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One-pot cascade reactions for the synthesis of dinitroalkanes in aqueous buffer†

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Dinitroalkanes are powerful synthetic building blocks because of the versatility of the 1,3-dinitro motif. Here, we show that dinitroalkanes can be synthesized from aliphatic aldehydes in a three-step cascade reaction catalysed by phosphate buffer and the amino acid lysine. We further show that this methodology can be expanded to limited alcohol substrates (1-butanol and 1-pentanol) with the inclusion of a biocatalysed alcohol oxidation. Simultaneous addition of all reagents gives a maximal yield of 52% of 3-(nitromethyl)hexane, derived from 1-butanol and nitromethane, whereas staggering the introduction of the amino acid catalyst and nitromethane substrate boosts the yield to 71% of 3-(nitromethyl)hexane with near-quantitative consumption of the *n*-butyraldehyde intermediate. Taken together, this work presents a mild synthetic method that couples multi-step catalytic cascades to generate 1,3-dinitroalkanes.

Introduction

Dinitroalkanes are key synthetic intermediates because they can be easily converted into other functional groups, including diamines, heterocycles, cyclic alkanes, poly-substituted phenols, and chiral cyclic thioureas.[1–5] However, the typical synthesis of dinitroalkane building blocks requires forcing synthetic conditions, high substrate concentrations, and high catalyst loadings. Two main synthetic routes are used to access dinitroalkanes. In the first approach, Michael addition of a nitroalkane to a nitroalkene yields the desired 1,3-dinitro motif. Alternatively, a tandem Henry/Michael cascade can be used, in which an aldehyde and excess nitroalkane undergoes a Henry reaction followed by dehydration and a Michael addition. In both approaches, a basic catalyst is required to activate the nitroalkane, often triethylamine or a metal-based catalyst (*e.g.*, Al_2O_3).[6–8] To bias the reaction toward the dinitroalkane, it is common to supply the nitroalkane in large excess (>50 molar equivalents)[9,10] or even as the reaction solvent.[3,7] Moreover, because of the multi-step nature of the reactions, the intermediate nitroalcohol and/or nitroalkene are typically isolated after each step, increasing complexity and resource requirements.

Complementary to this approach are a number of demonstrations that successfully produce nitroalcohols and dinitroalkanes in aqueous conditions. Common bases such as sodium hydroxide[11] and sodium bicarbonate[12] have been used to promote the Henry reaction and/or Michael addition in an aqueous environment. Several enzymatic biocatalysts have also been able to catalyse the Henry reaction, including hydroxynitrile lyase from *Hevea brasiliensis*[13] and protein-glutamine γ-glutamyltransferase (TGase) from *Streptoverticillium griseoverticillatum*.[14] However, a biphasic system is often necessary to achieve peak activity. Busto *et al.* synthesized various aromatic nitroalcohols (yields >46%) from aromatic aldehydes and nitromethane by using bovine serum albumin (BSA) as a catalyst.[15] In addition, some dinitroalkane products were formed in yields ranging from 3–25%. Bora *et al.* showed that phosphate buffer (PB) could be used to synthesize nitroalcohols from aromatic aldehydes and nitroalkanes in aqueous conditions.[16] A subsequent study revealed that at slightly elevated temperature (60 °C), phosphate buffer will also catalyse the Michael addition of nitroalkane to nitroalkene.[17] Both protein/amino acid or phosphate-based catalysis avoids the use of basic conditions or metal catalysts typically required for a Henry reaction/Michael addition to occur. In addition, the amount of excess nitroalkane required can often be minimized.

Given the past work established by others, we hypothesized that a dual catalytic system of buffer and amino acid could execute the full conversion of aliphatic aldehydes to dinitroalkanes in a one-pot process. Moreover, if sufficiently mild conditions could be identified, this catalytic cascade could potentially be merged with a biocatalysed oxidation step to expand the reaction sequence to alcohol

