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Mutation Research 492 (2001) 73–80

Genetic Toxicology and Environmental Mutagenesis

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Identification of 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4) as a potent mutagen in river water in Kyoto and Aichi prefectures, Japan

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Received 25 December 2000; received in revised form 7 February 2001; accepted 7 February 2001

Abstract

We have previously isolated five mutagens in blue rayon-adsorbed substances from water at a site below sewage plants in the Nishitakase River, in Kyoto, Japan, and identified two of them 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-[(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2). In the present study, we collected adsorbed materials on blue cotton (3 kg × 9 times) at the same location, and isolated a sufficient amount (97 μg) of one of the remaining three mutagens other than PBTA-1 and PBTA-2, for structural analysis, by multiple column chromatography. The structure of mutagen, accounting for 12% of the total mutagenicity of the blue rayon-adsorbed substances, was determined to be a PBTA-1 analogue, 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4). PBTA-4 is a potent mutagen, inducing 190,000 and 7,800,000 revertants of *Salmonella typhimurium* TA98 and YG1024 per microgram, respectively, in the presence of S9 mix. In addition to the water of the Nishitakase River, PBTA-4 was detected in water samples from two rivers that flow through other regions where textile-dyeing

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1383-5718/01/$ – see front matter
PII: S1383-5718(01)00147-4

industries have been developed. Like other PBTA analogues, PBTA-4 might also be produced from azo dyes during industrial processes in dyeing factories and treatment at sewage plants.

Keywords: Mutagen; PBTA-4; Azo dye; Blue cotton; River water

1. Introduction

Genotoxic compounds from industrial and domestic effluents are often discharged into surface waters such as rivers [1]. Water of the Yodo River, which serves as the main supply of drinking water for people living in the Osaka area, Japan, has been shown to be markedly mutagenic to *Salmonella typhimurium* TA98 and YG1024 with S9 mix [2–5]. We previously investigated blue rayon-adsorbed substances from the Nishitakase River, a tributary of the Yodo River, to clarify their chemical structures, and isolated five mutagens, compounds I–V. Among them, compounds I and II have been identified as 2-[2- (acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) [6] and 2-[2-(acetylamino)-4-[(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2) [7], respectively. PBTA-1 and PBTA-2 accounted for 21 and 17% of the total mutagenicity of the blue rayon-adsorbed substances, respectively. Based on synthesis studies, both PBTA compounds would be expected to have been formed from corresponding dinitrophenylazo dyes, used as industrial materials [7,8]. Dinitrophenylazo dyes might be converted to the non-chlorinated derivatives of PBTA compounds (non-ClPBTAs) in which a chlorine atom of PBTA compound is replaced with a hydrogen atom, by treatment with reducing reagents such as sodium hydrosulfite, employed in textile-dyeing processes. PBTA compounds could then be formed from the non-ClPBTAs by chlorination reagents such as sodium hypochlorite during treatment of industrial effluent at sewage plants, and discharged into rivers. This hypothesis is supported by results of quantification of PBTA-1 and PBTA-2 in the Yodo River system [9]. In addition, we have isolated another PBTA-type mutagen, 2-[2-(acetylamino)-4-[(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3), from the river water sample of the Nikko River in Aichi prefecture, Japan [10]. A similar route for PBTA-3 formation could be suggested, as for the PBTA-1 and PBTA-2 cases.

As described above, we previously determined the structures of two out of five mutagens, compounds I–V, which were isolated from the Nishitakase River in Kyoto. In the present study, among the three mutagens whose structures remained unclear, compound IV, which accounted for 12% of the total mutagenicity of blue rayon-adsorbed substances [6], could be collected in sufficient quantities for its structural analysis. As a result, it was determined to be 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4), by spectral analyses and chemical synthesis. The mutagenicity of PBTA-4 was comparable with those of the three previously identified PBTA-type mutagens. The distribution of PBTA-4 in river waters other than the Nishitakase River and the possible formation route are also described.

