Supporting Information

Different Mechanisms of Alkaline and Enzymatic Hydrolysis of the Insensitive Munition Component 2,4-Dinitroanisole Lead to Identical Products

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S1 Chemical analysis

S1.1 Quantification of DNAN and DNP

For alkaline hydrolysis reactions, 2,4-dinitroanisole (DNAN) and 2,4-dinitrophenol (DNP) concentrations were measured for 1 mL aliquots of the quenched reactions by ultra-performance liquid chromatography (UPLC, Waters) using an XBridge C18 column (Waters), a gradient method with methanol and nanopure water as eluents (both containing 0.1% formic acid), and an ultraviolet-visible detector (DNAN and DNP measured at wavelengths of 294 and 259 nm, respectively). DNAN and DNP eluted at retention times of 17.3 min and 16.8 min by this method, respectively. The ultraviolet-visible (UV-vis) spectra for a DNP standard and the hydrolysis samples containing DNP matched (an example is shown for the sample taken after 14 days of reaction time in Figure S1), indicating that concentration measurements for DNP were not affected by the presence of any intermediate products. For enzymatic hydrolysis reactions HPLC analysis was carried out as described previously. Briefly, measurements were carried out with an Agilent 1260 HPLC system and a Merck Chromolith RP-18e column (150 mm × 4.6 mm). The eluents were 0.1% trifluroacetic acid in water or acetonitrile (80:20) and were pumped at a flow rate 1.0 ml min⁻¹. DNAN was monitored at a wavelength of 260 nm.

![Figure S1 UV-vis absorbance spectra for DNP (retention time 16.8 min) for a DNP standard and a hydrolysis sample after 14 days of reaction time.](image)
S1.2 LC-HRMS analysis

Liquid chromatography high resolution mass spectrometry (LC-HRMS) was carried out using a high-resolution quadrupole orbitrap mass spectrometer (QExactive, Thermo Fisher Scientific Corporation, San Jose, US) by a previously published method by Men et al.\textsuperscript{5} to (1) confirm the presence of DNP as the primary transformation product and (2) determine if any intermediate products were present. Briefly, 100 μL sample (diluted 1 : 1000 with nanopure water) was loaded onto an Atlantis-T3 column (particle size 3 μm, 3.0 mm × 150 mm, Waters), and eluted at a flow rate of 300 μL min\textsuperscript{-1} by a gradient method. Mobile phases consisted of nanopure water and HPLC-grade methanol containing 0.1% formic acid (98-100%, Merck). HRMS analysis by electrospray ionization (ESI) was performed in full scan mode at a resolution of 140’000 at 200 m/z and a scan range of 50-750 m/z in positive/negative switching mode. The following samples were analyzed: a blank of the reaction solution with no DNAN added, a control with the DNAN starting material added to the reaction solution after the pH was adjusted to 7, and five reaction samples (time = 0, 2, 5, 9, and 14 days).

Example ESI negative chromatograms for the control and the 14-day reaction sample are shown in Figures S2a and S2b, respectively (none of the peaks visible in ESI positive mode appeared to change during the reaction, thus only ESI negative results are shown here). Each figure shows the total ion chromatogram (TIC) for a full scan with a mass range of 100-800, as well as an extracted ion chromatogram for the exact mass of negatively ionized DNP (183.0042) with a 2 ppm mass tolerance. The small peaks present in the control were presumably due to impurities in the starting material, as they were not present in the blank of the reaction solution and they did not change as the reaction progressed. The peak in the XIC for DNP (with an exact mass match within a 2 ppm tolerance and an isotope pattern consistent with a compound containing carbon and oxygen) grew as the reaction progressed. The MS2 fragmentation spectra obtained for DNP in the 14 day sample is shown in Figure S2c.

No intermediate reaction product was identified in any of the samples in either ESI negative or ESI positive mode. XICs were generated for expected masses of the Meisenheimer complex (m/z = 216.0382, 215.0304, and 217.0461 in neutral, negatively ionized, and positively ionized form, respectively), but revealed no visible peaks. However, it is still possible that a Meisenheimer product was generated and present at a concentration below its detection limit, as the samples had to be diluted 1000 fold prior to analysis by HRMS.

