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Adduct Formation at C-8 of Guanine on *in vitro* Reaction of the Ultimate Form of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine with 2′-Deoxyguanosine and Its Phosphate Esters

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We examined the reactivity of the *N*-hydroxyamino derivative of a carcinogenic heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), after its *O*-acetylation with four 2′-deoxyribonucleoside 3′-monophosphates. ^{32}P-Postlabeling analysis demonstrated that the levels of adducts with 2′-deoxyguanosine 3′-monophosphate were much higher than those with the other three nucleotides. ^{1}H-NMR, mass spectral and UV absorption spectral analyses of the major adducts formed by *N*-acetoxy-PhIP with 2′-deoxyguanosine and with its phosphate esters indicated that PhIP bound at the C-8 position of guanine, as previously demonstrated with other heterocyclic amines.

Key words: PhIP — *N*-OH-PhIP — Deoxyguanosine — dG-C8-PhIP

Various mutagenic and carcinogenic heterocyclic amines are produced during the cooking process of proteinaceous foods.[1,2] Among these, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was first isolated as the most abundant mutagenic compound by weight in fried ground beef.[3] Later PhIP was also found in other cooked foods at relatively high levels.[4] Moreover, it was shown to induce lymphomas in CDF_1 mice,[5] small and large intestinal carcinomas in Nagase analbuminemic rats[6] and large intestinal and mammary carcinomas in F344 rats.[7]

PhIP is metabolically activated by cytochrome P450-IA2 to 2-hydroxyamino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (*N*-OH-PhIP).[8] Then *N*-OH-PhIP is converted to the ultimate forms, the *N*-sulfate and *N*-acetoxy derivatives, by sulfotransferase and acetyltransferase, respectively,[9] and reacts with DNA to form adducts. ^{32}P-Postlabeling analysis of DNA adducts in F344 rats fed PhIP indicated that PhIP-DNA adducts were present in all organs tested, their levels being high in the lung, pancreas and heart, and low in the liver.[10] However, the structures of the DNA adducts formed by PhIP have not yet been determined. Therefore, in this study, using the ^{32}P-postlabeling method, we first examined which nucleotide(s) forms adducts with *N*-OH-PhIP in the presence of acetic anhydride, then attempted to identify the structure of the major adduct.

For preparation of *N*-OH-PhIP, 4 μmol of 1-methyl-2-nitro-6-phenylimidazo[4,5-*b*]pyridine (Nard Institute, Osaka) and 5 mg of 5% palladium on activated carbon (Pd/C) in 2 ml of tetrahydrofuran were stirred for 1 h at the ambient temperature under hydrogen. The Pd/C was removed by filtration through a 0.2-μm microfilter, and the *N*-OH-PhIP (98% pure as judged by HPLC) in tetrahydrofuran was treated with 4.4 μmol of acetic anhydride at 0°C for 2 min to give *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-acetoxy-PhIP), which could not be isolated because of its high reactivity. Then, one-tenth (200 μl) of the reaction mixture containing *N*-acetoxy-PhIP was added dropwise to 600 μl of degassed 20 m*M* Tris-HCl buffer (pH 7.4) containing 0.4 μmol of each 2′-deoxyribonucleoside 3′-monophosphate (3′-dGp, 3′-dAp, 3′-dCp or 3′-dTp; Sigma Chemical Co., St. Louis, MO) and the mixtures were incubated for 20 min at 25°C. Aliquots of the reaction mixtures were ^{32}P-labeled under standard conditions, and the adducts were separated and detected on PEI-cellulose sheets as described previously.[10] As shown in Fig. 1, after reaction of 3′-dGp with *N*-acetoxy-PhIP, two spots (1 and 2) of adducts were detected, their levels corresponding to 20 and 1.2 per 10^4 nucleotides, respectively. After reactions of the other three 2′-deoxyribonucleoside 3′-monophosphates (3′-dAp, 3′-dCp, and 3′-dTp) with *N*-acetoxy-PhIP, two to five spots of adducts were detected, but at much lower levels than those of the adducts of 3′-dGp and *N*-acetoxy-PhIP, being 10^3-fold less than that of the major adduct (spot 1) in Fig. 1.