2. Materials and methods

2.1. Isolation of compound IV from river water

Compound IV was originally isolated from the adsorbate on 24 g of blue rayon, which was hung in the river for 24 h at a site downstream of two sewage plants along the Nishitakase River, by HPLC as previously reported [6]. Using this original sample as a standard marker, a large amount of compound IV sufficient for structural determination was purified from river water samples, collected with 27 kg (3 kg × 9 times) of blue cotton from March 1995–June 1996 [6]. The materials adsorbed to each 3 kg of blue cotton, prepared as described previously [11], were extracted by stirring in a methanol/ammonia water solution, and the extract was then evaporated to dryness. The residue (2.6 g) was dissolved in 20 ml of methanol, filtered through a glass filter and applied to a Sephadex LH-20 column (50 mm × 877 mm, Pharmacia, Uppsala, Sweden). The materials were eluted

with methanol and the fractions at elution volumes of 2710–3220 ml were found to contain compound IV. These fractions were combined and evaporated. The residue (61 mg) was dissolved in 1.0 ml of methanol, and then applied again to a Sephadex LH-20 column (12 mm × 1322 mm) with methanol as a mobile phase. The fractions that contained compound IV were eluted at elution volumes of 215–221 ml, and then combined and evaporated. The residue was dissolved in 250 μl of methanol and finally purified by HPLC on a semi-preparative YMC-pack ODS-AM 324 column (5 μm particle size, 10 mm × 300 mm, YMC Co. Ltd., Kyoto). The mobile phase of 75% methanol was pumped in isocratically at a flow rate of 2 ml/min, and compound IV was found in peak fractions with retention times of 16–18 min. The above processes were repeated nine times to give 97 μg of compound IV.

The presence of a peak corresponding to authentic compound IV was confirmed by HPLC on an analytical YMC-Pack ODS-A 303 column (5 μm particle size, 4.6 mm × 250 mm; YMC Co. Ltd.) with a mobile phase of 35% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 1 ml/min.

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

2.2. 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 (1H: 500 MHz, 13C: 125 MHz) Fourier transform spectrometer. The following abbreviations are used: s: singlet, d: doublet, t: triplet, q: quartet, br: broad. Chemical shifts are shown in ppm 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.

2.3. Synthesis of 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide

The azo coupling reaction of 2-bromo-4,6-dinitrobenzenediazonium sulfate, which was prepared from 2-bromo-4,6-dinitroaniline (2.6 g, 10 mmol), sodium nitrite (800 mg, 11 mmol) and concentrated sulfuric acid (10 ml), with 3-amino-4-methoxyacetanilide (1.8 g, 10 mmol) was carried out in a manner similar to that described previously [8] to yield a green powder (4.08 g, 90%), mp: 240°C (decomp.). FABMS *m/z*: 453 ($M^{+}+1$, ^{79}Br), 455 ($M^{+}+1$, ^{79}Br). ^{1}H-NMR (DMSO-d_6) δ: 2.17 (3H, s, $NCOCH_3$), 3.40 (2H, br, NH_2), 3.84 (3H, s, OCH_3), 7.12 (1H, s, ArH), 7.80 (1H, s, ArH), 7.90 (1H, br, CONH), 8.66 (1H, d, J = 2.3 Hz, ArH), 8.68 (1H, d, J = 2.3 Hz, ArH).

2.4. Synthesis of 2-(2-acetylamino-4-amino-5-methoxyphenyl)-6-amino-4-bromo-2H-benzotriazole (non-ClPBTA-4)

Iron powder (3 g, 53 mmol) and magnesium chloride·$6H_2O$ (6.4 g, 31 mmol) were added to a solution of 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide (2.3 g, 5 mmol) in 200 ml of a mixed solvent (tetrahydrofuran/methanol/water = 2/2/1). The mixture was stirred at 80°C for 1 h. Insoluble materials were filtered off and washed with tetrahydrofuran. The filtrate was concentrated to 1/3 of its original volume under reduced pressure and extracted with dichloromethane. The organic solution was washed with brine and dried over magnesium sulfate. Removal of the solvent under reduced pressure gave 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 (150 mg, 7.7%), mp > 240°C. FABMS *m/z*: 391 ($M^{+}+1$, ^{79}Br), 393 ($M^{+}+1$, ^{81}Br). ^{1}H-NMR ($CDCl_3$) δ: 2.25 (3H, s, $NCOCH_3$), 3.95 (3H, s, OCH_3), 3.96 (2H, br, NH_2), 4.13 (2H, br, NH_2), 6.88 (1H, d, J = 2.0 Hz, ArH), 7.14 (1H, d, J = 2.0 Hz, ArH), 7.69 (1H, s, ArH), 8.04 (1H, s, ArH), 11.11 (1H, br, CONH). ^{13}C-NMR (DMSO-d_6) δ: 30.25, 55.65, 92.34, 105.85, 107.72, 109.10, 119.54, 123.23, 124.97, 137.61, 138.78, 142.47, 145.29, 148.56, 167.64.