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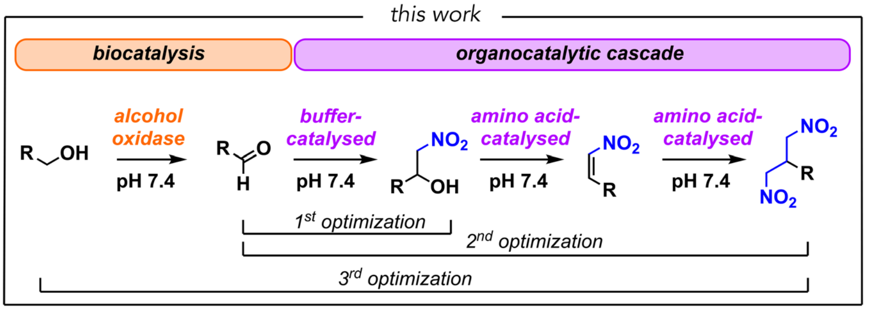

Fig. 1 We envisioned combining biocatalytic alcohol oxidation with a 3-step one-pot catalytic cascade to deliver 1,3-dinitroalkanes in aqueous buffer at room temperature.

substrates. Successful development of this approach would yield a one-pot four-step cascade of dinitroalkanes from abundant and potentially renewable alcohol substrates without the need for intermediate isolation.

Results and discussion

Optimization of tandem Henry/Michael reaction

In previous work, we successfully combined whole-cell (*G. oxidans*, *K. pastoris*) and enzyme-catalysed oxidation (alcohol oxidase (AO)) of aliphatic alcohols with a biocompatible organocatalysed aldol condensation to furnish industrially relevant α,β-unsaturated aldehydes.[18,19] Building off of this work, we sought to expand upon the products accessible through the merger of biocatalysis and organocatalysis. Previous efforts to combine organocatalysis and biocatalysis include the synthesis of optically active diols,[20] aminolactones,[21] indole derivatives,[22] and aldol condensation products,[23] among others.[24] Toward our goal of synthesizing dinitroalkanes under mild conditions, we envisioned using an enzyme or whole-cell biocatalyst to produce an aldehyde from an alcohol substrate. This reaction could be coupled to an organocatalysed reaction sequence to yield the dinitroalkane. We approached this challenge by breaking down the optimization into the three steps of the overall synthesis: 1) chemocatalysed aldehyde conversion to nitroalcohol; 2) chemocatalysed conversion of aldehyde to dinitroalkane; 3) biocatalytic oxidation coupled with chemocatalytic production of the dinitroalkane (Fig. 1).

Optimization of the chemocatalysed conversion of the aldehyde the nitroalcohol

We began by screening three buffers for their ability to catalyse the nitroaldol reaction of butyraldehyde and nitromethane in aqueous conditions at physiological pH 7.4 (Fig. 1, "1st optimization"). Previous demonstrations have shown that phosphate buffer will catalyse nitroaldol reactions under these conditions, although the addition of a cationic surfactant (CTAB) was necessary to facilitate the reaction between aliphatic aldehydes and nitroethane.[16] We also used Tris and HEPES buffers to determine if primary or tertiary amines, respectively, could catalyse the reaction. All buffers were tested at 100 mM and pH 7.4 with 5 mg mL^{-1} butyraldehyde and two equivalents nitromethane. Reactions were run for 24 hours, extracted with ethyl acetate, and the products quantified with gas chromatography-mass spectrometry (GC-MS) against an external calibration curve.

In the absence of any buffer, only trace amounts of the nitroalcohol product were formed (Fig. 2). Phosphate buffer and HEPES delivered 66% and 63% conversion to the nitroalcohol, respectively. No intermediate nitroalkene was observed, suggesting that the nitroalkene intermediate is rapidly consumed and converted to the dinitroalkane under these reaction conditions. Tris buffer was less effective than phosphate buffer and HEPES, delivering only 13% of the nitroalcohol. The lower activity of Tris likely results from reversible iminium ion formation with the primary amine, a strategy that has previously been used to isolate aldehydes from bacterial culture.[25]

