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Structures of mutagens produced by the co-mutagen norharman with *o*- and *m*-toluidine isomers

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Abstract

Norharman, abundantly present in cigarette smoke and cooked foods, is not mutagenic to *Salmonella typhimurium* strains. However, norharman shows mutagenicity to *S. typhimurium* TA98 and YG1024 in the presence of S9 mix when coexisting with aromatic amines, including aniline, *o*- and *m*-toluidines. We previously reported that the mutagenicity from norharman and aniline in the presence of S9 mix was due to the formation of a mutagenic compound, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman). In the present study, we analyzed the mutagens produced by norharman with *o*- or *m*-toluidine in the presence of S9 mix. When norharman and *o*-toluidine were reacted at 37°C for 20 min, two mutagenic compounds, which were mutagenic with and without S9 mix, respectively, were produced, and these were isolated by HPLC. The former mutagen was deduced to be 9-(4′-amino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole (amino-3′-methylphenylnorharman) on the basis of various spectral data, and this new heterocyclic amine was confirmed by its chemical synthesis. The latter mutagen was identified to be the hydroxyamino derivative. Amino-3′-methylphenylnorharman induced 41,000 revertants of TA98, and 698,000 revertants of YG1024 per μg with S9 mix. Formation of the same DNA adducts was observed in YG1024 when amino-3′-methylphenylnorharman or a mixture of norharman plus *o*-toluidine was incubated with S9 mix. These observations suggest that norharman reacts with *o*-toluidine in the presence of S9 mix to produce amino-3′-methylphenylnorharman, and this compound is metabolically activated to yield its hydroxyamino derivative. After activation by *O*-acetyltransferase, it might bind to DNA and exert mutagenicity in *S. typhimurium* TA98 and YG1024. When norharman and *m*-toluidine were reacted

Abbreviations: HCAs, heterocyclic amines; Aminophenylnorharman, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole; DMSO, dimethyl sulfoxide; Amino-3′-methylphenylnorharman, 9-(4′-amino-3′-methylphenyl) -9*H*-pyrido[3,4-*b*]indole; Hydroxyamino-3′-methylphenylnorharman, 9-(4′-hydroxyamino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole; RAL, relative adduct labeling; Amino-2′-methylphenylnorharman, 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole

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in the presence of S9 mix, 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole (amino-2′-methylphenylnorharman) was identified as a mutagen. Thus, the mutagenicity of norharman with *m*-toluidine may follow a mechanism similar to that with *o*-toluidine.

Keywords: Norharman; Toluidine isomers; Amino-3′-methylphenylnorharman; Amino-2′-methylphenylnorharman

1. Introduction

The development of human cancer is thought to be mainly associated with diet and smoking-related factors [1]. A non-mutagenic β-carboline compound, norharman, is widely distributed in our environment, such as cigarette smoke and cooked foods [2–4]. It is found at levels 11–730-fold higher than those of known mutagenic and carcinogenic heterocyclic amines (HCAs) [5]. Norharman is a co-mutagen, as demonstrated by its mutagenicity toward *Salmonella typhimurium* TA98 and YG1024 in the presence of both S9 mix and aromatic amines, including aniline [6–9]. Aniline is present in cigarette smoke condensates and some vegetables [10,11]. In addition, it has been reported that both of norharman and aniline were detected in human urine [12–14]. In a previous report, we studied the mechanism of the co-mutagenic action of norharman with aniline, and mutagenicity was found to be due to the formation of a mutagenic coupled compound of norharman with aniline, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman) [15]. Under the same conditions, an *N*-hydroxy derivative of aminophenylnorharman was also produced. Moreover, the same DNA adducts were observed in YG1024 when aminophenylnorharman or a mixture of norharman plus aniline was incubated with S9 mix. The hydroxyamino derivative also yielded the same DNA adducts in YG1024. Thus, the mechanism of the co-mutagenic action of norharman with aniline was suggested to be as follows: in the presence of S9 mix, a coupled mutagenic compound, aminophenylnorharman, is formed from norharman and aniline. This compound is then converted to the *N*-hydroxy derivative and forms DNA adducts after esterification to induce mutations in *Salmonella* strains [15].

Other aromatic amines, *o*- and *m*-toluidine, have also been shown to react with norharman to show mutagenicity in *S. typhimurium* TA98 and YG1024 in the presence of S9 mix. In contrast, the combination of norharman with *p*-toluidine did not show mutagenicity in *Salmonella* strains [6,8,9]. Moreover, good correlation was found between DNA adduct formation and the appearance of mutagenicity in *Salmonella* strains by norharman with toluidine isomers [9].

As mentioned above, norharman is present in cigarette smoke, and *o*- and *m*-toluidine are also detected at levels of 24–3919 ng per cigarette [11,16]. Moreover, *o*-toluidine has been found in human urine and milk samples [13,14,17]. Thus, it is likely that humans are simultaneously exposed to norharman and toluidine isomers in daily life. Therefore, it is important to elucidate the mechanisms of the co-mutagenicity of norharman with toluidine *o*- and *m*-isomers to understand the genotoxic effects of norharman with toluidine isomers in humans.

In the present study, we isolated mutagenic compounds produced from norharman with *o*- or *m*-toluidine in the presence of S9 mix, and then determined their structures by various spectrometric methods and by comparison with chemically synthesized authentic compounds. Possible metabolic activation processes of the mutagens are also discussed.

2. Materials and methods

2.1. Generals

Melting points were determined with a Yanagimoto micro melting-point apparatus and are uncorrected. ^{1}H and ^{13}C-NMR spectra were recorded with JEOL GX-400 and α600 with micro probe FT-NMR spectrometers, respectively. Tetramethylsilane was used as the internal standard for solutions in $CDCl_3$. EI-MS was recorded on a JEOL JMS-DX 300 mass spectrometer and UV absorbance spectra were measured with a PD-8020 photodiode array detector (Tosoh, Tokyo, Japan). Distillation for elemental analysis was

performed with a Micro Distillation and Sublimation apparatus (Yanagimoto CHN coder MT-3, Tokyo, Japan).

