement of $^1O_2$ in the formation of these products was ruled out for the reagent HOCl system under neutral conditions.[21] However, for the myeloperoxidase–H_2O_2–Cl^- system under mildly

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acidic conditions, the involvement of $^1O_2$ could not be excluded. There is a possibility that dD could be generated from dG by $^1O_2$ in addition to HOCl.

We have now studied the reaction of dG with a methylene blue photooxidation system under aerobic conditions, that is, with singlet oxygen, and found that dD is also generated in this reaction mixture.

Results

Formation of dD from dG by methylene blue photooxidation

When the mixture obtained from the methylene blue photooxidation of dG at pH 7.4 and 37 °C for 1 h under aerobic conditions was analyzed by RP-HPLC, several peaks were detected on the chromatogram in addition to unreacted dG (Fig. 1). Among the product peaks, four were identified as dS, dZ, dIz and 8-oxodG by coincidence of the retention time and UV spectrum of authentic samples. In addition to these previously reported products of the dG–methylene blue photooxidation system, we observed a peak (Fig. 1; **1**) with a retention time of 7.8 min. Product **1** exhibited a UV spectrum with λ_{max} 255 and 334 nm (Fig. 1, inset). The retention time and UV spectrum of product **1** were identical to those of the authentic diimino-imidazole nucleoside (dD) (Fig. 2), a nucleoside recently identified by our group as a product of the reaction of dG with HOCl.[21] Co-injection of the isolated compound and authentic dD to RP-HPLC gave a single peak with an identical UV spectrum. On the basis of these results, we conclude that dD is generated from dG by methylene blue photooxidation.

Time course of the reaction of dG with the methylene blue photooxidation system

We investigated the time course of the methylene blue photooxidation of dG. Figure 3A shows the concentrations of unreacted dG when 1 mM dG and 0.5 mM methylene blue were irradiated with visible light for various times (0–2 h) at pH 7.4 and 37 °C under aerobic conditions. The consumption of dG increased in a time-dependent manner. Figure 3B shows the changes in yield of the products (dD, dIz and 8-oxodG). The yields of dD and dIz increased in a time-dependent manner. The yield of 8-oxodG increased rapidly in the early stage of the reaction and reached a plateau, showing a profile characteristic of a reaction intermediate. We could not quantify dS and dZ in the reaction mixture, since both were eluted as broadened peaks near the chromatographic void volume on RP-HPLC analysis and may overlap with other peaks. For 1 h incubation, the concentrations of the products were 1.3 μM dD, 8.6 μM dIz and 9.1 μM 8-oxodG, with 127 μM dG consumed. Thus, the yields were 1.0% dD, 6.8% dIz and 7.2% 8-oxodG relative to the dG consumed. The ratio of the yield of dD to that of dIz (dD/dIz) was 0.15.

Effect of D_2O and NaN_3 on the reaction of dG with the methylene blue photooxidation system

To identify the reactive species generating dD from dG with the methylene blue photooxidation system, the reaction was performed in the presence of D_2O, an enhancer of singlet oxygen ($^1O_2$) reactions and NaN_3, a quencher of $^1O_2$. As shown in Table 1, the consumption of dG and the yields of dD and dIz increased when

