Reaction of 3′,5′-di-O-acetyl-2′-deoxyguansoine with hypobromous acid

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ABSTRACT

Hypobromous acid (HOBr) is formed by eosinophil peroxidase and myeloperoxidase in the presence of H_2O_2, Cl^-, and Br^- in the host defense system of humans, protecting against invading bacteria. However, the formed HOBr may cause damage to DNA and its components in the host. When a guanine nucleoside (3′,5′-di-O-acetyl-2′-deoxyguansoine) was treated with HOBr at pH 7.4, spiroiminodihydantoin, guanidinohydantoin/iminoallantoin, dehydro-iminoallantoin, diimino-imidazole, amino-imidazolone, and diamino-oxazolone nucleosides were generated in addition to an 8-bromoguanine nucleoside. The major products were spiroiminodihydantoin under neutral conditions and guanidinohydantoin/iminoallantoin under mildly acidic conditions. All the products were formed in the reaction with HOCl in the presence of Br^-. These products were also produced by eosinophil peroxidase or myeloperoxidase in the presence of H_2O_2, Cl^-, and Br^-. The results suggest that the products other than 8-bromoguanine may also have importance for mutagenesis by the reaction of HOBr with guanine residues in nucleotides and DNA.

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1. Introduction

Bromine is one of the ubiquitous trace elements, and exists as bromide (Br^-) in humans.[1] The concentration of Br^- in human plasma ranges over levels of 39–84 µM.[2] However, the essential roles of Br^- in cells have been difficult to demonstrate, since Br^- can replace Cl^- as a substrate in many biological processes including enzyme activation and inhibition.[1] The first identified use of Br^- by enzymes in humans appears to be a role in defense mechanisms against parasites mediated by the preferential oxidation of Br^- by eosinophil peroxidase (EPO).[3] EPO generates a reactive species, hypobromous acid (HOBr), from H_2O_2 and Br^-. An EPO/H_2O_2/Br^- system in the presence of a plasma concentration of Cl^- can react with nucleosides to form brominated nucleosides.[4–6] Meanwhile, myeloperoxidase (MPO), an enzyme secreted from neutrophil and monocytic cells, generates hypochlorous acid (HOCl) from H_2O_2 and Cl^-.[7,8] The formed HOCl is also of central importance in host defence mechanisms. Recently, it has been reported that an MPO/H_2O_2/Cl^- system in the presence of a plasma concentration of Br^- also generates brominated nucleosides.[9] HOCl formed in the MPO system would react with Br^-, generating a brominating reagent, HOBr. The reaction of 2′-deoxyguanosine (dGuo) with reagent HOCl has been investigated,

revealing that the reaction pathway is complex, including several products of spiroiminodihydantoin deoxyribonucleoside (dSph), guanidinohydantoin/iminoallantoin deoxyribonucleoside (dGh/dIa), diimino-imidazole deoxyribonucleoside (dDiz), amino-imidazolone deoxyribonucleoside (dIz), diamino-oxazolone deoxyribonucleoside (dZ), in addition to 8-chloro-2′-deoxyguanosine (8-Cl-dGuo).[10–12] These products are also formed by an MPO/H_2O_2/Cl^- system. 7,8-Dehydro-8-oxo-2′-deoxyguanosine (8-oxo-dGuo) was generated in the reaction systems but detected only in trace amounts, since further oxidation occurs generating dSph or dGh/dIa.[13–15] There is, however, little information about the reaction products of dGuo with HOBr other than 8-Br-dGuo.

In the present study, we investigated the reaction of dGuo with reagent HOBr and HOBr generating enzymatic systems including MPO and EPO using an acetylated derivative of dGuo (3′,5′-di-O-acetyl-2′-deoxyguansoine; AcdGuo) to improve the separation of the products by reversed phase high performance liquid chromatography (RP-HPLC). We report our identification of the products and effects of pH of the reaction systems on their concentrations.

