Fluorescence enhancement of ethanolic solution of nitroarenes and its analytical application

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Abstract

When ethanolic solutions of 1-nitropyrene (1-NP) and 3-nitrobenzanthorone (3-NBA) were irradiated with intense light they absorb for several minutes, the fluorescence characteristics of the solutions was significantly changed. After such preliminary irradiation, the fluorescence intensity of 1-NP increased immediately by a factor of $10^4$ with a blue shift of 100 nm and that of 3-NBA 700 with a red shift of 10 nm. The findings were applied to high performance liquid chromatography with a fluorescence detector so that the two nitroarenes were quantitatively analyzed by preliminary irradiation of their solutions before measurements. The calibration curves were linearly drawn over the concentration range from $1.0 \times 10^{-9}$ to $1.0 \times 10^{-7}$ M for 1-NP and $1.0 \times 10^{-8}$ to $1.0 \times 10^{-6}$ M for 3-NBA and the limits of detection were 2.3 pg for 1-NP and 28 pg for 3-NBA. From the results, fluorescence enhancement was found to be very effective for determining nitroarenes, being practically non-fluorescent and very important in environmental health-risk assessment, easily and sensitively. The mechanism of the fluorescence enhancement of 1-NP and 3-NBA was also discussed.

Keywords: Nitroarenes, 1-nitropyrene, 3-nitrobenzanthorone, irradiation, fluorescence enhancement, HPLC.
Introduction

Nitroarenes such as nitropyrenes and nitrobenzanthrones have been receiving a considerable amount of attention, because of their mutagenic activity and wide occurrence in the environment.\(^1\)\(^-\)\(^3\) They are especially abundant in exhaust gas and particles from vehicle engines and in smoke and soot from factories.\(^2\) Accordingly, it is very important in environmental analysis to detect them with high sensitivity. High performance liquid chromatography with a fluorescence detector (HPLC-FLD),\(^4\) gas chromatography / mass spectrometry (GC/MS)\(^5\)\(^,\)\(^6\) and liquid chromatography / mass spectrometry (LC/MS)\(^7\)\(^,\)\(^8\) are generally used for sensitive analysis of polycyclic aromatic compounds. Such conventional methods, however, have been barely successful to the analysis for nitroarenes, because nitroarenes are almost non-fluorescent\(^9\) and they have very low ionization probability.\(^10\)\(^,\)\(^11\) Nitroarenes are reduced to amino compounds and then detected by HPLC with FLD\(^12\) and chemiluminescence detector (CLD),\(^13\) attaining high analytical sensitivity\(^14\). The limit of detection (LOD) of HPLC-FLD is 2.8 pg for 1-nitropyrene (1-NP)\(^12\) and 20 pg for 3-nitrobenzanthorone (3-NBA)\(^15\) and that of HPLC-CLD is 9.8 pg for 1-NP\(^13\) and 82.5 pg for 3-NBA.\(^16\) In addition, nitroarenes can be also detected sensitively by means of gas-chromatography/tandem-mass-spectrometry with negative ion chemical ionization;\(^17\) LODs for 1-NP and 3-NBA are 0.12 pg and 0.11 pg, respectively. These methods, however, requires additional and expensive equipment and/or troublesome pretreatment is also necessary before the analysis\(^18\). Thus, a new method has been expected which enables to detect nitroarenes much easily with sufficient sensitivity.

On the other hand, benzanthrone, naphthanthrone, and their nitrated compounds are known to exhibit fluorescence enhancement. The fluorescence of the compounds in degassed organic solution significantly increases with intense-light irradiation in advance;\(^19\)\(^-\)\(^21\) degassing to remove oxygen in the solution is essential to the fluorescence enhancement. Recently, however, we have found that 1-NP (Fig. 1a), 3-NBA (Fig. 1b), 1-nitrobenzanthrone and
2-nitronaphthanthrone in alcohol give rise to the fluorescence enhancement completely without degassing. This prompted us to apply the fluorescence enhancement to analysis of nitroarenes. In the present paper, we report the fluorescence enhancement of ethanol solutions of 1-NP and 3-NBA, which are important in environmental health-risk assessment, and its application to quantitative analysis of the two compounds by HPLC-FLD. The chemical species causing the enhancement are also discussed on the basis of the data of LC/MS measurements and molecular orbital calculations.

