Identification of a New Mutagenic Polychlorinated Biphenyl Derivative in the Waka River, Wakayama, Japan, Showing Activation of an Aryl Hydrocarbon Receptor-Dependent Transcription

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Water samples from the Waka River, which runs through an area housing many chemical industry facilities in Wakayama, Japan, have been found to show significant mutagenicity, especially without a mammalian metabolic activation system (S9 mix) in the *Salmonella typhimurium* YG1024 strain. Mutagens in the river water were adsorbed to 3 kg of blue cotton, extracted with methanol/ammonia, and separated by several low- and high-pressure liquid chromatography steps with reversed-phase columns. One mutagen (0.6 mg), accounting for 50% of the total mutagenicity of the adsorbed materials, was isolated. On the basis of the mass, high-resolution mass, ^{1}H NMR and ^{13}C NMR spectra, the chemical was determined to have a polychlorinated biphenyl skeleton with nitro and amino substitution groups. Well-designed chemical synthesis of the putative mutagen revealed it to be 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl. This novel compound exerted strong mutagenicity without the S9 mix, inducing 66 000 and 140 000 revertants/nmol in *S. typhimurium* TA98 and YG1024, respectively. Moreover, this polychlorinated biphenyl derivative was proven to activate the human aryl hydrocarbon receptor-mediated transcription in a *lac Z* reporter gene assay with an efficiency almost the same as that of *β*-naphthoflavone, well-known to be a synthetic aryl hydrocarbon receptor agonist. It is possible that the mutagen is formed unintentionally via postemission modification of drainage water containing parent chemicals, such as 3,3′-dichlorobenzidine or 3,3′-dichloro-4,4′-dinitrobiphenyl, which are known to be raw materials in the manufacture of polymers and dye intermediates in chemical plants.

Introduction

In recent years, many countries have been burdened with serious problems concerning water pollution (*1*−*6*). This has adverse effects on aquatic organisms and may also pose a risk to human health because of the contamination of drinking water (*4*−*6*). The large-volume manufacture and commercial use of synthetic organic chemicals have resulted in their widespread dispersion as aquatic environmental contaminants from domestic, industrial, and agricultural sources (*2*, *3*, *7*). Moreover, postemission modification may complicate the chemical composition of environmental water. Several contaminating compounds have been identified to be mutagens or carcinogens; however, it is pointed out that there is a great possibility of the presence of many other unrecognized examples in an aquatic environment (*8*).

Polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene and benz[*a*]anthracene, and heterocyclic amines, such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)[1] and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), have been detected in concentrates of river water using blue cotton or blue rayon (*9*−*12*). We have recently identified five 2-phenylbenzotriazole (PBTA) compounds (i.e., 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) (*13*), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)-ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2) (*14*), 2-[2-(acetylamino)-4-[(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3) (*15*), 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4) (*16*), and 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6) (*17*)) as major mutagens in river water samples collected using blue cotton or blue rayon at sites in geographically different areas where textile-related industries are con-

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[1] Abbreviations: Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]-indole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; PBTA, 2-phenylbenzotriazole; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; COSY, correlation spectroscopy; PCB, polychlorinated biphenyl.

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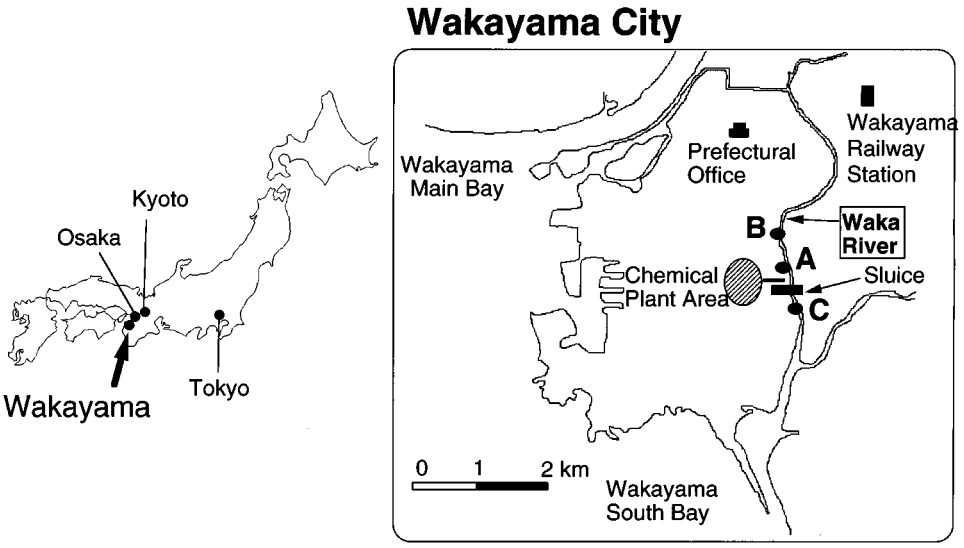


