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Detection and identification of mutagens and carcinogens as their adducts with guanosine derivatives

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ABSTRACT

For use in screening for environmental mutagens and carcinogens, a highly fluorescent derivative of guanosine, 2'-deoxy-2'-(2",3"-dihydro-2",4"-diphenyl-2"-hydroxy-3"-oxo-1"-pyrrolyl)guanosine (FG), was synthesized. When incubated with FG in aqueous solution, mutagens form adducts that can be analyzed with an HPLC-fluorescence detector-system. By this method, mutagens such as glyoxal, methylglyoxal, 2-(2-furyl)-3-(5-nitrofuryl)acrylamide and 4-nitroquinoline-N-oxide, used as model compounds, were detected rapidly with high sensitivity.

Reaction with isopropylideneguanosine (IPG), followed by isolation and characterization of the mutagen-IPG-adduct was found to be a useful method for identifying unknown mutagens in crude samples. This method was successfully applied in identification of the mutagens in heated glucose (200°C, 20 min); glyoxal-IPG and 8-hydroxy-IPG were identified in the reaction mixture.

INTRODUCTION

Many mutagens and carcinogens react with base residues in DNA, particularly guanine residues,[1] and so it is possible to detect them as adducts after treatment with a guanosine derivative. For this purpose, we synthesized a new fluorescent derivative of guanosine, 2'-deoxy-2'-(2",3"-dihydro-2",4"-diphenyl-2"-hydroxy-3"-oxo-1"-pyrrolyl)guanosine (FG) in which a fluorescent group is attached to the sugar moiety. After treatment of samples with this reagent with or without S9 mix, adducts could be detected with high sensitivity by high performance liquid chromatography (HPLC) in an apparatus connected with a fluorescence detector. Furthermore, all procedures, including preincubation with or without S9 mix could be complete within 1 hr.

For structural characterization of mutagens and carcinogens, we found that adducts with isopropylideneguanosine (IPG) were more suitable than those with FG. This method with IPG was applied to a sample of heated glucose, which has been reported to be mutagenic to Salmonella strain TA100.[2] The sample was treated with IPG, and the adducts were isolated by HPLC and

characterized by spectral measurements.

This communication shows that certain mutagens or carcinogens can be rapidly detected with high sensitivity with or without S9 mix by the former method. The identification of adducts formed by the reaction of IPG with heated glucose is given as an example of application of the latter method.

MATERIALS AND METHODS

Materials.

Glyoxal (Tokyo Kasei), methylglyoxal (Aldrich), benzo[a]pyrene (BP, Sigma), 4-nitroquinoline-N-oxide (4NQO, Dai-ichi Kagaku) and 2-aminoimidazo-[4,5-f]quinoline (IQ, Nard Institute) were used without further purification Purified samples of 2-(2-furyl)-3-(5-nitrofuryl)acrylamide (AF-2) and 2-acetylaminofluorene (AAF) were gifts from Dr. M. Nagao of our institute, and Dr. M. Nakadate of the National Institute of Hygienic Sciences, Tokyo, respectively. S9 was a gift from Dr. K. Wakabayashi of our Institute; it was prepared by the method of Ames et al.[3] from the livers of male Sprague-Dawley rats treated with polychlorinated biphenyls (Kanechlor 500). Isopropylideneguanosine and 8-bromoguanosine were purchased from Sigma. 2-Methoxy-2,4-diphenyl-3(2H)-furanone(MDPF) was obtained from Tokyo Kasei. 2'-Amino-2'-deoxyguanosine was a gift from Dr. F. Tomita (Kyowa Hakko Kogyo). Diaion HP-20 was purchased from Mitsubishi Chemical Industries.

Preparation of 2'-deoxy-2'-(2",3"-dihydro-2",4"-diphenyl-2"-hydroxy-3"-oxo-1"-pyrrolyl)guanosine (FG)

A solution of 2'-deoxy-2'-aminoguanosine (40 mg: 0.14 mmol) and triethylamine (14 mg: 0.14 mmol) in 5 ml of water/MeOH (1:1, v/v) was mixed with a solution of MDPF (40 mg: 0.15 mmol) in 2 ml of MeOH at 50°C. The mixture was kept for 10 min at 50°C and then cooled to room temperature. It was then diluted with 50 ml of water and passed through a column of Diaion HP-20 (1.5 cm x 20 cm). The column was washed with 200 ml of water and then material was eluted with a linear gradient obtained by placing 500 ml of water in the mixing chamber and 500ml of 50% aqueous MeOH in the reservoir. Fluorescent fractions were collected and evaporated to dryness to afford an amorphous solid, consisting of two diastereomeric components (major isomer: minor isomer=15:1, based on ^{1}H-NMR spectra). Attempts to separate the diastereomeric components by HPLC were unsuccessful due to rapid epimerization. Yield 35%. MS (permethyl derivative): m/z 600 (M^+), m/z 194 (b+2). ^{1}H-NMR (DMSO-d_6 + D_2O, major isomer): δ(ppm) 9.35 (1H, s, >N-CH=), 7.88 (2H, d, J=8.3 Hz, phenyl), 7.80 (1H, s, 8-H), 7.42 (2H, dd,

