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Biotransformation of 2,4-dinitroanisole by a fungal *Penicillium* sp

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Abstract

Insensitive munitions explosives are new formulations that are less prone to unintended detonation compared to traditional explosives. While these formulations have safety benefits, the individual constituents, such as 2,4-dinitroanisole (DNAN), have an unknown ecosystem fate with potentially toxic impacts to flora and fauna exposed to DNAN and/or its metabolites. Fungi may be useful in remediation and have been shown to degrade traditional nitroaromatic explosives, such as 2,4,6-trinitrotoluene and 2,4-dinitrotoluene, that are structurally similar to DNAN. In this study, a fungal *Penicillium* sp., isolated from willow trees and designated strain KH1, was shown to degrade DNAN in solution within 14 days. Stable-isotope labeled DNAN and an untargeted metabolomics approach were used to discover thirteen novel transformation products. *Penicillium* sp. KH1 produced DNAN metabolites resulting from *ortho*- and *para*-nitroreduction, demethylation, acetylation, hydroxylation, malonylation, and sulfation. Incubations with intermediate metabolites such as 2-amino-4-nitroanisole and 4-amino-2-nitroanisole as the primary substrates confirmed putative metabolite isomerism and pathways. No ring-cleavage products were observed, consistent with other reports that mineralization of DNAN is an uncommon metabolic outcome. The production of metabolites with unknown persistence and toxicity suggests further study will be needed to implement remediation with *Penicillium* sp. KH1. To our knowledge, this is the first report on the biotransformation of DNAN by a fungus.

Keywords

Insensitive munitions explosives; 2,4-dinitroanisole; DNAN; bioremediation; *Penicillium*

INTRODUCTION

Insensitive munitions explosives (IMX) are new formulations designed to minimize the risk of unintended detonation. IMX formulations are being implemented by the US Army to phase out traditional explosives containing trinitrotoluene (TNT) and hexahydrotrinitrotriazine (RDX) (Dodard et al. 2013). The increased safety of IMX mixtures

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comes at a potential environmental expense as the individual IMX constituents, such as 2,4-dinitroanisole (DNAN), are more water-soluble than TNT and RDX. For example, DNAN has a solubility in water of 276 mg/L compared to 100 mg/L for TNT (Boddu et al. 2008; Taylor et al. 2015). Army-approved formulation IMX-101 contains 43% DNAN, 37% nitroguanidine and 20% 3-nitro-1,2,4-triazol-5-one (NTO) by volume, while IMX-104 consists of 32% DNAN, 53% NTO, and 15% RDX (Taylor et al. 2015). The high percentage of DNAN in these formulations and the estimated 50 million acres affected by legacy explosives in the United States alone (Pichtel 2012) raise concerns about the widespread release of this compound and its transformation products (TPs). In addition, DNAN has a relatively unknown ecosystem fate and toxicity (Stanley et al. 2015). Therefore, the new explosives and their TPs pose a contamination risk to groundwater and ecosystems and require further characterization of transformation and transport in the environment (Boddu et al. 2008; Taylor et al. 2015).

Fungi of many taxa have been shown to degrade xenobiotics of all types, including nitroaromatic explosives (Fernando et al. 1990; Harms et al. 2011; Sheremata and Hawari 2000). Fungi can often mineralize recalcitrant compounds, but may also create previously unknown TPs (Golan-Rozen et al. 2015; Sheremata and Hawari 2000). Fungal bioreactors have been proposed for treating liquid waste streams (Harms et al. 2011), and fungal bioremediation (mycoremediation) has been demonstrated for various compounds in solid waste (Singh 2006). In addition, fungi are ubiquitous in the environment and play an important role in ecosystem nutrient cycling (Harms et al. 2011). Consequently, it is important to characterize fungal transformations of contaminants to assess new TPs and the possibility of remediation of soil and solids with fungal cultures.

Microorganisms isolated from inside plant tissues (i.e., endophytes) may prove useful for bioaugmentation and enhancing plant-based remediation (Doty 2008; Kang et al. 2012). Endophytic bacteria isolated from hybrid poplar and willow tree tissues have even been shown to degrade DNAN and other explosives (Schroer et al. 2015; Van Aken et al. 2004). While DNAN has been mineralized by a *Nocardioides* sp. (Fida et al. 2014), complete biological mineralization of DNAN remains unusual, and fungal TPs have not yet been identified. Here, we (1) isolate and identify an endophytic fungus that can degrade DNAN and (2) characterize the transformation products of DNAN from the isolated fungus.

EXPERIMENTAL SECTION

Chemicals

2,4-Dinitroanisole (98%) was purchased from Sigma (St. Louis, MO). [$^{13}\text{C}_6$]DNAN (>99%) and [$^{15}\text{N}_2$]DNAN (>99%) were synthesized as previously described (Schroer et al. 2015). A 2,4-dinitrophenol analytical standard (USPH1401), 4-methoxy-3-nitroaniline [4-ANAN (97%)], 2-methoxy-5-nitroaniline [2-ANAN (98+%)], 4-amino-2-nitrophenol (99%), and 2-amino-4-nitrophenol (99%) were purchased from Fisher Scientific (Waltham, MA). All reagents were ACS reagent grade or better and liquid chromatography solvents were Fisher Optima grade.