The structure of the material in spot 1 was determined as follows. First, we obtained cold 2′-deoxyguanosine 3′,5′-diphosphate (3′,5′-pdGp)-PhIP adduct using the ^{32}P-labeled spot 1 as a marker, and converted it to

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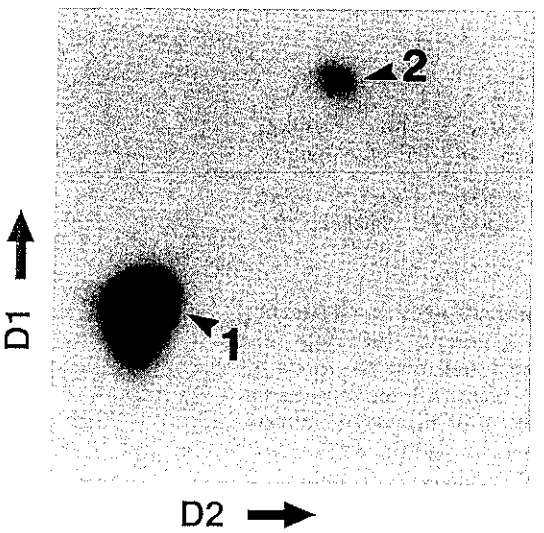

Fig. 1. Autoradiogram of adducts formed by reaction of 3′-dGp with *N*-OH-PhIP in the presence of acetic anhydride. Adducts were ^{32}P-labeled under standard conditions, and the X-ray film was exposed for 30 min at −80°C. Spots of adducts are numbered.

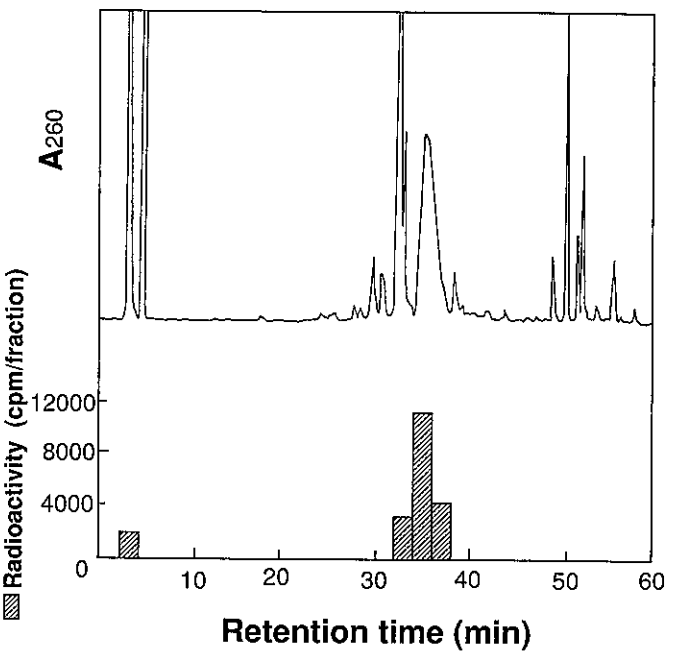

Fig. 2. HPLC chromatogram of compounds formed from 3′,5′-pdGp with *N*-OH-PhIP in the presence of acetic anhydride on a TSKgel ODS-80Ts column. Materials were eluted with the following solvent system at a flow rate of 1 ml/min at the ambient temperature with monitoring of UV absorbance at 260 nm: 0–10 min, 18% CH_3CN in 25 m*M* phosphate buffer (pH 2.0); 10–70 min, a linear gradient of 18 to 50% CH_3CN in 25 m*M* phosphate buffer (pH 2.0). The radioactivity of an aliquot of each fraction collected at 2 min intervals was measured in a Beckman LS8100 liquid scintillation counter.

the dephosphorylated derivative, the 2′-deoxyguanosine (dG)-PhIP adduct, by treatment with alkaline phosphatase. With this dG-PhIP adduct as an authentic compound, a large amount of the dG-PhIP was prepared for measurement of its mass and ^{1}H-NMR spectra, because spectroanalysis of nucleosides is much easier than that of nucleotides. The experimental procedures and results were as described below.

