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Deoxyribonucleic Acid Modification by Mutagenic 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole: The Chemical Events

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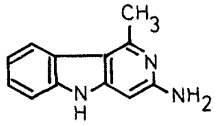
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) is a mutagen/carcinogen isolated from a pyrolysate of L-tryptophan. The active metabolite of Trp-P-2, 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (N-OH-Trp-P-2), was synthesized and the chemical reactions of N-OH-Trp-P-2 with deoxyribonucleic acid were investigated. The structure of the nucleic acid base covalently bound with Trp-P-2 was elucidated as 3-(C^8-guanyl)amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Gua-Trp-P-2) by comparison with a synthetic sample. The initial chemical events in carcinogenesis caused by Trp-P-2 were established.

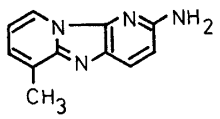
Keywords——3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2; DNA; mutagen; chemical carcinogenesis; DNA modification

Introduction

It is well established that some chemical carcinogens are metabolically activated to reactive forms (proximate or ultimate forms) which can be sources of electrophilic species and can react covalently with deoxyribonucleic acid (DNA).[1] The pathways of DNA modification by chemical carcinogens (for example, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole (Glu-P-1),[2] acetylaminofluorene,[3] dimethylaminoazobenzene,[4] benzo(*a*)pyrene,[5] 4-nitroquinoline-*N*-oxide,[6] naphthylamine,[7] *etc.*) have been well studied chemically. In recent years, the presence of mutagen/carcinogens in cooked foods has become a subject of concern, and some potent compounds have been isolated from amino acid pyrolysate or cooked foods (*e.g.*, from pyrolysates of L-tryptophan,[8] L-glutamic acid,[9] lysine,[10] ornithine,[11] broiled sardines,[12] soybean protein,[13] grilled beef,[14a] broiled cuttlefish,[14b] *etc.*). Most of these compounds have been characterized as heteroaromatic amines which possess an amino group at a position adjacent to the ring heteroatom.[15] We have been engaged in research on the initial event caused by these mutagens, especially by 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1).[2] Glu-P-1 is metabolically activated to the corresponding hydroxylamine, which is a proximate form of Glu-P-1, and is *O*-acylated in the cytosol to give the ultimate form of Glu-P-1.[2,16] This ultimate form of Glu-P-1 reacts with DNA *in vitro* to give 2-(C^8-guanyl)amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole after hydrolysis of the modified DNA.[2,17] The same modified nucleic acid base was isolated from liver DNA of rats treated with Glu-P-1.[2,18] Another important mutagen/carcinogen is 3-amino-1-methyl-5*H*-pyrido[4,-3-*b*]indole (Trp-P-2) isolated from a pyrolysate of tryptophan.[8] This compound shows extremely high mutagenicity toward *Salmonella typhimurium* TA98 in the presence of metabolizing systems (S-9 mix.), and is hepatocarcinogenic.[19] Recently, we established the binding of Trp-P-2 to nucleic acids in the presence of rat liver microsomal proteins.[20] Binding experiments using homopolymers suggested that metabolically activated Trp-P-2 attacks the guanine moiety in nucleic acids.[20] In this paper, we describe the path of DNA modification by Trp-P-2, that is, metabolic activation and identification of the active metabolite of Trp-P-2,[21] and the structural identification of the nucleic acid base modified with Trp-P-2.[22] We also describe the DNA modification in rats.[18]



Trp-P-2



Glu-P-1

Fig. 1. Structures of Trp-P-2 and Glu-P-1

Materials and Methods

Materials

Calf thymus DNA (type I, sodium salt), deoxyribonuclease (DNase, type I, from bovine pancreas), ribonuclease (RNase, type I-A, from bovine pancreas), phosphodiesterase I (type III, from *Crotalus adamanteus* venom), alkaline phosphatase (type I, from bovine intestine), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P-D), nicotinamide adenine dinucleotide phosphate (NADPH) and guanyl (3′-5′)cytidine (ammonium salt, GpC) were purchased from Sigma Co. Male Wistar rats (150 g) were purchased from Nippon Seibutsu Zairyo Center. Rat liver microsomes were prepared by the method of Kinoshita *et al.*[23] from rats which had been treated with polychlorinated biphenyls (PCB, KC-300) as described previously.[24,25]

Methods

High Performance Liquid Chromatography (HPLC)——A Shimadzu LC-2 apparatus equipped with an SPD-1 ultraviolet (UV) spectrophotometer as a detector was used (column, elution solvent, detection, flow rate and other conditions of analysis are given in the figure legends). UV spectra were obtained by the stopped-flow method in the SPD-1 apparatus. The UV spectra thus obtained depend on the elution solvent used for HPLC and are not identical with those obtained by the standard method in MeOH using a Shimadzu UV-200S double-beam spectrophotometer.