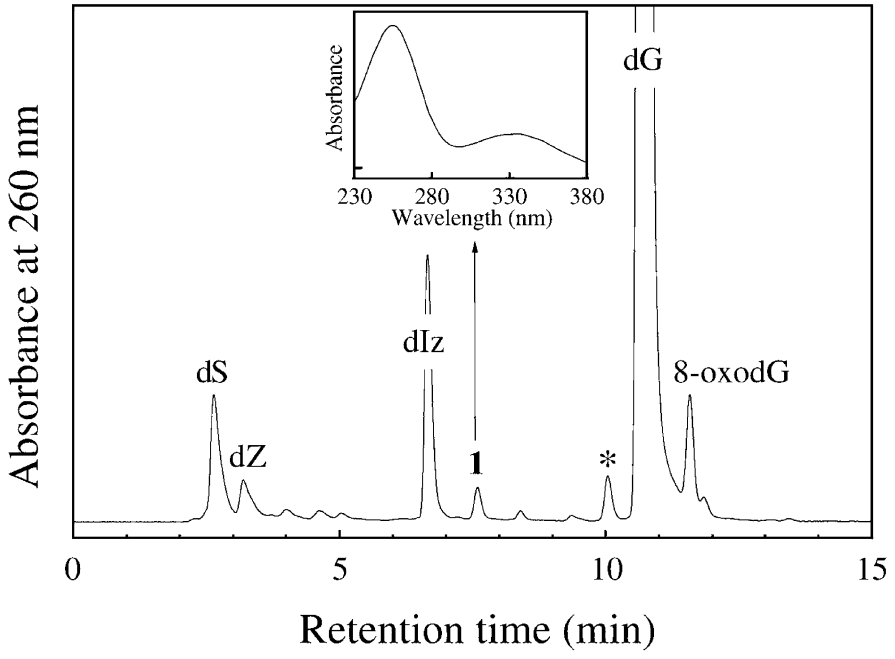


Figure 1. RP-HPLC chromatogram of a reaction mixture of dG with methylene blue photooxidation. A solution (1 mL) of 1 mM dG containing 0.5 mM methylene blue and 100 mM sodium phosphate buffer (pH 7.4) in an NMR tube (5 mm i.d.) was irradiated with visible light (550 nm cut-off filter) at 37 °C for 1 h under aerobic conditions. RP-HPLC analysis was conducted immediately after the reaction. Inset is an on-line UV spectrum of product **1**. The peak indicated by an asterisk is an impurity present in the starting dG.

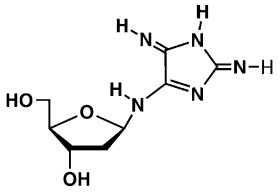


Figure 2. The structure of product **1**, a diimino-imidazole nucleoside, 2,5-diimino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-2*H*,5*H*-imidazole (dD).

the reactions were carried out in the presence D_2O and decreased in the presence of NaN_3. The dD/dIz ratio was increased by D_2O and decreased by NaN_3 (Table 1). These results suggest that $^1O_2$ is involved in the formation of dD from dG with the methylene blue photooxidation system.

Reaction of 8-oxodG with the methylene blue photooxidation system

In order to determine whether dD is generated via 8-oxodG, we analyzed by RP-HPLC the products of methylene blue photooxiation of 8-oxodG at pH 7.4 and 37 °C for 1 h under aerobic conditions. As shown in Table 2, dD was not detected (detection limit, 0.03 μM), while a large amount of dIz was formed. The reaction of 8-oxodG was enhanced by D_2O and suppressed by NaN_3; in neither case was dD detected.

Formation of dD by reaction of dG with other photooxidation systems

We examined the formation of dD from dG with other photooxidation systems. As shown in Table 3, rose bengal with visible light, another type II ($^1O_2$, energy transfer) dominant photooxidation system, generated dD with a dD/dIz ratio (0.10) similar to that in the methylene blue system. For type I (hydrogen atom abstraction or electron transfer) dominant photooxidation systems, we used riboflavin and benzophenone with UV light. Although dD was generated by the riboflavin system, the dD/dIz ratio (0.019) was one order of magnitude smaller than for the type II photooxidation systems. dD was not detected in the benzophenone system.

