Environ. Sci. Technol. **2008,** *42,* 734–739

OYE Flavoprotein Reductases Initiate the Condensation of TNT-Derived Intermediates to Secondary Diarylamines and Nitrite

ROLF-MICHAEL WITTICH,*,† ALÍ HAÏDOUR,†,‡ PIETER VAN DILLEWIJN, AND JUAN-LUIS RAMOS†

Departamento de Protección Ambiental, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Calle Profesor Albareda 1, E-18008 Granada, Spain, and Unidad de Resonancia Magnética Nuclear, Centro de Instrumentación Científica, Universidad de Granada, Paseo Juan Osorio s/n, E-18071 Granada, Spain

Received June 15, 2007. Revised manuscript received November 20, 2007. Accepted November 21, 2007.

Polynitroaromatic explosives such as 2,4,6-trinitrophenol (picric acid) and 2,4,6-trinitrotoluene (TNT) are toxic and recalcitrant environmental pollutants. They persist in the environment due to the highly inactivated π system of their aromatic rings, which are inaccessible to dioxygenases that normally initiate the bacterial aerobic catabolism of (nitro-) aromatic compounds. Aside from reductive transformation of nitro side groups to hydroxylamines, trinitroarenes are prone to aromatic ring reductions by some flavin reductases to yield Meisenheimer mono and dihydride complexes. Here we show that the simultaneous accumulation of Meisenheimer complexes and aromatic hydroxylamines derived from TNT gives rise to the condensation of both types of reactive intermediates to secondary diarylamines and nitrite as the end-products of this environmentally relevant reaction sequence. As a consequence, overall mass balances of aerobic biotransformations of TNT become possible for the first time. In our study, the process of TNT activation was enzymatically initiated by the xenobiotic reductase B (XenB)-like flavin reductase of *Pseudomonas putida* JLR11 and then completed chemically by autodimerization. The structures of the formed end products were unequivocally elucidated by NMR.

Introduction

The increasing number of publications on the old yellow enzyme (OYE) flavoprotein reductases of yeast and bacteria strongly suggests that their activity with regard to the reduction of cyto- and hepatotoxic nitroaromatic compounds is well established, and of considerable interest for applications in industrial and medical biotransformations, and whole cell bioremediation technologies (*1, 2*). Catabolic pathways for aerobic bacterial mineralization of mono and dinitroaromatic compounds, generally initiated by electrophilic dioxygenase systems, have long been known (*3*). However, trinitrated compounds such as 2,4,6-trinitrophenol (picric acid) and 2,4,6-trinitrotoluene (TNT) were regarded as extremely recalcitrant. Nevertheless, a convincing catabolic route was described in gram-positive *Rhodococcus* and *Mycobacterium* strains, whereby the aromatic rings of these trinitroarenes are reduced to Meisenheimer hydride complexes (*4–6*). These findings clearly demonstrated that, while mono and dinitroaromatics are preferentially attacked by bacterial oxygenase enzymes, the highly inactivated aromatic π system of trinitroarenes can only be activated by reductases such as those found in the aforementioned microorganisms. Low concentrations of azoxy- and azo condensates from intermediary hydroxylamine and nitroso derivatives have also been identified and in a few cases partially denitrated intermediates or end-products were reported as well, though their proposed structures were not always unequivocally confirmed (*3*).

In contrast to picric acid, which was shown to be completely mineralized through H^--trinitrophenol formation, further reduction by a hydride transferase and subsequent ring cleavage (*7*), TNT biotransformation by the above bacteria resulted in the formation of reduced aromatic ring systems, partially reduced nitro groups and concomitant nitrite release as end-products. However, the Meisenheimer dihydride complex formed by *Rhodococcus erythropolis* strain HL PM-1 has been described as having a very short half-life of only a few hours, whereas investigating its structure in a nuclear magnetic resonance spectrometer, this reactive compound was assumed to undergo rapid decomposition, presumably by hydrolytic denitration in deuterated water (*6*). Also important in this context and from an environmental point of view, is the immobilization of biologically generated reactive products into nonextractable polymeric soil structures. Thus, they could escape structural identification as potential environmental pollutants (*8, 9*).

In the meantime, the description of TNT-reducing bacteria has become legion (*10–12*). OYE flavoproteins have been identified in gram-negative bacteria such as *Enterobacter cloacae* from which pentaerythritol tetranitrate (PETN) reductase was purified and crystallized (*13, 14*). This enzyme not only can reduce nitro groups of nitrate esters but also of TNT. Additionally, it simultaneously reduces the aromatic ring leading to nitrite release for growth (*13*). Similarly, the so-called xenobiotic reductase B from *Pseudomonas fluorescens* I-C (*15, 16*) catalyzes analogous reactions, thereby confirming earlier observations (*17, 18*) of the reductive potential of bacteria of the genus *Pseudomonas*. Moreover, the OYE-like *N*-ethyl maleimide reductase from *Escherichia coli* demonstrates similar capacities (*2*) and has been shown to be responsible for allowing growth of this bacteria with TNT as sole nitrogen source (*19, 20*). However, in most of the abovementioned cases, the structures of the denitrated intermediate(s) and end products, information critical for determining the importance of these reactions for the bioremediation of TNT, still await unequivocal elucidation.

