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# Modification of the Amino Group of Guanosine by Methylglyoxal and Other α-Ketoaldehydes in the Presence of Hydrogen Peroxide

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**Methylglyoxal is directly mutagenic to *Salmonella typhimurium* TA100 and its mutagenicity is markedly enhanced in the presence of hydrogen peroxide. We found that methylglyoxal in phosphate buffer was decomposed easily by hydrogen peroxide at room temperature to yield acetic acid and formic acid as major products and diacetyl as a minor product; acetyl radical was detected in the solution by ESR spectroscopy by the use of a spin-trapping reagent, 5,5-dimethyl-1-pyrroline *N*-oxide. Furthermore, guanosine was converted into $N^2$-acetylguanosine by a combination of methylglyoxal and hydrogen peroxide in 0.1 M phosphate buffers (pH 6.1 to 7.4). This acetylation may be related to the enhancement of methylglyoxal mutagenicity by hydrogen peroxide. Other α-ketoaldehydes such as glyoxal and phenylglyoxal also yielded the corresponding acids and α-dicarbonyls upon reaction with hydrogen peroxide under the same conditions as above. These acids would have been produced through Baeyer–Villiger reaction or coupling of acyl radical with hydroxy radical, and dicarbonyls by dimerization of acyl radicals. In addition, when phenylglyoxal was used, the generation of benzoyl radical and the conversion of guanosine to $N^2$-benzoylguanosine were observed. However, it remains to be established whether the generation of acyl radicals is directly involved in the *N*-2 acylation of guanosine.**

**Keywords** methylglyoxal; hydrogen peroxide; $N^2$-acetylguanosine; mutagenicity; α-ketoaldehyde; guanosine

## Introduction

α-Dicarbonyl compounds formed during heating or pyrolysis of carbohydrates such as sugars and starches are mutagenic to *Salmonella typhimurium* TA100 without metabolic activation.[1)] They are contained in many kinds of foodstuffs and beverages, including not only roasted and baked foods but also fermented products.[2)] Coffee, which is one of the most potently mutagenic foodstuffs, contains large amounts of them.[2b)] Among α-dicarbonyl compounds, α-ketoaldehydes are more mutagenic than α-diketones, and methylglyoxal is the most potent of them.[2b)] Moreover, the mutagenicity of methylglyoxal is enhanced about 30 times by the presence of hydrogen peroxide.[3)] Coffee includes both methylglyoxal and hydrogen peroxide and the mutagenicity of the combination of these compounds accounts for most of the mutagenicity of coffee.[3)] In order to elucidate the mechanism of the enhancement of the mutagenicity, we first investigated the reactivity of methylglyoxal and other α-ketoaldehydes with hydrogen peroxide.

We had shown in a previous study that $N^2$-acetyl-2′-deoxyguanosine was formed when 2′-deoxyguanosine was treated with methylglyoxal in the presence of hydrogen peroxide.[4)] Based on the results obtained in the first part of the present investigation, we next examined in detail the reaction mechanism of *N*-2 acylation of guanine base by α-ketoaldehyde in the presence of hydrogen peroxide. Guanosine was used in this study instead of 2′-deoxyguanosine, because the *N*-2 acyl derivative formed from guanosine is much more stable than that from 2′-deoxyguanosine.

## Results and Discussion

α-Dicarbonyl compounds such as methylglyoxal and diacetyl were decomposed easily by hydrogen peroxide in phosphate buffer. Methylglyoxal and other α-dicarbonyl compounds were kept in 0.1 M phosphate buffer (pH 6.86) at 37 °C, with and without 10 eq of hydrogen peroxide, and the residual amounts of them were analyzed periodically after conversion to quinoxaline derivatives by reaction with *o*-phenylenediamine. The results are shown in Fig. 1. At the same time, the products in the reaction mixture of each α-dicarbonyl compound and hydrogen peroxide were examined by gas chromatography (GC) and found to be mainly the corresponding carboxylic acids, *e.g.*, acetic acid and formic acid from methylglyoxal, and acetic acid from diacetyl, as shown in Table I. It is inferred that these carboxylic acids were produced by the

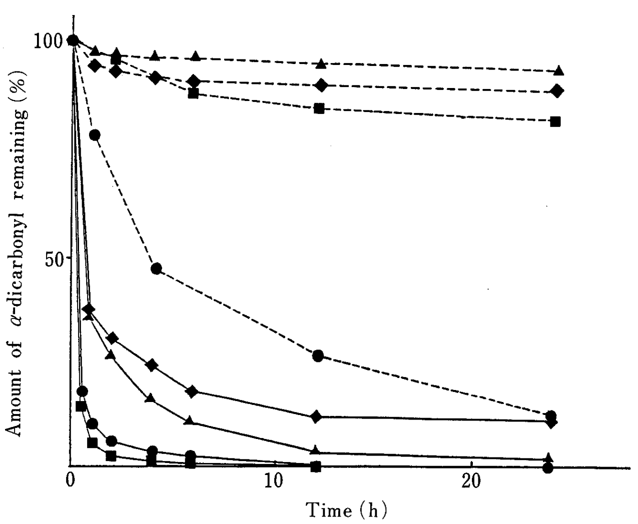

Fig. 1. Degradation of α-Dicarbonyl Compounds with or without Hydrogen Peroxide in 0.1 M Phosphate Buffer (pH 6.86) at 37 °C

