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Coupled chemoenzymatic transfer hydrogenation catalysis for enantioselective reduction and oxidation reactions

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Abstract—Stereoselective reductions of prochiral ketones were performed using a new thermophilic, NAD-dependent alcohol dehydrogenase from *Thermus* sp. (TADH). The enzyme was produced on 2L-scale from recombinant *Escherichia coli* and purified by a simple, one-step heat treatment procedure yielding 220 mg of pure enzyme. Regeneration of NADH was catalyzed by the organometallic complex $[Cp^*Rh(bpy)(H_2O)]^{2+}$ using formate as a reducing agent. The catalytic performance of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ in terms of total number of catalytic cycles and number of catalytic cycles per hour achieved herein (up to 1500 and more than 400 h^{-1}, respectively), are the highest reported for a non-enzymatic nicotinamide regeneration system so far. Chemoenzymatic reduction reactions in a two liquid phase setup were performed on a gramme-scale, for example, 1.3 g of enantiopure (1*S*,3*S*)-3-methylcyclohexanol was obtained after purification. The volumetric productivity reached up to 3.9 mM h^{-1} with an average of 2.6 mM h^{-1} (5.3 g L^{-1} d^{-1}) over 10 h. In addition, chemoenzymatic oxidations utilizing the same catalyst set and molecular oxygen as a terminal electron acceptor were performed. Thus, the preparative value of chemoenzymatic transfer hydrogenations with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a regeneration catalyst coupled especially to thermophilic ADHs was demonstrated.

1. Introduction

There is an increasing need for enantiopure biologically active compounds and their precursors. Among them, chiral alcohols are important building blocks used for pharmaceuticals,[1] agrochemicals,[2] and flavours.[3] As a result, much effort has been made in the development of stereoselective processes for the preparation of enantiopure compounds. Today, transition metal-based hydrogenations and transfer hydrogenations of ketones, as well as hydrolytic kinetic resolution of the racemic alcohols, are the approaches most preferred by industry and organic chemists.[4]

Alternatively, alcohol dehydrogenases (ADHs) are attractive catalysts because of their remarkable chemo-, regio- and stereoselectivities, their impressive catalytic efficiency and their reactivity in aqueous media. Early problems with ADH catalysis, such as the sometimes narrow substrate spectrum of a given ADH or exclusive preference for one product stereoisomer, can now be addressed with standard molecular biology tools, such as directed evolution.[5,6] ADH catalysis however necessitates stoichiometric amounts of costly and unstable nicotinamide cofactors, which so far has severely impaired the preparative usefulness of ADHs for enantioselective catalysis. Stoichiometric use of these cofactors is prohibitive, leaving in situ regeneration as one of few alternatives. Many specialized regeneration concepts based on enzymatic,[7,8] electro-, and photochemical approaches[9] have been reported. All these regeneration catalysts however share the major drawback of being very specific for one form of the nicotinamide cofactor (phosphorylated or non-phosphorylated as well as oxidized or reduced). Furthermore, the optimal reaction conditions of a given ADH do not necessarily show a large overlap with those from the putative regeneration catalyst in terms of pH, temperature and ionic strength of the reaction medium, often resulting in poor compromises under which the full potential of each catalyst cannot be exploited.

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Over a decade ago, Steckhan et al. introduced $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a catalyst for the catalytic regeneration of NADH.[10–12] Its advantages over the aforementioned regeneration approaches are its versatility with respect to the source of reducing equivalents, its high activity over a broad temperature and pH-range, as well as its identical activity towards phosphorylated and non-phosphorylated nicotinamides.[13] Furthermore, there are indications that the same catalyst might also be applicable for the catalytic regeneration of oxidized nicotinamides. Considering these features, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ appears to be an ideal catalyst to promote ADH-catalyzed enantiospecific reduction reactions as well as oxidative kinetic resolutions. However, a demonstration of its preparative applicability is still missing.

Herein, we report the practical usefulness of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ for this purpose. As biocatalyst we used a secondary ADH recently isolated from *Thermus* sp. (TADH), which is particularly useful for the conversion of cyclic ketones and alcohols.[14] Both catalysts are productively coupled via the nicotinamide cofactor to perform enantioselective reductions of ketones (Fig. 1).

