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One-pot Conversion of Cycloalkanes to Lactones

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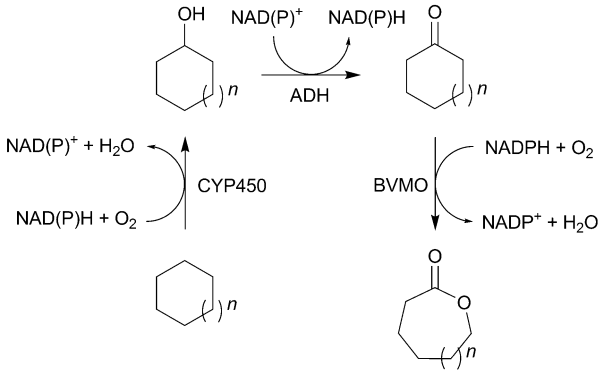
The one-pot conversion of cycloalkanes to their corresponding lactones was achieved through the use of a synthetic pathway consisting of a cytochrome P450 monooxygenase (CYP450) for initial oxyfunctionalization of the cycloalkane, an alcohol dehydrogenase for ketone production and a Baeyer–Villiger monooxygenase for lactone formation. Through variation of the cofactor dependence of the biocatalysts and the cofactor regeneration system, final product concentrations of nearly 3 g L^{-1} enantholactone (2-oxocanone) from cycloheptane was reached within 12 h with a total turnover number (TTN) of 4185 with respect to the CYP450.

Multi-enzyme one-pot reactions or synthetic cascades take advantage of the inherent operational compatibility of enzymes to perform transformations by mimicking "mini" metabolic pathways. In addition to the advantages offered by biocatalysts, these cascade reactions have the added benefit of circumventing the requirement to purify reaction intermediates, overcoming product inhibition and shifting the equilibrium of reversible reactions.[1–3] Although conceptually straightforward, construction of these synthetic cascades is no trivial matter, particularly if the pathway includes oxidoreductases. Different strategies have been employed including "designer cells" where all the enzymes are co-expressed simultaneously, as well as "strain-mixing" where the enzymes are produced separately but mixed in a single reaction vessel either as whole-cells, permeabilized cells or cell-free extracts (lysates).[4–7]

To date, most multi-enzyme systems in biocatalysis are limited to the biocatalyst of interest being used with an unrelated enzyme for co-factor regeneration. Recently oxidoreductases have been used in tandem reactions, whereby the product of the first reaction not only serves as substrate for the second reaction, but also renders the reaction redox-balanced or self-sufficient.[8–11] This approach has been used in two studies to convert cyclohexanol to ε-caprolactone (2-oxepanone), by combining a Baeyer–Villiger monooxygenase (BVMO) in a one-pot fashion with either a polyol dehydrogenase[12] or an alcohol dehydrogenase (ADH)[13] to convert cyclohexanol to cyclohexanone and also regenerate the co-factor NADPH required by the BVMO. Similarly the oxidation of (cyclo)alkanes to (cyclo)alkanones have been demonstrated.[14,15]

We report here a multi-enzyme biocatalytic pathway or cascade in which a non-activated cycloalkane is converted to a lactone (Scheme 1), an important class of building block for polymers.[16] This pathway or cascade consists of a cytochrome P450 monooxygenase (CYP450) for oxyfunctionalization of the cycloalkane, an alcohol dehydrogenase for conversion of the resulting alcohol to a ketone, and finally a Baeyer–Villiger monooxygenase for production of the corresponding lactone. For the first step, we employed a previously created CYP102A1 (BM3) mutant (CYP102A1_139-3C1)[17] with increased activity toward cycloalkanes, as well as its NADH dependent counterpart. Two alcohol dehyrogenases (TADH[18] and TeSADH[19]), which differed in their cofactor dependence, were used for the second step. The final Baeyer–Villiger oxidation step was catalyzed by a stabilized mutant of cyclohexanone monooxygenase (CHMO),[20] which is NADPH dependent. The biocatalysts for the first two steps were evaluated in all four combinations with CHMO (Scheme S1, Supporting Information).



Scheme 1. Multi-enzyme cascade for the production of lactones from cycloalkanes.

Although our initial attempts at creating "designer cells", with all three enzymes co-expressed in *E. coli*, were successful, the yields of the final lactone as well as the intermediate products were disappointingly low (generally less than 1 mM lactone). Analysis of the concentrations of the enzymes revealed the expression levels of all three recombinant enzymes to be considerably reduced (Figure S1, Supporting Information). This was most likely due to the metabolic burden caused by the simultaneous expression of three recombinant proteins. Unfortunately, not only were the expression levels reduced, but in an unpredictable fashion, with the alcohol dehydrogenase and the cytochrome P450 being more affected than the BVMO.