▲, methylglyoxal; ◆, glyoxal; ■, 1-phenyl-1,2-propanedione; ●, diacetyl; ---, without hydrogen peroxide; ——, with hydrogen peroxide.

hydrolysis of acetic formic anhydride or acetic anhydride derived from methylglyoxal or diacetyl through the Baeyer–Villiger reaction, respectively.[5)]

Next, one-tenth equivalent of hydrogen peroxide was added to methylglyoxal solution under the same conditions, and the products were analyzed by high-performance liquid chromatography (HPLC) after *o*-phenylenediamine treatment as before, except that HPLC eluent was changed to $CH_3CN$:0.1 N AcOH (35:65). A small amount of 2,3-dimethylquinoxaline was detected at the retention time of 14.5 min on the HPLC chromatogram, as shown in Fig. 2(A). The compound was collected by the repeated HPLC procedure and confirmed to be 2,3-dimethylquinoxaline by GC and GC-mass spectral (GC-MS) examination ($m/z$: 158 ($M^+$), 117 ($M^+-CH_3CN$, base peak), 77 ($C_6H_5$)). The peak was not observed in the reaction product obtained in the absence of hydrogen peroxide, as shown in Fig. 2(B). This result means that diacetyl was formed in the reaction mixture containing hydrogen peroxide, presumably through dimerization of acetyl radicals derived from methylglyoxal, as shown in Chart 1. The formation of acetyl radical was confirmed by the electron spin resonance (ESR) method using a radical-trapping reagent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The ESR spectrum (Fig. 3) was consistent with that of the DMPO-acetyl adduct of DMPO and acetyl radical generated by Mackor *et al.*[6)] from di-*tert*-butylperoxyoxalate (DBPO)[7)] and acetaldehyde.

TABLE I. Decomposition Products and Their Yields Formed from α-Dicarbonyl Compounds by Hydrogen Peroxide

| α-Dicarbonyl compound | Products (% yield) | |
|---|---|---|
| | With 10 eq of $H_2O_2$ | With 1/10 eq of $H_2O_2$ |
| Methylglyoxal | Acetic acid (81)<br>Formic acid (79) | Diacetyl (0.25) |
| Glyoxal | Formic acid (62) | |
| Diacetyl | Acetic acid (83) | |
| 1-Phenyl-1,2-propanedione | Benzoic acid (91)<br>Acetic acid (83) | Benzil (0.2) |

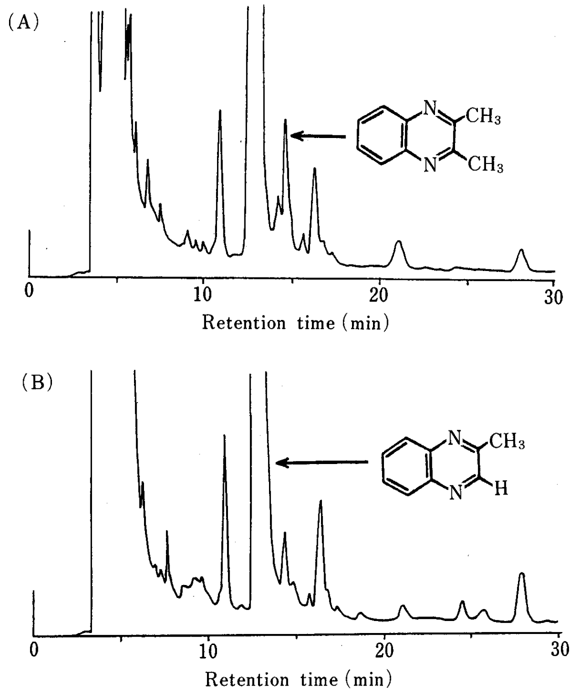

Fig. 2. HPLC Chromatogram of Reaction Mixture of Methylglyoxal and *o*-Phenylenediamine with (A) or without (B) Hydrogen Peroxide in 0.1 M Phosphate Buffer (pH 6.86) at 37 °C

HPLC conditions were as given in Experimental under "degradation of methylglyoxal by hydrogen peroxide" except that the eluent was changed to $CH_3CN$:0.1 N AcOH (35:65).

