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Orchestration of Concurrent Oxidation and Reduction Cycles for Stereoinversion and Deracemisation of *sec*-Alcohols

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Abstract: Black and white are opposites as are oxidation and reduction. Performing an oxidation, for example, of a *sec*-alcohol and a reduction of the corresponding ketone in the same vessel without separation of the reagents seems to be an impossible task. Here we show that oxidative cofactor recycling of $NADP^+$ and reductive regeneration of NADH can be performed simultaneously in the same compartment without significant interference. Regeneration cycles can be run in opposing directions beside each other enabling one-pot transformation of racemic alcohols to one enantiomer via concurrent enantioselective oxidation and asymmetric reduction employing defined alcohol dehydrogenases with opposite stereo- and cofactor-preference. Thus, by careful selection of appropriate enzymes, NADH recycling can be performed in the presence of $NADP^+$ recycling to achieve overall, for example, deracemisation of *sec*-alcohols or stereoinversion representing a possible concept for a "green" equivalent to the chemical-intensive Mitsunobu inversion.

Catalytic (asymmetric) tandem reactions[1] are processes in which multiple catalysts operate concurrently in one pot, circumventing the often time-consuming and yield-reducing isolation and purification of intermediates in multistep synthesis.[2] Unfortunately, it is still a challenge to run multiple reactions simultaneously in one pot in organic chemistry due to the diverging reaction conditions required for each transformation. This is especially true when oxidations and reductions should be performed concurrently.[3] Chemical reaction sequences involving oxidation and reduction for the deracemisation of secondary alcohols, whereby a racemic alcohol is converted to one single alcohol enantiomer in >99% yield and ee (enantiomeric excess), have only been achieved in a sequential fashion;[4] deracemisation of racemic secondary alcohols to yield optical pure products in 100% yield are highly demanded as indicated by the huge efforts devoted to the dynamic kinetic resolution of this class of substrate.[5] A biocatalytic deracemisation[6−8] protocol involving concurrent redox reactions has recently been reported involving the combination of whole cells (*Alcaligenes faecalis* DSM 13975) as nondefined "black box" for the enantioselective oxidation of the (*R*)-enantiomer to the corresponding ketone followed by (*S*)-stereoselective reduction by a recombinant alcohol dehydrogenase (ADH) to yield optically pure (*S*)-alcohol.[9] The enantioselective oxidation required molecular oxygen as the sole reagent. However, because the enzymes for the oxidation were not known, the method was

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not flexible and was limited to *A. faecalis*. To develop a tunable and general concept to obtain optically pure alcohols starting from their racemates, we performed experiments to understand the *A. faecalis*−ADH system. It was shown that the enzymes present in *A. faecalis* involved in the oxidation of the (*R*)-alcohol occurred due to the presence of NADH oxidase(s)[10,11] at the expense of molecular oxygen and NADH-dependent (*R*)-ADH(s). Consequently, we combined a cell-free extract of *A. faecalis* oxidizing the (*R*)-alcohol with a NAD**P**H-dependent (*S*)-selective alcohol dehydrogenase (ADH from *Thermoanaerobium brockii*) for reduction coupled with a NADPH cofactor-recycling system (NADPH-formate dehydrogenase, formate). To our delight, substrate *rac*-**1a** was transformed to (*S*)-**1a**, showing 48% ee with only traces of ketone (<1%) detectable within 16 h. The successful experiment suggested that two cofactor recycling systems, one for NAD^+ and one for NADPH, can be run in parallel in solution in opposite directions; thus, one system is recycling the oxidized nicotinamide cofactor NAD^+ and one system is recycling the reduced cofactor NADPH. Obviously, the enzymes need to possess a sufficient high cofactor preference; thus, each of the required nicotinamide-dependent enzymes has to accept mainly one of the two nicotinamide cofactor derivatives. Low cofactor preference would lead to unproductive cycles. Our experimental results also suggested that *A. faecalis* extracts are devoid of NAD**P**H oxidase(s).

Having set up a system deduced from nature for a simple mechanistic oxidation−reduction sequence for deracemisation involving defined enzymes, we searched for known enzymes in the literature to construct an artificial deracemisation system. The first step, the oxidation of alcohols at the expense of molecular oxygen,[12] requires a highly efficient and soluble NAD(P)H oxidase that can easily be produced in large quantities.

