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Stable Isotope-Enabled Pathway Elucidation of 2,4-Dinitroanisole Metabolized by *Rhizobium lichtii*

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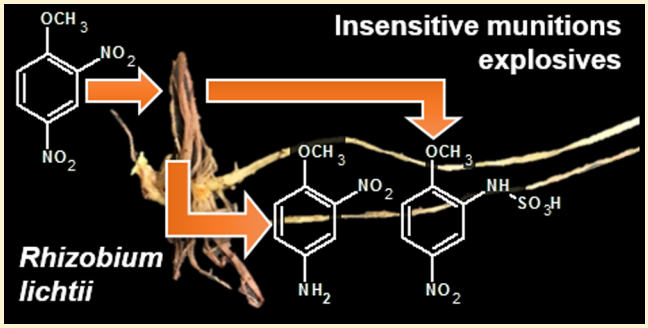
Supporting Information

ABSTRACT: Recently developed military explosives are safer to handle, but more environmentally mobile, than traditional explosives. The new insensitive munitions, including 2,4-dinitroanisole (DNAN), have been sparsely studied, making the use of bacterial and/or plant-based remediation approaches unfeasible without further information about the transformation of these compounds. To that end, *Rhizobium lichtii* isolated from willow tree tissues were studied and observed to completely degrade 5 mg L^{-1} DNAN within 24 h in liquid media with added carbon and nitrogen. The use of DNAN, [$^{13}C_6$]DNAN, and [$^{15}N_2$]DNAN enabled the identification of 11 previously unknown metabolites resulting from sulfation, acetylation, and/or methylation processes. Accurate mass analysis revealed DNAN metabolites with ion formulas of $C_7H_7N_2O_6S$, $C_7H_7N_2O_7S$, $C_9H_9N_2O_5$, $C_8H_7N_2O_4$, and $C_9H_9N_2O_4$. Further mass spectral analysis provided evidence of various combinations of amino and hydroxylamino DNAN metabolites. Nitro reduction was observed for the first time at the *para* position of DNAN to produce 4-amino-2-nitroanisole.



INTRODUCTION

Insensitive munitions explosives (IMX) are new formulations that are safer to handle and transport, with less risk of self-detonation, than traditional explosives such as 2,4,6-trinitrotoluene (TNT) and hexahydrotrinitrotriazine (RDX). The U.S. Army recently approved IMX-101 and IMX-104, which contain 2,4-dinitroanisole (DNAN), to protect soldiers.[1] Unfortunately, DNAN and other IMX constituents have aqueous solubilities and transport mobilities higher than those of other explosives, potentially increasing the risk to plants, earthworms, microbes, and rats that are negatively impacted by IMX.[1,2] In the few studies of DNAN degradation, the compound is transformed by anaerobic bacteria in sludge,[3,4] by endogenous soil bacteria supplemented with carbon and nitrogen,[5] by an aerobic *Nocardioides* sp. isolated from activated sludge treating DNAN,[6] and abiotically.[7,8] DNAN toxicity to soil-grown grasses has been suggested,[1,9] but amino derivatives of DNAN are considered less mobile and less toxic.[8,10]

Plant-based remediation (phytoremediation) has been studied extensively for traditional explosives[11−14] and may prove to be useful for IMX. Furthermore, the frequency of study of endophytes (i.e., organisms that inhabit plants asymptomatically) is increasing, given their ability to degrade xenobiotics naturally or via bioaugmentation.[15−17] In this study, we (1) isolated and identified a hybrid willow tree endophyte capable of degrading DNAN and (2) identified several DNAN transformation products using ^{15}N and ^{13}C stable isotope metabolic profiling.

EXPERIMENTAL SECTION

Chemicals. Dinitroanisole (98%) was from Sigma (St. Louis, MO). Liquid chromatography solvents (Optima), 2-methoxy-5-nitroaniline (2-ANAN) (98%), and 4-methoxy-3-nitroaniline (4-ANAN) (97%) were supplied by Fisher (Waltham, MA).

