韓國環境衛生學會誌
Kor. J. Env. Hlth. Soc.
Vol.17, No.2, 114~120, 1991

Synthesis of 1, 3-, 1, 6- and 1, 8-Dinitropyrenes and Evaluation of Their Mutagenic Activities

Young Sik Yoo

Dept. of Environmental Science and Technology, Seoul Health Junior College

1, 3, -1, 6- 및 1, 8-Dinitropyrene의 합성과 돌연변이원성의 평가

유 영 식

서울보건전문대학 환경관리과

요 지

대기부유입자상물질이나 diesel 배출가스중에 함유하여, 주요한 direct-acting mutagen의 하나로 작용하는 1-nitropyrene, 1,3-, 1,6-및 1,8-dinitropyrene을 합성하고, 고속액체 chromatography로 분리정제하여, *Salmonella typhimurium* TA 98, S9mix 비첨가의 계에서 돌연변이원성을 측정한 결과, 1-nitropyrene에 비하여, dinitropyrene은 강력한 돌연변이 원성을 나타내었고, 그 중에서도 1,8-dinitropyrene은 733,000 revertants/μg으로 최고의 돌연변이원 활성을 나타내었다.

I. Introduction

Organic solvent extracts of urban airborne particulates or diesel exhaust particulates are mixture of hundreds of polycylic aromatic hydrocarbons (PAHs) present in concentrations ranging from 0.01 to 10 ppm[1]. The extracts are directly active(S9 mix not required) in the Ames assay. Because the carcinogenic PAHs known to be in extracts are mutagenic in the Ames assay with S9 mix, direct-acting mutagenic activity implies that the extracts contain mutagens and therefore possible carcinogens, and differ from the "classical" PAH.

Very little can be said, however, about the potential human health effects of the direct-acting mutagens until they are identified.[2]

To assess the toxicological significance of direct-acting mutagenic activity in particulates, it is first necessary to establish the chemical identities of the mutagens.[3] A number of nitro polycyclic aromatic hydrocarbons(nitro-PAHs) have tentatively been identified in particulate emissions from mobile and stationary sources.[4,5] Some of these compounds have been shown to cause mutation in bacteria and are suspect carcinogens.[3] Nitration of pyrene under various conditions(*e. g.*, with a mixture of nitric acid and sulfuric acid, with nitric acid in acetic acid, with acetyl nitrate) yields 1,3-, 1,6-and 1,8-dinitropyrene with various amounts of 1-nitropyrene. However, separation of these isomers in preparative amounts in diffi-

cult because of the slight solubility of these compounds in light organic solvents and their similar polarity values. Since highly purified samples of these suspected nitro-PAHs are needed for analytical standards and subsequent biological testing for mutagenic/carcinogenic potencies, we have undertaken the synthesis and separation of dinitropyrenes by preparative HPLC to yield pure isomers of dinitropyrenes, and here report on their mutagenicities.

Ⅱ. Materials and Methods

1. Synthesis of Dinitropyrene

Pyrene was nitrated by the method of Vollman et al[6] to yield a mixture containing the three isomers of dinitropyrenes and substantial quantity of 1-nitropyrene. The synthesis of 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene, shown in Fig. 1 was accomplished by treating a 90℃ glacial acetic acid solution of pyrene with 70% nitric acid. The yellow precipitates were diluted with water and the products isolated by filtration.

2. HPLC Fractionation

The reaction products were purified by using a high performance preparative liquid chromatograph LC-09(Japan Analytical Industry Co., Ltd.) equipped with a normal phase silica column(JAIGEL-SIL) on a UV detctor at 254 nm.

The crystals were dissolved in a minimum amount of n-hexane : dichloromethane (8 : 2) and the solution was subjected to HPLC. The injection volume was 3 m*l* and the eluent was the same solution as above at a flow rate of 5.7 m*l*/min.

Fractions separated by HPLC were collected automatically in glass vials.

Each fraction was purified by recrystallization from a mixture of benzene and n-hexane. Collected peaks were identified using electron impact mass spectrometry and high resolution proton magnetic resonance spectrometry.