Spot 1 on the thin-layer chromatography (TLC) was cut off and extracted with 0.5 ml of 2-propanol–ammonia water (1:1, v/v) solution three times with shaking for 30 min. The extract was evaporated and the residue was dissolved in 200 μl of 50% methanol. In addition, cold 3′,5′-pdGp-PhIP adduct was prepared by treating 2.0 μmol of 3′,5′-pdGp in 3 ml of a degassed aqueous solution with 2.0 μmol of *N*-OH-PhIP and 2.2 μmol of acetic anhydride in 1 ml of tetrahydrofuran for 20 min at 25°C. A sample of 200 μl of this reaction solution containing cold 3′,5′-pdGp-PhIP adduct was then mixed with 20 μl (20,000 cpm) of the extract of the ^{32}P-labeled spot 1, and subjected to high-performance liquid chromatography (HPLC) on a TSKgel ODS-80Ts column (5 μm particle size, 4.6×250 mm, Tosoh Corp., Tokyo). Almost 75% of the radioactivity was recovered in fractions with retention times of 34–38 min (Fig. 2). These fractions were collected and evaporated, and the residue was dissolved in 400 μl of 50% methanol. A sample of 50 μl of the solution was purified further on a TSKgel CN-80Ts column (5 μm particle size, 4.6×150 mm, Tosoh Corp.). As shown in Fig. 3, on HPLC, this sample gave a single peak with a retention time of 18 min and 90% of the radioactivity was recovered in the peak fraction. Moreover, on a PEI-cellulose sheet, this peak fraction gave a single spot, detected by autoradiography, in the same position as spot 1 in Fig. 1.

The HPLC procedure was repeated and the compound, 3′,5′-pdGp-PhIP adduct, in the peak fraction was collected. Fig. 4 shows the UV absorption spectrum of the compound on a TSKgel CN-80Ts column, recorded with an SPD-M6A photodiode array UV-VIS detector (Shimadzu Corp., Kyoto). The yield of the 3′,5′-pdGp-PhIP adduct from 3′,5′-pdGp was about 0.2% as estimated from the UV absorption at 260 nm. For removal of inorganic salts in the fraction collected, 4 ml of the solution was passed through an ODS cartridge. The cartridge was washed with 2 ml of water, and then the adsorbed compound was eluted with 1 ml of methanol–ammonia water (1:1, v/v). To obtain the authentic dG-PhIP adduct, we incubated ~5 μg of the compound with

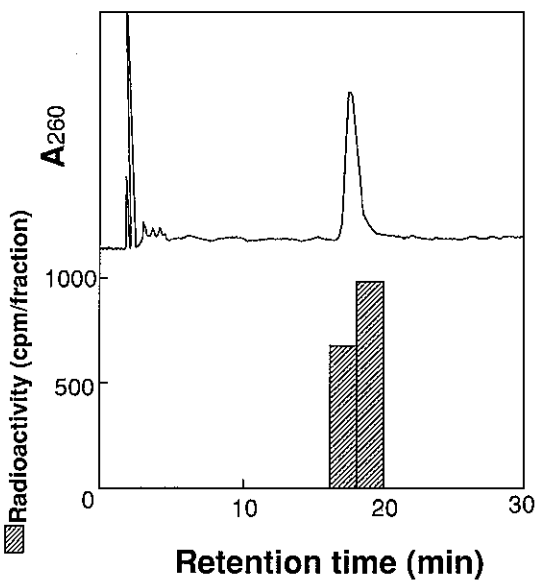

Fig. 3. HPLC chromatogram of the 3′,5′-pdGp-PhIP adduct on a TSKgel CN-80Ts column. The mobile phase of 10% CH_3CN in 25 m*M* phosphate buffer (pH 2.0) was pumped in at a flow rate of 1 ml/min at the ambient temperature. The UV absorbance at 260 nm and radioactivity are indicated in the upper and lower plots, respectively.