2.2. Materials

Norharman was purchased from Katsura Chemical Co. (Tokyo, Japan). *o*-Toluidine, NADPH, G6P, 5-fluoro-2-nitrotoluene and potassium carbonate were from Wako Pure Chemical Industries (Osaka, Japan). Micrococcal nuclease and phosphodiesterase II were purchased from Worthington Biochemical Co. (Freehold, NJ). [γ-^{32}P]ATP, T4 polynucleotide kinase, nuclease P1 and phosphodiesterase I were obtained from ICN Biochemicals (Irvine, CA), Takara Shuzo Co. (Kyoto, Japan), Yamasa Shoyu Co. (Choshi, Japan) and Worthington Biochemical Co. (Freehold, NJ), respectively. Silica gel 60 for column chromatography (spherical, 100–210 mesh) was from Kanto Chemical Co. (Tokyo, Japan) and Kiesel gel GF_{254} for thin layer chromatography was from Merck Japan Ltd. (Tokyo, Japan). All other chemicals used were of analytical grade.

2.3. Isolation of mutagens produced by norharman with o- or m-toluidine in the presence of S9 mix

Norharman·HCl (40 mg) and *o*-toluidine (20 mg) dissolved in 20 ml of water were mixed with 10 ml of S9 mix, and incubated for 20 min at 37°C. After incubation, 30 ml of cooled acetonitrile was added and the mixture was centrifuged at 10,000 rpm and 4°C to remove the proteins. The supernatant was evaporated in vacuo and the residue dissolved in 2 ml of 50% methanol was then separated by HPLC with a semi-preparative TSKgel ODS-120A column (10 μm particle size, 7.8 mm × 300 mm; Tosoh) as reported previously [15]. An aliquot of each 4 min fraction (8 ml) was tested for mutagenicity using *S. typhimurium* YG1024 in the presence or absence of S9 mix.

In the presence of S9 mix, mutagenicity was mainly observed in fractions with retention times of 48–52 min. These were evaporated and the residue was dissolved in 2 ml of 50% methanol. An aliquot of the solution was injected into an analytical-grade TSKgel ODS-80Ts column (5 μm particle size, 4.6 mm × 250 mm, Tosoh) and a mobile phase of 25% acetonitrile in 25 mM phosphate buffer (pH 2.0) was pumped in isocratically at a flow rate of 1 ml/min. The mutagenic fractions eluted at retention times of 15–17 min were further purified on the same TSKgel ODS-80Ts column. Applied material was eluted with a mobile phase of 50% acetonitrile in water with 0.1% diethylamine adjusted to pH 6.0 with acetic acid at a flow rate of 1 ml/min.

In the absence of S9 mix, mutagenicity was observed in fractions with retention times of 48–64 min on the semi-preparative TSKgel ODS-120A column. The mutagenic fractions with retention times of 56–64 min were combined, and further purified on an analytical-grade TSKgel ODS-80Ts column with a mobile phase of 25% acetonitrile in 25 mM phosphate buffer (pH 2.0) under the same conditions used for the separation of mutagenic compounds that showed activity with S9 mix.

Isolation of the mutagen, produced by the reaction of norharman with *m*-toluidine in the presence of S9 mix, was performed with the same procedures as described above. Norharman·HCl (40 mg) and *m*-toluidine (20 mg) were incubated with S9 mix at 37°C for 20 min, then the mutagen showing activity with S9 mix was isolated using semi-preparative ODS column. The major mutagenic activity was observed in the fractions with retention times of 52–56 min. Then, these fractions were further purified using an analytical grade ODS column with the following two eluent conditions, 25% acetonitrile in 25 mM phosphate buffer (pH 2.0) and 50% acetonitrile in water with 0.1% of diethylamine adjusted to pH 6.0 with acetic acid.

All the above HPLC procedures were performed at the ambient temperature by monitoring of UV absorbance of the eluate at 254 nm.

2.4. Chemical synthesis of 9-(3′-methyl-4′-nitrophenyl)-9H-pyrido[3,4-b]indole

A mixture of norharman (168 mg, 1 mmol), 5-fluoro-2-nitrotoluene (465 mg, 3 mmol) and anhydrous potassium carbonate (140 mg) in *N*,*N*-dimethylformamide (2 ml) was heated at 100°C with stirring under an argon atmosphere for 10 h. The reaction mixture was poured into 100 ml of water and extracted twice with 100 ml of ethyl acetate. The organic layer was washed with water, dried over magnesium sulfate,

and evaporated to dryness in vacuo. The residue was dissolved in dichloromethane and chromatographed on a silica gel column (2.5 cm × 20 cm) using dichloromethane–methanol (50:1, v/v) as the eluent, and 9-(3′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*] indole was obtained as a yellow powder (242 mg, 80%); m.p. 165–168°C; silica gel TLC (developing solvent; dichloromethane–methanol, 10:1, v/v) Rf 0.63; ^{1}H NMR ($CDCl_3$) δ 8.93 (1H, s, H-1), 8.58 (1H, d, J = 5.2 Hz, H-3), 8.30 (1H, d, J = 9.2 Hz, H-5′), 8.21 (1H, d, J = 7.9 Hz, H-5), 8.03 (1H, d, J = 5.2 Hz, H-4), 7.59–7.64 (3H, m, H-2′, H-6′, H-7), 7.55 (1H, d, J = 7.9 Hz, H-8), 7.41 (1H, t, J = 7.3 Hz, H-6), 2.76 (3H, s, CH_3). Anal. calcd. for $C_{18}H_{13}N_3O_2$: C, 71.28; H, 4.32; N, 13.85. Found C, 71.27; H, 4.13; N, 13.83.