J=8.3, 7.4 Hz, phenyl), 7.21 (1H, dd, J=7.4, 7.4 Hz, phenyl), J=7.15 (1H, dd, J=7.4, 7.4 Hz, phenyl), 7.01 (2H, dd, J=7.4, 7.4 Hz, phenyl), 6.91 (2H, d, J=7.4 Hz, phenyl), 6.53 (1H, d, J=9.2 Hz, 1'-H), 4.62 (1H, dd, J=9.2, 5.1 Hz, 2'-H), 4.33 (1H, d, J=5.1 Hz, 3'-H), 4.22 (1H, m, 4'-H), 3.71 (2H, ABX, 5'H). IR (KBr): 1667, 1625, 1597, 1563 cm^{-1}.

Reaction of mutagens with FG

Solutions (100 µl) of the direct acting mutagens glyoxal and methyl glyoxal (50 µg/100 µl DMSO) were each mixed with 100 µl of solution of FG (50 µg/100 µl H_2O) and 500 µl of sodium phosphate buffer (100 mM, pH 6.8) in a test tube (1 x 8 cm). The solutions (total 0.7 ml) were shaken at 37°C for 20 min and then 5 µl samples were analyzed by HPLC in an apparatus equipped with a fluorescent detector [instrument: Shimadzu LC-3A, column: Merck Hibar column LiChrosorb RP-18, 5µ, 0.4 x 25 cm, solvent: 30% aqueous methanol containing 10 mM NH_4OAc (pH 5.3), detector: JASCO FP-110 fluorescence spectrofluorometer, excitation at 365 nm, emission at 480 nm]. In experiments with 4NQO, AF-2, AAF, BP and IQ, 100 µl of solutions of these mutagens (50 µg/100 µl DMSO) were mixed with 100 µl of solution of FG (50 µg/100 µl H_2O) and 500 µl of S9 mix (pH 7.4). S9 mix consisted of 30 µl of S9, 2 µmol NADPH and 2.5 µmol glucose-6-phosphate, 4 µmol $MgCl_2$, 16.5 µ mol KCl and 50 µmol sodium phosphate buffer (pH 7.4) in a total of 0.5 ml.

Reaction of isopropylideneguanosine with heated glucose and isolation of adducts by HPLC

Glucose (10 g) in a 500 ml Erlenmeyer flask was heated on an oil bath at 200°C. Heating was continued for 20 min after all the glucose had melted. The heated glucose was cooled to room temperature and dissolved in 100 ml of a solution of 100 mg of isopropylideneguanosine in 25 mM sodium phosphate buffer (pH 6.8). The brown solution obtained was kept in an incubator at 37°C for 15 hrs.

The reaction mixture (100 ml) was extracted with ethyl acetate (100 ml each x 5) and the extract was evaporated to dryness. The residue was suspended in 15% MeOH solution (5 ml) and centrifuged. The supernatant was fractionated by HPLC [instrument: Waters M-45 solvent delivery system, column: Waters, semi-preparative µBondapak C_{18} (0.78 x 30 cm), solvent: 15% aqueous methanol, flow rate: 6 ml/min, detector: Varian Aerograph UV detector, 254 nm]. In this way two adducts, adduct-1 (3.5 A_{260} units) and adduct-2 (2.0 A_{260} units) were obtained.

Mass spectrometry

Adduct-2 was converted to the trimethylsilyl derivative as described

previously.[4] A dried sample of 0.2 A_{260} unit of adduct-2 was dissolved in 10 µl of N,O-bis(trimethylsilyl)trifluoroacetamide-pyridine-trimethylsilyl-chlorosilane (100:10:1, v/v/v), and heated for 1 hr at 100°C. Mass spectra were recorded in a JEOL 01SG-2 instrument with an ionizing electron energy of 75 eV and an ion source temperature of 200°C. The sample was introduced by direct probe after removal of silylation reagents under vacuum.

