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An Enzymatic Toolbox for Cascade Reactions: A Showcase for an In Vivo Redox Sequence in Asymmetric Synthesis

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Single-step transformations of non-natural substrates by enzymes have been successfully established in the last decades as highly valuable techniques for the synthesis of chiral building blocks.[1] Owing to their properties, biocatalysts can be employed as catalytic tools for the facile transformation of functional groups in organic synthesis. By exploiting the manifoldness of enzymes and their different catalytic activities, it is possible to design new artificial biosynthetic pathways on the basis of the "retrosynthetic approach"[2] that is commonly applied in chemical synthesis and that was only very recently proposed as a novel concept for biocatalysis.[3] This design principle is used in the strategic planning of organic syntheses by transforming a target molecule into simpler precursors in which molecular complexity is reduced by manipulation of functional groups.

The concept of multistep one-pot reactions has caught the attention of synthetic chemists in recent years.[4] To address the increasing demands by society to further improve the sustainability of chemical processes, one-pot cascades of reaction sequences can substantially decrease the amount of chemicals used for each reaction and subsequent down-stream processing by concomitantly optimizing the energy requirements and operation expenses. As pointed out in a recent review,[4a] such cascades not only improve processes by saving time and reducing waste, but they also offer advantages if unstable or toxic intermediates are involved, as these do not accumulate. Nature uses the design principle in a highly successfully manner, as all metabolic pathways are interconnected and conducted within the "single-vessel" environment of a cell. The concerted interaction of numerous enzymes within the cell allows exceptionally high yields in multistep biosynthetic pathways.[4a,5] However, this approach is largely limited to already-existing metabolic reaction sequences (e.g., 1,3-propane diol[6] and 1,4-butane diol[7]) and does not necessarily deliver the high structural diversity of compounds utilized by the chemical industry.

The major difference between biocatalysis and classical fermentation is the extension of the enzymatic transformations to non-natural substrates. Consequently, the extension of single-step biotransformations, which are particularly powerful in asymmetric synthesis, is a logical development.[4a,8] In this context, we recently reported the successful conversion of unsaturated fatty acids into medium-chain alcohols, ω-hydroxy acids, and dicarboxylic acids through the combination of appropriate enzymes in a cascade reaction by employing a combined in vivo/in vitro strategy towards nonchiral products.[9]

Very recently, Schrewe et al.[10] presented an interesting three-step synthetic approach including two enzymes for the production of terminal alkylamino functionalization in a recombinant *E. coli* strain. Notably, however, only a single substrate was investigated, but even so, the versatility of the presented enzyme was highlighted. Another approach was published very recently by the group of Li[11] who applied a two-strain-mixed-culture strategy for the synthesis of δ-lactones. Both studies showed the potential of redox cascades in living organisms and at the same time pointed out their limitations. A simple transfer from a single-step biotransformation (including broad substrate acceptance and high selectivity) to enzyme cascades still remains a challenge. The prime novelty and major aim of the present study was to combine the efficiency of biosynthetic redox pathways, the modularity of synthesis by simple functional group transformations, and the substrate promiscuity of enzymes. Designing and evaluating the feasibility of a multienzyme-catalyzed cascade process in living microbial cells enabled the creation of an artificial "mini"-metabolic pathway connected to primary metabolism through redox-cofactor regeneration. This approach was applied in an in vivo environment and concomitantly provided access to diverse chemical entities through divergent reaction pathways.