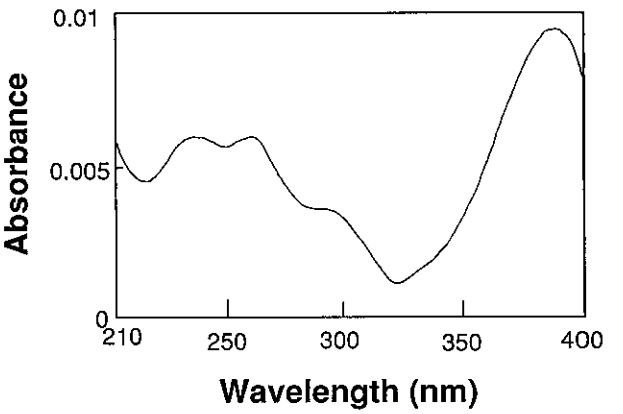

Fig. 4. UV absorption spectrum of the 3′,5′-pdGp-PhIP adduct. A sample of a fraction containing ~200 ng of the adduct was examined with a photodiode array detector on a TSKgel CN-80Ts column. The mobile phase of 10% CH_3CN in 25 m*M* phosphate buffer (pH 2.0) was pumped in at a flow rate of 1 ml/min.

0.2 unit of alkaline phosphatase (type III, Sigma Chemical Co.) in the presence of 1.0 m*M* $MgCl_2$ in a total volume of 200 μl of 10 m*M* Tris-HCl buffer (pH 7.6) for 3 h at 37°C, and then analyzed an aliquot by HPLC on a TSKgel CN-80Ts column under the same conditions as shown for Fig. 3. The peak corresponding to the 3′,5′-pdGp-PhIP adduct had almost disappeared, and instead, a single peak with a retention time of 12 min was detected. The UV absorption pattern of the compound (dG-PhIP adduct) in this fraction was identical to that of the 3′,5′-pdGp-PhIP adduct.

Next we prepared a large quantity of dG-PhIP adduct, by treating 60 μmol of dG (Sigma Chemical Co.) in 90 ml of a degassed aqueous solution with 60 μmol of *N*-OH-PhIP in the presence of 66 μmol of acetic anhydride in 30 ml of tetrahydrofuran for 20 min at 25°C. The reaction mixture was evaporated to dryness and the residue was dissolved in 4 ml of 50% methanol. The dG-PhIP adduct was purified by the following HPLC procedures with monitoring of the UV absorbance of eluted materials at 260 nm at the ambient temperature. One-tenth of the 50% methanol solution was injected into a semi-preparative TSKgel ODS-120T column (10 μm particle size, 7.8×300 mm, Tosoh Corp.), and material was eluted at a flow rate of 1 ml/min with the following concentration of CH_3CN in 25 m*M* phosphate buffer (pH 2.0): 0–10 min, 18%; 10–70 min, a linear gradient of 18 to 50%. The fractions with retention times of 40–44 min contained a compound showing the same elution position on HPLC and the same UV absorption spectrum as those of the authentic dG-PhIP adduct. The above HPLC procedure was repeated and the fractions containing this compound were collected. Inorganic salts were removed in the ODS cartridge, and the dG-PhIP adduct obtained (430 μg) was used for spectral analyses.