S2 Details for cell growth and enzyme partial purification

Cells of \textit{Nocardioides} sp. strain JS1661 were grown and enzyme preparations were made as described previously,\textsuperscript{3} with modifications. Briefly, cells were grown to late exponential phase in 1/4-strength trypticase soy broth, harvested by centrifugation, washed with 20 mM phosphate buffer (pH 7.0) and stored on ice until used in whole cell transformation experiments or for partial purification of DNAN O-demethylase. For partial purification of DNAN O-demethylase cells were lysed by passage through a French pressure cell at 40’000 psi and the exudate was
Figure S2 ESI negative LC-HRMS analysis of (A.) a control sample (DNAN starting material in pH 7 buffer), and (B.) a hydrolysis sample after a reaction time of 14 days. The total ion chromatograms (TIC) show full scan results (mass range 100-800), and the extracted ion chromatograms (XIC) show extracted results for the exact mass of negatively ionized DNP (m/z = 183.0042) with a 2 ppm mass tolerance. The small peaks in the control TIC are presumably impurities in the starting material, as they were not present in the blank and did not change during the reaction. (C.) MS2 fragmentation spectra of DNP from the 14-day hydrolysis sample.
centrifuged at 175'000 g for 30 min. The clarified supernatant was subjected to ammonium sulfate precipitation and the fraction that precipitated between 27% and 43% was dissolved in phosphate buffer and subjected to ultrafiltration (100 kDa Amicon Ultra-15 EMD Millipore, Billerica, MA). The retentate was stored on ice until used for DNAN transformation experiments.

S3 Stable isotope analysis

S3.1 Standard materials

The DNAN used as standard for C and N isotope analysis by gas chromatography / isotope ratio mass spectrometry (GC/IRMS) as well as the starting material for alkaline hydrolysis experiments was obtained from Alpha Aesar (98%). The $\delta^{13}C$ and $\delta^{15}N$ values were determined by elemental analyzer / IRMS as $-37.4\pm0.1\%e$ and $-2.4\pm0.1\%e$, respectively. The DNAN as the starting material for aerobic biodegradation and enzymatic experiments was also obtained from Alfa Aesar (98%) but originated from a different lot ($\delta^{13}C = -26.5\pm0.2\%e$, $\delta^{15}N = -0.4\pm0.2\%e$). The $\delta^{13}C$ and $\delta^{15}N$ values of the DNP standard used for isotopic analysis by GC/IRMS were $-27.6\pm0.3\%e$ and $-3.9\pm0.5\%e$, respectively. Hydrogen isotope ratios were calibrated using different alkanes as reference compounds in the $\delta^2H$-range between $-50\%e$ to $500\%e$.

S3.2 SPME-GC/IRMS procedures

For isotopic analysis, DNAN and DNP were extracted by solid phase micro extraction (SPME) according to previously published procedures as well as using the PAL SPME arrow. Samples were initially diluted with 10 mM phosphate buffer at pH 7.2 (DNAN) or pH 2.1 (DNP) to obtain concentrations within a range of linear response. Carbon and nitrogen isotope analysis was carried out using a gas chromatograph (Trace GC; Thermo Electron Corp.) equipped with a Rtx-5MS capillary column (0.32 mm ID, 1 $\mu$m film thickness, 30 m length) and coupled to an isotope ratio mass spectrometer (IRMS; DeltaPLUS XL, Thermo Electron Corp.) through a combustion interface (GC Combustion III, Thermo) equipped with a customized Ni/Pt reactor.

During SPME-GC-IRMS analysis of DNAN and DNP the GC was operated in splitless mode (splitless time 6 minutes for SPME arrow and 3 minutes for conventional SPME, purge flow 50 ml min$^{-1}$) with the following temperature program: 50 $^\circ$C for 1 minute, ramp 10 $^\circ$C min$^{-1}$ to 250 $^\circ$C, hold for 5 minutes. Analytes were measured against standard laboratory gases that were introduced at the beginning of each run (CO$_2$ and N$_2$). Subsets of six samples were bracketed with in-house C and N isotope standards (see above) in triplicate to allow correction for any drift in signal observed during each run. Alkaline hydrolysis samples were run in duplicate (isotope signatures are ported as individual values) whereas samples from biodegradation experiments were run in triplicate and the isotope signatures are reported as the arithmetic mean ±1 standard deviation. DNAN samples and standards analyzed by the SPME arrow were determined at concentrations of 1 $\mu$M and 5 $\mu$M for C and N isotope analysis, respectively. DNP samples and
standards for C and N isotope analysis were run at concentrations of 5 μM and 50 μM. Peak amplitudes for DNAN and DNP analyzed at these concentrations were between 1500 and 8000 mV. Hydrogen isotope analysis was carried out for a small subset of samples with the identical instrumental setup and procedures except for the use of a chromium containing pyrolysis reactor according to Renpenning et al. 7