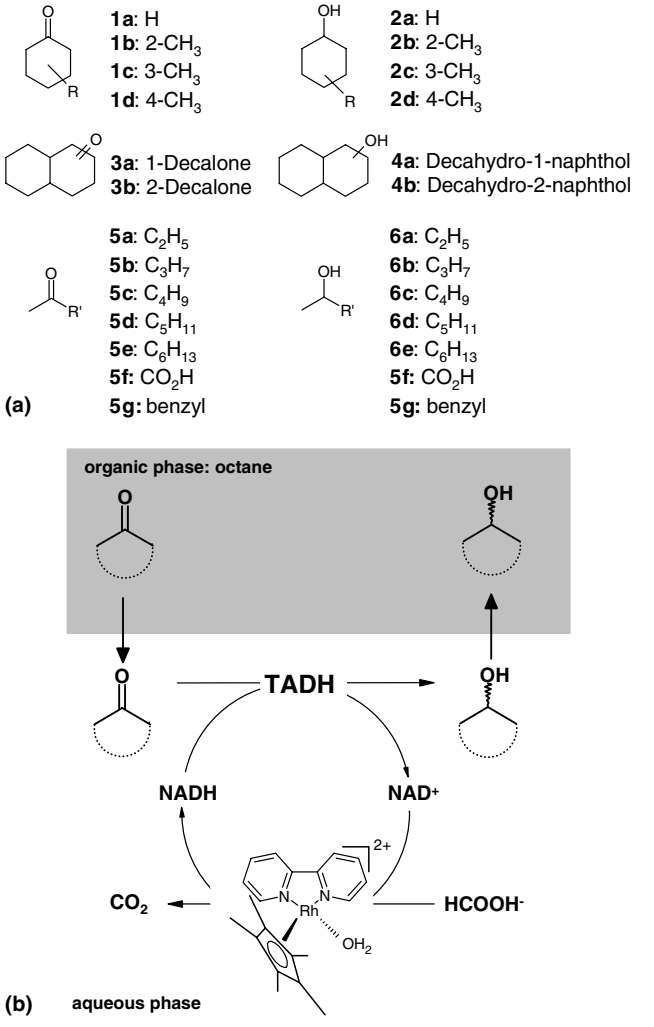

Figure 1. (a) Substrates and products used for chemoenzymatic reduction in a two-liquid-phase system as shown in (b). The nomenclature is used throughout the text. (b) Chemoenzymatic reduction of various ketones in a two-liquid-phase system catalyzed by TADH coupled to $[Cp^*Rh(bpy)(H_2O)]^{2+}$-promoted regeneration of NADH.

2. Results and discussion

2.1. Catalyst preparation

A prerequisite for the practical applicability of the chemoenzymatic reaction sequence is the facile and inexpensive preparation of the catalysts. [Cp*Rh(bpy)Cl]Cl was prepared in one step, starting from commercially available $[(Cp^*Rh)_2Cl_2]$ and bipyridine in near quantitative yield.[15] Aqueous stock solutions containing [Cp*Rh(bpy)Cl]Cl readily hydrolyzed and yielded the catalytically active complex; these were stored for several months at ambient temperature without detectable loss of activity.

TADH was prepared in a similarly facile fashion using well-established *Escherichia coli* BL21 (DE3).[16] From one 2L lab-scale fermentation, approximately 3.6 g cell dry weight with more than 2.2 g of total protein were obtained (Table 1). To purify TADH from the host proteins, heat denaturation of the mesophilic *E. coli* proteins (80 °C for 15 min) proved to be a facile and efficient procedure yielding more than 220 mg of essentially pure TADH (Fig. 2). Interestingly, heat treatment even increased the total TADH activity. It was speculated that if under the mesophilic growth conditions of *E. coli*, TADH did not fold into in its native, catalytically most active, ternary- and quaternary structure and that increasing the temperature would allow refolding into a catalytically more active structure; alternatively host proteins present in the crude cell lysate might inhibit TADH. Further investigations to clarify this phenomenon are currently in progress.

Table 1. Purification characteristics of TADH from *Escherichia coli* BL21(DE3) (pASZ2)

	Crude extract	Supernatant after heat denaturation
Total protein (mg)	2244	228
Total activity (U)[a]	673	1072
Specific activity (U mg^{-1})	0.3	4.7
Purification factor (-fold)	1	15.6
Recovery (%)	100	159

Recombinant *E. coli* were grown in standard LB-medium supplemented with 0.5% (w/v) glucose at 37 °C to a final cell density of 1.8 g CDW L^{-1}, TADH expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at a cell concentration of 0.24 gCDW L^{-1}.

[a] The TADH activity was determined spectrophotometrically based on the formation of NADH ($\lambda = 340$ nm, $\varepsilon = 6220\ M^{-1}\ cm^{-1}$) from NAD^+ (1mM) during the oxidation of 1-butanol (100 mM) at 60 °C and pH 9.0; 1 international unit (U) corresponds to the formation of 1 μmol product per minute.

TADH was stored at −20 °C at a concentration of 1.9 mg mL^{-1}, where it retained full activity over at least 5 months. Initial characterization of TADH revealed its suitability for the conversion of cyclohexanone