Reaction of dD with the photooxidation systems

We have reported that dD is not stable in a neutral solution, hydrolysing gradually with a half-life of 4.9 h at pH 7.4 and 37 °C, to give rise to dIz and its base moiety (Iz).[21] To examine whether dD reacts with photooxidation systems and disappears readily from the reaction mixture, authentic dD was incubated with photooxidation systems and the reaction was monitored by RP-HPLC. Figure 4 shows the change in dD concentration when 18.5 μM dD was incubated in 100 mM sodium phosphate buffer at pH 7.4 and 37 °C for 0–2 h with or without photooxidation systems. The methylene blue photooxidation system did not affect the rate of dD

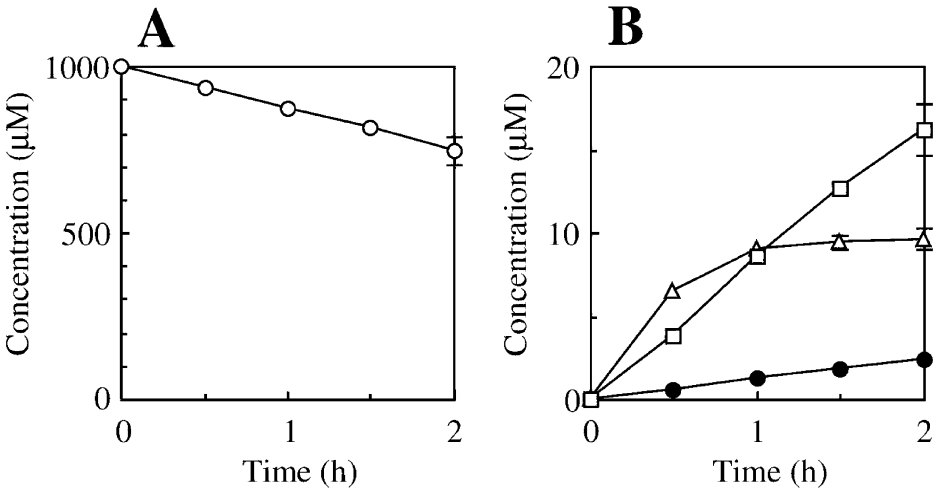


Figure 3. Time course of methylene blue photooxidation of dG. (A) concentration of unreacted dG (open circles). (B) Formation of dD (closed circles), dIz (open squares), and 8-oxodG (open triangles). A solution (1 mL) of 1 mM dG containing 0.5 mM methylene blue and 100 mM sodium phosphate buffer (pH 7.4) in an NMR tube (5 mm i.d.) was irradiated with visible light (550 nm cut-off filter) at 37 °C for 0–2 h under aerobic conditions. The concentrations were determined by RP-HPLC analysis, conducted immediately after the reaction. Means ± SD ($n = 3$) are presented.

Table 1. Effects of D_2O and NaN_3 on the reaction of dG with the methylene blue photooxidation system[a]

Additives	dD (μM)	dIz (μM)	8-oxodG (μM)	dG (μM)	dD/dIz
None	1.3 ± 0.1	8.6 ± 0.2	9.1 ± 0.2	873 ± 7	0.15
+ D_2O (75%)	5.4 ± 0.0	19.3 ± 0.1	7.2 ± 0.1	647 ± 8	0.28
+ NaN_3 (10 mM)	0.06 ± 0.01	0.6 ± 0.1	1.1 ± 0.1	996 ± 6	0.10

[a]A solution containing 1 mM dG, 0.5 mM methylene blue, and 100 mM sodium phosphate buffer (pH 7.4) was irradiated with visible light (550 nm cut-off filter) in the presence of 75% D_2O or 10 mM NaN_3 at 37 °C for 1 h under aerobic conditions. The reaction mixture was analyzed by RP-HPLC immediately after the reaction. Means ± SD ($n = 3$) are presented.