Nucleic bases were expected to be modified by the highly reactive species generated from methylglyoxal and hydrogen peroxide. So, guanosine was treated with methylglyoxal with or without hydrogen peroxide in 0.1 M phosphate buffer (pH 6.8 to 7.4) at 37 °C. When the reaction mixture without hydrogen peroxide was analyzed by HPLC, new peaks at retention times of 13.5 and 18.5 min were found, as shown in Fig. 4(A). Shapiro *et al.* reported that guanosine produced cyclic adducts with methylglyoxal, and the reaction was reversible.[8)] When excess hydrogen peroxide was added to the reaction mixture containing the cyclic adducts, the peaks of cyclic adducts disappeared and a new peak [compound A] appeared at a retention time of 48 min, as shown in Fig. 4(B).

Compound A was collected by preparative HPLC and its structure was elucidated. The $^1H$- and $^{13}C$-nuclear magnetic resonance (NMR) spectra of compound A measured in dimethyl sulfoxide-$d_6$ (DMSO-$d_6$) suggested that this compound was $N^2$-acetylguanosine. This was confirmed by spectral comparison with authentic $N^2$-acetylguanosine that was synthesized by partial deacetylation with barium methoxide of tetraacetylguanosine[9a)] followed by purification through Diaion HP-20 column chromatography.

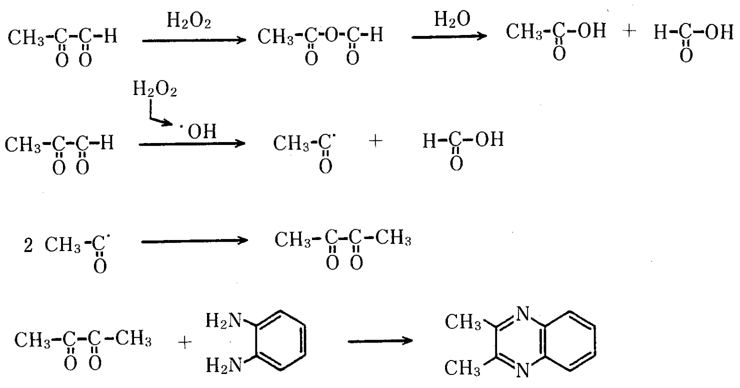

Chart 1. Generation of Acetyl Radical and Diacetyl, and Determination of Diacetyl by the *o*-Phenylenediamine Method

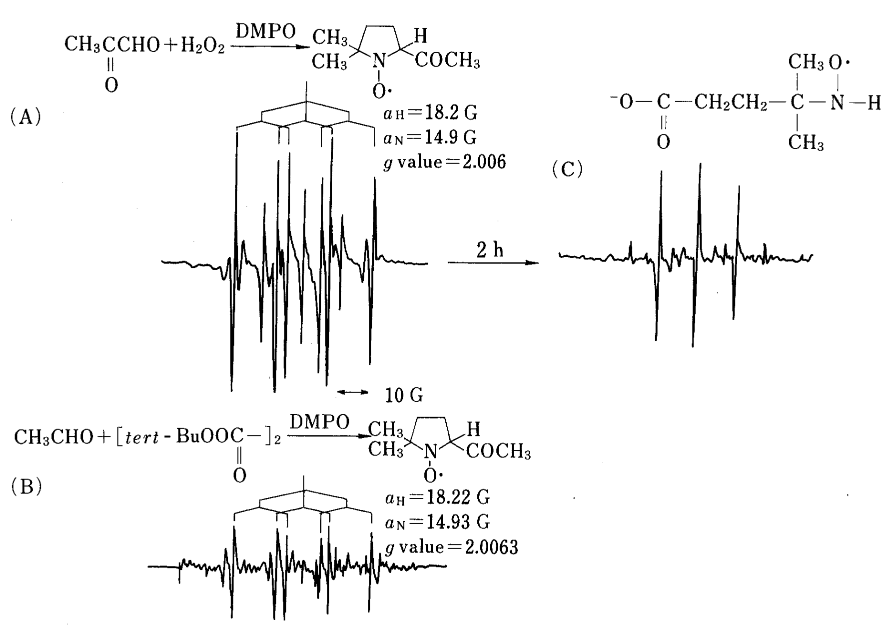

Fig. 3. ESR Spectra of DMPO Adduct (A) from the Reaction Mixture of Methylglyoxal with Hydrogen Peroxide and DMPO-Acetyl Adduct (B) from that of Di-*tert*-butylperoxyoxalate and Acetaldehyde

ESR signals of the DMPO adduct disappeared after 2 h and those of the decomposition product of DMPO remained, as shown in (C).