Synthesis and Characterization of Isotope-Labeled DNAN. [$^{13}C_6$]DNAN and [$^{15}N_2$]DNAN (Figure 1) were synthesized by nitration of the corresponding anisole with propionyl nitrate generated *in situ* from propionic anhydride and nitric acid. Treatment of [$^{13}C_6$]anisole with fuming nitric acid gave [$^{13}C_6$]DNAN (purity >99%), and treatment of nonlabeled anisole with [^{15}N]nitric acid gave [$^{15}N_2$]DNAN

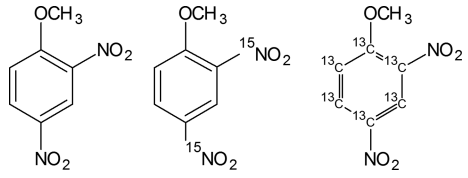


Figure 1. Unlabeled DNAN and stable isotope-labeled versions of DNAN used in this study.

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(>99% pure). Structural confirmation and purity analysis were conducted by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, liquid chromatography−high resolution mass spectrometry, and gas chromatography−mass spectrometry (see the Supporting Information for details).

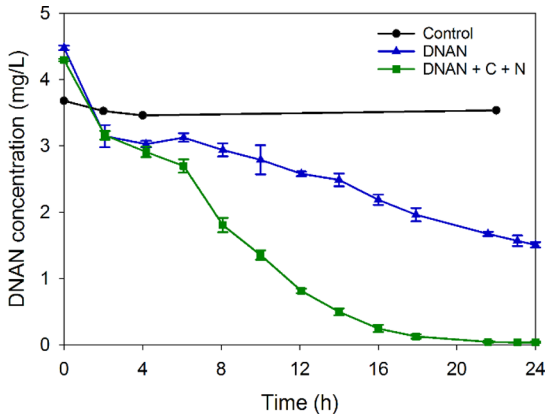

Figure 2. Degradation over 24 h by *R. lichtii* exposed to 5 mg L^{-1} DNAN with and without 5 mM fructose (C) and ammonium nitrate (N). Control indicates DNAN + C + N without *R. lichtii*. Error bars denote one standard deviation (n = 3).

Endophyte Isolation and Identification. Willow tree cuttings (Salix "Iowa Willow") were obtained from Ecolotree (North Liberty, IA). Portions of the leaves, stems, and roots were removed and surface sterilized to kill epiphytic bacteria, and endophytes were isolated as previously described.[18−20] Full details are outlined in the Supporting Information. Isolated colonies that grew on plates with 5 mg L^{-1} DNAN as the sole source of carbon and nitrogen were streaked onto R2A medium and stored in the dark at 30 °C. Genomic DNA was extracted using a PowerWater DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) followed by 16S rRNA polymerase chain reaction (universal 1492r, 27f primers) (Mastercycler Model Epgradient, Eppendorf, Hauppauge, NY). Annealing conditions were 2 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, before 7 min at 72 °C prior to cooling at 4 °C. The amplified DNA was purified (QIAquick PCR Purification Kit, QIAGEN, Valencia, CA) and sequenced at the Iowa Institute of Human Genetics Genomics Division. The sequenced DNA was identified as *Rhizobium* by the blastn suite (National Center for Biotechnology Information, Bethesda, MD) and deposited under accession number KT881311 as *Rhizobium lichtii*. The cultures were maintained in a minimal medium solution (without agar) supplemented with 40 g/L glucose and 1.20 g/L ammonium nitrate for

Table 1. Accurate Masses of DNAN Metabolites and Corresponding Molecular Formulas As Determined by ESI$^-$ LC-MS/QToF