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We therefore turned to a strategy whereby the separately expressed proteins are mixed as cell-free extracts (lysates) to perform the cascade in a one-pot fashion. This approach not only retained the high-level expression, but also allows for the regulation of each step depending on the specific activity of the particular biocatalyst. Since our cascade was not redox balanced, the mixing of cell-free extracts had the additional advantage that native *E. coli* proteins and endogenous NAD(P)H were available for co-factor regeneration. Glucose and glycerol were therefore included in the reaction mixture to drive co-factor regeneration by these native enzymes. Cycloheptane was used as a model substrate, since the CYP450 showed the best activity with cycloheptane.[17] The concentration of cycloheptane used (165 mM) far exceeded its aqueous solubility (≈0.3 mM[21]). As reported by us[17] and others,[22,23] CYP450 activity greatly benefits if these hydrophobic substrates are added as a second phase, thereby also serving as a co-solvent.

Gratifyingly, all four combinations of biocatalysts yielded enantholactone from cycloheptane (Figure 1 and Figure S2 of the Supporting Information). The highest concentration of total product was obtained when the NADH-dependent mutant of the CYP450 was used. This could be attributed to the higher levels of endogenous NADH as well as *E. coli*'s ability to more efficiently regenerate NADH using glucose and glycerol as substrates. The highest concentration of enantholactone (3.2 mM, 0.41 g L^{-1}) was produced in combination IV (Scheme S1, Supporting Information), where the NADH-dependent CYP450 was paired with TeSADH and CHMO, which are both NADP(H) dependent. In this combination, *E. coli* regenerates NADH for the hydroxylation step, with the last two NADP(H)-dependent steps being redox balanced (self-sufficient). CYP450 BM3 and its mutants also often have low coupling efficiencies whereby reducing equivalents are wasted by

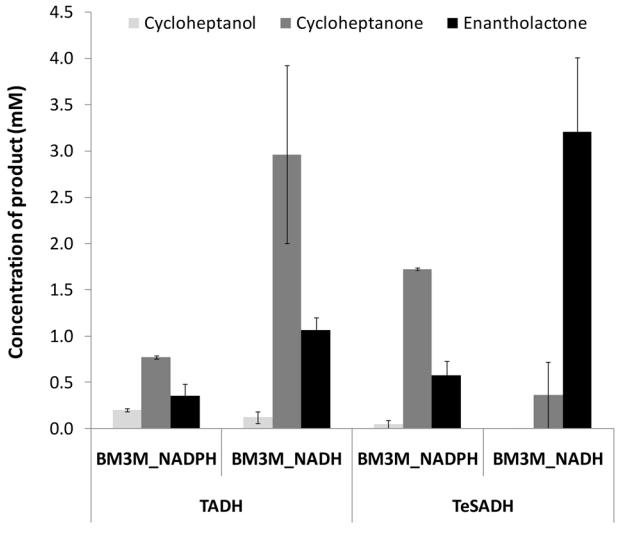

Figure 1. One-pot conversion of cycloheptane to enantholactone by four different combinations of biocatalysts. Reaction conditions: 1 mL reaction volumes with 20 μL substrate added neat, containing 1.33 μM BM3 M_NADPH and 1.77 μM BM3 M_NADH (CFE of 0.02 gWCW ml^{-1}), CFE of 0.04 gWCW ml^{-1} TeSADH or TADH, CFE of 0.02 gWCW ml^{-1} CHMO_M16, 100 mM glucose, glycerol, 200 mM Tris-HCl (pH 8), 30 °C, 200 rpm, 20 h.

unproductive catalytic cycles.[24] This wasting will result in the requirement for additional co-factor regeneration by the native-metabolism of *E. coli*. Nevertheless, a turnover frequency (TOF, calculated over the first 2 h) of 11.2 min^{-1} was achieved for the CYP450 with respect to total oxygenated products formed with a total turnover number (TTN) of 2011 after 20 h.