2.5. Chemical synthesis of 9-(4′-amino-3′-methylphenyl)-9H-pyrido[3,4-b]indole

A mixture of 9-(3′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*]indole (70 mg, 0.23 mmol) and 5% Pd–C (50 mg) in 7 ml of ethanol–acetic acid (5:2, v/v) was stirred under a hydrogen atmosphere for 1 h at 20°C. After the catalyst was removed by filtration, the filtrate was condensed under reduced pressure. The residue was dissolved in dichloromethane and chromatographed on a silica gel column (2.5 cm × 20 cm) using dichloromethane–methanol (20:1, v/v) as an eluent to yield 9-(4′-amino-3′-methylphenyl)-9*H*-pyrido [3,4-*b*]indole (amino-3′-methylphenylnorharman) (55 mg, 87%); m.p. 164–167°C; silica gel TLC (developing solvent; dichloromethane–methanol, 10:1, v/v) Rf 0.56; UV λ max (methanol) 238, 288, 357 nm; EI-MS *m/z* 273 (M^+); 1H NMR ($CDCl_3$) δ 8.77 (1H, s, H-1), 8.49 (1H, d, J = 4.5 Hz, H-3), 8.18 (1H, d, J = 7.5 Hz, H-5), 7.99 (1H, d, J = 4.5 Hz, H-4), 7.52 (1H, t, J = 7.5 Hz, H-7), 7.41 (1H, d, J = 7.5 Hz, H-8), 7.31 (1H, br.t, J = 7.5 Hz, H-6), 7.21 (1H, s, H-2′), 7.19 (1H, d, J = 8.0, H-6′), 6.86 (1H, d, J = 8.0 Hz, H-5′), 3.86 (2H, br.s, NH_2), 2.26 (3H, br.s, CH_3); ^{13}C NMR ($CDCl_3$) δ 144.6 (C-4′), 142.3 (C-8a), 139.3 (C-3), 137.5 (C-9a), 133.4 (C-1), 129.0 (C-2′), 128.4 (C-4a), 128.3 (C-7), 127.0 (C-1′), 125.8 (C-6′), 123.6 (C-3′), 121.6 (C-5), 121.1 (C-4b), 120.1 (C-6), 115.6 (C-5′), 114.4 (C-4), 110.6 (C-8). Anal. calcd. for $C_{18}H_{15}N_3$: C, 79.10; H, 5.53; N, 15.37. Found C, 79.09; H, 5.31; N, 14.93.

2.6. Chemical synthesis of 9-(4′-hydroxyamino-3′-methylphenyl)-9H-pyrido[3,4-b]indole

A mixture of 9-(3′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*]indole (50 mg, 0.16 mmol) and 5% Pd–C (30 mg) in 5 ml of tetrahydrofuran was stirred under a hydrogen atmosphere for 30 min at 20°C. After removing Pd–C by filtration, the filtrate was concentrated at 30°C and subjected to preparative thin layer chromatography with silica gel (20 cm × 20 cm plate) (developing solvent; dichloromethane–methanol, 10:1, v/v) to yield 9-(4′-hydroxyamino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole (hydroxyamino-3′-methylphenylnorharman) (20 mg, 43%); silica gel TLC (developing solvent; dichloromethane–methanol, 10:1, v/v) Rf 0.40; UV λ max (methanol) 251, 300, 388 nm; EI-MS *m/z* 289 (M^+).

2.7. Chemical synthesis of 9-(2′-methyl-4′-nitrophenyl)-9H-pyrido[3,4-b]indole

A mixture of norharman (168 mg, 1 mmol), 2-fluoro-5-nitrotoluene (155 mg, 1 mmol) and anhydrous potassium carbonate (140 mg) in *N*,*N*-dimethylformamide (2 ml) was heated at 100°C with stirring under an argon atmosphere for 10 h. The reaction mixture was poured into 100 ml of water and extracted twice with 100 ml of ethyl acetate. The organic layer was washed with water, dried over magnesium sulfate, and evaporated to dryness in vacuo. The residue was dissolved in dichloromethane and chromatographed on a silica gel column (2.5 cm × 20 cm) using dichloromethane–methanol (50:1, v/v) as the eluent, and 9-(2′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*] indole was obtained as a yellow powder (268 mg, 88%); m.p. 129–130°C; silica gel TLC (developing solvent; dichloromethane–methanol, 10:1, v/v) Rf 0.63; ^{1}H NMR ($CDCl_3$) δ 8.57 (1H, d, J = 5.5 Hz, H-3), 8.50 (1H, s, H-1), 8.41 (1H, d, J = 2.3 Hz, H-3′), 8.31 (1H, dd, J = 2.3, 8.5 Hz, H-5′), 8.24 (1H, d, J = 7.5 Hz, H-5), 8.06 (1H, d, J = 5.5 Hz, H-4), 7.61 (1H, d, J = 7.5 Hz, H-6″), 7.57 (1H, t, J = 7.5 Hz, H-7), 7.40 (1H, t, J = 7.5 Hz, H-6), 7.10 (1H, d, J = 7.5 Hz, H-8), 2.16 (3H, s, CH_3). Anal. calcd. for $C_{18}H_{13}N_3O_2$: C, 71.28; H, 4.32; N, 13.85. Found C, 71.27; H, 4.13; N, 13.83.