2. Results

2.1. Identification of products

A 100 µM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 100 µM HOBr at 37 °C for 30 min and terminated by addition of 1 mM N-acetyl-L-cysteine

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(AcCys). The reaction mixture was analyzed by RP-HPLC equipped with a UV–vis photodiode-array detector. In the chromatogram detected at 245 nm, several product peaks were observed as shown in Figure 1. These products were isolated and subjected to ESI-TOF/MS. Products **1** and **2** were identified as diastereomers of 3′,5′-di-O-acetyl derivative of spiroiminodihydantoin deoxyribonucleoside (AcdSph). **3** and **4** were a 3′,5′-di-O-acetyl derivative of guanidinohydantoin or iminoallantoin deoxyribonucleoside (AcdGh or AcdIa). Compound **5** was a 3′,5′-di-O-acetyl derivative of diamino-oxazolone deoxyribonucleoside (AcdZ). Compound **6** was a 3′,5′-di-O-acetyl derivative of amino-imidazolone deoxyribonucleoside (AcdIz). Compound **7** was a 3′,5′-di-O-acetyl derivative of diimino-imidazole deoxyribonucleoside (AcdDiz). Compound **8** was a 3′,5′-di-O-acetyl derivative of dehydro-iminoallantoin deoxyribonucleoside (AcdIa^ox). Compound **9** was a 3′,5′-di-O-acetyl derivative of 8-bromo-2′-deoxyguanosine (8-Br-AcdGuo). A 3′,5′-di-O-acetyl derivative of 7,8-dehydro-8-oxo-2′-deoxyguanosine (8-oxo-AcdGuo) with the RP-HPLC retention time of 28.2 min was not detected (detection limit; 50 nM). The products were identified on the basis of coincidence of their UV and MS spectra reported in reaction systems of AcdGuo with HOCl or other oxidizing reagents.[10–12] Authentic samples were also synthesized for AcdSph, AcdGh/AcdIa, AcdIa^ox, and 8-Br-AcdGuo, and confirmed to accord with the corresponding products in the present AcdGuo/HOBr reaction for the RP-HPLC retention time, UV spectrum, and MS spectrum. Table 1 summarizes the characteristics of products **1–9**. For the reaction of AcdGuo with HOCl, a 3′,5′-di-O-acetyl derivative of 8-chloro-2′-deoxyguanosine (8-Cl-AcdGuo) with the RP-HPLC retention time of 39.6 min, λ_{max} = 259 nm, and
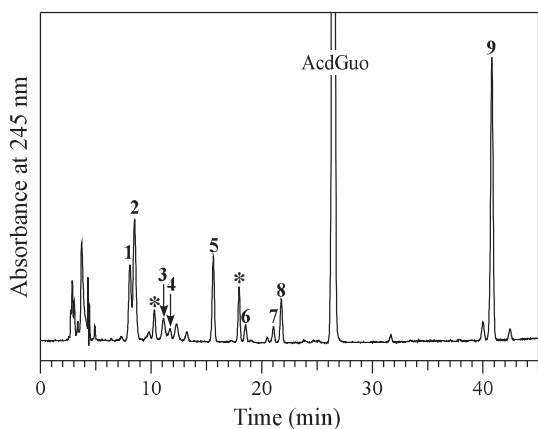

Figure 1. RP-HPLC chromatogram of a reaction mixture of AcdGuo with HOBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. The RP-HPLC chromatogram was detected at 245 nm. The peaks indicated by asterisks are impurities present in the starting AcdGuo.

Table 1
Characteristics of the products formed by the reaction of AcdGuo with HOBr[a]

Products	t_R (min)	λ_{max} (nm)	m/z (negative)
1, AcdSph (fast)	8.1	230 (shoulder)	382
2, AcdSph (slow)	8.5	230 (shoulder)	382
3, AcdGh/AcdIa (fast)	11.1	224	356
4, AcdGh/AcdIa (slow)	11.7	220	356
5, AcdZ	15.6	232	329
6, AcdIz	18.5	254, 320	311
7, AcdDiz	21.0	257, 336	310
8, AcdIa^ox	21.8	236	354
9, 8-Br-AcdGuo	40.8	262	428, 430

[a] A solution of 100 μM AcdGuo in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 100 μM HOBr at 37 °C and terminated by addition of 1 mM AcCys.
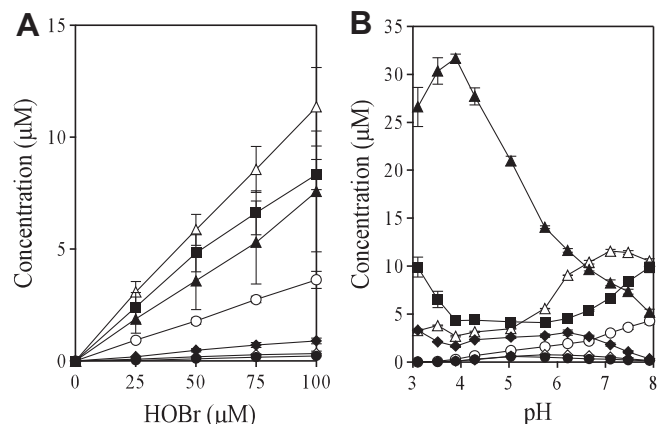