2.5. Synthesis of PBTA-4

An aqueous solution of sodium hypochloride (1 ml, available chlorine minimum of 1%) was added dropwise to a stirred solution of non-ClPBTA-4 (40 mg, 0.1 mmol) in dichloromethane (100 ml) at room temperature. After stirring for 1 h, 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 column chromatography on Sephadex LH-20 (eluent: methanol) to yield a yellow powder (35 mg, 82%), mp > 230°C. UV max (MeOH): 219, 264, 389 nm. FABMS *m/z*: 425 (M^++1, ^{79}Br, ^{35}Cl) 427 (M^++1, ^{81}Br, ^{35}Cl & ^{79}Br, ^{37}Cl) 429 (M^++1, ^{81}Br, ^{37}Cl). ^{1}H-NMR ($CDCl_3$) δ: 2.26 (3H, s, $COCH_3$), 3.97 (3H, s, $COCH_3$), 4.16 (2H, brs, NH_2), 4.34 (2H, brs, NH_2), 7.21 (1H, s, ArH), 7.74 (1H, s, ArH), 8.06 (1H, s, ArH), 11.13 (1H, brs, NHCO). ^{13}C-NMR (DMSO-d_6) δ: 30.26, 55.63, 96.15, 106.00, 107.92, 119.78, 122.59, 124.74, 127.78, 137.45, 139.14, 142.54, 144.02, 151.35, 167.66.

2.6. Detection of the mutagen in water samples from rivers other than the Nishitakase River

Samples were collected from the Nikko River in Aichi in September 1997 and from the Uji River in Kyoto in February 1998. At each sampling site, 50 g of blue cotton was hung for 24 h in the river and then collected and treated by the method described above. Blue cotton extracts were separated by Sephadex LH-20 column chromatography (12 mm × 1322 mm) with methanol as a mobile phase. The fractions at elution volumes of 215–221 ml were evaporated to dryness and the residue was dissolved in methanol and finally purified by HPLC on a semi-preparative YMC-pack ODS-AM 324 column. The mobile phase of 75% methanol was pumped in isocratically at a flow rate of 2 ml/min.

The presence of a peak corresponding to standard PBTA-4 was confirmed by HPLC on an analytical YMC-Pack ODS-A 303 column (5 μm particle size, 4.6 mm × 250 mm; YMC Co. Ltd.) with a mobile phase of 35% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 1 ml/ min.

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

2.7. Mutagenesis assay

All test samples were dissolved in 100 μl of 50% dimethyl sulfoxide and examined for mutagenicity by the preincubation method [12] using *S. typhimurium* TA98, TA100, YG1024 and YG1029 with or without S9 mix. *S. typhimurium* YG1024 and YG1029, produced by introducing plasmids containing the acetyltransferase gene from TA1538 into TA98 and TA100, respectively [13], were kindly provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo. The S9 mix contained 5 μl of S9 (25 mg protein/ml) (Kikkoman Co. Ltd., Noda, Japan), which was prepared from the livers of male Sprague–Dawley rats treated with phenobarbital and 5,6-benzoflavone, in a total volume of 500 μl. Mutagenic activities of the test samples were calculated from the linear portions of the dose-response curves, obtained with four or five doses, with duplicate plates, in two independent experiments.

3. Results and discussion

3.1. Isolation and structural analysis of compound IV

From material adsorbed to 27 kg of blue cotton, mutagenic compounds were separated by Sephadex LH-20 column chromatography twice and finally compound IV (97 μg) was isolated as a single UV absorption peak by HPLC on semi-preparative ODS columns, as shown in Fig. 1. The UV absorption spectrum of compound IV was found to be similar to those of PBTA-1, PBTA-2 and PBTA-3 [6,7,10], suggesting possession of a 2-phenylbenzotriazole skeleton (Fig. 2). Analysis of compound IV, using high-resolution mass spectrometry, indicated its molecular formula to be $C_{15}H_{14}BrClN_6O_2$ (*m/z*, 424.0050, calcd. 424.0051). Compared with the structure of PBTA-1, compound IV has the same number of nitrogen atoms, but six less carbon atoms, 12 less protons and two less oxygen atoms.

