Modification of DNA with Potent Mutacarcinogenic 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole Isolated from a Glutamic Acid Pyrolysate: Structure of the Modified Nucleic Acid Base and Initial Chemical Event Caused by the Mutagen

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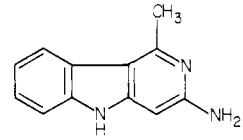
Abstract: 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), a potent mutacarcinogen isolated from a glutamic acid pyrolysate, reacted with DNA in vitro and in vivo. The structure of the modified nucleic acid base was determined as 2-(8-guanylylamino)-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Gua-Glu-P-1) by comparison with synthetic material. The metabolically activated form of Glu-P-1 was identified with synthetic 2-(hydroxyamino)-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (N-OH-Glu-P-1). N-OH-Glu-P-1 is a proximate form of Glu-P-1, and its ultimate form was suggested to be its O-acyl derivative.

An important step in carcinogenesis is thought to be initial attack on the DNA molecule by a chemical or a so-called ultimate carcinogen, which is formed metabolically from a parent carcinogen. The covalent binding of the ultimate carcinogen to the DNA bases creates a premutational lesion that is processed by repair, replication, and recombination enzymes in vivo and eventually may by converted to a mutation.

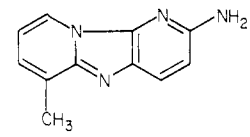
Many carcinogens in our environment have been detected as mutagens by using *Salmonella typhimurium* TA 98 as a tester strain.[1] Recent studies showed that pyrolysis products of foods, proteins, and amino acids contain strong mutagens, and the active compounds were isolated and their structures determined.[2] Among these compounds, 3-amino-5*H*-pyrido[4,3-*b*]indoles (Trp-P) from a pyrolysate of tryptophan[2b] and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazoles (Glu-P) from a pyrolysate of glutamic acid[3] are strong mutagens and are widely destributed in various heat-treated foods.[4] The carcinogenicities of these compounds have also been demonstrated.[5]

Elucidation of the structures of DNAs modified with mutacarcinogens is essential in understanding the molecular basis for alteration of gene expression in mutacarcinogenesis. Studies on the chemical structures of DNAs modified with (acetylamino)-fluorene, benzopyrene, 4-nitroquinoline *N*-oxide, and aflatoxin B_1 have been pioneering works in this field.[6] In particular, much attention has been paid to benzopyrene.[7] A very recent report in this area concerning the synthesis of guanine–aflatoxin B_1 adducts appeared in this journal.[8] Since the fraction of modified nucleotides in DNA is quite small, studies encounter various difficulties, which could be overcome by improved techniques.

Since isolation and synthesis of the unique mutacarcinogenic heteroaromatic amines Trp-P-2 and Glu-P-1,[9] we have continued



Trp-P-2



Glu-P-1

studies on the initial process caused by mutacarcinogens. Recently, we reported the structures of the active form of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and of nucleic acid base modified by this compound.[10]

This paper reports the structure of the major modified nucleic acid base in DNA treated with 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1). Identification of the active form as a microsomal metabolite in vitro and chemical modification of DNA by this active form are also reported.

Results

Covalent Binding of Glu-P-1 with DNA. Glu-P-1 shows very high mutagenic activity toward *Salmonella typhimurium* TA 98 in the presence of microsomal proteins. Consistent with this fact, Glu-P-1 binds to DNA only in the presence of microsomes. This binding is covalent, since reprecipitation, gel filtration, or dialysis of the mutagen–DNA complex do not liberate free Glu-P-1. The amount of Glu-P-1 bound to DNA was enhanced by addition of NADPH to incubation mixture, although only to one molecule

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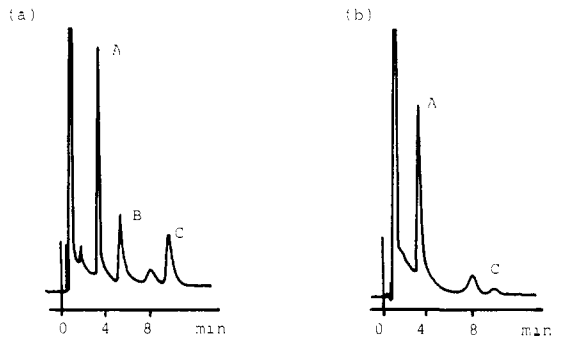
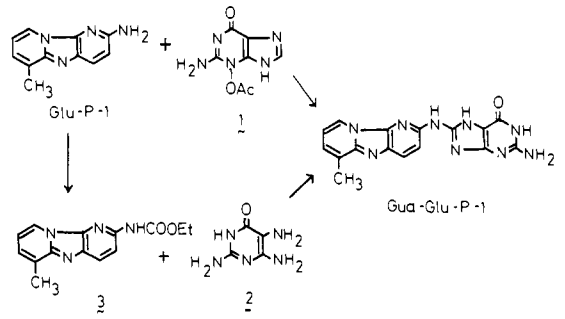


Figure 1. HPLC of the hydrolysis mixtures of the modified DNA. (a) DNA was modified with Glu-P-1 in vitro in the presence of microsomal proteins. (b) DNA was modified chemically with N-OAc-Glu-P-1. Column: Polygosil $_5C_{18}$, 4.6 mm i.d. × 250 mm. Solvent: H_2O–CH_3-OH– concentrated NH_4OH (59:40:1). Flow rate: 1.0 mL/min. Detection: absorbance 254 nm. Peak A, Gua-Glu-P-1; peak B and C, minor products (probably modified nucleic acid bases).