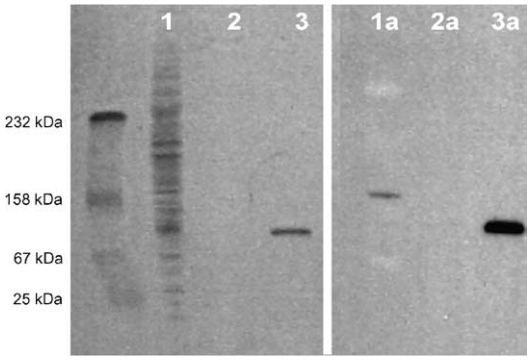

Figure 2. Native PAGE analysis of TADH-purification. Lines 1,1a: cell crude extracts from non-induced recombinant *Escherichia coli*; 2,2a: heat-treated cell crude extracts from non-induced recombinant *E. coli*; 3,3a: heat-treated cell crude extracts from induced recombinant *E. coli*. Lines 1–3 Coomassie brilliant blue staining, lines 1a–3a qualitative in situ ADH-activity assay.[17] General conditions: Gel-electrophoresis under non-denaturating conditions was performed using a PhastGel PAGE-System (Amersham Pharmacia, Freiburg, Germany) (native gradient gel 8–25%) following the recommendations of the supplier. In situ alcohol dehydrogenase activity was determined qualitatively by immersion of the native gradient PAGE gels for 5 min at 60 °C in a solution containing 0.2 mg mL^{-1} blue nitrotetrazolium-chloride, 0.08 mg mL^{-1} *N*-methylphenazoniummethylsulfate, 1 mM NAD^+ and 1% (v/v) ethanol in 100 mM Tris (pH 8).[17]

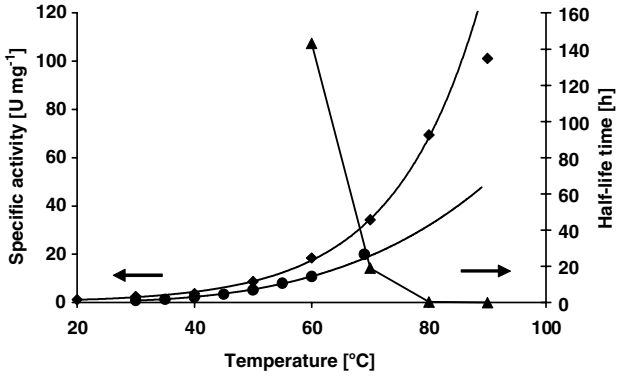

Figure 3. Influence of temperature on the TADH-activity (♦) and TADH-stability (▲) during the oxidation of 1-butanol compared to the activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (●) during NADH regeneration.[13] General conditions: Assays were performed in potassium phosphate buffer (50 mM, pH 9.0), c(TADH) = 0.19 μM (30.3 μg mL^{-1}), $c(NAD^+)$ = 1 mM, c(1-butanol) = 100 mM, activity calculations are based on the initial 30 s of the reaction. Stability assays of TADH were performed by incubating the enzyme (1.9 mg mL^{-1} in potassium phosphate buffer) at the indicated temperature. At intervals samples were withdrawn and analyzed at 60 °C.

derivatives, a detailed study on its substrate range and catalytic properties will soon be reported elsewhere.

Overall, TADH was obtained in pure form and free from competing NAD-dependent enzymes present in *E. coli* following a simple and scalable protocol. Its preparation was completed within 3 days from inexpensive and non-toxic starting materials without tedious chromatographic steps.

2.2. Optimization of chemoenzymatic reductions

In the first set of experiments, we investigated the influence of temperature on stability and activity of the catalysts. As shown in Figure 3, TADH activity exponentially increased with temperature, which was counteracted by the exponentially decreasing half-life of the biocatalyst. Therefore, we chose an operation temperature of 60 °C as at this temperature the product of TADH activity and TADH half life was at its highest (Fig. 3). pH 7 was chosen as a compromise between TADH activity and stability of the nicotinamide cofactor. In the reductive direction, TADH activity was highest at pH 6 with approximately 80% of this activity at pH 7. NADH, on the other hand had a half life of 38 and 139 min at pH 6 and pH 7 in 50 mM phosphate buffer, respectively (data not shown).

Under these conditions, $[Cp^*Rh(bpy)(H_2O)]^{2+}$exhibited a specific activity of approximately 11 U mg^{-1} with a half life exceeding the time-scale of TADH stability (at 80 °C a half life of over 15 days was determined).[13]

To evaluate the scope of the chemoenzymatic reaction, we performed catalytic reduction reactions as indicated in Figure 3; results are shown in Table 2.

Interestingly, none of the experiments gave full conversion of the starting material even though from a thermodynamic point of view the equilibrium should be far on the side of the products (>99.5% conversion as calculated from the group contribution method[18,19]). This could be explained by assuming that TADH is subject to product inhibition, which is quite common amongst ADHs.[20,21] The significantly decreased 'initial' rates of the chemoenzymatic reactions (determined after 15 min and up to 20% conversion) compared to the rates determined by steady state kinetic measurements further support this assumption (Table 2).

In fact, kinetic studies revealed a pronounced product inhibition, for example, for **2c** (isolated from preparative-scale chemoenzymatic reactions, vide infra) ($K_i = 8.7 \pm 1.4$ mM). Furthermore, TADH stability was significantly impaired in the presence of substrate (data not shown). Any inhibitory effects of the reactants (alcohols and/or ketones) on $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was excluded (data not shown).