Figure 1. Geographic locations of the water sampling sites on the Waka River in Wakayama City, Japan.

centrated in Japan. These polycyclic aromatic hydrocarbons, heterocyclic amines, and PBTA derivatives require metabolic activation by the S9 mix in order to exert mutagenicity in the *Salmonella* assay. While 1-nitropyrene, which shows mutagenicity without the S9 mix, was detected in concentrates of river water using the XAD-2 resin column method (*18*), its contribution to the total mutagenicity under these conditions was only 1% without the S9 mix. The chemical structures of other major mutagens in river water remain unclear.

During assessment of the mutagenicity of river water in Japan, we found that blue rayon-adsorbed material taken at sites along the Waka River in Wakayama showed strong mutagenicity toward *Salmonella typhimurium* YG1024 in the absence of the S9 mix. In this study, we isolated a major mutagen in the water samples and identified it as a novel compound, 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl. The mutagenicity and aryl hydrocarbon receptor (AhR)/aryl hydrocarobon receptor nuclear translocator (Arnt)-mediated transcriptional activity of this compound and its distribution, along with its possible origin, are also described in this paper.

Experimental Procedures

Chemicals. Blue rayon was obtained from Funakoshi Co. Ltd. (Tokyo, Japan), and blue cotton was prepared according to the method reported previously (*19*). HPLC grade acetonitrile and methanol were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Ozone was generated with a Nippon Ozone Co. Ltd. type ON-1-2 apparatus at a rate of 10 mmol/h with an oxygen flow of 10 L/h and an applied voltage of 80 V. All other chemicals used were of analytical grade.

Instrumentation. Melting points were determined on a Yanagimoto hot stage apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FT-IR DR 8000/8100 infrared spectrophotometer, and only prominent peaks in the region 2000−700 cm^{-1} were recorded. ^1H NMR and ^{13}C NMR spectra were obtained in $CDCl_3$ with Varian Gemini-200 (200 MHz for ^1H NMR) and JEOL-JNM-A500 (500 MHz for ^1H NMR; 75.45 MHz for ^{13}C NMR) spectrometers. Electron impact mass spectra (EI-MS) were recorded on a Shimadzu GC−MS QP-2000A spectrometer at 70 eV and chemical ionization mass spectra (CI-MS) on a Shimadzu GC−MS QP-5000 with DI-50 using isobutane as a reacting gas. An HPLC system, consisting of a Shimadzu LC10A pump and an SPD10A UV spectrometer, was used for mutagen purification and quantification.

Mutagenicity Assay. The mutagenicity assay was carried out by the preincubation method (*20*, *21*), using *S. typhimurium* TA98 (*20*), TA100 (*20*), YG1021 (*22*), YG1024 (*23*), and YG1029 (*23*). YG1021 is derived from TA98 and has elevated nitroreductase activity (*22*), whereas YG 1024 and YG1029 are derivatives of TA98 and TA100, respectively, and have elevated *O*-acetylation activity (*23*). The S9 mix contained 50 μL of S9, prepared from livers of male Sprague−Dawley rats treated with phenobarbital and β-naphthoflavone, in a total volume of 500 μL. Mutagenic activities of test samples were calculated from the linear portions of dose−response curves obtained with four or five doses with duplicate plates in two independent experiments.

AhR/Arnt-Mediated *lac Z* Reporter Gene Assay. *Saccharomyces cerevisiae* YCM3, expressing the human AhR, the aryl hydrocarbon receptor nuclear translocator (Arnt), and a *lac Z* reporter plasmid containing the aryl hydrocarbon response element (pTXRE-Z) system, were provided by Dr. C. A. Miller, III, (Tulane University, New Orleans, LA) (*24*). Briefly, YCM3 is a derivative of W303a, engineered with genomically integrated human AhR and Arnt genes and a stable reporter plasmid containing five aryl hydrocarbon response elements (*24*). The β-galactosidase assay for YCM3 has been described elsewhere (*24*).

Collection of Water Samples. Sample collection was carried out in August 1997 at the three sites in the Waka River shown in Figure 1. At each sampling site, 15 g of blue rayon (5 g × 3) was hung in the river for 24 h. Then, the recovered blue rayon was washed with distilled water several times. The materials adsorbed to the blue rayon were extracted by shaking in 80 mL of methanol/ammonia (50:1) per gram of blue rayon for 30 min twice. The extracts were combined and evaporated to dryness, and the residue was dissolved in dimethyl sulfoxide for assay of mutagenicity.