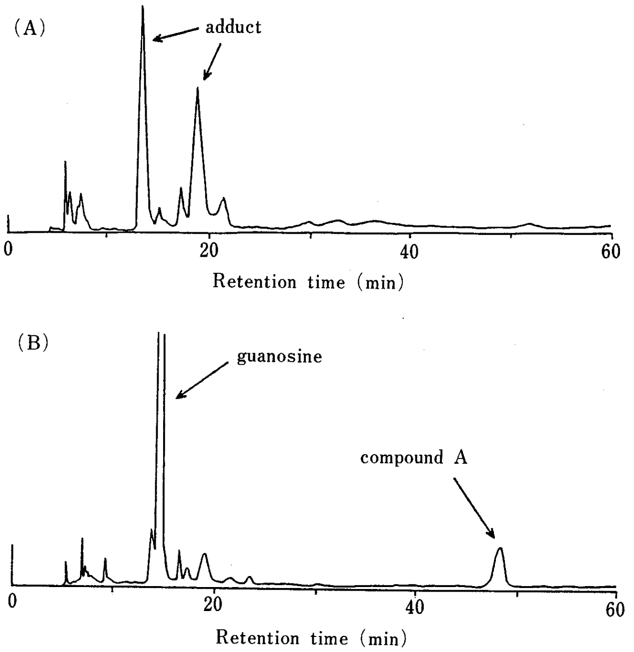

Fig. 4. HPLC Chromatograms of the Reaction Mixture of Guanosine and Methylglyoxal without (A) or with (B) Hydrogen Peroxide

HPLC conditions are given in Experimental.

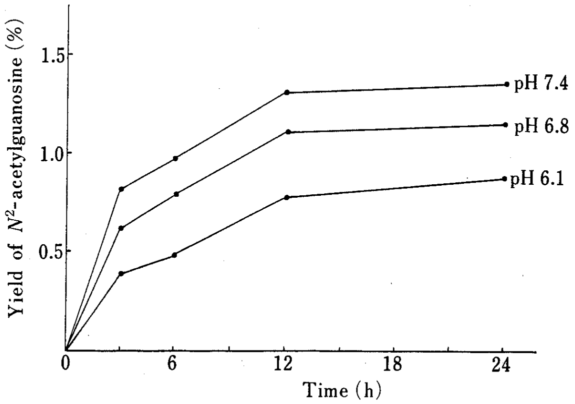

Fig. 5. Yield of $N^2$-Acetylguanosine in the Reaction of Guanosine with Methylglyoxal and Hydrogen Peroxide in 0.1 M Phosphate Buffer of Various pH Values at 37 °C

The time course of $N^2$-acetylguanosine formation from guanosine with methylglyoxal and hydrogen peroxide was examined in 0.1 M phosphate buffer of various pH values at 37 °C. The amount of $N^2$-acetylguanosine was calculated from a standard curve obtained with the authentic sample, and the results are shown in Fig. 5. The amount increased with increase of incubation time and at higher pH value, and was about 1.4% at pH 7.4 after 24 h. On the other hand, 8-hydroxyguanosine, a common product of guanosines modification by peroxides, was not detected. The above acetylation could be related to the enhancement of mutagenicity of methylglyoxal by hydrogen peroxide. However, the mutagenicity of methylglyoxal with hydrogen peroxide was not inhibited by the presence of a radical-trapping reagent such as ethyl alcohol, so it remains uncertain whether the generation of acetyl radical in this medium is a significant factor.

This acetylation of guanosine was found to occur in a solution of coffee in phosphate buffer, as shown in Fig. 6. Namely, a solution of guanosine and instant coffee in phosphate buffer (pH 6.86) was stirred at 37 °C, then applied to a Diaion HP-20 column. The adsorbate eluted with methanol was subjected to HPLC to detect the peak of $N^2$-acetylguanosine at the retention time of 11 min. The peak was confirmed to be due to $N^2$-acetylguanosine by simultaneous injection of an authentic sample. The fast atom bombardment mass spectrum (FAB-MS) of the compound in this peak fraction collected by the repeated HPLC showed ion peaks at *m/z* 326 $(M+H)^+$ and 194 $(M-\text{ribose}+2H)^+$. The yield of this compound from guanosine was 0.17%. This compound was not detected

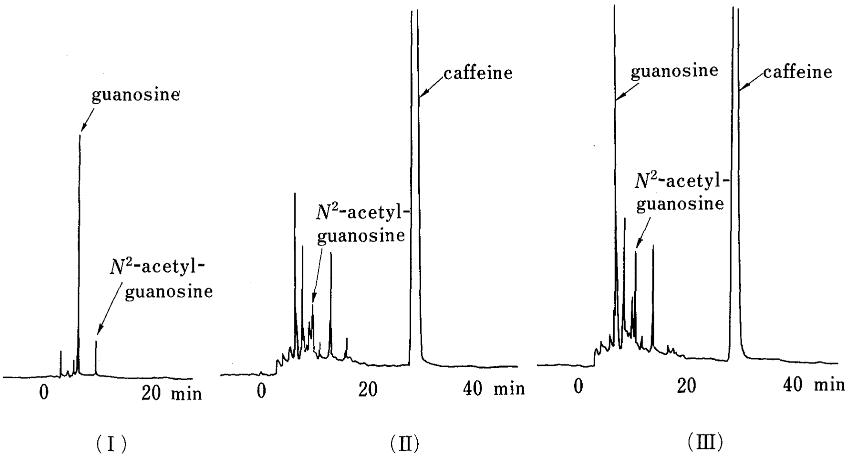

Fig. 6. Acetylation of Guanosine in a Solution of Coffee in 0.1 M Phosphate Buffer (pH 6.86) at 37 °C