With the goal of efficiently shifting the transfer hydrogenation equilibrium to the side of the desired products we evaluated the influence of in situ NADH regeneration (catalyzed by $[Cp^*Rh(bpy)(H_2O)]^{2+}$) and in situ product removal on the reaction progress (Fig. 4). In the case of stoichiometric NADH addition (Fig. 4■) only 1.9 mM of product were formed corresponding to 38% of the theoretical yield [exclusively considering that exclusively (*S*)-3-methyl cyclohexanone is converted by TADH at significant rates]. $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed regeneration of the reduced nicotinamide coenzyme had a positive but not very pronounced effect on the yield (2.9 mM product formed, corresponding to 58% of the theoretical yield, Fig. 4▲). If however, an organic phase (here heptane) was added, TADH turnover increased drastically (Fig. 4♦).

Table 2. Chemoenzymatic reduction of the ketones **1**, **3** and **5**

Substrate	Initial rate (U mg^{-1})		Yield[a] (%)	TN ([Cp*Rh(bpy)(H$_2$O)]$^{2+}$)[e]	ee-value[b] (%)
	After 15 min[b]	Initial rate studies[c]			
1a	3.6	6.2	57.3	228	—
1b	n.d.[d]	1.0	Traces	n.d.[d]	n.d.[d]
rac-**1c**	2.0	12.4	36.4	110	99.6[g]
(*R*)-**1c**	—	0.2	—	—	—
rac-**1c**[f]	3.4	12.4	40.6	—	n.d.[d]
1d	1.0	3.5	19.2	76	14.9
3b	1.7	3.1	27.9	112	n.d.[d]
5a–d	n.d.[d]	0.2–0.9	Traces	n.d.[d]	n.d.[d]

General reaction conditions: 1 mL-scale, 20 mM Tris–HCl buffer (pH 7), $T = 60$ °C, c(substrate) = 20 mM, $c(NAD^+) = 5$ mM, c([Cp*Rh(bpy)(H$_2$O)]$^{2+}$) = 0.05 mM, $c(NaHCO_2) = 150$ mM.

[a] Determined after 3.5 h reaction time with residual reactant concentration.

[b] Determined from chemoenzymatic reactions via GC.

[c] From initial rate studies determined spectrophotometrically.

[d] Not determined.

[e] Calculated from the amount of product formed.

[f] Formate dehydrogenase [20 U mL^{-1} (at 25 °C)] was used for NADH-regeneration.

[g] Determined according to $(V_{rac} - V_S) \times (V_{rac} - V_R)^{-1}$ [due to the low activity with (*R*)-**1c**, we approximated values determined for the racemate solely to the (*S*)-enantiomer].

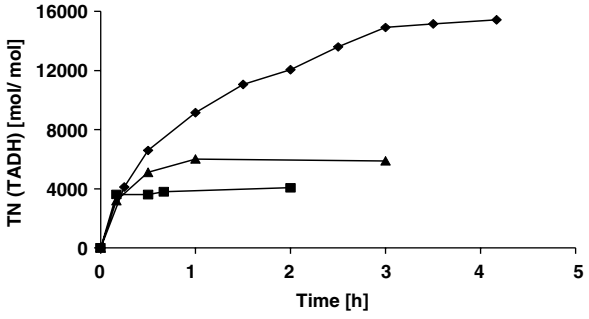

Figure 4. Influence of NADH-regeneration and in situ product removal on the reduction of 3-methylcyclohexanone. General conditions: 50 mM potassium phosphate buffer pH 6.0, $T = 60$ °C, $c(NaHCO_2) = 150$ mM. The reactions were performed in 2 mL Eppendorf cups in a thermoshaker (1500 rpm) with a total volume of 1 ml; (■) Stoichiometric NADH: c(TADH) = 0.484 μM, c(NADH) = c(**1c**) = 10 mM; (▲) NADH-regeneration: c(TADH) = 0.484 μM, $c(NAD^+) = 0.5$ mM, c([Cp*Rh(bpy)(H$_2$O)]$^{2+}$) = 0.1 mM, c(3-methylcyclohexanone) = 10 mM; (♦) in situ-product removal: aqueous concentrations c(TADH) = 0.242 μM, $c(NAD^+) = 0.5$ mM, c([Cp*Rh(bpy)(H$_2$O)]$^{2+}$) = 0.05 mM, c(3-methylcyclohexanone) = 250 mM$_{heptane}$≅19 mM$_{aq}$; phase ratio heptane:buffer = 1.5. Due to the varying TADH concentrations applied, different product formation rates were obtained. In order to make the experiments comparable, normalization on the turnover number of TADH [TN(TADH)] were performed.

The second organic phase extracts the product formed, and thus decreases the effect of product inhibition on the enzymatic reduction reaction. Therefore, we concentrated on the biphasic reaction media. Among the solvents tested with respect to their compatibility with TADH octane was found to be the most suitable organic phase (data not shown). Compounds **1c** and **2c** partitioned between octane and the buffer (phase ratio 0.5) at 13:1 and 10:1, respectively. Emulsions were formed to assure a sufficiently high transfer rate of substrates and products over the phase interface. Unfortunately, TADH turned out to be very sensitive towards the mechanically demanding reaction conditions, necessary for forming an emulsion. When the reaction was mixed by shaking (e.g., Fig. 4♦), TADH activity was lost after approximately 3 h. If, however, stirring was necessary (e.g., to form emulsions on larger scale, vide infra) already after 30 min stirring no residual TADH activity was detected. To stabilize TADH it was covalently attached to Eupergit C™.[22] Immobilization of TADH, however, resulted in a dramatically decreased biocatalyst activity, which we could assign to an increase of the apparent K_M-value for NADH by at least a factor of 500 (Fig. 5). However, we accepted this drawback in the light of the expected stability increase for TADH. Currently, we are evaluating various alternative approaches to stabilize TADH, without impairing its catalytic efficiency.