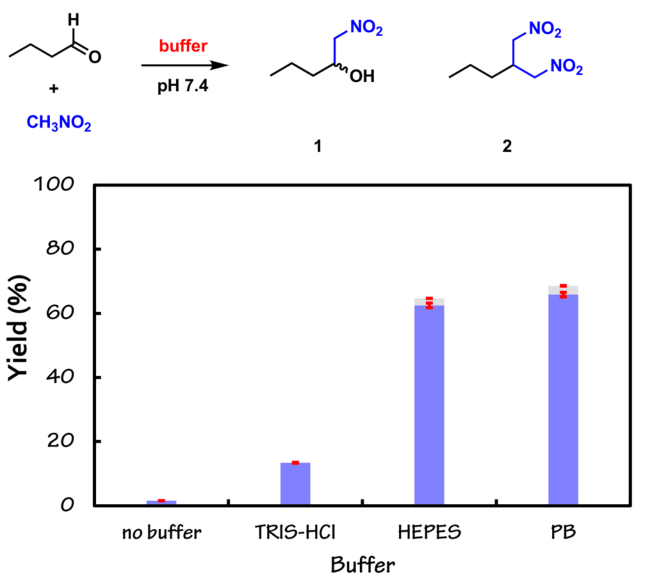

Fig. 2 Nitroalcohol (**1**) (blue) and dinitroalkane (**2**) (light grey) yields as a function of buffer. Reaction conditions: buffer (100 mM buffer, pH 7.4), butyraldehyde (5 mg mL^{-1}), nitromethane (2 equivalents). Reactions were run for 24 h at room temperature, and yields of nitroalcohol and dinitroalkane yields were determined by GC-MS. Each data point represents the average ± SD of three separate reactions.

Optimization of the chemocatalysed conversion of the aldehyde the dinitroalkane

In order to drive conversion of the nitroalcohol to the dinitroalkane, we next explored the addition of an amino acid catalyst (Fig. 1, "2nd optimization"). While probing protein-catalysed nitroaldol reactions, Busto *et al.* showed that lysine gave low yields of dinitroalkanes (25%) from aromatic aldehydes.[15] We hypothesized that an amino acid organocatalyst would promote dehydration to the nitroalkene and the subsequent Michael addition of another equivalent of nitromethane. Amino acid catalysis—the use of an amino acid to catalyse a chemical reaction—is an important subset of organocatalysis and has strong precedence for aldehyde and/or ketone activation. Early precedence for amino acid catalysis was provided by Prout, who showed that leucine, glycine, methionine and other amino acids could catalyse the Knoevenagel condensation between acetone and ethyl cyanoacetate.[26] Recognizing that amino acids may also impart stereocontrol led to the development of the proline-catalysed aldol addition by MacMillan and List,[27,28] igniting the field of organocatalysis.[29,30] To determine if amino acid catalysis could deliver dinitroalkanes from aliphatic aldehydes in this cascade, we initially selected three amino acids to pair with our system, including lysine, leucine, and phenylalanine (Fig. 3). These amino acids were chosen to include potentially catalytic side chains (lysine), non-polar aliphatic side chains (leucine), and non-polar aromatic side chains (phenylalanine). The amino acids (50 mM) were prepared in PB (100 mM) and tested at the previous substrate loadings of butyraldehyde (5 mg mL^{-1}), and nitromethane (2 equivalents). While the addition of the amino acid decreased overall conversion of the butyraldehyde substrate, dinitroalkane yields increased.

Reactions with phenylalanine gave 52% conversion with 16% of the dinitroalkane, leucine converted 53% overall with 14% yield of the dinitroalkane, and lysine peaked at 51% conversion with 14% of the dinitroalkane.

Because each amino acid catalyst performed similarly across the three amino acids tested, we chose lysine because of our previous work using lysine as a biocompatible organocatalyst.[18,19] To further drive the reaction toward the dinitroalkane, we increased the relative stoichiometry of nitromethane (Fig. 4). A steady increase in conversion was observed as nitromethane increased from 2 to 16 equivalents. Additionally, the proportion of the dinitroalkane within the product profile increased with increased nitromethane. Yields improved from 45% overall conversion with 12% yield of the dinitroalkane at two equivalents of nitromethane to 91% conversion with 73% yield of the dinitroalkane at 16 equivalents of nitromethane. Because the previous experiments indicate that lysine is a necessary catalyst to yield the dinitroalkane, we did not explore the effect of increased nitromethane stoichiometry in the absence of lysine catalyst. A probe of the aldehyde scope revealed that a range of aliphatic aldehydes, including *n*-propanal, *n*-pentanal, and *n*-hexanal, as well as a representative aryl aldehyde, benzaldehyde, could be converted to their corresponding dinitroalkanes in yields ranging from 42–62% using this optimized methodology (ESI† Fig. S1).

