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Detection of Mutagenic 1-Chloro-3-, -6-, and -8-Nitropyrenes in Surface Soil Collected in Kyoto, Japan

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Mutagenic 1-chloro-3-nitropyrene (1,3-CNP), 1,6-CNP and 1,8-CNP were detected in surface soil collected in Kyoto, Japan. First, 1,3-, 1,6- and 1,8-CNPs were synthesized by chlorination of 1-nitropyrene. Mutagenic activities obtained by Ames assay using *Salmonella typhimurium* strain TA98 under conditions without S9 mix were 1.22 revertants per nmol for 1,3-CNP, 1.14 for 1,6-CNP and 0.83 for 1,8-CNP. Miller's aryl hydrocarbon receptor (AhR) yeast reporter gene assay showed that the three CNPs had no AhR ligand activity. Surface soil was collected in Kyoto, Japan and analyzed for 1,3-, 1,6- and 1,8-CNPs by two-dimensional high-performance liquid chromatography with fluorescence detection and a zinc on-line catalytic column. 1,3-CNP, 1,6-CNP and 1,8-CNP were detected in the ranges 9.2–13.8, 3.4–4.6 and 4.3–5.0 pg per gram of soil ($n = 2$), respectively.

Key words —— chloronitropyrene, soil, mutagenicity, aryl hydrocarbon receptor

INTRODUCTION

Nitroarenes are produced by the incomplete combustion of organic compounds such as fossil fuels,[1–3] and are also produced in the atmosphere from parent arenes and nitrogen oxides.[4–6] Nitroarenes are widespread environmental pollutants[7–9] and some are suspected human carcinogens.[10]

Recently, we reported that chlorination and nitration of pyrene occurred in the presence of a metal oxide such as titanium oxide under xenon lamp irradiation.[11,12] Since metal oxides are contained in surface soil, chlorination and nitration of pyrene could occur in surface soil. Therefore, it is necessary to determine the toxicity and occurrence of chloronitropyrenes (CNPs) in surface soil, to evaluate the risk to humans. However, there are no published reports on CNPs.

In this study, we synthesized 1-chloro-3-nitropyrene (1,3-CNP), 1,6-CNP and 1,8-CNP (Fig. 1) by chlorination of 1-nitropyrene (1-NP). Then, we assayed their mutagenic activities by Ames test using *Salmonella typhimurium* strain TA98 and aryl hydrocarbon receptor (AhR) ligand activities using the yeast recombinant assay established by Miller *et al*.[13–15] Finally, we analyzed 1,3-, 1,6- and 1,8-CNPs in surface soil collected in Kyoto, Japan, using a two-dimensional high-performance liquid chromatograph (HPLC) with a fluorescence detector and a zinc on-line catalytic column.

MATERIALS AND METHODS

Equipment —— Melting points (mp) were measured with a Yanaco MP micro-melting-point apparatus without correction. ^{1}H-NMR and ^{1}H-^{1}H correlation spectroscopy (COSY) spectra were measured on a Varian UNITY INOVA 400NB (400 MHz) spectrometer and chemical shifts δ are reported in ppm. Mass spectra (MS) were measured on a Shimadzu GCMS-QP5050A gas chromatograph/mass spectrometer equipped with a J&W DB-5MS (30 m × 0.25 mm × 0.25 μm) capillary column. An HPLC system for the separation of CNP isomers consisted of a Shimadzu LC-6A pump, a Rheodyne 7125 sample injector, a Shimadzu CTO-6A column oven and a Shimadzu SPD-6A UV detector. An HPLC system for the clean-up of surface soil consisted of

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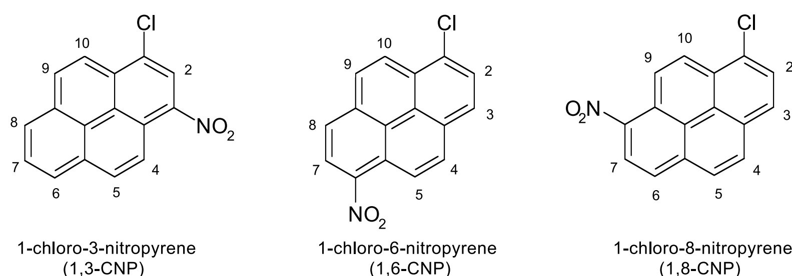