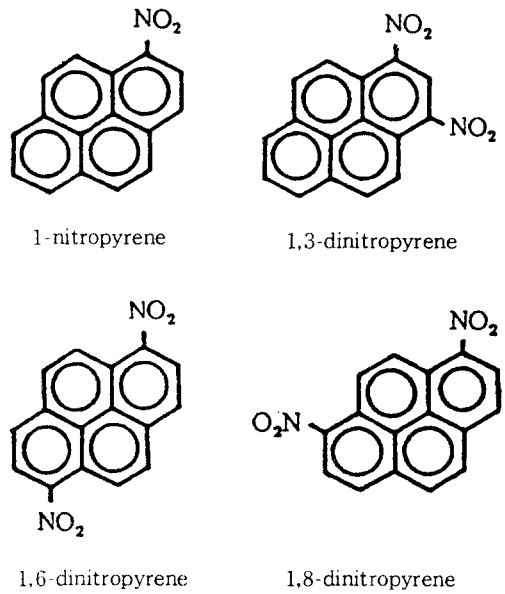

Fig. 1. Chemical structures of isomers of nitropyrenes.

3. Capillary Gas Chromatography with ECD

A gas chromatography(Shimadzu Model : GC 7A) with an electron capture detector (ECD) and solventless injection system was used for the analysis of nitropyene. High resolution gas chromatography was performed with a 25 m × 0.31 mm i.d. fused silica methyl silicone OV-1 capillary column with 0.52 μm film thickness. Typical run conditions comprised an initial oven temperature of 150℃ for 2 min followed by a 4℃/min temperature programed to 250℃, hold for 5 min.

4. High Resolution Capillary Gas Chromatography/Mass Spectrometry

GC/MS was performed in the elctron impact mode with a JEOL DX 303 mass spec-

trometer interfaced to a JEOL GCG 06 gas chromatograph equipped with a 25 m × 0.31 mm i.d. fused silica methylsilicone OV-1 capillary column with 0.52 μm film thickness. Temperature programming conditions consisted of an initial oven temperature of 220 ℃ followed by a 4 ℃ /min program to 250 ℃. Mass spectra acquired at 70-eV electron ionization conditions were processed with an JEOL DA 5,000 data system. Masses from 50 to 400 amu were scanned every 0.5s.

5. Nuclear Magnetic Resonance Spectrometry

Fourier-transformed 400 MHz ^{1}H-NMR spectra of nitropyrenes in $CDCl_3$ were obtained using a JEOL-GX 400 spectrometer. The chemical shifts of each resonance peak were, calibrated against tetramethylsilane (TMS) as an internal reference, and expressed in ppm on the δ scale.

6. Mutagenic Assay

Mutagenic activity was measured in histidine requiring strains of *Salmonella typhimurium*[7] by the plate incorporation method,[8] using strain TA98 kindly provided from Dr. Bruce N. Ames of the Univerisity of California. Duplicate plates were used in all assays. Mutagenic activity, corrected for the spontaneous number of revertants, is expressed as net revertants per plate.

7. Chemicals

Pyrene was purchased from Aldrich Chemicals Co., Inc. Its purity was checked by gas chromatography. Acetic acid and nitric acid were reagent grade products of Wako Pure Chemicals Co., Inc. The organic solvents were also of guraranteed reagent grade.

Ⅲ. Results and Discussion

1. HPLC Fractionation

Fig. 2 shows the elution profile for nitropyrenes using the preparative HPLC procedure. As shown in Fig. 2, the separation efficiency of the HPLC system used for the fractionation was quite satisfactory. 1-Nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene were eluted in that order. Each yellow nitropyrene fraction was evaporated and purified by recrystallization from a mixture of benzene and n-hexane to give yellow needles. Identification of the HPLC fraction was confirmed by GC/MS and ^{1}H-NMR spectrometry.

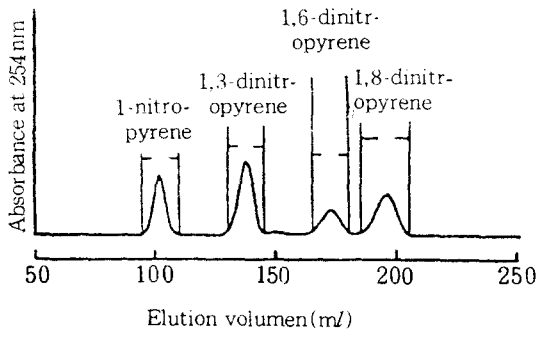

Fig. 2. HPLC fractionation profiles for 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene, utilizing normal phase silica column(JAL GEL SIL) with UV at 254 nm.

2. Capillary Gas Chromatography with ECD

Fig. 3 presents the chromatogram of the mixture of nitropyrenes obtained by gas chromatography with ECD. Complete separtion of 1,3-, 1,6- and 1,8-dinitropyrenes was achieved.