In an attempt to construct a redox-balanced cascade, we included an enzyme for co-factor regeneration. Glucose dehydrogenase is an often employed choice as it efficiently regenerates both NADH and NADPH.[25] This however led to much higher cycloheptanol concentrations, but without the concomitant production of cycloheptanone and enantholactone. Both the alcohol dehydrogenase and glucose dehydrogenase requires oxidized cofactor [NAD(P)$^+$] for activity, resulting in the glucose dehydrogenase competing with the alcohol dehydrogenases for oxidized co-factor. We therefore decided to uncouple the co-factor dependence of the different steps of the cascade by changing the co-factor regenerating enzyme to formate dehydrogenase (FDH).[26] Unlike glucose dehydrogenase that can accept both NAD^+ and $NADP^+$,[27] FDH can only accept NAD^+. This resulted in much higher levels of product formation, with combination IV again giving the highest level of enantholactone (10.6 mM, 1.4 g L^{-1}, Figure S3, Supporting Information) with the TTN reaching 6915 and a TOF of 33.7 min^{-1} calculated over the first 2 h. Similar to when native *E. coli* enzymes were regenerating the required co-factor for the initial CYP450 step, the first step is uncoupled from the second and third steps by means of co-factor dependence: step 1 is self-sufficient in that FDH recycles the required NADH, and steps 2 and 3 are self-sufficient in the oxidation and reduction of NADPH. Similar uncoupling of cofactor dependency between different biocatalytic steps have previously been shown for the successful two-step amination[28] or deracemization[29] of *sec*-alcohols.

To determine if the endogenous levels of co-factor is limiting the cascade, we included additional NAD^+ and $NADP^+$ (0.1 mM each) in the bioreaction mixture. This resulted in an approximately 20% increase in enantholactone production for combination IV (Figure S4, Supporting Information) with the TOF now reaching 49.7 min^{-1} (TTN of 6023). Also less than 1% of the total products formed was of the alcohol and ketone intermediates.

Finally, in an effort to improve the overall performance of the cascade we increased the biocatalyst concentrations by using highly concentrated cell suspensions for the preparation of the cell-free extracts. Time-course analysis revealed maximum lactone formation after approximately 8–12 h (Figure 2). This is followed by a decrease in the intermediate alcohol levels, and an increase in the corresponding ketones (Figure S5, Supporting Information) implicating both the BVMO and CYP450 as unstable under the prolonged reaction times. Nonetheless, yields of more than 23 mM (~3 g L^{-1}; Table 1) enantholactone were achieved. Likewise more than 0.5 g L^{-1} of caprolactone and caprylolactone (2-oxanone) was produced from cyclohexane and cyclooctane respectively.

In summary, we have developed a redox-balanced one-pot synthetic cascade employing four oxidoreductases for the syn-

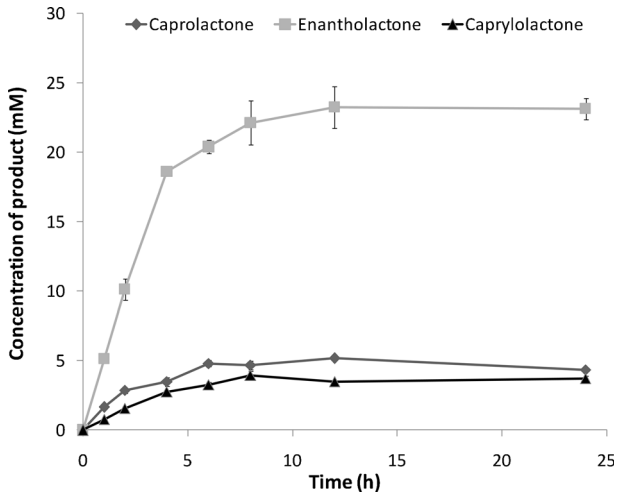

Figure 2. Time-resolved one-pot conversion of C6-C8 cycloalkanes to their corresponding lactones by a multi-step biocatalytic cascade. Reaction conditions: 1 mL reaction volumes with 20 μL substrate added neat, containing 6.3 μM P450 (CFE of 0.075 gWCW ml^{-1}), CFE of 0.15 gWCW ml^{-1} TeSADH, CFE of 0.075 gWCW ml^{-1} CHMO_M16, CFE of 0.075 gWCW ml^{-1} CboFDH, 100 mM glucose, glycerol and sodium formate, 0.1 mM $NAD(P)^+$, 200 mM Tris-HCl (pH 8), 30 °C, 200 rpm.

Table 1. One-pot conversion of cycloalkanes to the corresponding lactones using P450 BM3 M_NADH, TeSADH and CHMO_M16.