Fig. 1. Structures of CNPs

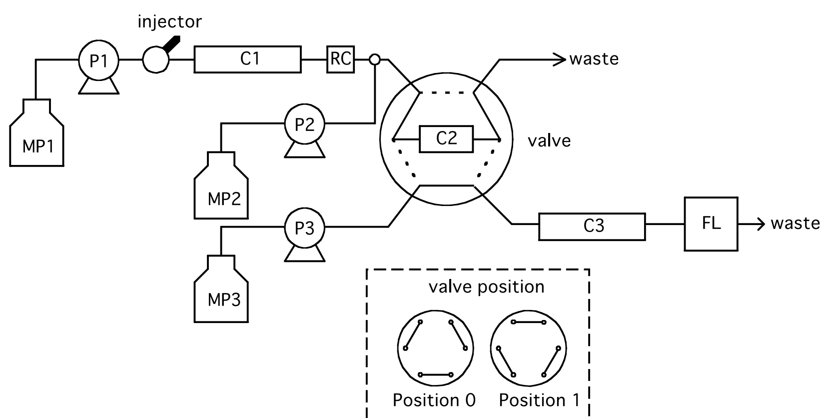

Fig. 2. Schematic Diagram of HPLC System
Abbreviations and conditions are described in the text.

a Shimadzu LC-10AD$_{VP}$ pump, a Rheodyne 7125 sample injector, a Shimadzu CTO-10A$_{VP}$ column oven and a Shimadzu SPD-10A$_{VP}$ UV detector. An HPLC system for the analysis of the three CNPs (Fig. 2) consisted of three Shimadzu LC-10AD$_{VP}$ pumps, a Rheodyne 7125 sample injector, a Shimadzu CTO-10A$_{VP}$ column oven, a Valco 6-port 2-position valve with an electronic actuator and a JASCO FP-1520S fluorescence detector.

Synthesis of CNPs ——— To obtain 1,3-, 1,6- and 1,8-CNPs, 200 mg of 1-NP, 8 ml of monochlorobenzene and 0.2 g of anhydrous copper chloride were mixed and refluxed in an oil bath (150°C) for 1.5 hr.

The reactant was filtered and applied to a silica gel column (50 × 2.6 cm i.d.). Materials were eluted with 200 ml of *n*-hexane followed by *n*-hexane/toluene (3 : 1, v/v). The *n*-hexane/toluene fraction was collected and evaporated to dryness. The residue was dissolved in methanol and then the solution was subjected to semi-preparative HPLC. A Nacalai Tesque Cosmosil 5C$_{18}$ARII (25 × 2 cm, i.d.) column was used as the stationary phase, and methanol was used as the mobile phase at a flow rate of 18 ml min^{-1}. The temperature of the column oven was set at 30°C, and the wavelength of the UV detector was set at 254 nm. Five peaks (peaks 1–5) were observed in the chromatogram shown in Fig. 3. Each peak was collected and evaporated to dryness.

Peak 2 (1,6-CNP): mp: 229–232°C; MS *m/z*: 281 (M^+), 251 (M^+-NO), 235 (M^+-NO_2) and 200 (M^+-Cl-NO_2); ^{1}H-NMR ($CDCl_3$): δ: 8.16 (1H, d, J = 8.2 Hz), 8.20 (1H, d, J = 9.3 Hz), 8.22 (1H, d, J = 8.6 Hz), 8.23 (1H, d, J = 8.2 Hz), 8.27 (1H, d, J = 9.5 Hz), 8.65 (1H, d, J = 9.2 Hz), 8.69 (1H, d, J = 8.6 Hz), 8.87 (1H, d, J = 9.5 Hz).

Peak 3 (1,8-CNP): mp: 208–211°C; MS *m/z*: 281 (M^+), 251 (M^+-NO), 235 (M^+-NO_2) and 200 (M^+-Cl-NO_2); ^{1}H-NMR ($CDCl_3$): δ: 8.03 (1H, d, J = 9.0 Hz), 8.09 (1H, d, J = 8.2 Hz), 8.13 (1H, d, J = 9.0 Hz), 8.14 (1H, d, J = 8.4 Hz), 8.16 (1H, d, J = 8.1 Hz), 8.63 (1H, d, J = 8.4 Hz), 8.64 (1H, d, J = 9.7 Hz), 8.91 (1H, d, J = 9.7 Hz).