Isolation of a Mutagen in the Waka River. To obtain large amounts of mutagens, 3 kg of blue cotton (150 g × 20) was hung in the river for 24 h at point A in Figure 1, and the recovered blue cotton was washed twice with 40 L of distilled water. After the excess water was removed in a spin-drier, the adsorbed materials were extracted with 60 L of methanol/ammonia (50:1) (20 L × 3). The extracts were combined and evaporated, and the residue was suspended in methanol and filtered. The filtrate was then evaporated to dryness to give 1.0 g of residue. Mutagens in this sample were purified by low- and high-pressure liquid chromatography under the following conditions. First, the sample in 60% acetonitrile was applied to a COSMOSIL 40 C_{18}-PREP column (1.0 × 43 cm) (Nacalai Tesque, Kyoto, Japan), and the materials were eluted with a mobile phase of 60% acetonitrile at a flow rate of 2.5 mL/min. Eluate was

collected, and an aliquot of each was tested for mutagenicity in *S. typhymurium* YG1024 without the S9 mix. The major mutagenic activity was observed in the fractions eluted from 8 to 12 min. These mutagenic fractions were collected, evaporated, applied to a TSK-GEL ODS-120A column (7.8 × 300 mm, Tosoh, Co., Tokyo, Japan), and then eluted with a mobile phase of 65% acetonitrile at a flow rate of 2.5 mL/min. Fractions with retention times of 15−20 min, showing mutagenicity, were further purified using a LUNA 5μ phenyl-hexyl column (4.6 × 250 mm, Phenomenex, Co., Torrance, CA) with a mobile phase of 60% acetonitrile at a flow rate of 0.7 mL/min. Mutagenic fractions with retention times of 20−22 min were finally applied to an STR ODS-II column (4.6 × 250 mm, Shinwa Chemical Ind., Kyoto, Japan), and the materials were eluted with 60% acetonitrile at a flow rate of 0.7 mL/min. A single peak with mutagenicity was detected at a retention time of 26.5 min.

Synthesis Procedures. (1) 3,3′-Dichlorobenzidine (1). 3,3′-Dichlorobenzidine dihydrochloride (5 g; Sigma Chemical Co., St. Louis, MO) was dissolved in 80% aqueous ethanol under an argon atmosphere at 50 °C (*25*). After 30 min, 4.6 g of anhydrous potassium carbonate was added in small portions with stirring, which was then continued for an additional 30 min. The precipitate was collected, dried under vacuum, and used immediately in the next step.

(2) 3,3′-Dichloro-4,4′-dinitrobiphenyl (2) (*25*). 3,3′-Dichlorobenzidine **1** (5 g) was added in small portions to a mixture of acetic acid (100 mL) and 30% hydrogen peroxide (30 mL) at 100 °C. A mixture of acetic acid (50 mL) and hydrogen peroxide (15 mL) was then added, and the reaction mixture was stirred for 10 h under reflux. After being cooled, the yellow precipitate was collected (50%): yellow powder, mp 224−225 °C; IR (KBr) ν (cm^{-1}): 1591, 1572, 1518, 1338; ^1H NMR (200 MHz, δ): 7.6 (dd, J = 8.4, 1.9 Hz, 2H, H = 6, 6′), 7.7 (d, J = 1.9 Hz, 2H, H = 2, 2′), 8.0 (d, J = 8.4 Hz, 2H, H = 5, 5′); MS m/z (%): 312 (28), 314 (18), 282 (18) 284 (12), 220 (26), 222 (14), 224 (11), 150 (100).

(3) 4-Amino-3,3′-dichloro-4′-nitrobiphenyl (3). A methanol solution of sodium hydrosulfide was added dropwise to a suspension of 3,3′-dichloro-4,4′-dinitrobiphenyl **2** (2 g) in toluene and methanol (1:5, v/v, 50 mL) (*26*). After the starting material disappeared on TLC analysis, the reaction mixture was poured into water and extracted with chloroform. The extract was washed with water and dried over sodium sulfate, and the solvent was evaporated in vacuo to leave a residue, used for the next step without further purification. For comfirmation of the structure of the aminobiphenyl compound **3**, a small portion of the residue was purified by recrystallization from ethyl acetate: yellow powder, mp 166−167 °C; IR (KBr) ν (cm^{-1}): 1589, 1572, 1460, 1334; ^1H NMR (200 MHz, δ): 6.8 (d, J = 8.4 Hz, H = 5), 7.3 (dd, J = 8.4, 2.0 Hz, 1H, H = 6′), 7.5 (m, 2H, H = 2, 6), 7.6 (d, J = 2.0 Hz, 1H, H = 2′), 7.9 (d, J = 8.4 Hz, 1H, H = 5′); MS m/z (%): 282 (87), 252 (54), 201 (100).