As seen in Fig. 5, the fast atom bombardment (FAB) mass spectrum of the compound (~30 μg) showed ion peaks at *m/z* 490 $(M+H)^+$ and 374 $(M\text{-sugar}+2H)^+$. The ^{1}H-NMR spectrum of the compound in dimethyl sulfoxide-d_6, measured in a JEOL JNM-GSX FTNMR apparatus operated at 270 MHz, indicated the presence of the following protons; δ (ppm) 2.05 (m, 1H), 3.25 (m, 1H), 3.54 (dd, 1H, J=11.5, 5.5 Hz), 3.66 (s, 3H), 3.71 (dd, 1H, J=11.5, 5.4 Hz), 3.81 (m, 1H), 4.50 (m, 1H), 6.39 (s, 2H), 6.51 (t, 1H, J=7.4 Hz), 7.39 (t, 1H, J=7.2 Hz), 7.50 (dd, 2H, J=7.2, 8.1 Hz), 7.77 (d, 2H, J=8.1 Hz), 8.01 (d, 1H, J=2.0 Hz) and 8.42 (d, 1H, J=2.0 Hz). Of these protons, the three at 3.66 ppm were assigned to the *N*-methyl group of PhIP, the two at 6.39 to the *N*-2 amino group of guanine, and the seven at 7.39–8.42 ppm to aromatic protons of PhIP. The following seven 2′-deoxyribose carbon-bound protons were also observed: C-1 at 6.51 ppm, C-2 at 2.05 and 3.25 ppm, C-3 at 4.50 ppm, C-4 at 3.81 ppm and C-5 at 3.54 and 3.71 ppm. Moreover, the NMR data revealed the absence of a C-8 proton of guanine and protons of

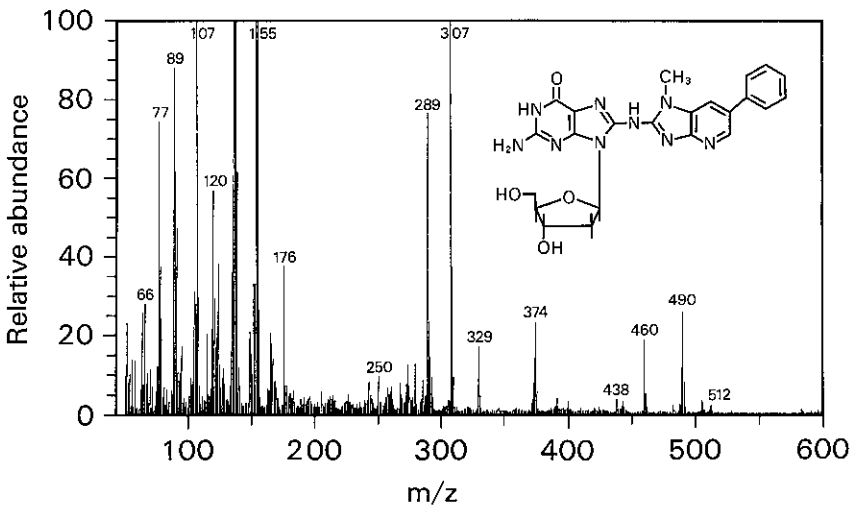

Fig. 5. FAB mass spectrum of dG-C8-PhIP. 3-Nitrobenzyl alcohol was used as a matrix. The ion peaks at *m/z* 154, 176, 289, 307, 329 and 460 were derived from 3-nitrobenzyl alcohol.

the N-2 amino group of PhIP. From the above results, the structutre of the dG-PhIP adduct was concluded to be *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP).

In the present study, we found that an ultimate form of PhIP reacted with 2′-deoxyguanosine or its phosphate esters to form adducts at the C-8 position of guanine. These results are in accordance with the data which were only recently published by Frandsen *et al.*[11] We previously obtained several spots of adducts from the organs of rats fed PhIP by ^{32}P-postlabeling analysis under standard conditions.[10] The position of one of these spots on TLC coincided with that of the 3′,5′-pdGp-C8-PhIP adduct. However, the problem of whether the other spots are those of different mononucleotide adducts from 3′,5′-pdGp-C8-PhIP or are undigested dinucleotides and oligonucleotides including this adduct remains to be solved. In addition, no data on the nature of the compound in spot 2 (Fig. 1) are presently available. Other heterocyclic amines, such as 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), have been shown to react with DNA after *O*-acylation of their *N*-hydroxy derivatives and to produce adducts at the C-8 position of guanine.[12–14] It will be very interesting to determine whether the biological effects, including induction of DNA conformational changes and mutagenic activity, caused by these four heterocyclic amines through formation of DNA adducts at the C-8 position of guanine are similar or not.

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