Peak 4 (1,3-CNP): mp: 217–232°C; MS *m/z*: 281 (M^+), 251 (M^+-NO), 235 (M^+-NO_2) and 200 (M^+-Cl-NO_2); ^{1}H-NMR ($CDCl_3$): δ: 8.18 (1H, t, J = 7.7 Hz), 8.33 (1H, d, J = 9.5 Hz), 8.37 (1H, d, J = 7.2 Hz),

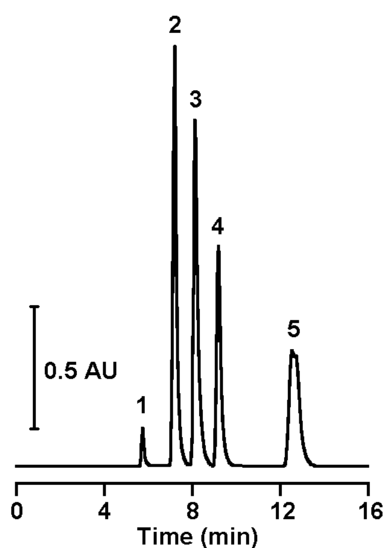

Fig. 3. Chromatogram of Reaction Solution
Peaks: 1, 1-NP; 2–4, CNPs; 5, di-CNPs.

8.37 (1H, d, J = 9.2 Hz), 8.39 (1H, d, J = 7.3 Hz), 8.53 (1H, d, J = 9.2 Hz), 8.77 (1H, s), 8.87 (1H, d, J = 9.3 Hz).

Ames Assay ——— CNPs were dissolved in Me_2SO and assayed for mutagenicity by the pre-incubation method in the absence of S9 mix. *Salmonella typhimurium* strain TA98 was kindly provided by Dr. B. N. Ames of the University of California. CNPs were assayed at four doses with duplicate plates at each dose. Revertant colonies were counted after incubation for two days at 37°C. The slope of the dose–response curve (revertants per nmol), which was calculated by least-squares linear regression from the first linear portion of the dose–response curve, was adopted as the mutagenic potency.

AhR Ligand Assay ——— The yeast recombinant assay developed by Miller *et al.*[13–15] was used in this study. The yeast strain was YCM3, which co-expressed the human AhR and AhR nuclear translocator proteins. YCM3, which was kindly gifted by Miller, was grown overnight at 30°C in a shaking incubator in synthetic glucose medium. Sample solution (10 μl), overnight culture (50 μl) and synthetic galactose medium were mixed in a test tube and incubated at 30°C for 18 hr. Culture (10 μl), Z-buffer (70 μl, containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol and 0.2% salkosil) and 4 mg/ml *o*-nitrophenol-β-D-galactopyranoside solution (20 μl) were mixed and incubated at 37°C for 20 min. The absorbance at 420 nm was then measured. The positive control (PC) was 10 μM BaP, and the negative control (NC) was only vehicle.

Sampling and Pretreatment of Surface Soil ——— Surface soil was collected at a sampling point of the Kyoto Pharmaceutical University (5 Nakauchi-cho Misasagi Yamashina-ku, Kyoto, Japan) in January 2004. The soil was sieved, and organic matter was extracted from 350 g of the fine soil with 500 ml of acetone using a Soxhlet extractor for 24 hr. The solvent was evaporated to dryness, and the residue was dissolved in 10 ml of toluene. This solution was applied to a Waters Sep-Pak Vac 20 cc (5 g) cartridge and then the material was eluted with 20 ml of toluene. The solvent was evaporated to dryness, and the residue was dissolved in 10 ml of *n*-hexane. This solution was applied to a Waters Sep-Pak Vac 20 cc (5 g) cartridge and then the material was eluted with 10 ml of *n*-hexane, 20 ml of *n*-hexane/toluene (3 : 1, v/v), 20 ml of *n*-hexane/toluene (1 : 1, v/v) and 20 ml of toluene. The toluene fraction was collected and evaporated to dryness. The residue was dissolved in 4 ml of acetone. This solution was subjected to HPLC using a Cosmosil 5PYE column (Nacalai Tesque, 25 cm × 4.6 mm i.d.) at 30°C. The mobile phase was acetone at a flow rate of 1 ml min^{-1}. Effluent from 5.5 to 8.0 min was collected and the solvent was evaporated to dryness. The residue was dissolved in 1 ml acetone. This solution was then subjected to HPLC using a Cosmosil 5PBB column (Nacalai Tesque, 25 cm × 4.6 mm i.d.) at 30°C. The mobile phase was acetone at a flow rate of 1 ml min^{-1}. Effluent from 6.7 to 7.8 min was collected and the solvent was evaporated to dryness. The residue was dissolved in 1 ml methanol. This solution was then subjected to HPLC using a Cosmosil 5NPE column (Nacalai Tesque, 25 cm × 4.6 mm i.d.) at 30°C. The mobile phase was methanol at a flow rate of 1 ml min^{-1}. Effluent from 5.6 to 7.5 min was collected and the solvent was evaporated to dryness. The residue was dissolved in 0.2 ml methanol, and this solution was subjected to the HPLC system shown in Fig. 2.