The ^{1}H-NMR spectrum of compound IV in chloroform-*d* indicated the presence of 14 protons in the molecule, and most of the chemical shifts

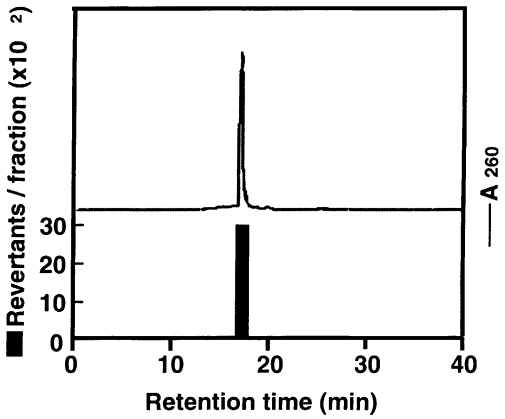


Fig. 1. Purification of mutagenic compound IV by HPLC. Mutagenic fractions from the second Sephadex LH-20 column with elution volumes of 215–221 ml were purified on a YMC-Pack ODS-AM 324 column. A single UV absorption peak showing mutagenicity was detected at a retention time of 17 min. The UV absorbance and mutagenicity are shown by the upper line and lower bar, respectively.

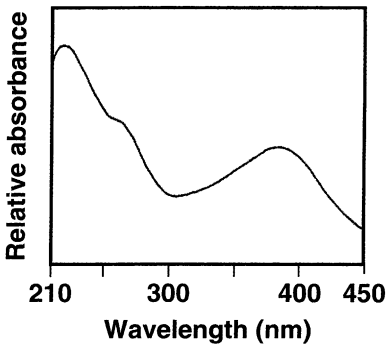


Fig. 2. UV absorption spectrum of the mutagen on the first YMC-Pack ODS-AM 324 column with a photodiode array detector. The material was eluted with 75% methanol.

were similar to those of PBTA-1, as shown in Fig. 3. Three aromatic protons were observed at 7.22, 7.74 and 8.06 ppm as singlets, and two singlets at 2.26 (3H) and 3.97 (3H) ppm were assigned to acetyl and methoxy groups, respectively. Exchangeable protons due to a primary amino and an amido groups were observed as broad singlets at 4.33 (2H) and 11.12 (1H) ppm, respectively. Moreover, a broad singlet of an exchangeable proton was observed at 4.15 (2H) ppm, indicating the presence of a primary amino group instead of the $-N(CH_2CH_2OCH_3)_2$ in PBTA-1. Based on the results of the spectral analyses described above, the chemical structure of the mutagen was deduced to be PBTA-4 (Fig. 3).

To confirm the structure of compound IV, PBTA-4 was synthesized from an azo dye, 5-amino-2-