The procedure is described in Experimental. The figures show the HPLC profiles of authentic guanosine and $N^2$-acetylguanosine (I), the Diaion HP-20 column eluate from the reaction mixture (II), and a mixture of the two (III).

in the instant coffee solution without an addition of guanosine in the manner described above.

Furthermore, we examined whether other α-dicarbonyl compounds in combination with hydrogen peroxide modify the *N*-2 amino group of guanosine in the same way that methylglyoxal does. It was found that phenylglyoxal converted guanosine to $N^2$-benzoylguanosine,[9] and the generation of benzoyl radical in the reaction was detected by the ESR method using DMPO. However, α-diketones such as diacetyl and 1-phenyl-1,2-propanedione did not cause such modification, though acetyl and benzoyl radicals, respectively, were detected in the reaction mixture. This acylation at the *N*-2 amino group of guanosine was concluded to be specific to α-ketoaldehydes with hydrogen peroxide. 2′-Deoxyguanosine was acetylated in this system and also in a solution of coffee in phosphate buffer.[4] Since methylglyoxal is produced in mammalian cells,[10] it is important to determine whether this modification occurs *in vivo* at guanine residues on deoxyribonucleic acid.

Methylglyoxal was demonstrated to have not only tumor-initiating activity[11] but also tumor-promoting activity.[12] Further, methylglyoxal is carcinogenic in rats when given by subcutanous injection,[13] though not when given to animals in their drinking water.[14] Hydrogen peroxide itself is weakly mutagenic, and induces tumors of the duodenum on oral administration to mice.[15] It will be of interest to examine the carcinogenicity of the combination of methylglyoxal and hydrogen peroxide, in view of the possibility that α-ketoaldehydes in the presence of hydrogen peroxide can chemically modify guanine residues in DNA.

## Experimental

$^1$H-NMR and $^{13}$C-NMR spectra were measured in a JEOL JNM-GSX FTNMR (270.05 MHz) apparatus in DMSO-$d_6$ with tetramethylsilane as an internal standard and the chemical shifts are given in δ (ppm) values. ESR spectra were measured in a JEOL JES.3BS-X apparatus. GC-MS and FAB-MS were measured in JEOL JMS-AX505W and JEOL JMS-SX 102 mass spectrometers, respectively. A melting point was measured on a Yanaco micro melting point apparatus and is uncorrected.

**Degradation of Methylglyoxal by Hydrogen Peroxide** To initiate the reaction, 0.65 ml of 31% aqueous hydrogen peroxide (0.62 mmol) was added to 0.096 ml of 40% aqueous methylglyoxal (6.25 mmol) in 25 ml of 0.1 M phosphate buffer (pH 6.86) at 37 °C. The change of methylglyoxal content with time was analyzed after conversion of methylglyoxal to 2-methylquinoxaline by reaction with *o*-phenylenediamine: a 0.5 ml aliquot of the reaction mixture was added to 4.5 ml of aqueous *o*-phenylenediamine (3 mg/ml) and the mixture was stirred at 37 °C for 30 min. The content of 2-methylquinoxaline was quantified by HPLC on a YMC A-324 ODS column (10 × 300 mm) eluted with $CH_3CN:H_2O$ (40:60) at 3 ml/min. Detection, UV 235 nm.

**Analysis of Carboxylic Acids** Carboxylic acids except benzoic acid in the reaction mixture were analyzed by GC (Hitachi 263-70) on a PEG-6000 10% Flusin P column (0.3 × 200 cm) with He as the carrier gas at a flow rate of 50 ml/min. Oven temperature, 140 °C; injection temperature, 170 °C; detection, thermal conductivity detector (TCD) (100 mA).

Benzoic acid was analyzed by HPLC on a YMC A-324 ODS column (10 × 300 mm) eluted with $CH_3CN$:0.1 N AcOH (1:1) at 3 ml/min. Detection, UV 220 nm.