S4 Data evaluation

S4.1 DNAN hydrolysis kinetics

The rate constants of enzymatic and alkaline DNAN hydrolysis and concomitant formation of DNP were derived from pseudo-first order rate law and the concentration dynamics of DNAN and DNP follow from eqs. S1 and S2.

\[
\begin{align*}
    c_{\text{DNAN}}(t) &= c_{\text{DNAN}}(t_0) \cdot e^{-k_{\text{DNAN}}^{\text{obs}} \cdot t} \\
    c_{\text{DNP}}(t) &= c_{\text{DNAN}}(t_0) \cdot (1 - e^{-k_{\text{DNP}}^{\text{obs}} \cdot t})
\end{align*}
\]

where \(c_{\text{DNAN}}(t)\) and \(c_{\text{DNP}}(t)\) are the concentrations of DNAN and DNP at time \(t\) and \(c_{\text{DNAN}}(t_0)\) is the initial DNAN concentration. A bimolecular rate constant for the alkaline hydrolysis of DNAN, \(k_{\text{OH}^-}\), was derived at a single pH value (pH 12) with eq. S3.

\[
k_{\text{OH}^-}^{\text{DNAN}} = k_{\text{obs}}^{\text{DNAN}} / 10^{(14 - \text{pH})}
\]

S4.2 Stable isotope data processing

Evaluation of isotope data and substrate and product fractionation was carried out according to procedures described in detail in Pati et al. 6 Isotope signatures were calculated according to eq S4.

\[
\delta^hE = \frac{R(\text{H/E})_{\text{sample}}}{R(\text{H/E})_{\text{standard}}}
\]

where \(\delta^hE\) is the isotope signature of the elements carbon or nitrogen, and \(R(\text{H/E})_{\text{sample}}\) and \(R(\text{H/E})_{\text{standard}}\) are the isotope ratios of the heavy (\(h\)) and light (\(l\)) isotopes. Isotope enrichment factors, \(\epsilon_E\), of DNAN and DNP were calculated according to eqs. 1 and 2 in the manuscript by non-linear regression. Substrate isotope fractionation from replicate experiments was reconciled using the Pitman estimator method described in Scott et al. 9 The comparison of C and N isotope enrichment factors of DNAN calculated by linear and nonlinear regression from selected data sets prior to combination with the Pitman estimator is shown in Table S1. The results illustrate that both approaches lead to identical results within uncertainty (95% confidence intervals). The comparison isotope of C and N isotope enrichment factors derived
Table S1 C and N enrichment factors for DNAN calculated according to a linear and nonlinear regression according to Pati et al. Uncertainties represent 95% confidence intervals.

<table>
<thead>
<tr>
<th>System</th>
<th>( \epsilon_{C, \text{linear}} )</th>
<th>( \epsilon_{C, \text{nonlinear}} )</th>
<th>( \epsilon_{N, \text{linear}} )</th>
<th>( \epsilon_{N, \text{nonlinear}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocardioides sp. JS1661</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cell experiments</td>
<td>(-3.2 \pm 0.2)</td>
<td>(-3.2 \pm 0.4)</td>
<td>(-2.6 \pm 0.2)</td>
<td>(-2.7 \pm 0.2)</td>
</tr>
<tr>
<td>Partially purified enzyme</td>
<td>(-4.3 \pm 0.2)</td>
<td>(-4.3 \pm 0.6)</td>
<td>(-2.5 \pm 0.2)</td>
<td>(-2.5 \pm 0.6)</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>(-5.8 \pm 0.2)</td>
<td>(-6.1 \pm 0.4)</td>
<td>(-2.7 \pm 0.2)</td>
<td>(-2.7 \pm 0.4)</td>
</tr>
</tbody>
</table>

from DNAN vs. DNP by eqs. 1 and 2 is shown in Table S2.