HPLC Analysis ——— The HPLC system consisted of three pumps (P1–3), an injector, a 6-port 2-position valve controlled by P2 and a fluorescence detector (FL) set at wavelengths of 288 nm for excitation and 450 nm for emission. C1, C2 and C3 are Nacalai Tesque Cosmosil $5C_{18}$ARII (25 cm × 4.6 mm i.d.), Cosmosil $5C_{18}$MSII (1 cm × 4 mm i.d.) and Cosmosil $5C_{18}$MSII (25 cm × 4.6 mm i.d.) columns, respectively. The reduction column (RC) was a SUS316 chromatographic tube (1 cm × 4 mm i.d.) dry packed with zinc/glass beads (1 : 1, w/w). Mo-

bile phase (MP) 1 was 10 mM imidazole-perchloric acid buffer solution (pH 6.8)/acetonitrile (2 : 8, v/v) at a flow rate of 1 ml min^{-1}, and MP3 was 10 mM imidazole-perchloric acid buffer solution (pH 6.8)/acetonitrile (5 : 3, v/v) at a flow rate of 1.5 ml min^{-1}. MP2 was 10 mM ascorbic acid solution at a flow rate of 4 ml min^{-1}. P2 was operated from 14.85 to 15.65 min for the determination of 1,6- and 1,8-CNPs and from 17.17 to 17.97 min for 1,3-CNP. The valve was rotated from position 0 to position 1 at 15.65 min for the determination of 1,6- and 1,8-CNPs and at 17.97 min for 1,3-CNP. The valve rotated back to position 0 at 16.65 and 18.97 min for 1,6- and 1,8-CNPs and 1,3-CNP, respectively.

RESULTS AND DISCUSSION

Synthesis of CNPs

Three CNP isomers, 1,3-, 1,6- and 1,8-CNP, were synthesized by chlorination of 1-NP. A chromatogram of the reaction solution is shown in Fig. 3. Five peaks (peaks 1–5) were observed in the chromatogram. Peak 1 was identified as 1-NP by its retention time and its mass spectrum. MS of peaks 1–5 were obtained by GC/MS. Peaks 2–4 were identified as CNPs, because signals at *m/z* 281 (M^+), 251 (M^+-NO), 235 (M^+-NO_2) and 200 (M^+-Cl-NO_2) were found in their mass spectra (Fig. 4). Peak 5, containing at least two peaks, was identified as being due to di-CNPs, because its mass spectrum showed signals at *m/z* 314 (M^+) (data not shown).

^{1}H-NMR and ^{1}H-^{1}H COSY spectra of peaks 2, 3 and 4 were measured (Fig. 5). In the case of peak 4, a singlet signal and a triplet signal were observed. These signals were considered to be derived from C_2 and C_7 of 1,3-CNP. In the cases of peaks 2 and 3, eight doublet signals were observed. For peak 3, the signal at a chemical shift of 8.91 ppm was correlated with that at 8.64 ppm, indicating that peak 3 is due to 1,8-CNP, which has two protons located at the peri position to chlorine and nitro groups in the ortho position. The NMR results are summarized in Table 1.

Minabe *et al.* have synthesized 1-bromo-3-, -6- and -8-nitropyrenes (BNPs) and measured their ^{1}H NMR chemical shifts (Table 1).[16] Chemical shifts of peaks 2, 3 and 4 were very similar to those of 1,6-, 1,8- and 1,3-BNPs, respectively. This result also shows that peaks 2, 3 and 4 were due to 1,6-, 1,8- and 1,3-CNPs, respectively.