Scheme I

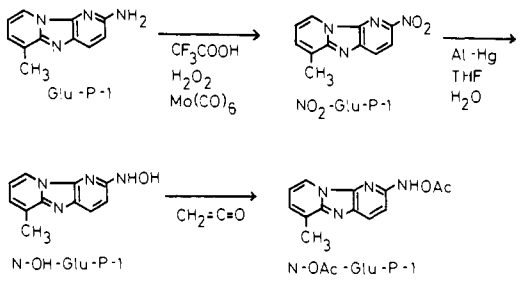


per about 5000 nucleotides in DNA even when the incubation was repeated 3 times.

Structure of the Modified Nucleic Acid Base. Modified nucleosides (and modified nucleic acid bases) were separated from normal nucleosides by Sephadex LH-20 column chromatography (MeOH–H_2O) after enzymatic hydrolysis. The fastest moving fraction was fluorescent, and therefore consisted of modified nucleosides, while the following fraction, partly superimposed on the fluorescent fraction, consisted of normal nucleosides. Fractions eluted with 60–100% MeOH were again fluorescent. The fastest moving fractions were lyophilized and hydrolyzed with 0.05 N HCl at room temperature to give a similar mixture to the last fluorescent fractions. Therefore, the last fractions were nucleic acid bases modified by Glu-P-1. Later, we found that hydrolysis to bases under very mild conditions may be interpreted by the lability of the glycosidyl bond of the modified DNA or the modified nucleotide. Analysis of the modified nucleic acid base mixture by high-performance liquid chromatography (HPLC) showed that there were three modified nucleic acid bases (Figure 1a, peak A, B, and C), and the main component (peak A in Figure 1a) was isolated in a chromatographically pure state.

Hydrolysis of the purified peak fraction with 1 N NaOH at 100 °C for 3 h gave Glu-P-1 and uric acid in good yield.[11] These results suggested that the major modified nucleic acid base was a Glu-P-1–guanine adduct whose binding sites were position 8 of guanine and a heteroatom of Glu-P-1. Therefore, we synthesized Gua-Glu-P-1, whose binding sites are position 8 of guanine and the nitrogen atom of the 3-amino group of Glu-P-1, for the first time, as the most plausible compound. Gua-Glu-P-1 was prepared in good yield by nucleophilic substitution of 3-acetoxyguanine (**1** Scheme I) at position 8[12] with Glu-P-1. This synthetic method has been applied in syntheses of nucleic acid bases modified with Trp-P-2,[10a] naphthylamine,[13] and (dimethylamino)azobenzene.[14] The structure of the synthetic Gua-Glu-P-1 was deduced from the following lines of evidence: (1) Its ^1H NMR spectrum showed no signal of a proton at position 8 of guanine, and all other protons bound to carbons could be assigned completely. (2) Its ^{13}C NMR spectrum showed 16 signals for carbon (3) On mass spectroscopy it gave M^+ 347 (Glu-P-1–guanine adduct), *m/e* 198 (Glu-P-1), and *m/e* 151 (guanine). (4) Hydrolysis of Gua-Glu-P-1 with 1 N NaOH at 100 °C for 3 h gave Glu-P-1 and uric acid in good yield.

Scheme II



The binding site of the Glu-P-1 moiety is ambiguous (the binding site could be another nitrogen atom). A methylation–hydrolysis experiment to determine the binding site of the Glu-P-1 moiety was unsuccessful. We tried an alternative method of synthesis of Gua-Glu-P-1: synthesis of the compound in 20% yield by ring condensation of 2,4,5-triamino-6-hydroxypyrimidine (**2**) with the N^3-carboethoxy derivative (**3**) of Glu-P-1 at the melting temperature. The binding site of the Glu-P-1 moiety of Gua-Glu-P-1 synthesized by this method was definitely the nitrogen atom at position 3 of Glu-P-1, because the structure of the intermediate, the N^3-carboethoxy derivative (**3**) of Glu-P-1, has been established by X-ray analysis.[15]

The major nucleic acid base modified by Glu-P-1 in vitro was identified with authentic Gua-Glu-P-1 as follows: (1) Its retention times in two HPLC systems were same as those of authentic Gua-Glu-P 1. (2) The UV spectra and fluorescence spectra of the modified nucleic acid base in neutral, acidic, and basic conditions were the same as those of authentic Gua-Glu-P-1. (3) Both the modified nucleic acid base and authentic Gua-Glu-P-1 gave Glu-P-1 and uric acid in good yield when treated with 1 N NaOH at 100 °C for 3 h. These results show that the nucleic acid base modified with Glu-P-1 in vitro is Gua-Glu-P-1.[16]