Figure 2. (A) HOBr dose dependence of concentration of products in the reaction of AcdGuo with HOBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 0–100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. (B) pH dependence of concentration of products in the reaction of AcdGuo with HOBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 3–8) was reacted with 100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. AcdSph (open triangle), AcdGh/AcdIa (closed triangle), AcdZ (open circle), AcdIz (closed circle), AcdDiz (open rhombus), AcdIa^ox (closed rhombus), 8-Br-AcdGuo (closed square). The nucleoside concentrations were determined by RP-HPLC analysis detected at 245 nm. Means ± SD (n = 3) are shown.

m/z (negative) = 384 and 386 was formed besides 8-Br-AcdGuo (data not shown). 8-Cl-AcdGuo in the present reaction mixture was identified by accordance with data reported in an AcdGuo/HOCl reaction system for UV spectrum and MS spectrum.[10,12]

2.2. Reaction of AcdGuo with HOBr

Figure 2A shows the concentrations of the products in the reaction of AcdGuo with various concentrations of HOBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 0–100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. The concentrations of all the products increased linearly with increasing HOBr dose. Figure 2B shows the pH dependence of concentrations of the products in the reaction of AcdGuo with HOBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 3–8) was reacted with 100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. The concentrations of the products varied greatly depending on the reaction pH. While AcdSph was the major product under neutral conditions, AcGh/AcdIa were the major products under mildly acidic conditions.

2.3. Reaction of 8-oxo-AcdGuo with HOBr

To obtain information about the reaction pathway of formation of the products, the reaction of 8-oxo-AcdGuo, a typical oxidative damage product of AcdGuo, with HOBr was investigated. Figure 3 shows the pH dependence of concentrations of the products in the reaction of 8-oxo-AcdGuo with HOBr. A 100 μM 8-oxo-AcdGuo solution in 100 mM potassium phosphate buffer (pH 3–8) was reacted with 100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. AcdSph was the major product under neutral conditions, and AcGh/AcdIa were the major products under mildly acidic conditions.

2.4. Reaction of AcdGuo with HOCl in the presence of Br⁻

The effects of addition of Br⁻ in the reaction of AcdGuo with HOCl were investigated. Figure 4 shows the concentrations of the
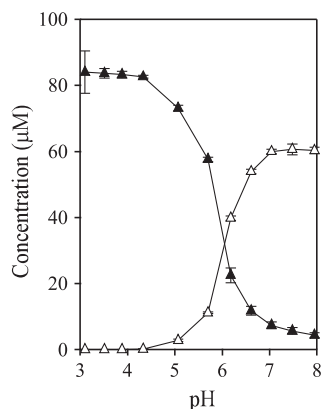
Figure 3. pH dependence of concentration of products in the reaction of 8-oxo-AcdGuo with HOBr. A 100 μM 8-oxo-AcdGuo solution in 100 mM potassium phosphate buffer (pH 3–8) was reacted with 100 μM HOBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. AcdSph (open triangle), AcdGh/AcdIa (closed triangle). The nucleoside concentrations were determined by RP-HPLC analysis detected at 245 nm. Means ± SD (n = 3) are shown.
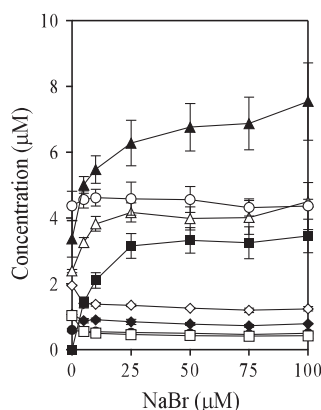

Figure 4. NaBr dose dependence of concentration of products in the reaction of AcdGuo with HOCl. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 100 μM HOCl in the presence of 0–100 μM NaBr at 37 °C for 30 min and terminated by addition of 1 mM AcCys. AcdSph (open triangle), AcdGh/AcdIa (closed triangle), AcdZ (open circle), AcdIz (closed circle), AcdDiz (open rhombus), AcdIa^{ox} (closed rhombus), 8-Br-AcdGuo (closed square), 8-Cl-AcdGuo (open square). The nucleoside concentrations were determined by RP-HPLC analysis detected at 245 nm. Means ± SD (n = 3) are shown.

