One-Step Isolation and Identification of Hydroxylamino-Dinitrotoluenes, Unstable Products from 2,4,6-Trinitrotoluene Metabolites, with Thin-Layer Chromatography and Laser Time-of-Flight Mass Spectrometry

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Abstract

Two kinds of hydroxylamino-dinitrotoluenes (HADNTs), 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), are known to be major metabolites produced from 2,4,6-trinitrotoluene (TNT) by bacteria. These chemicals could not be identified as TNT metabolites produced by *Pseudomonas* sp. strain TM15 because the mass spectra of these chemicals could not be obtained by liquid chromatography–mass spectrometry (MS) or gas chromatography–MS, which are the classic methods for identifying the metabolites of xenobiotics. However, these problems are overcome by isolating 2HADNT and 4HADNT from TNT metabolites with one-step thin-layer chromatography using dichloromethane as the developing solvent, and individually extracting them into acetonitrile by collecting spots of 2HADNT and 4HADNT. The purity of each HADNT was approximately 98%, based on the results of high-performance liquid chromatographic analyses. 2HADNT and 4HADNT are identified by obtaining their mass spectra with laser time-of-flight MS. 2HADNT and 4HADNT dissolve in distilled water and are spontaneously broken down with time. Also, heat treatment (increasing temperatures) and dissolved oxygen accelerate the destruction of HADNTs. This technique may be applicable for the identification and exact quantitative analysis of unstable and fragile compounds such as HADNTs.

Introduction

Since the Industrial Revolution, massive amounts of chemicals have been produced and released into the environment around the world (1,2). Various living organisms such as bacteria, fish, and mammalians (including human) biotransform these chemicals into innocent forms via conjugation with glutathione or the breakdown of their structures. Various endogenous enzymes in biological fluid often produce unstable metabolic intermediates such as epoxides (3–5) and carbinolamine intermediates (6) in the process of metabolism. Unstable products are spontaneously broken down for a short interval of time and during the measurement. Therefore, identification and strict quantitation of unstable products requires a crucial technique. Many researchers must overcome these problems when they identify and quantitate unstable metabolic intermediates.

The highly energetic chemical 2,4,6-trinitrotoluene (TNT), one of the xenobiotics, is toxic because the symmetric location of the nitro groups on its aromatic ring limits attack by enzymes that normally metabolize aromatic compounds (7). Because of this special feature, TNT is persistent in the environment, presenting the risk of toxicity, mutagenicity, and carcinogenicity to animals and humans (8–10). TNT-metabolizing bacteria were screened from TNT-polluted soils in the Yamada Green Zone (Kitakyushu, Japan), where abnormal frogs have appeared, and one strain was found (11). This strain was designated as *Pseudomonas* sp. strain TM15 and could efficiently biodegrade the TNT present at 250 or 500 mg/L. In the culture medium of strain TM15, high-performance liquid chromatographic (HPLC) analyses detected two unidentified products, which have retention times of 10.7 and 11.4 min, respectively, as major TNT metabolites. As shown in Figure 1, these unidentified products were spontaneously disappearing with time, even though the conditions were abiotic. These compounds could not be identified because their mass spectra could be obtained with liquid chromatography (LC)–mass spectrometry (MS) and gas chromatography (GC)–MS. These compounds were unclear metabolites produced by *Pseudomonas* sp. strain TM15 in our studies on the degradation of TNT. In this paper, these metabolites were identified by a combination of thin-layer chromatography (TLC) (one-step isolation) and laser time-

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of-flight (TOF)-MS (mass spectrum), and the stability of 2HADNT and 4HADNT was examined in water under various conditions.

Experimental

Chemicals

As chemical standards, TNT (a gift of Chugoku Kayaku Co. Ltd., Tokyo, Japan); 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) (a gift of Dr. Y. Kumagai, Tsukuba University, Tsukuba-shi Ibaraki-ken, Japan); 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) (a gift of Dr. R. Spanggord, SRI International, Tokyo, Japan); 2-amino-4,6-dinitrotoluene (2ADNT); and 4-amino-2,6-dinitrotoluene (4ADNT) (AccuStandard Inc. New Haven, CT) were used. All chemicals were of the highest purity commercially available.

Culture condition

The cells of *Pseudomonas* sp. strain TM15 were aerobically grown until the late logarithmic phase in Luria-Bertani broth (10 g tryptone, 5 g yeast extract, and 5 g sodium chloride), containing 100 mg/L TNT at 30°C in the dark with shaking (120 rpm). Cells were washed twice with 50mM sterilized phosphate buffer (pH 7.0) and resuspended in the same buffer. Cell suspensions were mixed into the TNT solution (100 mL) with M8 minimal medium (12) containing TNT (100 mg/L) and acetate (10mM) as nitrogen and carbon sources, and the mixture was then aerobically incubated at 30°C in the dark with shaking (120 rpm).