**Experimental**

1-NP was purchased from Wako Pure Chemical, Tokyo, and purified by vacuum sublimation. 3-NBA was synthesized by direct nitration of benzoanthrone with nitric acid in acetic acid. After recrystallization in ethanol, it was purified by vacuum sublimation. The structure was confirmed by $^1$H-NMR spectroscopy. Each compound was dissolved in ethanol; the concentration of the solutions ranged from $1.0 \times 10^{-9}$ to $1.0 \times 10^{-7}$ M and $1.0 \times 10^{-8}$ to $1.0 \times 10^{-6}$ M for 1-NP and 3-NBA, respectively. For comparison, degassed solutions were also prepared. The degas treatment was performed by the freeze-pump-thaw cycle method by means of a glass vacuum line in a dark room; on two or three repetitions of the cycle, the solution was thoroughly freed from oxygen. The solution was irradiated with a 500 W Xe-lamp (Ushio, Tokyo, Japan) through an L-39 sharp-cut-filter (Toshiba, Tokyo, Japan); the irradiation time was 10 minutes. After the preliminary irradiation, spectral measurements were performed with a FluoroMax spectrophotometer (Spex, Edison, USA). Monochromatic light was also used for preliminary irradiation. Light from the Xe-lamp (150 W) of the spectrophotometer was filtered through its monochromator for excitation and incident upon the solution for 10 min, and then fluorescence intensity was measured as a function of the irradiation wavelength at every 10 nm from 300 to 500 nm.
The analysis of chemical compounds in the irradiated solution was carried out by means of an Agilent 1260 series HPLC system (Agilent, California, USA) equipped with a photo-diode-array detector (DAD) and FLD. HPLC separations were conducted on a ZORBAX-Eclipse-XDB-C18 column (4.6×150 mm, 2.5 μm) with an ethanol-water gradient elution at a flow rate of 0.25 mL/min. The mobile-phase composition was maintained initially at 50/50 (v/v) of ethanol/water (0 – 5 min), changed linearly to 100/0 (5 – 20 min), and then held 100/0 (20 – 30 min). The injection volume was 20 μL and the column temperature was maintained at 30 ºC. An LC/MS system (LCMS-2010 EV, Shimadzu, Kyoto, Japan) equipped with an electrospray-ionization interface was used for the analysis of the compounds produced on irradiation. The mass scan was over the range of mass-to-charge ratios, m/z, 50 – 500 in the positive and negative ion mode.

Molecular orbital calculations were performed on the software package, SCIGRESS in Version 2 (Fujitsu). The molecular structures were optimized by the MM3 molecular mechanics and PM6 semi-empirical molecular orbital method and the energy and oscillator strength of electronic transitions were calculated by Zerner’s intermediate neglect of differential overlap (ZINDO) method. In the calculations default settings were used; the number of configuration interactions in ZINDO was 20.

Results and Discussion

Figure 2 shows the fluorescence spectra of an ethanol solution of 1-NP (1.0×10⁻⁵ M) before and after irradiation. The fluorescence of 1-NP before irradiation is extremely weak and the magnified (×10³) spectrum (broken line) exhibits a broad band with a maximum at 480 nm; no fluorescence was measured in low concentrations smaller than 10⁻⁵ M. After preliminary irradiation for 10 min, the spectrum (solid line) of the solution is significantly enhanced and largely blue-shifted by about 100 nm, showing a clear vibrational structure with peaks at 385,
405, and 430 nm. The solution once caused to induce fluorescence enhancement continues to exhibit the intense fluorescence more than several days no longer with preliminary irradiation. Similar enhancement of fluorescence can be seen for an ethanol solution of 3-NBA (Fig. 3). The spectrum (solid line) after irradiation becomes about 700 times as large as that (broken line) before irradiation, maximizing at 560 nm; contrary to the case of 1-NP, the enhanced spectrum for 3-NBA is only slightly red-shifted by 10 nm, showing no vibrational structure.

