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A biocatalytic cascade for the amination of unfunctionalised cycloalkanes

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Here we describe a one-pot, three-enzyme, cascade involving a cytochrome P450 monooxygenase, an alcohol dehydrogenase and a reductive aminase for the synthesis of secondary amines from cycloalkanes. Amine product concentrations of up to 19.6 mM were achieved. The preparative scale amination of cyclohexane was also demonstrated with a space-time yield of 2 g L^{-1} d^{-1}.

A large number of biologically active molecules contain nitrogen functionalities,[1,2] and consequently there is demand for the development of efficient synthetic routes to access these valuable chemicals. Biocatalytic routes have been developed for the synthesis of chiral amines using transaminases,[3,4] ammonia lyases,[5,6] amine dehydrogenases,[7] monoamine oxidases[8,9] and imine reductases.[10–12] Recently, the toolbox of enzymes for amine synthesis has been expanded by the discovery of an NADP(H)-dependent reductive aminase (RedAm) from *Aspergillus oryzae* (*Asp*RedAm). This enzyme is capable of catalysing not only the reduction of preformed imines, but also the coupling of amines and ketones followed by the subsequent reduction of the imine intermediate.[13] All of these enzymes require electrophilic centres to insert nitrogen atoms, whereas a more elegant route would be the direct replacement of an unactivated C-H with a C-N bond or its synthetic equivalent.

In organic chemistry, C-H activation is typically enabled by metal complexes and requires the presence of auxiliary groups.[14–20] Rare enzymatic counterparts have been found in nature to carry out this attractive chemistry,[21] and only recently, heme-proteins have been engineered to perform C(sp^3)-H amination reactions. This biocatalytic amination reaction was achieved through the introduction of mutations at residues essential for the native mono-oxygenation activity of a cytochrome P450 or through the installation of synthetic metal complexes in place of the natural heme.[22–26] Even though these studies have extended the potential for biocatalytic C-H functionalisation remarkably, these novel catalysts either rely on pre-functionalized molecules possessing both the nitrogen functionality and the target C-H bond (intramolecular C-H amination), or on pre-activated aminating agents (*e.g.*, tosyl azides).

Oxidative enzymes have previously been successfully combined with amination biocatalysts to functionalise C(sp^3)-H bonds,[28,29] alkenes[30] or to convert alcohols to amines.[31,32] Moreover, biocatalytic redox-neutral hydrogen-borrowing processes have been demonstrated for the conversion of alcohols into the corresponding primary amines.[33,34,35] Thus far, these studies are limited to the synthesis of primary amines due to the nature and specificity of the amination biocatalyst. However, recently we have demonstrated the synthesis of aliphatic and aromatic secondary amines starting from primary and secondary alcohols *via* hydrogen-borrowing employing a single alcohol dehydrogenase (ADH) and the newly characterised *Asp*RedAm.[36]

Herein, we report a new biocatalytic cascade for the conversion of cycloalkanes to secondary amines employing a cytochrome P450 mono-oxygenase for the first oxidation step followed by amination using an ADH in combination with a RedAm (Scheme 1). As a model substrate, we selected cyclohexane **1** for P450-catalyzed hydroxylation to the corresponding alcohol cyclohexanol **2** followed by ADH mediated oxidation to cyclohexanone **3** and subsequent amination with *Asp*RedAm.

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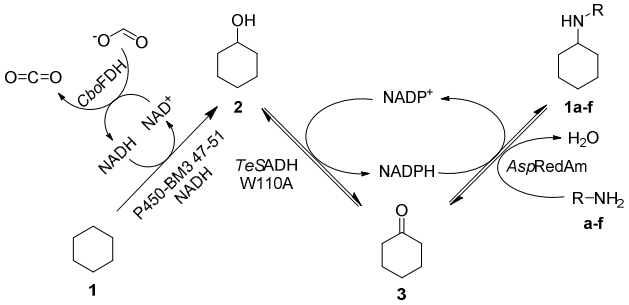

Scheme 1. Biocatalytic cascade for the amination of cycloalkanes.

For the initial C-H activation step, we selected the P450-BM3 mutant R47L/Y51F which was previously shown to have high activity towards cyclohexane.[37] In particular, it was observed that the hydroxylation of cyclohexane improved when the reaction was carried out in a biphasic system, with the substrate acting as co-solvent.[38] In order to uncouple the cofactor dependence of the two steps and avoid competition between the P450 and the RedAm for reducing equivalents, the two amino acid substitutions R966D and W1046S were introduced in the reductase domain of the self-sufficient P450-BM3 to increase its affinity towards NADH.[38,39] Cofactor regeneration for the oxyfunctionalisation step was performed by the formate dehydrogenase from *Candida boidinii* (*Cbo*FDH).[40] Finally, for the amination step *via* hydrogen-borrowing we selected a variant of the ADH from *Thermoanaerobacter ethanolicus* (*TeS*ADH W110A)[41] and *Asp*RedAm, as previously reported by our group.[36]

In an attempt to exploit all the selected enzymes in a one-step process, we initially investigated the effect of amines **a–f** at different substrate concentrations on the performance of the P450, expressed as residual total turnover numbers (TTNs, Figure 1). The negative effect on P450 performance was most pronounced at 250 mM amine, with only small primary amines, such as ammonia **a** and methylamine **b**, being tolerated. At 50 mM amine, P450 hydroxylation activity was still significantly affected, especially with propargylamine **c**.