2.8. Chemical synthesis of 9-(4′-amino-2′-methylphenyl)-9H-pyrido[3,4-b]indole

A mixture of 9-(2′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*]indole (106 mg, 0.35 mmol) and 5% Pd–C (80 mg) in 7 ml of ethanol-acetic acid (5:2, v/v) was stirred under a hydrogen atmosphere for 1 h at 20°C. After the catalyst was removed by filtration, the filtrate was condensed under reduced pressure. The residue was dissolved in dichloromethane and chromatographed on a silica gel column (2.5 cm × 20 cm) using dichloromethane–methanol (20:1, v/v) as an eluent to give 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido [3,4-*b*]indole (amino-2′-methylphenylnorharman) (80 mg, 84%); m.p. 131–132°C; silica gel TLC (developing solvent; dichloromethane–methanol, 10:1, v/v) Rf 0.55; UV λ max (methanol) 239, 289, 356 nm; EI-MS *m/z* 273 (M^+); ^{1}H NMR ($CDCl_3$) δ 8.52 (1H, s, H-1), 8.49 (1H, d, J = 5.2 Hz, H-3), 8.19 (1H, d, J = 7.9 Hz, H-5), 8.01 (1H, d, J = 5.2 Hz, H-4), 7.52 (1H, dd, J = 7.9, 8.2 Hz, H-7), 7.31 (1H, t, J = 7.9 Hz, H-6), 7.14 (1H, d, J = 8.2 Hz, H-8), 7.12 (1H, d, J = 8.2 Hz, H-6′), 6.75 (1H, d, J = 3.5 Hz, H-3′), 6.68 (1H, dd, J = 3.5, 8.2 Hz, H-5′), 2.76 (3H, s, CH_3), 3.89 (2H, br.s, NH_2); ^{13}C NMR ($CDCl_3$) d 147.2 (C-4′), 142.4 (C-8a), 139.3 (C-3), 138.0 (C-2′), 137.6 (C-9a), 133.3 (C-1), 129.9 (C-6′), 128.4 (C-7), 128.3 (C-4a), 125.4 (C-1′), 121.7 (C-5), 121.0 (C-4b), 120.0 (C-6), 117.2 (C-3′), 114.5 (C-4), 113.6 (C-5′), 110.6 (C-8), 17.4 (CH_3). Anal. calcd. for $C_{18}H_{15}N_3$: C, 79.10; H, 5.53; N, 15.37. Found C, 79.25; H, 5.62; N, 15.30.

2.9. Measurement of mutagenic activity

The mutation assay was carried out as described previously [18], using *S. typhimurium* TA98, TA100, YG1024 and YG1029. *S. typhimurium* YG1024 and YG1029, produced by introducing plasmids containing the acetyltransferase gene from TA1538 into TA98 and TA100, respectively [19], were kindly provided by Dr. T. Nohmi, National Institute of Hygienic Sciences, Tokyo. All test samples except those obtained by HPLC separation were dissolved in 100 μl of dimethyl sulfoxide (DMSO). For materials at various steps in HPLC purification, 100 μl of 50% DMSO was used. The S9 mix contained 50 μl of S9, unless otherwise stated, in a total volume of 500 μl.

2.10. Preparation of DNA samples from S. typhimurium YG1024 treated with norharman and o-toluidine, or amino-3′-methylphenylnorharman

Ten milliliter of an overnight culture of *S. typhimurium* YG1024 in Oxoid No. 2 (Unipath Ltd., Hampshire, England) were pelleted by centrifugation and resuspended in 4 ml of 0.1 M sodium phosphate buffer (pH 7.4). This suspension was mixed with 2 ml of water containing 8 mg of norharman·HCl plus 4 mg of *o*-toluidine or 2 ml of 25% DMSO solution containing 5 μg of amino-3′-methylphenylnorharman with 20 ml of S9 mix. After incubation for 6 h at 37°C, the bacteria were collected by centrifugation and the bacterial pellet was washed with 4 ml of distilled water. Bacterial cell clumps were mixed with buffer containing 25 mM Tris–HCl (pH 8.0), 1.7 mM sodium chloride, 4 mM potassium chloride, 0.3 mM disodium hydrogenphosphate, 10 mM EDTA and 0.4% SDS. DNA was then obtained by standard procedures involving the enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v). DNA concentration was estimated spectrophotometrically at 260 nm and adjusted to 2 mg per milliliter of 0.01 × SSC buffer.

2.11. ^{32}P-postlabeling method

Bacterial DNA was digested with micrococcal nuclease and phosphodiesterase II, and the digest was ^{32}P-postlabeled under modified adduct intensification conditions, as reported previously [9].

3. Results

3.1. Isolation and structural determination of mutagens formed from norharman with o-toluidine in the presence of S9 mix

The reaction products from norharman with *o*-toluidine in the presence of S9 mix were separated by HPLC on a semi-preparative ODS column. Fig. 1 shows HPLC profiles for UV absorbance and mutagenicity. Mutagenicity of each fraction using *S. typhimurium* YG1024 was examined in the presence of S9 mix, and the major mutagenic activity

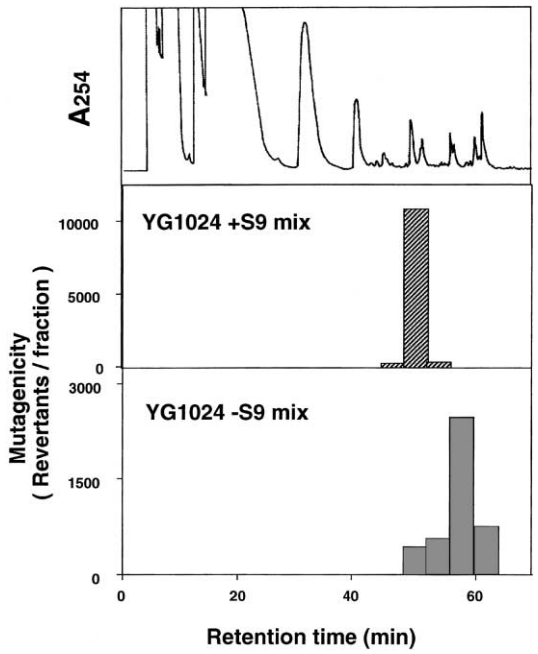


Fig. 1. Separation of mutagens formed from norharman with *o*-toluidine in the presence of S9 mix by HPLC. A sample of the reaction mixture was applied to a semi-preparative ODS column, and an aliquot of each 4 min fraction was tested for mutagenicity in *S. typhimurium* YG1024 with or without S9 mix. The UV absorbance of the eluate was monitored at 254 nm. Elution positions of norharman and *o*-toluidine under these conditions were 16 and 10 min, respectively.