Substrate[a]	TTN[b]	TOF[c] [min^{-1}]	Oxygenated Product Concentrations Alcohol [mM]	Ketone [mM]	Lactone [mM]	Lactone [g L^{-1}]
cyclohexane	822	4.37	0	0	5.18	0.59
cycloheptane	4185	15.26	0.48	2.63	23.25	2.95
cyclooctane	1017	5.59	0.27	2.40	3.74	0.53

[a] Reaction conditions: 1 mL reaction volumes with 20 μL substrate added neat, containing 6.3 μM P450 (CFE of 0.075 gWCW), CFE of 0.15 gWCW TeSADH, CFE of 0.075 gWCW CHMO_M16, CFE of 0.075 gWCW CboFDH, 100 mM glucose, glycerol and sodium formate, 0.1 mM $NAD(P)^+$, 200 mM Tris-HCl (pH 8), 30 °C, 200 rpm, 24 h. [b] TTN: Total turnover number (maximum μmol total oxygenated products formed within 24 h μmol^{-1} P450). [c] TOF: (maximum μmol total oxygenated product/μmol P450) per min after 1 h.

thesis of lactones from C6 to C8 cycloalkanes. Moreover we have addressed the importance of uncoupling co-factor requirements between multiple oxidation reduction steps to ensure that no competition for co-factors occurs. Further process development is in progress. This involves optimization of biocatalyst concentrations and ratios, as well as in situ product removal to overcome product inhibition. The creation of more stable biocatalysts will also allow for longer reaction times thereby increasing final lactone titres.

Experimental Section

All substrates were obtained from Sigma–Aldrich and used without any purification.

The cytochrome P450 monooxygenase mutants and cyclohexanone monooxygenase were expressed from the pET28b(+) and pET22b(+) vectors (Novagen) respectively. The alcohol, glucose and formate dehydrogenases were expressed from the pETDuet-1 vector (Novagen). Co-expression of the three biocatalysts in *E. coli* was achieved by expressing the alcohol dehydrogenase and Baeyer–Villiger monooxygenase from pETDuet-1 and the cytochrome P450 from the pCDFDuet-1 vector (Novagen).

Expression constructs were transformed into *E. coli* BL21-Gold (DE3) competent cells (Stratagene) and plated on LB plates containing an appropriate antibiotic: ampicillin (100 mg mL^{-1}; pET22 and pETDuet-1), kanamycin (30 mg mL^{-1}; pET28), or streptomycin (50 mg mL^{-1}; pCDFDuet-1). Expression was performed by using ZYP-5052 auto-induction medium.[30] Cells were cultured for 36 h at 25 °C, after which they were harvested through centrifugation (8000×g, 10 min) and 1 g cells (wet weight) resuspended in 5 mL (or 2 mL for a high-concentration cell suspension) of 200 mM Tris-HCl (pH 8) containing EDTA-free protease inhibitor cocktail (Roche) and DNAse. Cells were disrupted by a single passage through a cell disrupter (Constant Systems) at 30 kPSI. The cell-free extracts (lysates) containing the biocatalysts were obtained as the supernatant following centrifugation (8 000×g, 10 min) of the broken cells.

Biotransformations were performed in 40 mL capped amber glass vials. The biotransformation reaction mixture (BRM, 1 mL) was prepared by mixing equal volumes (0.5 mL) of the mixed lysates (1:2:1:1 volumetric ratios of CYP450:ADH:BVMO:DH) and the biotransformation buffer (200 mM Tris-HCl buffer pH 8). When highly concentrated cell suspensions were used, 0.75 mL of the mixed lysates were added to 0.25 mL biotransformation buffer. Glucose and glycerol (and sodium formate when formate dehydrogenase was used for co-factor regeneration) were included at 100 mM each. The reactions were started by the addition of 20 μL substrate and shaken (200 rpm) at 30 °C. The content of the each vial was extracted using an equal volume of ethyl acetate containing 2 mM 2-decanol as internal standard. GC-MS analysis was carried out on a Finnigan Trace GC ultra (ThermoScientific) equipped with a FactorFour VF-5 ms column (60 m×0.32 mm×0.25 μm, Varian).

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Keywords: biocatalysis · cascade reactions · cytochrome P450 monooxygenase · lactone · one-pot synthesis

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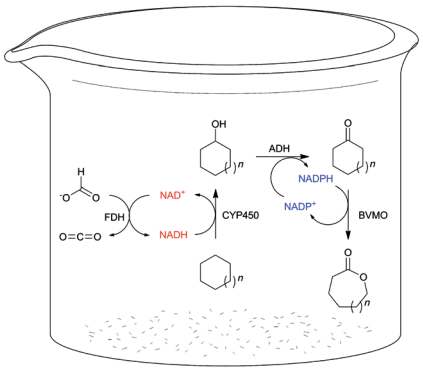
One-pot recipe: One-pot conversion of cycloalkanes to their corresponding lactones through the use of a synthetic pathway consisting of a cytochrome P450 for initial oxyfunctionalization of the alkane, an alcohol dehydrogenase for ketone production and a Baeyer–Villiger monooxygenase for lactone formation.



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