Table 2. Effects of D_2O and NaN_3 on the reaction of 8-oxodG with the methylene blue photooxidation system[a]

Additives	dD (μM)	dIz (μM)	8-oxoG (μM)
None	<0.03	39.1±4.4	679±14
+D_2O (75%)	<0.03	129.3±5.5	283±18
+NaN_3 (10 mM)	<0.03	2.5±0.2	931±10

[a]A solution containing 1 mM 8-oxodG, 0.5 mM methylene blue, and 100 mM sodium phosphate buffer (pH 7.4) was irradiated with visible light (550 nm cut-off filter) in the presence of 75% D_2O or 10 mM NaN_3 at 37 °C for 1 h under aerobic conditions. The reaction mixture was analyzed by RP-HPLC immediately after the reaction. Means±SD ($n=3$) are presented.

hydrolysis. The riboflavin photooxidation system accelerated the hydrolysis of dD, but not greatly.

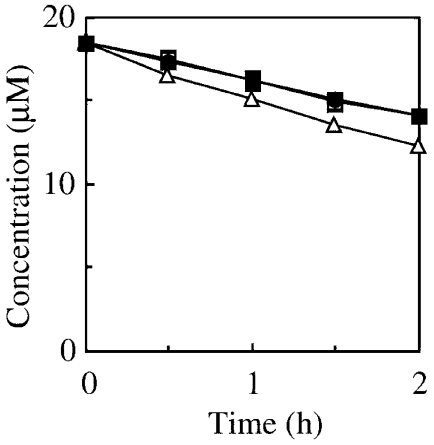


Figure 4. Effect of photooxidation on the hydrolysis of dD. Concentrations of dD without sensitizer, light and air bubbling (closed circles), with methylene blue, visible light and air bubbling (open squares), with riboflavin, UV light and air bubbling (open triangles), are plotted against the incubation time. A solution (1 mL) of 18.5 μM dD and 100 mM sodium phosphate buffer (pH 7.4) with or without 0.5 mM photosensitizers was incubated in the NMR tube at 37 °C for 0–2 h. The solution was irradiated with visible light (>550 nm) for methylene blue photooxidation and UV light (366 nm) for riboflavin photooxidation under aerobic conditions. The concentrations of dD were determined by RP-HPLC analysis, conducted immediately after the reaction. Means±SD ($n=3$) are presented.

Discussion

In the present study, we identified one of the products formed from dG by methylene blue photooxidation as a dD, which was recently reported as a novel product in the reaction of dG with HOCl.[21] The yield of dD from dG with the methylene blue photooxidation system increased in the presence of D_2O and was decreased by addition of azide. No dD was formed from 8-oxodG by the methylene blue photooxidation system. The riboflavin photooxidation system generated only a small amount of dD relative to that of dIz. dD does not react with the methylene blue photooxidation system. dD reacted with the riboflavin photooxidation system, but only slowly. On the basis of these combined observations, we conclude that dD is formed by singlet oxygen directly from dG, not via 8-oxodG. A possible reaction path is shown in Scheme 1