Synthesis and Identification of N-OH-Glu-P-1. The structure of the modified nucleic acid base Gua-Glu-P-1 suggests that the metabolically activated form of Glu-P-1 is the corresponding hydroxylamine, N-OH-Glu-P-1, because N-OH-Glu-P-1 could attack the C^8 atom of guanine in DNA electrophilically. We thought that N-OH-Glu-P-1 is probably not stable enough to be isolated from the incubation mixture because it is a 2-(hydroxyamino)pyridine derivative. Therefore, we investigated the synthesis of N-OH-Glu-P-1. Synthesis of N-OH-Glu-P-1 was achieved by partial reduction of the nitro derivative of Glu-P-1 (NO_2-Glu-P-1), which was obtained by oxidation of Glu-P-1. Oxidation of Glu-P-1 was achieved in good yield in a H_2O_2–CF_3COOH–$Mo(CO)_6$ system.[17] The NO_2-Glu-P-1 obtained was stable (Scheme II).

Partial reduction of NO_2-Glu-P-1 to N-OH-Glu-P-1 was difficult: Many reduction conditions, including catalytic hydrogenation or treatment with phenylhydrazine, ascorbic acid with

(11) Compounds were identified by comparing their retention times and UV spectra with those of authentic samples. Yields were calculated from the peak heights on HPLC.

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(17) Oxidation with MCPBA, Caro's acid, or *t*-BuOOH was not successful, yielding the azoxy derivative as the major product. Addition of Na_2WO_4 or $VO(acac)_2$ in place of $Mo(CO)_6$ did not increase the yield of NO_2-Glu-P-1.

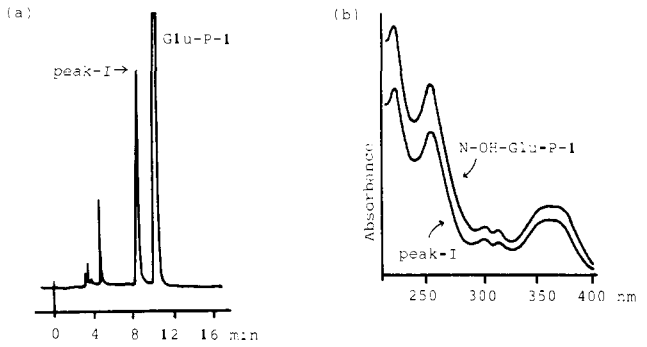


Figure 2. Identification of microsomal metabolities of Glu-P-1: (a) HPLC of microsomal metabolites of Glu-P-1. Column: Zorbax SIL 4.6 mm i.d. × 250 mm. Solvent: CH_2Cl_2–CH_3CN–MeOH (75:20:5). Flow rate: 1.0 mL/min. Detection: absorbance 254 nm. (b) UV spectra of peak I and synthetic N-OH-Glu-P-1.

base, or Zn dust–NH_4Cl, reduced N-OH-Glu-P-1 faster than NO_2-Glu-P-1. However, on reduction with aluminum amalgam at low temperature, NO_2-Glu-P-1 gave the expected N-OH-Glu-P-1. N-OH-Glu-P-1 could be recrystallized and gave correct analytical values, though it was unstable with a half-life of 1–2 h. The structure of N-OH-Glu-P-1 was deduced from its ^1H NMR spectrum (complete assignment of all hydrogens on carbon atoms), mass spectrum (M^+ 214), UV spectrum (similar to that of Glu-P-1), elemental analysis, and its further reduction to Glu-P-1. In addition, N-OH-Glu-P-1 reacted with nitrosobenzene in THF to give the phenylazoxy derivative in 74% yield, confirming its structure.

On analysis of the incubation mixture of Glu-P-1 (HBr salt) in the presence of microsomal proteins, only one peak of metabolite was observed (Figure 2, peak I). This peak was smaller when the incubation time or workup time was longer, and was increased by addition of NADPH to the incubation mixture. This major metabolite of Glu-P-1 was identified with synthetic N-OH-Glu-P-1 by comparison of their retention times on HPLC and UV spectra (Figure 2). Addition of nitrosobenzene to the metabolite mixture caused disappearance of peak I and formation of another major peak, which was identified as that of the phenylazoxy derivative of N-OH-Glu-P-1.[11] These results suggest that Glu-P-1 was converted metabolically to the corresponding hydroxylamine, N-OH-Glu-P-1. About 25% of the substrate, Glu-P-1, was metabolized to N-OH-Glu-P-1 under the conditions used. The other peaks observed when the incubation time or workup time was longer were probably those of decomposition products of N-OH-Glu-P-1 or of secondary products formed metabolically from N-OH-Glu-P-1.