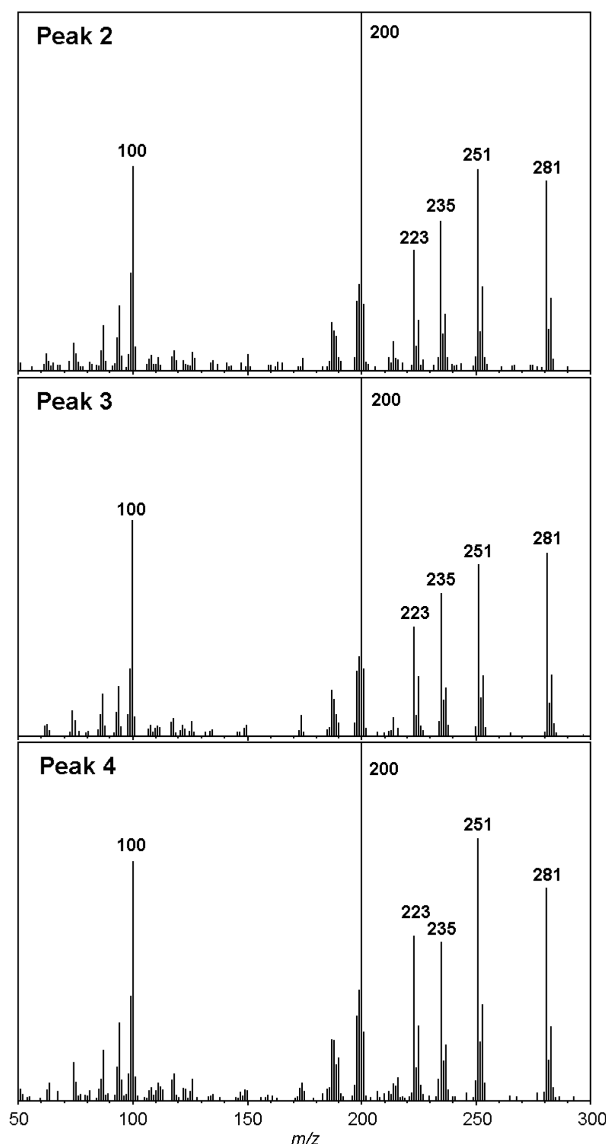

Fig. 4. Mass Spectra of Peaks 2, 3 and 4

Mutagenic Activities of CNPs

Mutagenic activities of 1,3-, 1,6- and 1,8-CNPs were tested by Ames Salmonella assay using *Salmonella typhimurium* strain TA98 under the conditions without S9 mix.

Mutagenicities were determined by testing in the 0.025–0.2 ng per plate range. The dose–response curves obtained are shown in Fig. 6. All CNP isomers showed dose–dependent responses. Mutagenic activities were calculated from curve fitting in the linear portions of dose–response curves. Mutagenicities of 1,3-, 1,6- and 1,8-CNPs were calculated to be 1.22, 1.14 and 0.83 revertants per nmol, respectively. These mutagenicities were higher than that of 1-NP (0.47 revertants per nmol) or 1-chloropyrene