Here, we demonstrate that stabilization of the TNT-derived Meisenheimer dihydride complex originating either from in vitro biological enzyme reactions with a recombinant flavoprotein reductase of *Pseudomonas putida* JLR11, or generated chemically, occurs through the condensation with biologically or chemically prepared reactive hydroxylamines to form stable secondary diarylamines.

Materials and Methods

Chemicals. Analytical standards of amino- and hydroxylaminodinitrotoluenes as well as azoxy and azo adducts were from AccuStandard (New Haven, CT). All other nitroaromatics

* Corresponding author phone: +34-958-181 600 X138; fax: +34-958-129 600; e-mail: rolf.wittich@eez.csic.es.
† Consejo Superior de Investigaciones Científicas.
‡ Universidad de Granada.

10.1021/es071449w CCC: $40.75

Published on Web 01/03/2008

were from Aldrich Chemie, Steinheim, Germany, and all other chemicals (p.a. grade) from Fluka, Buchs, Switzerland. TNT was obtained from Unión Española de Explosivos (Madrid, Spain).

Construction of an Expression Vector for Recombinant Protein Production. *Pseudomonas putida* JLR11 (no. PSC 44, EEZ, *Pseudomonas* Reference Culture Collection, Granada) was grown routinely at 30 °C in M9 minimal medium supplemented with 0.5% (wt/vol) glucose or 10 mM sodium benzoate as a carbon source (*21*). Chromosomal DNA from *P. putida* JLR11 was obtained using an AquaPure genomic DNA isolation kit (Bio-Rad). To obtain the *xenB* homologue gene of *P. putida* JLR11, genomic DNA was used as a template for amplification by PCR using 5′-TTTGGATCCATAAAAG-CACTGGCCCAC-3′ as a forward primer and 5′-AAAAAGCT-TCAACCGCGGATAATCGATG-3′ as a reverse primer. The amplification product was then digested with the restriction enzymes *Bam*HI and *Hin*dIII prior to ligation into a pET28b(+) vector (Novagen) which likewise had been digested with *Bam*HI and *Hin*dIII. The resulting plasmid contained the coding sequence in frame with a DNA sequence encoding a His_6-tag at its 3′ end which was verified by DNA sequencing. The nucleotide sequence of the xenobiotic reductase B of *P. putida* JLR11 is 100% identical to the sequence of the same name (Genebank ID AAN66545) in *P. putida* KT2440.

Purification and Assay of XenB-like Reductase. For protein-His_6 purification, the pET28b+ derivative plasmid was transformed into *E. coli* BL21 pLysS (Novagen). The cells were grown in several one-liter batches at 30 °C in 2 × YT culture medium (*21*) with 50 μg mL^{-1} kanamycin to an A_{660} between 0.5 and 0.7 and then induced with 1 mM IPTG. Cells were harvested after overnight induction at 18 °C, resuspended in 500 mM NaCl, 10% (vol/vol) glycerol, 25 mM sodium phosphate buffer pH 7.5, 0.1 mM EDTA, and 1 μM FMN and protease inhibitor cocktail (complete, Roche) and disrupted by treatment with 20 μg mL^{-1} of lysozyme and French press. Following centrifugation at 20000*g* for 30 min, the protein was found predominantly (more than 80%) in the soluble fraction. XenB-His_6 was purified by nickel affinity chromatography and eluted with a continuous imidazole gradient. Peak fractions were pooled and the protein concentration determined using the Bio-Rad Protein Assay kit. The molecular mass was determined by SDS-PAGE using defined protein standards (Bio-Rad). Results of the purification are shown in Supporting Information (SI) Figure 1).

The enzyme reaction contained in a total volume of 1 mL: 0.85 mL of 50 mM sodium potassium phosphate buffer of pH 7.0 saturated with TNT (overnight with an excess of mortar-ground TNT followed by filtration before starting the experiment, final concentration 0.49 mM at 25 °C), 0.02 mL of isopropanol as the substrate for alcohol dehydrogenase, also saturated with TNT and giving a total concentration of 0.9 mM of TNT in the assay; 0.01 mL of NADPH (10 mM), and 0.1 mL of secondary alcohol dehydrogenase (5 U) from *Thermoanaerobium brockii* (Sigma) for recycling of formed $NADP^+$. The reaction was initiated by the addition of 0.02 mL (2.25 μM) of purified recombinant xenobiotic reductase B of the bacterial strain *Pseudomonas putida* JLR11 and proceeded at 25 °C in the autosampler of the HPLC system, which served to monitor the course of the enzymatic reaction (concentration of NADPH and NADP), the depletion of TNT (**1**), and the transient formation of the monohydride complex of TNT (**2**), the subsequent dihydride complex (**3**), and the two isomeric secondary diarylamines **9** and **10** over time (see Figure 1).