products in the reaction of AcdGuo with HOCl in the presence of various concentration of NaBr. A 100 μM AcdGuo solution in 100 mM potassium phosphate buffer (pH 7.4) was reacted with 100 μM HOCl in the presence of 0–100 μM NaBr at 37 °C for 30 min and terminated by the addition of 1 mM AcCys. The concentration of 8-Br-AcdGuo increased with increasing NaBr dose up to 25 μM and was then constant for 25–100 μM. In contrast, the concentration of 8-Cl-AcdGuo decreased by half with addition of 5 μM NaBr and was constant for 5–100 μM NaBr. The concentrations of AcdSph and AcdGh/AcdIa increased with increasing NaBr dose.

2.5. Reaction of AcdGuo with an MPO/H$_2$O$_2$/Cl$^-$ system in the absence and presence of Br$^-$

Figure 5A shows the pH dependence of concentrations of the products in the reaction of AcdGuo with an MPO/H$_2$O$_2$/Cl$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$ and 100 mM NaCl in the presence of 1 unit/mL MPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction

terminated by addition of 1 mM AcCys. The products were formed under mildly acidic conditions. The major product was AcdIz at pH 5 and AcdSph at 6. AcdDiz was formed with a relatively high concentration at pH 5 and 6. In addition to 8-Cl-AcdGuo, 8-Br-AcdGuo was observed. The concentrations of all the products were very low at pH 7 and 8. Figure 5B shows the pH dependence of concentrations of the products in the reaction of AcdGuo with an MPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$, 100 mM NaCl, and 100 μM NaBr in the presence of 1 unit/mL MPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction terminated by addition of 1 mM AcCys. The reaction products were observed even under neutral conditions. The major product was AcdGh/AcdIa under mildly acidic conditions and AcdSph under neutral conditions. 8-Br-AcdGuo was also formed with a relatively high concentration comparative to AcdGh/AcdIa or AcdSph.

2.6. Reaction of AcdGuo with an EPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system

Figure 6 shows the pH dependence of concentrations of the products in the reaction of AcdGuo with an EPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$, 100 mM NaCl, and 100 μM NaBr in the presence of 19 μg/mL EPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction terminated by addition of 1 mM AcCys. The major product was 8-Br-AcdGuo. AcdDiz was also generated with a relatively high concentration at pH 6 and 7. In addition, AcdGh/AcdIa under mildly acidic conditions or AcdSph under neutral conditions was formed.

3. Discussion

In the present study, we found that several products formed in the reaction of AcdGuo with HOBr in addition to 8-Br-AcdGuo. The concentrations of products varied greatly with pH (Fig. 2B). The major products were AcdSph at neutral pH and AcdGh/AcdIa at mildly acidic pH. 8-Oxo-AcdGuo readily reacted with HOBr resulting in AcdSph at neutral pH and AcdGh/AcdIa at mildly acidic
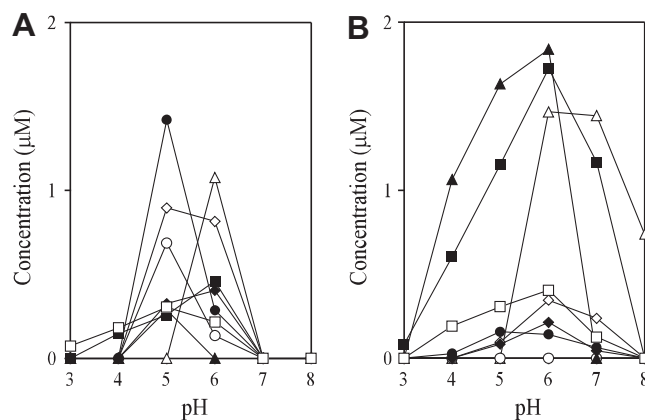