Figure 1 illustrates the straightforward concept of this work. In lieu of looking at different specific target compounds, for which one synthetic step is performed biocatalytically, we reduced the complexity of the molecule to the functional groups of the compound regardless of the residual structure. From a synthetic point of view, oxidation of a simple allylic alcohol starting material to the corresponding α,β-unsaturated ketone

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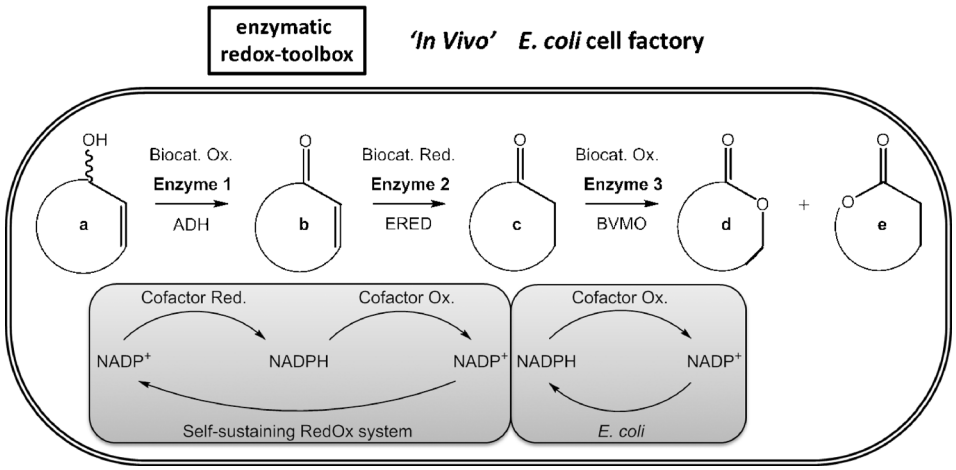


Figure 1. Basic concept of the developed biocatalytic redox toolbox.

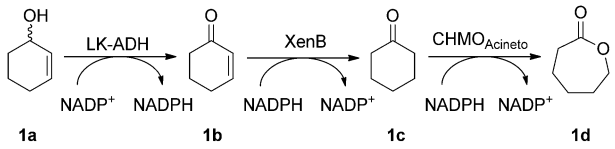
needed to be achieved. In the second step, (stereo)selective reduction of the double bond in the presence of a ketone functionality was required. Finally, the obtained saturated ketone needed to be further oxidized to the corresponding target molecule, a chiral lactone.

Each transformation is quite well known in classical synthetic chemistry, but the combination of these transformations in a one-pot fashion is rather difficult to achieve. A simple reason for that is the possible need for different solvents, reaction temperatures, reaction atmosphere, and, predominantly, suitable catalysts with desired substrate scope, specific activity, and chemo-, regio-, and stereoselectivity. Judicious choice of the enzymes also offers a major advantage over chemical processes, which often lack the required chemoselectivity if more than one functional group is present in the target molecule. This can only be circumvented by either the introduction of protecting groups or the design of "domino reactions". The latter type of reaction has a major disadvantage in that the subsequent reaction can only be started once the preceding transformation is complete to avoid byproduct formation.

We developed a three-step artificial metabolic "mini"-pathway comprising three types of biocatalysts with broad substrate scopes that are naturally not connected in a single organism in a metabolic context:[12] An alcohol dehydrogenase (ADH) for the oxidation of an allylic secondary cyclic alcohol to the corresponding α,β-unsaturated ketone, an enoate reductase (ERED) for the subsequent reduction of the double bond, and a Baeyer–Villiger monooxygenase (BVMO) for the formation of the corresponding lactone (as exemplified for substrate **1a** in Scheme 1). This particular sequence was realized as a proof-of-concept study to demonstrate the principal feasibility of the construction of enzymatic cell factories for the synthesis of fine chemicals and to determine and resolve limiting aspects.

