Articles

Isolation and Identification of a New 2-Phenylbenzotriazole-Type Mutagen (PBTA-3) in the Nikko River in Aichi, Japan

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We have previously determined the chemical structures of two 2-phenylbenzotriazole mutagens (PBTA-1 and PBTA-2) in blue cotton-adsorbed material from the Nishitakase River in Kyoto, Japan. In the present study, further analysis of mutagenic substances in the Nikko River, which flows through Aichi Prefecture in Japan, allowed the isolation of a new mutagen. Material (2.2 g) adsorbed on blue cotton (3 kg) at a site below the sewage plant on the Nikko River was purified by various column chromatographies, and a mutagen (120 μg) accounting for 11% of the total mutagenicity was isolated. On the basis of data from UV, mass, and ^{1}H NMR spectra of the mutagen, the compound was deduced to be a PBTA-1 analogue. As with PBTA-1, the mutagen was able to be synthesized from the azo dye 2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[(2-hydroxyethyl)amino]acetanilide by reduction and chlorination. Since all spectra of the mutagen isolated from the river water were the same as those of the synthesized form, the structure was concluded to be 2-[2-(acetylamino)-4-[(2-hydroxyethyl)-amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3). PBTA-3 is a potent mutagen, inducing 81 000 and 3 000 000 revertants per microgram of *Salmonella typhimurium* TA 98 and YG1024 respectively, in the presence of an S9 mix. In addition to its detection in the water of the Nikko River, PBTA-3 was detected in water samples from three other rivers flowing through regions where dyeing industries have been developed. Like PBTA-1 and PBTA-2, PBTA-3 might have also been produced from azo dyes during industrial processes in dyeing factories and/or through treatment at sewage plants.

Introduction

Since environmental genotoxic compounds may play some role in the development of various diseases such as cancer, their identification is an important step to our understanding of their significance for human health. Although various kinds of genotoxic compounds have been detected in river water, drinking water, and industrial/domestic wastewater (*1*−*5*), many sources of genotoxic contamination still remain to be clarified.

Recently, we isolated five mutagens in water samples from the Nishitakase River below sewage plants in Kyoto with 100−600 times the mutagenicity of river water in Tokyo via adsorption onto blue rayon or blue cotton (*6*) using *Salmonella typhimurium* YG1024 with an S9 mix (*7*). Among the isolated mutagens, two were identified as 2-phenylbenzotriazole derivatives, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) and 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2) (*6*, *8*). Furthermore, on the basis of synthesis

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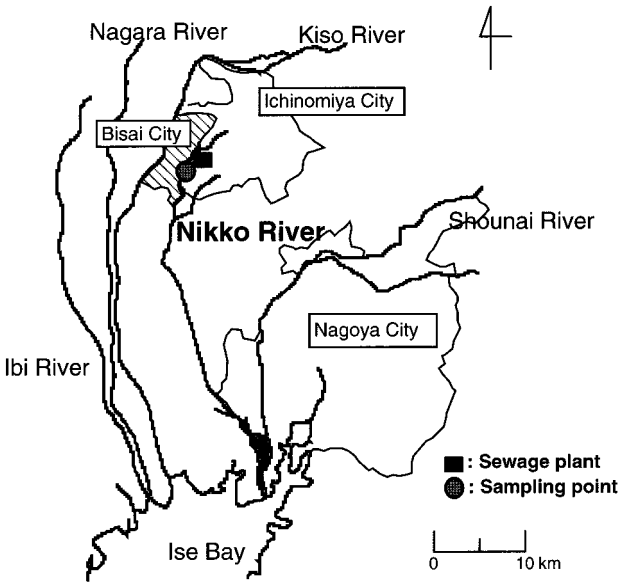


Figure 1. Geographic locations of the water sampling site and the sewage plant on the Nikko River in Aichi Prefecture.

studies, it was suggested that both mutagens can be formed from dinitrophenylazo dyes via reduction with sodium hydrosulfite and subsequent chlorination with sodium hypochlorite (*9*). Thus, it is postulated that these mutagens are produced from azo dyes during industrial processes in dyeing factories and through treatment at sewage plants and are then discharged into the river. Actually, PBTA-1 and PBTA-2 were also detected at many other points near dyeing factories in the Yodo river system, in Japan (*10*).

In addition, the structure−activity relationships, based on results for PBTA-1, its congeners, and five related 2-phenylbenzotriazoles, suggested that a primary amino group, along with the planarity of the 2-phenylbenzotriazole ring and halogen groups, plays an essential role in the mutagenic activity (*11*).