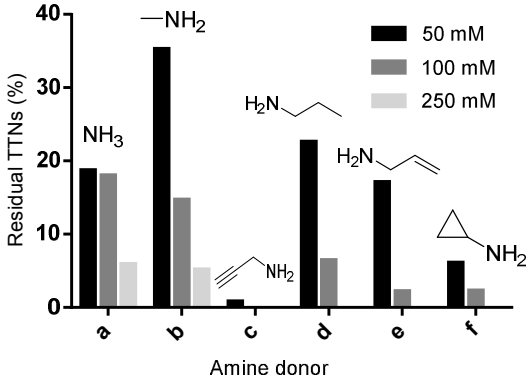

Figure 1. Effect of various amine donors selected for the amination cascade on the performance of the P450. See Supporting Information for full experimental conditions.

In the light of these initial results, we incubated crude enzyme preparations (the exception being *Asp*RedAm, used in purified form) with **1** in the presence of 1 mM NAD^+ and 1 mM $NADP^+$, 250 mM sodium formate and 250 mM methylamine, but unfortunately no amine formation was detected. We speculated that the cofactors required for the hydrogen-borrowing step ($NADP^+$/NADPH) were intercepted by endogenous *E. coli* enzymes, leading to imbalanced cofactor regeneration.[33,34] Thus, the amount of lysate added for the hydroxylation step was reduced and, gratifyingly, conditions were found to carry out the reaction as a one-step process (4.5 mM *N*-methylcyclohexylamine **1b** produced, Table S1). Next, we decided to extend the one-step cascade for the amination of **1** using a panel of six amine nucleophiles at different loadings (250, 100, 50, 25 and 10 mM, as reported in Table S1), guided by our initial investigation on the effect of the selected amines on hydroxylation activity (Figure 2 and Table S1). The outcome of the amination cascade was observed to be a trade-off between the inhibitory effect of the amine donor on the P450 and the reactivity of the same amine nucleophile in the *Asp*RedAm-catalysed reductive amination.[13] 3.6 mM *N*-propargylcyclohexylamine **1c** and 2.1 mM *N*-cyclopropylcyclohexylamine **1f** were produced when propargylamine **c** and cyclopropylamine **f** were used as reacting partners in the one-step process, with 0.7 mM cyclohexanone **3** left. Interestingly, the highest product concentration was observed with **e** (8.3 mM), although a considerable amount of **2** (4.7 mM) and **3** (5.9 mM) remained unreacted. Moreover, 9.6 mM cyclohexanone **3** accumulated with just 1 mM $NADP^+$ when **a** was employed as the amine donor, although only a low concentration of cyclohexylamine **1a** was produced (0.2 mM, Table S2). These results suggest that $NADP^+$ regeneration might also be supported by the P450 (which can still accept the phosphorylated cofactor)[38] and/or by other enzymes in the lysate. Moreover, coupling efficiency between cofactor consumption and substrate oxidation in the hydroxylation step cannot be underestimated.[38,42]

To test these hypotheses, experiments with purified proteins were performed using **e** as the amine donor, and almost total conversion of **3** to **1e** was achieved (92%). On the one hand, this result indicates that endogenous enzymes in *E.coli* might compete with *Asp*RedAm for reducing equivalents. On the other hand, there was also a substantial amount of **2** left (70% of total products formed), which might suggest competition between the P450 and *Asp*RedAm for NADPH, leading to a buildup of the ketone intermediate when less reactive amine donors were employed (Figure S1).Taking into account some of the inherent limitations associated with a one-step approach and the application of purified enzymes, we decided to explore the potential of the one-pot cascade as a two-step process with crude enzyme preparations. This approach made it possible to increase the enzyme loading in the first step without impacting on the presence of the amine donor for the second step (Table S2). With this setup, production of *N*-propargylcyclohexylamine **1c** reached 19.6 mM, and good results were also obtained with allylamine **e** and cyclopropylamine **f** (15.4 and 17.1 mM product, respectively),

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with **3** accounting for 19%, 35% and 16% of total products formed (Figure 2 and Table S2). Less pronounced improvements were achieved when amines **a**, **b** and **d** were employed in the two-step process, with 50%-80% products represented by **3**.