(4) 4-Acetylamino-3,3′-dichloro-4′-nitrobiphenyl (4). Crude 4-amino-3,3′-dichloro-4′-nitrobiphenyl **3** was dissolved in dichloromethane. To this solution were added dropwise triethylamine (0.3 mL) followed by acetyl chloride (0.5 mL), and the reaction mixture was poured into water. The organic layer was washed with water, dried over sodium sulfate, and evaporated in vacuo to afford an oily residue. This was suspended with a small amount of ethyl acetate and filtered to give almost pure 4-acetylamino-3,3′-dichloro-4′-nitrobiphenyl **4**: yellow powder, mp 241−242 °C; IR (KBr) ν (cm^{-1}): 1699, 1522, 1334, 1307; ^1H NMR (200 MHz, δ): 2.2 (s, 3H, CH_3), 7.5 (dd, J = 8.4, 2.1 Hz, 1H, H = 6′), 7.6 (dd, J = 8.6, 1.9 Hz, 1H, H = 6), 7.7 (d, J = 2.0 Hz, 2H, H = 2, 2′), 8.0 (d, J = 8.4 Hz, 1H, H = 5), 8.5 (d, J = 8.4 Hz, 1H, H = 5′); MS m/z (%): 324 (10), 282 (39), 201 (100).

(5) 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl (6). To a solution of 4-acetylamino-3,3′-dichloro-4′-nitrobiphenyl **4** (100 mg) in acetonitrile (30 mL) kept at 0 °C was added 1 mL of nitrogen dioxide. Ozonized oxygen was passed through this solution slowly, as described previously (*27*). After 10 min, the reaction mixture was evaporated. The resulting oily residue containing 4-acetylamino-3,3′-dichloro-5,4′-dinitrobiphenyl **5** was then dissolved in a mixture of hydrochloric acid and methanol and stirred under reflux. After the reaction was complete, the organic material was extracted with chloroform. The organic layer was dried and evaporated, and the residue was chromatographed using silica gel to give a yellow powder. Spectral data are shown in the Results section.

Table 1. Mutagenicities of Water Samples from the Waka River[a]

sampling site	YG1024 (revertants/g of blue rayon)	
	−S9 mix	+S9 mix
A	1 150 000	301 000
B	28 800	45 200
C	270	3 400

[a] Sample collection was carried out on August 21, 1997.

Quantification of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl (6) in the Waka River. Water samples (1 L) were collected at the three sites shown in Figure 1 on August 4, 2000. Each was passed through a glass column (2 × 5 cm) with 20 mL of Supelpack 2 resins (Sigma Chemical Co., St. Louis, MO) at a flow rate of 10 mL/min. The resins were then rinsed with 100 mL of distilled water twice, and adsorbed materials were eluted with 100 mL of methanol twice. The eluates were combined and evaporated to dryness, and the residues were dissolved in 0.5 mL of 85% methanol and separated by HPLC using a semipreparative YMC-pak ODS-AM324 column (10 × 300 mm, YMC Co., Kyoto, Japan) with 85% methanol as a mobile phase at a flow rate of 1.6 mL/min. Fractions corresponding to 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl **6** with retention times of 23−24 min were further purified on a LUNA 5μ phenyl-hexyl column (4.6 × 250 mm). A mobile phase of 55% acetonitrile was pumped in at a flow rate of 1 mL/min, and fractions corresponding to **6** with retention times of 24−25 min were then applied to an analytical STR ODS-II column (4.6 × 250 mm) with a mobile phase of 55% acetonitrile at a flow rate of 1 mL/min. A single peak due to **6** was detected at a retention time of 25.5 min.

All HPLC procedures were carried out at ambient temperature, and the UV−vis absorption spectra of eluates were monitored for structural confirmation with a Shimadzu SPD-M10AV photodiode array detector.