Merging biocatalysis with the three-step chemocatalysed cascade

We next sought to merge whole-cell biocatalytic alcohol oxidation with the chemocatalytic cascade (Fig. 1, "3rd optimization").

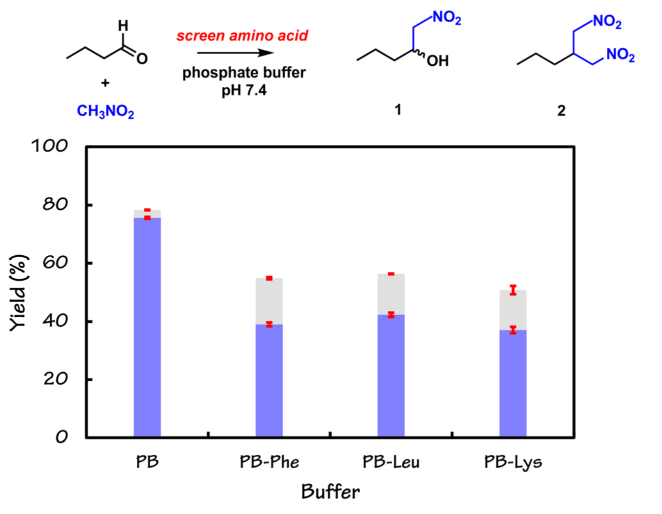

Fig. 3 Nitroalcohol (**1**) (blue) and dinitroalkane (**2**) (light grey) yields with buffer and amino acid catalysts. Reaction conditions: buffer (100 mM buffer, pH 7.4), amino acid (50 mM) butyraldehyde (5 mg mL^{-1}), nitromethane (2 equivalents). Reactions were run for 24 h at room temperature, and nitroalcohol and dinitroalkane yields were determined by GC-MS. Each data point represents the average ± SD of three separate reactions.

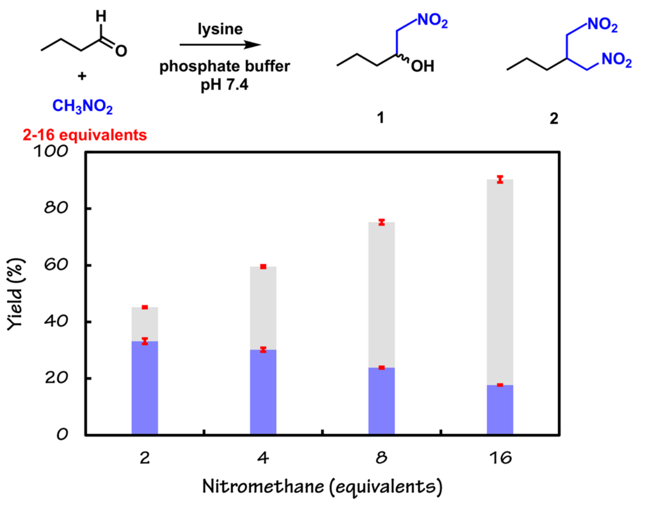

Fig. 4 Nitroalcohol (**1**) (blue) and dinitroalkane (**2**) (light grey) yields of as a function of nitromethane. Reaction conditions: phosphate buffer (PB) (100 mM, pH 7.4), lysine (50 mM), butyraldehyde (5 mg mL^{-1}), nitromethane (2, 4, 8, or 16 equivalents). Reactions were run for 24 h at room temperature and nitroalcohol and dinitroalkane yields were determined by GC-MS. Each data point represents the average ± SD of three separate reactions.