To estimate the potential of [Cp*Rh(bpy)(H$_2$O)]$^{2+}$-catalyzed regeneration of NADH, chemoenzymatic reduction reactions with immobilized TADH were performed under [Cp*Rh(bpy)(H$_2$O)]$^{2+}$-limited conditions: a total turnover number of [Cp*Rh(bpy)(H$_2$O)]$^{2+}$ of approximately 1500 was achieved at turnover frequencies of up to 475 ± 6 turnovers per hour. Compared to literature values, these represent an increase of at least one order of magnitude (Table 3).

Despite the high catalytic turnover of [Cp*Rh(bpy)(H$_2$O)]$^{2+}$ observed (Table 3), it did not reach the values determined in the absence of TADH (up to 1000 h^{-1}). We assigned this to an inhibitory effect of TADH on [Cp*Rh(bpy)(H$_2$O)]$^{2+}$. It is possible that nucleophilic residues present in TADH can coordinate to [Cp*Rh(bpy)(H$_2$O)]$^{2+}$, thereby gradually decelerating the rate of the formate-driven regeneration reaction. Similar effects have been observed previously.[23] This phenomenon was attenuated by the addition of nucleophilic buffer components such as Tris. Principally, the interaction between TADH and [Cp*Rh(bpy)(H$_2$O)]$^{2+}$ can be eliminated completely by more nucleophilic addi-

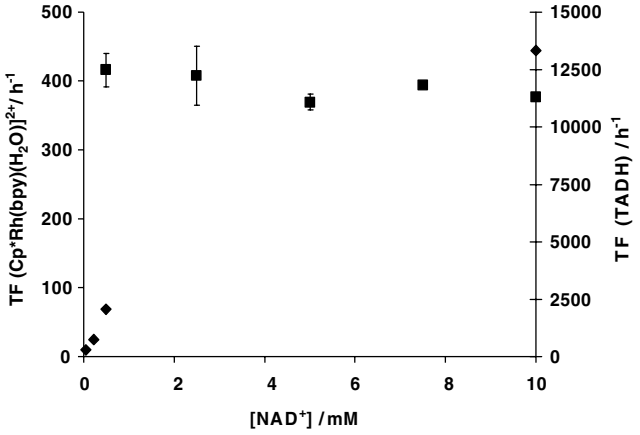

Figure 5. Comparison of the influence of NAD^+ concentration on the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed NADH regeneration rate (■) and on the rate of the chemoenzymatic reduction of 3-methylcyclohexanone in terms of turnover rate of the immobilized biocatalyst (♦). General conditions: 20 mM Tris buffer (pH 7), $T = 60$ °C, $c(NaHCO_2) = 150$ mM; $TF([Cp^*Rh(bpy)(H_2O)]^{2+})$ (5 μM) was determined via NADH formation ($\lambda = 340$ nm).

Table 3. Catalytic parameters determined for $[Cp^*Rh(bpy)(H_2O)]^{2+}$ from previous studies

	$c([Cp^*Rh(bpy)(H_2O)]^{2+})$ (mM)	TN_{max}	TF_{max} (h^{-1})	Scale (ml)
5g[12]	0.5	50	10	10
5g[25]	0.21	30	1.25	10
5f[11]	0.5	14	6	50
1a[26]	0.5	200	2.5	n.d.
1c[a]	0.001–0.1	1500	470	Up to 750

[a] This study.

tives making electrochemical regeneration of the active hydridorhodium species an option.[13] Further investigations are currently underway.

2.3. Gramme-scale preparation of (1*S*,3*S*)-3-methylcyclohexanol ((1*S*,3*S*)-2*c*)

Preparative-scale reactions (750 mL-scale emulsion process at 2:1 ratio of organic:aqueous phase) were performed. Reaction parameters from reductions of **1c** and **3b** are listed in Table 4.

The NMR spectrum of purified **2c** indicated at least 97.2% enantiomeric excess for the *trans*-substituted isomer (the diastereomeric ratio was determined by the integrals of the carbinol protons, δ 3.45 and 3.97 ppm for the *cis* and *trans*, respectively).[28] Considering the high preference of TADH for (3*S*)-**1c**, it can be asserted that (1*S*,3*S*)-**2c** is the predominant product from the chemoenzymatic reduction reaction. As shown in Table 4, respectable catalytic performances of both catalysts were obtained, especially for $[Cp^*Rh(bpy)(H_2O)]^{2+}$ outnumbering previous results by far (Table 3). Also, the mass-based yield for both catalysts is promising.