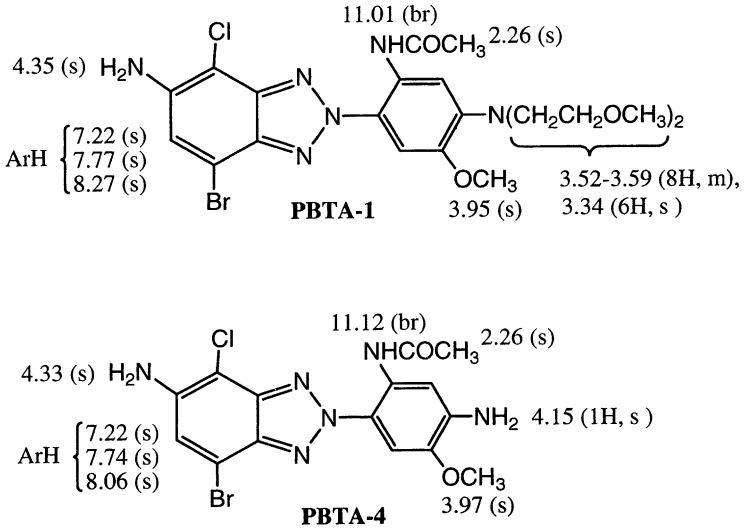
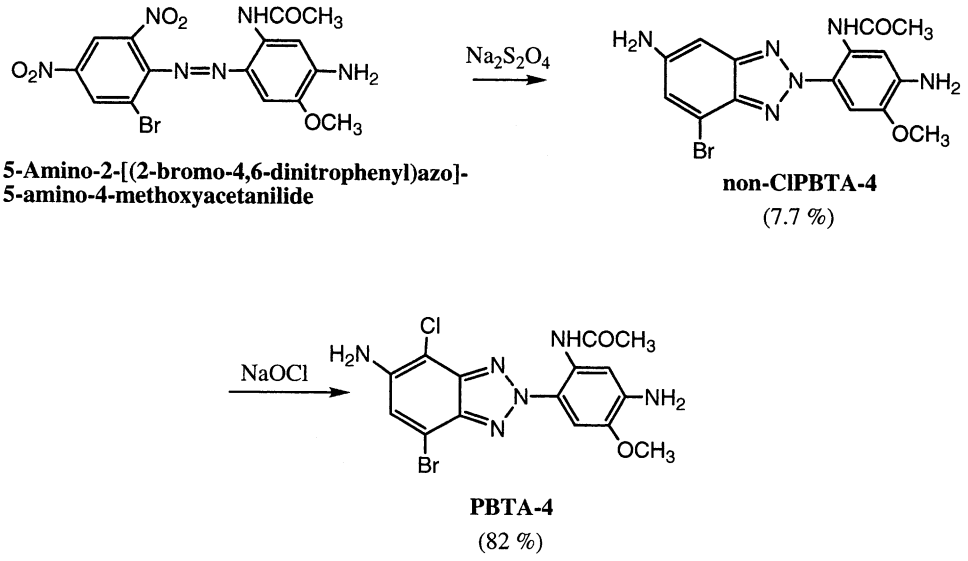


Fig. 3. Chemical structures of PBTA-1 and PBTA-4. ^{1}H-NMR chemical shifts in chloroform-*d* are presented as ppm; s: singlet, t: triplet, q: quartet, br: broad.



5-Amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide

non-ClPBTA-4
(7.7 %)

PBTA-4
(82 %)

Scheme 1. Synthesis of PBTA-4.

[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide, according to the method used for PBTA-1 [6], as shown in Scheme 1.

Iron powder reduction of 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide yielded a 2-phenylbenzotriazole skeleton product, non-ClPBTA-4, at a yield of 7.7%. As reported previously [8], synthesis of non-ClPBTA-4 was also carried out by the reduction of 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide with sodium hydrosulfite, with a yield down to 1.5%. The monochlorinated product, PBTA-4, was produced from the non-chlorinated derivative (non-ClPBTA-4) by treatment with sodium hypochlorite, at a yield of 82%. The UV, MS and ^{1}H-NMR spectra of the synthesized compound, PBTA-4, were the same as those of compound IV, isolated from water samples from the Nishitakase River. The ^{13}C-NMR spectrum was also consistent with the corresponding structure. Thus, we concluded the new PBTA-type mutagen isolated from the Nishitakase River to be PBTA-4.

3.2. Detection of PBTA-4 in water of rivers other than the Nishitakase River

To investigate the distribution of PBTA-4 in rivers other than the Nishitakase River, water samples were collected as blue cotton-adsorbed materials from the Nikko River and the Uji River in Aichi and Kyoto prefectures, respectively. These rivers flow through regions where dyeing industries have been developed. The blue cotton extracts were separated by Sephadex LH-20 column chromatography, and fractions corresponding to authentic PBTA-4 were then further purified by HPLC on a YMC-Pack ODS-AM 324 column. A single UV absorption peak was observed at a retention time of 17 min. The retention time and the UV spectrum of each peak fraction were identical to those of PBTA-4. Moreover, the retention time of the peak fraction from each river sample on a YMC-Pack ODS-A 303 column was 26 min, which is the same as that of authentic PBTA-4. Thus, PBTA-4 was detected in the water samples from the Nikko and Uji Rivers, at concentrations of 21 and 33 ng/g of blue cotton, respectively. Under the same separation conditions, the level of PBTA-4 in the Nishitakase River was estimated to be 32 ng/g of blue cotton.