Synthesis of N-OH-Trp-P-2——One gram of Trp-P-2 (CH_3COOH salt) was added in portions to 80 ml of 30% H_2O_2 containing $Na_2WO_4 \cdot 2H_2O$ (150 mg), and the mixture was stirred at room temperature for 12 h, then extracted with AcOEt. A small quantity of PtO_2 was added to the organic extract to decompose excess H_2O_2, and the extract was dried over Na_2SO_4. The mixture was filtered and then evaporated. The resulting residue was separated by silica gel column chromatography with a mixture of AcOEt–CH_2Cl_2–hexane (2 : 1 : 1). The major yellow fraction was evaporated and the residue was recrystallized from AcOEt to give 1-methyl-3-nitro-5*H*-pyrido[4,3-*b*]indole (NO_2-Trp-P-2, 91.5 mg) in a yield of 11%: mp 285—290 °C; M^+ 277; IR (KBr): 3150, 2360, 1500, 1350, 1300 cm^{-1}; UV (nm in MeOH): 235, 252, 280, 330. *Anal.* Calcd for $C_{12}H_9N_3O_2$: C, 63.42; H, 4.00; N, 18.50; Found: C, 63.44; H, 4.05; N, 18.45. When the oxidation was performed in CH_3CN, the yield was increased to about 60%. Ten milligrams of NO_2-Trp-P-2 was dissolved in 10 ml of tetrahydrofuran (THF). Aluminum amalgam (Al–Hg, prepared from 200 mg of aluminum foil by the usual method)[26] was added under ice cooling. The mixture was stirred vigorously for 3 min at 0 °C, then Na_2SO_4 was added. The mixture was filtered and dried under a vacuum at 0 °C. The residue was dissolved in CH_2Cl_2 containing 1% MeOH. Concentration by flushing with N_2 gas yielded a pale yellow precipitate of pure 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (N-OH-Trp-P-2, 1 mg) in a yield of 14%: mp 176—177 °C (dec.); M^+ 213; UV (nm in MeOH and molar extinction coefficient): 204 (3.1×10^4), 218 (sh), 237 (3.7×10^4), 256 (4.7×10^4), 288 (0.7×10^4), 300—320. N-OH-Trp-P-2 is an unstable compound having a half-life time of about 30 min in MeOH at room temperature, and has a weak fluorescence (less than one-tenth of that of Trp-P-2) and shows no fluorescence on silica gel thin-layer chromatography (TLC). N-OH-Trp-P-2 was converted to 1-methyl-3-(phenyl-*ONN*-azoxy)-5*H*-pyrido[4,3-*b*]indole easily by addition of nitrosobenzene in THF containing 1% CH_3COOH: mp 258—270 °C. *Anal.* Calcd for $C_{18}H_{14}N_4O$: C, 71.51; H, 4.66; N, 18.54; Found: C, 71.41; H, 4.67; N, 18.32 (Fig. 5).

Synthesis of Gua-Trp-P-2——Guanine-N^3-oxide[27] (250 mg) was suspended in a mixture of dimethylsulfoxide (DMSO) and dimethylformamide (DMF) (50 ml–50 ml), and $(CH_3CO)_2O$ (140 μl) was added under cooling. The mixture was stirred for 5 min, then 100 mg of Trp-P-2 (CH_3COOH salt) was added. The whole was stirred for 12 h at room temperature, then evaporated under a vacuum, and the residue was washed with water. The residue was separated by cellulose column chromatography (eluted with DMSO) or HPLC (LiChroprep RP 18, DMSO–H_2O–NH_4OH = 45 : 54 : 1). Chromatographically pure 3-(C^8-guanyl)amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Gua-Trp-P-2, 20 mg) was obtained in a yield of 15%: mp >300 °C; M^+ 346.1255 (Calcd for $C_{17}H_{14}N_8O$: 346.1288); IR (KBr): 3400, 1715, 1650, 1620, 1550, 1500 cm^{-1}; ^{1}H-NMR (DMSO-d_6): 3.02 (s, 3H), 7.39 (s, 1H), 8.12 (d, 1H), 7.3—7.6 (m, 3H), 12.60 (s, 1H); UV (nm, ε): pH 1: 256 (1.9×10^4), 280 (sh), 293 (3.0×10^4), 325 (2.0×10^4), 336 (2.1×10^4); pH 7: 245, 263 (2.5×10^4), 293—305 (*ca.* 1.1×10^4), 320 (1.3×10^4), 333 (1.7×10^4); pH 13: 243, 255, 292, 330 (Fig. 3).