Starting with the simple racemic substrate 2-cyclohexenol (**1a**), the first aim of this study was dedicated to the identification of suitable enzymes for all functional group transformations that are conducted in this reaction sequence. In an initial study (see the Supporting Information), two suitable and preferentially nonselective ADHs were investigated, that is, LK-ADH from *Lactobacillus kefir*[13] and RR-ADH from *Rhodococcus ruber*. According to the literature and our preliminary results, LK-ADH was the most suitable for the first reaction step. Alcohol **1a** was fully oxidized to corresponding cyclohexenone **1b** with concomitant consumption of nicotinamide adenine dinucleotide phosphate ($NADP^+$). Subsequent reduction of the α,β-unsaturated ketone was performed by an ERED from *Pseudomonas* sp., that is, XenB.[14] The artificial "mini"-pathway was completed by the well-known cyclohexanone monooxygenase ($CHMO_{Acineto}$) from *Acinetobacter* sp.[15]

Having all catalysts identified, a first one-pot in vivo experiment—by expressing all enzymes at once in *E. coli*—gave promising results, and reasonable amounts of final lactone **1d** (Figure 2, Table 1) were formed. The possible rate-limiting step of the cascade, the ADH-catalyzed oxidation to unsaturated ketone **1b**, was successfully shifted by subsequent reduction of the C=C bond by the ERED to yield **1c**. This further under-



Scheme 1. Artificial metabolic pathway composed of an alcohol dehydrogenase (LK-ADH), an enoate reductase (XenB), and a Baeyer–Villiger-monooxygenase ($CHMO_{Acineto}$) starting from 2-cyclohexenol. All enzymes were expressed recombinantly in *E. coli*. See the Supporting Information for details.

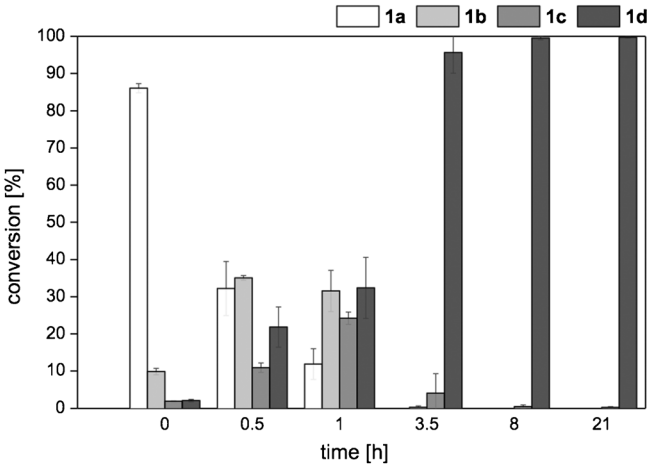
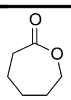
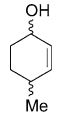
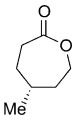
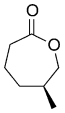
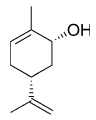
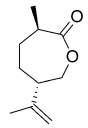
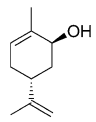
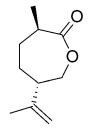
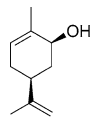
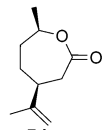


Figure 2. In vivo cascade starting from 2-cyclohexenol (**1a**) to yield lactone **1d**.

Table 1. Results of the enzymatic cascade reactions.

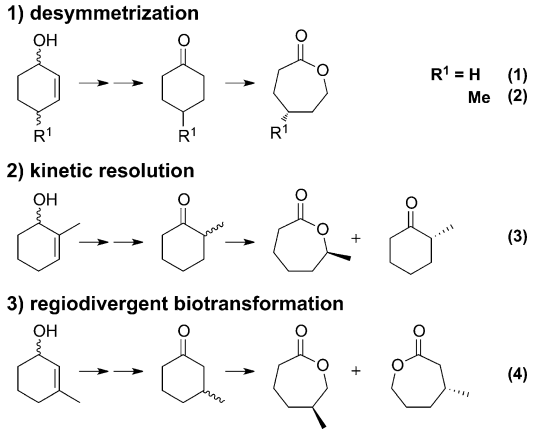
Substrate[a]	Product	Conv. [%]	*ee*/*de*[b] [%]	*t* [h]
H  **1a**	 **1d**	>99	n.a.[c]	21
4-Me  **2a**	 **2d**	>99	>99	20
2-Me  **3a**	 **3d**	64	>99	20
3-Me  **4a**	 **4d**	n.a.[c,d]	n.a.[c]	n.a.[c]
	 **4e**	86	>99	20
carveol  **5a** (1*R*,5*R*)	 **5d**[e]	63	>99	20
carveol  **6a** (1*S*,5*R*)	 **6d**[e]	93	>99	20
carveol  **7a** (1*S*,5*S*)	 **7d**	>99	>99	20