ion formula	proposed ion	metabolite	retention time (min)	accurate mass (m/z)	deviation (mDa) (ppm)
$C_7H_7N_2O_6S$	$[M - H]^-$	M1a	0.81	247.0058	3.3 (13.4)
		$[^{15}N_2]$-		*a*	*a*
		$[^{13}C_6]$-		253.0264	3.8 (15)
		M1b	1.23	247.0037	1.2 (4.9)
		$[^{15}N_2]$-		249.0012	4.6 (18.5)
		$[^{13}C_6]$-		253.0253	2.7 (10.7)
		M1c	1.39	247.0031	0.6 (2.4)
		$[^{15}N_2]$-		248.9986	2.0 (8.0)
		$[^{13}C_6]$-		253.0237	1.1 (4.3)
		M1d	1.42	247.0026	0.1 (0.4)
		$[^{15}N_2]$-		248.9978	1.2 (4.8)
		$[^{13}C_6]$-		253.0234	0.8 (3.2)
		M1e	1.75	247.0036	1.1 (4.5)
		$[^{15}N_2]$-		248.9965	−0.1 (−0.4)
		$[^{13}C_6]$-		253.0226	0.0 (0.0)
$C_7H_7N_2O_7S$	$[M - H]^-$	M2a	1.30	262.9984	1.0 (3.8)
		$[^{15}N_2]$-		264.9904	−1.1 (−4.2)
		$[^{13}C_6]$-		269.0167	−0.8 (3.0)
		M2b	1.37	262.9979	0.5 (1.9)
		$[^{15}N_2]$-		264.9914	−0.1 (−0.4)
		$[^{13}C_6]$-		269.0191	1.6 (5.9)
$C_9H_9N_2O_5$	$[M - CH_3]^-$	M3	2.49	225.0492	−1.9 (−8.4)
		$[^{15}N_2]$-		227.0456	0.4 (1.8)
		$[^{13}C_6]$-		231.0734	2.1 (9.1)
$C_8H_7N_2O_4$	$[M - CH_3]^-$	M4a	2.75	195.0410	0.4 (2.1)
		$[^{15}N_2]$-		197.0360	1.3 (6.6)
		$[^{13}C_6]$-		201.0615	0.8 (4.0)
		M4b	3.23	195.0430	2.4 (12.3)
		$[^{15}N_2]$-		197.0348	0.1 (0.5)
		$[^{13}C_6]$-		201.0590	−1.7 (−8.5)
$C_8H_7N_2O_4$	$[M - CH_3]^-$	M5	2.98	209.0602	4.0 (19.1)
		$[^{15}N_2]$-		211.0521	1.8 (8.5)
		$[^{13}C_6]$-		215.0765	0.1 (0.5)

[a]Below the detection limit.

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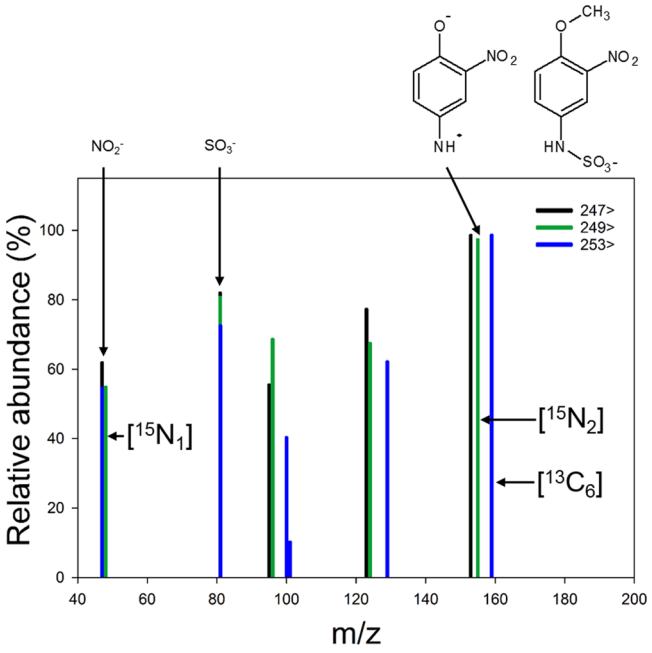

Figure 3. Sulfated amine of DNAN (m/z 247) evidenced by ESI$^-$ LC-MS/MS mass fragments at m/z 152, 122, 80, and 46 (black bars) corresponding to the molecular structures shown. A sulfated amine of [$^{15}N_2$]DNAN (m/z 249) had fragments at m/z 154, 123, 80, and 47 (green bars), and a sulfated amine of [$^{13}C_6$]DNAN (m/z 253) had fragments at m/z 158, 128, 80, and 46 (blue bars).

growing biomass or streaked onto R2A plates and stored in the dark at 30 °C.

Degradation of DNAN, [$^{13}C_6$]DNAN, and [$^{15}N_2$]DNAN. Pure *R. lichtii* cultures were grown overnight in rich media and exposed to 5 mg L^{-1} DNAN in triplicate with and without added carbon and nitrogen. Samples were taken every 2 h, filtered (0.2 μm), and diluted 4:1 with methanol. The bioreactors were sterilized and foam-stoppered, and serum vials (30 mL) containing minimal medium (25 mL) were inoculated by 1 mL of the *R. lichtii* solution with three cultures amended with fructose and ammonium nitrate (5 mM). Controls were inoculated with 1 mL of sterile deionized water, and all bioreactors were incubated at 30 °C in the dark. In a second experiment to identify DNAN metabolites, *R. lichtii* was incubated separately with DNAN, [$^{13}C_6$]DNAN, or [$^{15}N_2$]DNAN amended with 5 mM fructose and ammonium nitrate. After 24 h, three extracellular samples, each incubated with a different stable isotope of DNAN, were filtered (0.2 μm), combined to a final volume of 200 μL containing 10% acetonitrile, and analyzed for accurate mass data.