Synthesis of 1,2,5-Trimethyl-3-oxo-3*H*-pyrido[4,3-*b*]indole——Trp-P-2 (CH_3COOH salt, 250 mg) was suspended in 100 ml of dry CH_3CN, and 150 mg of $NOBF_4$ (Aldrich) was added under ice cooling. The mixture was stirred for 10 min, and then a small portion of Cu powder was added. Stirring was continued for 30 min at room temperature and 50 ml of water was added. The reaction mixture was extracted with AcOEt and the extract was evaporated. The

residue was separated by silica gel column chromatography to give 100 mg (yield: 50%) of 3-acetylamino-1-methyl-5*H*-pyrido[4,3-*b*]indole. (This reaction pathway confirms the acetylation position of Trp-P-2.) The product was treated with $(CH_3O)_2SO_2$ (20 ml) at 100 °C for 1 h, and then evaporated to dryness under a vacuum. The residue was treated with 30 ml of 1 N NaOH under an N_2 atmosphere at 100 °C for 3 h. The mixture was evaporated and the residue was separated by silica gel column chromatography (AcOEt–MeOH) to give 1,2,5-trimethyl-3-oxo-3*H*-pyrido[4,3-*b*]indole (yield: 20%): M^+ 226.1103 (Calcd for $C_{14}H_{14}N_2O$: 226.1103); ^{1}H-NMR ($CDCl_3$): 2.84 (s, 3H), 3.00 (s, 3H), 3.80 (s, 3H); 6.24 (s, 1H), 7.2—7.5 (m, 3H), 7.93 (d, 1H). (Fig. 3).

Isolation of Modified Nucleic Acid Base Prepared *in Vitro*——Calf thymus DNA (1 g) was allowed to react with Trp-P-2 (CH_3COOH salt, 25 mg) in the presence of liver microsomes (300 mg protein) prepared from rats treated with PCB in a Bis-Tris buffer[28] (100 mM, pH 7.5, 500 ml) at 37 °C for 30 min. The incubation mixture contained $MgCl_2$ (1.5 mmol), Na_2SO_4 (1.5 mmol), ethylenediaminetetraacetic acid (EDTA, 50 μmol), G-6-P (5 mmol), G-6-P-D (100 units) and NADPH (0.5 mmol). One liter of phenol was added to the mixture. The aqueous phase was collected and 1 l of cold EtOH was added to precipitate the modified DNA. The modified DNA thus obtained was incubated twice more under the same conditions. After the final work-up, 850 mg of modified DNA was recovered. The amount of Trp-P-2 bound to DNA was 230 μmol/mol P, as estimated from the intensity of fluorescence (370 nm, excited at 315 nm). The modified DNA thus obtained was dissolved in 1 l of 0.01 M Tris buffer (pH 7.5) containing 0.01 M $MgCl_2$ and DNase I (26×10^4 units) was added. After 6 h at 37 °C, 1 l of 0.1 M Tris buffer (pH 9.0) and phosphodiesterase (100 units) were added and the mixture was incubated at 37 °C for 50 h. Then, 2500 units of alkaline phosphatase was added, and the mixture was incubated at 37 °C for 50 h. The mixture was lyophilized and the residue was separated by Sephadex LH 20 column chromatography (2 i.d. × 100 cm, eluted with MeOH–H_2O gradient). Fluorescent fractions (nucleosides modified with Trp-P-2 eluted after the normal nucleosides) were collected and lyophilized. The residue was treated with 0.05 N HCl to give a modified nucleic acid base mixture. The mixture was separated by HPLC (Fig. 2). The peak which is fluorescent and has absorbance at 310 nm (characteristic for Trp-P-2 moiety), that is peak I in Fig. 2, was collected and lyophilized. The modified base (Gua-Trp-P-2, *ca.* 1 μg) was obtained as a chromatographically pure compound.

Metabolite of Trp-P-2——Trp-P-2 (CH_3COOH salt, 2.0 mg) was dissolved in 0.05 M Bis-Tris buffer (pH 7.5, 10.0 ml), and NADPH (15.0 mg), G-6-P (30.0 mg), G-6-P-D (1.0 unit) and rat liver microsomes (80 mg -protein) were added. The mixture was incubated at 37 °C for 10 min, and then cooled in an ice bath (0 °C). Then, ice-cold acetone (10 ml) was added and the whole was centrifuged. The supernatant was deaerated by passing N_2 gas through it under ice cooling, and then analyzed by HPLC (Fig. 6).