Reaction of N-OH-Glu-P-1. N-OH-Glu-P-1 itself did not react with DNA under neutral or acidic conditions. However, when N-OH-Glu-P-1 was acetylated with ketene, the resulting N-OAc-Glu-P-1 bound to DNA effectively at 0 °C. The amount of Glu-P-1 bound to DNA was about one molecule per 300–400 nucleotides, as determined from the UV or fluorescence spectrum of the modified DNA. The N-OAc-Glu-P-1 could not be isolated because of its high reactivity. When the acetylated mixture of N-OH-Glu-P-1 was reduced by Raney nickel, Glu-P-1 was obtained in 40% yield as the major product, but N-acetyl-Glu-P-1 was not detected. This suggests that the acetylation of N-OH-Glu-P-1 with ketene in THF at low temperature occurred at the oxygen atom of the compound. Preferential acetylation at the oxygen atom rather than the nitrogen atom of the aromatic hydroxylamine might be partly attributed to the contribution of the oxime tautomer of N-OH-Glu-P-1 in the solution.

Acid hydrolysis of the modified DNA with N-OAc-Glu-P-1 gave Gua-Glu-P-1 as the major modified nucleic acid base (Figure 1b, peak A).[11] The amount of Gua-Glu-P-1 thus obtained accounts for almost all the covalent binding of N-OAc-Glu-P-1 to DNA. Therefore, N-OAc-Glu-P-1 is an appropriate model of the activated form of Glu-P-1 in vitro and in vivo (vide infra): The modified base, Gua-Glu-P-1, is probably formed from the

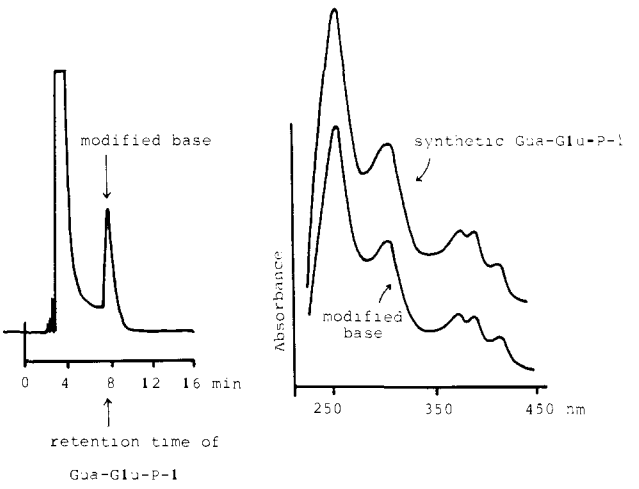


Figure 3. HPLC and UV spectra of Gua-Glu-P-1 liberated from Liver DNA of Rats Treated with Glu-P-1. (a) HPLC of Gua-Glu-P-1 liberated from liver DNA of rats treated with Glu-P-1. Column: Polygosil $_5C_{18}$, 4.6 mm i.d. × 250 mm. Solvent: H_2O–CH_3OH–concentrated NH_4OH (59:40:1). Flow rate: 1.0 mL/min. Detection: absorbance 254 nm. (b) UV spectra of the modified nucleic acid base isolated from liver DNA of the rats treated with Glu-P-1 and synthetic Gua-Glu-P-1. Measured in 47% HBr aqueous Me_2SO–H_2O (5:1:94).

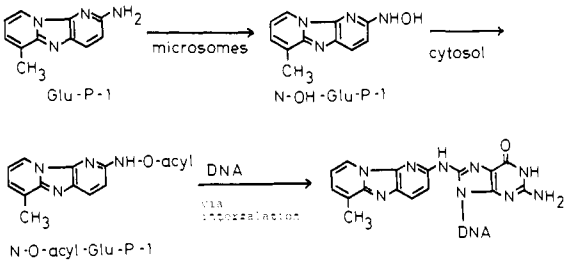
O-acyl (possibly the acetyl, aminoacyl, phosphoryl, or sulfate) derivative of the hydroxylamine. Hydroxylamine itself may not be the reactive form, even in vivo. Consistent with this idea, the mutagenic activity of N-OH-Glu-P-1 toward *Salmonella typhimurium* TA 98 was not strong, but addition of the cytosol fraction, which contains esterifying enzymes to the incubation mixture (without microsomes), greatly enhanced the mutagenicity.[18] Thus, it may be concluded that N-OH-Glu-P-1 is a proximate form of Glu-P-1 and that its ultimate form is an *O*-acyl derivative, which possesses enhanced reactivity for N–O bond heterolysis. The nucleophilic attack of guanine moieties in DNA on N-OAc-Glu-P-1 is probably an S_N2-like reaction, because disappearance of N-OAc-Glu-P-1 was much faster when DNA or strong nucleophiles were present.