Synthesis of 2-(N-acetyl)amino-4-nitroanisole

2-(N-acetyl)amino-4-nitroanisole (2-NAC-NAN) was synthesized as described by Ayyangar and Srinivasan (Ayyangar and Srinivasan 1984). Briefly, 2-amino-4-nitroanisole (50 mmol) was combined with 50 mmol pyridine and 55 mmol acetic anhydride and the mixture was stirred and heated in a boiling water bath for 3 hours. The mixture was poured into water, and the product was filtered and washed with water. The product was recrystallized twice with water:ethanol (1:1, v/v) to produce 8.63 g (82% yield) of yellow solid, mp 178–179 °C [lit. 178 °C, (Ayyangar and Srinivasan 1984)]. The purity was >99% as determined by liquid chromatography mass spectrometry (LC-MS) with positive electrospray ionization (ESI⁺). High resolution, quadrupole time-of-flight mass spectrometry (LC-QToF) (ESI⁺) revealed an exact mass of 211.0719, consistent with the calculated mass of 211.0723 for the expected formula [C₉H₁₀N₂O₄ + H]⁺. ¹H and ¹³C NMR spectra were recorded on a Bruker DRX-400 spectrometer in the University of Iowa Central NMR Research Facility (Iowa City, IA, USA) using tetramethylsilane as internal standard. The ¹H and ¹³C chemical shifts are consistent with the proposed structure: ¹H NMR (400 MHz, CDCl₃): δ 9.26 (s, 1H), 7.96 (d, *J* = 8.7 Hz, 1H), 7.81 (s, 1H), 6.92 (d, *J* = 8.7 Hz, 1H), 4.00 (s, 3H), 2.25 (s, 3H) ppm. ¹³C NMR (100 MHz, CDCl₃) δ 168.3, 152.2, 141.6, 127.8, 119.7, 114.9, 109.1, 56.4, 24.8 ppm (see Figures S3 and S4).

Fungus isolation and identification

Fungal biomass was isolated from surface-sterilized portions of a two-week old *Salix* 'Iowa Willow' cutting and identified as described previously (Schroer et al. 2015). Full details can also be found in the supplementary information (SI). DNA extraction was done as previously described (Dean et al. 2004) and polymerase chain reaction amplification primers were EF4-fung5 for 18S rDNA (Smit et al. 1999). The DNA sequence results were submitted to the blastn suite (National Center for Biotechnology Information, Bethesda, MD) to identify the fungus and the sequence was submitted to GENBANK under accession number KU866421 as *Penicillium* sp. strain KH1.

Biodegradation pathway elucidation experiment

The medium used for this study was a simple glucose peptone (GP) medium composed of 20 g glucose, 5 g peptone, 2 g yeast extract, 1 g K₂HPO₄, and 0.5 g MgSO₄•7H₂O per liter of deionized (DI) water (Golan-Rozen et al. 2015). The incubations (30 °C) were performed in sterilized amber serum bottles (125 mL) containing 20 mL of the medium and 100 µL of a solution of *Penicillium* sp. KH1 spores (1.21 × 10⁶ colony forming units per mL). The serum bottles were sealed with Micropore tape (3M, St. Paul, MN) and loosely covered with aluminum foil. After seven days of biomass pre-growth, the medium was decanted while retaining the biomass, and new medium (20 mL) was added. Six total bioreactors were treated with DNAN: three contained medium with 5 mg/L DNAN and 5 mg/L [¹³C₆]DNAN, while three contained medium with 5 mg/L DNAN and 5 mg/L [¹⁵N₂]DNAN. Three bioreactors containing fungus but no DNAN served as biological controls with an absence of DNAN metabolites. A second spike of the same media (10 mL) was added to each reactor after seven days of DNAN exposure.

Samples were collected at 2, 4, 6, 8, 16, 24, 32, 40, and 48 hours and then 1–3 day intervals. For each sample, 0.5–1.5 mL of medium was collected with a needle syringe and filtered through a 0.2 µm nylon filter (Pall Life Sciences, Port Washington, NY). For analysis, 400 µL of filtrate from the sample after 10 days of exposure was added to 600 µL of solvent (95% DI water and 5% acetonitrile containing 5 mM ammonium acetate). The degradation of DNAN over time and information on additional controls for the metabolite elucidation experiment can be found in the SI and Fig. S1.