Figure 5. (A) pH dependence of concentration of products in the reaction of AcdGuo with an MPO/H$_2$O$_2$/Cl$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$ and 100 mM NaCl in the presence of 1 unit/mL MPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction terminated by addition of 1 mM AcCys. (B) pH dependence of concentration of products in the reaction of AcdGuo with an MPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$, 100 mM NaCl, and 100 μM NaBr in the presence of 1 unit/mL MPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction terminated by addition of 1 mM AcCys. AcdSph (open triangle), AcdGh/AcdIa (closed triangle), AcdZ (open circle), AcdIz (closed circle), AcdDiz (open rhombus), AcdIa^{ox} (closed rhombus), 8-Br-AcdGuo (closed square), 8-Cl-AcdGuo (open square). The nucleoside concentrations were determined by RP-HPLC analysis detected at 245 nm.
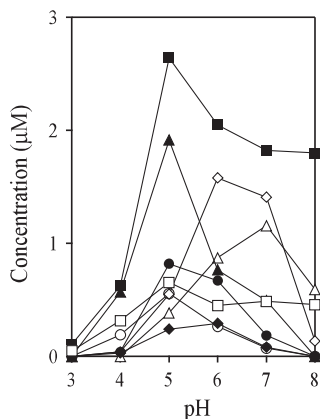
Figure 6. pH dependence of concentration of products in the reaction of AcdGuo with an EPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system. A 100 μM AcdGuo solution with 100 μM H$_2$O$_2$, 100 mM NaCl, and 100 μM NaBr in the presence of 19 μg/mL EPO in 100 mM potassium phosphate buffer (pH 3–8) was incubated at 37 °C for 30 min and the reaction terminated by addition of 1 mM AcCys. AcdSph (open triangle), AcdGh/AcdIa (closed triangle), AcdZ (open circle), AcdIz (closed circle), AcdDiz (open rhombus), AcdIa^{ox} (closed rhombus), 8-Br-AcdGuo (closed square), 8-Cl-AcdGuo (open square). The nucleoside concentrations were determined by RP-HPLC analysis detected at 245 nm.

pH (Fig. 3). 8-Oxo-AcdGuo is highly reactive toward further oxidation. Thus, AcdSph and AcdGh/AcdIa would be generated via 8-oxo-AcdGuo, although 8-oxo-AcdGuo was not detected in the reaction of AcdGuo with HOBr. Further oxidation of AcdIa would result in AcdIa^{ox}. In contrast, AcdDiz, AcdIz, AcdZ, and 8-Br-AcdGuo would be generated directly and independently from AcdGuo. Since it was reported that AcdDiz is hydrolyzed to AcdIz with a half-life of 4.9 h at 37 °C and pH 7.4, and that AcdIz is hydrolyzed to AcdZ with a half-life of 2.0 h at 37 °C,[11] AcdIz and AcdZ were partly generated from AcdDiz and AcdIz, respectively. A possible reaction pathway of AcdGuo with HOBr is summarized in Figure 7. In the present study, enzymatic systems generating HOBr were also investigated. No product was observed at neutral pH in the MPO/H$_2$O$_2$/Cl$^-$ system, but addition of 100 μM Br$^-$ enhanced the

reaction especially at neutral pHs (Fig. 5). This implies that the damage to guanosine nucleosides by MPO at neutral pHs is greatly affected by the concentration of Br$^-$. In the AcdGuo/MPO/H$_2$O$_2$/Cl$^-$ system (Fig. 5A), 8-Br-AcdGuo was also generated comparative to 8-Cl-AcdGuo, probably due to a trace amount of Br$^-$ contained in NaCl, potassium phosphate buffer, or MPO as an impurity. In the AcdGuo/EPO/H$_2$O$_2$/Cl$^-$/Br$^-$ system (Fig. 6), the reaction occurred at neutral and mildly acidic pHs. 8-Br-AcdGuo was the product with the largest concentration with comparative concentrations of AcdSph and AcdGh/AcdIa. In addition, AcdDiz was also a major product in the reaction system.