Instrumentation and Analyses. Nuclear magnetic resonance (NMR) spectra were measured on a Varian 500 MHz spectrometer at room temperature in deuterated acetonitrile. Infrared (IR) spectra were recorded on a NICOLET model 20SXB FT-IR spectrometer. Mass spectral data were obtained in the negative ionization mode with a Varian model 1200L quadrupole spectrometer coupled to a Varian Prostar HPLC system. Routine HPLC analyses were performed on a Hewlett-Packard model 1050 chromatograph equipped with a PDA at 25 °C on a Waters Nova-Pak C-8 column (5 μm, 3.9 × 150 mm). Solvent A of eluent system I consisted of water containing 5 mM tetrabutylammonium phosphate (pH 7.0, Fluka) as the ion-pairing agent and solvent B was gradient-grade acetonitrile. The compounds mentioned below (See also Figure 1) were separated at a flow rate of 0.85 mL min^{-1}. The gradient program was as follows: 0−13% B in 3 min, then to 25% in 17 min, and to 62% in 23 min; reequilibration was for 9 min. Retention times (min) and maxima of selected UV–vis spectra (nm) were as follows: nitrite, 3.3; nitrate, 4.8; the five isoforms of the Meisenheimer dihydride complex (**3**), **3a** 9.1 (231, 422), **3b** 9.9 (236, 469), **3c** 12.5 (265, 438), **3d** 14.4 (263, 502), **3e** 15.9 (262, 496); TNT (**1**), 16.3; 2-hydroxylamino-4,6-dinitrotoluene (**6**), 13.8; 4-hydroxyl-amino-2,6-dinitrotoluene (**5**), 14.8; 2-amino-4,6-dinitrotoluene, 15.1; 4-amino-2,6-dinitrotoluene, 15.7; Meisenheimer monohydride complex of TNT (**2**) (253, 476), 21.3; minor (3–5% of (**2**)) Meisenheimer monohydride complex of TNT (hydrogen enters geminal with regard to the methyl group) 25.2 (208, 248, 470); the 3 new compounds (**7**), 29.6 (200, small shoulders at 230 and 292); (**9**), 30.7 (201, 276, shoulder at 320); and (**10**), 31.5 (204, shoulder at 232, 268, shoulder at 320); 4,4′,6,6′-tetranitro-2,2′-azoxytoluene, 33.3; 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 33.9. (Bio-) transformation reactions were routinely monitored at 210, 230, and 450 nm, and UV–vis spectra recorded within the range from 200 to 600 nm were stored on a PC connected to the instrument. Purifications of compounds **7**, **9**, and **10** were carried out on a semipreparative Nova-Pak HR C-18 column (6 μm, 7.8 × 300 mm, Waters) with acetonitrile-water (40:60%, v:v, system II) at ambient temperature (22 °C). Retention times were as follows: 38.0 min for **7**, 47.2 min for **10**, and 55.0 min for **9**.

Reduction Products from TNT and Synthesis of the Dimers. Standard procedures (syntheses of compounds **3**, **5**, and **6**, see Figure 1): Due to the low solubility of TNT in salts-buffered water (113 mg, 0.49 mmol at 25 °C) (*22*), an excess of the mortar-ground compound was dispersed in 2 L of water on a rotary shaker (120 rpm) overnight at 30 °C before being kept at 25 °C. To the resulting saturated solution obtained after filtration, small quantities of KBH_4 were added slowly under stirring. The formation of **2** (reddish-brown) and **3** (orange-yellow) was monitored by ion-pair reversed-phase HPLC (C-18) after every increment of KBH_4 until only **3** was present (monitored as its five isoforms; it has to be mentioned that **3** is being formed from **2** much faster than **2** from **1**, meaning that the presence of **2** is only transient and **3** always present in aqueous solution). We have previously shown that compound **3** remains stable in aqueous solution for several weeks while very slowly degrading to 2-hydroxylamino-6-nitrotoluene **4** (*19*). Since the synthesis of **3** was performed stoichiometrically from **1** and the UV spectra of each of the isoforms differ only slightly, the sum of the isoforms was used to calculate the concentration of **3.** On the other hand, in a 100 mL Erlenmeyer flask flushed with nitrogen, **1** (681 mg, 3.0 mmol in 20 mL of ethanol) and ammonium chloride (1.0 g, 18.7 mmol in 10 mL of water) were combined under constant stirring. Zinc powder (800 mg, 12.3 mmol) was added stepwise and the solution was kept stirring under temperature control (50 °C). The formation of compounds **5** and **6** was monitored by HPLC over the entire time of reaction as had been done for the production of compound **3**. Calculation of the concentrations and stoichiometry was achieved using the respective reference compound supplied by AccuStandard (New Haven, CT).