3.3. Mutagenicities of PBTA-4, non-ClPBTA-4, and the azo dye

In the presence of S9 mix, synthesized PBTA-4, non-ClPBTA-4, and the azo dye, 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyac-

Table 1
Mutagenicities of azo dye, non-ClPBTA-4 and PBTA-4 in four *Salmonella* strains with S9 mix

Compound	Revertants[a]/μg			
	TA98	TA100	YG1024	YG1029
Azo dye[b]	600	19	2700	750
Non-ClPBTA-4	950	35	370000	1400
PBTA-4	190000	310	7800000	15000

[a] Numbers of revertants are averages obtained from two independent experiments.

[b] Azo dye: 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide.

etanilide were all mutagenic in four strains (TA98, TA100, YG1024 and YG1029) of *S. typhimurium*, as shown in Table 1. PBTA-4 induced 190,000 revertants for *S. typhimurium* TA98, 310 for TA100,7,800,000 for YG1024 and 15,000 for YG1029 per microgram in the presence of S9 mix. Compared to TA98 and TA100, YG1024 and YG1029, which originate from TA98 and TA100, respectively, with a high *O*-acetyltransferase activity, were more sensitive to PBTA-4. Therefore, acetyltransferase seems to be required for the mutagenicity of PBTA-4. In addition, PBTA-4 showed a higher mutagenicity in *S. typhimurium* TA98 and YG1024, detectors of frame-shift mutations, than in *S. typhimurium* TA100 and YG1029, detectors of base-pair-change mutations. The mutagenic potency was found to be about twice as high as those of PBTA-1, PBTA-2 and PBTA-3 [7,10]. Non-Cl PBTA-4 and the azo dye were also mutagenic in the four strains in the presence of S9 mix, again especially high in the strains detecting frame-shift mutations. The mutagenic activities of PBTA-4 in TA98 and YG1024 were 200 and 21 times higher than those of non-ClPBTA-4, and 320 and 900 times higher than those of the azo dye, respectively. In the absence of S9 mix, none of the three compounds were mutagenic in any of the strains, at up to 10 μg.

We have successfully synthesized PBTA-4 from the azo compound, 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide, through non-ClPBTA-4. In the present study, synthesis of non-ClPBTA-4 was carried out with the reducing reagent sodium hydrosulfite, as well as iron powder, and sodium hypochlorite was used for the chlorination of non-ClPBTA-4. Sodium hydrosulfite is used for discharge printing in dyeing factories, and the chlorinating reagent sodium hypochlorite is used as a disinfecting agent in sewage plants. The azo dye used for PBTA-4 synthesis, 5-amino-2-[(2-bromo-4,6 -dinitrophenyl)azo]-5-amino-4-methoxyacetanilide, is listed as an industrial material [14], like other azo dyes, which are possible precursors of PBTA-1 and PBTA-2 [7,8]. Therefore, PBTA-4 could be formed from the azo dye 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide during industrial activity and disinfecting processes at sewage plants, and then discharged into rivers. In addition, the possibility that 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide is formed by the dehydroxyethylation of azo dyes, such as 2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[bis(2-acetoxyethyl)amino]acetanilide (Color Index Name, Disperse Blue 79:1), cannot be ruled out, because similar dehydroxyethylation has been reported to occur for ethanolamines on treatment with hypochlorous acid or chlorine dioxide [15]. Since dinitrophenylazo dyes such as Disperse Blue 79:1 are very popular in dyeing factories throughout the world, it is plausible that the formation of PBTA-4 from azo dyes results in pollution not only of river water in Japan, but also worldwide, wherever there are textile-related industries.

In order to estimate the influence of PBTA compounds on the aquatic ecosystem and human health, quantification of the levels of these mutagens in the water of various rivers and determination of their biological activities, including carcinogenicity in rodents, are needed. Furthermore, the development of new methods that prevent the formation and promote the degradation of PBTA compounds is very important.

Acknowledgements

This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan and funds under a contract with the Environment Agency of Japan.

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