Reportedly, when a dSph containing template is replicated in *Escherichia coli*, the replication is strongly blocked at the dSph moiety.[16] When replication occurs over the lesions, both G to T and G to C transversions occur, suggesting predominant insertion of dAMP and dGMP opposite the lesions by DNA polymerase. dSph moiety was detected in vivo using *E. coli*.[17] dGh/dIa in DNA were bypassed as efficiently as 8-oxo-Gua but were highly mutagenic, causing an almost exclusive G to C transversion.[16] Several repair enzymes can excise the dSph and dGh/dIa moiety, but these efficiencies are relatively low.[18,19] In contrast, an addition of 5′-triphosphates of dSph and dGh/dIa did not significantly increase the mutation frequency of *E. coli*.[20] dZ lesion in DNA induces dAMP insertion, suggesting that dZ formed in DNA causes G to T mutation.[21,22] Little information is available regarding the mutagenicity of dDiz and dIz, probably due to their low stabilities for hydrolysis. For 8-Br-dGuo, an in vitro study showed that human DNA polymerases incorporated dGMP, dAMP, and dTMP in addition to one-base deletion opposite to an 8-Br-dGuo residue in an oligodeoxynucleotide, suggesting that 8-Br-dGuo in DNA is a mutagenic lesion.[23] Recently, 8-Br-dGuo was reportedly detected in urine from healthy volunteers with a similar concentration of 8-Cl-dGuo, while urinary 8-Br-dGuo and 8-Cl-dGuo levels from diabetic patients were 8-fold higher than the levels in healthy volunteers.[24] This implies that 8-Br-dGuo exists in healthy humans and that inflammatory diseases greatly increase its level. However, there is little information available about the formation of dSph, dGh/dIa, dDiz, dIz, dZ in humans. The present study showed that reagent HOBr and MPO and EPO systems generated these products
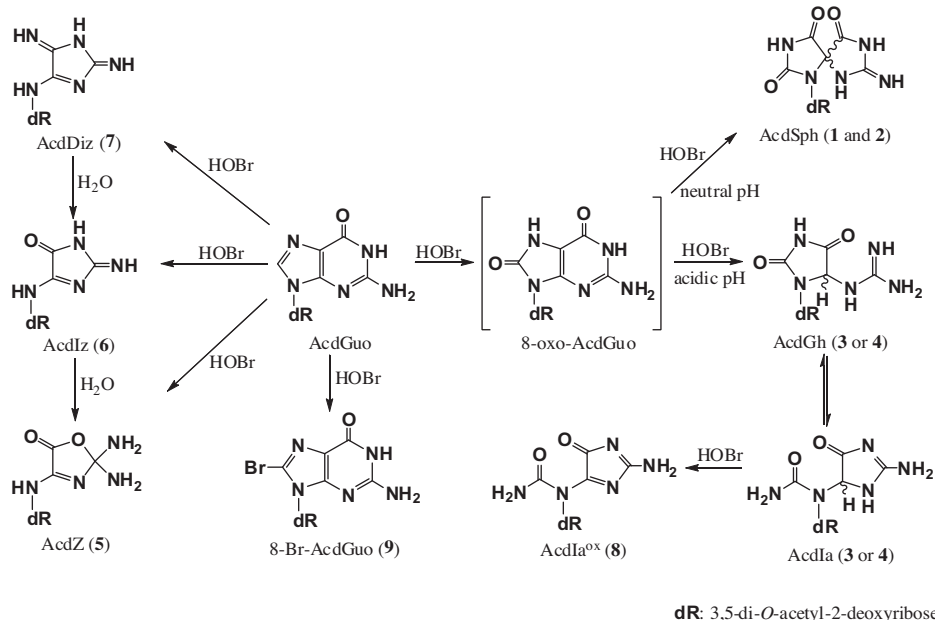

dR: 3,5-di-O-acetyl-2-deoxyribose

Figure 7. Proposed reaction pathway for the reaction of AcdGuo with HOBr.

in addition to 8-Br-dGuo in nucleoside, suggesting that these products are also important for the genotoxicity of HOBr.

4. Conclusion

We investigated the reaction of AcdGuo with HOBr or peroxidase systems including MPO and EPO. In all the reaction systems, several products including AcdSph, AcdGh/AcdIa, dDiz, dIz, and dZ were generated in addition to 8-Br-AcdGuo. The present results draw attention to the contribution of these products in addition to the bromine derivative in damaging guanine nucleosides, guanine nucleotides, and guanine residues in DNA by HOBr.