Results

Isolation of a Mutagen from Water of the Waka River. Data for mutagenicities of concentrate samples of the Waka River water in *S. typhimurium* YG1024 with or without the S9 mix are summarized in Table 1. The sample from site A showed the most potent mutagenicity (1 150 000 revertants/g of blue rayon) in *S. typhimurium* YG1024 in the absence of the S9 mix. The mutagenicity of the same sample with the S9 mix is around 4 times less than that without the S9 mix. Without the S9 mix, the mutagenicities of samples collected at site B, downstream of site A, were lower than that from site A. The sample collected at site C, which is on the opposite side of the sluice from site A, exhibited weak mutagenicity without the S9 mix. The mutagenic potencies of samples from sites B and C were increased by the addition of the S9 mix, but their potencies were less than 10% of the activity found for the sample from site A in the absence of the S9 mix. The mutagenicities of samples collected at sites A−C in *S. typhymurium* YG1029 with or without the S9 mix were much lower than those in *S. typhimurium* YG1024 (data not shown).

For the isolation and structural determination of mutagens which did not require S9 mix activation, sample collection was performed at site A using 3 kg of blue cotton, and 1.1 g of concentrate was obtained. This

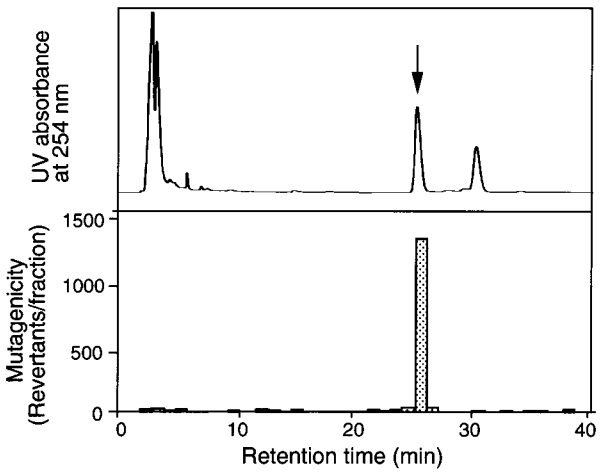


Figure 2. Purification of the mutagen by HPLC. Mutagenic fractions from a LUNA 5μ phenyl-hexyl column with retention times of 20−22 min were purified on an STR ODS-II column. A mutagen was eluted at a retention time of 26.5 min as a single peak, indicated by an arrow. Its UV absorbance and mutagenicity are shown by the upper line and lower bar, respectively.

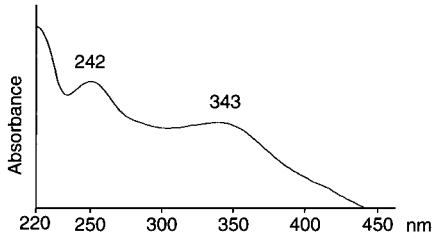


Figure 3. UV−vis absorption spectrum of the mutagen. The UV−vis spectrum was measured in 60% acetonitrile using a photodiode array detector.

concentrate sample exerted potent mutagenicity toward *S. typhimurium* YG1024 without the S9 mix, inducing 420 000 revertants/mg. First, this sample was separated by low-pressure liquid chromatography using a column packed with COSMOSIL 40C_{18}-PREP. Although several fractions exhibited mutagenicity without the S9 mix, the most potent mutagenic activity was observed with retention times of 8−12 min, which accounted for 65% of the total mutagenicity of the blue cotton adsorbate. The mutagenic fraction was then separated by HPLC successively on a semipreparative TSK-GEL ODS-120A column, a LUNA 5μ phenyl-hexyl column, and an STR ODS-II column, monitoring the mutagenic activity in *S. typhimurium* YG1024 without the S9 mix. A single UV peak exhibiting mutagenicity was observed at a retention time of 26.5 min on the STR ODS-II column (Figure 2), and this mutagenic fraction accounted for about 50% of the total mutagenicity of the concentrate from Waka River water without the S9 mix. A total of 0.6 mg of the mutagen was obtained from materials adsorbed on 3 kg of blue cotton.

Structural Analysis of the Mutagen. The UV−vis absorption spectrum of the mutagen isolated from the Waka River exhibited absorption maxima at 242 and 343 nm (Figure 3). As shown in Figure 4, the mass spectrum of this mutagen showed a parent ion peak at *m/z*: 327. From mass fragment patterns, this compound was presumed to have two nitro groups (*m/z*: 297 $[M - NO]^+$, 281 $[M - NO_2]^+$, 235 $[M - 2 \times NO_2]^+$), two chloro substituents (*m/z*: 200 $[M - 2 \times NO_2 - Cl]^+$, 164 $[M - 2 \times NO_2 - Cl - HCl]^+$), and one amino group (observed