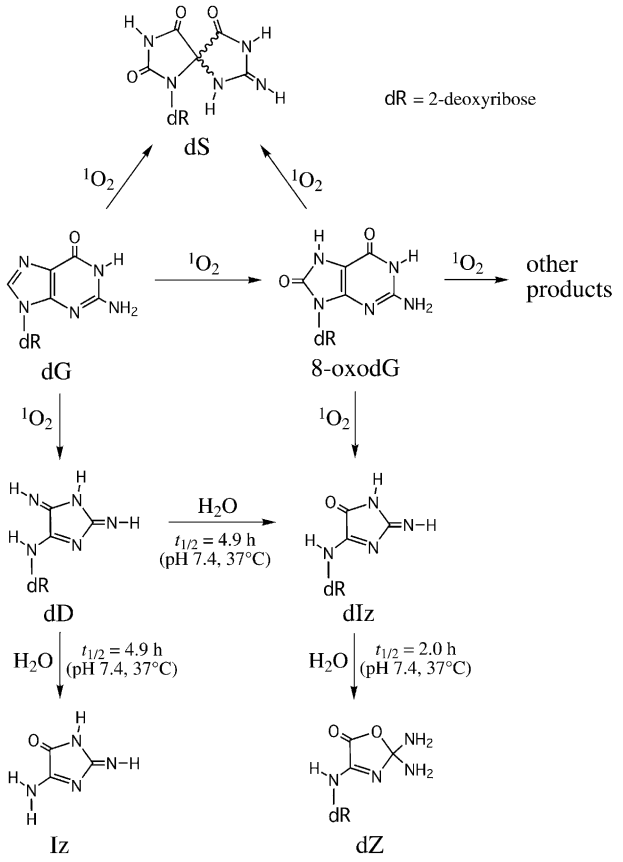
The reaction mechanisms of dG and 8-oxodG with $^1O_2$ were studied by Foote's group exclusively by means of direct measurements of NMR signals for intermediates in organic solvents at low temperature.[22–25] In the reaction of 8-oxodG with $^1O_2$, diastereomers of an endoperoxide intermediate were formed, which upon warming were converted to a variety of products.[23] The mechanism of the reaction between dG and $^1O_2$ is not fully known. No endoperoxide intermediate was detected, while two carbamic acids were observed as intermediates.[25] However, the carbamic acids were not converted to dS in the organic solvents upon warming. dS is a remarkable product with respect to the reaction mechanism of dG with $^1O_2$, since it is a major product in aqueous medium and should be generated directly from dG.[4,5] In the present study, we showed another product, dD, is formed from dG by the reaction with $^1O_2$, but not via 8-oxodG, while dS can be generated not only from dG but also from 8-oxodG.[7] The direct formation of dD from dG may provide new information concerning the mechanism of the reaction of dG with $^1O_2$.

No information is available with regard to the formation and occurrence of dD in cells. The half-life of dD was 4.9 h at pH 7.4 and 37 °C, giving rise to dIz and its base moiety, Iz (Scheme 1).[21] If a similar conversion occurs in DNA, it will result in a dIz moiety and an abasic site in the DNA strand. An abasic site is potentially mutagenic.[26] It has been reported that dIz is hydrolyzed in neutral solution with a half-life of 2.0 h (pH 7.4 and 37 °C) to form dZ (Scheme 1), which is stable under physiological conditions.[21,27] dZ in an oligodeoxynucleotide induces mainly dAMP insertion by DNA polymerases.[28] Thus, the formation of dZ in DNA should lead to a G to T transversion if the lesion is not repaired. On the other hand, no information is available on genotoxic effects of dD.

Table 3. Yields of dD, dIz and 8-oxodG in the reaction of dG with various photooxidation systems[a]

Photooxidation systems	dD (μM)	dlz (μM)	8-oxodG (μM)	dG (μM)	dD/dlz
Methylene blue (550 nm)	1.3±0.1	8.6±0.2	9.1±0.2	873±7	0.15
Rose bengal (550 nm)	0.15±0.03	1.5±0.1	<0.03	951±20	0.10
Riboflavin (366 nm)	1.7±0.1	88.4±3.0	<0.03	287±9	0.019
Benzophenone (366 nm)	<0.03	2.0±0.5	<0.03	944±11	<0.015

[a]A solution containing 1 mM dG, 0.5 mM photosensitizers (except for benzophenone, used as a saturated solution), and 100 mM sodium phosphate buffer (pH 7.4) was irradiated with visible light (550 nm cut-off filter) for methylene blue and rose bengal or with UV light (366 nm) for riboflavin and benzophenone at 37 °C for 1 h under aerobic conditions. The reaction mixture was analyzed by RP-HPLC immediately after the reaction. Means±SD ($n=3$) are presented.



Scheme 1. Proposed pathway for the reaction of dG with the methylene blue photooxidation system.

Conclusion

In the present study, we found that the reaction of dG with singlet oxygen yielded dD in addition to dIz, dZ, dS and 8-oxodG. dD was generated from dG directly, not via 8-oxodG. Further studies are necessary to clarify the mechanism of the formation of dD and to elucidate the biological significance of dD in singlet oxygen-mediated genotoxicity.