During the assessment of mutagenicity of river water in Japan, we found that blue rayon-adsorbed material taken at a site below the sewage plant of the Nikko River in Aichi Prefecture showed strong mutagenicity toward *S. typhimurium* YG1024 in the presence of an S9 mix, and we attempted to isolate the mutagens in the water sample. In this paper, isolation and chemical synthesis from an azo dye of the third PBTA-type mutagen is described. Detection of this novel mutagen in other rivers is also described along with possible formation routes, on the basis of chemical synthesis studies.

Experimental Procedures

Isolation of the Mutagen from Water of the Nikko River. Five net bags of blue rayon (5 g per bag; Funakoshi Co., Tokyo, Japan) were attached to a floating board and were hung in the Nikko River for 24 h in April 1997 at a site downstream from a sewage plant (Figure 1). The blue rayon from different bags was combined and washed twice with 500 mL of water. Adsorbed materials were extracted by shaking the blue rayon in 1 L of methanol/ammonia water (50:1, v/v) three times for 1 h (*12, 13*). The combined extracts were evaporated to dryness, and the residue originating from the 25 g of blue rayon was dissolved in 6 mL of methanol. One-fifth of the solution was applied to a semipreparative YMC-Pack ODS-AM 324 column (5 μm particle size, 10 × 300 mm; YMC Co., Kyoto, Japan) for HPLC (Tosoh Corp., Tokyo, Japan) and then eluted with the following gradient system of methanol in distilled water: 0−40 min, 75%; 40−50 min, a linear gradient of 75−85%; 50−104 min, 85%, at a flow rate of 2 mL/min. An aliquot of each 2 min fraction was tested for mutagenicity. The above HPLC procedure was repeated four more times.

Fractions with a retention time of 13−20 min were combined and evaporated to dryness. Residues were dissolved in 40% acetonitrile in 25 mM phosphate buffer (pH 2.0) and injected into a YMC-Pack ODS-A 303 column (5 μm particle size, 4.6 × 250 mm; YMC Co.) with a mobile phase of 40% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 1 mL/min. Fractions with a retention time of 16−17 min were purified on a CAPCELL PAK C_{18} column (UG80, 5 μm particle size, 4.6 × 250 mm; Shiseido Co., Tokyo, Japan) with a mobile phase of 30% acetonitrile in 25 mM Tris-HCl buffer (pH 8.5) at a flow rate of 1 mL/min. A mutagenic compound was isolated from the fraction with a retention time of 30−32 min. Its purity was confirmed on the second YMC-Pack ODS-A 303 column with a mobile phase of 38% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 0.5 mL/min.

All HPLC procedures were carried out at ambient temperature, and eluates were monitored for absorbance at 260 nm.

Preparation of a Large Quantity of the Mutagen. A large amount of the mutagen, required for structural analysis, was isolated as follows, using the original compound described above as a standard marker. A total of 3 kg of blue cotton, prepared as described previously (*14*), was divided equally into 22 net bags, which were hung for 24 h in the Nikko River, then combined, and washed twice with distilled water. After undergoing dehydration in a spin-dryer, the adsorbed materials were extracted by being stirred once in 18 L of methanol/ammonia water (50:1, v/v) for 3 h and twice more in 12 L of the same solution. The extract was then evaporated to dryness, and the residue (2.2 g) was dissolved in 30 mL of methanol, filtered through a glass filter, and applied to a Sephadex LH-20 column (50 × 870 mm, Pharmacia, Uppsala, Sweden). The materials were eluted with methanol, and fractions of 85.5 mL each were collected. Fractions at elution volumes of 2300−2560 mL were combined and evaporated. The residue (44.2 mg) was dissolved in 2 mL of methanol and applied again to a Sephadex LH-20 column (23 × 1360 mm) with methanol as a mobile phase, and fractions of 3.4 mL each were collected. Fractions containing the mutagen, eluting at elution volumes of 442−479 mL, were combined and evaporated. The residue, dissolved in 300 μL of methanol, was finally purified by HPLC on a semipreparative YMC-Pack ODS-AM 324 column, followed by HPLC on a semipreparative TSK gel ODS-120A column (10 μm particle size, 7.8 × 300 mm, Toso Corp). The mobile phases of 63 and 65% methanol were pumped in isocratically for the YMC-Pack ODS-AM 324 column and the TSK gel ODS-120A column, respectively, at a flow rate of 2 mL/min. The mutagen was found in the peak fractions with retention times of 28−30 and 26−28 min, respectively. Using the above processes, we obtained about 120 μg of the mutagen.