Analytical Methods. Samples were analyzed using an Agilent (Santa Clara, CA) liquid chromatography system and 6460 triple-quadrupole mass spectrometer (LC-MS/MS). For DNAN, an 80:20 methanol/water mixture with 1 mM ammonium acetate (0.25 mL min^{-1}) through a Zorbax C18 column (3 mm × 150 mm, 5 μm) at 35 °C and a 10 min run time was used. For nonlabeled DNAN, the MS/MS transition was 183 > 109 in negative ion electrospray ionization (ESI$^-$) mode.[21] For metabolites, full and product ion scans were typically run with a linear gradient (10 to 90% methanol) over 15 min, with a 10 min hold and a 10 min return to 10% methanol. For positive ion mode (ESI$^+$) analysis, 5 mM formic acid was used with methanol as the organic solvent.

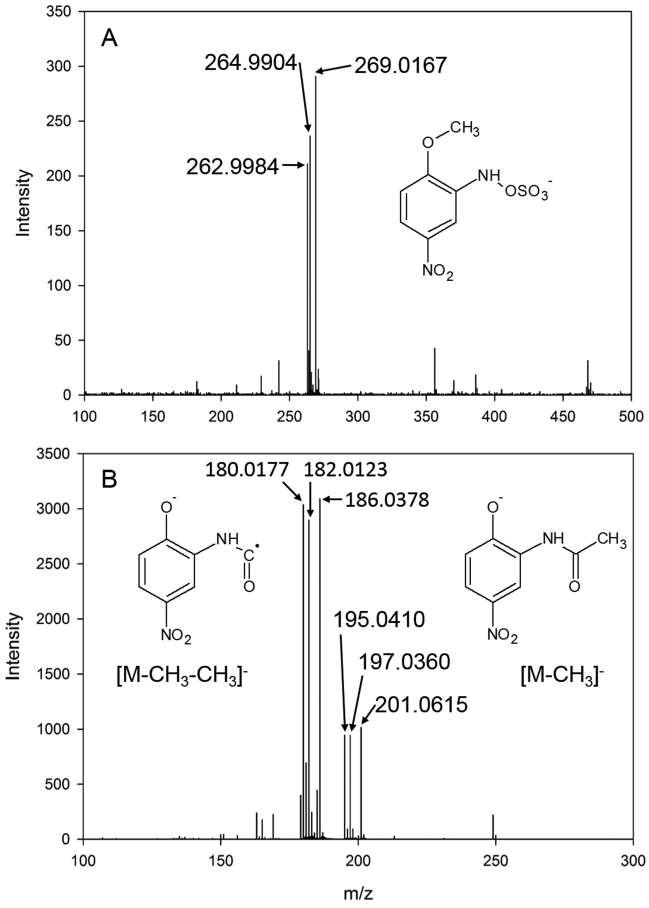

Figure 4. (A) Sulfated hydroxylamines of DNAN (m/z 262.9984), [$^{15}N_2$]DNAN (m/z 264.9904), and [$^{13}C_6$]DNAN (m/z 269.0167) were evidenced by ESI$^-$ LC-MS/QTof. (B) Acetylated amines of DNAN (m/z 195.0410), [$^{15}N_2$]DNAN (m/z 197.0360), and [$^{13}C_6$]DNAN (m/z 201.0615) showed methyl group losses resulting in ions at m/z 180.0177, 182.0123, and 186.0378, respectively.

Accurate mass determinations were made on a Waters (Milford, MA) Acquity ultraperformance liquid chromatography system followed by a Waters QTof-premier mass spectrometer. An Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) heated to 60 °C (0.6 mL min^{-1}) was used. Leucine enkephalin was infused (10 μL min^{-1}) as the lock mass. The mobile phase was 95% deionized water (A) and 95% acetonitrile (B), both containing 5 mM ammonium acetate. The gradient was as follows: 0 min, 0% B; linear to 10 min, 100% B; 15 min, 100% B. ESI$^-$ mode, a mass to charge (m/z) range of 50−600, a scan rate of 0.15 s scan^{-1}, and a cone voltage of 35 V were utilized.