Apparent \(^{13}\)C and \(^{15}\)N kinetic isotope effects, \(^{13}\)C and \(^{15}\)N AKIEs, for the transformation of DNAN were derived from C and N enrichment factors according to eq. S5.

\[
h_{E, \text{AKIE}} = \frac{1}{1 + n/x \cdot z \cdot \epsilon_E}
\]

where \(n_C = 7\) and \(x = z = 1\) for calculation of \(^{13}\)C-AKIE for DNAN degradation by both alkaline hydrolysis and DNAN biodegradation. For the calculation of the \(^{15}\)N-AKIE, \(n, x,\) and \(z\) always equals 1. None of the N atoms participate in the reaction and \(^{15}\)N-AKIE represents the average of the secondary isotope effects of the two \(\text{NO}_2\) substituents.

The C and N isotope fractionation factors of DNP was obtained by eq. 2 in the main manuscript and these data were compared against \(\epsilon_E\) values at low substrate conversion where \(c/c_0\) approaches unity according to eq. S6.

\[
\lim_{c/c_0 \to 1} \epsilon_E \approx \delta^{hE}_{p, \text{ini}} - \delta^{hE}_{S, 0}
\]

where \(\delta^{hE}_{S, 0}\) and \(\delta^{hE}_{p, \text{ini}}\) represent the initial substrate isotope signatures as well as those of the averaged product isotope signatures at less than 10% substrate conversion.

Correlations of C and N isotope fractionation were compared based on the correlation slope, \(\Lambda^{N/C}\), which is proportional to the corresponding isotope enrichment factors, \(\epsilon_C\) and \(\epsilon_N\), as well as the AKIEs (eq. S7).

\[
\Lambda^{N/C} = \frac{\Delta^{15}N}{\Delta^{13}C} \approx \frac{\epsilon_N}{\epsilon_C} \approx \frac{(n/x)_N}{(n/x)_C} \cdot \frac{^{15}\text{N-KIE} - 1}{^{13}\text{C-KIE} - 1} \cdot \frac{1 + ^{13}\text{C-KIE} \cdot (z_C - 1)}{1 + ^{15}\text{N-KIE} \cdot (z_N - 1)}
\]
Table S2 Comparison of C and N enrichment factors calculated from substrate and product isotope fractionation according to Pati et al.\textsuperscript{6} Uncertainties represent 95\% confidence intervals.

<table>
<thead>
<tr>
<th>Evaluation procedure</th>
<th>\textit{Nocardioides} sp. JS1661</th>
<th>DNAN O-demethylase (partially purified)</th>
<th>alkaline hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C isotope fractionation}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\epsilon_C$ (DNAN, eq. 1, non-linear fit)</td>
<td>$-2.8 \pm 0.1$</td>
<td>$-3.7 \pm 0.1$</td>
<td>$-6.0 \pm 0.5$</td>
</tr>
<tr>
<td>$\epsilon_C$ (DNP, eq. 2)</td>
<td>$-2.2 \pm 0.1$</td>
<td>$-4.3 \pm 0.9$</td>
<td>$-8.9 \pm 1.4$</td>
</tr>
<tr>
<td>\textit{N isotope fractionation}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\epsilon_N$ (DNAN, eq. 1, non-linear fit)</td>
<td>$-2.5 \pm 0.1$</td>
<td>$-3.2 \pm 0.1$</td>
<td>$-2.7 \pm 0.4$</td>
</tr>
<tr>
<td>$\epsilon_N$ (DNP, eq. 2)</td>
<td>$-2.2 \pm 0.3$</td>
<td>$-1.9 \pm 2.2$</td>
<td>$-3.6 \pm 1.0$</td>
</tr>
</tbody>
</table>
S4.3 Intramolecular $^{13}\text{C}/^{12}\text{C}$ ratio distribution in DNAN

Intramolecular $\delta^{13}\text{C}$ values of DNAN pertinent to the 6 aromatic C atoms, $\delta^{13}\text{C}_{\text{aromatic}}$, and the aliphatic C atom in the methoxy substituent, $\delta^{13}\text{C}_{\text{OCH}_3}$, were derived with two approaches. Note that we used DNAN specimen from different producers for biodegradation vs. alkaline hydrolysis experiments (Section S3.1).