Detection of the Mutagen in Water Samples from Rivers Other Than the Nikko River. Sample collection was carried out at the Asuwa River in Fukui in March 1998 and at the Katsura and Nishitakase rivers in Kyoto in May 1998. At each sampling site, 15 g of blue rayon was hung for 24 h in the river and then collected and treated as described above. Blue rayon extracts were separated by HPLC on a semipreparative YMC-Pack ODS-AM 324 column with a mobile phase of 75% methanol at a flow rate of 1.6 mL/min. Fractions with a retention time of 19−20 min were evaporated to dryness, dissolved in 400 μL of 50% methanol, and further applied to a CAPCELL PAK C_{18} column. A mobile phase of 35% acetonitrile in 25 mM Tris-HCl buffer (pH 6.5) was pumped in at a flow rate of 1 mL/min. Fractions with a retention time of 20−21 min were evaporated to dryness, and residues were dissolved in 400 μL of 50% methanol and applied to a YMC-Pack ODS-A 303 column with a mobile phase of 35% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 1.0 mL/min. Peaks

due to the same mutagen as that isolated from the Nikko River were detected at 19.5 min.

All HPLC procedures were carried out at ambient temperature, and the eluates were monitored for absorbance at 260 nm using a Shimadzu SPD-10AV spectrometric detector (Kyoto, Japan).

Spectral Measurement. UV absorption spectra were measured with a Tosoh PD-8020 photodiode array detector and a Beckman DU 640 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were taken of solutions in chloroform-*d* and DMSO-d_6 with a JEOL JNM-GSX270 (^{1}H, 270 MHz; ^{13}C, 67.5 MHz) or a JNM-GSX500 (^{1}H, 500 MHz,; ^{13}C, 125 MHz) Fourier transform spectrometer. Chemical shifts are shown in parts per million using tetramethylsilane as an internal standard. High-resolution mass spectra were measured using a JEOL JMS-DX300 or a JEOL JMS-AX505w mass spectrometer, equipped with a direct inlet system. Fast atom bombardment mass spectra (FAB-MS, matrix, *m*-nitrobenzyl alcohol) were measured with a JEOL JMS-SX103 mass spectrometer. All melting points were obtained on a Yazawa micro melting point apparatus (5Y-1) and are given as uncorrected values.

Synthesis of 3-[(2-Hydroxyethyl)amino]-4-methoxynitrobenzene. A solution of 3-amino-4-methoxynitrobenzene (15.6 g, 0.1 mol; Tokyo Kasei Co., Tokyo, Japan), benzaldehyde (15.9 g, 0.15 mol), and *p*-toluenesulfonic acid (0.1 g) in benzene (200 mL) was heated under reflux in a Dean-Stark apparatus for 3 h with azeotropic distillation of water. The solvent was removed under reduced pressure to yield a crude product, *N*-benzylidene-2-methoxy-5-nitroaniline, to which was added 2-iodoethanol (25.8 g, 0.15 mol; Tokyo Kasei Co.) . The mixture was stirred at 80 °C for 5 h. After the mixture was cooled, 100 mL of water was added, and the mixture was heated at 100 °C with stirring for 1 h. The aqueous layer was separated, and the organic layer was extracted with 2 N HCl. To remove the resultant benzaldehyde, we extracted the combined aqueous layer three times with 50 mL of ether, alkalinized the aqueous layer with sodium hydroxide, and then extracted it with dichloromethane. The organic solution was dried over magnesium sulfate and concentrated under reduced pressure to yield an oily residue, which was subjected to column chromatography on silica gel (E. Merck Co., Darmstadt, Germany) (eluent, 2% methanol in chloroform) to yield a solid (9.54 g, 45%). MS *m*/*z*: 212 (M^+). ^{1}H NMR ($CDCl_3$): δ 1.82 (1H, t, J = 5.2 Hz, OH), 3.39 (2H, q, J = 5.2 Hz, CH_2), 3.92 (2H, q, J = 5.2 Hz, CH_2), 3.95 (3H, s, OCH_3), 4.77 (1H, br, t, NH), 6.77 (1H, d, J = 8.8 Hz, ArH), 7.41 (1H, d, J = 2.9 Hz, ArH), 7.65 (1H, dd, J = 2.9, 8.8 Hz, ArH). ^{13}C NMR ($CDCl_3$): δ 45.44, 56.05, 60.98, 103.90, 108.07, 113.59, 138.36, 142.42, 151.86.

Synthesis of 3-[(2-Hydroxyethyl)amino]-4-methoxyacetanilide. A solution of trifluoroacetic anhydride (12.6, 60 mmol; Wako Pure Chemical Industries Co., Osaka, Japan) in dichloromethane (10 mL) was added dropwise to a stirred mixture of 3-[(2-hydroxyethyl)amino]-4-methoxynitrobenzene (4.2 g, 20 mmol) and potassium carbonate (6 g) in dichloromethane (100 mL) at room temperature. After the mixture was stirred for 1 h, insoluble material was filtered off. The filtrate was washed with cooled brine and dried over magnesium sulfate. After removal of the solvent under reduced pressure, an oily residue including the *N*,*O*-bistrifluoroacetylated nitrobenzene derivative was used in the next step without further purification.