[a] In all cases the substrate concentration was 4 mM, with the exception of substrate **5a** (2.5 mM). [b] *de* = diastereomeric excess. [c] n.a. = not applicable. [d] No formation of proximal lactone **4d** was observed. [e] **5d** = **6d**, owing to the stereopreference of XenB.

scored the advantages enabled in a cascade reaction setting. Moreover, as the involved enzymes are not evolutionary interconnected, there was no regulation process and the synthetic pathway towards the final product was not restricted. This is a particularly important feature for exploitation in chemical synthesis.

LK-ADH converts $NADP^+$ into NADPH, which serves as a cofactor for XenB and BVMO. Consequently, the first and the second steps of the cascade are self-sustaining with respect to cofactor recycling, but the final oxygenation step requires an additional equivalent of NADPH (Scheme 1). Implementation of the multistep cascade in the viable microorganism *E. coli* provides all redox cofactors and thus can close the gap in the redox cycle, and this leads to improved performance of this multistep biotransformation and reduces the metabolic burden of the cell significantly.

Given that the first aim, a proof of concept, was pervaded successfully, we next addressed the questions of flexibility and modularity of our approach and increased the complexity of the process by also considering stereoselective transformations. Selection of various substrates enabled us to address all important reaction types: an achiral transformation, desymmetrization, and kinetic resolution (Scheme 2). For this, we synthesized a set of cyclohexenol derivatives for sequential biocatalytic reactions with stepwise-increased complexity of these transformations (Table 1).



Scheme 2. Substrate scope of the proposed enzyme cascade including all important biocatalytic reactions. Classifications of transformations are based on the BVMO-mediated step. All substrates and intermediates were synthesized according to literature or adapted protocols (see the Supporting Information).

Introduction of chirality by desymmetrization of prochiral substrates is of common interest in chiral synthesis, as a 100% theoretical yield can be achieved directly with high optical purity. (*cis/trans*)-4-Methylcyclohexenol (**2a**) was chosen as a model substrate for this type of transformation and oxidation by LK-ADH gave racemic **2b** in very good yield (see the Supporting Information). Subsequent reduction of the double bond by the ERED XenB to prochiral 4-methylcyclohexanone (**2c**) and final desymmetrization by the BVMO led to lactone **2d** in excellent GC yield (100%) and perfect optical purity [>99% enantiomeric excess (*ee*)].

On the basis of these promising results, attention was shifted to 2-substituted cyclohexenol derivatives by employing kinetic resolution in the BVMO-mediated step (Table 1). Oxidation of **3a** by LK-ADH gave **3b**, and reduction of **3b** with the ERED delivered optically pure **3c**, which was then converted stereoselectively into chiral lactone **3d** catalyzed by $CHMO_{Acineto}$ (64% GC yield, >99% *ee*). By applying this cascade

sequence we were able to circumvent classical kinetic resolution, which resulted in very low E values for several tested BVMOs (data not shown), to obtain enantiomerically pure lactone **3d**. Again, we demonstrated the particular advantages of this atom-efficient multistep redox cascade, which yielded 64% (GC) of the desired product after three steps.