Degradation kinetics and pathway elucidation using metabolites as substrates

To resolve isomeric metabolites and to determine metabolite formation over time, *Penicillium* sp. KH1 (20 µL of spore solution) was pre-grown for seven days at 30 °C in GP medium (2 mL) in sterilized test tubes (100 × 13 mm) with a glass wool plug and a metal cap. After biomass growth, samples were decanted and refilled with medium containing 10 mg/L DNAN (n=36). Controls included autoclaved biomass dosed with 10 mg/L DNAN (n=36) and spiked medium containing no fungal spores (n=21). At each time point, three bioreactors from each condition were sacrificially sampled by freezing at –80 °C until further processing. Sample preparation involved thawing the test tubes in a 20 °C water bath before filtering the media with a 0.22 µm PES syringe filter (Chemglass Life Sciences, Vineland, NJ). For LC-MS analyses, 250 µL of sample filtrate was added to 50 µL of acetonitrile and 700 µL of DI water. One sample each from the treatment was lost for days 5, 6, and 14 during sample preparation for analysis.

Separate bioreactors were dosed with 5 mg/L of each of the following (n=2, each): 2,4-dinitrophenol (DNP), 2-amino-4-nitroanisole (2-ANAN), 4-amino-2-nitroanisole (4-ANAN), 2-amino-4-nitrophenol (2-ANP), 4-amino-2-nitrophenol (4-ANP) and 2-(*N*-acetyl)amino-4-nitroanisole (2-NAc-NAN). Each of these metabolites was observed in the initial biodegradation pathway experiment and served as primary substrates to further elucidate and confirm the metabolic pathways (Golan-Rozen et al. 2015). One bioreactor from each of the alternative primary substrates was sacrificially sampled after eight and 16 hours of incubation. Samples were frozen at –80 °C and filtered and diluted as above. Spores used for all experiments were never previously exposed to DNAN, ensuring that the fungal enzymatic systems were not previously adapted to or induced by DNAN degradation.

To correct for media evaporation, a follow-up experiment was conducted under the same conditions with no added DNAN. Three bioreactors contained autoclaved fungus, three contained live fungus and one contained only media. The reactors were weighed periodically, and a linear regression was applied to each condition and used to correct the observed concentrations and peak areas of metabolites.

Analytical methods

Tandem mass spectrometry (LC-MS/MS) was performed on an Agilent (Santa Clara, CA) 1260 liquid chromatograph coupled to an Agilent 6460 triple-quadrupole MS with a Jetstream ESI source. Samples in the auto-sampler tray were maintained at 8 °C. An Agilent Zorbax XDB (2.1 × 50 mm, 3.5 µm) column (35 °C) at 500 µL/min was used with mobile phase (A) as 95% DI water with 5% acetonitrile (ACN) and mobile phase (B) as 95% ACN

and 5% DI water. Both mobile phases contained 5 mM of ammonium hydroxide and ammonium acetate. For DNAN analysis, the mobile phase gradient was: 0 min, 10% B; linear to 5 min, 95% B; 7 min, 95% B; 7.1 min 10% B; 12 min 10% B. DNAN and other metabolites were quantified in ESI⁻ and ESI⁺ modes and qualified with a second fragment ion using the mass transitions outlined in the SI, Table S1. Samples were quantitated using a six-standard, external calibration curve run during each batch. Agilent MassHunter Quantitative Analysis software was used to fit a power regression model to the curve in order to extend the range of quantification to 3–4 orders of magnitude of concentration ($R^2 > 0.998$ for all analytes). The limits of detection (LODs) are listed in the SI, Table S1. Three to four quality control injections of the standards were made during each batch run to ensure consistent detector response, and samples were injected in a random order during each batch of 25–75 samples. A DI water blank was run every five samples to ensure there was no carry-over or contamination during analysis runs. For quantitation of reduced DNAN metabolites, the solvent system consisted of (A) DI water and (B) acetonitrile both containing 0.1% formic acid with the same column and flow rate. The mobile phase gradient was: 0 min, 5% B; 2 min, 5% B; linear to 8 min, 35% B; linear to 10 min, 95% B; 10.1 min 5% B; 15 min 5% B.

Accurate mass data were collected on a Waters (Milford, MA) Acquity ultraperformance liquid chromatograph (UPLC) followed by a Waters QToF Premier MS. An Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) heated to 60 °C (0.6 mL/min) was used. Leucine enkephalin was infused (10 μL/min) as the lock mass. Samples were analyzed (in separate runs) by both ESI⁻ and ESI⁺ mode. A mass to charge (m/z) range of 40–600, a scan rate of 0.2 s/scan and a sampling cone voltage of 30 were used for ESI⁻ mode. A scan rate of 0.15 s/scan and a sampling cone voltage of 23 were used for ESI⁺ mode. Samples in the auto-sampler tray were maintained at 8 °C. The mobile phase was (A) 95% DI water with 5% acetonitrile and (B) 95% acetonitrile and 5% DI water, both containing 5 mM ammonium acetate for ESI⁻. For ESI⁺ mode, 0.05% formic acid was added to both mobile phases. The mobile phase gradient was: 0 min, 0% B; linear to 7 min, 100% B; 9 min, 100% B; 9.1 min 0% B; 13 min 0% B. The desolvation gas (400 °C) flow was 800 L/h and the capillary voltage was 2.5 kV.