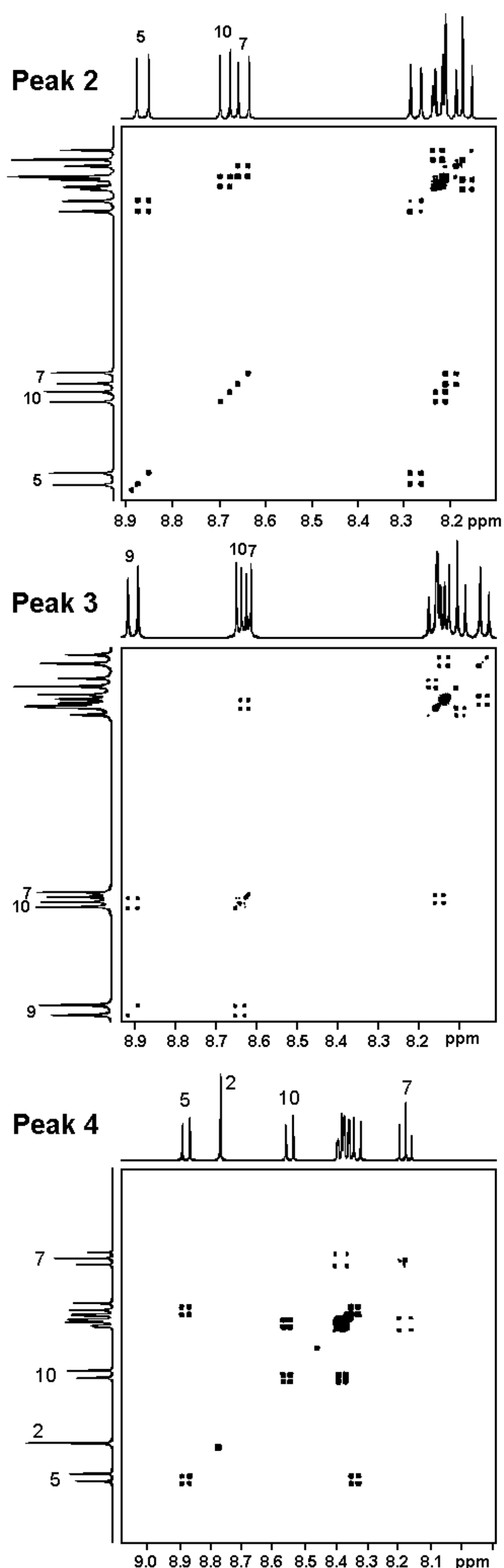

Fig. 5. ^{1}H-^{1}H COSY Spectra of Peaks 2, 3 and 4
Peak numbers indicate the positions of carbon atoms in the CNPs.

(mutagenicity was not observed up to 33.33 nmol per plate[17]). Therefore, chlorination of 1-NP was found to increase its mutagenic activity.

AhR Ligand Activities of CNPs

AhR ligand activities of 1,3-, 1,6- and 1,8-CNPs were tested using the recombinant yeast reporter gene assay established by Millar *et al.*[13–15] The concentration — response curves obtained are shown in Fig. 7. The three CNPs showed no AhR ligand activity, whereas 1-NP showed weak activity. This result suggests that chlorination of 1-NP decreases its AhR ligand activity.

Analytical Method for the Determination of the CNPs

Although CNPs have no fluorescence, their reduced products, aminochloropyrenes (CAPs), have strong fluorescence. Fluorescence spectra of CAPs, which were obtained by reduction of CNPs using zinc as a reducer, are shown in Fig. 8. Their fluorescence spectra were very similar to each other. Excitation and emission spectra showed maximal intensities at 288 and 450 nm, respectively. Therefore we used these wavelengths for the determination of CAPs.

The HPLC system used for the analysis of the CNPs is shown in Fig. 2. After sample injection, the sample components were roughly separated on a polymeric C_{18} column (C1). Then, CNPs were reduced by passing the sample through a reduction column packed with zinc particles (RC). Platinum black heated to 75°C as a catalyst and methanol as a mobile phase also reduced CNPs, but not to a high enough extent. After reduction, CAPs were concentrated onto a concentrator column (C2) by increasing the water concentration of the mobile phase. Then, the valve was rotated, and the target compounds were separated on a monomeric C_{18} column (C3) and detected using a FL.

The calibration graphs were linear from 1 to 100 ng ml^{-1}, and detection limits (3σ) were 0.2 ng ml^{-1} for 1,3-CNP and 0.1 ng ml^{-1} for 1,6- and 1,8-CNP.

Detection of CNPs in Surface Soil Collected in Kyoto

Since the matrix of surface soil is complex, several clean-up steps are necessary, to remove interfering compounds that appear near the target compounds in the chromatogram and to dissolve the