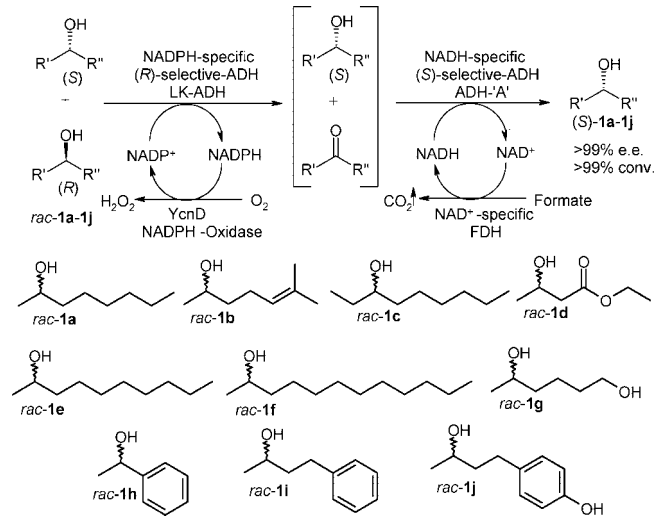

Figure 1. Artificially designed reaction pathway combining simultaneous concurrent tandem oxidation and reduction cycles with opposite cofactor- and stereopreference in one pot to access enantiopure (*S*)-alcohols from the racemates. LK-ADH: ADH from *Lactobacillus kefir*; ADH-'A': ADH from *Rhodococcus ruber* DSM 44541.

For this purpose, we chose the FMN-dependent YcnD from *Bacillus subtilis* which prefers NADPH, although it has never been used in bio-organic chemistry. The recombinant protein can be produced in large amounts.[13]

Because YcnD from *Bacillus subtilis* has a NADPH-cofactor preference, we had to employ a NADPH-dependent (*R*)-enantioselective ADH (*Lactobacillus kefir* LK-ADH) in the oxidation step, while a NADH-dependent (*S*)-ADH (ADH-'A' from *Rhodococcus ruber* DSM 44541) and a NAD-specific formate dehydrogenase (FDH) for NADH recycling were used for the reduction step (Figure 1). All enzymes employed are commercially available, except YcnD.

We were gratified to find that all racemic alcohols *rac*-**1a**−**1j** investigated were transformed to enantiopure (*S*)-alcohol (ee >99%) with no detectable trace of ketone (Table 1, Figure 1). Additionally it proved that recycling of oxidized $NADP^+$ is feasible in presence of a NADH recycling system. Furthermore, it clearly demonstrated that opposing redox cycles can be performed without any compartmentalization. Hydrogen peroxide, which is a coproduct of the NADPH oxidase did not inhibit the other enzymes, neither the two ADHs nor the FDH. However, since biological systems are intrinsically sensitive to H_2O_2, addition of catalase may be advantageous. In our system, the presence of catalase as a fifth enzyme did neither have a positive nor a negative effect.

Following the basic concept of this (deracemization) system, the enzymes transforming the substrate (ADHs) can be exchanged for instance by related enzymes showing opposite

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Table 1. Simultaneous Tandem Oxidation and Reduction Cycles for the Deracemisation of *sec*-Alcohols According to Figures 1 and 2[a]

		(*S*)-system (Figure *1*)			(*R*)-system (Figure *2*)		
entry	substrate[b]	time [h]	alcohol [%][c]	ee [%][d]	time [h]	alcohol [%][c]	ee [%][d]
1	*rac*-**1a**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
2	*rac*-**1b**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
3	*rac*-**1c**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
4	*rac*-**1d**	6	>99	>99 (*S*)	24	>99	>99 (*R*)
5	*rac*-**1e**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
6	*rac*-**1f**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
7	*rac*-**1g**	6	>99	>99 (*S*)	24	>99	>99 (*R*)
8	*rac*-**1h**	3	>99	>99 (*S*)	6	>99	>99 (*R*)
9	*rac*-**1i**	3	>99	>99 (*S*)	24	>99	>99 (*R*)
10	*rac*-**1j**	3	>99	>99 (*S*)	24	>99	>99 (*R*)