Reaction of N-OH-Trp-P-2 with Nucleic Acid——a): N-OH-Trp-P-2 (30 mg) was dissolved in THF (30 ml) under ice cooling, then $(CH_3CO)_2O$ (15 μl) was added to the mixture. After 50 s, a solution of calf thymus DNA (200 mg/100 ml 0.1 M citrate buffer, pH 7.0) was added. The mixture was allowed to react for 10 min at room temperature, then 700 ml of cold EtOH was added and the precipitated modified DNA was collected by centrifugation. The modified DNA was purified by reprecipitation from a mixture of EtOH–H_2O or by Sephadex G-50 gel column chromatography. The amount of the modified DNA thus obtained was 165 mg. The resulting modified DNA was dissolved in H_2O (20 ml) and CF_3COOH (20 ml) was added. The mixture was heated at 100 °C for 1 h, and evaporated under a vacuum. The residue was separated by HPLC. The amount of Trp-P-2 bound to DNA was 1.6 mmol/mol P. The reactions of N-OAc-Trp-P-2 with guanine, guanosine, guanylic acid, poly G and GpC were performed similarly. The reaction mixture was analyzed by HPLC after acid hydrolysis with aqueous CF_3COOH.

b): N-OH-Trp-P-2 (3 mg/ml THF) was added to an aqueous solution of calf thymus DNA (2 mg/ml 0.1 M phosphate buffer, pH 5.0) and the mixture was incubated for 2 h at room temperature. The work-up was performed as described in method a.

DNA Modification by Trp-P-2 *in Vivo*——Trp-P-2 (CH_3COOH salt, 25 mg/kg) suspended in corn oil (25 mg/ml) was injected into male Wistar rats (150 g) intraperitoneally. Some rats died from the toxic effect of Trp-P-2 within an hour. Two hours later, the liver was removed from the remaining rats and homogenized. Nucleic acids were extracted by Kirby's PAS–phenol method.[29] The DNA fraction and ribosomal ribonucleic acid (rRNA) fraction were treated with RNase and DNase, respectively. The yields of DNA and rRNA from 10 g of liver were about 10 and 50 mg, respectively. The nucleic acid thus obtained were dissolved in dilute CH_3COOH (pH 4) and heated at 100 °C for 1 h. This treatment results in quantitative liberation of Gua-Trp-P-2 from the modified DNA.[18] The mixture was cooled, an equal volume of cold EtOH was added, and the precipitated nucleic acids were removed by centrifugation. The supernatant was concentrated under a vacuum, and then analyzed by HPLC (Fig. 4).

Results and Discussion

Trp-P-2 bound to DNA only in the presence of microsomal protein. The amount of Trp-P-2 bound to DNA was estimated to be 230 μmol/mol P from the fluorescence spectrum of the modified DNA after the incubation had been repeated three times successively. The modified DNA was enzymatically hydrolyzed to nucleosides and separated by Sephadex LH-20 column

chromatography as described in Materials and Methods. Fluorescent fractions (excited at 315 nm, characteristic of the Trp-P-2 moiety) were eluted later than the nonfluorescent unmodified nucleosides with 5—20% methanol–water. The fluorescent fractions were hydrolyzed with dilute HCl to obtain the bases and analyzed by HPLC (Fig. 2). Three major peaks were observed. Since peak I in Fig. 2 was fluorescent (excited at 315 nm) and possessed absorption at 310 nm, which are characteristic of the Trp-P-2 moiety, this peak (peak I) was separated. The other peaks (peak II and III) were not fluorescent and showed UV spectra which do not resemble that of Trp-P-2. In addition, only the compound corresponding to peak I was isolated as a modified nucleic acid base from the liver DNA of rats treated with Trp-P-2 (*vide infra*). Therefore, peak II and III may represent decomposed materials or artifacts. Hydrolysis of peak I with 1 N NaOH at 100 °C for 1 h gave Trp-P-2, 8-hydroxyguanine and uric acid (Fig. 3), which were shown to be identical with authentic samples by comparison of their retention times on HPLC and their UV spectra. The result suggested that the modified nucleic acid base was Trp-P-2-guanine adduct and that the binding sites are the heteroatom of Trp-P-2 and position 8 of guanine. We synthesized 3-(C^8-guanyl)amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Gua-Trp-P-2) as the most plausible structure of the modified nucleic acid base (Fig. 3). The synthesis was performed by nucleophilic substitution by Trp-P-2 at position 8 of 3-acetoxyguanine.[27] The structure of the synthetic Gua-Trp-P-2 was proved