Table 1. NMR Results

CNP		BNP[a]	
δ	COSY	Site	δ
CNP2 (1,6-CNP)			1,6-BNP
8.16 (1H, d, J = 8.2 Hz)	A	3	8.18 (1H, d, J = 8.2Hz)
8.20 (1H, d, J = 9.3 Hz)	B	9	8.23 (1H, d, J = 9.4Hz)
8.22 (1H, d, J = 8.6 Hz)	C	8	8.26 (1H, d, J = 8.7Hz)
8.23 (1H, d, J = 8.2 Hz)	A	2	8.38 (1H, d, J = 8.2Hz)
8.27 (1H, d, J = 9.5 Hz)	D	4	8.31 (1H, d, J = 10.0Hz)
8.65 (1H, d, J = 9.2 Hz)	B	10	8.66 (1H, d, J = 9.4Hz)
8.69 (1H, d, J = 8.6 Hz)	C	7	8.71 (1H, d, J = 8.7Hz)
8.87 (1H, d, J = 9.5 Hz)	D	5	8.92 (1H, d, J = 10.0Hz)
CNP3 (1,8-CNP)			1,8-BNP
8.03 (1H, d, J = 9.0 Hz)	A	5	8.14 (1H, d, J = 8.8Hz)
8.09 (1H, d, J = 8.2 Hz)	B	3	8.17 (1H, d, J = 8.4Hz)
8.13 (1H, d, J = 9.0 Hz)	A	4	8.22 (1H, d, J = 8.8Hz)
8.14 (1H, d, J = 8.4 Hz)	C	6	8.24 (1H, d, J = 8.5Hz)
8.16 (1H, d, J = 8.1 Hz)	B	2	8.36 (1H, d, J = 8.4Hz)
8.63 (1H, d, J = 8.4 Hz)	C	7	8.71 (1H, d, J = 8.5Hz)
8.64 (1H, d, J = 9.7 Hz)	D	10	8.70 (1H, d, J = 9.8Hz)
8.91 (1H, d, J = 9.7 Hz)	D	9	8.99 (1H, d, J = 9.8Hz)
CNP4 (1,3-CNP)			1,3-BNP
8.18 (1H, t, J = 7.7 Hz)	A	7	8.18 (1H, t, J = 7.6Hz)
8.33 (1H, d, J = 9.5 Hz)	B	5	8.36 (1H, d, J = 9.6Hz)
8.37 (1H, d, J = 7.2 Hz)	A	6	8.36 (1H, d, J = 7.6Hz)
8.37 (1H, d, J = 9.2 Hz)	C	9	8.38 (1H, d, J = 9.2Hz)
8.39 (1H, d, J = 7.3 Hz)	A	8	8.40 (1H, d, J = 7.6Hz)
8.53 (1H, d, J = 9.2 Hz)	C	10	8.52 (1H, d, J = 9.2Hz)
8.77 (1H, s)		2	8.95 (1H, s)
8.87 (1H, d, J = 9.3 Hz)	B	4	8.87 (1H, d, J = 9.6Hz)

a) Reference 16.

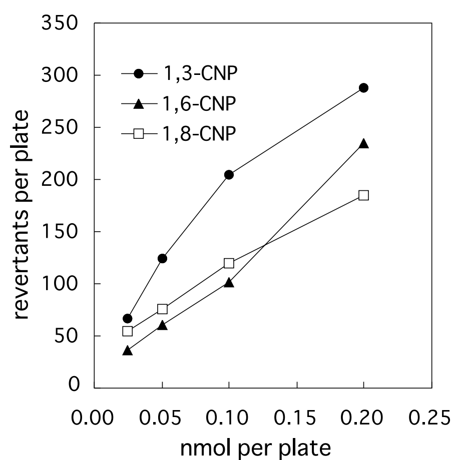

Fig. 6. Mutagenic Activities of CNPs

Mutagenic activities were assayed by Ames test in *Salmonella typhimurium* TA98 strain without S9 mix condition.

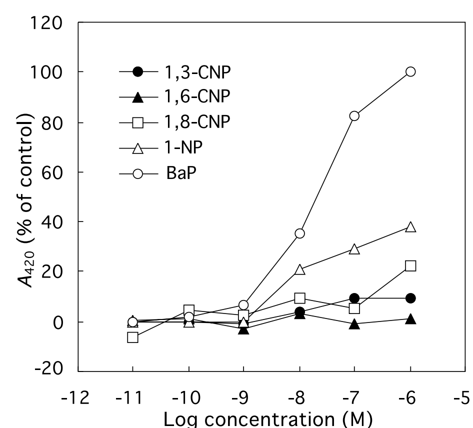

Fig. 7. AhR Ligand Activities of CNPs

AhR ligand activities were assayed by Miller's yeast recombinant assay. Benzo[*a*]pyrene (BaP) was used as positive control.