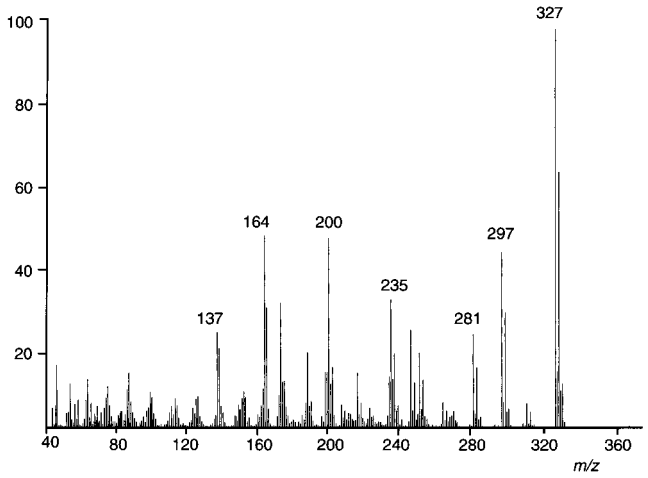


Figure 4. EI-MS spectrum of the mutagen.

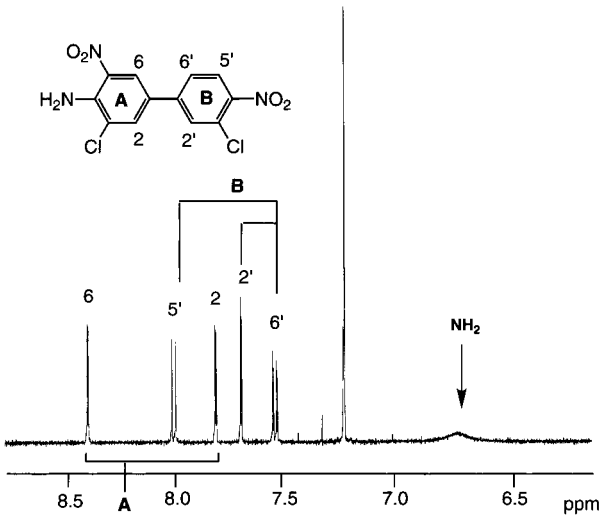


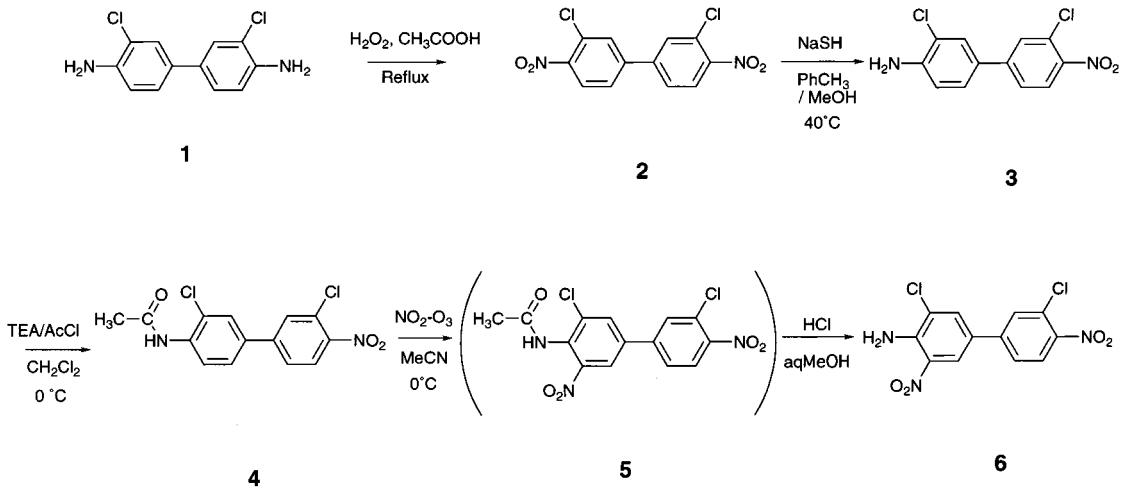
Figure 5. ^{1}H NMR spectrum of the mutagen in $CDCl_3$ and its chemical structure. Chemical shifts are reported in parts per million (ppm) with respect to tetramethylsilane as an internal standard. Proton assignment in aromatic ring systems A and B and also the amino proton (NH_2) are indicated.

as an HCN elimination, *m/z*: 137 $[M - 2 \times NO_2 - Cl - HCl - HCN]^+$). One chloride was eliminated as hydrogen chloride (HCl, $\Delta m/z$: 36) derived from *m/z*: 200−164. The elimination of a protonated chloride is usually observed only when chloride is positioned ortho to an amino group (*28*).