RESULTS AND DISCUSSION

DNAN Degradation and Metabolite Identification. *R. lichtii* cultures with added fructose and ammonium nitrate completely removed 5 mg L^{-1} DNAN within 24 h, while cultures without added carbon or nitrogen removed approximately half of the DNAN (Figure 2).

Accurate mass analysis revealed DNAN metabolites with ion formulas of $C_7H_7N_2O_6S$, $C_7H_7N_2O_7S$, $C_9H_9N_2O_5$, $C_8H_7N_2O_4$, and $C_9H_9N_2O_4$ (Table 1). The formulas representing additions of sulfur and oxygen suggest sulfation of DNAN ($C_7H_6N_2O_5$) after nitro group reduction to an amine [M1a−e (Table 1)] (Figure 3) or hydroxylamine [M2a and M2b (Table 1)]

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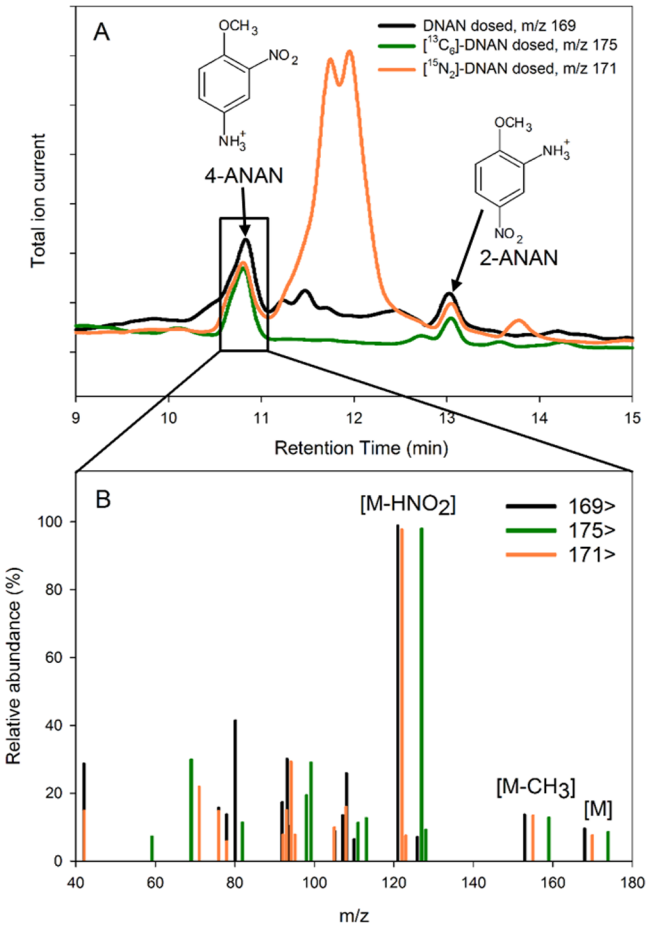

Figure 5. (A) ESI$^+$ LC-MS/MS provided evidence of two monoamino metabolites of DNAN (m/z 169, black line), [$^{15}N_2$]DNAN (m/z 171, orange line), and [$^{13}C_6$]DNAN (m/z 175, green line) corresponding to the structures shown. (B) The *para*-substituted amino metabolite (4-ANAN) of DNAN was confirmed by mass fragments corresponding to methyl loss (M − CH_3) and nitro group loss (M − HNO_2). Both 2-ANAN and 4-ANAN were confirmed by true standards.

(Figure 4A). The formulas representing additions of carbon, hydrogen, and oxygen suggest an acetylation product [M3−5 (Table 1)] (Figure 4B). Two metabolites ($C_8H_7N_2O_4$ and $C_9H_9N_2O_4$) appeared to be combinations of acetylation and/or methylation [M3 and M5 (Table 1)].

Five DNAN metabolite isomers [M1a−e (Table 1)], corresponding to the $C_7H_8N_2O_6S$ formula determined by accurate mass, were detected by ESI$^-$ LC-MS/QTof. These isomers exhibited similar mass spectra, including identical nitro group (m/z 46) fragments for DNAN and [$^{13}C_6$]DNAN and a single mass-shifted (m/z 47) nitro group loss for [$^{15}N_2$]DNAN (Figure 3). The sulfate group fragment (m/z 80) was identical for DNAN, [$^{15}N_2$]DNAN, and [$^{13}C_6$]DNAN. Other mass fragments confirmed an intact aromatic ring and a nitro group reduction to the corresponding amine (Figure 3). The data suggest an unknown SO_3^- addition for metabolites M1a, M1b, and M1d and amine group sulfation for metabolites M1c and M1e (Figure 3), but mass spectrometry does not enable exact structural determination.