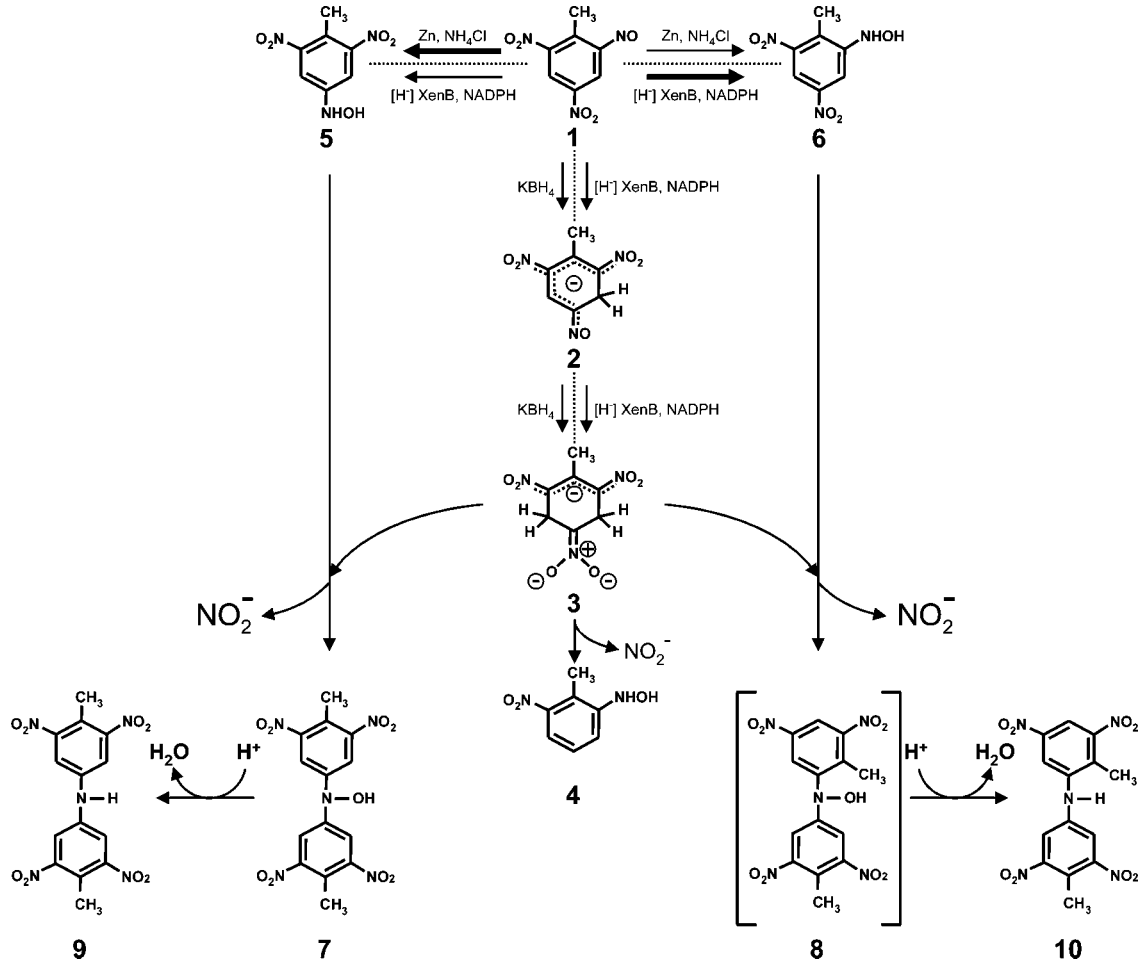

FIGURE 1. Release of nitrite by rearomatization of 2,4,6-trinitrotoluene-derived Meisenheimer dihydride complexes through condensation with TNT-derived hydroxylamines. Enzymatic reductions as well as chemical reductions furnish identical products which undergo the shown condensation reactions.

Upon completion of the reduction of TNT to both hydroxylaminodinitrotoluenes, the solution containing compounds **5** (78%) and **6** (22%) was cooled to 25 °C, diluted 10-fold with distilled water, neutralized to pH of 7.0 and a stoichiometric amount of compound **3** added, to simulate the conditions in the biological system described above. The reaction mixture was stirred in the presence of air and started to turn turbid after several minutes, indicative for the formation of the dimers, which precipitated nearly completely within 4 h due to their low solubility in water. Samples were analyzed every 55 min by HPLC. After 2 more hours of incubation, the crude products were collected by filtration and the filtrate extracted with ethylacetate, dried over anhydrous sodium sulfate and the solvent evaporated in vacuum. The final yield was 323 mg of a red-brownish solid, which was dissolved in acetonitrile. The mixture of the newly formed compounds containing minute amount of residual TNT was analyzed by LC-MS and separated by semipreparative HPLC, using a mixture of acetonitrile-water (40:60, v:v). Fractions from eight separations containing compounds **7**, **9**, and **10** were pooled, saturated with sodium chloride to achieve transfer into the organic phase, and the aqueous phase discarded. The organic phases were dried over anhydrous magnesium sulfate and the solvent evaporated in vacuum, yielding in all cases light-yellow semicrystalline compounds which were further analyzed by NMR and FT-IR spectrometry.

Results and Discussion

We describe here the condensation (dimerization) of the Meisenheimer dihydride complex (compound **3** in Figure 1) with hydroxylaminodinitrotoluenes (compounds **5** and **6**) derived from the chemical or enzymatic reduction of TNT, to form the corresponding secondary diarylamines (compounds **9** and **10**) with the concomitant release of significant, nearly stoichiometric amounts of nitrite (Figures 2, 3). In both cases identical condensation products were formed, which confirms that this process of nitrite elimination is basically a chemical S_N reaction and thus not enzyme-catalyzed. Products obtained from both the enzymatic and chemical dimerization were analyzed by LC-MS, ^{1}H NMR, ^{13}C NMR, and FT-IR. However, we could not find any evidence for the occurrence of a carbon-carbon coupling reaction suggested previously to furnish isomeric aminodimethyltetranitrobiphenyls (*16, 20*). In contrast to several reports on C−C coupling reactions starting from Meisenheimer complexes, those reported to yield (rearomatized) diaryl amines are rare. These polyfunctional amines are of interest since they represent building blocks for many pharmaceuticals, natural products, and other chemicals. Apart from the relevance of above-mentioned microbially induced processes for environmental biogeochemistry, the herein reported structures may also be of potential interest as highly energetic and insensitive explosives and/or propellant formulations (*23*).