Regiodivergent biotransformations are a particularly valuable feature of BVMOs, as this conversion enables access to regioisomeric products from antipodal precursors. Substrate **4a** was easily oxidized by LK-ADH to 3-methylcyclohexenone (**4b**), but XenB did not accept **4b**, and this stopped the cascade at this point. To solve this issue, we investigated an alternative ERED from *Saccharomyces carlsbergensis*—also named old yellow enzyme (OYE1)[16]—previously reported to accept **4b**. Indeed, optically pure (*S*)-3-methylcyclohexanone (**4c**) was obtained by applying OYE1, and this cyclohexanone served as a substrate for the final Baeyer–Villiger oxidation with the use of $CHMO_{Acineto}$ to ultimately obtain distal lactone **4e** (86% GC yield, >99% *ee*).[17]

Having now established a toolbox of recombinantly expressed enzymes in one cell in proper combination (two ADHs, two EREDs, and one BVMO; see the Supporting Information), we approached the cascade conversion of three different isomers of carveol (i.e., **5a**–**7a**) representing the most complex substrates for this study. Applying our designed pathway offers the advantage that different diastereomers of carveol can be directly converted into the desired product in a stereoselective manner by choosing the appropriate enzymes. Furthermore, the availability of BVMOs with opposite regioselectivity[18] can be exploited to afford different regioisomeric lactones. Notably, both ADHs showed excellent stereopreference with respect to the alcohol group and were completely unselective towards the stereogenic center in the 5-position. RR-ADH converted exclusively (1*R*,5*R*)-carveol (**5a**), whereas LK-ADH accepted only the (1*S*,5*R*) and (1*S*,5*S*) stereoisomers, that is, **6a** and **7a**, respectively. Experiments with resting *E. coli* cells containing the appropriate enzymes revealed the formation of the desired product after 20 h for all three carveol isomers, as exemplified in Figure 3 for substrate **7a**.

The expected intermediates from the cascade reaction were detected only in low amounts as a result of fast biotransformation. A closer look at each individual reaction step revealed that an *E. coli* background transformation competes with the enoate reductase step in our cascade. A literature survey identified *N*-ethylmaleimide reductase (NemR) as a disturbing reductase.[19] After a first glance, we found that both EREDs showed the same stereopreference for both carvone substrates and that NemR did not accept the other α,β-unsaturated substrates of the cascades (i.e., **1**–**4b**). Nevertheless further extension of the cascade, including the accessibility of different isomers, would require a Δ*nemA E. coli* strain. First, in vivo studies with BL21(DE3)Δ*nemA* showed the complete absence of the background reaction (Table S2, Supporting Information).

Ultimately, to demonstrate the applicability of the presented concept for the synthesis of high-value compounds for the chemical industry, we performed two preparative-scale experiments. Thus, freshly prepared resting cells supplemented with

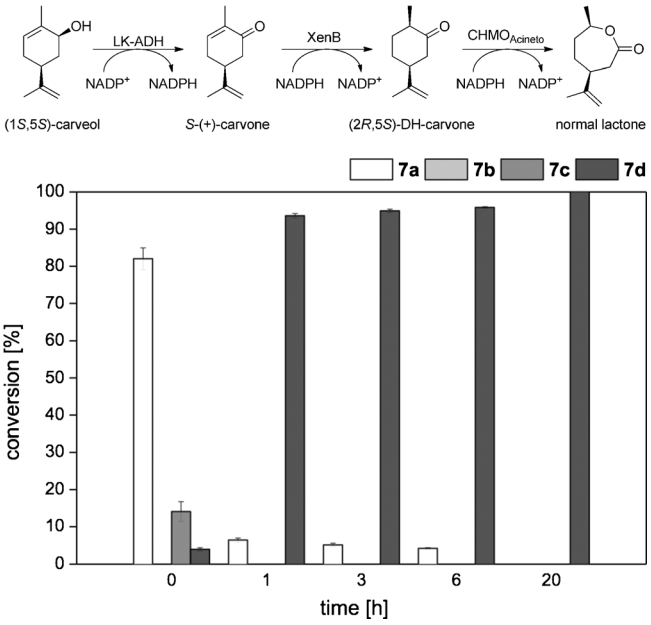


Figure 3. In vivo cascade for the conversion of (1*S*,5*S*)-carveol (**7a**) into corresponding lactone **7d**.