However, comparably high concentrations of the nicotinamide cofactor (10 mM) were necessary to guarantee high activity of the immobilized biocatalyst. Currently, we are evaluating alternatives to Eupergit© in order to stabilize the biocatalyst under the mechanically demanding conditions. Thus, a significant decrease in the NAD^+ demand is expected as the K_M-value of diffusible TADH towards NADH is only about 10 μM (data not shown), which will further increase the economic attractiveness of the chemoenzymatic approach. Overall, we have for

Table 4. Gram-scale chemoenzymatic reduction reactions of **1c** and **3b**[a]

	1c	**3b**
Product		
Final concentration in octane (mM)	21.4	18.5
Enantiomeric excess (% ee)	>97	n.d.
Isolated product (g)[b]	1.3 (95% pure)[d]	1.8 (95% pure)[d]
Maximal productivity[c] [mmol L^{-1} h^{-1}] ([g L^{-1} h^{-1}])	3.9 (0.445)	2.1 (0.354)
TADH		
TF_{max} (h^{-1})	4300	2210
TN	28,000	24,200
g g^{-1}[e]	0.012	0.011
$[Cp^*Rh(bpy)(H_2O)]^{2+}$		
TF_{max} (h^{-1})	120	95
TN	233	146
g g^{-1}[e]	0.035	0.035

General conditions: 250 mL Tris/phosphate-buffer (20/50 mM; $pH_{60} = 7.0$), $T = 60$ °C, $c(NaHCO_2) = 150$ mM, $c(TADH) = 1.53$ μM (60 mg immobilized on Eupergit C), $c(NAD^+) = 10$ mM, $c([Cp^*Rh(bpy)(H_2O)]^{2+})_0 = 5$ μM; 500 mL octane, c(**1c**) = 250 mM, c(**3a**) = 1.4 M, respectively, c(dodecane) = 2 mM (internal standard for GC analysis); $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was continuously added to the reaction to compensate the gradual $[Cp^*Rh(bpy)(H_2O)]^{2+}$-inhibition (1.15 mL h^{-1} of a 5 mM stock-solution corresponding to an approximate increase of the aqueous concentration of 23 μM h^{-1}).

[a] Determined via NMR.[28]

[b] After chromatographic purification.

[c] Based on the organic phase.

[d] As determined via GC-analysis.

[e] Mass-based ratio: mass of catalyst consumed per gramme of product ($M(TADH) = 47 \times 10^3$ g mol^{-1}; $M([Cp^*Rh(bpy)(H_2O)]Cl_2) = 483$ g mol^{-1}) per gramme of product.

the first time applied chemoenzymatic catalysis using an organometallic and an enzymatic catalyst for preparative production of chiral alcohols. Though the results obtained so far are already promising, further significant increases of the catalytic activity of especially NAD is expected by optimized reaction conditions (i.e., non-heterogeneous stabilization of the biocatalyst).[24]

2.4. Chemoenzymatic oxidation of racemic 3-methylcyclohexanol *rac*-2c

From a synthetic point-of-view, it may be more desirable to produce enantiopure alcohols via kinetic resolution of the racemate than by enantioselective reduction of the prochiral ketone. In this case, an additional driving force is required, since the NAD^+-promoted oxidation of alcohols is thermodynamically unfavourable.[18,19] Recently, we reported evidence that a system consisting of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and flavin in an O_2-containing media might be suitable for regenerating $NAD(P)^+$. The very high oxidation potential of the O_2/H_2O_2 couple is exploited to shift the redox equilibrium NAD(P)H/$NAD(P)^+$ to the side of the desired products.[13] Studies on chemoenzymatic kinetic resolution of *rac*-**2c** were performed on mL-scale using soluble TADH (Fig. 7).

The chemoenzymatic oxidation following the reaction cascade proposed in Figure 6 was shown to be feasible. Direct oxidation of the alcohols via $[Cp^*Rh(bpy)(H_2O)]^{2+}$ could be excluded since no ketone formation was observed in the absence of the biocatalyst. Furthermore, the oxidation reaction preceded stereoselectively (Fig. 7) indicating that the oxidation step was catalyzed by TADH. Approximately 2 mM **1c** was formed after 2.5 h. This corresponds to 80% of the theoretical yield assuming an even distribution of the four stereoisomers within the substrate and a high preference of TADH towards (1*S*,3*S*)-**2c**. The initial oxidation rate under non-optimized conditions was 1.92 mM h^{-1} (2.2 s^{-1} and 38.5 h^{-1} for TADH and $[Cp^*Rh(bpy)(H_2O)]^{2+}$, respectively). Further characterization of this reaction system is expected to lead to optimized catalyst performances.