The formation of acetylated DNAN metabolites (M3, M4a, M4b, and M5) has been previously overserved[5] and could have been catalyzed by aryl-*N*-acetyltransferases (NATs) known to acetylate aryl-amines and aryl-hydroxylamines.[22] The NAT gene in *Mycobacterium tuberculosis* is located in the same operon as enzymes capable of degrading aromatics.[23] NAT genes have been found across all domains of life and even in the Rhizobiales, *Mesorhizobium loti*.[24]

Novel *para* Position Transformation of DNAN to 4-Amino-2-nitroanisole. In this study, we detected 2-amino-4-nitroanisole (2-ANAN), which is a common transformation product of DNAN.[3,8,10] Conversely, the formation of 4-amino-2-nitroanisole (4-ANAN) resulting from *para* nitro group reduction of DNAN has not been reported. True standards and ESI$^+$ LC-MS/MS confirmed the presence of 2-ANAN and 4-ANAN in the DNAN-incubated samples (Figure 5). The peaks at 10.8 and 13.1 min (Figure 5A) had mass shifts consistent with an aminonitroanisole (m/z 169), a [$^{15}N_2$]-aminonitroanisole (m/z 171), and a [$^{13}C_6$]aminonitroanisole (m/z 175). Tandem mass spectra confirmed the structure of each aminonitroanisole [4-ANAN (Figure 5B)]. Similarly, a DNAN metabolite detected at m/z 183, 185, and 189 is proposed to be 2-hydroxylamino-4-nitroanisole (2-HA-NAN) or 4-hydroxylamino-2-nitroanisole (4-HA-NAN) within the putative DNAN transformation pathway (Figure 6).

The ability to culture *R. lichtii* on solid and in liquid media suggests promise for bioaugmenting soils contaminated with DNAN. A longer incubation with DNAN as the sole carbon and nitrogen source could result in DNAN mineralization, especially considering that the microbe could grow on DNAN

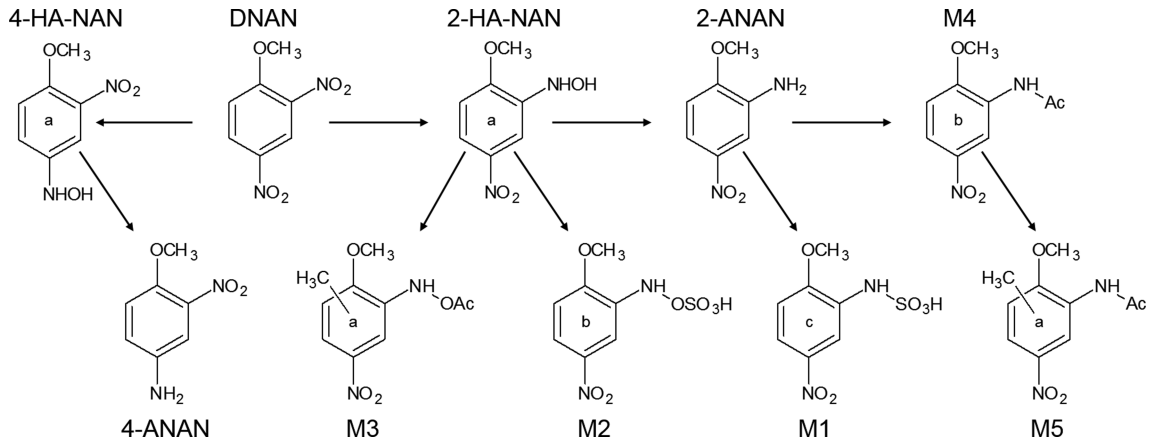

Figure 6. Putative DNAN transformation pathway of *R. lichtii*. M1−5 could result from reduction of either nitro group, although *ortho* reduction is indicated in the diagram. Letters indicate the number of isomers detected: a = 1, b = 2, and c = 5.

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as the sole source of carbon and nitrogen. However, the broad range of DNAN metabolites identified in this study and the lack of aromatic ring alteration with carbon and nitrogen added in this short assay suggest that the degradation pathway should be more fully elucidated before field-scale implementation of phytoremediation or bioaugmentation with *R. lichtii*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00278.

Stable isotope-labeled DNAN synthesis and characterization and surface sterilization and endophyte isolation (PDF)

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Notes

The authors declare no competing financial interest.

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