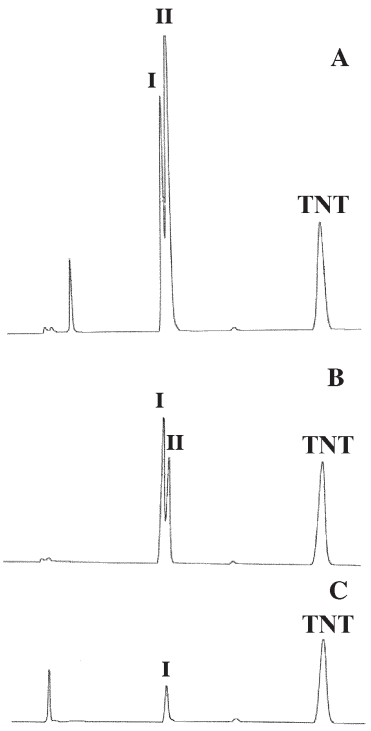

Figure 1. Spontaneous degradation of two unidentified metabolites produced from TNT by *Pseudomonas* sp. strain TM15. The cells were removed from the TNT reaction solution by centrifugation at 5000 × *g* for 10 min and with a membrane filter (0.45 µm) after 2-h cultivation at 30°C. The culture fluid was analyzed with HPLC after 0 h (A), 6 h (B), and 21 h (C), respectively. The two unidentified metabolites (I and II) and TNT have retention times of 10.7, 11.4, and 20.8 min, respectively.

Biotransformation of TNT and extraction of TNT metabolites

TNT solution was autoclaved (110°C, 10 min). The cells (~ 5×10^8 cells/mL) were aerobically incubated for different periods at 30°C and were removed by centrifugation at 5000 × *g* for 10 min. The culture fluid (pH 7.0) was extracted twice with 50 mL dichloromethane. The extracts were dried over anhydrous sodium sulfate, and excess solvent was removed by rotary evaporation under pressure at 30°C. The samples were used for TLC analysis.

Analyses or isolations of TNT metabolites with TLC

The sample (20 µL) was spotted on a silica gel sheet (20 × 20 cm) (Sillica gel 60 TLC aluminum sheet, Merck Ltd., Tokyo, Japan). This was subsequently developed once by the ascending method with a solvent system consisting of benzene–hexane–chloroform (1:1:1, v/v/v) or dichloromethane for a distance of 16 cm (total time 1 or 2 h). Spots of intermediates were visualized under UV illumination.

Identification of hydroxylamino-dinitrotoluenes with TOF-MS

Spots of the unidentified metabolites were extracted with acetonitrile. The purity of each sample was immediately assayed with HPLC. HPLC analyses were performed on an Inertsil ODS-2 column (GL Sciences Inc., Tokyo, Japan) with acetonitrile–water (40:60) as the mobile phase and a flow rate of 0.4 mL/min. TNT metabolites were detected at 254 nm with a Shimadzu (Kyoto, Japan) SPD-10AVP UV–vis detector. These samples were dropped on the sample plate and dried at room temperature, and then they were measured with laser TOF-MS (the conditions were: extraction mode, delayed; polarity, negative; extraction delay time, 100 ns; and laser intensity, 2000) (voyager DE, PerSeptive Biosystem Inc., Foster City, CA).

Stability test of the unstable TNT metabolites, 2HADNT and 4HADNT

2HADNT and 4HADNT were extracted from their spots on the TLC plate with distilled water, and then the extracts were incubated at 25°C and 90°C to monitor the stability of the unstable TNT metabolites. The concentrations of 2HADNT and 4HADNT were determined by measuring the absorbance at 254 nm with a UV–vis spectrophotometer V-530 (Jasco, Osaka, Japan). Also, 2HADNT and 4HADNT were dissolved in distilled water treated by argon gas (Taiyo Toyo Sanso, Osaka, Japan) with a SEC-4400M mass flow meter (STEC Inc., Kyoto, Japan) for 1 h (50 mL/min) at 25°C. To test the effect of dissolved oxygen on their destruction, these samples were also measured by a method similar to that mentioned previously.