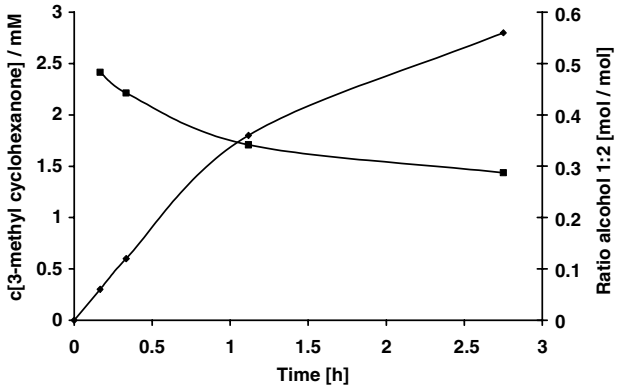

Figure 7. Oxidation of racemic 3-methylcyclohexanol (mixture of all four stereoisomers) in the system TADH/$[Cp^*Rh(bpy)(H_2O)]^{2+}$/FAD/O_2. General conditions: 1 mL of 50 mM potassium phosphate buffer (pH 9.0), c(TADH) = 0.242 μM (38 μg mL^{-1}), c(NADH) = 5 mM, $c([Cp^*Rh(bpy)(H_2O)]^{2+})$ = 50 μM, c(FAD) = 0.2 mM, c(catalase) = 0.64 μM; (♦) **1c**, (■) ratio of **2c**-enantiomers (alcohols 1 and 2, see Section 4) detected by GC. The reactions were performed in Eppendorf cups placed in a thermoshaker. Air input was performed by rotating the reaction vessels at 1400 rpm.

Overall, we have shown for the first time that an NAD^+ regeneration system based on $[Cp^*Rh(bpy)(H_2O)]^{2+}$/flavin/O_2 can be successfully applied to a dehydrogenase-catalyzed kinetic resolution of alcohols.

3. Conclusion

Herein, we have demonstrated the synthetic practicability of chemoenzymatic redox reactions. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is an efficient and versatile regeneration catalyst for reduced and oxidized nicotinamide cofactors. Especially coupled to thermophilic ADHs, it can deploy its high catalytic potential. The use of thermophilic ADHs bears further practical advantages such as very easy production and purification. Overall, we believe that the chemoenzymatic approach will render biocatalytic procedures more attractive to preparative organic chemists thus becoming a new tool for enantioselective synthesis.

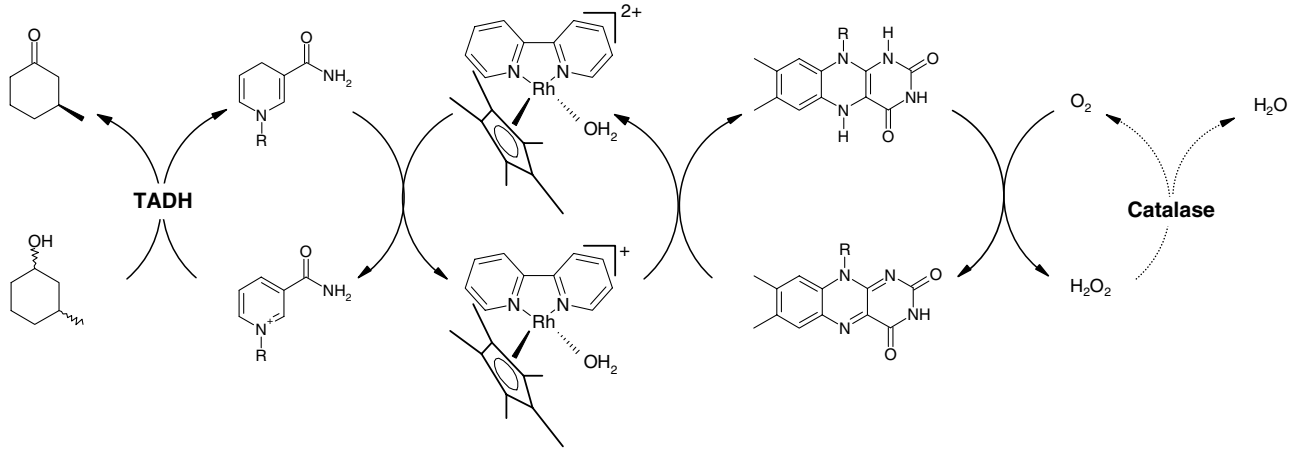

Figure 6. Chemoenzymatic oxidation.

4. Experimental

4.1. Chemicals, bacterial strains and plasmids

Chemicals were purchased from Fluka (Buchs, Switzerland) or Aldrich (Steinheim, Germany) in the highest quality available and used without further purification. $\{Cp^*RhCl(\mu\text{-}Cl)\}_2$ was a gift from DEGUSSA, Hanau, Germany. Appropriate safety precautions were taken, especially when working with rhodium and organic solvents at elevated temperatures. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was synthesized according to literature methods[15] by addition of 2 equiv of 2,2′-bipyridine to a suspension of $\{Cp^*RhCl(\mu\text{-}Cl)\}_2$ in methanol. [Cp*Rh(bpy)Cl]Cl was precipitated upon addition of diethyl ether. Aqueous stock solutions were stored at room temperature for several months without detectable loss of transfer hydrogenation activity.

Unless indicated otherwise, all pH values were adjusted to 60 °C.