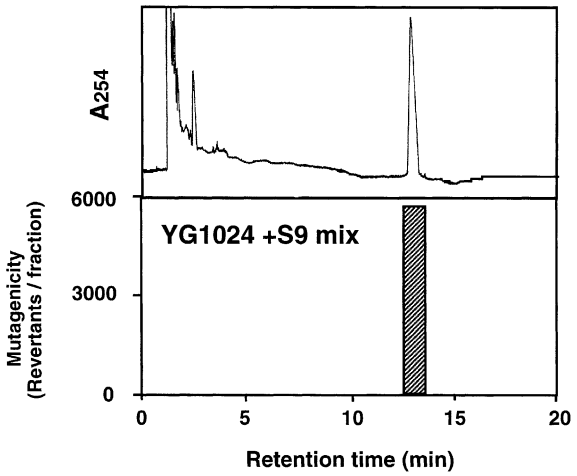


Fig. 2. HPLC chromatogram of mutagenic compound I. Column: analytical ODS-80Ts, mobile phase: 50% acetonitrile in water with 0.1% diethylamine adjusted to pH 6.0 with acetic acid. The upper line shows UV absorbance at 254 nm, and the lower bar shows mutagenicity of each 1 min fraction in *S. typhimurium* YG1024 with S9 mix.

was recovered in fractions with retention times of 48–52 min. On the other hand, when the mutation test was carried out in the absence of S9 mix, mutagenicity was observed in fractions with retention times of 48–64 min. The elution positions of these mutagenic fractions on HPLC were different from those of norharman and *o*-toluidine.

The mutagenic fraction, which showed activity with S9 mix, was separated by HPLC on a TSKgel ODS-80Ts column. Several UV absorption peaks were detected and the mutagenicity was mainly recovered in fractions with retention times of 15–17 min. This mutagenic fraction was further purified on the same column with a mobile phase of 50% acetonitrile in 0.1% diethylamine-water solution adjusted to pH 6.0 with acetic acid, and a single peak fraction with mutagenicity (compound I) was detected at retention times of 13–14 min (Fig. 2). The above procedure was repeated more than 10 times and about 30 μg of compound I was collected for the measurement of UV absorption, mass and ^{1}H-NMR spectra for structural analysis. The yield of compound I from norharman was around 0.06%.

The UV-absorption spectrum of the mutagen, compound I, obtained on the ODS column with a photodiode array detector, showed absorption maxima at 238, 288 and 357 nm (Fig. 3). This UV absorption pattern was similar to that of aminophenylnorharman [15]. The mass spectrum of compound I exhibited a molecular ion peak at *m/z* 273. The ^{1}H-NMR spectrum in $CDCl_3$ is shown in Table 1. It showed the presence of the following protons: δ (ppm) 2.27 (3H), 6.83 (1H), 7.19 (1H), 7.21 (1H), 7.31 (1H), 7.41 (1H), 7.53 (1H), 8.14 (1H), 8.18 (1H), 8.48 (1H), 8.76 (1H). Among these, seven protons were assigned to the norharman moiety, H-1 at 8.76, H-3 at 8.48, H-4 at 8.14, H-5 at 8.18, H-6 at 7.31, H-7 at 7.53 and H-8 at 7.41 ppm, three to the aromatic protons of the *o*-toluidine moiety, H-2′ at 7.21, H-5′ at 6.83 and H-6′ at 7.19 ppm, and the three at 2.27 ppm corresponded to the methyl group. Based on the UV, mass and ^{1}H-NMR spectra of compound I, its structure was deduced to be amino-3′-methylphenylnorharman.

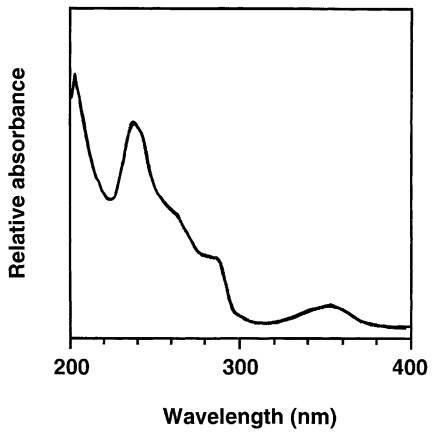


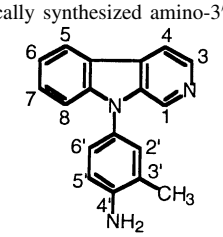
Fig. 3. UV absorption spectrum of compound I. The spectrum was measured on an ODS column with a photodiode array detector. The material was eluted with the same mobile phase described for Fig. 2.

On the other hand, the fractions, which showed activity without S9 mix, with retention times of 56–64 min on the semi-preparative ODC column (Fig. 1) were further purified by HPLC under the same conditions as for compound I. As shown in Fig. 4, several UV-absorption peaks were detected, and mutagenicity was only found in a peak fraction (compound II) with retention times of 14–15 min. The UV absorption maxima of compound II were observed at 250, 301 and 387 nm. Based on the present and previous observations [15], the structure of compound II was deduced to be hydroxyamino-3′-methylphenylnorharman.

3.2. Structural determination of compound I

To confirm the structure of compound I, we tried to chemically synthesize amino-3′-methylphenylnorharman which were the deduced mutagen produced by norharman and *o*-toluidine. When norharman was reacted with three molar equivalents of 5-fluoro-2-nitrotoluene in the presence of potassium carbonate, 9-(3′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*]indole was produced at a yield of 80%. This nitro derivative was then reduced to obtain amino-3′-methylphenylnorharman under a hydrogen atmosphere in the presence of Pd–C. The yield of amino compound from nitro compound was 87%. The UV and mass spectra of the synthetic compound were the same as those of the mutagenic compound I formed by the reaction

Table 1
^{1}H-NMR spectral data of compound I and chemically synthesized amino-3′-methylphenylnorharman in $CDCl_3$[a]



	Compound I	Synthetic compound
1	8.76 (1H, s[b])	8.77 (1H, s)
3	8.48 (1H, br.s)	8.49 (1H, d, $J = 4.5$ Hz)
4	8.14 (1H, br.s)	7.99 (1H, d, $J = 4.5$ Hz)
5	8.18 (1H, d, $J = 7.5$ Hz)	8.18 (1H, d, $J = 7.5$ Hz)
6	7.31 (1H, br.t, $J = 7.5$ Hz)	7.31 (1H, br.t, $J = 7.5$ Hz)
7	7.53 (1H, br.t, $J = 7.5$ Hz)	7.52 (1H, t, $J = 7.5$ Hz)
8	7.41 (1H, d, $J = 7.5$ Hz)	7.41 (1H, d, $J = 7.5$ Hz)
2′	7.21 (1H, br.s)	7.21 (1H, s)
5′	6.83 (1H, d, $J = 8.0$ Hz)	6.86 (1H, d, $J = 8.0$ Hz)
6′	7.19 (1H, d, $J = 8.0$ Hz)	7.19 (1H, d, $J = 8.0$ Hz)
CH_3	2.27 (3H, br.s)	2.26 (3H, br.s)
NH_2		3.86 (2H, br.s)

[a] ^{1}H-NMR chemical shifts are presented as parts per million.
[b] s: singlet; d: doublet; t: triplet; and br: broad.