A mixture of the crude product described above and 5% palladium on charcoal (330 mg; Wako Co.) in ethyl acetate (100 mL) was stirred overnight under a hydrogen atmosphere. The catalyst was removed by filtration. Potassium carbonate (1 g) was added to the filtrate, and then acetic anhydride (3 g, 30 mmol) was added dropwise to the stirred mixture in an ice bath. After being stirred for an additional hour, the solvent was removed under reduced pressure to yield an oily residue including the *N*,*O*-bistrifluoroacetylated acetanilide derivative, to which methanol (20 mL) and potassium carbonate (0.5 g) were added. The mixture was stirred for 2 h in an ice bath. After removal of the solvent, the oily residue was subjected to column chromatography on silica gel (eluent, AcOEt) to yield colorless crystals (670 mg, 15%). Mp: 133−134 °C. MS *m*/*z*: 224 (M^+). ^{1}H NMR (DMSO-d_6): δ 1.98 (3H, s, $COCH_3$), 3.07 (2H, q, J = 5.8 Hz, CH_2), 3.62 (2H, q, J = 5.8 Hz, CH_2), 3.75 (3H, s, OCH_3), 4.75 (1H, t, J = 5.8 Hz, OH or NH), 4.77 (1H, t, J = 5.8 Hz, OH or NH), 6.67 (1H, d, J = 8.7 Hz, ArH), 6.79 (1H, br, ArH), 6.80 (1H, dd, J = 2.4, 8.7 Hz, ArH), 9.54 (1H, br, NH). ^{13}C NMR (DMSO-d_6): δ 23.84, 45.32, 55.36, 59.32, 101.66, 106.32, 109.38, 133.23, 137.96, 142.48, 167.45.

Synthesis of 2-[(2-Bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[(2-hydroxyethyl)amino]acetanilide (AZO DYE-3). The azo coupling reaction of 2-bromo-4,6-dinitrobenzenediazonium sulfate [prepared from 2-bromo-4,6-dinitroaniline (2.9 g, 11 nmol; Aldrich Co., Milwaukee, WI), sodium nitrite (760 mg, 11 mmol), and concentrated sulfuric acid (20 mL)] with 3-[(2-hydroxyethyl)amino]-4-methoxyacetanilide (2.2 g, 10 mmol) was carried out as described previously (*6*) to yield a green powder (4.2 g, 85%). Mp: 254−256 °C. HRMS for $C_{17}H_{17}BrN_6O_7$ *m*/*z*: found, 496.0332; calcd, 496.0341. ^{1}H NMR (DMSO-d_6): δ 2.20 (3H, s, $COCH_3$), 3.39 (2H, q, J = 5.7 Hz, CH_2), 3.65 (2H, q, J = 5.7 Hz, CH_2), 4.95 (1H, t, J = 5.7 Hz, OH), 7.16 (1H, s, ArH), 7.70 (1H, t, J = 5.7 Hz, NH), 7.78 (1H, s, ArH), 8.64 (1H, d, J = 2.0 Hz, ArH), 8.65 (1H, d, J = 2.0 Hz, ArH)., 9.1 (1H, br, CONH). ^{13}C NMR (DMSO-d_6): δ 24.60, 45.29, 55.44, 58.66, 97.41, 115.40, 119.53, 121.24, 128.18, 130.11, 132.14, 140.11, 142.50, 144.26, 147.04, 149.85, 168.29.

Synthesis of 2-[2-(Acetylamino)-4-[(2-hydroxyethyl)-amino]-5-methoxyphenyl]- 6-amino-4-bromo-2*H*-benzotriazole (non-ClPBTA-3). Sodium hydrosulfite (10 g; Kanto Chemical Co., Tokyo, Japan) was added in small portions to a vigorously stirred solution of AZO DYE-3 (5 g, 10 mmol) in 500 mL of mixed solvent (3:1:1 tetrahydrofuran/methanol/water) at room temperature. The mixture was stirred for an additional hour, insoluble material was filtered off, and the resulting mixture was washed with tetrahydrofuran. The filtrate was concentrated to $^1/_3$ of its original volume under reduced pressure, and the solution was extracted with dichloromethane. The organic solution was washed with brine and dried over magnesium sulfate. Removal of the solvent under reduced pressure yielded a dark-colored oil, which was subjected to column chromatography on silica gel (eluent, 2% methanol in chloroform) and Sephadex LH-20 (eluent, methanol) to yield a yellow powder (230 mg, 5.3%). Mp: 172−174 °C. HRMS for $C_{17}H_{19}BrN_6O_3$ *m*/*z*: found, 434.0704; calcd, 434.0701. UV max (MeOH): 220, 264, 393 nm. ^{1}H NMR (DMSO-d_6): δ 2.03 (3H, s, $COCH_3$), 3.19 (2H, q, J = 5.5 Hz, CH_2), 3.68 (2H, q, J = 5.5 Hz, CH_2), 3.89 (3H, s, OCH_3), 4.83 (1H, t, J = 5.5 Hz, OH or NH), 5.27 (1H, t, J = 5.5 Hz, OH or NH), 5.64 (2H, s, NH_2), 6.72 (1H, d, J = 1.7 Hz, ArH), 7.23 (1H, d, J = 1.7 Hz, ArH), 7.28 (1H, s, ArH), 7.36 (1H, s, ArH), 10.22 (1H, br, CONH). ^{13}C NMR (DMSO-d_6): δ 24.13, 45.10, 55.73, 59.13, 92.34, 103.70, 105.03, 109.11, 119.26, 123.25, 125.22, 137.66, 138.77, 142.70, 145.33, 148.61, 167.86.