**Mass balance approach**

$\delta^{13}\text{C}_{\text{aromatic}}$ values were obtained from measurements of $\delta^{13}\text{C}$ of DNP after conversion of DNAN to DNP exceeded 95% (Figures 1a and 1b in the main manuscript). $\delta^{13}\text{C}_{\text{OCH}_3}$ followed from eq. S8 by taking into account the initial $\delta^{13}\text{C}$ of DNAN, $\delta^{13}\text{C}_{\text{DNAN,0}}$.

$$\delta^{13}\text{C}_{\text{OCH}_3} = 7 \cdot \delta^{13}\text{C}_{\text{DNAN,0}} - 6 \cdot \delta^{13}\text{C}_{\text{aromatic}}$$  \hspace{1cm} (S8)

The intramolecular $\delta^{13}\text{C}$ of aliphatic vs. aromatic C atoms of DNAN used in alkaline hydrolysis experiments is discussed in the main manuscript. The initial $\delta^{13}\text{C}$ of DNAN ($-26.4 \pm 0.5\%_e$) used in biodegradation experiments and the final $\delta^{13}\text{C}$ of DNP at 95% DNAN conversion ($-26.8 \pm 0.7\%_e$) were the same within analytical error, despite loss of the methoxy C during the demethylation reaction. The calculated $\delta^{13}\text{C}$ of the OCH$_3$ substituent of DNAN of $-25.1 \pm 0.5$ (eq. S8) suggests that aliphatic and aromatic C atoms of DNAN were nearly isotopically identical.

**Carbon isotope fractionation of reaction product**

The initial $\delta^{13}\text{C}$ of DNAN in alkaline hydrolysis experiments was also derived from fitting $\delta^{13}\text{C}$ of DNP to equation 2 of the main manuscript ($\delta^{13}\text{C}_{\text{DNAN,0}} = -25.8 \pm 0.8\%_e$).
S5  Kinetics of DNAN transformation to DNP for alkaline and enzymatic hydrolysis

![Graph showing kinetics of DNAN transformation to DNP](image)

**Figure S3** Kinetics for DNAN transformation to DNP by (a) *Nocardiooides* sp. JS1661 whole cells and (b) partially purified DNAN O-demethylase. Fitted lines correspond to Solid lines show the pseudo-first order kinetic description of concentration dynamics according to eqs. S1 to S2 with $k_{\text{DNAN}}^{\text{obs}} = 0.018 \pm 0.002 \text{ min}^{-1}$ and $k_{\text{DNP}}^{\text{obs}} = 0.020 \pm 0.002 \text{ min}^{-1}$. Parameter uncertainties and coloured areas in panel (b) stand for 95% confidence intervals of the fit.
Figure S4 Kinetics for alkaline hydrolysis of DNAN at pH 12 and formation of DNP. Solid lines show the pseudo-first order kinetic description of concentration dynamics according to eqs. S1 to S2. The rate constant of DNAN hydrolysis, $k_{\text{DNAN}}^{\text{obs}}$, equals $(2.2 \pm 0.1) \cdot 10^{-6}$ s$^{-1}$ is almost identical to the one of DNP formation $k_{\text{DNP}}^{\text{obs}}$ is $(2.8 \pm 0.2) \cdot 10^{-6}$ s$^{-1}$ (uncertainties are 95% confidence intervals). The dashed lines represent the fit for DNAN hydrolysis to DNP with a single $k_{\text{obs}}$ that corresponds to a bimolecular rate constant of DNAN hydrolysis by OH$^-$, $k_{\text{DNAN}}^{\text{OH}^-}$, of $(2.5 \pm 0.2) \cdot 10^{-4}$ M$^{-1}$ s$^{-1}$ (eq. S3).
Isotope fractionation for reaction with partially purified DNAN \( O \)-demethylase

**Figure S5** (a) C isotope fractionation of DNAN and DNP during enzyme-catalyzed hydrolysis by the partially purified DNAN \( O \)-demethylase and (b) the corresponding N isotope fractionation. Solid and dotted lines indicate the substrate and product isotope fractionation calculated according to eqs. 1 and 2 in the main manuscript, respectively (data in Tables 1 and S2). The shaded areas indicate the 95% confidence interval of the fit.
Figure S6 Correlation of C and N isotope fractionation of DNAN biodegradation by *Nocardoides* sp. JS1661 vs. enzyme-catalyzed hydrolysis by the partially purified DNAN O-demethylase. The slopes denote $\Lambda^{N/C}$ values ± 95% confidence intervals (shaded areas).

Figure S7 H isotope fractionation of DNAN associated with transformation by the partially purified DNAN O-demethylase in two experiments (A and B). Shaded areas indicate ± 95% confidence intervals of the fit with eq. 1 of the main manuscript corresponding to an $\epsilon_H$ of $-4 \pm 4\%$. The tentative $^2$H-AKIE calculated with $n = 6$, $x = z = 3$ is $1.026 \pm 0.023$. 
S7 References