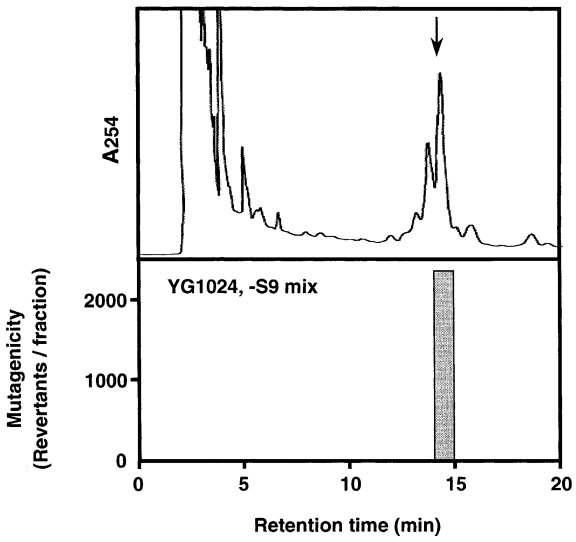


Fig. 4. HPLC profile of mutagenic compound II. The fractions with mutagenic activity in YG1024 without S9 mix, as shown in Fig. 1, were combined and applied to an analytical ODS 80Ts column. The material was eluted with a mobile phase of 25% acetonitrile in 25 mM phosphate buffer (pH 2.0). The upper line shows UV absorbance of the eluate at 254 nm, and the lower bar shows mutagenicity of each 1 min fraction in *S. typhimurium* YG1024 without S9 mix. A UV absorption peak showing mutagenicity is indicated by an arrow.

of norharman with *o*-toluidine in the presence of S9 mix. When the ^{1}H-NMR spectrum of the synthetic compound in $CDCl_3$ was measured, 15 protons were observed. Among these, 13 protons indicating aromatic protons from the norharman and *o*-toluidine moieties and methyl protons coincided with those of compound I (Table 1). In addition, two protons at 3.86 ppm assigned to an amino group were detected in the synthetic compound. Based on these results, compound I was considered to be amino-3′-methylphenylnorharman, formed by coupling of norharman at the N-9 position with *o*-toluidine at the *para* position of an amino group.

3.3. Structure of compound II, showing activity without S9 mix

Aminophenylnorharman and its *N*-hydroxy derivative have been previously reported to be produced by reacting norharman with aniline in the presence of S9 mix [15]. Therefore, compound II, which showed mutagenic activity without S9 mix, was tentatively considered to be hydroxyamino-3′-methylphenylnorharman. To confirm its structure, authentic hydroxyamino-3′-methylphenylnorharman was synthesized from 9-(3′-methyl-4′-nitrophenyl)-9*H*-pyrido[3,4-*b*] indole by its reduction. The elution position and UV spectrum of this synthetic hydroxyamino-3′-methylphenylnorharman in HPLC on an ODS column were the same as those of compound II produced by norharman and *o*-toluidine in the presence of S9 mix. Based on these results, compound II was determined to be hydroxyamino-3′-methylphenylnorharman.

3.4. Mutagenicity and DNA adduct formation by amino-3′-methylphenylnorharman

The mutagenicity of synthesized amino-3′-methylphenylnorharman was tested in *S. typhimurium* TA98, TA100, YG1024 and YG1029 with S9 mix containing 5 μl of S9 per plate. Amino-3′-methylphenylnorharman was mutagenic in all four strains in a dose-dependent manner. Similar to aminophenylnorharman, amino-3′-methylphenylnorharman showed higher mutagenicity in *S. typhimurium* TA98 and YG1024, detectors of frameshift mutations, than in *S. typhimurium* TA100 and YG 1029, detectors of base-pair-change mutations, in the presence of S9 mix. Amino-3′-methylphenylnorharman induced 41,000 revertants of TA98 and 1,980 revertants of TA100 per microgram. YG1024 and YG1029, which have high acetyltransferase activity, were more sensitive to this mutagen, and 1 μg of the compound gave 698,000 revertants for YG1024 and 8,640 revertants for YG1029. In the absence of S9 mix, amino-3′-methylphenylnorharman was not mutagenic in either strain.

A sample of 5 μg of amino-3′-methylphenylnorharman was incubated with an overnight culture of *S. typhimurium* YG1024 in the presence of S9 mix for 6 h at 37°C, and the resulting DNA adducts were analyzed by the ^{32}P-postlabeling method under modified adduct intensification conditions. As shown in Fig. 5A, three adduct spots, two major (spots 1 and 2) and one minor (spot 3), were detected, and their RALs were estimated to be 0.7, 0.4 and 0.1 adducts per 10^8 nucleotides, respectively, with a total RAL value of 1.2 per 10^8 nucleotides. A mixture of norharman·HCl (8 mg) and *o*-toluidine (4 mg) in the presence of S9 mix was incubated with YG1024, and DNA adducts