Synthesis of 2-[2-(Acetylamino)-4-[(2-hydroxyethyl)-amino]- 5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3). An aqueous solution of sodium hypochlorite (1 mL, available chlorine minimum of 1%; Wako Co.) was added dropwise to a stirred solution of non-ClPBTA-3 (130 mg, 0.3 mmol) in dichloromethane (30 mL) at room temperature. After the solution was stirred for 5 min, the organic layer was separated, washed with brine, and dried over magnesium sulfate. The solvent was removed under reduced pressure to yield a pale yellow solid, which was subjected to medium-pressure liquid chromatography (Nippon Seimitsu Kagaku Co., Tokyo, Japan) with a silica gel column (eluent, 2% methanol in chloroform) and then with an ODS silica gel column (eluent, 70% methanol) to yield a yellow powder (105 mg, 75%). Mp: 231−234 °C. UV max (MeOH): 219, 264 (sh), 381 nm. FABMS *m*/*z*: 468, 470, 472 (M^+, 74:100:29 relative abundance). ^{1}H NMR (DMSO-d_6): δ 1.99 (3H, s, $COCH_3$), 3.19 (2H, q, J = 5.6 Hz, CH_2), 3.65 (2H, t, J = 5.6 Hz, CH_2), 3.89 (3H, s, OCH_3),

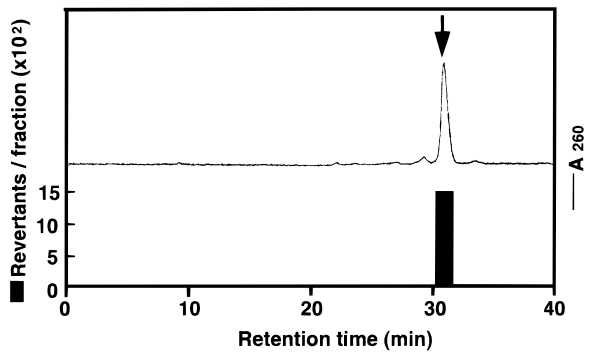


Figure 2. Purification of a new PBTA-type mutagen by HPLC. Mutagenic fractions from a YMC-Pack ODS-A 303 column with retention times of 16−17 min were purified on a CAPCELL PAK C_{18} ODS column. The mutagen was obtained at a retention time of 31 min. The UV absorbance and mutagenicity are shown by the upper line and lower bar, respectively.

4.87 (1H, br, OH or NH), 5.33 (1H, t, J = 5.6 Hz, OH or NH), 5.93 (2H, s, NH_2), 7.13 (1H, s, ArH), 7.32 (1H, s, ArH),7.40 (1H, s, ArH), 10.03 (1H, br, CONH). ^{13}C NMR (DMSO-d_6): δ 23.81, 45.05, 55.79, 59.10, 96.16, 104.23, 105.34, 108.00, 119.74, 122.66, 125.41, 137.55, 139.10, 142.87, 142.90, 144.09, 167.97.

Mutagenesis Assay. All test samples were dissolved in 100 μL aliquots of 50% dimethyl sulfoxide, and mutagenicity was examined by the preincubation method (*15*) using *S. typhimurium* YG1024 in the presence of an S9 mix. *S. typhimurium* YG1024, produced by introducing plasmids containing the acetyltransferase gene from TA1538 into TA98 (*16*) was kindly provided by T. Nohmi from the National Institute of Health Sciences, Tokyo. The S9 mix contained 5 μL of S9 (25 mg of protein/mL), prepared from livers of male Sprague-Dawley rats intraperitoneally administered phenobarbital and 5,6-benzoflavone, at a total volume of 500 μL. Mutagenic activities of test samples were calculated from the linear portions of the dose−response curves, obtained with four or five doses, and duplicate plates, in two independent experiments.