Initial attempts with whole cell biocatalysis of *n*-butanol with a tandem Henry/Michael reaction were unsuccessful. We first used *Komagataella pastoris* ATCC® 28485™, a yeast that expresses alcohol oxidase (AO) in peroxisomes. AO expression was induced by growing *K. pastoris* in media containing 10 g L^{-1} methanol, and OD_{600} = 1.0 (0.8 g L^{-1} dry-cell weight) *K. pastoris* cells were tested in 100 mM buffer (PBS or HEPES) with either 50 mM lysine or phenylalanine organocatalyst and with either 5 or 10 molar equivalents nitromethane. Coupling the Henry/Michael reactions to the *K. pastoris* bio-oxidation resulted in poor dinitroalkane yields (<5%) (ESI† Fig. S2). We hypothesized that the low yield may be a result of nitromethane toxicity. To explore this possibility, nitromethane toxicity was investigated by culturing *K. pastoris* in media containing nitromethane concentrations equivalent to 0–10 equivalents with 5 mg mL^{-1} *n*-butanol for 48 hours at 30 °C. Because our previous experiments indicate that the reaction proceeds a minimum of 200 mM nitromethane to proceed, we did not explore lower concentrations than 200 mM in the toxicity test. The cultures were plated onto YPD agar and incubated at 30 °C for another 48 hours. No cell growth was seen in all samples containing nitromethane, suggesting that nitromethane toxicity to *K. pastoris* may be the origin of low product yield with whole-cell biocatalysts (ESI† Fig. S3). Follow-on experiments probing the ability of heat-killed *K. pastoris* and live *K. pastoris* to oxidize *n*-butanol revealed only live cells were effective biocatalysts (ESI† Fig. S4).

Switching from whole cell *K. pastoris* to isolated alcohol oxidase (AO) (EC 1.1.3.13) considerably improved yields. We tested a range of concentrations of nitromethane (2, 4, 8, and 16 equivalents). Yields were maximized at 8 equivalents nitromethane (68% overall conversion; 52% yield of the dinitroalkane product) (Fig. 5). We saw similar trends to the 3-step cascade from an aldehyde substrate with higher relative stoichiometry of nitromethane driving the reaction. However, at the highest relative stoichiometry (16 equivalents) yields begin to fall (51% overall conversion with 44% yield of the dinitroalkane product). Because there is no evidence of side product formation, as indicated by GC-MS, these data suggest the higher nitromethane concentration is impairing the enzyme-catalysed alcohol oxidation step, potentially *via* competitive inhibition and/or enzyme denaturation in the nitromethane/PB mixture. To probe the biocatalysis step, we added a second aliquot of butanol after 24 h. No additional product was seen with additional alcohol substrate, further supporting our hypothesis that the conditions were impeding alcohol oxidase activity. Finally, quantitative NMR (qNMR) analysis revealed that nitromethane diminished alcohol oxidase activity in a concentration-dependent manner (ESI† Fig. S5).

To understand the dynamics of the integrated system, we tracked the production of the nitro alcohol and dinitroalkane products as a function of time (Fig. 6). Time-course yields for the integrated system (bio-oxidation and Henry/Michael reaction) were compared to time-course production of the dinitroalkane product from butyraldehyde (Henry/Michael reaction) (ESI† Fig. S6). Starting from butyraldehyde (5 mg mL^{-1}, 8 equivalents nitromethane) the reactions reach a plateau after 4 h at 76% conversion with 55% dinitro

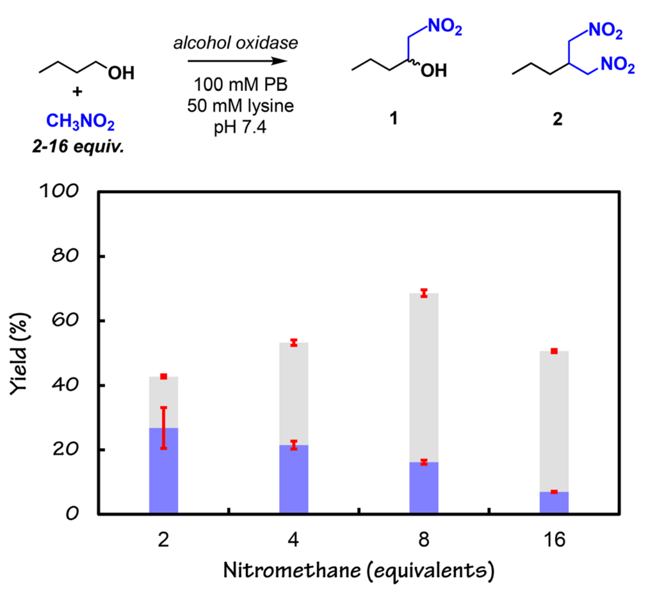

Fig. 5 Nitroalcohol (**1**) (blue) and dinitroalkane (**2**) (light grey) yields as a function of nitromethane. Reaction conditions: phosphate buffer (100 mM, pH 7.4), lysine (50 mM), alcohol oxidase (6 units mL^{-1}), catalase (1 mg mL^{-1}), butanol (5 mg mL^{-1}), nitromethane (2, 4, 8, 16 equivalents). Reactions were run for 24 h at room temperature, and nitroalcohol and dinitroalkane yields were determined by GC-MS. Each data point represents the average ± SD of three separate reactions.