Accurate mass, full-scan data were collected for ten minutes, converted to netCDF format by Databridge software and uploaded to XCMS Online (Gowda et al. 2014) for an untargeted metabolomics approach to metabolite elucidation. The XCMS software takes raw, full-scan mass spectrometric data and reconstructs chromatograms of each ion detected. The reconstructed chromatograms were compared to determine differences between control and dosed fungus media based on magnitude, retention time, and mass-to-charge ratio for every ion detected in the samples. This study only examined compounds resulting from DNAN as confirmed by stable-isotope labeled substrates, and, as such, was not a comprehensive metabolomics investigation. Full XCMS Online parameters are detailed in the SI, Table S2. Two combinations of DNAN isotopes were used to allow unambiguous identification of metabolites derived from DNAN and to provide orthogonal data sets. Elemental formulas were assigned using Waters MassLynx software and data from the averaged accurate mass data from all biological replicates.

Statistical analysis

Raw, time-series data were compared for significant differences using a two-sided, matched-pairs Student's t-test ($\alpha = 0.05$, $p < 0.05$). An F-test ($\alpha = 0.05$) was used to test linear regressions for significant deviations ($p < 0.05$) from a null slope. Figures were produced and statistical analysis was conducted using GraphPad Prism 7.00.

RESULTS AND DISCUSSION

DNAN reduction, demethylation, and degradation kinetics

A fungal sp. isolated from willow tree cuttings that was able to degrade DNAN in rich medium was identified as a *Penicillium* sp. by the 18s ribosomal DNA gene. *Penicillium* is in the phylum Ascomycota and fellow ascomycetes have been shown to mineralize lignin and possess laccases, powerful oxidative enzymes useful for bioremediation (Liers et al. 2006; Baldrian 2006). While most of the studies on fungal metabolism of xenobiotics focuses on *Phanerochaete chrysosporium* (Fernando et al. 1990; Sheremata and Hawari 2000), *Penicillium* have been shown to degrade 100% of TNT in liquid media and to detoxify aromatic amines in soil (Scheibner et al. 1997; Martins et al. 2009).

A nominal quantity of 10 mg/L DNAN was degraded quickly in solution by *Penicillium* sp. KH1 (Fig. 1a). The degradation followed pseudo-first-order kinetics with a rate constant of $0.47 \pm 0.03 \text{ d}^{-1}$. This rate ($t_{1/2} = 35 \text{ h}$) is considerably slower than that observed by an isolated DNAN-mineralizing bacteria, *Nocardioides* sp. JS1661 ($t_{1/2} < 1 \text{ h}$); however, the half-life of DNAN degradation by the *Penicillium* sp. was on the same order of magnitude as aerobic soil slurries with added carbon and nitrogen ($t_{1/2} \approx 96 \text{ h}$) (Perreault et al. 2012) and aerobic sludge with added carbon ($t_{1/2} \approx 50 \text{ h}$) (Olivares et al. 2013).

An initial 8-hour sorption phase was observed in both control conditions. Up to eight hours, DNAN sorbed to the glassware in the negative control, but the slope of a least-squares linear regression from eight hours until 14 d was not significantly different from zero ($p = 0.6745$, $n = 21$), indicating that the concentration of DNAN did not change. There was also a significant difference ($p < 0.0001$, $n = 7$ pairs) in DNAN concentrations between the two control conditions, indicating that less DNAN was in the bulk media when the inactive biomass was present. The killed control provided evidence for sorption – rather than transformation – because molar concentrations of reduced and/or demethylated metabolites represented less than 0.9% of the original DNAN (Fig. 1a). The slope of the killed control was different from zero after eight hours ($p = 0.0191$, $n = 12$). This finding indicates that some spores may have survived autoclaving or that some enzymes could still be intact to degrade DNAN. However, DNAN concentrations in the treatment were significantly different than the killed control over 14 days ($p < 0.0001$, $n = 12$ pairs).

Our previous work demonstrated the biotic production of 4-ANAN in a *Rhizobium* sp. (Schroer et al. 2015). Here, 4-ANAN and 2-ANAN were also observed to result from nitroreduction of DNAN (Fig. 1b). 2-ANAN is the most commonly detected transformation product of DNAN and has been observed to result from soil and sludge cultures, as well as abiotic reduction (Hawari et al. 2014; Olivares et al. 2013; Perreault et al. 2012). Note that 2-ANAN and 4-ANAN have also been abbreviated as MENA and iMENA, respectively, in

other literature (Olivares et al. 2016); here, we chose this convention to more easily relate isomeric TPs explicitly to the *ortho* and *para* nitro groups of DNAN. DNP, resulting from demethylation of the methoxy group of DNAN, is a TP of DNAN in mammalian systems, (Dodard et al. 2013; Hoyt et al. 2013) and was observed transiently in the treatment along with low levels of 4-ANP and 2-ANP (Table 1).