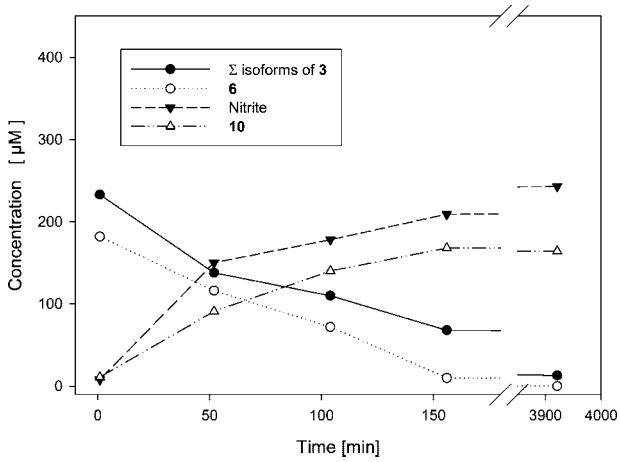

FIGURE 2. Chemical autocondensation of 2-hydroxylamino-4,6-dinitrotoluene (6) with the Meisenheimer dihydride complex of TNT represented by the overall sum of the five isoforms (3) over time in aqueous solution as monitored by HPLC. Samples were diluted with acetonitrile (1:1, vol) prior to analysis in order to redissolve the partially precipitated secondary diarylamine (10).

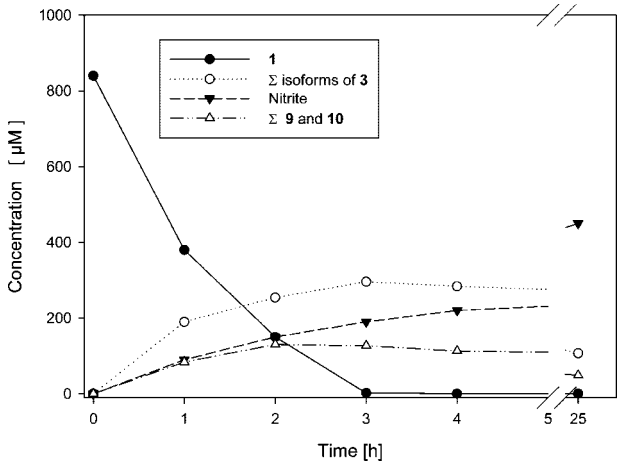

FIGURE 3. Time course by HPLC analysis of the enzymatic transformation of 1 by recombinant xenobiotic reductase from *Pseudomonas putida* JLR11. Within 3 h the target compound 1 is transformed to the predominant compounds 3, 9, and 10. The reaction does not appear to be fully stoichiometric because the secondary diarylamines and azoxy toluenes (always less than 11% of corresponding 9 and 10, not shown) started to precipitate after about 20−30 min of the reaction. Dissolving the reaction mixture with acetonitrile (1:1 by vol) resulted in their almost stoichiometric recovery, of at least 89% with regard to eliminated nitrite. Very small amounts of 5 and 6, as well as the corresponding amines were also detected (not shown).

The present in-depth study of TNT transformation was motivated by the observation of the formation of a relatively large amount of an unexpected brownish-yellow precipitate at the end of an in vitro reaction with a nitro reductase from the TNT-converting *Pseudomonas putida* strain JLR11 which shares 88% identity with the xenobiotic reductase B (XenB) of *Pseudomonas fluorescens* (*15*). However, monitoring of this enzyme reaction over several hours by HPLC using conditions described previously (*16*), only showed the production of small quantities of Meisenheimer dihydride complexes and very minute amounts of azoxy derivatives; the latter only appearing after several hours. Hydroxyl-amine derivatives of TNT were hardly detectable. We optimized the conditions of the reaction by increasing the substrate concentration by about 9-fold from the initial 0.1 mM by using TNT-saturated buffer solution as well as TNT-saturated isopropanol which is the substrate of the secondary alcohol dehydrogenase used to recycle NADPH (*2*). Under these conditions, additional peaks close to the known azoxy adducts became detectable although much less hydroxylamine or amino derivatives of TNT appeared than expected from literature. Further analyses of the precipitated material revealed that its two distinct peaks found by HPLC corresponded to the aforementioned newly formed peaks (see SI Figure 2). Since nitrite formation was nearly half of that expected stoichiometrically with regard to the depletion of TNT, we therefore hypothesized that the expected hydroxylamine derivatives of TNT were intercepted by a condensation reaction, whose products form part of the observed precipitate.