Results and Discussion

Isolation of a Mutagen from Water of the Nikko River. The materials adsorbed to 1 g of blue rayon from the water of the Nikko River were found to induce 3 900 000 revertants of *S. typhimurium* YG1024 in the presence of an S9 mix. Mutagens extracted from 25 g of blue rayon were separated by HPLC on a semipreparative YMC-Pack ODS-AM 324 column using a gradient solvent system. Many fractions showed mutagenicity, and the fractions recovered at this HPLC step showed 85% of the total mutagenicity. Among them, the most potent mutagenic activity was observed in the fraction with retention times of 13−20 min, and this fraction accounted for 34% of the total mutagenicity of the blue rayon-adsorbed material. Mutagenic activity in each fraction with other retention times was much lower than that in the fraction with retention times of 13−20 min. Two minor mutagenic fractions were also observed at retention times of 24−28 and 20−24 min, which corresponded to those of PBTA-1 and PBTA-2, accounting for 1.2 and 4.5% of the total mutagenicity, respectively.

A mutagenic fraction with retention times of 13−20 min was successively purified by HPLC on a YMC-Pack ODS-A 303 column and a CAPCELL PAK C_{18} ODS column. A single UV absorption peak exhibiting mutagenicity was observed at a retention time of 31 min on the CAPCELL PAK C_{18} ODS column (Figure 2), and this mutagen accounted for 11% of the total mutagenicity of the blue rayon-adsorbed material. Figure 3 shows the UV absorption spectrum of this mutagen, obtained on the second YMC-Pack ODS-A 303 column with a photodiode array detector. UV absorption maxima were observed at 219, 264 (sh), and 381 nm. Several other mutagenic fractions were also observed at the second HPLC step on a YMC-Pack ODS-A 303 column; however, each of their contributions to the total mutagenicity was much smaller than that of the above isolated compound.

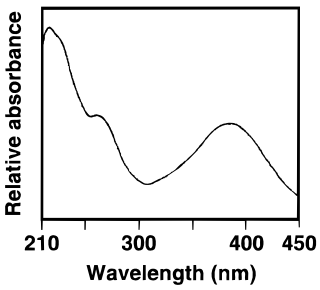


Figure 3. UV absorption spectrum of the mutagen on the second YMC-Pack ODS-A 303 column with a photodiode array detector. The material was eluted with 38% acetonitrile in 25 mM phosphate buffer (pH 2.0).

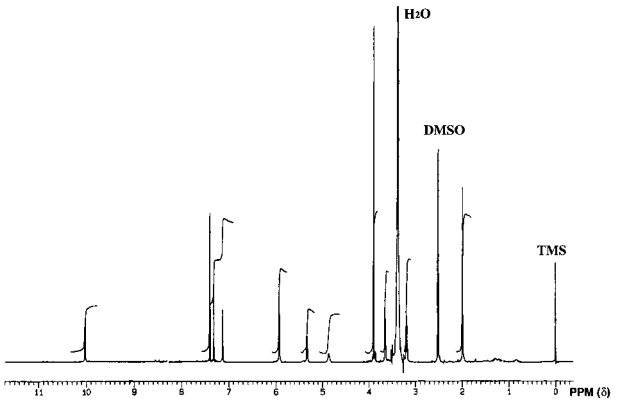


Figure 4. ^{1}H NMR spectrum of the mutagen in DMSO-d_6.

With the new mutagen isolated from the adsorbate to blue rayon as a marker, about 120 μg of the mutagen could be isolated by Sephadex LH-20 column chromatography and HPLC on semipreparative ODS columns from materials adsorbed to 3 kg of blue cotton.

Structural Analysis of the Mutagen. The UV absorption spectrum of the new mutagen shown in Figure 3 is similar to those of PBTA-1 and PBTA-2 (*6*, *8*). Analysis using high-resolution mass spectrometry indicated the molecular formula to be $C_{17}H_{18}BrClN_6O_3$ (m/z: found, 468.0320; calcd, 468.0312). Compared with the structure of PBTA-1, the mutagen has four fewer carbon atoms, eight fewer protons, and one fewer oxygen atom but the same number of nitrogen atoms.