When strain KH1 was incubated with DNP as the starting substrate, 2-ANP and 4-ANP were formed, while separate incubations with 2-ANAN yielded 2-ANP as well (Table 1).

However, when the starting substrate was 4-ANAN, the compound was not demethylated to form 4-ANP. These incubations indicate that demethylation and *o*-nitroreduction are parallel degradation pathways that can occur simultaneously and *p*-nitroreduction precludes demethylation. In general, 2-ANAN was observed in quantities at least two orders of magnitude greater than those of DNP and 4-ANAN (Fig. 1b, DNP not shown), indicating that *ortho* reduction of DNAN was the most dominate transformation pathway as previously observed abiotically and biologically, both in the presence and absence of O₂ (Hawari et al. 2014; Olivares et al. 2013; Olivares et al. 2016; Perreault et al. 2012). DNAN dimers have been observed in other studies (Olivares et al. 2013; Olivares et al. 2016) and are quite commonly formed from TNT (Haidour and Ramos 1996; Wang et al. 2000); however, no DNAN dimers were observed in our study.

Metabolite identification

After ten days of incubation with DNAN, including an additional spike after seven days, bioreactors were analyzed for unknown metabolite identification. Accurate mass data and untargeted metabolomics-based analysis revealed 16 additional compounds that were in the treatments but absent in the fungal reactors without DNAN (Table 2). Each compound contained the corresponding mass shifts consistent with the stable isotope labeling of the starting substrate, confirming that these were metabolites of DNAN. The accurate mass data for the ¹⁵N₂- and ¹³C₆-labeled DNAN metabolites can be found in the SI in Tables S3 and S4, respectively. The proposed *Penicillium* metabolic pathway for DNAN is presented in Scheme 1.

DNAN is acetylated and hydroxylated by *Penicillium*

The metabolite detected at *m/z* 211.0729 (along with the corresponding mass label shifts) was hypothesized to be 2-(*N*-acetyl)amino-4-nitroanisole (2-Nac-NAN), and the compound was confirmed by a synthesized standard (M210, Table 2). This compound has been observed in two of the three previous investigations of DNAN degradation by an isolated culture (Perreault et al. 2012; Schroer et al. 2015). After initial formation over the first five days of exposure, the concentration of 2-Nac-NAN (M210) decreased and then plateaued (Fig. 1b). When strain KH1 was incubated with 2-ANAN, direct amine acetylation was observed to form M210. This is in contrast to the findings of Perreault and co-workers, who observed hydroxylamine acetylation with subsequent de-hydroxylation to form 2-Nac-NAN (Perreault et al. 2012). The *N*-acetylation reaction is known to be catalyzed by *N*-acetyltransferases, which are ubiquitous in fellow plant-derived Ascomycota fungi (Karagianni et al. 2015) and detoxify a range of other aromatic amines (Martins et al. 2009). An ascomycete fungus detoxified soil artificially contaminated by 3,4-dichloroaniline by *N*-

acetylation to the extent that plants could then grow, indicating that this reaction likely reduces the toxicity of the DNAN metabolite (Martins et al. 2009).

2,4-Diaminoanisole was not detected by high resolution mass spectrometry, which is consistent with previous studies in aerobic systems (Hawari et al. 2014; Olivares et al. 2013; Olivares et al. 2016). However, two observed metabolites may have resulted from dual nitro group reductions (M208a and M208b, Table 2). Since 2-NAc-NAN was not demethylated by strain KH1 (Table 1), metabolites 208a and 208b are proposed to result from demethylation of DNAN followed by two successive acetylations of the primary amine group. These *N*-acetyl groups would withdraw electrons from the aromatic ring and promote further reduction of the remaining nitro group to yield M208a and M208b. The intermediate nitro-containing compounds were not detected, but these intermediates would be subject to amide hydrolysis to yield M196e and M196f or reduction to yield the isomers of M208. A third metabolite (M252) was detected that is proposed to result from multiple *N*-acetylations; this specific case is *N*-acetylation of 2-NAc-NAN (M210). Since 4-(*N*-acetyl)amino-2-nitroanisole was not detected, it is logical that only one isomer of M252 (presumably *o*-reduced) was detected. An additional metabolite (M226) had the formula $C_9H_9N_2O_5$, and is proposed to result from ring hydroxylation of 2-NAc-NAN (M210).

Six isomers of M196 have ambiguous structures

Six isomers (m/z 195), designated M196a to M196f, were detected in ESI⁻ mode using selected ion monitoring (SIM) LC-MS (Fig. 2) and/or LC-QToF. Multiple primary substrates were utilized to facilitate metabolite elucidation. M196a, c, and d were formed by demethylation and subsequently reduction at the *para* position because these metabolites resulted from 4-ANP and DNP, but not 4-ANAN. After strain KH1 was incubated with DNP, 2-ANAN, and 2-ANP, the same two isomers (M196b and e) of m/z 195 were detected. This demonstrated that M196b and M196e resulted from both demethylation and *o*-nitroreduction of DNAN in parallel. Finally, incubations with 2-NAc-NAN and 4-ANAN yielded no isomers of M196, indicating that demethylation of DNAN occurs before *p*-nitroreduction and *o*-acetylation (Fig. 2).