3. Capillary Gas Chromatography/ Mass Spectra

Fig. 4 shows the mass spectra of 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene. The mass spectrum of 1-nit-

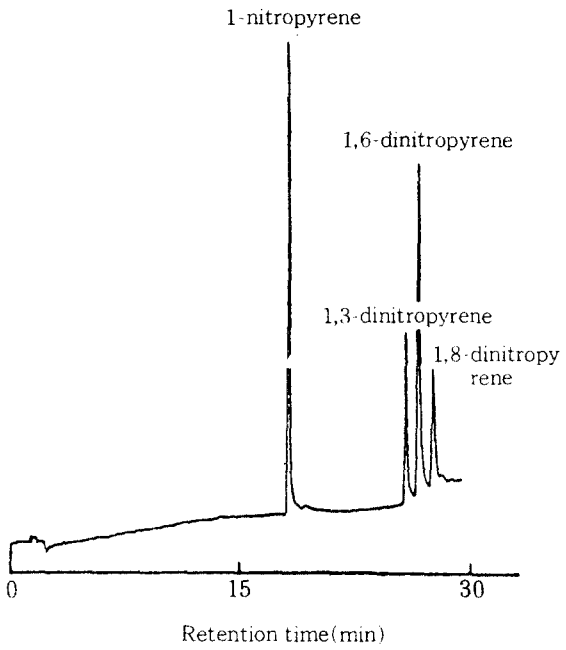

Fig. 3. Gas chromatograms for 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene, obtained with a 25m × 0.31 mm i. d. fused silica methyl silicone OV-1 capillary column with an electron capture detector.

ropyrene showed a molecular ion at m/e 247 $(M)^+$ and fragment ions at m/e 217$(M-NO)^+$, m/e 201$(M-NO_2)^+$, m/e 200$(M-HNO_2)^+$ and m/e 189$(M-CNO_2)^+$ ions. The mass spectra of dinitropyrenes showed a molecular ions at m/e 292$(M)^+$ and fragment ions at m/e 262 $(M-NO)^+$, m/e 232$(M-2NO)^+$ m/e 216$(M-N_2O_3)^+$ and m/e 200$(M-2NO_2)^+$ ions. Mass spectra of 1,6- and 1,8-dinitropyrene characteristically yielded abundant$(M)^+$, $(M-2NO)^+$ and $(M-2NO_2)^+$ ions. Although there are some spectral differences among dinitropyrenes, the mass spectra of the three are so similar that no positive identification can be made on the basis of mass spectra only.

4. ^{1}H-NMR-Spectra

Structural assignments were accomplished using ^{1}H-NMR spectra. Fig. 5 shows the Fourier-transformed 400MHz nuclear magnetic resonace spectra of nitropyrenes. The spectroscopic data were as follows : 1-nitropyrene : δ 8.76 (d, 1, J (9, 10)=9.5 Hz, $C_{10}H$), δ8.56 (d, 1, J (2,3)=8.5 Hz C_2H), δ8.2-7.9 (m, 7, ArH).

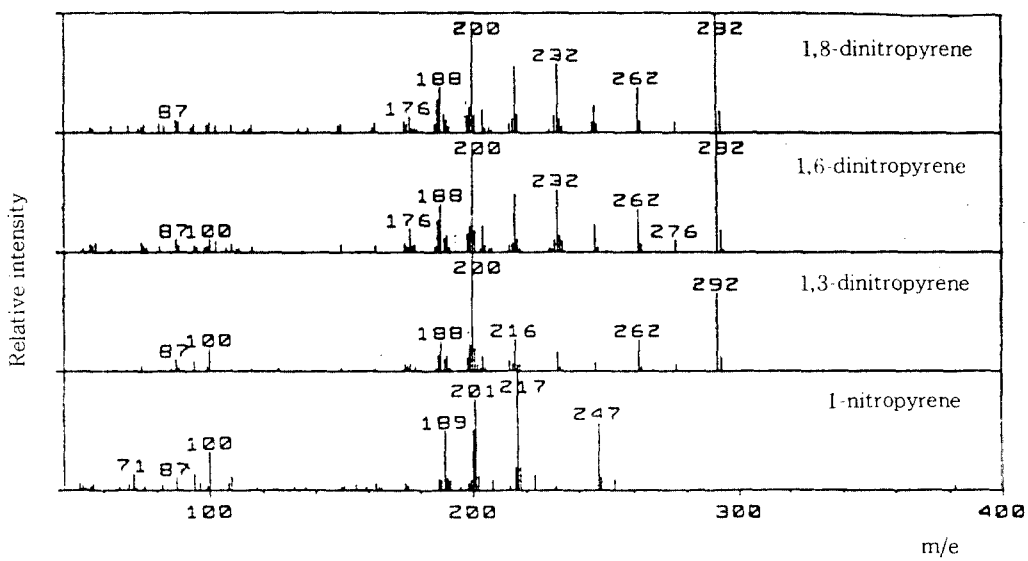

Fig. 4. Mass spectra of 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene.