Figure 4 shows the ^{1}H NMR spectrum of the mutagen in DMSO-d_6, indicating the presence of 18 protons in the molecule, which is consistent with the results of high-resolution mass spectrometry. Aromatic protons and several other protons were observed at chemical shifts similar to those of PBTA-1 as shown in Figure 5, suggesting the presence of similar substituents at the same positions. For example, broad singlets at 5.93 (2H) and 10.03 ppm (1H) indicated the presence of exchangeable protons due to a primary amino and an amido group, respectively. Two singlets at 1.99 (3H) and 3.89 ppm (3H) suggested acetyl and methoxy groups, respectively. The

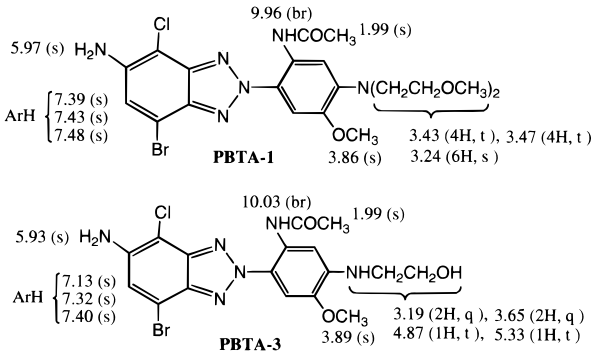
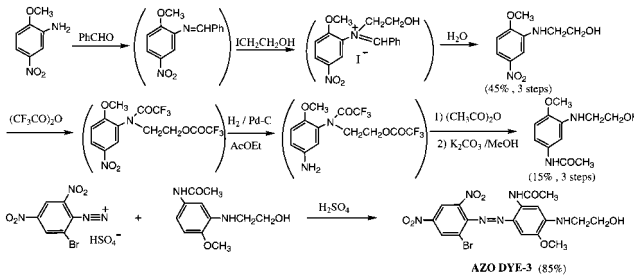


Figure 5. Chemical structures of PBTA-1 and PBTA-3. ^{1}H NMR chemical shifts in DMSO-d_6 are presented as ppm. s = singlet, t = triplet, q = quartet, br = broad.

Scheme 1. Synthesis of AZO DYE-3

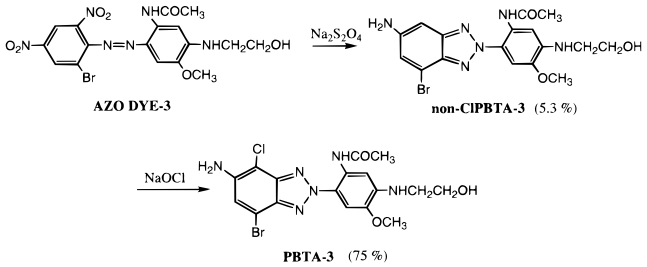


absorbance pattern due to aromatic protons at 7.13, 7.32, and 7.40 ppm was similar to that of PBTA-1. On the other hand, the triplets at 5.33 (1H) and 4.87 ppm (1H) and quartets at 3.19 (2H) and 3.65 ppm (2H) predicted the presence of a hydroxyethylamine moiety ($NHCH_2CH_2$-OH), where both hydroxy and amine protons display coupling with the adjacent methylene protons instead of with $N(CH_2CH_2OCH_3)_2$ for PBTA-1.

On the basis of results of the spectral analyses described above, the chemical structure of the mutagen was deduced to be PBTA-3 (Figure 5). To further confirm its structure, we conducted synthesis from 2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[(2-hydroxyethyl)amino]-acetanilide (AZO DYE-3) by the method used for PBTA-1 (*9*). AZO DYE-3 was prepared by the coupling reaction of 2-bromo-4,6-dinitrobenzenediazonium sulfonate with 3-[(2-hydroxyethyl)amino]-4-methoxyacetanilide prepared as shown in Scheme 1. The molecular formula, $C_{17}H_{17}BrN_6O_7$, of synthesized AZO DYE-3 was determined by high-resolution mass spectrometry (*m*/*z*: found, 496.0332; calcd, 496.0341). In the ^{1}H NMR (DMSO-d_6) spectrum, peaks due to four aromatic protons were observed as two singlets at 7.16 and 7.78 ppm and two meta doublets (J = 2.0 Hz) at 8.64 and 8.65 ppm, suggesting that azo coupling occurred at the 6 position of the acetanilide derivative. Two methylene protons appeared as A_2B_2X system quartets at 3.39 and 3.65 ppm. Peaks due to protons of acetamide, amino, and hydroxyl groups were observed at 9.1 (br), 7.70 (t), and 4.95 ppm (t), respectively.

