

Identification of Spontaneous Conversion Products of Unstable 2,4,6-Trinitrotoluene Metabolites, Hydroxylamino-dinitrotoluenes, by Combination of Thin-Layer Chromatography and Laser Time-of-Flight Mass Spectrometry

Toshinari Maeda¹, Nobuaki Nagafuchi¹, Akira Kubota¹, Kiwao Kadokami^{1,2}, and Hiroaki I. Ogawa^{1,*}

¹Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan and ²Aqua Research Center, Graduate School of Environmental Engineering, The University of Kitakyushu, 1-2-1 Shinike, Tobata-ku, Kitakyushu 804-0082, Japan

Abstract

Two kinds of hydroxylamino-dinitrotoluenes, 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. The amounts of 2HADNT and 4HADNT in distilled water are found to spontaneously decrease with time, albeit abiotic conditions, and many white precipitates are educed in the processes of this phenomenon; however, how these compounds are converted is unclear. We evinced the mystery of this phenomenon by using thin-layer chromatography (TLC) and laser time-of-flight mass spectrometry (TOFMS). TLC analyses in the spontaneous conversion processes of 2HADNT, 4HADNT, and 2HADNT plus 4HADNT demonstrates that three novel spots emerge on the TLC plate, respectively. These products are individually extracted into acetonitrile by collecting each spot. The purity of these extracts, which have retention times of 14.0, 17.7, and 15.4 min, is approximately 98%, judging from the results of high-performance liquid chromatographic analyses. The spontaneous conversion products of 2HADNT, 4HADNT, and 2HADNT plus 4HADNT are identified as 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'AZT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZT), and 4,2',6,6'-tetranitro-2,4'-azoxytoluene (2,4'AZT) by obtaining their mass spectra with laser TOFMS. It is confirmed that most of the spontaneous conversion products are 2,2'AZT, 4,4'AZT, or 2,4'AZT, judging from the results of mass balance in the spontaneous conversion processes of 2HADNT, 4HADNT, and 2HADNT plus 4HADNT.

Introduction

The highly energetic chemical 2,4,6-trinitrotoluene (TNT) is a nitroaromatic explosive that is released into soil and water ecosystems mainly due to military activities. TNT has been manufactured on a massive scale in the past. It is estimated that TNT is produced in amounts close to 1,000,000 kg per year (1). Presently, soil and groundwater contamination by the explosive is a serious problem in countries such as the United States, Germany, and Canada (2,3). Sediments and soils beneath some industrial sites contain large amounts of nitroaromatics, with up to 10 g of TNT per kg of soil being reported for some sites (4–6). TNT, a man-made compound, 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).

Many living organisms such as humans, plants, algae, yeast, and microorganisms commonly reduced TNT, resulting in the production of 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2-amino-4,6-dinitrotoluene (2ADNT), and 4-amino-2,6-dinitrotoluene (4ADNT) (11–15). TNT-polluted soils were isolated from the Yamada Green Zone (Kitakyushu, Japan), where abnormal frogs have appeared, and one strain was found (14). This strain, designated as *Pseudomonas* sp. strain TM15, also biotransformed TNT into 2HADNT, 4HADNT, 2ADNT, and 4ADNT. The amount of 2HADNT and 4HADNT in distilled water was found to spontaneously decrease with time, whereas bacteria and enzymes were absent in water (16) and many white precipitates were educed in the spon-

* Author to whom correspondence should be addressed: e-mail ogawahi@life.kyutech.ac.jp.

taneous conversion processes of 2HADNT and 4HADNT. However, how these compounds are converted is unclear. Although many studies of TNT biodegradation have been reported (11–19), to our knowledge, only limited numbers of literature data are available on the destruction of 2HADNT and 4HADNT. The elucidation of this phenomenon is essential for understanding complete TNT-degradation mechanism by *Pseudomonas* sp. strain TM15, because the spontaneous conversion of 2HADNT and 4HADNT habitually occurs in the biodegradation process by bacteria. In this paper, our objective was to reveal this phenomenon by using the combination of thin-layer chromatography (TLC) and laser time-of-flight mass spectrometry (TOFMS).