Results and Discussion

One-step isolation with TLC and identification with TOF-MS

2ADNT and 4ADNT were previously identified as minor products with GC–MS analyses (11). Though two unidentified

metabolites (Figure 1), which were produced from TNT by *Pseudomonas* sp. strain TM15, were detected with HPLC, their mass spectra could be not obtained with LC–MS. Also, GC or GC–MS analyses were not adequate for their identification, whereas two unidentified products were extracted with dichloromethane or ethyl ether. It was assumed that they were fragile and unstable metabolites and may have been destroyed by high temperatures in electrospray ionization in LC–MS or in an injection chamber in GC. Therefore, the unstable TNT metabolites were isolated with TLC for their identification (Figure 2), and it was determined which spots are the unidentified metabolites as shown in Figure 1, with HPLC analyses after extracting each spot into acetonitrile. Two unidentified products could be separated by the use of dichloromethane as the developing solvent (Figure 2B), although they were motionless in a solvent system consisting of benzene–hexane–chloroform (1:1:1, v/v/v) as shown in Figure 2A. The purity of each metabolite dissolved in acetonitrile was 98.1% ± 1.26% and 98.5% ± 2.69%, respectively. They were measured with TOF-MS, which can softly ionize the samples without destroying their structure, and their mass spectra were obtained (Figure 3). Their unidentified metabolites were 4HADNT and 2HADNT produced by four-electron reduction, judging from a comparison of the retention time and mass spectrum between samples and standard chemicals (4HADNT and 2HADNT). Thus, the identification of the TNT metabolites was successful with the combination of TLC and TOF-MS. Although a previous study aimed at identifying 2HADNT and 4HADNT has been reported (13), the used method, which reduces each HADNT with a saturated ethanolic ammonium sulfide solution to the corresponding amine, was not clear-cut. Our technique may be applicable for the identification of metabolites (intermediates) of various chemicals when the structures of the targets could not be determined with GC–MS and LC–MS.

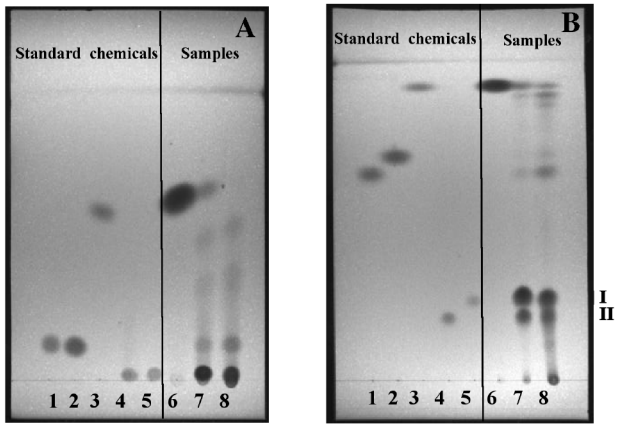

Figure 2. Thin-layer chromatograms of standard chemicals (lane 1–5) and samples (lane 6–8). The solvent systems for TLC were benzene–hexane–chloroform (1:1:1) (A) and dichloromethane (B). Standard chemicals were 4-amino-2,6-dinitrotoluene (lane 1), 2-amino-4,6-dinitrotoluene (lane 2), 2,4,6-trinitrotoluene (lane 3), 4-hydroxylamino-2,6-dinitrotoluene (lane 4), and 2-hydroxylamino-4,6-dinitrtotoluene (lane 5). Samples were prepared from the TNT reaction solution after 0-h (lane 6), 2-h (lane 7), and 9-h (lane 8) cultivation in the presence of *Pseudomonas* sp. strain TM15. I and II are the same unidentified metabolites as those of Figure 1.

Stability of 2HADNT and 4HADNT in various conditions

The absorbance was set at 254 nm because the aromatic ring allowed for monitoring the stability of 2HADNT and 4HADNT. As shown in Figure 4, the amount of these chemicals in distilled water was spontaneously decreasing with time, whereas bacteria and enzymes were absent of water, however, they were stable in acetonitrile. Also, the destruction of 2HADNT and 4HADNT was accelerated by heat treatment (Figure 5) but was suppressed with argon gas treatment (Figure 6), indicating that the cause of the breakdown of 2HADNT and 4HADNT may be dissolved oxygen. The fact that absorbance at 254 nm derived from 2HADNT and 4HADNT was decreased with time suggests that these chemicals may be converted into other products, which have molar absorbance coefficients lower than that of 2HADNT and 4HADNT, or the aromatic rings of these compounds may be cleaved.

Judging from the results, which were immediately accumulated after cultivation, 2HADNT and 4HADNT were the initial products produced from TNT by *Pseudomonas* sp. strain TM15.