As shown in Scheme 2, sodium hydrosulfite reduction of AZO DYE-3 yielded a 2-phenylbenzotriazole skeleton product, with a UV absorption pattern similar to that of PBTA-1, at a yield of 5.3%. The high-resolution mass spectrum of this product indicated a molecular formula of $C_{17}H_{19}BrN_6O_3$ (*m*/*z*: found, 434.0704; calcd, 434.0701), which corresponded to a compound in which a chlorine atom of PBTA-3 is replaced with a hydrogen. In the ^{1}H

Scheme 2. Synthesis of PBTA-3



NMR (DMSO-d_6) spectrum, aromatic protons were observed as two meta doublets (J = 1.7 Hz) at 6.72 and 7.23 ppm of the benzotriazole ring protons and two singlets at 7.28 and 7.36 ppm of the 2-phenyl moiety. Two quartets (J = 5.5 Hz) due to ethylene protons were observed at 3.19 and 3.68 ppm. Protons of acetamide and primary amine were noted at 10.22 (br) and 5.64 ppm (s), respectively. Two triplets (J = 5.5 Hz) of 5.27 and 4.83 ppm were assigned to protons of secondary amino and hydroxy groups. Two singlets at 2.03 and 3.89 ppm were due to methyls of acetyl and methoxy groups, respectively. On the basis of these findings and the ^{13}C NMR spectrum, this compound was determined to be non-ClPBTA-3.

The monochlorinated product, PBTA-3, was produced at a yield of 75% from a nonchlorinated derivative of PBTA-3 (non-ClPBTA-3) by treatment with sodium hypochlorite. The UV, MS, and ^{1}H NMR spectra of the prepared compound matched those of the mutagen, PBTA-3, isolated from water samples from the Nikko River. The ^{13}C NMR spectrum was also consistent. Thus, we concluded that the third PBTA-type mutagen isolated from the Nikko River is PBTA-3.

Detection of PBTA-3 in Water from Rivers Other Than the Nikko River. To investigate the distribution of PBTA-3 in water of other rivers in Japan, we collected water samples as blue rayon-adsorbed products from the Asuwa River flowing through Fukui Prefecture and the Katsura and Nishitakase rivers in Kyoto Prefecture. These rivers flow through the regions where dyeing industries have been developed. The blue rayon extracts were separated by HPLC on a YMC-Pack ODS-AM 324 column. Fractions with retention times of 19−20 min were further analyzed with CAPCELL PAK C_{18} and YMC-Pack ODS-A 303 columns. The retention times of single peaks observed with both columns were identical to those of PBTA-3, and the UV−spectra also matched. The concentrations of PBTA-3 detected in the Asuwa, Katura, and Nishitakase rivers were 38, 35, and 22 ng/g of blue rayon, respectively. Using the same method, we found the level of PBTA-3 in the Nikko River to be 140 ng/g of blue rayon.

In the present study, we identified a new mutagen, PBTA-3, as a major PBTA-type mutagen in water of the Nikko River. PBTA-3 induced 81 000 and 3 000 000 revertants/μg of *S. typhimurium* TA98 and YG1024, respectively; it is a strain originating from TA98 with a high *O*-acetyltransferase activity in the presence but not in the absence of an S9 mix. This mutagenic potency is comparable to those of PBTA-1 and PBTA-2 (*6*, *8*). Furthermore, we successfully synthesized PBTA-3 from the azo compound, AZO DYE-3, through non-ClPBTA-3. Although AZO DYE-3 is not listed as an industrial material, as are AZO DYE-1 (*9*) and AZO DYE-2 (*8*), it

is possible that it is formed by dehydroxyethylation of the azo dye, 2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[bis(2-acetoxyethyl)amino]acetanilide (Color Index Name, Disperse Blue 79:1), which is very commonly used in dyeing factories. Similar dehydroxyethylation was reported to occur for tri-, di-, and monoethanolamines by treatment with hypochlorous acid or chlorine dioxide (*17*). We also detected a small amount of the acetic acid ester of AZO DYE-3 in commercially available Disperse Blue 79:1, suggesting contamination of AZO DYE-3 or its ester in the process of industrial production.

The reduction agent sodium hydrosulfite is generally used in dyeing factories for discharge printing and bleaching, while the chlorination reagent sodium hypochlorite is employed in sewage plants for disinfection. AZO DYE-3 is thought to be converted to the corresponding 2-phenylbenzotriazole derivative, non-ClPBTA-3, with reducing reagents such as hydrosulfite during industrial dyeing processes. Therefore, PBTA-3 could be produced from non-ClPBTA-3 by subsequent chlorination during disinfection in sewage plants before being discharged into rivers. Since various dinitrophenylazo compounds are known to be used as industrial materials, it is plausible that other PBTA-type mutagens are also formed from azo dyes and contaminate the river water near dyeing factories. Quantification of the levels of these mutagens in the water of various rivers and determination of their carcinogenic effects as well as genotoxic activities in organisms other than *Salmonella* strains are necessary to fully estimate the impact of PBTA compounds on human health. Moreover, it is important to design new methods that prevent the formation of PBTA compounds and enhance its degradation.

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