Experimental

Chemicals

As chemical standards, 2HADNT, 4HADNT, 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'AZT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZT), and 4,2',6,6'-tetranitro-2,4'-azoxytoluene (2,4'AZT) (a gift of Dr. R. Spanggord, SRI International, Menlo Park, CA) were used. All chemicals were of the highest purity commercially available.

Spontaneous conversion of 2HADNT and 4HADNT and extraction by organic solvent

2HADNT and 4HADNT, dissolved in distilled water, were incubated at 30°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). Then, their fluids (1 mL) in the process of spontaneous conversion, were extracted twice with 0.5 mL dichloromethane. The samples were used for TLC analysis.

Analyses or isolation of the spontaneous conversion products with TLC

The sample (20 μ L) was spotted on a silica gel sheet (10 by 10 cm) (Silica gel 60 TLC aluminum sheet, Merck, Tokyo, Japan). This was subsequently developed once by the ascending method with a solvent system comprised of benzene–hexane–chloroform (1:1:5 by volume) for a distance of 8 or 16 cm. Spots of intermediates were visualized under UV illumination.

Identification of the spontaneous conversion products with TOFMS

The unidentified spots were extracted with acetonitrile. Purity of each sample was immediately assayed with high-performance liquid chromatography (HPLC). HPLC analyses were performed on an Inertsil ODS-2 column (4.0 \times 150 mm, 5- μ m particle size; GL Sciences, Tokyo, Japan) with acetonitrile–water (60:40) as the mobile phase, with a flow rate of 0.4 mL/min. The compounds were detected at 254 nm with a Shimadzu (Tokyo, 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 TOFMS under the following conditions: extraction mode, delayed; polarity, negative; extraction delay time, 100 nanoseconds; laser intensity, 2000 (voyager DE, PerSeptive Biosystem Inc., Foster City, CA).

Mass balance analyses

The concentrations of 2HADNT, 4HADNT, and the spontaneous conversion products were determined by an HPLC method similar to that mentioned earlier. 2HADNT, 4HADNT, and 2HADNT plus 4HADNT, dissolved in distilled water, were incubated at 30°C, and then their fluids (1 mL) in the process of spontaneous conversion were extracted twice with 1 mL dichloromethane to extract all compounds including the white precipitates (spontaneous conversion products). Then, organic solvent (dichloromethane) in the extracts was completely removed by drying it at 25°C in the desiccator, and the forming powders were dissolved into acetonitrile (1 mL). The samples were used for HPLC analyses.

Results and Discussion

Identification of the spontaneous conversion products of 2HADNT and 4HADNT

The absorbance was measured at 254 nm due to the aromatic ring, which allowed us to monitor the stability of 2HADNT or 4HADNT, and it was found that 2HADNT and 4HADNT, which are produced from TNT by *Pseudomonas* sp. strain TM15, were fragile and unstable TNT metabolites in abiotic conditions, as

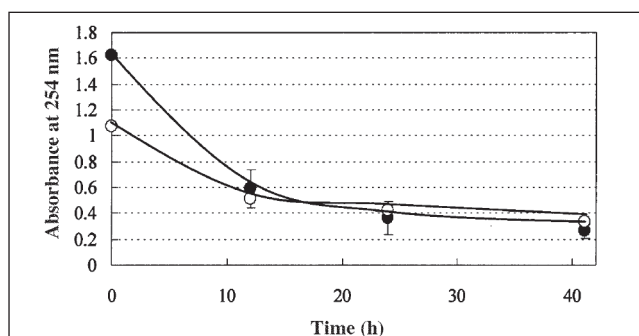


Figure 1. The spontaneous conversion of 2HADNT (●) and 4HADNT (○) in distilled water. These chemicals were dissolved in distilled water and were incubated at 30°C. Data shown are averages of three independent measurements; error bars indicate the standard errors of the means.