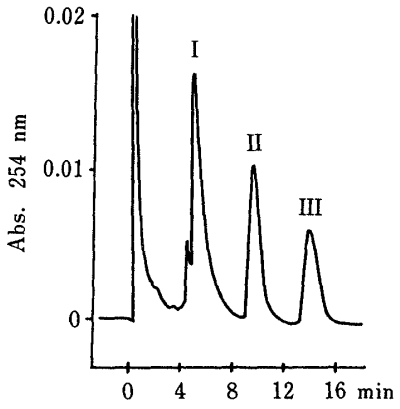


Fig. 2. HPLC of the Fraction Containing Trp-P-2 Bound to Nucleic Acid Base

Column: Zorbax ODS, 4.6 i.d. × 150 mm.
Solvent: MeOH–H_2O–NH_4OH (45 : 54 : 1).
Flow rate: 1.0 ml/min.
Detection: absorption at 254 nm.

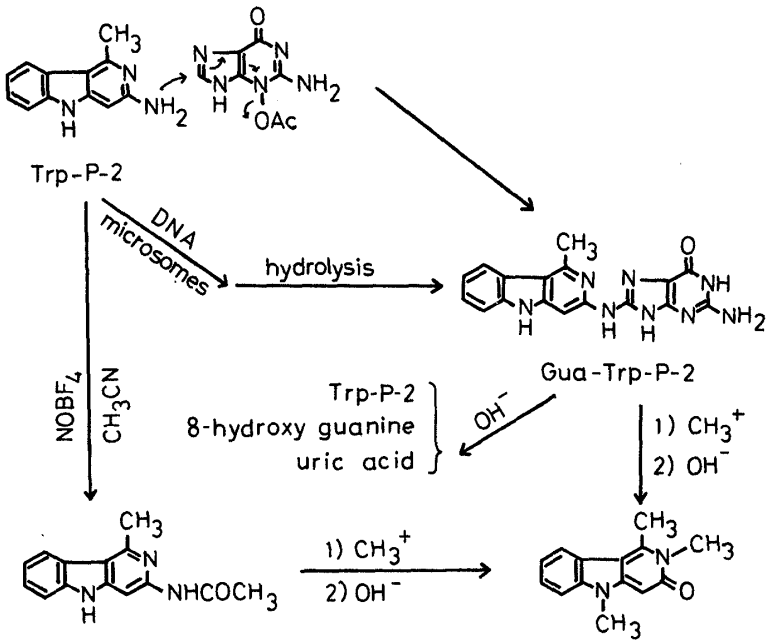


Fig. 3. Synthesis and Reactions of Gua-Trp-P-2

by its high resolution mass spectrum (MS) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra. Chemically, alkaline hydrolysis under degassed conditions gave Trp-P-2, 8-hydroxyguanine and uric acid. The binding site of the Trp-P-2 moiety was confirmed to be the 3-amino group by alkaline hydrolysis after methylation of synthetic Gua-Trp-P-2 with dimethylsulfate, giving 1,2,5-trimethyl-3-oxo-3*H*-pyrido[4,3-*b*]indole as the major product (Fig. 3). The modified nucleic acid base (peak I in Fig. 2) was concluded to be identical with synthetic Gua-Trp-P-2 on the basis of the following lines of evidence: (i) retention times (t_R) of peak I in various HPLC systems (columns: Zorbax ODS for reversed phase, Zorbax CN for reversed and normal phases, Zorbax NH_2 for normal phase)[22] were identical with those of synthetic Gua-Trp-P-2; (ii) fluorescence and UV spectra of peak I in neutral, acidic and basic conditions were identical with those of synthetic Gua-Trp-P-2;[22] (iii) dilute solutions of both peak I and synthetic Gua-Trp-P-2 were unstable under alkaline conditions in air, and the HPLC of their decomposed products gave the same chromatographic pattern. During the experiment, we found that the glycosidyl bond of the modified nucleoside or nucleotide is so labile that simple heating of the modified DNA or RNA in water causes quantitative liberation of Gua-Trp-P-2.