Experimental

Materials

dG was obtained from Fluka (Buchs, Switzerland). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) was purchased from Sigma (MO, USA). All other chemicals of reagent grade were purchased from Sigma, Fluka or Aldrich (WI, USA), and used without further purification.

High-performance liquid chromatography (HPLC) conditions

The HPLC system consisted of an HP1050 series pumping system (Hewlett Packard, CA, USA). On-line UV spectra were obtained with a Spectra Focus UV–visible photodiode-array detector (Spectra Physics, CA, USA). For reversed-phase HPLC (RP-HPLC), an Ultrasphere octadecylsilane (ODS) column (4.6×250 mm with a particle size of 5 μm, Beckman, CA, USA) was used. The eluent used was 20 mM sodium phosphate buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 12.5% over 15 min in a linear gradient mode. The column temperature was 30 °C and the flow rate 1 mL/min.

Reaction conditions

A solution (1 mL) of 1 mM dG or 8-oxodG containing 0.5 mM photosensitizers (except for benzophenone, used as a saturated solution) in 100 mM sodium phosphate buffer (pH 7.4) in an NMR tube (5 mm i.d.) was irradiated with visible light generated by a 100 W tungsten lamp through a 550 nm cut-off filter or with UV light of wavelength 366 nm at 37 °C for 1 h. During the irradiation, the solution was kept saturated with oxygen by continuous air bubbling (7 mL/min). RP-HPLC analysis was conducted immediately after the reaction. All experiments were carried out in triplicate.

Preparation of authentic samples

The authentic dD was prepared by incubation of 1 mM dG with 1 mM NaOCl in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C for 15 min and subsequent termination of the reaction with *N*-acetylcysteine.[21] dD was isolated from reaction mixtures by RP-HPLC. ^{1}H NMR (DMSO-d_6): δ (ppm/TMS) 8.19 (br, 1H), 7.95 (br, 1H), 7.68 (br, 1H), 5.62 (dd, 1H), 4.89 (br, 2H), 4.10 (m, 1H), 3.67 (m, 1H), 3.41 (m, 2H), 2.06 (m, 1H), 1.63 (m, 1H). ESI-MS: m/z 228 (M + H). UV: λ_{max} 255 and 334 nm (pH 7.0). The authentic dIz was prepared by irradiating a solution of 5 mM dG containing 0.6 mM riboflavin with UV light of wavelength 366 nm for 2 h at room temperature under aerated conditions.[27,29] The authentic dZ was prepared by incubating a solution of dIz in 100 mM sodium phosphate buffer (pH 7.4) at 70 °C for 30 min. dIz and dZ were isolated from each reaction mixture by RP-HPLC. Authentic dS was prepared by incubation of 1 mM 8-oxodG with 1 mM NaOCl in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C for 15 min and was purified by normal-phase HPLC.[18] We confirmed that the on-line UV and electrospray mass spectrometry spectra of dIz, dZ and dS were consistent with reported values.

Quantitative procedures

The concentrations of products were evaluated from the integrated peak areas of HPLC chromatograms and molar extinction coefficients (ε) at 260 or 330 nm. The values of ε at 260 nm used were 1.60×10^4 M^{-1} cm^{-1} for dD, 1.99×10^4 M^{-1} cm^{-1} for dIz and 1.15×10^4 M^{-1} cm^{-1} for dG.[21,30] We used ε values of 7.20×10^3 M^{-1} cm^{-1} for 8-oxodG at 260 nm and of 2.49×10^3 M^{-1} cm^{-1} for dD and 4.23×10^3 M^{-1} cm^{-1} for dIz at 330 nm, which were calculated on the basis of the reported ε values[21,31] and on-line UV spectra of 8-oxodG, dD and dIz.

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