Reaction of N-OAc-Glu-P-1 with Guanylyl(3′–5′)cytidine (GpC). The reactions of N-OAc-Glu-P-1 with other guanylic acid derivatives were also investigated. N-OAc-Glu-P-1 reacted with guanosine, guanylic acid, poly(G), and a mixture of guanylic acid and cytidylic acid to give Gua-Glu-P-1 only in low yields (0.01–0.02%). However, with guanylyl(3′–5′)cytidine (GpC), which is known to exist in equilibrium with a complementary dimer in solution that has similar cavity between the G–C pairs as the cavity formed in base pairs in DNA, N-OAc-Glu-P-1 reacted effectively to give Gua-Glu-P-1 (2.5%) after acid hydrolysis. These findings suggest that intercalation of N-OAc-Glu-P-1 of the ultimate form of Glu-P-1 into G–C base pairs precedes the covalent reaction of the activated compound with position 8 of guanine. The intercalation seems to be an important step in modification of DNA with Glu-P-1.

Modified Nucleic Acid Base in Vivo. As mentioned above, we elucidated the pathway of modification of DNA by Glu-P-1 in vitro and reproduced the reaction chemically by use of the model activated form. It is important to know whether the same modification of DNA occurs in vivo. Therefore, we injected Glu-P-1 (HBr salt) intraperitoneally into rats and extracted modified DNA from the liver. As described previously, the glycosidyl bond of Gua-Glu-P-1 in the modified DNA is so labile that only heating of the modified DNA in water causes quantitative liberation of Gua-Glu-P-1. This procedure made the isolation of Gua-Glu-P-1 from the modified DNA quite easy. The modified DNA extracted from the liver of the rats injected with Glu-P-1 was treated as described in the Experimental Section, and the modified nucleic acid base was analyzed by HPLC (Figure 3). The structure of

(18) M. Nagao, unpublished results.

Scheme III



the modified nucleic acid base liberated from the liver DNA of rats treated with Glu-P-1 was identified by comparison of its retention time on HPLC and UV spectrum with those of synthetic Gua-Glu-P-1 (Figure 3). The amounts of Glu-P-1 bound to nucleic acids were 2.5×10^{-5} mol/mol P of DNA and 3.0×10^{-6} mol/mol P of rRNA, determined from the peak heights on HPLC.

Discussion

The mutagenic Glu-P-1 binds covalently to DNA in vivo and in vitro. The digested mixture of the modified DNA was subjected to column chromatography, and the fluorescent fraction was separated by HPLC. The chemical behavior of the major modified nucleic acid base suggested that it is a guanine–Glu-P-1 adduct, bound through C^8 of the guanine and a nitrogen atom of the Glu-P-1. The same compound was synthesized unambiguously. The structure established suggested that the microsomal metabolite is a hydroxylamine derivative, which was identified with an authentic specimen. Some hydroxylamines bound to DNA under acidic conditions; naphthylhydroxylamines bound to DNA in slightly acidic conditions.[19] N-OH-Trp-P-2 also bound to DNA, though the binding was not effective.[10b] N-OH-Glu-P-1, however, did not bind to DNA in neutral or acidic conditions. O-Acetylation, which enhances the heterolytic cleavage of the N–O bond, facilitated the binding. Similar observations were made on the reactions of *N*-(hydroxyacetylamino)fluorene (requiring O-sulfonation)[20] and 4-(hydroxyamino)quinoline-*N*-oxide (requiring aminoacylation).[21]

Binding between the amino nitrogen atom and the C^8 position of guanine seems to be a general mode of reaction of carcinogenic aromatic amines. (Acetylamino)fluorene[20] and (dimethylamino)azobenzene[14] bind to DNA in a similar fashion. Trp-P-2 also binds at the C^8 position.[10a] The nucleic acid bases modified by the supposed active forms of 1-naphthylamine and 2-naphthylamine, 1-(hydroxyamino)naphthalene and 2-(hydroxyamino)naphthalene, respectively, are also C^8–N adducts.[13] Recently, 2-(hydroxyamino)quinoline *N*-oxide was reported to react at the C^8 posittion of guanine.[22] Thus, examples of C^8–N binding are accumulating, suggesting that this modification is very important in chemical carcinogenesis.

In vivo modification and chemical modification by N-OAc-Glu-P-1 yielded Gua-Glu-P-1 as a major product. In in vitro experiments, three products were obtained. No information on the structures of the other two (not necessarily modified nucleic acid base) has been obtained, though one of them (peak C in Figure 1) was found as a minor product on chemical modification by N-OAc-Glu-P-1.

The importance of intercalation was shown in the reaction of N-OAc-Glu-P-1 with GpC. The intercalation of the parent Glu-P-1 and its derivatives into DNA has been shown by the fluorescence and UV spectra, flow dichroism, and unwinding of circular DNA.[23] The conformation of the intercalated complex must be an important factor in determining the distance between reaction sites and the course of the reaction with nucleic acid bases in DNA. We are now studying the energy potential of GpC–Glu-P-1 interactions.

From the results obtained in this study, the overall pathway of modification of DNA by Glu-P-1 in vivo and in vitro was established to be as shown in Scheme III.