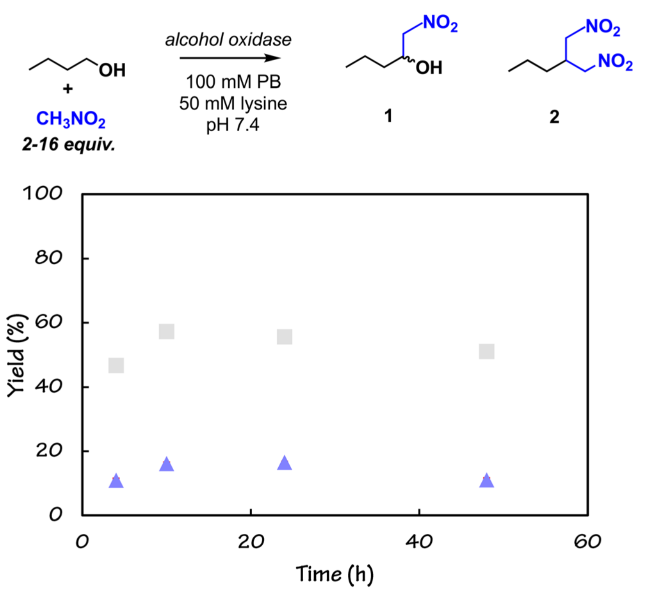

Fig. 6 Time-course yields of the one-pot biocatalysed oxidation of *n*-butanol with organocatalytic cascade. Reaction conditions: phosphate buffer (100 mM, pH 7.4), lysine (50 mM), alcohol oxidase (6 units per mL), catalase (1 mg mL^{-1}), butanol (5 mg mL^{-1}), nitromethane (8 equivalents). Reactions were stopped at the indicated time point, and nitroalcohol (**1**) (blue triangle) and dinitroalkane (**2**) (grey square) yields were determined by GC-MS. Each timepoint represents a separate reaction. Each data point represents the average ± SD of duplicate reactions.

product, while the integrated system peaked at roughly 73% conversion (57% dinitro product) after 10 hours. A brief substrate screen showed no measurable product from 1-propanol; however, 1-pentanol gave the corresponding dinitroalkane in 24% yield. This is likely a consequence of several factors including high volatility of the aldehyde intermediates.

In previous studies, increasing the initial substate loading has delivered higher titers;[18] however, higher substrate loadings would require a proportional increase in nitromethane loadings, which will decrease enzyme performance. An increase in temperature would likely improve the organocatalysed cascade of butyraldehyde to the dinitroalkane while maintaining current nitromethane loadings; however, alcohol oxidase performs optimally at 30 °C.[18,31] Because we do not see any overoxidation to the carboxylic acid in our experimental conditions, we next investigated how a sequential reaction one-pot reaction compared to a reaction with all reagents added at the onset. We anticipated that this may help alleviate incompatibilities between the organocatalytic cascade and bio-enzymatic oxidation step. Thus, we next sought to temporally separate the biocatalysed aldehyde production from the tandem Henry/Michael addition, anticipating that this would minimize any interference between the two steps and allow for increased nitromethane concentration to drive the Henry/Michael toward formation of the desired product. The sequential systems were run with 2, 5, or 10 mg mL^{-1} butanol in PB (100 mM) with AO (6 units per mL) and catalase (1 mg mL^{-1}). After 24 hours, lysine (50 mM) and nitromethane (16 equivalents) were added, and the reactions were allowed to continue for another 10 hours. All reactions were extracted with ethyl acetate, and the dinitroalkane was quantified by GC-MS against an external calibration curve (Fig. 7). At 2 mg mL^{-1} butanol loading, we saw near-quantitative conversion of *n*-butanol (98%), giving 33% nitroalcohol and 65% dinitroalkane. Increasing the butanol loading to 5 mg mL^{-1} decreased overall conversion to 89% but increased the dinitroalkane yield to 71%. Increasing the *n*-butanol loading to 10 mg mL^{-1} further decreased conversion (67%) and the yield of dinitroalkane (55%) (Fig. 7A). However, increasing the alcohol loading did improve overall titers, with the highest *n*-butanol loading yielding 13.1 mg mL^{-1} of the dinitroalkane (Fig. 7B).