The permethyl derivative of synthetic FG was prepared using methyl-sulfinyl carbanion and methyl iodide as described previously.[5]

Nuclear magnetic resonance spectroscopy

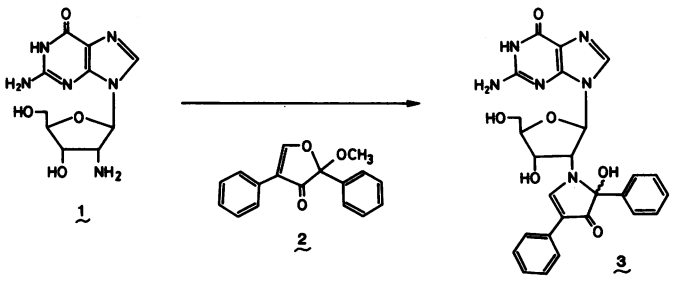
First 2 A_{260} units of adduct-2 was lyophilized from 99.5% CD_3OD and dissolved in 99.95% CD_3OD (Merk) in a glove bag filled with nitrogen gas. The proton NMR spectrum of adduct-2 in CD_3OD was recorded in a Bruker CXP-300 spectrometer at 23°C. Chemical shifts were measured relative to the residual CHD_2OD signal (3.40 ppm).

Mutation assay

S. typhimurium mutant TA100 was used for mutation assay. Assays were performed by the preincubation method without S9 mix,[6] using two plates for each concentration of sample. Specific activity was calculated from the data within the linear dose-response range.

RESULTS AND DISCUSSION

2-Methoxy-2,4-diphenyl-3(2H)-furanone (MDPF, structure 2) has been used for fluorescent labeling of amino groups in protein.[7] We chose it for fluorescent labeling of 2'-amino-2'-deoxyguanosine (structure 1) because it reacts selectively with the 2'-amino group in the sugar moiety, and does not react with the 2-amino group of the guanine base. As MDPF is not itself fluorescent, and on reaction it gives a single fluorescent product, this product was easily separated by column chromatography with fluorescence



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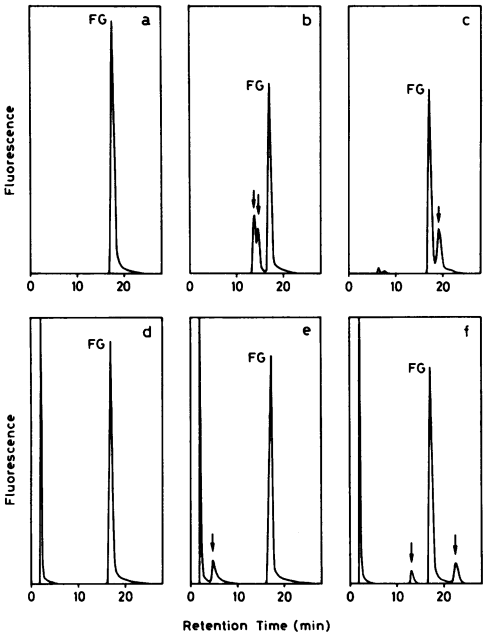


Fig. 1. Detection of mutagen-FG-adducts by HPLC.
Mutagens were incubated with FG in the presence or absence of S-9 mix and analyzed by HPLC as described in the experimental section. a) control FG, b) glyoxal + FG, c) methylglyoxal + FG, d) control FG + S9 mix, e) 4NQO + FG + S9 mix, f) AF-2 + FG + S9 mix. Peaks of adducts are indicated by arrows.

monitoring. The fluorescent product (FG, structure 3) was found to be extremely stable over a wide range of pH values. It could be extracted from the aqueous reaction mixture with an organic solvent such as ethyl acetate.

When the typical direct acting mutagens glyoxal[8] and methylglyoxal[9] were incubated with a solution of FG and then analyzed by HPLC in an apparatus equipped with a fluorescent detector, they gave extra peaks in the chromatograms (Fig. 1b and c) besides that of control FG (Fig. 1a). These extra peaks probably correspond to adducts formed between the mutagens and FG, because they were not seen when control FG solution or mutagen solution alone was analyzed by HPLC. This method was also applied to mutagens and carcinogens which require metabolic activation for their mutagenic action. The conditions used for preincubation of samples with S9 mix were exactly the same as those for the bacterial mutagenicity test,[6] except that the bacterial cell suspension were replaced by FG solution (see experimental

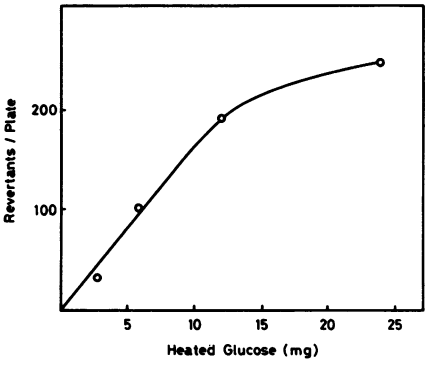


Fig. 2. Mutagenicity of heated glucose (200°C, 20 min) to Salmonella strain TA100.