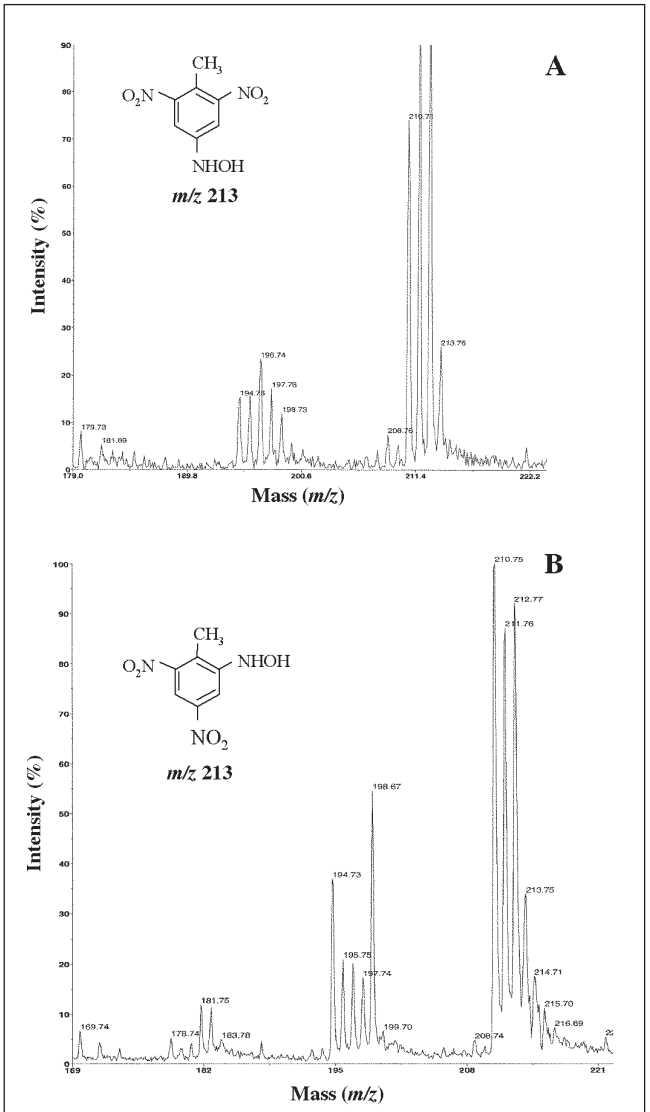

Figure 3. Mass spectra of 4HADNT (A) and 2HADNT (B). These chemicals were identified by TOF-MS analyses.

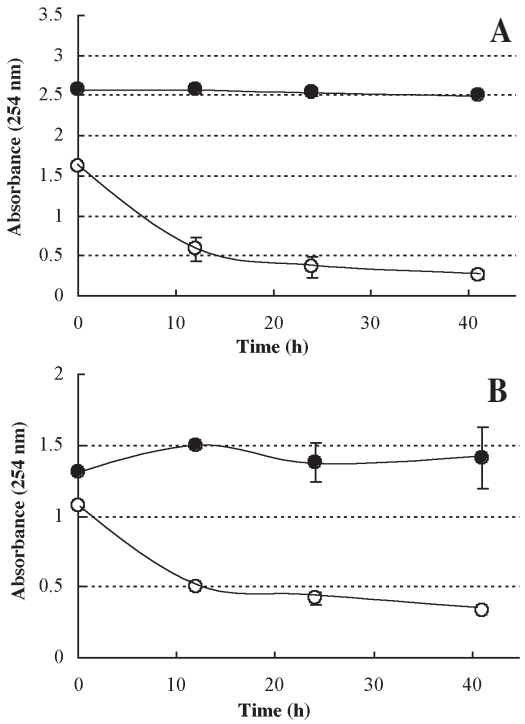

Figure 4. Decrease of the amount of 2HADNT (A) and 4HADNT (B) dissolved in distilled water (○) or acetonitrile (●). Data shown are averages of three independent measurements; error bars indicate the standard errors of the means.

Also, the amounts of 2ADNT and 4ADNT were gradually increasing with time, indicating that the strain TM15 converted 2HADNT and 4HADNT into 2ADNT and 4ADNT, respectively (Figure 2). The 2HADNT and 4HADNT were found to be unstable metabolites, which were spontaneously broken down in abiotic conditions. This destruction may be a significant phenomenon because this event habitually occurs in biodegradation processes by bacteria. Therefore, the elucidation of this phenomenon is essential for understanding the complete TNT-degradation mechanism by *Pseudomonas* sp. strain TM15. Although many studies of the biodegradation of TNT have been reported (14–16), to our knowledge, only a limited number of literature data are available on the destruction of 2HADNT and 4HADNT.