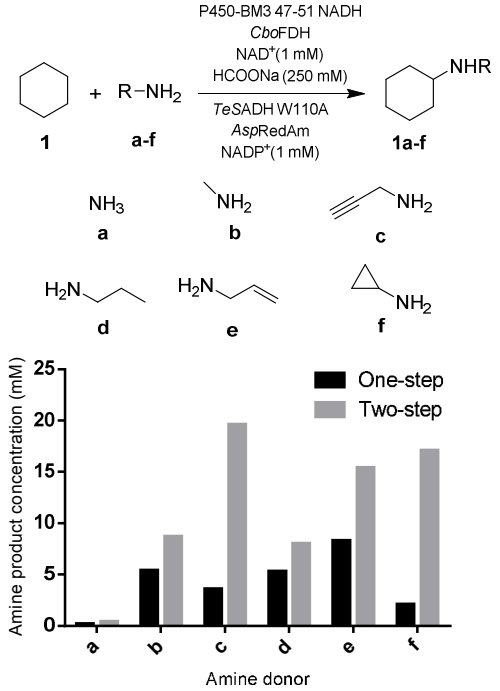

Figure 2. One-step and two-step cascade for the amination of cyclohexane using a panel of primary amines. See Supporting Information for full experimental conditions.

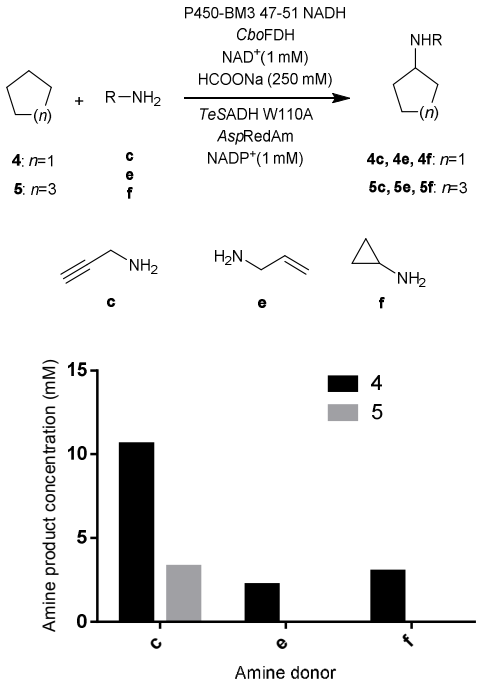

Figure 3. Two-step cascade for the amination of cyclopentane and cycloheptane. See Supporting Information for full experimental conditions.

The P450 variant employed in this study can accept a variety of cycloalkanes[37] and, similarly, *TeS*ADH has already been employed with a range of cyclic alcohols.[39] *Asp*RedAm can also catalyse the reductive amination of cyclopentanone **8**, albeit less efficiently than that of **3**.[13] Thus, we explored the possibility of expanding the scope of the cascade to other cycloalkanes (Figure 3 and Table S3). When employing amine donors **c**, **e** and **f**, product formation was detected when starting from cyclopentane **4** and cycloheptane **7**. For cyclooctane **10**, even though product formation was detected when *Asp*RedAm was incubated with cyclooctanone **11** and **e** (40 % conversion, Figure S2), unfortunately, the multi-step amination of **10** was unsuccessful.

When **c** was reacted with **4**, 10.6 mM *N*-propargylcyclopentylamine **4c** was produced (50% of total products formed), whereas **e** and **f** gave only 2.2 mM *N*-allylcyclopentylamine **4e** and 3.0 mM *N*-cyclopropylcyclopentylamine **4f**, with the accumulation of the intermediate oxidised products, which represented 85 and 79% of total products formed, respectively. Modest product concentrations and an excess cycloheptanone **9** (61% total products formed) were obtained when the bulkier cycloheptane **7** was used, with 3.3 mM *N*-propargylcycloheptylamine **5c** produced with **c** and no product detected with both **e** and **f**. We speculate that the reactivity of *Asp*RedAm towards specific carbonyl acceptors and amine partners influences the outcome of the amination cascade both in terms of yield and conversion of the intermediate ketone to the amine.

To examine the scalability of the process, we selected the two-step cascade for the amination of cyclohexane with **c** to form **1c**. A 2-fold improvement in TTN for the hydroxylation step was found when the reaction was carried out in a flask with orbital shaking instead of a round-bottom flask with magnetic stirring (5707 *vs.* 2976), probably due to a better mass-transfer and/or oxygenation (Figure S3).[38] Following this, the amination step yielded 50 mg of the final product **1c** giving a space-time yield of 2 g L^{-1} d^{-1} (Figure S4).

In summary, we have demonstrated a biocatalytic cascade for the preparation of a set of secondary amines from cycloalkanes. Unlike chemical approaches for the addition of amines across aliphatic C-H bonds, the presented cascade is carried out in aqueous buffer, does not produce any toxic by-products and does not require additional deprotection steps to obtain the final amine product. The plethora of information available for oxidoreductases, combined with the broad substrate tolerance of reductive aminases, will allow for a larger application of the presented cascade to access a variety of different amines starting from unfunctionalized compounds.

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Conflict of interests

There are no conflicts of interest to declare.

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