Conclusion

The initial process of modification of DNA with mutacarcinogenic Glu-P-1 was established. Glu-P-1 was metabolically activated to the corresponding hydroxylamine, N-OH-Glu-P-1, by microsomes. N-OH-Glu-P-1 is a proximate form of Glu-P-1, because it does not react with DNA. The hydroxylamine is further activated chemically by acetylation, or by O-acylation with cytosol enzymes, and the resulting N-O-acyl-Glu-P-1 reacts effectively with DNA. Consequently, N-O-acyl-Glu-P-1 is an ultimate form of Glu-P-1 that reacts with DNA at the C^8 position of guanine. Intercalation of the ultimate form of Glu-P-1 into DNA is suggested to be the important step in modification of DNA.

Experimental Section

Materials. Calf thymus DNA (type I, sodium salt), DNase (type I, from bovine pancreas), RNase (type I-A, from bovine pancreas), phosphodiesterase I (type III, from *Crotalus adamanteus* venom), alkaline phosphatase I (type I, from bovine intestine), glucose 6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P-D), NADPH, and GpC (ammonium salt) were purchased from Sigma Co. Male Wistar rats (150 g) were purchased from Nippon Seibutsu Zairyo Center. Glu-P-1 was prepared by our method described previously.[9b] Rat liver microsomes were prepared by the method of Kinoshita et al.[24] from rats treated with polychlorinated biphenyls (KC-300) as described previously.[25]

Methods. High-Performance Liquid Chromatography (HPLC). A Shimadzu LC-2 apparatus equipped with an SPD-1 UV spectrophotometer as a detector and a Polygosil $_5C_{18}$ column (35% MeOH–1% NH_4OH–H_2O) or a Zorbax SIL column (CH_2Cl_2–CH_3CN–MeOH, 75:20:5) was used for analysis. UV spectra were obtained by the stop-flow method in an SPD-1 apparatus.

Isolation of Modified Nucleic Acid Base in Vitro. Glu-P-1 bound DNA was obtained by thrre repeated incubations at 37 °C for 30 min of DNA (1 g) with Glu-P-1 (50 mg, HBr salt) in the presence of rat liver microsomes (300 mg protein) in bis(2-hydroxyethyl)-Tris–HCl buffer (bisTris, 0.1 M, 500 mL, pH 7.5).[26] The incubation mixture also contained NADPH (0.5 mmol), G-6-P (5 mmol), G-6-P-D (100 units), $MgCl_2$ (1.5 mmol), Na_2SO_4 (1.5 mmol), and EDTA (50 μmol) in 500 mL. To the mixture 500 mL of phenol was added, and to the aqueous phase 1 L of cold EtOH was added. The resulting precipitate of modified DNA was collected by centrifugation. The amount of Glu-P-1 bound to DNA was roughly one molecule per 5000 nucleotides, estimated from the intensity of fluorescence of the modified DNA (excitation at 360 nm, emission at 450 nm). The Glu-P-1 bound DNA (850 mg) thus obtained was dissolved in 1 L of 0.01 M Tris (pH 7.5) containing $MgCl_2$ (0.01 M) and hydrolyzed in the usual way with 26×10^4 K units of DNase at 37 °C for 6 h. To this mixture were added 1 L of 0.1 M Tris (pH 9.0) and 100 units of phosphodiesterase, and the mixture was incubated at 37 °C for 50 h. Then 2500 units of alkaline phosphatase was added and the mixture incubated at 37 °C for 50 h. The hydrolysate was lyophilyzed, applied to a Sephadex LH-20 column (2.0 $\phi \times 80$ cm), and eluted with a stepwise gradient of H_2O–MeOH. The modified bases were separated and purified by HPLC (Polygosil $_5C_{18}$, 35% MeOH–1% NH_4OH–H_2O). About 1 μg of the major modified nucleic acid base (Gua-Glu-P-1) was obtained.

2-(8-Guanylyl)amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Gua-Glu-P-1): **A.** One gram of guanine N^3-oxide[12] was suspended in a mixture of Me_2SO and DMF (150–50 mL). To the suspension 0.6 mL (6 equiv) of $(CH_3CO)_2O$ was added dropwise at 0 °C. The mixture was stirred vigorously at room temperature for 10 min. To the resulting brown mixture 200 mg (1 equiv) of Glu-P-1 was added, and the mixture was stirred at room temperature for 12 h. Then the mixture was concentrated to about 10 mL under reduced pressure at 70–80 °C, and 100 mL of H_2O was added. The resulting brown precipitate was collected by centrifugation and dried. The residue was dissolved in a minimum