section). When 4NQO or AF-2 was assayed by this method adducts were detected on the chromatograms (Fig. 1e and f). These adducts were absent when 4NQO or AF-2 was incubated with FG in the absence of S9 mix or incubated with S9 mix in the absence of FG. These compounds were mutagenic in the Salmonella test in the absence of S9 mix because bacteria have nitroreductases and other enzymes that convert the nitro group to a hydroxylamino group and further to its ester form. With the frameshift mutagens AAF, BP and IQ no adduct was detected, suggesting that polymer DNA may be required for their reaction with guanine residues and that they do not react with a monomer derivative of guanine, such as FG.

For final identification of the original mutagens, the structures of the adducts must be determined. Even when the adducts are detected by HPLC as fluorescent peaks by the FG-method, it is not easy to characterize them, because they have a rather large fluorescent chromophore in their molecule, which influences their mass, nmr and UV spectra. Thus for characterization of adducts, it is better to use isopropylideneguanosine (IPG, structure 4) instead of FG as a starting reagent. As an example, we used IPG to characterize the mutagens in heated glucose. When glucose was heated at 200°C for 20 min, it became mutagenic to Salmonella strain TA100 without S9 mix (Fig. 2), and its specific activity under optimum conditions was about 16,000 revertants/g. This specific activity is much weaker than that (6,000 revertants/mg) reported for the tar obtained by heating glucose at much higher temperature.[2] The heating conditions (200°C, 20 min) used in this experiments were chosen because they are similar to cooking conditions and

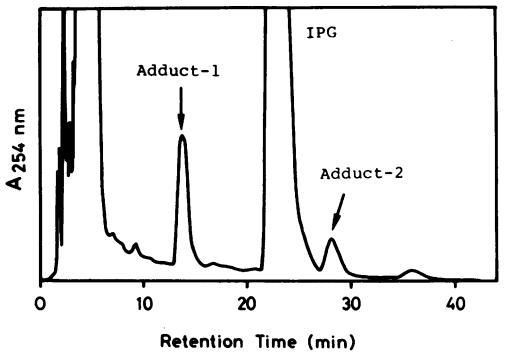
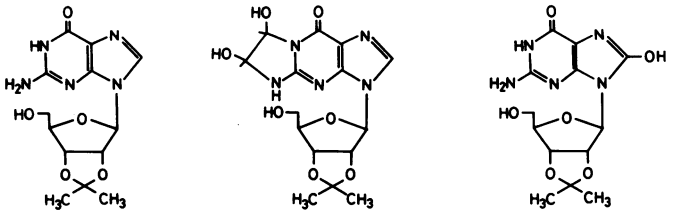


Fig. 3. Fractionation of heated glucose-IPG-adducts by HPLC.

because glucose heated at higher temperature contains many UV-absorbing materials that hinder the detection and isolation of IPG-adducts by HPLC. The HPLC profile of the reaction mixture of heated glucose with IPG is shown in Fig. 3. The chromatogram shows two peaks of IPG-adducts (adduct-1 and adduct-2) eluted before and after the large IPG peak, respectively. These two peaks were not seen with control IPG solution or heated glucose solution alone, confirming that they were those of reaction products between IPG and components in heated glucose. Adduct-1 was unambiguously identified as glyoxal-IPG adduct (structure 5) from similarity of its UV spectrum and retention time on HPLC to those of authentic glyoxal-IPG adduct which was prepared from glyoxal and IPG according to Shapiro and Hachmann.[10] This adduct was degraded gradually to IPG during its purification by repeated HPLC.

The UV spectra of adduct-2 were very characteristic (Fig. 4). The spectrum in H_2O had two λ_{max} at 245 nm and 293 nm. This spectrum was very similar to that of 8-hydroxyguanosine reported by Ikehara et al.[11] The nmr spectrum of adduct-2 measured in methanol-d_4 showed the following signals:



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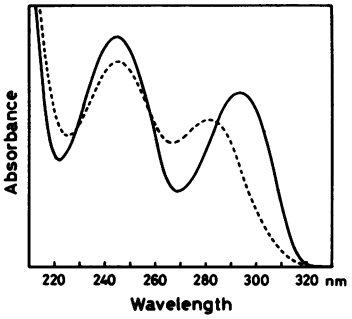

Fig. 4. UV spectra of adduct-2; ——— pH 2 and H_2O, ----- pH 10.