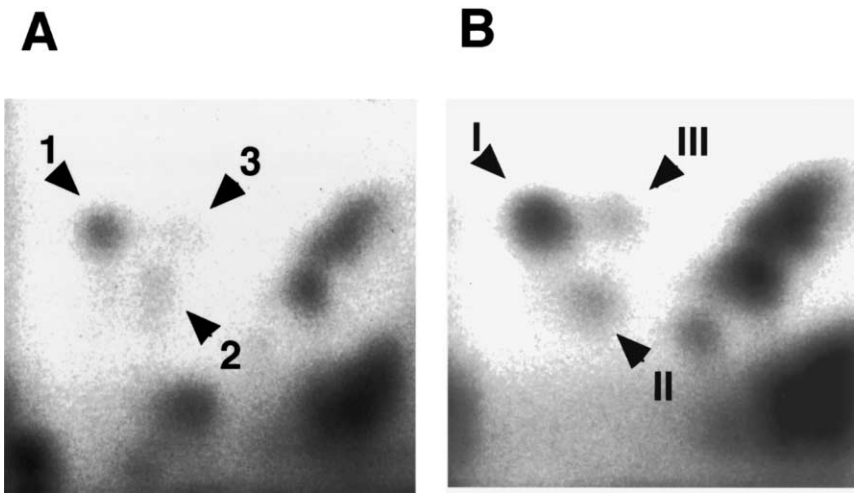


Fig. 5. Autoradiograms of DNA adducts in *S. typhimurium* YG1024 treated with amino-3′-methylphenylnorharman or a mixture of norharman and *o*-toluidine in the presence of S9 mix. Amino-3′-methylphenylnorharman (A) or a mixture of norharman plus *o*-toluidine (B) was incubated with an overnight culture of *S. typhimurium* YG1024 and S9 mix for 6 h at 37°C, and DNA adduct formation was analyzed under modified adduct intensification conditions. The imaging plates were exposed for 24 h. DNA adducts derived from amino-3′-methylphenylnorharman and the mixture of norharman and *o*-toluidine are indicated by arrowheads.

were analyzed under the same conditions as for amino-3′-methylphenylnorharman. The combination of norharman with *o*-toluidine yielded the same DNA adduct pattern as amino-3′-methylphenylnorharman, with a total RAL value for the three adduct spots (I, II, III) of 186 per 10^8 nucleotides (Fig. 5B). No adduct spots were detected in the absence of S9 mix with either amino-3′-methylphenylnorharman or a mixture of norharman and *o*-toluidine.

3.5. Isolation and structural determination of mutagen formed from norharman with m-toluidine in the presence of S9 mix

Based on the above results, it is expected that amino-2′-methylphenylnorharman would be produced when a mixture of norharman and *m*-toluidine was incubated in the presence of S9 mix. Thus, authentic amino-2′-methylphenylnorharman was chemically synthesized from norharman and 2-fluoro-5-nitrotoluene as starting materials. Then, the formation of amino-2′-methylphenylnorharman in a reaction mixture of norharman and *m*-toluidine with S9 mix at 37°C for 20 min was analyzed by HPLC as follows. The mutagen in the reaction mixture was purified by HPLC under the same three conditions as were used for the separation of amino-3′-methylphenylnorharman. At the third HPLC step with a TSKgel ODS-80Ts column, a single UV absorption peak showing mutagenicity in YG1024 with S9 mix was detected at a retention time of 12.5 min. Under this condition, authentic amino-2′-methylphenylnorharman was eluted at the same position. These procedures were repeated several times and about 50 ng of this mutagen was collected for measurement of the UV absorption spectrum on the ODS column with a photodiode array detector. Fig. 6A shows the UV spectrum of the mutagen produced from norharman with *m*-toluidine in the presence of S9 mix. Absorption maxima of the mutagen were seen at 238, 283 and 356 nm, and its spectrum coincided with that of authentic amino-2′-methylphenylnorharman (Fig. 6B). Thus, the mutagen produced by norharman and *m*-toluidine was concluded to be amino-2′-methylphenylnorharman. The yield of this mutagen from norharman was 0.02%. Amino-2′-methylphenylnorharman induced 140 revertants of TA98, 16 revertants of TA100, 3000 revertants of YG1024 and 69 revertants of YG1029 per microgram in the presence of S9 mix. These mutagenic activities were much lower than those of the 3′-methyl

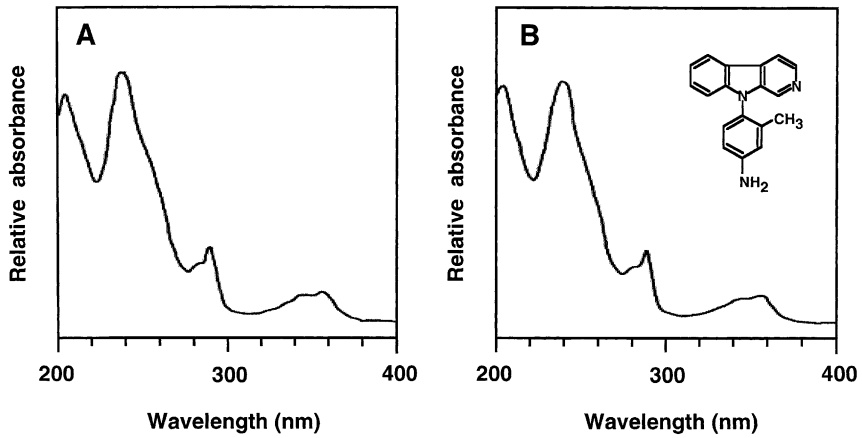


Fig. 6. UV absorbance spectra of mutagen formed from norharman with *m*-toluidine, and authentic amino-2′-methylphenylnorharman. Mutagen (A) produced by norharman with *m*-toluidine and authentic amino-2′-methylphenylnorharman (B) were examined on an analytical ODS 80Ts column with a photodiode array detector. The materials were eluted with a mobile phase of 50% acetonitrile in water with 0.1% diethylamine adjusted to pH 6.0 with acetic acid.

derivative. Amino-2′-methylphenylnorharman was not mutagenic in either strain without S9 mix.