1,3-dinitropyrene : δ 9.38 (s, 1, C_2H), δ8.99 (d, 2, J (4,5)=J (9,10)=9.5 Hz, C_4H and $C_{10}H$), δ 8.55 (d, 2, C_5H and C_9H), δ8.53 (d, 2, J (6,7) and J (7,8)=7.5Hz, C_6H and C_8H), δ8.30 (t, 1, C_7H. 1,6-dinitropyrene : δ9.02 (d, 2, j (4,5)=J (9,10)=9.6 Hz, C_5H and $C_{10}H$), δ8.76 (d, 2, J (2, 3)=J (7,8)=8.4Hz, C_2H and C_7H), δ8.40 (d, 2, C_3H and C_8H), δ8.40 (d, 2, C_4H and C_9 H). 1,8-dinitropyrene : δ 9.12 (d, 2, C_9H and $C_{10}H$), δ8.78 (d, 2, J (2,3)=J (6,7)=8.40Hz, C_2H and C_7H), δ8.40 (d, 2, J (2, 3)=J (6,7) =8.40 Hz, C_3H and C_6H), δ8.33 (d, 2, C_4H and C_5H).

5. Mutagenicity of Nitropyrenes

Each of the synthetic nitropyrene compounds was subjected to a mutation test using *Salmonella typhimurium* TA 98 and TA 100. The specific mutagenicity of 1-nitropyrene was 2,340 revertants/μg, and those of 1,3-dinitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene were 270,000, 288,000, 733,000, respectively. It was noted that the mutagenicity of dinitropyrenes was higher than that of mononitropyrene. Of them 1,8-dinitropyrene was the highest in mutagenicity. The mutagenicities of nitropyrenes were eliminated almost completely by the addition of S9 mix to the preincubation medium. All nitropyrenes were more mutagenic toward TA 98 than TA 100.

Since these dinitropyrenes are the highest in mutagenic activity reported in the environmental samples, it is important and necessary to clarify the extent to which the direct-acting Ames assay mutagenicity of the urban airborne particulates or diesel exhaust particulates can be assigned to these compounds[9-12]. Assuming that emission of nitrated PAHs into the environment is inherent in various combustion processes, it might be possible to influnece the degree of nitration through