The precipitate after being dissolved in acetonitrile produced two mayor peaks in HPLC-DAD analysis using the ion-pair eluent system I. Each peak gave a pseudo molecular mass of 376 $[M-H]^-$ when analyzed by HPLC-MS using the solvent system II in the negative ionization mode, (SI Figure 3 A) matching an empirical formula of $C_{14}H_{11}N_5O_8$. Since little evidence for possible solid structures of the condensed products or for their synthesis pathways were found in the literature (*16, 20*), we decided to elucidate the corresponding structures by establishing a chemical model reaction by de novo synthesis. We started with the chemically synthesized Meisenheimer dihydride complex of TNT (**3**) dissolved in water (the absence of TNT or the monohydride complex had previously been confirmed by HPLC-DAD and ^{1}H NMR) and pure (AccuStandard) 4-hydroxylamino-2,6-dinitrotoluene or 2-hydroxylamino-4,6-dinitrotoluene dissolved in acetonitrile (**5** or **6**, respectively, Figure 1). The condensation of **3** and **5** and of **3** and **6** and the concomitantly released nitrite were followed by HPLC-DAD analysis over time. The formation of **10** from **3** and **6** is shown in Figure 2 and clearly demonstrated the stoichiometry of the reaction sequence depicted in Figure 1. The condensation of **3** and **5** to generate **9** gave a similar image (not shown). The peaks shared by chemical and biological reactions exhibited identical retention times and UV–vis spectra. Also LC-MS $[M-H]^-$ analysis furnished identical pseudo molecular masses of $m/z = 376$ ($C_{14}H_{11}N_5O_8$) for each of the two new peaks (SI Figure 3C and D).

The scaled up synthesis aimed for the isolation and in-depth identification of the new compounds unexpectedly furnished three instead of only the two main peaks found previously in the optimized enzymatic and in the chemical reaction using the authentic standard compounds. The solution containing the three precipitated condensation products was filtered, the precipitate dissolved in acetonitrile, purified by preparative RP-HPLC, and subsequently analyzed in more detail. The identical molecular mass and the very similar IR spectra of compounds **9** and **10**, respectively, suggested that compound **10** is an isomer of compound **9**, which was confirmed by its ^{1}H NMR spectrum. Either compound exhibited two equivalent protons as a singlet, two coupled allylic protons, but only in compound **10**, two separated methyl protons, indicative for its nonsymmetric structure. The third new peak in the HPLC chromatogram, representing compound **7** (SI Figure 3B), was only observed in the preparative chemical reaction. It eluted first in RP-HPLC and provided a pseudomolecular mass of 392 with LC-MS, equivalent to a molecular mass of $m/z = 393$. ^{1}H NMR of compound **7** revealed four aromatic protons as a singlet and six aliphatic protons corresponding to the methyl groups, similar to compound **9**. However, the FT-IR spectrum of compound **7** showed a very broad band at 3304 cm^{-1} whereas the IR spectra of the other two compounds **9** and **10** exhibited significantly smaller bands at 3382 cm^{-1}, altogether indicative of a secondary hydroxylamine and two secondary amines, respectively, and confirming mass spectral data: the differentiation between these structures was

deduced from results obtained by LC-MS and IR spectrometry. Results from analyses by ^{13}C NMR, in which all carbon signals were unequivocally assigned by HSQC and HMBC correlations, allowed the definitive assignment of substituents of all three compounds, which were finally identified as the symmetric *N,N*-bis(3,5-dinitrotolyl) hydroxylamine (**7**), *N*-(2-methyl-3,5-dinitrophenyl)-4-methyl-3,5-dinitroaniline (**10**), and the symmetric *N,N*-bis(3,5-dinitrotolyl) amine (**9**). The individual detailed analytical data of compounds **7**, **9**, and **10** were as follows:

(**7**): MS (HPLC-MS, low resolution): 392 $[M-H]^-$. ^1H NMR (500 MHz, CD_3CN, 25 °C, TMS): $\delta = 2.47$ (s, 6H; CH_3), 7.93 (s, 4H; ArH), 8.82 (s, 1H, =NOH); ^{13}C NMR (125 MHz, CD_3CN, 25 °C, TMS): $\delta = 14.21$ (C-7,7′), 118.74 (C-2,2′,6,6′), 122.25 (C-1,1′), 146.64 (C-4,4′), 152.58 (C-3,3′,5,5′). FT-IR (ν cm^{-1}): 3304 (=NOH very br.), 3095 (Ar-H), 2919, 2851 (−CH_3), 2261, 2115 (=N−OH def.), 1623 (−NH def.), 1537, 1349 (NO_2 str.); UV λ^{ACN} nm, 200 (log ϵ 4.87).

(**9**): MS (HPLC-MS, low resolution): 376 $[M-H]^-$. ^1H NMR (500 MHz, CD_3CN, 25 °C, TMS): $\delta = 2.42$ (s, 6H; 2 × CH_3), 7.77 (s, 4H; ArH 2,2′6,6′), NH > 9.0 (var.); ^{13}C NMR (125 MHz, CD_3CN, 25 °C, TMS): $\delta = 13.95$ (C-7,7′), 116.85 (C-2,2′,6,6′), 119.13 (C-1,1′), 141.57 (C-4,4′), 152.83 (C-3,3′,5,5′). FT-IR (ν cm^{-1}): 3376 (−NH br.), 3089 (Ar−H), 2922, 2853 (−CH_3), 1624 (−NH def.), 1537, 1347 (NO_2 str.); UV λ^{ACN} nm, 201 (log ϵ 4.67), 275 (log ϵ 4.49).