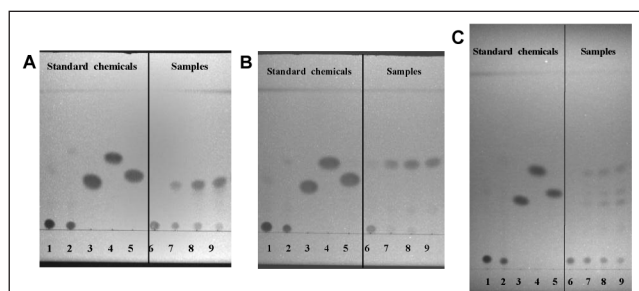


Figure 2. Thin-layer chromatograms of standard chemicals (lane 1–5) and samples (lane 6–9). TLC was developed once by the ascending method with a solvent system comprised of benzene–hexane–chloroform (1:1:5 by volume) for a distance of 8 cm (A and B) or 16 cm (C). Standard chemicals were 2HADNT (lane 1), 4HADNT (lane 2), 2,2'AZT (lane 3), 4,4'AZT (lane 4), and 2,4'AZT (lane 5). Samples were prepared from the reaction solution of 2HADNT (A), 4HADNT (B), and 2HADNT plus 4HADNT (C) after 0-day (lane 6), 1-day (lane 7), 2-day (lane 8), and 3-day (lane 9) cultivation.

shown in Figure 1, and it was also found that many white precipitates were educed in the processes of this phenomenon. Although many studies of TNT biodegradation have been reported (11–19), to our knowledge, only limited numbers of literature data are available on the destruction of 2HADNT and 4HADNT, and no literature data reports on the white precipi-

tates. Therefore, to isolate the spontaneous conversion products formed from 2HADNT, 4HADNT, and 2HADNT plus 4HADNT, TLC analyses were performed for the scientific elucidation. The results of TLC analyses showed producing three unidentified spots on the TLC plate (Figure 2). Three unidentified products were able to be separated by the use of benzene–hexane–chloroform (1:1:5 by volume) as developing solvents for a distance of 8 cm or 16 cm. The purity of each metabolite dissolved in acetonitrile was $98.1\% \pm 0.86\%$, $98.5\% \pm 1.22\%$, and $97.9\% \pm 1.51\%$, respectively. Because the mass spectra for these unidentified metabolites could not be obtained by using GC–MS, they were measured with TOFMS, which can softly ionize the samples without the destruction of their structure, resulting in obtaining their mass spectra (Figure 3). Their unidentified metabolites were 2,2'AZT, 4,4'AZT, and 2,4'AZT, judging from a comparison of the retention time and mass spectrum between samples and standard chemicals (2,2'AZT, 4,4'AZT, and 2,4'AZT). Although many studies have reported that 2,2'AZT, 4,4'AZT, and 2,4'AZT were produced in the processes of TNT degradation and composting (17–21), no direct evidence for the production of these tetranitroazoxytoluenes have been reported; although only one previous study has reported that these azoxytoluenes are produced from partial TNT reduction metabolites in the presence of molecular oxygen (21). The results obtained by the method, which reduces TNT with ammonium sulfide, were uncertain and not clear-cut because these solutions are mixtures, which include HADNTs, amino-nitrotoluenes, and other reduction products, and because actual mass balance between HADNTs and azoxy compounds does not match theoretical values quantitatively (a molecules of azoxytoluenes should be produced from two molecules of HADNTs). Our results clearly showed that spontaneous conversion products of 2HADNT and 4HADNT are 2,2'AZT, 4,4'AZT, and 2,4'AZT by using the combination of TLC and TOFMS. Understanding the mechanism of spontaneous conversion for these compounds is essential for understanding the complete TNT-degradation mechanism by *Pseudomonas* sp. strain TM15 because the spontaneous conversion of 2HADNT and 4HADNT habitually occurs in the TNT-biodegradation process by bacteria. A possible mechanism for the spontaneous production of azoxytoluenes has been suggested in previous literature (21). Also, our previous study showed that dissolved oxygen is responsible for the spontaneous conversion of HADNTs (16); this is consistent with previous results that azoxy compounds were determined to spontaneously form in the presence of molecular oxygen from partial TNT reduction products (21). However, it must be determined which bacterial degradation or spontaneous conversion is a preferential pathway in TNT or HADNT degradation; this knowledge will be significant for developing the TNT remediation technologies.