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amount of dry Me_2SO and purified by HPLC (Polygosil $_5C_{18}$, 45% Me_2SO–1% NH_4OH–H_2O). The fraction containing Gua-Glu-P-1 was collected and evaporated under reduced pressure at 70–80 °C, and then the residue was reprecipitated from 100 mL of MeOH containing 50 mL of 47% HBr, to yield 247 mg of Gua-Glu-P-1 (57%) as the HBr salt: mp, >300 °C; M^+ 347; NMR (Me_2SO-d_6) 2.75 (s, 3 H, 6-CH_3), 7.70 (d, J = 8 Hz, 3-H), 7.72 (t, J = 6 Hz, 8-H), 8.15 (d, J = 6 Hz, 7-H), 8.45 (d, J = 8 Hz, 4-H), 9.45 (d, J = 6 Hz, 9-H), 11.82 (br s); UV λ_{max}, nm, (ϵ) at pH 1, 237 (2.3 × 10^4), 302 (1.2 × 10^4), 360 (1.0 × 10^4); at pH 7, 260 (2.3 × 10^4), 300 (1.3 × 10^4), 323 (0.5 × 10^4), 360 (0.5 × 10^4), 372 (0.6 × 10^4), 392 (0.5 × 10^4).

B. Glu-P-1 (300 mg) was dissolved in dry pyridine (10 mL), and $ClCOOC_2H_5$ (1 mL) was added to the solution. The mixture was stirred at room temperature for 5 min and evaporated under reduced pressure. The residue was washed with water and the precipitate was recrystallized from benzene–hexane to give the *N*-carboethoxy derivative (**3**, 300 mg, 73%), mp 136–137 °C, M^+ 270, correctly analyzed.[15] The 50 mg of finely powdered **3** thus obtained was mixed with finely powdered 2,4,5-triamino-6-hydroxypyrimidine (**2**, 100 mg) with grinding. The mixture was heated at 150–180 °C for 1 h to allow it to melt. The mixture was cooled and washed with $CHCl_3$ to remove the unreacted **3**. The residue was dissolved in a minimum amount of dry Me_2SO and purified as described in method A. The 35 mg of Gua-Glu-P-1 obtained in 20% yield was identified by comparison of its IR spectrum with that of the compound prepared by method A.

2-Nitro-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (NO_2-Glu-P-1). To a mixture of 100 g of $(CF_3CO)_2O$ and 10 mL of CF_3COOH was added 20 mL of 30% H_2O_2 dropwise under ice-cold conditions. To the mixture was added in small portions at 0 °C[27] 2 g of finely powdered $Mo(CO)_6$. Then a solution of Glu-P-1 (3 g) in 250 mL of CH_2Cl_2 and 10 ml of CF_3COOH was added dropwise over more than 20 min with stirring at 0 °C. The mixture was then warmed to room temperature and stirred for 5 min. To the resulting mixture was added concentrated K_2CO_3 to make the mixture basic, and then the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was separated and purified by silica gel column chromatography (AcOEt). Fractions containing NO_2-Glu-P-1 were combined, evaporated, and recrystalyzed from AcOEt to give 2.5 g of NO_2-Glu-P-1 (73%) as yellow plates: mp 221–224 °C; M^+ 228; NMR ($CDCl_3$) δ 2.72 (s, 3 H, 6-CH_3), 7.02 (t, J = 8 Hz, 8-H), 7.52 (d, J = 8 Hz, 7-H), 8.84 (d, J = 8 Hz, 9-H), 8.40 and 8.54 (d, J = 8 Hz, 3-H and 4-H); UV (MeOH) λmax nm 205, 220, 280, 290; IR (KBr) 1530 cm^{-1}. Anal. Found: C, 57.86; H, 3.51; N, 24.56. Calcd: C, 57.82; H, 3.49; N, 24.45.

2-(Hydroxyamino)-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (N-OH-Glu-P-1). NO_2-Glu-P-1 (100 mg) was dissolved in THF (70 mL), and 1 mL of H_2O was added. To the mixture, Al–Hg, prepared from 700 mg of aluminum foil by the usual method,[28] was added at 0 °C, with vigorous shaking for 5 min. Then Na_2SO_4 was added and the mixture was filtered. The filtrate was evaporated at 10 °C under reduced pressure and the residue was washed with CH_2Cl_2. Then MeOH was added and insoluble material was removed by filtration. The filtrate was again evaporated at 10 °C under reduced pressure to yield 75 mg of N-OH-Glu-P-1 (purity 95%, yield 40%) as a yellow powder. The powder was suspended in CH_2Cl_2 and dissolved by addition of a minimum amount of MeOH at room temperature. Addition of *n*-hexane gave pure N-OH-Glu-P-1 as yellow needles: mp 175–185 °C dec; M^+ 214; NMR (CD_3OD) 2.59 (s, 3H; 6-CH_3), 6.91 (t, J = 7 Hz, 8-H), 7.22 (d, J = 8 Hz, 3-H), 7.33 (d, J = 7 Hz, 7-H), 8.08 (d, J = 8 Hz, 4-H), 8.62 (d, J = 7 Hz, 9-H); UV (MeOH) λmax, nm, (ϵ) 213 (2.1 × 10^4), 255 (2.0 × 10^4), 301 (sh), 312 (0.7 × 10^4), 365 (0.9 × 10^4). Anal. Found: C, 61.96; H, 4.73; N, 25.99. Calcd: C, 61.68; H, 4.71; N, 26.16.