Conclusion

GC–MS and LC–MS analyses could not identify 4HADNT and 2HADNT, the initial TNT metabolites produced by *Pseudomonas* sp. strain TM15, because they are unstable and breakdown under high temperatures in a classic apparatus. These problems were overcome by isolating 4HADNT and 2HADNT with TLC and identifying them by obtaining their mass spectra with TOFMS. The amount of 4HADNT and 2HADNT in distilled water solution spontaneously decreased with time, although only a slight destruction could be observed in acetonitrile solution. The destruction of 2HADNT and 4HADNT is accelerated by increasing temperatures and the presence of oxygen.

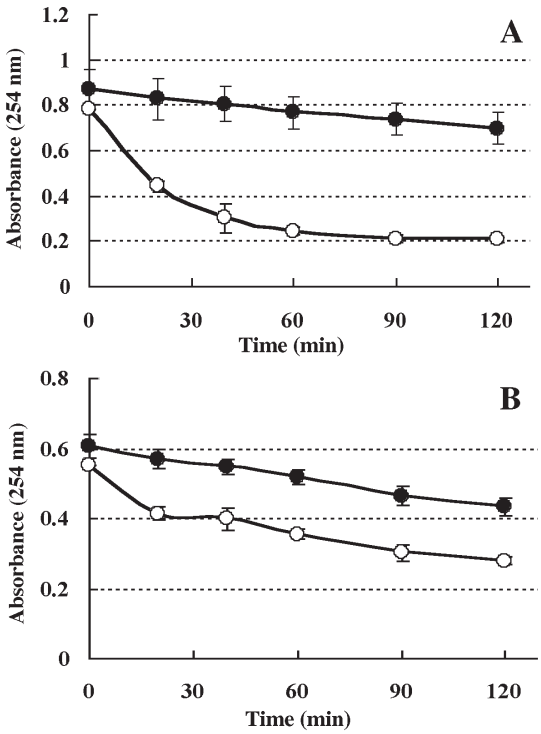

Figure 5. Effects of heat treatment on the destruction of 2HADNT (A) and 4HADNT (B). These chemicals were dissolved in distilled water and incubated at 25°C (●) or 90°C (○). Data shown are averages of three independent measurements; error bars indicate the standard errors of the means.

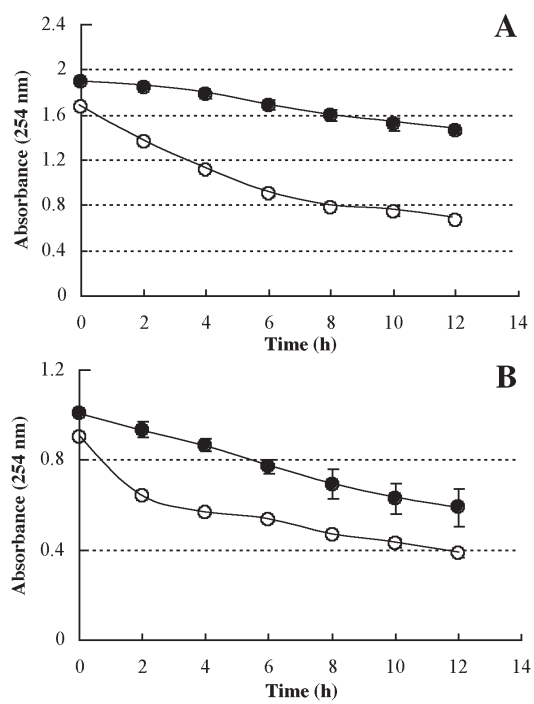

Figure 6. Effects of dissolved oxygen on the destruction of 2HADNT (A) and 4HADNT (B). These chemicals were dissolved in distilled water treated with argon gas (●). Also, they were dissolved in untreated distilled water (○) as control. Data shown are averages of three independent measurements; error bars indicate the standard errors of the means.

Acknowledgments

We are grateful to Chugoku Kayaku Co. Ltd., Japan, for their gift of TNT, and to Yoshito Kumagai, Department of Environmental Medicine, Institute of Community Medicine, Tsukuba University, for his gift of 4HADNT. We thank Junya Ogawa, Louis A. Watanabe, Hitoshi Asakawa, Tamaki Kato, Tetsuya Haruyama, and Norikazu Nishino of the Department of Biological Functions and Engineering, Graduate School of Life Sciences and Systems Engineering, Kyushu Institute of Technology, for their support in the TOF-MS analyses.

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Manuscript received July 27, 2005;
revision received December 13, 2005.