To close, we have reported a single-pot approach to convert either aldehyde or alcohol substrates to their corresponding dinitroalkane products. From alcohol substrates, alcohol oxidase oxidizes C_4–C_5 alcohols to their corresponding C_4–C_5 aldehydes, followed by an organocatalysed Henry/Michael cascade with nitromethane. Beginning from aliphatic aldehydes, the scope includes C_3–C_6 *n*-aliphatic aldehydes, as well as benzaldehyde. The reaction can be run in one-pot as either a tandem or sequential reaction under mild (pH 7.4, room temperature), aqueous conditions. The substrate scope is limited by alcohol oxidase to short unbranched alcohols from C_1–C_5;[32,33] however, other oxidative biocatalysts may show greater substrate tolerance and could be merged with this methodology. Finally, while exploring common extractants, we discovered that the nitroalkene could be selectively extracted with cyclohexane or TPGS-750M. This opens the door for other Michael-type additions in the organic phase that could be paired with the aqueous bio-oxidation/Henry reaction/dehydration reaction sequence. Taken together, our study highlights that merging biocatalysis with *in situ* organocatalytic upgrading enables access to the complexity and breadth of products available from bioprocesses.

Author contributions

Kelsey N. Stewart: formal analysis (equal); investigation; methodology (equal); writing – original draft preparation

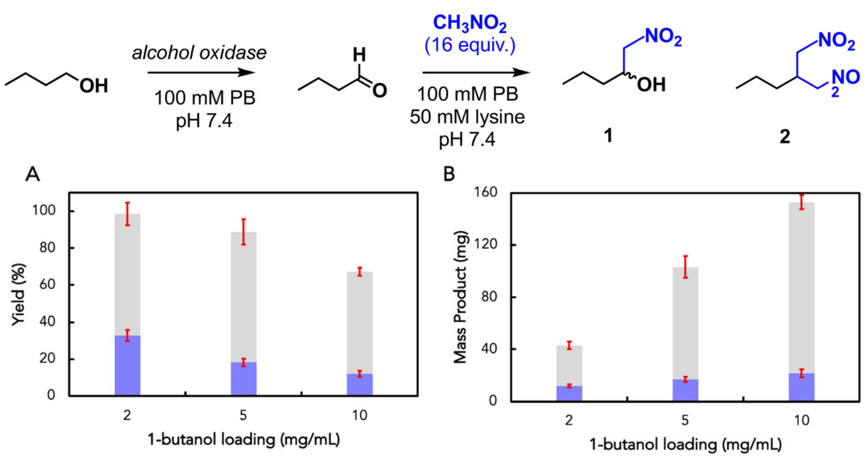

Fig. 7 Nitroalcohol (**1**) (blue) and dinitroalkane (**2**) (light grey) sequential reaction yields of as a function of butanol loading. Expressed as (A) % conversion of the butanol substrate or (B) total product yield (mg). Reaction conditions: phosphate buffer (100 mM, pH 7.4), alcohol oxidase (6 units per mL), catalase (1 mg mL^{-1}), butanol (5 mg mL^{-1}). The bio-oxidation was run for 24 h at room temperature. After 24 h lysine (50 mM) and nitromethane (16 equivalents) were added and the reaction proceeded for an additional 24 h at room temperature. Nitroalcohol and dinitroalkane yields were determined by GC-MS. Each data point represents the average ± SD of three separate reactions.

(equal); writing – review & editing (equal). Kendyll G. Hawkins: formal analysis (equal); investigation; methodology (equal); writing – original draft preparation (equal); writing – review & editing (equal). Campbell M. Andersen: formal analysis; investigation; methodology; writing – review & editing. Dylan W. Domaille: conceptualization; funding acquisition; project administration; supervision; writing – review & editing (lead).

Conflicts of interest

There are no conflicts to declare.

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