2-Phenyl(*N*,*N*,*O*)azoxy-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Phenylazoxy Derivative of Glu-P-1). To a solution of N-OH-Glu-P-1 (20 mg) in THF (10 mL) were added CH_3COOH (0.2 mL) and nitrosobenzene (100 mg). After 2 h at room temperature, the solution was evaporated and separated by silica gel column chromatography to give the title compound as a yellow powder (21 mg, 74%): mp 148–150 °C; M^+ 303; NMR ($CDCl_3$) 2.76 (s, 3H, 6-CH_3), 7.08 (t, J = 8 Hz, 8-H), 7.55 (d, J = 8 Hz, 7-H), 7.50–7.65 and 8.30–8.45 (m, 5H, phenyl), 8.42 (d, J = 8 Hz, 4-H), 8.76 (d, J = 8 Hz, 3-H), 9.00 (d, J = 8 Hz, 9-H).

Metabolic Activation of Glu-P-1 in Vitro. Rat liver microsomes were prepared as described above. Glu-P-1 (HBr salt, 3 mg) was dissolved in 10 mL of 0.05 M bis Tris (pH 7.5) containing NADPH (10 mg) and microsomal protein (60 mg). The mixture was incubated at 37 °C for 10 min and then extracted with cold AcOEt (10 mL). The organic layer was evaporated at below 0 °C under reduced pressure. The residue was dissolved in a known amount of THF and analyzed by HPLC (Zorbax SIL, CH_2Cl_2–CH_3CN–MeOH 75:20:5, Figure 2). Longer incubation made the metabolite mixture more complex.

Reaction of N-OH-Glu-P-1 with DNA after O-Acetylation. N-OH-Glu-P-1 (30 mg) was dissolved in THF–MeOH (20 mL:1 mL). When ketene gas was bubbled through it at 0 °C for 6–8 min, the spot of the starting material on the silica gel plate (AcOEt) disappeared. The resulting solution of 2-(acetoxyamino)-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole (N-OAc-Glu-P-1) was added dropwise to an aqueous solution of DNA (30 mg/15 mL) at 0 °C. Then the mixture was warmed to room temperature and stirred for 15 min. Then 50 mL of cold EtOH and 0.1 mL of saturated NaCl were added to precipitate the modified DNA, which was collected by centrifugation. The modified DNA was purified by reprecipitation from aqueous EtOH or gel filtration column chromatography on Sephadex G-50. The amount of Glu-P-1 bound to DNA was about one molecule per 300–400 nucleotides, estimated from its UV spectrum (ratio of absorbance 254 nm to that of 350 nm). The modified DNA was dissolved in 10 mL of H_2O, and 10 mL of CF_3CO-OH was added to hydrolyze the former at 100 °C for 1 h. The hydrolysate was analyzed by HPLC (Figure 1-b). The reactions of N-OAc-Glu-P-1 with guanosine, guanylic acid, poly(G) and GpC were performed similarly, except that the modified nucleotides were not purified before hydrolysis.

Modification of DNA in Vivo. Glu-P-1 (HBr salt) suspended in corn oil–Me_2SO mixture was injected intraperitoneally into male Wistar rats (150 g, 25 mg/kg).[29] The animals were killed 6 h later and their liver was removed. The livers were homogenized and nucleic acids were extracted by Kirby's PAS–phenol method.[30] The DNA fraction and the rRNA fraction were treated with RNase and DNase, respectively. The purities of these fractions were checked by measuring their UV spectra. The yields of DNA and rRNA from 10 g of liver were about 10 and 50 mg, respectively. The modified nucleic acids extracted from the liver were dissolved in dilute CH_3COOH (pH 4) and heated at 100 °C for 1 h. This treatment results quantitative liberation of Gua-Glu-P-1.[19] The mixtures were cooled, an equal volume of cold EtOH was added, and precipitated nucleic acids were removed by centrifugation. The supernatant was concentrated under reduced pressure, the residue was dissolved in a known amount of 10% CF_3COOH–Me_2SO and analyzed by HPLC, and the modified nucleic acid base, Gua-Glu-P-1, was purified. The amounts of Glu-P-1 bound to DNA and rRNA were about one molecule per 2.5 × 10^4 and 3.0 × 10^5 nucleotides, respectively, estimated from the peak heights of Gua-Glu-P-1 on HPLC (Figure 3).

Registry No. **1**, 72553-23-2; **2**, 1004-75-7; **3**, 83692-81-3; Glu-P-1, 67730-11-4; Gua-Glu-P-1, 72553-24-3; NO_2-Glu-P-1, 83692-82-4; N-OH-Glu-P-1, 73341-53-4; Glu-P-1 (phenylazoxy derivative), 83692-83-5; N-OAc-Glu-P-1, 76206-39-8; guanine N^3-oxide, 18905-29-8.

(27) **Caution**: Dangerous if not cooled.
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