(**10**): MS (HPLC-MS, low resolution): 376 $[M-H]^-$. ^1H NMR (500 MHz, CD_3CN, 25 °C, TMS): $\delta = 2.426$ (s, 3H; CH_3), 2.432 (s, 3H; CH_3), 7.33 (s, =NH), 7.66 (s, 2H; ArH2 and ArH6), 8.26 (d, 1H; ArH6′, $J = 2.23$ Hz), 8.32 (d, 1H; ArH4′, $J = 2.23$ Hz); ^{13}C NMR (125 MHz, CD_3CN, 25 °C, TMS): $\delta = 14.23$ (C-7), 14.67 (C-7′), 114.41 (C-4′), 116.75 (C-2,6), 118.74 (C-6′), 118.79 (C-1,1′), 132.64 (C-2′), 143.54 (C-4), 143.88 (C-3′), 147.49 (C-5′), 153.19 (C-3,5). FT-IR (ν cm^{-1}): 3382 (−NH br.), 3091 (Ar−H), 2923, 2852 (−CH_3), 1632 (-NH def.), 1535, 1347 (NO_2 str.); UV λ^{ACN} nm, 204 (log ϵ 4.58), 268 (log ϵ 4.34).

We consider symmetrical compound **7** as the more stable intermediate in the formation of compound **9** since we did not find any clear evidence for an analogous hydroxylamine precursor (**8**) of compound **10**. The conversion of chemically generated diaryl hydroxylamines to the corresponding secondary amines in the presence of oxygen has already been reported to proceed via nitroxyl radicals as intermediates, possibly through an unusual disproportioning (*24*), but we failed to identify further oxidized products and tentatively consider the mechanism an autoreduction similar to that of aromatic hydroxylamines to the corresponding amines. As already stated above, in the enzymatic reaction with XenB, mainly products **9** and **10** could be identified (Figure 3) but with the help of the spectroscopic data (HPLC) of **7** we were able to identify small amounts of this compound within small overlapping peaks of yet unidentified minor compounds. The fact that only very small amounts of secondary hydroxylamine could be found in the enzymatic reaction can be explained by the relatively slower and, therefore, rate-limiting velocity of the biological reaction. In contrast to the chemical reduction of TNT with Zn in the presence of NH_4Cl, which generates predominantly (around 80%) 4-hydroxylamino-2,6-dinitrotoluene, the enzymatic reduction of TNT predominantly furnishes 2-hydroxylamino-4,6-dinitrotoluene, which is reflected by the significantly higher concentration of the corresponding adduct **10** (data not shown). This suggests that the condensation reaction to compound **10** is probably faster compared to that yielding compound **9** through compound **7,** or that the reaction exhibits certain selectivity. On the other hand, HPLC-purified compound **7** dissolved in acetonitrile appears to be slightly unstable since it was partially (around 35%) transformed into compound **9** after about two months in the dark at room temperature. Self-reduction represents a common feature of aromatic hydroxylamines.

RP-HPLC analyses of aqueous solutions of the chemically synthesized Meisenheimer dihydride complex of TNT over time only allowed the identification of its isoforms and of the adducts described here, but never resulted in the identification of hydroxylamines or other rearomatized species except after several weeks that of compound **4** on which we reported recently (*19*). On the other hand, upon further reduction with KBH_4, the colorless product obtained from the orange-yellow dihydride complex, yielded predominantly 4-amino-2,6-dinitrotoluene upon slow rearomatization without any detectable release of nitrite (not shown).

In Figure 2 the self-condensation reaction of compounds **3** and **6** to yield compound **10** under stoichiometric release of nitrite is shown over time. Similar patterns are obtained when **3** condenses with **5** to yield **9** (data not shown). Finally, the prepared amounts of purified compounds **9** and **10** allowed the full quantification for the mass balance of the enzymatic reaction initiated by the XenB homologue (Figure 3). Results presented in this figure clearly show that also here the release of nitrite is nearly stoichiometrically related to the end-products formed. Thus, by knowing the concentrations of compounds **9** and **10**, the concentrations of compounds **5** and **6** intercepted by the condensation can be calculated and the final mass balance of the overall reaction, which is only initiated enzymatically by this OYE flavin reductase, correctly computed.

Our earlier observations (*18, 19*) together with the aforementioned results now allow us to clearly explain why some microorganisms can utilize TNT as a sole source of nitrogen. The results also clearly confirm that nitrite release from TNT degradation is not enzyme-catalyzed but a secondary chemical reaction. Furthermore, we have found that nitrodiarylhydroxylamines and nitrodiarylamines can be produced in aqueous solvents from Meisenheimer complexes of TNT and hydroxylaminodinitrotoluenes without the need of aryl halides as starting material and (transition) metal catalysts. In addition, the identification of the above compounds may explain why only low concentrations of compounds **5** and **6** (if at all) and the corresponding amines were detected in biological TNT degradation experiments; they were intercepted (scavenged) by the dimerization reaction with the reactive dihydride complex of TNT. Currently, we are characterizing a number of bacterial OYE reductase gene products with the help of the quantitative and qualitative data obtained in the present work.