[a] Reaction conditions: Two ADHs with complementary cofactor preference and stereoselectivity were employed (see Figures 1 and 2). The reaction was performed in TRIS Buffer (0.5 mL, pH 7.5, 50 mM), *rac*-substrate, and catalytic amounts (0.5 mM) of cofactors (NADPH and NADH) were added. $NADP^+$, required in the oxidation reaction, was recycled by YcnD oxidoreductase; NADH, required in the reduction reaction, was provided by a NADH-dependent FDH. The reaction mixture was shaken in Eppendorf tubes (2.5 mL) at 30 °C and 350 rpm on an Eppendorf Thermoshaker. [b] Five microliter substrate (3−5 mg, 38−60 mM). [c] GC-yield. [d] Measured by GC employing a chiral stationary phase.

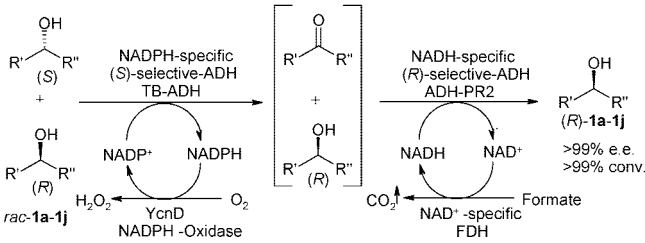

Figure 2. (*R*)-Alcohols obtained from the racemates via inversion of the (*S*)-configured alcohol by a concurrent oxidation and reduction in one pot. TB-ADH: ADH from *Thermoanaerobium brockii*; ADH-PR-2: commercial ADH.

stereopreference but the same cofactor preference, which would allow accessing the mirror-image (*R*)-enantiomer in optical pure form (Figure 2).

This was achieved by the combination of the commercial NADPH-dependent (*S*)-ADH (*Thermoanaerobium brockii* TB-ADH) with an NADH-dependent (*R*)-ADH (ADH-PR-2) using again YcnD and NADH-specific FDH for cofactor recycling (Figure 2). After tuning the amount of enzymes in this second "artificial" pathway, the racemic substrates *rac*-**1a**−**1j** were deracemised to yield enantiopure (*R*)-alcohols (ee >99%, Table 1, System Figure 2). In all cases, the asymmetric reduction step ensured that no trace of ketone as possible side product was detectable (Table 1, System Figure 2). Employing the same reaction conditions, but increasing the substrate concentration to 20 g L^{-1}, we could show that complete deracemisation was still achieved at increased reaction times.

Next, we extended our approach to stereoinversion of an enantioenriched *sec*-alcohol which is in organic chemistry performed by the Mitsunobu inversion which requires toxic and expensive reagents. The time course of the stereoinversion of enantiopure (*R*)-alcohol **1a** by monitoring the formation of (*S*)-alcohols was followed employing the system described in Figure 1 (for time course, see Supporting Information). Alcohol (*R*)-**1a** was completely inverted to yield (*S*)-**1a** (ee >99%) within three hours. Therefore, the stereoinversion presented here resembles a concept for a "green" alternative to the Mitsunobu reaction,[14] which shows a low atom economy.[15]

Finally, deracemisation of *rac*-1-phenylethanol (*rac*-**1h**, 50 mg) on a preparative scale employing the system of Figure 1 confirmed the stereoinversion of one enantiomer into the other. After 16 h of shaking at 30 °C, we obtained enantiopure (*S*)-1-phenylethanol in 90% isolated yield (45 mg, >99% ee).