**Analysis of Quinoxaline Derivatives by GC** Quinoxaline derivatives were analyzed by GC (Shimadzu GC-3BF) on a 3% silicon OV-17 Chromosorb W column (0.3 × 120 cm) with $N_2$ as carrier gas at a flow rate of 43 ml/min; oven temperature, 120 °C; injection temperature, 160 °C; detection, flame ionization detector (FID). Retention times: quinoxaline, 3.2 min; 2-methylquinoxaline, 5.0 min; 2,3-dimethylquinoxaline, 8.8 min.

**Identification of Acetyl Radical in the Reaction Mixture of Methylglyoxal with Hydrogen Peroxide** A mixture of 40% aqueous methylglyoxal (30.8 μl, 0.2 mmol), 31% aqueous hydrogen peroxide (19.8 μl, 0.2 mmol) and DMPO (22.3 μl, 0.2 mmol) was kept at 37 °C for 5 min in the dark. An aliquot (15 μl) was taken in a hematocrit tube for measurement of the ESR spectrum.

**Generation of an Acetyl Radical and Synthesis of DMPO-Acetyl Adduct** Synthesis of DBPO[7]: Pentane (5 ml) and pyridine (0.4 ml, 5 mmol) were added to a solution of *tert*-butyl hydroperoxide (630 mg, 5 mmol) in benzene (1.8 ml). The mixture was cooled in an ice bath and a solution of oxalyl chloride (0.22 ml, 2.5 mmol) in pentane (2.5 ml) was dropped into it over 20 min. The solution was allowed to warm to room temperature, the precipitate of pyridinium chloride was removed, and the filtrate was cooled in a solid carbon dioxide–acetone bath. The precipitated DBPO crystals were filtered off and recrystallized from pentane to yield white needles (99 mg, 85% yield).

Synthesis of DMPO-Acetyl Adduct: A mixture of DBPO (23.4 mg, 0.1 mmol), acetaldehyde (100 μl, 2.0 mmol) and purified water (50 μl) was allowed to react at room temperature for 5 min. Then DMPO (5.6 μl, 0.05 mmol) was added to the reaction mixture and the solution was kept for 5 min. A 15 μl aliquot of the mixture was taken in a hematocrit tube for measurement of the ESR spectrum.

**Isolation of a Modified Guanosine, Compound A** A mixture of guanosine (81 mg, 0.286 mmol) in 50 ml of 0.1 M phosphate buffer (pH 6.86), 4.2 ml (28.6 mmol) of 40% aqueous methylglyoxal and 2.86 ml (28.6 mmol) of 31% aqueous hydrogen peroxide was stirred at 37 °C for 24 h. The reaction mixture was diluted with 300 ml of water and subjected to HP-20 column chromatography (150 ml). The column was washed with 2 l of water, then the adsorbate was eluted with 500 ml of methanol. The eluate was evaporated to give 140 mg of residue, which was separated by preparative HPLC on a Nomura ODS-5 column

(10×250 mm) eluted with $CH_3CN:H_2O$ (5:95) at a flow rate of 2 ml/min (detection, UV 254 nm), to afford 5.2 mg of a light yellow powder, compound A. The structure of compound A was determined as $N^2$-acetylguanosine by direct comparisons of spectra (MS, UV, and $^1$H-NMR) and the retention time on HPLC with a synthetic sample.

**Analysis of $N^2$-Acetylguanosine Formation** A mixture of guanosine (1.80 mg, 6.25 $\mu$mol) in 5.0 ml of 0.1 M phosphate buffer (pH 6.1, 6.8, or 7.4), 19.3 $\mu$l of 40% aqueous methylglyoxal (0.125 mmol) and 12.8 $\mu$l of 31% aqueous hydrogen peroxide (0.125 $\mu$mol) was stirred at 37 °C. To determine the amount of $N^2$-acetylguanosine produced, aliquots were taken periodically and analyzed by HPLC on a Nomura ODS column (10×250 mm) eluted with $CH_3CN:H_2O$ (10:90) at a flow rate of 2 ml/min. Detection, UV 254 nm.