Biologically, it is important to know whether the same modified nucleic acid base is formed *in vivo*. We found that Trp-P-2 bound to rat liver DNA *in vivo* after intraperitoneal injection into rats (25 mg/kg). Trp-P-2 is toxic and some of the rats died within 1 h after the injection. However, modified DNA was extracted from the liver of the remaining rats. As mentioned above, heating of the modified nucleic acid causes quantitative liberation of the modified nucleic acid base. Using this method, we could separate the modified nucleic acid bases. The major modified nucleic acid base was identified by comparison of its retention time and UV spectrum with those of authentic Gua-Trp-P-2 (Fig. 4). The amounts of bound Trp-P-2 were 2.0×10^{-5} mol/mol P for DNA and 5.0×10^{-6} mol/mol P for rRNA, as determined from the peak heights of the modified base on HPLC.

The structure of the modified nucleic acid base (Gua-Trp-P-2) suggested that the metabolically activated form of Trp-P-2 is the corresponding hydroxylamine (N-OH-Trp-P-2), because N-OH-Trp-P-2 can act as an electrophile and can attack position 8 of guanine residues in DNA.[22] We expected N-OH-Trp-P-2 to be unstable because it is a derivative of 2-hydroxyaminopyridine, and in fact, the major metabolite of Trp-P-2 by rat liver microsomes

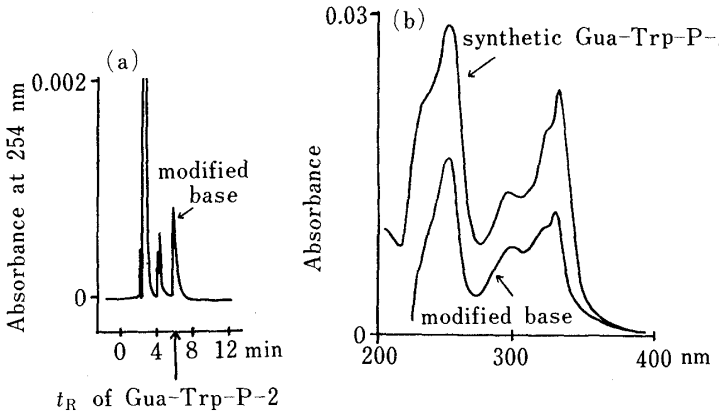


Fig. 4. HPLC (a) and UV Spectra (b) of Gua-Trp-P-2 Liberated from Liver DNA of Rats Treated with Trp-P-2

(a) Column: Polygosil $_5C_{18}$, 4.6 i.d. $\times$250 mm.
Solvent: MeOH–H_2O–NH_4OH (40:59:1).
Flow rate: 1.0 ml/min.
Detection: absorption at 254 nm.

(b) UV spectra were obtained by a stopped-flow method (SPD-1 apparatus) in MeOH–H_2O–NH_4OH (40:59:1).

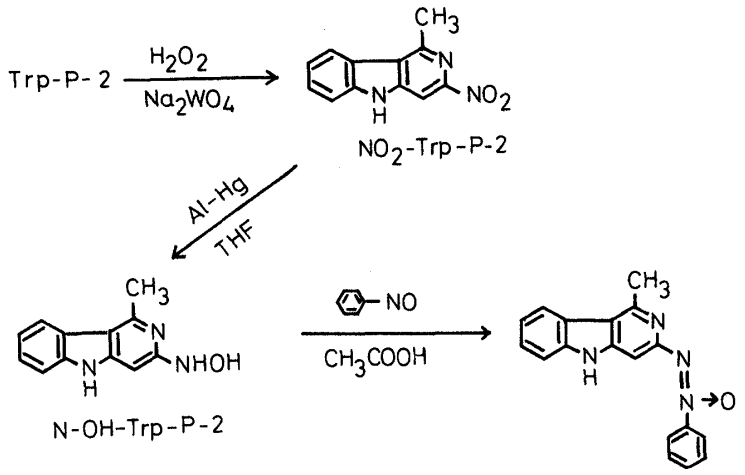


Fig. 5. Synthesis and Transformation of N-OH-Trp-P-2

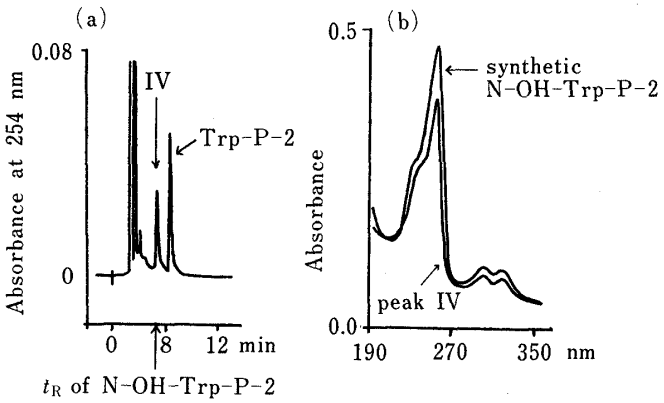


Fig. 6. HPLC of Metabolite Mixture Derived from Trp-P-2 (a) and UV Spectra of the Major Metabolite and Authentic N-OH-Trp-P-2 (b)