Four of the isomers of M196 were detected by LC-QToF after ten days of incubation with DNAN and were assigned the formula $C_8H_8N_2O_4$. M196b showed a neutral loss of H_2O , as previously observed for aromatic hydroxyl groups (Fabre et al. 2001). In addition, M196b had a fragment of $[M-Ac-NO]^-$ ($C_6H_5NO_2$), consistent with the nitroso-containing structures presented in Fig. 3a and c. None of the M196 isomers were detected in ESI⁺ mode which strongly suggests an absence of primary amine functional groups. The lack of ESI⁺ ionization, and the observation that M196a-e resulted from demethylated substrates points to the formation of three pairs of analogous *para*- and *ortho*-reduced isomers structures (Fig. 3). The peak areas of the M196a-e isomers were monitored over time on LC-MS (SIM), but only M196b and M196d were detected in measurable quantities when DNAN was the initial substrate (Fig. 4). Similar to the proportion of 2-ANAN to 4-ANAN, *o*-nitroreduced M196 isomers were formed in more substantial quantities than their analogous *para* isomers.

Nitro-reduction products of DNAN are sulfated

A metabolite ($C_8H_8N_2O_7S$), designated M276 (Table 2), is hypothesized to result from sulfation of M196f. This compound had a major fragment of m/z 195.0414, $[M-H-HSO_3]^-$, with unlabeled isotopes and 197.0376 and 201.0616 with the $^{15}N_2$ - and $^{13}C_6$ -labels, respectively. A fragment of $[M-H-HSO_3]^-$ is diagnostic of a sulfate attached to an sp^2 carbon (Yi et al. 2006), which suggests the phenolic group was sulfated. The *ortho*-nitro group of M276 was reduced as evidenced by incubations with DNAN, DNP, 2-ANP, 2-ANAN that yielded this compound and incubations with 4-ANP and 4-ANAN that did not produce M276 (Table 1). Metabolite M196f was the only isomer of M196 with a longer retention time than M276 which is consistent with an expected increase in polarity due to sulfation. M276 contained a nitro group (Table 2), suggesting that M196f was the precursor to M276 with the structure shown in Fig. 3d. Finally, M276 was not observed when strain KH1 was incubated with 2-Nac-NAN, suggesting that DNAN is demethylated initially, and then acetylated and sulfated to form M276.

A metabolite, designated M306 ($C_9H_9N_2O_8S$), is proposed to result from *ortho*-nitroreduction, acetylation, hydroxylation of the aromatic ring, and then sulfation of the phenolic group. M306 had the diagnostic fragment, $[M-H-HSO_3]^-$, that is characteristic of a sulfate group directly attached to an aromatic carbon, and M306 would directly result from sulfation of M226. Sulfated metabolite peak areas increased over time suggesting they are “dead-end” products (Fig. 5a). Sulfation is a well-known detoxification strategy for xenobiotics, especially sulfation of hydroxyl groups (Yi et al. 2006). This reaction greatly increases solubility, making elimination of the product easier, and, therefore, sulfated products are generally not subject to further degradation (Harms et al. 2011).

Amino-nitroanisoles are malonylated

Two additional isomeric metabolites (M254a and M254b), concluded to be malonylated amino-nitroanisoles, were detected in ESI^+ and ESI^- and assigned the formula $C_{10}H_{10}N_2O_6$. Evidence for the structure of these metabolites was collected *via* LC-MS/MS. In ESI^- mode, the $[M-H]^-$ ion was detected at m/z 253. With higher source energy, the predominant signal was $[M-H-44]^-$ at m/z 209, which has been shown to be diagnostic of a loss of the carboxyl group in *N*-malonylated lysine in peptides (Peng et al. 2011). In fact, the $[M-H]^-$ ion was below detection for M254a (Table 2) on the LC-QToF, while $[M-H-CO_2]^-$ was the base peak. An LC-MS/MS product scan of the parent $[M-H]^-$ ions revealed fragments of m/z 167 and 152 resulting from both isomers. These fragments represent a loss of malonate (m/z 167) and a loss of malonate and a methyl group (m/z 152).

A recent study of amine transformation in sludge found that the secondary amine of ortho-chlorophenylpiperazine most likely underwent malonylation. This and other *N*-acylations were implicated as important and previously overlooked xenobiotic amine transformation pathways (Gulde et al. 2016). Plant-associated fungi have been shown to perform the *N*-malonylation reaction on structurally similar aryl amines produced by plants to kill unwanted microorganisms. The fungi have adapted to detoxify these aryl amines, so M254a and b likely result from this known malonyl-transferase-mediated reaction (Karagianni et al. 2015). All of the *N*-acylated metabolites are likely to undergo amide hydrolysis and be back-

transformed to primary and secondary amines (Helbling et al. 2010), which may explain why most did not accumulate in the media (Fig. 1b, 4b, and 5b).