**Synthesis of $N^2$-Acetylguanosine** Reese and Saffhill reported that $N^2$-acetylguanosine was prepared by treatment of tetraacetylguanosine with sodium methoxide in anhydrous dioxane and methanol, though the compound was not obtained in crystalline form.[9a)] Because complete physical data for this compound have not been given, we improved on the synthesis and purification, as follows. A mixture of a solution of guanosine (2.0 g, 7 mmol) in 10 ml of pyridine and 10 ml of acetic anhydride was stirred at 60 °C for 24 h. The reaction mixture was poured into water and evaporated. The residue was subjected to silica gel column chromatography (5% methanol–chloroform) to give 3.0 g of 2′,3′,5′,$N^2$-tetraacetylguanosine.[9a)] A solution of this compound (100 mg, 0.25 mmol) in 10 ml of methanol was treated with 100 $\mu$l of 0.1 M $Ba(OCH_3)_2$ in methanol and the mixture was stirred for 5 h in an ice bath. $CO_2$ gas was bubbled into the reaction mixture to neutralize it. Evaporation gave a residue, which was dissolved in 20 ml of water and centrifuged. The supernatant was subjected to HP-20 column chromatography. The column was washed with 700 ml of water, then the adsorbate was eluted with 300 ml of methanol. The solvent was evaporated off and the residue was recrystallized from aqueous methanol to give 40 mg (56%) of $N^2$-acetylguanosine as white crystals, mp 226 °C (dec.). FAB-MS *m/z*: 326 [$(M+H)^+$], 194 [$(M-\text{sugar}+2H)^+$]. *Anal.* Calcd for $C_{12}H_{15}N_5O_6$: C, 44.31; H, 4.65; N, 21.53. Found: C, 44.21; H, 4.62; N, 21.41. $^1$H-NMR (in DMSO-$d_6$) $\delta$: 12.04 (1H, s), 11.73 (1H, s), 8.26 (1H, s, 8-H), 5.79 (1H, d, $J$=5.8 Hz, 1′-H), 5.46 (1H, d, $J$=5.8 Hz, 2′-OH), 5.16 (1H, d, $J$=4.6 Hz, 3′-OH), 5.02 (1H, t, $J$=5.4 Hz, 5′-OH), 4.43 (1H, m, 2′-H), 4.12 (1H, m, 3′-H), 3.90 (1H, m, 4′-H), 3.59 (2H, m, 5′-H), 2.18 (3H, s, $COCH_3$). UV $\lambda_{\max}^{\text{EtOH}}$ nm (log $\varepsilon$): 254 (4.18), 277 (4.04).

**Modification of Guanosine in a Solution of Coffee in Phosphate Buffer** Several brands of instant coffee purchased in Japan included the same kinds of $\alpha$-dicarbonyl compounds as regular coffee and generated the same quantity of hydrogen peroxide in phosphate buffer (pH 7.0). Guanosine (3.0 mg, 11 $\mu$mol) was added to a solution of 100 mg of instant coffee in 5 ml of 0.1 M phosphate buffer (pH 6.86), and the mixture was stirred at 37 °C for 24 h. The reaction mixture was diluted with 45 ml of water and applied to a Diaion HP-20 column (50 ml). The column was washed with 1500 ml of water to remove unreacted guanosine and eluted with 500 ml of methanol. Evaporation of the eluate gave 19.9 mg of residue, which was dissolved in methanol and analyzed by HPLC on a Nomura ODS-5 column (10×250 mm) eluted with $CH_3CN:H_2O$ (10:90) at a flow rate of 2 ml/min (detection, UV 254 nm). The peak of $N^2$-acetylguanosine was observed at the retention time of 11 min. The compound in the peak fraction was collected by repeated HPLC and analyzed by FAB-MS.

**Identification of $N^2$-Benzoylguanosine** A mixture of guanosine (3.6 mg, 0.013 mmol) and phenylglyoxal (19.0 mg, 0.125 mmol) in 5.0 ml of 0.1 M phosphate buffer (pH 7.4) and 12.8 $\mu$l (0.125 mmol) of 31% aqueous hydrogen peroxide was stirred at 37 °C for 24 h. The reaction mixture was analyzed by HPLC on YMC A-324 ODS column (10×300 mm) eluted with a gradient of $CH_3CN:H_2O$ (5 min with 5:95; from 5 to 40 min with a linear gradient from 5:95 to 50:50) at a flow rate of 3 ml/min (detection, UV 254 nm). The peak fraction at a retention time of 20.5 min was collected by repeated HPLC and evaporated to give a white powder. This product was shown to be identical with authentic $N^2$-benzoylguanosine by comparisons of UV spectrum and retention time on HPLC.

$N^2$-Benzoylguanosine[9)]: This was synthesized by partial debenzoylation with barium methoxide of tetrabenzoylguanosine[9a)] in $CHCl_3:MeOH$ (1:4) in a similar manner to $N^2$-acetylguanosine and recrystallized from water to give white crystals (64%), mp 233—236 °C (dec.) (lit.[9b)] 246—248 °C). *Anal.* Calcd for $C_{17}H_{17}N_5O_6$: C, 52.71; H, 4.42; N, 18.08. Found: C, 52.59; H, 4.36; N, 18.18. $^1$H-NMR (in DMSO-$d_6$) d: 12.32 (1H, br s), 11.94 (1H, br s), 8.28 (1H, s), 8.03 (2H, d, $J$=7.9 Hz), 7.56 (3H, m), 5.88 (1H, d, $J$=5.7 Hz), 5.46 (1H, d, $J$=5.7 Hz), 5.18 (1H, d, $J$=4.2 Hz), 5.02 (1H, t, $J$=5.0 Hz), 4.46 (1H, m), 4.13 (1H, m), 3.91 (1H, m), 3.65 (2H, m). UV $\lambda_{\max}^{\text{MeOH}}$ nm (log $\varepsilon$): 236 (4.02), 264 (3.98), 294 (3.99).