100 mg of either 3-methylcyclohexanone (**4a**) or carveol (**7a**) were used to produce desired lactones **4e** and **7d** (Table 2) at a conversion of 94 and 93%, respectively. Classical extractive workup was performed followed by column chromatography to yield **4e** and **7d** in 55 and 60% (isolated material) yield, respectively. In summary, we conducted a three-step enzymatic redox cascade and achieved an average of 82% (for **4a**→**4e**) and 84% (for **7a**→**7d**) yield in each individual step.

Table 2. Preparative-scale cascade biotransformations.

Substrate (4 mM)		Product	Yield[a] [%]	*ee*/*de*[b] [%]	*t* [h]
3-Me	**4a**	**4d**[c]	n.a.[d]	n.a.[d]	n.a.[d]
		4e	55	>99	24
carveol	**7a** (1*S*,5*S*)	**7d**	60	>99	18

[a] Yield of isolated product after column chromatography. [b] *de* = diastereomeric excess. [c] No formation of proximal lactone **4d** was observed. [d] n.a. = not applicable.

By this proof-of-concept study, we were able to design and create a modular enzymatic multistep redox toolbox in the microbial host *E. coli*. The design principle of this artificial "mini"-metabolic pathway was predicated on the basis of the retrosynthetic approach derived from strategic planning of organic syntheses. By increasing the complexity, we managed to cover the full range of important reaction types known in chiral synthesis. Hence, by selection of proper enzymes—not present in a single natural microorganism—we enabled the efficient three-step synthesis of various chiral building blocks in a one-pot fashion simply by using resting *E. coli* cells. We met the challenge of combining relatively unselective enzymes in the

first reaction step, the oxidation of a secondary alcohol to an unsaturated ketone, by reducing the complexity and removing one chiral center. Moreover, in the subsequent reduction step we exploited the promiscuity of the applied EREDs (i.e., XenB and OYE1), and finally we took advantage of the oxygenation capabilities of the BVMO $CHMO_{Acineto}$. The different types of biotransformations achievable were demonstrated with model substrates (cyclohexenol derivatives) as case studies; taken together with the already demonstrated broad substrate profiles of the enzymes involved, this demonstrates the versatility of this concept, in general.

Apart from the clear advantages of a cascade reaction setup, the "mini"-pathway offers an advantage over stepwise transformations in that the oxidation step can be significantly improved by shifting the equilibrium. Future work will focus on investigating structurally more diverse substrates, optimizing fermentation conditions, changing the expression host to the Δ*nemA* strain, and improving and tightening the control of protein expression and the interplay of the artificial "mini"-metabolic pathway with the central carbon metabolism of the *E. coli* host. We envisage to create a "designer cell" that is capable of simply exchanging particular parts of the enzymatic toolbox derived from retrosynthetic analysis to expand the applicability of biocatalysis in the synthesis of complex chiral compounds in an atom-efficient and environmentally benign manner.

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Keywords: artificial pathway · biocatalysis · domino reactions · redox chemistry · retrosynthesis

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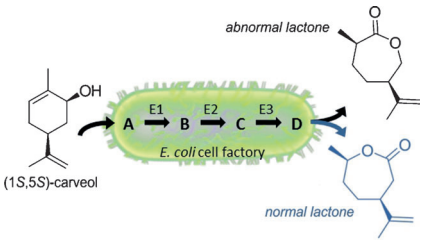
An Enzymatic Toolbox for Cascade Reactions: A Showcase for an In Vivo Redox Sequence in Asymmetric Synthesis



Joined forces: Alcohol dehydrogenase, enoate reductase, and Baeyer–Villiger monooxygenase are combined in a cascade reaction by coexpression in *E. coli* to have a recombinant whole-cell biocatalyst. Such an artificial metabolic "mini"-pathway provides access to functionalized chiral compounds in high yields and optical purities as exemplified for kinetic resolutions, desymmetrizations, and regiodivergent biotransformations.