5. Experimental

5.1. Materials

2'-Deoxyguanosine (dGuo) was obtained from Sigma (MO, USA). 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) was purchased from Berry & Associates (MI, USA). NaBr (99.999%) and NaCl (99.99%) were obtained from Aldrich (WI, USA) and Sigma, respectively. All other chemicals of reagent grade were purchased from Sigma, Aldrich, Nacalai Tesque (Tokyo), TCI (Tokyo), Wako (Osaka, Japan), and Cica (Tokyo), and used without further purification. Water was distilled and then purified with a Millipore Milli-Q deionizer. Bromide-free hypobromous acid (HOBr) was prepared by addition of silver nitrate and subsequent distillation as previously reported.[9,25] The concentration of HOBr was determined spectrophotometrically at 331 nm in 10 mM NaOH using a molar extinction coefficient of $315 \, M^{-1} \, cm^{-1}$.[25] Chloride-free sodium hypochlorite (NaOCl) was prepared using ethyl acetate abstraction by the method previously reported.[26] The concentration of NaOCl was determined spectrophotometrically at 290 nm in 10 mM NaOH using a molar extinction coefficient of $350 \, M^{-1} \, cm^{-1}$.[27] AcdGuo and 8-oxo-AcdGuo were synthesized from dGuo and 8-oxo-dGuo, respectively, by acetylation using acetic anhydride as previously described.[28] Human MPO was purchased from Enzo Life Sciences (NY, USA). Human EPO was purchased from Acris Antibodies (Herford, Germany).

5.2. HPLC and MS conditions

The HPLC system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. For RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6×250 mm and particle size 5 μm (GL Sciences, Tokyo) was used. For analyses, 20 mM ammonium acetate buffer (pH 7.0) containing methanol was used as the eluent. The methanol concentration was increased from 7.5% to 30% for 45 min in linear gradient mode. The column temperature was 40 °C and the flow rate was 1.0 mL/min. The ESI-TOF/MS measurements were performed on a Bruker MicroTOF spectrometer (Bremen, Germany) in the negative mode. The sample isolated by RP-HPLC using 20 mM ammonium acetate buffer (pH 7.0) containing methanol as the eluent was directly infused into the MS system by a syringe pump without a column at a flow rate of 2 μL/min.

5.3. Preparation of the authentic samples

5.3.1. Diastereomers of AcdSph

The reaction was carried out as previously reported.[29] Briefly, 8-oxo-AcdGuo (30 mg, 82.4 μmol) dissolved in 100 mM potassium phaosphate buffer (pH 7.0, 11.1 mL). CoCl$_2$·6H$_2$O (0.3 mg, 1.3 μmol) and potassium peroxymonosulfate (24 mg, 80 μmol as KHSO$_5$) were added, and stirred for 1 h. After lyophilization, the

products were purified by RP-HPLC. AcdSph (fast): ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 1.99 (s, 3H, CH$_3$), 1.99 (s, 3H, CH$_3$), 2.02 (m, 1H, H2' or 2''), 2.80 (m, 1H, H2' or 2''), 3.95 (m, 1H, H4'), 3.99 (dd, 1H, H5' or 5''), 4.17 (dd, 1H, H5' or 5''), 5.01 (dd, 1H, H1'), 5.12 (m, 1H, H3'), 7.97 (s, 1H, NH), 8.28 (s, 1H, NH), 8.44 (s, 1H, NH), 11.37 (s, 1H, NH); MS (negative): m/z = 382; UV: λ_{max} = 230 nm (shoulder). AcdSph (slow): ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 2.03 (s, 3H, CH$_3$), 2.03 (s, 3H, CH$_3$), 1.91 (m, 1H, H2' or 2''), 2.21 (m, 1H, H2' or 2''), 3.92 (m, 1H, H4'), 4.00 (dd, 1H, H5' or 5''), 4.05 (dd, 1H, H5' or 5''), 5.03 (m, 1H, H3'), 5.51 (dd, 1H, H1'), 7.98 (s, 1H, NH), 8.22 (s, 1H, NH), 8.29 (s, 1H, NH), 11.46 (s, 1H, NH); ESI-TOF/MS (negative): m/z = 382; UV: λ_{max} = 230 nm (shoulder).