High-resolution MS indicated a molecular formula of $C_{12}H_7N_3O_4Cl_2$ (observed *m/z*: 326.9810, calcd: 326.9818). As described previously, this mutagen has two NO_2, two Cl, and one NH_2 substituent. The remaining $C_{12}H_5$ suggests the presence of a biphenyl moiety.

On the basis of the ^{1}H NMR and ^{1}H-^{1}H COSY spectra (see Supporting Information), this compound was shown to have five protons in the aromatic region (Figure 5). A downfield peak at δ: 8.4 was meta-coupled, with absorptions at δ: 7.8. A double-doublet peak at δ: 7.5 was ortho-coupled, with a signal at δ: 8.0, and also meta-coupled, with a doublet peak at δ: 7.7. A broad signal with integral height of two protons at around δ: 6.7 was proton-exchangeable by D_2O treatment, confirming the presence of an amino substituent. By ^{13}C NMR measurement, five proton-enhanced carbons in the aromatic region (δ: 133.3, 129.3, 126.6, 125.2, 123.6) were found, indicating an absence of aliphatic protons.

Scheme 1. Chemical Synthesis of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl[a]



[a] Abbreviations are as follows: MeOH, methanol; $PhCH_3$, toluene; TEA, triethylamine; MeCN, acetonitrile.

Table 2. Mutagenicity of Nitrobiphenyl Derivatives

	mutagenicity (revertants/nmol)[a]							
	TA98		TA100		YG1024		YG1021	
compound	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
2	90	10	2	3	640	80	97	50
3	80	40	4	30	410	130	80	40
4	160	50	20	30	3400	360	170	100
6	66 000	1 700	880	90	140 000	2900	7 700	3000

[a] Data for revertants are averages obtained from at least two independent experiments.

From these results, the mutagen was deduced to be 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (Figure 5).

Chemical Synthesis of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl. To confirm the chemical structure of the mutagen isolated from the Waka River, we independently synthesized the presumed mutagen (Scheme 1). 3,3′-Dichlorobenzidine **1** was oxidized by peracetic acid to give dinitrobiphenyl derivatives **2** (*25*). Compound **2** was smoothly monoreduced with sodium hydrosulfide in methanol/toluene system (*26*). The mono amine **3** was easily converted to acetylamide **4** by a conventional procedure. For the ortho-selective nitration of **4**, Kyodai nitration was used (*27*). This is a highly selective method for ortho nitration to an acetylamide group. The desired 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl **6** was obtained in moderate yield by acid hydrolysis of **5**. The NMR, UV, and mass spectral data and the HPLC retention time of **6** were perfectly coincident with those of the mutagen isolated from the Waka River.

Mutagenicity of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl (6) and Related Compounds. The mutagenicity of **6** and a series of nitrobiphenyls, **2**−**4**, which were obtained at each step of chemical synthesis of **6**, were assayed using *S. typhimurium* TA98, TA100, YG1021, and YG1024 (Table 2). These four compounds were mutagenic toward each strain, both with and without the S9 mix. Among them, **6** was the most potent mutagen toward each strain, and its activity in each case was higher without the S9 mix. Mutagenic activity of **6** in TA98, a detector of frame-shift mutations, was 75 times as high as that in TA100, a detector of base-pair change mutations. The mutagenicity of **6** without the S9 mix in YG1024 was 2-fold higher than that in TA98, suggesting that *O*-acetyltransferase is required for its mutagenicity. Compound **2** showed mutagenic activity toward each strain that was very similar to that in **3** without the S9 mix. In the absence of the S9 mix, the mutagenic activity of **4** was about twice as high as those of **2** or **3** in TA 98 and about 5 ∼ 8 times higher in YG1024, probably because of acetyl protection of the amino group which lowers the reduction potential of the nitro group at the 4-position.

Transactivation Activity of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl (6) to Human AhR. 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl **6** has a polychlorinated biphenyl (PCB) moiety, which would be predicted to bind to the human AhR (*29*−*31*). Thus, we tested this ability of **6** using a yeast YCM3 in which an AhR−Arnt complex system is expressed. Transactivation activity is shown as β-galactosidase activity, calculated from the absorbance of *o*-nitrophenol generated by this assay. As expected, **6** could activate the human AhR-mediated transcription in a dose-dependent manner, ranging from 0.1 to 1000 μM (Figure 6). Its transactivation activity was almost the same as that of β-naphthoflavone, used as a positive control. No *lac* Z activity was found with the yeast W303a transfected with a plasmid encoding *lac Z* but not possessing the AhR.