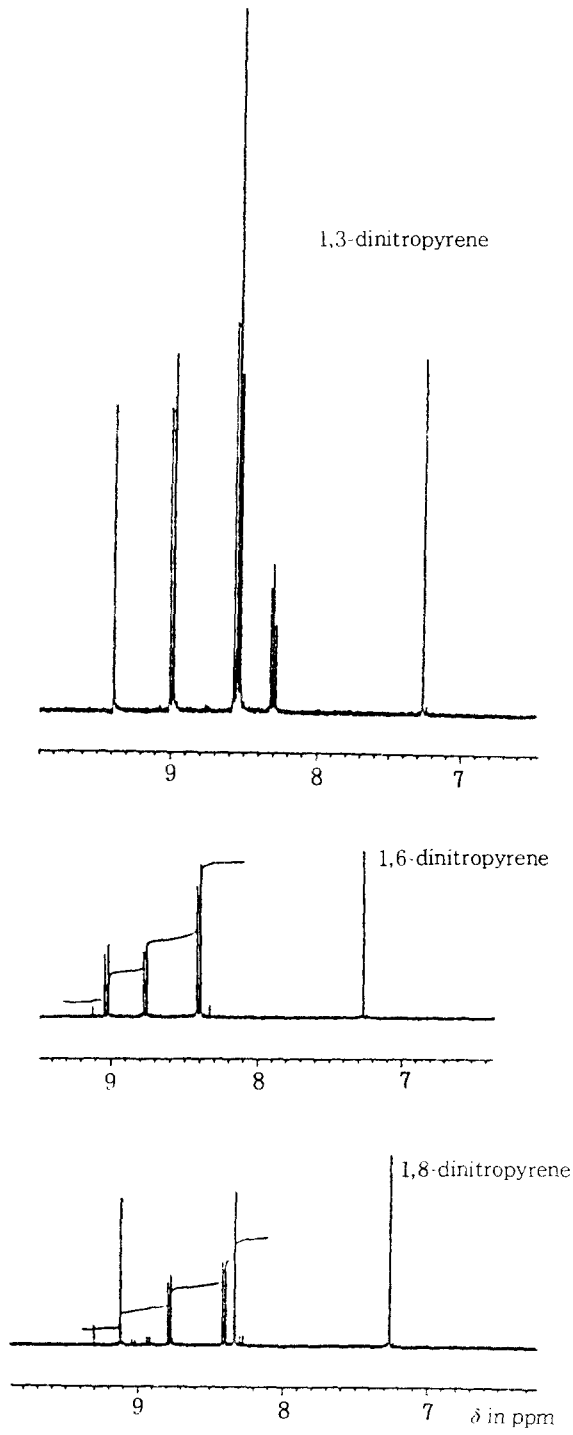

Fig. 5. 400 MHz ^{1}H-NMR spectra of 1,3-dinitropyrene, 1,6-dinitroypyrene and 1,8-dinitropyrene.

modification of the comustion process. The availability of PAH molecules nitrated to different degrees (*e. g.*, mono-, tri-, tetra-substituted) and determination of their mutagenicity might provide some indication as to whether optimization of the combustion process to minimize the emission of highly mutagenic species in a plausible approach. It is apparent that nitropyrenes may account for a significant portion of the direct-acting mutagenicity, better bioassay and chemical quantitation of nitropyrenes are required.

To understand the relationship between chemical composition and mutagenicity, larger scale HPLC separations and more sensitive mutagenic assays are needed. By combining HPLC fractioation methods and Ames assay, the potent and interesting families of environmental mutagens can be isolated.

Summary

Isomers of dinitropyrenes were synthesized and separated via preparative HPLC. Each of the molecular structures of the dinitropyrenes was confirmed by gas chromatography/mass spectrometry and NMR spcetrometry.

1-Nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene were subjected to a mutation test using *Salmonella typhimurium* TA 98 without S9 mix. Of them, 1,8-dinitropyrene was the most potent mutagen, producing 733,000 revertant/μg, whereas 1-nitropyrene showed the lowest mutagenicity.

Acknowledgement

The author gratefully acknowledge many helpful discussions with and the technical assistance of Dr. Koichi Kuroda and Dr. Taro Yoshikura, Osaka City Institute of Public Health and Environmental Sciences, Japan. Special thanks are due to Dr. Yoshihiro Takubo, University of Osaka for performing this work and the ^{1}H-NMR spectral measurements.

References

1) Yoo, Y. S. and Kim, M. Y. et al. : The evaluation of airborne particualtes with mutagenic activity as a carcinogenic indicator, Report submitted to Korea Ministry of Science and Technology, 1990.

2) Kuroda, K. and Yoo, Y. S. : Mutagenicity of airborne particulates, Annual Rep. of Osaka city Inst. of Public Health and Environ. Sci. **46**, 19~26, 1984.

3) Yoo, Y. S. : Nitroarenes, recently recognized air pollutants, Korea J. Env. Hlth. Soc. **15**, 1~9, 1989.

4) Nielsen, T. : Isolation of polycyclic aromatic hydrocarbons and nitro derivatives in complex mixtures by liquid chromatography, Anal. Chem. **55**, 286~290, 1983.

5) Schuetzle, D., Riley, T. L., Harvey, T. M. and Hunt, D. F. : Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particles, Anal. Chem., **54**, 265~271, 1982.

6) Vollman, H., Becker, H., Corell, M. and Streeck, H. : Justus Liebigs Annalen Der Chemie, **531**, 1~159, 1937.

7) Ames, B. N., McCann, J. and Yamasaki, E. : Mehtods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test, Mutation Res., **31**, 347~364, 1975.

8) Yahagi, T., Nagao, M., Seino, Y., Matsushima, T. and Sugimura, T. : Mutagenicities of N-nitrosoamies in *Salmonella*, Mutation Res. **48**, 121~130, 1977.

9) Rosenkranz, H. S. and McCoy, E. C., et al. : Nitropyrenes : Isolation, indentification and reduction of mutagenic impurities in carbon black and toners, Science **209**,

1039~1043, 1980.
10) Tokiwa, H. and Otofuji, T., et al. : 1,6-dinitropyrene : Mutagenicity in Salmonella and carcinogenicity in BALB/C mice, J. Natl. Cancer Inst., **76**, 92~96, 1986.
11) Salmeen, I. and Durisin, A. M., et al. : Contribution of 1-nitropyrene to direct-acting Ames assay mutagenicities of diesel particulate extracts, Mutation Res. **104**, 17~23, 1982.
12) Gibson, T. L. : Nitro derivatives of polynuclear aromatic hydrocarbons in airborne and source particulate, Research Publication GMR-3836, ENV#18, 1981.