5.3.2. AcdGh/AcdIa

The reaction was carried out as previously reported.[30] Briefly, 8-oxo-AcdGuo (30 mg, 82.4 μmol) dissolved in H$_2$O (11.1 mL). CoCl$_2$·6H$_2$O (0.3 mg, 1.3 μmol) and potassium peroxymonosulfate (32.5 mg, 110 μmol as KHSO$_5$) were added, and stirred for 1 h. After lyophilization, the products were purified by RP-HPLC as a mixture of AcdGh and AcdIa. AcdGh/AcdIa (component 1): ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 2.03 (s, 3H, CH$_3$), 2.05 (s, 3H, CH$_3$), 2.05 (m, 1H, H2' or 2''), 2.17 (m, 1H, H2' or 2''), 3.85–4.28 (m, 2H, H5',5''), 3.94 (m, 1H, H4'), 5.03 (d, 1H, H3'), 5.40 (s, 1H, CH), 5.78 (m, 1H, H1'); ESI-TOF/MS (negative): m/z = 356; UV: λ_{max} = 222 or 225 nm. AcdGh/AcdIa (component 2): ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 2.01 (s, 3H, CH$_3$), 2.05 (s, 3H, CH$_3$), 2.08 (m, 1H, H2' or 2''), 2.67 (m, 1H, H2' or 2''), 3.85–4.28 (m, 2H, H5',5''), 3.91 (m, 1H, H4'), 5.11 (dd, 1H, H3'), 5.51 (s, 1H, CH), 5.52 (m, 1H, H1'); ESI-TOF/MS (negative): m/z = 356; UV: λ_{max} = 222 or 225 nm.

5.3.3. AcdIa^{ox}

The reaction was carried out as previously reported.[30] Briefly, 8-oxo-AcdGuo (8.1 mg, 22.1 μmol) dissolved in H$_2$O (2.85 mL). A 200 μL aqueous solution of Na$_2$IrCl$_6$ (54.2 mg, 97.1 μmol) was added in 10 portions during 2 min. After 30 min, the reaction solution was lyophilized for 1 h. DMSO-d_6 (750 μL) was added to dissolve the residue and the mixture was centrifuged for 1 min. The supernatant liquid was analyzed. ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 2.01 (s, 3H, CH$_3$), 2.05 (s, 3H, CH$_3$), 2.30 (m, 1H, H2' or 2''), 2.98 (m, 1H, H2' or 2''), 4.06 (dd, 1H, H5' or 5''), 4.13 (m, 1H, H4'), 4.28 (dd, 1H, H5' or 5''), 5.28 (m, 1H, H3'), 5.97 (dd, 1H, H1'), 8.33 (s, 2H, NH$_2$), 8.42 (s, 2H, NH$_2$); ESI-TOF/MS (negative): m/z = 354; UV: λ_{max} = 263 nm.

5.3.4. 8-Br-AcdGuo

The reaction was carried out as previously reported.[31] Briefly, bromine-water was added to a suspension of AcdGuo (50 mg, 140 μmol) dissolved in H$_2$O (600 μL) until the yellow color did not disappear. The filtrated crude product was recrystallized from H$_2$O. ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm/TMS) 1.99 (s, 3H, CH$_3$), 2.08 (s, 3H, CH$_3$), 2.42 (m, 1H, H2' or 2''), 3.49 (m, 1H, H2' or 2''), 4.18 (m, 1H, H4'), 4.19 (dd, 1H, H5' or 5''), 4.39 (dd, 1H, H5' or 5''), 5.42 (m, 1H, H3'), 6.18 (dd, 1H, H1'), 6.55 (s, 2H, NH$_2$), 10.85 (s, 1H, NH); ESI-TOF/MS (negative): m/z = 428, 430; UV: λ_{max} = 262 nm.

5.4. Quantitative procedures

The concentrations of the products were evaluated from integrated peak areas on RP-HPLC chromatograms detected at 245 nm and the molecular extinction coefficients at 245 nm ($\varepsilon_{245 \, nm}$). The $\varepsilon_{245 \, nm}$ values of the authentic samples were determined from integration of H1' or H3' proton signal of NMR and the HPLC peak area detected at 245 nm relative to those of AcdGuo

($\varepsilon_{245\,nm}$ = 12,400 M^{-1} cm^{-1})[12] in the mixed solution. The estimated $\varepsilon_{245\,nm}$ values were 5480 M^{-1} cm^{-1} for diastereomers of AcdSph, 1790 M^{-1} cm^{-1} for AcdGh/AcdIa, 12,840 M^{-1} cm^{-1} for AcdIa^{ox}, 15,560 M^{-1} cm^{-1} for 8-oxo-AcdGuo, and 9800 M^{-1} cm^{-1} for 8-Br-AcdGuo. The reported $\varepsilon_{245\,nm}$ values used were 6000 M^{-1} cm^{-1} for AcdZ, 20,500 M^{-1} cm^{-1} for AcdIz, 14,000 M^{-1} cm^{-1} for AcdDiz, 11,800 M^{-1} cm^{-1} for 8-Cl-AcdGuo.[12]

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