CONCLUSIONS

This is the first report we are aware of that demonstrated DNAN degradation by a fungus. *Penicillium* sp. KH1 isolated from willow tree cuttings quickly degraded DNAN in solution. Thirteen previously unknown degradation products were identified and confirmed with stable isotope-labeled substrate. DNAN was transformed *via* many reaction pathways including *ortho*- and *para*-nitroreduction, demethylation, acetylation, hydroxylation, malonylation, and sulfation. Transformation of DNAN by the fungus did result in degradation of the parent compound, but also formed metabolites of unknown persistence and toxicity. Further work would be needed to implement remediation with *Pencillium* sp. KH1. Finally, incubation with metabolites as primary substrates is an effective way to confirm metabolite isomerism and elucidate metabolic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Time series of (a) 10 mg/L DNAN degradation and (b) metabolite formation over fourteen days of incubation at 30 °C with *Penicillium* sp. KH1 (n=3, ^adenotes n=2). The sum of 2-ANAN, 2-ANP, 4-ANAN, 4-ANP, DNP and 2-NAC-NAN in the killed control is represented with closed blue triangles (n=1). Error bars represent one standard deviation from the mean

Fig. 2. LC-MS chromatogram of m/z 195 in ESI^- mode illustrating that isomeric starting substrates confirm five isomers of M196 resulting from either *ortho* (*o*) or *para* (*p*) nitro-reduction of DNAN

Fig. 3.
Possible structures for the six isomers of M196 and reactions required for formation from the corresponding amino-nitrophenol

Fig. 4. LC-MS (SIM) peak areas over time of m/z 195 in ESI⁻ mode for metabolite (a) M196b and (b) M196d produced from DNAN by *Penicillium* sp. KH1. Error bars are one standard deviation from the mean ($n=3$, $n=2$ days 5, 6, and 14). The open triangles are the concentrations in the killed control media ($n=1$)

Fig. 5. Concentration trends of (a) sulfated and (b) malonylated DNAN metabolites as demonstrated by LC-MS/MS peak areas. Error bars represent one standard deviation from the mean (n=3, n=2 days 5, 6 and 14)

Scheme 1.

Proposed DNAN transformation pathways for *Penicillium* sp. KH1. Brackets indicate the intermediate compound was not detected. An “X” over an arrow indicates the reaction was not observed when incubated with the starting substrate for 8 and 16 hours. An “a” indicates an isomer of M196 of unknown structure

Table 1
Transformations of various DNAN metabolites resulting from 16 hours of incubation of strain KHI

Starting substrate	Resulting metabolite					
	2-ANP	2-ANAN	4-ANP	4-ANAN	2-NAC-NAN	M276
DNAN	Yes	Yes	Yes	Yes	Yes	Yes
DNP	Yes	No	Yes	No	No	Yes
2-ANP	N/A	No	No	No	No	Yes
2-ANAN	Yes	N/A	No	No	Yes	Yes
4-ANP	No	No	N/A	No	No	No
4-ANAN	No	No	No	N/A	No	No
2-NAC-NAN	No	No	No	No	N/A	No

Table 2 DNAN metabolites determined from LC-QToF and XCMS online data analysis. Each accurate mass value is the average of six biological replicates