(a) Column: Polygosil $_5C_{18}$, 4.6 i.d. × 250 mm. Solvent: CH_3CN–0.02 M KH_2PO_4 (4:6). Flow rate: 0.7 ml/min. Detection: absorption at 254 nm.
(b) UV spectra were obtained by a stopped-flow method (SPD-1 apparatus) in CH_3CN–0.02 M KH_2PO_4 (4:6).

(peak IV in Fig. 6) was an unstable compound. However, we were able to synthesize N-OH-Trp-P-2 (Fig. 5). N-OH-Trp-P-2 was prepared by partial reduction of 1-methyl-3-nitro-5*H*-pyrido[4,3-*b*]indole (NO_2-Trp-P-2), which was itself prepared by oxidation of Trp-P-2 with aluminum amalgam in aqueous THF. The structure was confirmed by its mass spectrum, UV and ^{1}H-NMR spectra, further reduction to Trp-P-2, and derivatization by reaction with nitrosobenzene to a phenylazoxy derivative which gave the expected analytical values. Synthetic N-OH-Trp-P-2 was a crystalline compound, but was unstable in methanol solution with a half-life time of about 30 min at room temperature, and it has weak fluorescence compared to Trp-P-2. The major metabolite (peak IV in Fig. 6) was shown to be identical with synthetic N-OH-Trp-P-2 by comparison of their retention times on HPLC and their UV spectra (Fig. 6). The yield of this metabolite (N-OH-Trp-P-2) *in vitro* was fairly good (*ca.* 30%) when the work-up process was rapid and the metabolite solution was kept cool during the process. Usual work-up not only decreased the yield of N-OH-Trp-P-2, but also yielded many decomposed products of the initial metabolite. Therefore a longer incubation time did not increase the yield. This finding of N-OH-Trp-P-2 confirmed a previous suggestion regarding its formation from the base structure of DNA modified with Trp-P-2.[22] There are recent papers which have claimed that this compound is also a microsomal metabolite of Trp-P-2.[30]

Nonenzymatic binding of the metabolite (N-OH-Trp-P-2) to nucleic acids was examined. N-OH-Trp-P-2 did not bind to DNA under neutral conditions. However, it bound to DNA under slightly acidic conditions (pH 5, *O*-protonation of N-OH-Trp-P-2 makes the heterolytic cleavage of the N–O bond more facile) to give Gua-Trp-P-2 after hydrolysis of the modified

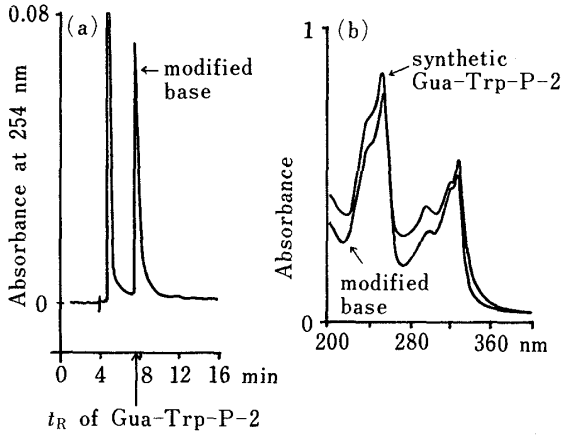


Fig. 7. HPLC of a Hydrolysate of DNA Modified with N-OAc-Trp-P-2 (a) and UV Spectra of the Major Modified Base and Authentic Gua-Trp-P-2 (b)

(a) Column: Polygosil $_5C_{18}$, 4.6 i.d. × 250 mm. Solvent: MeOH–H_2O–NH_4OH (45 : 54 : 1). Flow rate: 1.0 ml/min. Detection: absorption at 254 nm.
(b) UV spectra were obtained by a stopped-flow method (SPD-1 apparatus) in MeOH–H_2O–NH_4OH (45 : 54 : 1).