Acknowledgments

This work was supported by grant contract MADOX QLRT-2001-00345 from the European Union. We thank Dr. Fernando Lafont from the University of Córdoba for molecular mass determinations by LC-MS and the Unión Española de Explosivos for providing TNT.

Supporting Information Available

A photograph of an SDS-PAGE gel showing protein purification steps of the XenB protein, termed Figure S1. Figure S2 shows a representative separation by HPLC with diode array detection of metabolites derived from TNT reduction and the formed condensation products. Figure S3A−D shows ESI-MS spectra of newly described condensation products from TNT-derived Meisenheimer dihydride complexes and hydroxylaminodinitrotoluenes. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Williams, R. E.; Bruce, N. C. 'New uses for an old enzyme'–the Old Yellow Enzyme family of flavoenzymes. *Microbiology* **2002**, *148,* 1607–1614.

(2) Williams, R. E.; Rathbone, D. A.; Scrutton, N. S.; Bruce, N. C. Biotransformation of explosives by the Old Yellow Enzyme family of flavoproteins. *Appl. Environ. Microbiol.* **2004**, *70*, 3566–3574.
(3) Marvin-Sikkema, F. D.; de Bont, J. A. M. Degradation of nitroaromatic compounds by microorgnisms. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 499–507.
(4) Lenke, H.; Knackmuss, H.-J. Initial hydrogenation during catabolism of picric acid by *Rhodococus erythropolis* HL 24–2. *Appl. Environ. Microbiol.* **1992**, *58*, 2933–2937.
(5) Vorbeck, C.; Lenke, H.; Fischer, P.; Knackmuss, H.-J. Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. *J. Bacteriol.* **1994**, *176*, 932–934.
(6) Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J. C.; Knackmuss, H.-J. Initial reductive reaction in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Appl. Environ. Microbiol.* **1998**, *64*, 246–252.
(7) Hofmann, K. W.; Knackmuss, H.-J.; Heiss, G. Nitrite elimination and hydrolytic ring cleavage in 2,4,6-trinitrophenol (picric acid) degradation. *Appl. Environ. Microbiol.* **2004**, *70*, 2854–2860.
(8) Heiss, G.; Knackmuss, H.-J. Bioelimination of nitroaromatic compounds: immobilization versus mineralization. *Curr. Opin. Microbiol.* **2002**, *5*, 282–287.
(9) Weiss, M.; Geyer, R.; Russow, R.; Richnow, H. H.; Kästner, M. Fate and metabolism of [^{15}N] 2,4,6-trinitrotoluene in soil. *Environ. Toxicol. Chem.* **2004**, *23*, 1852–1860.
(10) Esteve-Núñez, A.; Caballero, A.; Ramos, J. L. Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 335–352.
(11) Stenuit, B.; Eyers, L.; El Fantroussi, S.; Agathos, S. N. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. *Rev. Environ. Sci. Bio/Technol.* **2005**, *4*, 39–60.
(12) Symons, Z. C.; Bruce, N. C. Bacterial pathways for degradation of nitroaromatics. *Nat. Prod. Rep.* **2006**, *23*, 845–850.
(13) French, C. E.; Nicklin, S.; Bruce, N. C. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Appl. Environ. Microbiol.* **1998**, *64*, 2864–2868.
(14) Khan, H.; Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C. E.; Scrutton, N. S. Kinetic and structural basis of reactivity of pentaerythritol tetranitrate reductase with NADPH, 2-cyclohexenone, nitroesters, and nitroaromatic explosives. *J. Biol. Chem.* **2002**, *277*, 21906–21912.
(15) Blehert, D. S.; Fox, B. G.; Chambliss, G. H. Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. *J. Bacteriol.* **1999**, *181*, 6254–6263.
(16) Pak, J. W.; Knoke, K. L.; Noguera, D. R.; Fox, B. G.; Chambliss, G. H. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. *Appl. Environ. Microbiol.* **2000**, *66*, 4742–4750.
(17) Haïdour, A.; Ramos, J. L. Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. *Environ. Sci. Technol.* **1996**, *30*, 2365–2370.
(18) Esteve-Núñez, A.; Ramos, J. L. Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. *Environ. Sci. Technol.* **1998**, *32*, 3802–3808.
(19) González-Pérez, M. M.; van Dillewijn, P.; Wittich, R.-M.; Ramos, J. L. *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. *Environ. Microbiol.* **2007**, *9*, 1535–1540.
(20) Stenuit, B.; Eyers, L.; Rozenberg, R.; Habib-Jiwan, J.-L.; Agathos, S. N. Aerobic growth on 2,4,6-trinitrotoluene (TNT) as sole nitrogen source by *Escherichia coli* and evidence of TNT denitration with whole cells and cell-free extracts. *Appl. Environ. Microbiol.* **2006**, *72*, 7945–7948.
(21) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
(22) Prak, D. J. L.; O'Sullivan, D. W. Solubility of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene in seawater. *J. Chem. Eng. Data* **2006**, *51*, 448–450.
(23) Agrawal, J. K. Some new high energy materials and their formulations for specialized applications. *Propellants Explos. Pyrotech.* **2005**, *30*, 316–328.
(24) Yost, Y.; Gutmann, H. R. Carbonyl compounds and secondary amines from diarylhydroxylamines via nitroxides. *J. Org. Chem.* **1973**, *38*, 165–167.

ES071449W