	proposed structure	confidence level ^a	RT (min)	ESI	ion formula	proposed ion	accurate mass	deviation (mDa (ppm))	fragment mass ^b	fragment formula	proposed fragment ion ^d
M210, 2-NAc-NAN		1. Synthesized standard, HR-MS, MS/MS, RT	2.78	+	C ₉ H ₁₁ N ₂ O ₄	[M+H] ⁺	211.0713	-0.6 (-2.8)	169.0627	C ₇ H ₉ N ₂ O ₃	[M+H ₂ -Ac] ⁺
				-	C ₉ H ₉ N ₂ O ₄	[M-H] ⁻	209.0561	-0.1 (-0.5)	177.0299	C ₈ H ₈ N ₂ O ₃	[M-OCH ₃] ⁻
M276		2b. HR-MS, MS/MS, diagnostic fragment, relative RT, putative pathways	1.86	-	C ₈ H ₇ N ₂ O ₅ S	[M-H] ⁻	274.9976	0.2 (0.7)	195.0411	C ₈ H ₇ N ₂ O ₄	[M-H-HSO ₃] ⁻
									153	N/A	[M-Ac-SO ₃] ⁻
									123	N/A	Ambiguous
									46	N/A	[NO ₂] ⁻
M306		3. HR-MS, MS/MS, diagnostic fragment	1.50	-	C ₉ H ₉ N ₂ O ₈ S	[M-H] ⁻	305.0063	-1.7 (-5.6)	225.0498	C ₉ H ₉ N ₂ O ₅	[M-H-HSO ₃] ⁻
									153.0212	C ₆ H ₅ N ₂ O ₃	[M-OCH ₃ -Ac-HSO ₃] ⁻
									210	N/A	[M-H-HSO ₃ -CH ₃] ⁻
									168	N/A	[M-HSO ₃ -CH ₃ -Ac] ⁻
									180	N/A	[M-HSO ₃ -NO ₂] ⁻
M254a		3. HR-MS, MS/MS, diagnostic fragment	2.04 ^c	+	C ₁₀ H ₁₁ N ₂ O ₆	[M+H] ⁺	255.0648	3.1 (12.2)	237	N/A	[M+H-H ₂ O] ⁺
									209	N/A	[M+H-CH ₂ O ₂] ⁺
			1.45	-	C ₉ H ₉ N ₂ O ₄	[M-H-CO ₂] ⁻	209.0564	0.2 (1.0)	96	N/A	Ambiguous
									167.0450	C ₇ H ₇ N ₂ O ₃	[M-H-C ₃ H ₅ O ₃] ⁻
									152.0219	C ₆ H ₄ N ₂ O ₃	[M-H-C ₃ H ₅ O ₃ -CH ₃] ⁻
M254b		3. HR-MS, MS/MS, diagnostic fragment	2.28 ^c	+	C ₁₀ H ₁₁ N ₂ O ₆	[M+H] ⁺	255.0669	5.2 (20.4)			
									191	N/A	Ambiguous
			1.72	-	C ₁₀ H ₉ N ₂ O ₆	[M-H] ⁻	253.0431	-3.0 (-11.9)	167.0456	C ₇ H ₇ N ₂ O ₃	[M-H-C ₃ H ₅ O ₃] ⁻
									152.0219	C ₆ H ₄ N ₂ O ₃	[M-H-C ₃ H ₅ O ₃ -CH ₃] ⁻

	proposed structure	confidence level ^a	RT (min)	ESI	ion formula	proposed ion	accurate mass	deviation (mDa (ppm))	fragment mass ^b	fragment formula	proposed fragment ion ^d
M208a		4. HR-MS	0.67	–	C ₁₀ H ₁₁ N ₂ O ₃	[M–H] [–]	207.0755	–1.5 (–7.2)	165.0560	C ₈ H ₉ N ₂ O ₂	[M–H–H ₂ O] [–]
M208b		4. HR-MS	0.72	–	C ₁₀ H ₁₁ N ₂ O ₃	[M–H] [–]	207.0756	–1.4 (–6.8)			
M208a or b	N/A	4. HR-MS	1.31 ^c	+	C ₁₀ H ₁₃ N ₂ O ₃	[M+H] ⁺	209.0939	1.3 (6.2)			
M196a	See Fig. 3	4. HR-MS, MS/MS	1.00	–	C ₈ H ₇ N ₂ O ₄	[M–H] [–]	195.0411	0.5 (2.6)	153.0311	C ₆ H ₅ N ₂ O ₃	[M–Ac] [–]
M252		4. HR-MS	1.09	+	C ₁₁ H ₁₃ N ₂ O ₅	[M+H] ⁺	253.0802	–2.2 (–8.7)			
M196b	See Fig. 3	4. HR-MS, MS/MS	1.13	–	C ₈ H ₇ N ₂ O ₄	[M–H] [–]	195.0393	–1.3 (–6.7)	177.0294	C ₈ H ₅ N ₂ O ₃	[M–H–H ₂ O] [–]
M196d	See Fig. 3	4. HR-MS	1.31	–	C ₈ H ₇ N ₂ O ₄	[M–H] [–]	195.0405	–0.1 (–0.5)	123.0325	C ₆ H ₅ NO ₂	[M–Ac–NO] [–]
M196f		4. HR-MS, RT relative to M276	2.00	–	C ₈ H ₇ N ₂ O ₄	[M–H] [–]	195.0404	–0.2 (–1.0)			
M226		4. HR-MS, putative pathways	2.29	–	C ₉ H ₉ N ₂ O ₅	[M–H] [–]	225.0513	0.2 (0.9)			

	proposed structure	confidence level ^a	RT (min)	ESI	ion formula	proposed ion	accurate mass	deviation (mDa (ppm))	fragment mass ^b	fragment formula	proposed fragment ion ^d
M280	Ambiguous	5. HR-MS	2.30	–	Ambiguous	[M+H] ⁺	278.9735	N/A			
M368	Ambiguous	5. HR-MS	2.18	+	Ambiguous	[M+H] ⁺	369.1450	N/A			

^aConfidence levels assigned as suggested by Schymanski et al. (2014)

^bNominal mass fragment indicates LC-MS/MS data. Accurate mass indicates LC-QToF fragment

^cIndicates a different retention time was observed in the separate positive and negative ionization chromatography runs. Because the pH of the mobile phases was lower for positive ionization, these compounds were presumably protonated in the positive ionization run condition, resulting in longer retention times

^dAc represents the acetyl moiety of the formula CH₃CO