Mass balance of tetranitroazoxytoluenes from 2HADNT and 4HADNT

This study examined the mass balance of 2,2'AZT, 4,4'AZT, and 2,4'AZT, which are produced from 2HADNT and 4HADNT, by measuring these compounds with HPLC. As shown in Figure 4, it was confirmed that most of the spontaneous conversion products were tetranitroazoxytoluenes. Concentrations of 2,2'AZT and 4,4'AZT accounted for approximately half of those of

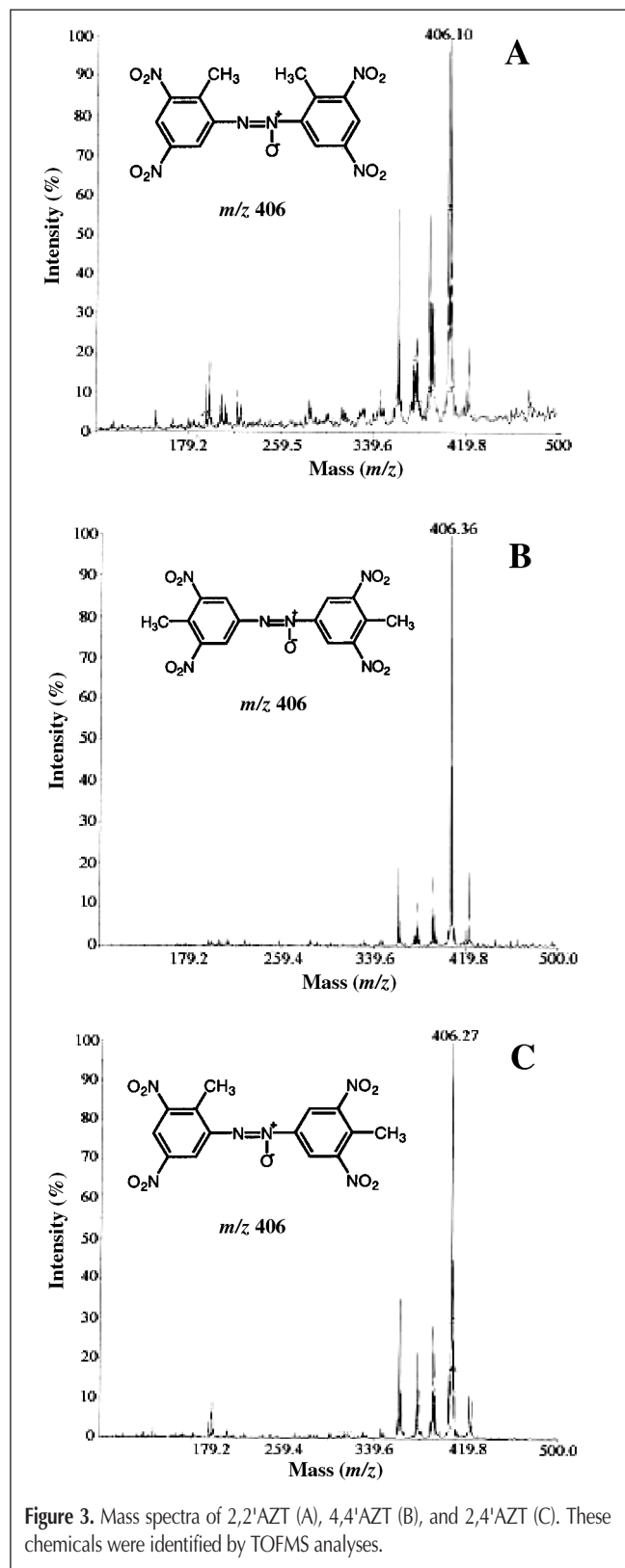
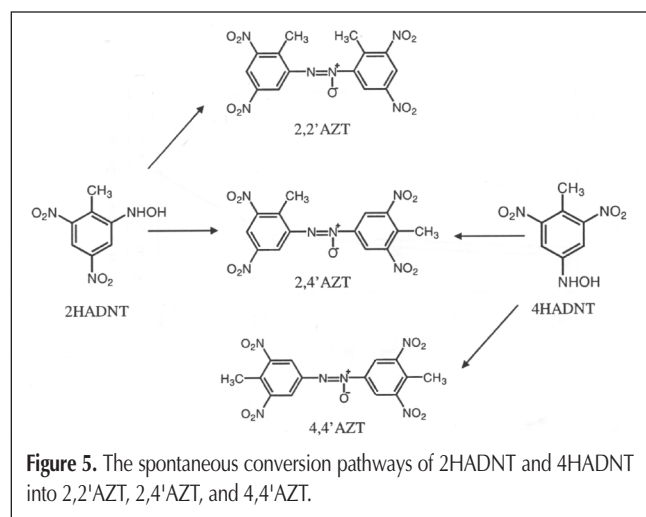
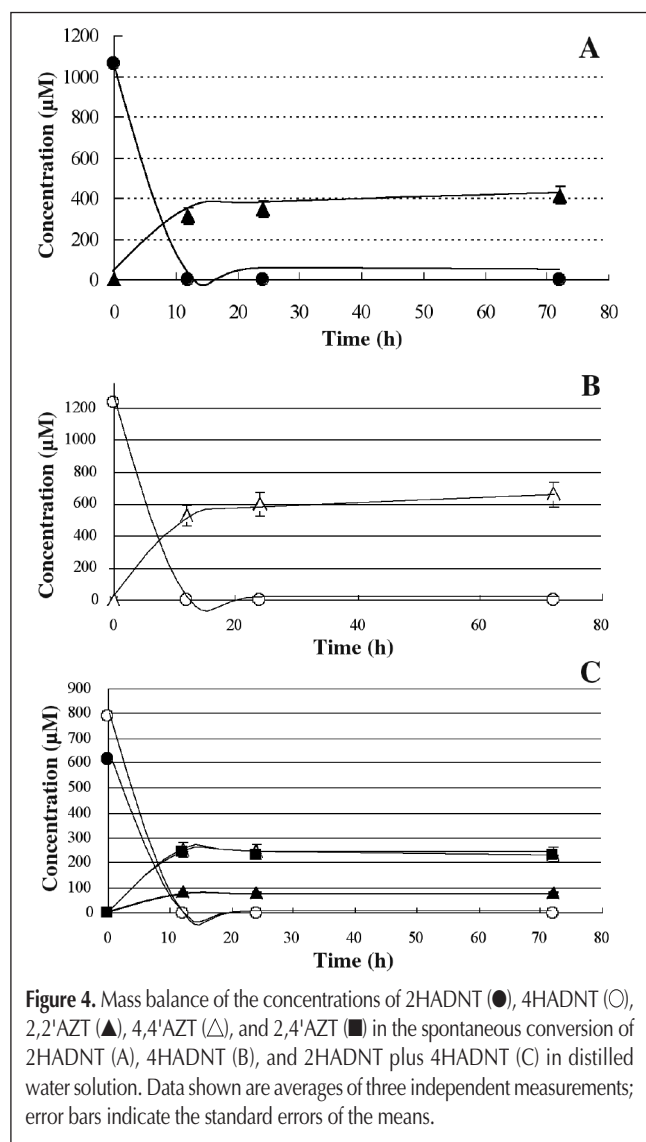


Figure 3. Mass spectra of 2,2'AZT (A), 4,4'AZT (B), and 2,4'AZT (C). These chemicals were identified by TOFMS analyses.

2HADNT and 4HADNT, respectively. These results are reasonable because a molecule of 2,2'AZT or 4,4'AZT are produced from two molecules of 2HADNT or 4HADNT. Also, the spontaneous conversion of 2HADNT plus 4HADNT accumulated 4,4'AZT and

2,4'AZT in preference to 2,2'AZT, which was an asymmetrical conformation. These results may contribute to the stable conformation of tetranitroazoxytoluenes.



Conclusion

2HADNT and 4HADNT in distilled water solutions were spontaneously decreasing with time albeit abiotic conditions. As shown in Figure 5, it was demonstrated that the spontaneous conversion products of 2HADNT and 4HADNT were 2,2'AZT, 4,4'AZT, and 2,4'AZT by using the combinations of TLC and TOFMS. The results of mass balance indicated that most of the spontaneous conversion products were tetranitroazoxytoluenes.

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