In summary, we have shown that NAD(P)H-dependent oxidation and reduction reaction cycles can be run simultaneously in one pot in parallel without compartmental separation. Although in Nature cells engage extensive cellular compartmentalization to avoid metabolic disorders, both nicotinamide derivatives are present in the same compartment and their utilization is determined by the cofactor specificity of the enzymes employed. The biomimetic reaction system described here needs enzymes with sufficient high nicotinamide cofactor specificity. The oxidized cofactor e.g. $NADP^+$ can be regenerated by an NADPH-oxidase employing molecular oxygen O_2 in the presence of a cofactor specific regeneration system for reduced NADH (e.g., by NADH-formate dehydrogenase). The generality of the system was exemplified for the deracemisation of *rac-sec*-alcohols (**1a**−**1j**) via stereoinversion yielding the optically pure (*R*)- as well as (*S*)-enantiomer by choosing the matching pairs of dehydrogenases. The oxidation of the substrate and the reduction of the corresponding ketone are catalyzed by ADHs with complementary cofactor preference and stereoselectivity. The presented deracemisation approach leading to the unprotected optically pure *sec*-alcohol is a pure biocatalytic alternative for the dynamic kinetic resolution of *sec*-alcohols, which leads to the acylated alcohol. Additionally, it represents a concept for a "green" alternative for Mitsunobu stereoinversion of alcohols. Due to the generality of the system, other cofactor recycling systems are feasible (e.g., glucose dehydrogenase), as well as is employing other ADHs for other types of *sec*-hydroxy compounds such as α-hydroxy acids or sugars. Because the general concept for the system is now understood, each enzyme can be designed and adapted, making the whole system controllable and tunable. Therefore, the proposed biocatalytic method leads to the development of a new tool for (industrial) chemistry for the preparation of optically pure compounds.[16]

Experimental Section

Typical Procedure for the Derazemisation of *sec*-Alcohols Employing Isolated Enzymes: To obtain enantiopure (S)-alcohols, the racemic alcohol (3−5 mg) was added to TRIS buffer (0.5 mL, pH 7.5, 50 mM) and a mixture of the following enzymes: NADPH-dependent (*R*)-LK-ADH (1 mg, 0.4 U), YcnD-oxidoreductase (10 μL, 13 μM), and catalytic amounts of NADPH (0.5 mM) for the oxidation step and for the reduction of the ketone to the (*S*)-enantiomer: NADH-dependent (S)-ADH-'A' (10 μL, 0.7 U), and NADH-recycling system [NADH-dependent-formate dehydrogenase (FDH, 10 μL, 2 U), formate (10 mg, 0.3 M), 0.5 mM NADH]. The mixture was shaken at 30 °C and 350 rpm for the specified

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time (3−24 h). Afterward, the biotransformation was stopped by addition of ethyl acetate (600 μL) and centrifugation (13 000 rpm, 5 min). The organic phase was dried (Na_2SO_4), and acetylated derivatives of the alcohol were prepared for chiral analysis by the addition of acetic anhydride (250 μL, 2.5 mM) and a catalytic amount of DMAP (4-dimethylaminopyridine, 0.02 mM). This reaction mixture was shaken at room temperature (25 °C) for 1 h at 170 rpm. Water (300 μL) was then added, as well as a solution of 1-decanol in ethyl acetate (50 mg mL^{-1}; 1 mg, 20 μL) as an internal standard, and the resulting solution was centrifuged (2 min). The organic phase was dried (Na_2SO_4) and analyzed by GC.

Preparative Scale: rac-1-Phenylethanol (rac-**1h**, 50 mg) was added to a mixture of LK-ADH (50 mg, 20 U), YcnD-oxidoreductase (250 μL, 340 μM), RE-ADH (250 mg, 8.5 U), NADH recycling system [FDH (500 μL, 100 U), formate (0.5 g, 15 M)], and catalytic amounts of cofactor (NADH 0.5 mM, NADPH 0.5 mM) in TRIS buffer (25 mL, pH 7.5, 50 mM). A 50 mL Falcon tube was employed as reaction vessel and the mixture was shaken at 30 °C and 170 rpm. The biotransformation was stopped after 16 h reaction time by addition of ethyl acetate (25 mL) and the solution was centrifuged (10 min, 10 000 rpm). For analysis of the deracemisation process, a sample (1 mL) of the reaction mixture (ethyl acetate phase) was taken, derivatized, and analyzed by chiral GC. For the determination of yield, the residual organic phase (24 mL) was transferred into a round shank and ethyl acetate was removed by evaporation under reduced pressure to yield 45 mg (90%) final optical pure (*S*)-**1h** (ee >99%).

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Supporting Information Available: Experimental procedures and analytical data for all compounds; enzymes and microorganisms; large tables; extensive figures; blank experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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