4. Discussion

Recently, we reported that the mechanism of the appearance of mutagenicity by norharman with aniline in the presence of S9 mix was suggested to be as follows [15]; norharman reacts with aniline and produces the mutagen, aminophenylnorharman. This compound is metabolically activated by S9 mix and converts to a hydroxyamino derivative. Then, hydroxyaminophenylnorharman is further activated to its ultimate forms and reacts with DNA bases to induce mutation in *Salmonella* strains.

In the present study, we determined the structures of mutagenic compounds produced from norharman with toluidine isomers. When norharman and *o*-toluidine were incubated with S9 mix, two mutagens, showing mutagenicity with and without S9 mix, respectively, were produced. The mutagen that showed activity with S9 mix was determined to be a new HCA, amino-3′-methylphenylnorharman, and the other was confirmed to be its *N*-hydroxyamino derivative. Amino-3′-methylphenylnorharman exhibited higher mutagenic activities in *S. typhimurium* TA98 and YG1024, detectors of frameshift mutations, than in *S. typhimurium* TA100 and YG1029, detectors of base-pair-change mutations. In addition, this compound gave higher mutagenic activities in YG1024 and YG1029, which have high *O*-acetyltransferase activity, than in TA98 and TA100, suggesting that acetyltransferase is required for the mutagenicity of amino-3′-methylphenylnorharman, as with aminophenylnorharman and other known carcinogenic HCAs [5,15,20]. The mutagenic activity of amino-3′-methylphenylnorharman is comparable to those of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) [5]. Amino-3′-methylphenylnorharman yielded the same DNA adducts in *S. typhimurium* YG1024 as those observed with the mixture of norharman and *o*-toluidine. Based on these observations, proposed mechanisms of the co-mutagenic action of norharman with *o*-toluidine in the presence of S9 mix are shown in Fig. 7. First, the N-9 position of norharman couples with *o*-toluidine at the *para* position of the amino group by enzymatic reaction to produce amino-3′-methylphenylnorharman. Next, the exocyclic amino group of this compound may be oxidized to an *N*-hydroxyamino derivative by P-450s, and further metabolically activated to form the *N*-acetoxy derivative by the action of acetyltransferase. The ultimate form reacts with DNA bases to induce mutations in *Salmonella* strains. In addition,

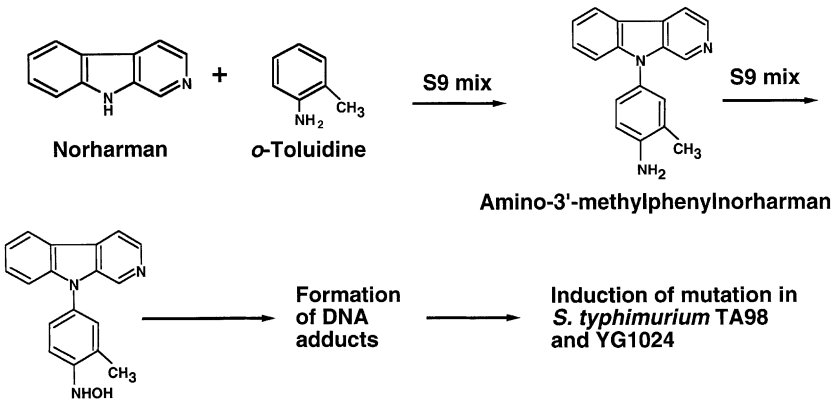


Fig. 7. Mutagen formation from norharman with *o*-toluidine in the presence of S9 mix, and its metabolic activation processes to induce mutation in *Salmonella* strains.

norharman also reacted with *m*-toluidine in the presence of S9 mix to yield amino-2′-methylphenylnorharman. A similar mechanism (Fig. 7) is expected for the appearance of mutagenicity of the mixture of norharman with *m*-toluidine in the presence of S9 mix.

We previously reported that the mixture of norharman (200 mg) with *o*- or *m*-toluidine (100 mg) induced 6990 and 62 revertants of TA98, respectively, in the presence of S9 mix [9]. In the present study, the mutagenic activity of amino-3′-methylphenylnorharman was more than 200-fold that of amino-2′-methylphenyl norharman. Moreover, the yield of amino-3′-methylphenylnorharman from norharman was three times larger than that of the 2′-methyl derivative. These diversities may be associated with the difference between in the mutagenicities of norharman and *o*- or *m*-toluidine. In contrast, a mixture of norharman with *p*-toluidine in the presence of S9 mix has been shown to be not mutagenic in *S. typhimurium* TA98 [9]. Thus, the methyl group of *p*-toluidine may interfere with the coupling reaction of norharman with *p*-toluidine. Moreover, we cannot exclude the possibility that even though coupled compound(s), such as 9-(2′-amino-5′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole and 9-(5′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*] indole, were formed from norharman with *p*-toluidine, they may not show any mutagenicity, although the mutagenicities of these derivatives have not yet been examined.

In conclusion, mutagenicity of a mixture of norharman, *o*- or *m*-toluidine and S9 mix was due to the formation of a new mutagenic HCA compound, either amino-3′-methylphenylnorharman or amino-2′-methyl phenylnorharman, as in the generation of aminophenylnorharman by the reaction of norharman and aniline [15]. When aminophenylnorharman was given to F344 rats by i.g. intubation, severe testicular damage was observed [21]. Moreover, we have recently reported that the development of glutathione S-transferase placental form (GST-P)-positive foci, a putative preneoplastic lesion, in the liver of rat was increased in a dose dependent manner by administration of aminophenylnorharman in diet at a dose of 10, 20 and 50 ppm for 4 weeks [22]. This suggests that aminophenylnorharman could induce liver tumors in rats. Aminomethylphenylnorharman, being an analogue of aminophenylnorharman, might also possess similar biological activities. As mentioned above, norharman and toluidine isomers, such as *o*- and *m*-toluidines, are present together in our environment. Therefore, it is very important to examine the formation of aminomethylphenylnorharman derivatives from norharman and toluidine isomers in vivo and to determine what enzymes might be involved in the reaction of norharman with these aromatic amines. Moreover, it is also important to evaluate the toxicity and carcinogenicity of aminomethylphenylnorharman derivatives or mixtures of norharman with toluidine isomers in rodents.

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