Figure 4 shows the dependence of the enhanced fluorescence at 385 nm on the wavelength of preliminary irradiation light for ethanol solution of 1-NP. The fluorescence intensity (diamond points) begins to increase from 450 nm and exhibits a maximum at 380 nm. The dotted line resembles the absorption spectrum (solid line) of 1-NP. This finding indicates that the excitation of 1-NP initiates the enhancement; namely, an excited state of 1-NP produces a strongly fluorescent compound. Similar result was obtained for ethanol solution of 3-NBA.

The fluorescence of the two nitroarenes increased with irradiation time up to about 30 min, but further irradiation caused the intensity to decrease without any change in the spectral shape. The fluorescence enhancement for the nitroarenes occured both in aerated and in degassed solution; the spectral shape of both enhanced fluorescence was identical and their intensities were almost the same. Thus, the features is markedly different from that for polycyclic aromatic ketones (PAKs) such as benzanthrone and naphthanthrone (details are described in Supporting Information).

Figure 5-a shows the chromatograms of ethanol solutions of 1-NP, which were recorded on DAD. At a high concentration of $1.0 \times 10^{-6}$ M, a peak appears at a retention time, $t$, of 25.2 min (Fig. 5-a1) before irradiation. After irradiation for 10 min, a new peak appears at $t = 22.1$ min and the peak at $t = 25.2$ min decreases compared with that before irradiation (Fig. 5-a2), indicating that the compound giving the latter peak undergoes a photochemical reaction to change into a product giving the former. Therefore, the peak at $t = 25.2$ min is assigned to 1-NP.
and that at $t = 22.1$ min to the fluorescent product, described above, causing fluorescence enhancement of 1-NP. At concentrations lower than $10^{-6}$ M, no peaks can be observed in the chromatogram on DAD regardless of irradiation; the chromatogram for $1.0 \times 10^{-8}$ M solution after irradiation is shown in Fig. 5-a3. On fluorescence detection, however, the fluorescent product is clearly detected at $t = 22.1$ min in the chromatogram even at a low concentration of $1.0 \times 10^{-8}$ M (Fig. 5-b2); before irradiation, no signals can be observed (Fig. 5-b1) because of extremely weak fluorescence of 1-NP. Similar results were obtained for 3-NBA. Calibration curves were drawn by plotting the fluorescence intensity against the concentration in the range from $1.0 \times 10^{-9}$ to $1.0 \times 10^{-7}$ M for 1-NP and $1.0 \times 10^{-8}$ to $1.0 \times 10^{-6}$ M for 3-NBA; the coefficients of determination $R^2$ were 0.9995 for 1-NP and 1.000 for 3-NBA. LODs were calculated as three times the standard deviation of the background noise and found to be 2.3 pg for 1-NP and 28 pg for 3-NBA. The LODs are almost the same as those of HPLC-FLD for reduced nitroarenes. Thus, the present results indicate that fluorescence enhancement is a promising method for determining nitroarenes, which are almost non-fluorescent and very important in environmental health-risk assessment, easily and sensitively.