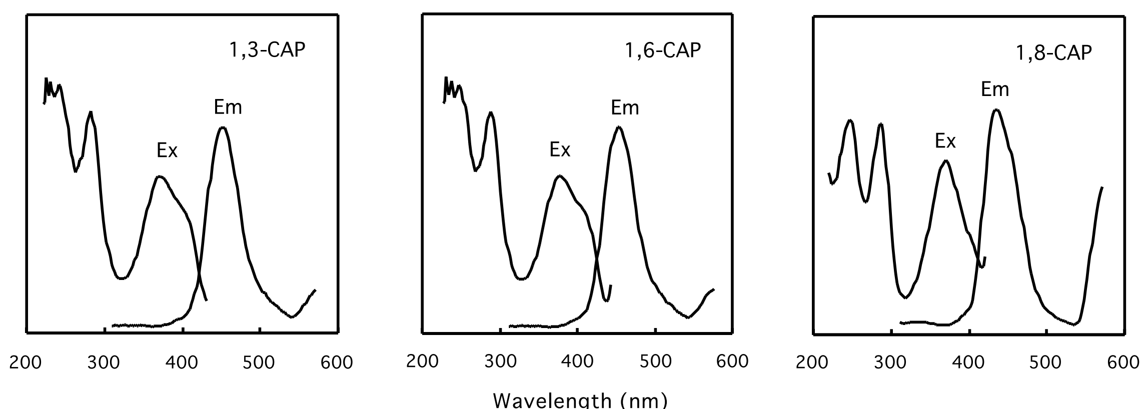

Fig. 8. Excitation (Ex) and Emission (Em) Spectra of CAPs
CAPs were obtained by reduction from CNPs with zinc powder.

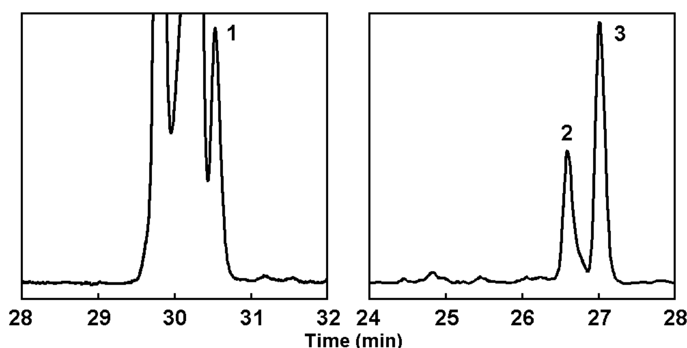

Fig. 9. Chromatograms of Surface Soil Extract
Peaks: 1, 1,3-CAP; 2, 1,6-CAP; 3, 1,8-CAP. Chromatographic conditions were described in the text.

sample in a small volume of solvent. First, a silica gel stationary phase and a toluene mobile phase were used to remove polar components. Second, a silica gel stationary phase and a stepwise gradient of *n*-hexane, *n*-hexane/toluene (3 : 1, v/v), *n*-hexane/toluene (1 : 1, v/v) and toluene as mobile phases were used to remove non-polar and weakly polar components. Then, pyrenylethyl, pentabromobenzyl and nitrophenylethyl stationary phases were used because the retention behaviors of polycyclic aromatic hydrocarbons and nitroarenes on these three stationary phases were different from those on a C_{18} stationary phase.[18–20] Extracts from 350 g of surface soil could be dissolved in 10 ml of toluene. After the silica gel clean ups, the samples could be dissolved in 4 ml of acetone, and after the pyrenylethyl, pentabromobenzyl and nitrophenylethyl clean ups, the samples could be dissolved in 1 ml of acetone, 1 ml of methanol and 0.2 ml of methanol, respectively. Recoveries of 1,3-, 1,6- and 1,8-CNPs after these five clean-up procedures using 10 ng of standard compounds were 99 ± 3%, 96 ± 1% and 97 ± 3% (mean ± S.D., $n = 3$), respectively.

We collected surface soil near a major road in Kyoto, Japan. After the pretreatments described above, we determined CNP concentrations by HPLC. Chromatograms of the surface soil extract are shown in Fig. 9. 1,3-CNP, 1,6-CNP and 1,8-CNP concentrations were found to be 9.2–13.8, 3.4–4.6 and 4.3–5.0 pg per g of soil ($n = 2$), respectively. This is the first time CNPs have been detected in environmental samples.

We previously found mutagenic 1,3-, 1,6- and 1,8-dinitropyrenes in surface soils collected in the Kanto, Chubu and Kinki areas, which are megalopolis districts in Japan.[21] The dinitropyrenes were detected in the range of 12–6809 pg per g of soil. Although these samples were different from those examined in the present study, CNP concentrations were lower than those of dinitropyrenes. Considering that the mutagenic activities of the dinitropyrenes were much higher than those of the CNPs, the CNP

contribution to the mutagenic activity of surface soil seems to be much smaller than that of the dinitropyrenes.

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