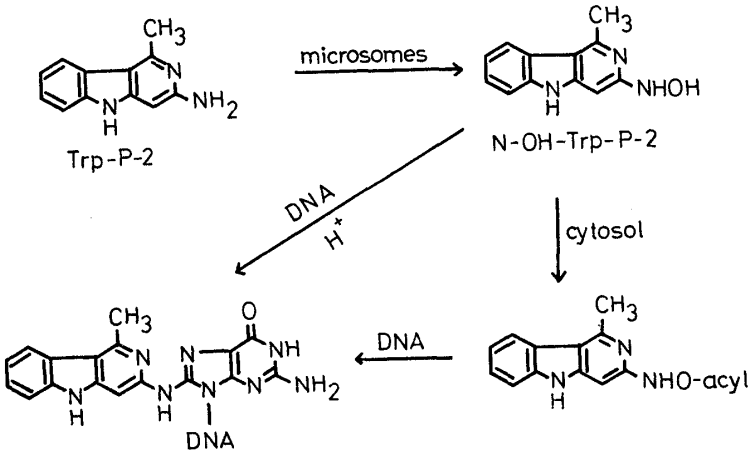


Fig. 8. The Pathway of DNA Modification by Trp-P-2

DNA, though in a very low yield (6×10^{-3}% based on guanine residues in DNA). When N-OH-Trp-P-2 was acetylated with acetic anhydride, the very reactive *O*-acetylated derivative, 3-acetoxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (N-OAc-Trp-P-2), was formed. N-OAc-Trp-P-2 reacted rapidly with DNA under neutral conditions to give Gua-Trp-P-2 in a yield of about 1% from guanine residues in DNA after hydrolysis of the modified DNA. *O*-Acetylation of an aromatic hydroxylamine is known to facilitate the heterolysis of the N–O bond of the hydroxylamine. Gua-Trp-P-2 was the only product (modified nucleic acid base) obtained in the DNA modifications (Fig. 7). The efficiency of the modification of DNA with N-OAc-Trp-P-2 is more than 30 times that of the microsome-mediated modification of DNA with Trp-P-2. This suggests that *O*-esterification may be involved in *in vivo* modification of DNA, though the free hydroxylamine itself must also contribute to the modification to some extent. These results are presented in Fig. 8 as a proposed path of DNA modification by Trp-P-2. In accordance with this path, N-OH-Trp-P-2 itself was found to be a direct mutagen toward *Salmonella typhimurium* TA98. However, the addition of cytosol, which contains large amounts of esterifying enzymes, enhances the mutagenicity.[31] The chemistry of DNA modification with Glu-P-1 is slightly different, in contrast. An active metabolite of Glu-P-1, 2-hydroxyamino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole, did not react with DNA even under acidic conditions. It reacts with DNA only after esterification.[2]

N-OAc-Trp-P-2 reacted with GpC, and on hydrolysis of the products gave Gua-Trp-P-2 in 1.7% yield. On the other hand, under similar conditions, guanine, guanosine, guanylic acid, a mixture of guanylic acid and cytidylic acid, and poly G were modified to extents of less than

0.3%. This suggests that intercalation of the activated mutagen into a complementary nucleotide base pair is important for the covalent binding.[2] In fact, Trp-P-2, which is considered to be a structural model compound of the ultimate form of Trp-P-2, was confirmed to intercalate into DNA by spectroscopic methods.[32] In the case of Glu-P-1, the intercalative character of Glu-P-1 into DNA is known to be very important for its covalent binding to DNA.[2,16] These results suggest that the size and shape of these fusèd heteroaromatic amines are important for their biological effects.[33] For example: (i) bulky alkyl substituent(s) on Trp-P-2 reduced the mutagenicity;[34] (ii) mutagenicity of Glu-P-1 derivatives is variable according to the position of the methyl group(s);[35] (iii) the position of an additional benzene ring fused to Glu-P-1 affects the mutagenicity.[36] Similar results were reported on mutagenic/carcinogenic aromatic epoxides.[37]

The conformation of the modified base in DNA is a biologically important problem. Experiments on quenching of the fluorescence of the modified DNA with inorganic ions could provide information of the position of bound Trp-P-2 in the modified DNA.[38] The fluorescence of the modified DNA was quenched by Hg^{2+} cation, but not by I^- anion. This suggests that Trp-P-2 moiety is bound to DNA at a position inside the DNA double helix.[38]

In conclusion, we established the pathway of DNA modification by Trp-P-2. The site of the modification of DNA is position 8 of guanine residues in DNA. This position seems to be a general site of modification by mutagenic/carcinogenic aromatic amines such as acetylaminofluorene, dimethylaminoazobenzene, naphthylamine, Glu-P-1, *etc.*

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