δ=6.05 ppm (1H, d, J=2.6 Hz, 1'-H), 5.45 (1H, dd, J=2.6, 6.1 Hz, 2'-H), 5.09 (1H, dd, J=3.3, 6.1 Hz, 3'-H), 4.27 (1H, m, 4'-H), 3.81 (2H, m, 5'-H), 1.65 (3H, s, CH_3) and 1.46 (3H, s, CH_3). This spectrum differed from that of IPG, only in having no signal of the C-8 proton of the guanine ring (8.03 ppm in IPG); other protons were detected at almost the same positions as in the spectrum of IPG. This finding strongly suggested that the modification in adduct-2 is at the C-8 position of IPG. The structure of adduct-2 was established by mass spectrometry of its trimethylsilyl derivative (Fig. 5). The molecular ion at m/z 627 corresponded to that of the tetra-timethylsilyl derivative of 8-hydroxy-IPG (structure 6). Fragment ions were also observed at m/z 412 (b+CH_2O) and m/z 383 (b+H). The structure of adduct-2 was confirmed by direct comparison of the adduct with synthetic 8-hydroxy-IPG prepared from 8-bromoguanosine;[11] the two compounds gave the same spectral data, Rf values and retention times on thin-layer chromatography and HPLC.

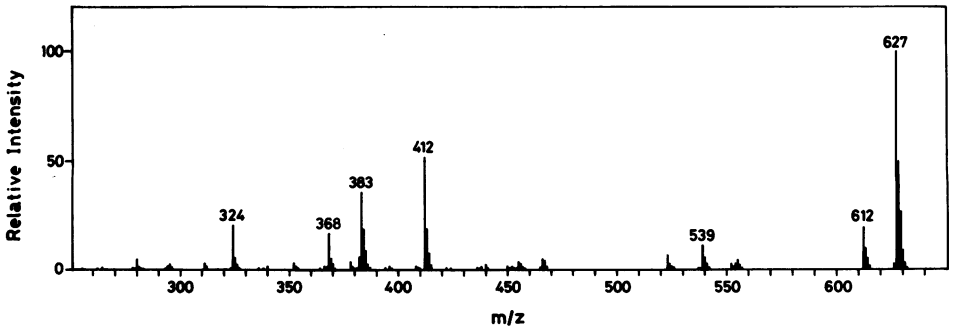

Fig. 5. Mass spectrum of the trimethylsilyl derivative of adduct-2.

The two new methods; FG-method for detection and IPG-method for identification of mutagens described above have the unique characteristic. They are based on chemical reactions of mutagens with a derivative of guanine, the most reactive component of DNA. Randerath et al. reported a new method for detecting DNA-mutagen-adducts by ^{32}P-post-labeling.[12]; their method is also based on the chemical reactivity of mutagens with DNA bases. A correlation between mutagenic or carcinogenic activities and reactivities with guanosine was first reported by Wislocki et al.[13] They monitored the electrophilic reactivity of ultimate carcinogens with ^{14}C-guanosine by measuring loss of radioactivity. In our method, a low concentration of the fluorescent reagent FG was enough for detection of small amounts of mutagens; the minimum quantity of mutagen detectable was about 5 - 50 ng. Another advantage of our method is that all procedures can be finished within 1 hr.

In our second method IPG is used for identifying mutagens present in crude extract, because its adduct can be easily extracted from aqueous reaction mixture with an organic solvent such as ethyl acetate. This extraction procedure selectively separated IPG and IPG-adducts from most other components of heated glucose. Thus the heated glucose-IPG-adducts could be isolated very easily. Furthermore, it was easy to determine whether the extra peaks separated by HPLC were those of derivatives of IPG or components of heated glucose by measuring their UV spectra.

The detection of 8-hydroxy-IPG in the reaction mixture of IPG and heated glucose raises interesting questions. What component in heated glucose is responsible for the C-8 hydroxylation of IPG? What is the mechanism of the hydroxylation? How much is this reaction involved in mutagenesis? Recently, we observed that various reducing agents, such as ascorbic acid, hydroxylamine, hydrazine, sodium bisulfite, dihydroxymaleic acid and acetol, hydroxylate guanosine or deoxyguanosine at the C-8 position in the presence of oxygen.[14] Therefore, it is possible that various reducing agents produced by heating glucose, for example acetol[15] and other reductones, might be responsible for the hydroxylation of IPG by heated glucose. It was also found that ascorbic acid hydroxylates guanine residues in DNA.[14] Studies are now in progress to elucidate the biological significance of this modification of DNA.

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