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## References

1) *a*) M. Nagao, T. Yahagi, T. Kawachi, Y. Seino, M. Honda, N. Matsukura, T. Sugimura, K. Wakabayashi, K. Tsuji, and T. Kosuge, "Progress in Genetic Toxicology," ed. by D. Scott, B. A. Bridges, and F. H. Sobels, Elsevier/North-Holland Biomedical Press, 1977, p. 259; *b*) M. Nagao, M. Honda, Y. Seino, T. Yahagi, T. Kawachi, and T. Sugimura, *Cancer Letters*, **2**, 335 (1977).

2) *a*) H. Kasai, K. Kumeno, Z. Yamaizumi, S. Nishimura, M. Nagao, Y. Fujita, T. Sugimura, H. Nukaya, and T. Kosuge, *Jpn. J. Cancer Res.* (*Gann*), **73**, 681 (1982); *b*) M. Nagao, Y. Fujita, K. Wakabayashi, H. Nukaya, T. Kosuge, and T. Sugimura, *Environ. Health Perspect.*, **67**, 89 (1986); *c*) M. Nagao, Y. Fujita, and T. Sugimura, "The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis," IARC Scientific Publications No. 70, ed. by B. Singer and H. Bartsch, Int. Agency Res. Cancer, Lyon, 1986, pp. 283—291; *d*) T. Sugimura and S. Sato, *Cancer Res.*, **43**, (Suppl.) 2415s (1983); *e*) A. Kosugi, M. Nagao, Y. Suwa, K. Wakabayashi, and T. Sugimura, *Mutat. Res.*, **116**, 179 (1983); *f*) M. Nagao, Y. Takahashi, H. Yamanaka, and T. Sugimura, *Mutat. Res.*, **68**, 101 (1979).

3) Y. Fujita, K. Wakabayashi, M. Nagao, and T. Sugimura, *Mutat. Res.*, **144**, 227 (1985).

4) H. Nukaya, T. Iwami, H. Ishida, K. Tsuji, Y. Suwa, K. Wakabayashi, M. Nagao, T. Sugimura, and T. Kosuge, *Mutat. Res.*, **245**, 251 (1990).

5) J. E. Leffler, *J. Org. Chem.*, **16**, 1785 (1951); C. A. Bunton, *Nature* (London), **163**, 444 (1945); W. Frankvoort, *Thermochimica Acta*, **25**, 35 (1978).

6) A. Mackor, Th. A. J. W. Wajer, and Th. J. De Boer, *Tetrahedron*, **24**, 1623 (1968).

7) P. D. Bartlett, E. P. Benzing, and R. E. Pincock, *J. Am. Chem. Soc.*, **82**, 1762 (1960).

8) R. Shapiro, B. I. Cohen, S.-J. Shiuey, and H. Maurer, *Biochemistry*, **8**, 238 (1969).

9) *a*) C. B. Reese and R. Saffhill, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 2937; *b*) S. Chladek and J. Smrt, *Coll. Czech. Chem. Comm.*, **29**, 214 (1964) [*Chem. Abstr.*, **60**, 8114*g* (1964)].

10) J. Sato, Y-M. Wang, and J. van Eys, *J. Biol. Chem.*, **255**, 2046 (1980).

11) *a*) M. Nagao, Y. Suwa, H. Yoshizumi, and T. Sugimura, "Coffee and Health," ed. by B. MacMahon and T. Sugimura, Banbury Report 17, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984, pp. 69—77; *b*) F. Nakasato, M. Nakayasu, Y. Fujita, M. Nagao, M. Terada, and T. Sugimura, *Mutat. Res.*, **141**, 109 (1984).

12) *a*) C. Furihata, Y. Sato, T. Matsushima, and M. Tatematsu, *Carcinogenesis*, **6**, 91 (1985); *b*) M. Takahashi, H. Okamiya, F. Furukawa, K. Toyoda, H. Sato, K. Imaida, and Y. Hayashi, *Carcinogenesis*, **10**, 1925 (1989); *c*) L. L. Yang, V. M. Maher, and J. J. McCormick, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5933 (1980).

13) Y. Fujita, K. Wakabayashi, M. Nagao, and T. Sugimura, *Mutat. Res.*, **142**, 145 (1985).

14) Y. Fujita, K. Wakabayashi, H. Ohgaki, M. Nagao, and T. Sugimura, Proceedings of the 45th Annual Meeting of the Japanese Cancer Association, Sapporo, 1986, p. 64.

15) A. Ito, H. Watanabe, M. Naito, and Y. Naito, *Jpn. J. Cancer Res.* (*Gann*), **72**, 174 (1981).