Amount of 4-Amino-3,3′-dichloro-5,4′-dinitrobiphenyl (6) in the Waka River. To assess the distribution of **6** in the Waka River, river water was collected at three sites, A−C, on August 4, 2000 (Figure 1), and the amounts of **6** detected by HPLC were corrected for the recoveries (65%) of the compound during the purification process in the river water samples. Compound **6** was detected in water samples collected at sites A and B, with the level in water from site B (1.5 ng/L) being 7% of that from site A (21.6 ng/L). The amount of **6** was less than the detection limit (0.1 ng/L) in water sample from site C.

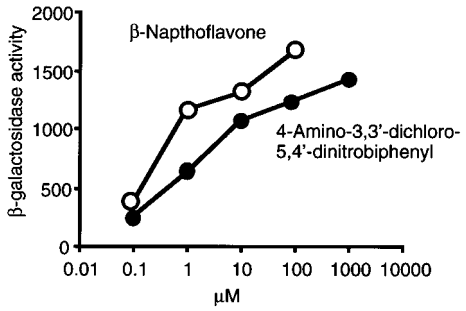


Figure 6. *Lac Z* reporter activity of yeast strain YCM3 after treatment with *β*-naphthoflavone (open circle) and 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (closed circle).

Discussion

In the present study, we identified a major mutagen in Waka River water as 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl **6**. This chemical is a novel polychlorinated biphenyl derivative, possessing amino and nitro substituents, and exerts potent mutagenicity and AhR transactivation activity. The mutagenic activity of **6** toward TA98 without the S9 mix is similar to that of 1,3,6-trinitropyrene and 4-nitro-*6H*-dibenzo[*b*,*d*]pyran-6-one, found in air-borne particulates (*32*, *33*). The mutagenicity of mono- or polynitrobiphenyls or biphenylamines has been widely studied over the past decade (*34*−*36*). Some trinitrobiphenyl or tetranitrobiphenyl derivatives have been reported to show mutagenic activity without the S9 mix (*35*, *36*), the degree depending on the positions of the nitro group(s). Substituents at both the 4- and 4′-positions and the lack of a substituent at the 2- or 2′-position appear necessary for enhanced mutagenicity (*35*, *36*). Consistent with these observations, the structure of 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl **6** certainly satisfies the requirements for exhibiting mutagenicity.

This novel compound has a PCB moiety and could have a coplanar conformation because of no substituent in the 2- or 2′-positions. Efficient transactivation of **6** to human AhR using the *lac Z* reporter gene assay might be caused by the coplanarity. Compounds **2**−**4** also have a coplanar PCB moiety and would be expected to transactivate human AhR. A recent study showed that this is the case for coplanar PCB, which suggests that it may disrupt the endocrine system in vivo (*37*).

The results of quantification of **6** suggested that this compound is discharged from chemical plants close to site A into the Waka River and is diluted, sedimentated, or decomposed while moving down the river. It could either be an intermediate in some chemical process or be formed spontaneously as a byproduct. 3,3′-Dichlorobenzidine **1** and 3,3′-dichloro-4,4′-dinitrobiphenyl **2**, used as starting materials for our synthesis of **6**, are known to be polymer and dye intermediates. Thus, it is possible that **6** is generated unintentionally via postemission modification of drainage water containing parent chemicals, such as 3,3′-dichlorobenzidine or 3,3′-dichloro-4,4′-dinitrobiphenyl which are known to be raw materials in the manufacture of polymers and dye intermediates in chemical plants. To evaluate the formation mechanisms, we are now attempting to determine the levels of the synthetic intermediates, including compounds **1** and **2** in the Waka River system. Furthermore, parent compounds **1** and **2** are also known to be widely distributed environmental contaminants (*38*); thus, it is quite possible that compound **6** is present in other rivers or in our environment. Further studies concentrating on their distributions in the natural environment are necessary to limit exposure of humans.

The Waka River runs through Wakayama city, and this river water is directly emitted to the sea. There might be a variety of aquatic biota exposed to this chemical. At present, the biological effects of compound **6**, especially to mammalians, are unknown. However, taking into account of the potential toxicity or carcinogenicity of 3,3′-dichlorobenzidine **1** and related compounds to mammalians, it is important to elucidate the biological effects of **6** or other synthetic intermediates on biota in the Waka River and its in vivo toxicity, including carcinogenicity in rodents.

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Supporting Information Available: $^1H-^1H$ COSY spectrum of the isolated mutagen. This information is available free of charge via the Internet at http://pubs.acs.org.

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