Finally, we investigated the photochemical product responsible for the fluorescence enhancement using LC/MS. The mass spectra for ethanol solution of 1-NP after irradiation showed a dominant peak at $m/z = 217$ in the negative mode; the $m/z$ value corresponds to a deprotonated hydroxypyrene ion. This finding indicates that irradiation of 1-NP replaces the nitro group by a hydroxy group. Similarly, irradiation of 3-NBA in ethanol solution was found to give a negative ion with $m/z = 245$, corresponding to a deprotonated hydroxybenzanthrone ion. These findings suggest that irradiation of the nitroarenes initiates the rearrangement of the nitro-group to a nitrite-group, followed by elimination of nitric oxide and then by hydrogen abstraction from the solvent, as shown in the following scheme:
Here, Ar-NO\textsubscript{2} denotes nitroarenes and R-H solvent. From the reaction scheme, 1-hydroxypyrene (1-HP) and 3-hydroxybenzantrone (3-HBA) are assumed to give rise to the fluorescence enhancement of 1-NP and 3-NBA, respectively. In fact, the validity of the assumption for 1-NP was verified by the measurements with an authentic sample of 1-HP (Wako Pure Chemical, Tokyo, Japan); the fluorescence spectrum and retention time of 1-HP were in good agreement with those of 1-NP after irradiation. Therefore, the peak at \( m/z = 217 \) in the mass spectrum is certainly assigned to the deprotonated 1-HP ion. On the other hand, the assumption for 3-HBA could not be experimentally verified because its authentic sample was unavailable. As an alternative, molecular orbital calculations were performed (the results are described in Supporting Information and Table S1). From the ZINDO calculations, 3-HBA is predicted to fluoresce very weakly at wavelengths shorter than those for 3-NBA (Table S1), which disagrees with the experimental results. Thus, 3-HBA is hardly identified as the expected fluorescent product. The peak with \( m/z = 245 \), corresponding to HBA, in the mass spectrum may be due to ions produced in the ionization process. The ZINDO calculations also predict that 3-nitritebenzanthrone should fluoresce strongly at wavelengths longer than those for 3-NBA (Table S1), which qualitatively agrees with the experiment. Accordingly, 3-nitritebenzanthrone is much likely to be the expected fluorescent product. Since its chemical stability, consistent with the duration (longer than several days) of enhanced fluorescence, is not yet confirmed, we are now trying to isolate the fluorescent product from the irradiated solution of 3-NBA.

Detailed studies are also in progress on the photochemical reaction of various nitroarene/solvent systems and on the analysis of nitroarenes in the environment.

Supporting Information
Supporting information contains: Explanation of the fluorescence enhancement for 1-NP and 3-NBA, Results of the molecular orbital calculations, and Table S1. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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**Figure Captions**

Fig. 1 Structures of 1-NP (a) and 3-NBA (b).

Fig. 2 Fluorescence spectra of an ethanol solution of 1-NP ($1.0 \times 10^{-5}$ M) before and after preliminary irradiation. The spectrum before irradiation is magnified by a factor of $10^3$ and drawn with a broken line; the excitation wavelength is 400 nm. The spectrum after irradiation is drawn with a solid line; the excitation wavelength is 234 nm.

Fig. 3 Fluorescence spectra of an ethanol solution of 3-NBA ($1.0 \times 10^{-5}$ M) before and after preliminary irradiation. The spectrum before irradiation is magnified by a factor of $10^2$ and drawn with a broken line; the excitation wavelength is 440 nm. The spectrum after irradiation is drawn with a solid line; the excitation wavelength is 440 nm.

Fig. 4 Dependence of the enhanced-fluorescence intensity on the wavelength of preliminary-irradiation light for ethanol solution of 1-NP ($1.0 \times 10^{-6}$ M). The increase in the fluorescence intensity due to 10 min irradiation of light was measured and plotted against the wavelength (diamond points); the excitation and emission wavelengths of the fluorescence measurement were 234 and 385 nm, respectively. For comparison, the absorption spectrum of the solution is also drawn with a solid line.

Fig. 5 Chromatograms of ethanol solutions of 1-NP with an ethanol-water gradient elution. (a) On photo-diode-array detection at 234 nm: (a1) $1.0 \times 10^{-6}$ M solution before irradiation, (a2) $1.0 \times 10^{-6}$ M solution after irradiation, and (a3) $1.0 \times 10^{-8}$ M solution after irradiation. (b) On fluorescence detection with excitation and emission wavelengths of 234 and 385 nm, respectively: (b1) $1.0 \times 10^{-6}$ M solution before irradiation, (b2) $1.0 \times 10^{-8}$ M solution after irradiation.
Fig. 1 Structures of 1-NP (a) and 3-NBA (b).

Fig. 2 Fluorescence spectra of an ethanol solution of 1-NP (1.0×10^{-5} M) before and after preliminary irradiation.
Fig. 3 Fluorescence spectra of an ethanol solution of 3-NBA (1.0×10^{-5} M) before and after preliminary irradiation.

Fig. 4 Dependence of the enhanced fluorescence at 385 nm on the wavelength of preliminary irradiation light for ethanol solution of 1-NP.
Fig. 5 Chromatograms of an ethanol solution of 1-NP with an ethanol-water gradient elution.