E. coli BL21(DE3)[16] was used as a host organism for the overexpression of the TADH gene from derivatives of plasmid pET11aEco (a derivative of pET11a, Novagene, carrying an additional EcoRI site (5′-AAGAAGGAGAATTCCATATG-3′) between the NdeI site and the RBS of pET11a). In previous work, the TADH gene was localized on the chromosome of *Thermus* sp. and subcloned into a pUC20-vector. From this vector it was now amplified by polymerase chain reaction (PCR) using the DyNazyme EXT kit (Finnzymes) and the primers 5′-GAAGGAATTCCATGCGCGCAGTGGTTTTTGA-3′ and 5′-ATGCTCCTCGGATCCCAGAAGTA-3′. The forward primer was designed to introduce an EcoRI site whereas the reverse primer contained a BamHI site. The resulting PCR product was digested with EcoRI and BamHI and ligated into the EcoRI and BamHI digested expression vector pET11aEco to give pASZ2.

4.1.1. Production of TADH. For overexpression of the TADH genes recombinant *E. coli* BL21(DE3) carrying pASZ2 were grown in Luria-Bertani (LB) medium (1% w/v Bacto Tryptone, 0.5% w/v yeast extract, 1% NaCl, pH 7.5) containing chloramphenicol (20 μg/mL) and ampicillin (200 μg/mL) at 37 °C. At $OD_{450} = 0.8$ (optical density at $\lambda = 450$ nm) 1 mM isopropyl β-D-thiogalactoside (IPTG) was added to induce protein expression. The cells were grown until the late exponential phase (OD_{450} about 9), harvested by centrifugation and stored at −80 °C after shock-freezing using liquid nitrogen.

4.1.2. Purification and immobilization of TADH. Frozen cells were resuspended in 20 mM Tris buffer (pH 7.5) and disrupted by two passages through a French press (1200 psi). After centrifugation (150,000*g* at 4 °C for 30 min) the supernatant was incubated at 80 °C for 15 min. Subsequent centrifugation (150,000*g* at 4 °C for 30 min) resulted in essentially pure TADH in the supernatant.

For immobilization, 12 mL TADH solution (1.9 mg mL^{-1}) was added to 2 g Eupergit C (Röhm, Darmstadt, Germany) and incubated for 48 h at room temperature. Afterwards, the beads were washed with 50 mM potassium phosphate buffer (pH 7.0) and stored in the same buffer at 4 °C. Eleven milligrammes of TADH per gramme Eupergit were completely immobilized (determined from the protein content in the supernatant) after 2–3 days of incubation at room temperature. Catalytic activity of immobilized TADH was determined using a thermostatted flow through glass column (volume 2.5 mL), containing 250 mg of TADH–Eupergit. The specific activity of immobilized TADH for cyclohexanone conversion (c(cyclohexanone) = 7 mM, c(NADH) = 5 mM, residence time 1 min) was determined to be 17.2 U g^{-1} (Eupergit) and 1.5 U mg^{-1} (TADH) corresponding to 25% of the specific activity of soluble TADH with cyclohexanone.

4.2. Analytical methods

Initial catalyst activities were determined spectrophotometrically in a thermostable Carey 1E UV-spectrophotometer (Varian, Switzerland) and were based on accumulation or depletion of NADH ($\varepsilon = 6230\ M^{-1}\ cm^{-1}$). All components of the reaction (except the catalysts) were incubated for 2 min at the indicated temperature. Assays were started by adding TADH and $[Cp^*Rh(bpy)(H_2O)]^{2+}$, respectively. Protein concentrations were determined according to the method of Bradford.[27] Affinity constants (K_M-values) were calculated using the Enzfitter software (Elsevier-Biosoft, 1987) by weighted linear regression.

For gas chromatography (GC), aqueous samples were extracted with an aliquot of diethyl ether containing 2 mM dodecane as internal standard. The organic phase was dried over anhydrous sodium sulfate and analyzed. GC analysis was done using a GC (Fisons Instruments, Milano, Italy) equipped with a Lipodex A capillary column (25 m; inner diameter 0.25 mm) from Machery-Nagel (Düren, Germany) with split injection (1:20) and hydrogen as carrier gas. The following temperature profiles were applied. Cyclohexanones: 3 min isotherm at 60 °C, from 60 to 120 °C at 5 °C min^{-1}; decalones: 2 min isotherm at 60 °C, from 60 to 160 °C at 5 °C min^{-1}. Compounds were detected using a flame ionization detector.

Resolution of isomers (retention time (min)): 3-methylcyclohexanol: 10.03 and 10.34 (ratio: 1:1.95); 3-methylcyclohexanone 8.98; 2-decalone: 14.03 and 15.1 (ratio: 1:3.3); 2-decahydronaphthol: 15.3, 16.2, and 16.3 (ratio: 1:1.2:2.9).

Chemoenzymatic reactions: 1–2 mL scale chemoenzymatic reduction and oxidation reactions were performed in Eppendorf reaction tubes in an Eppendorf 5436 thermomixer (Vaudaux-Eppendorf, Schönenbuch, Switzerland). Unless indicated otherwise (especially for chemoenzymatic oxidation reactions), the reaction mixtures were degassed by nitrogen purging and equilibrated at 60 °C. Reactions were started by addition of $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

Biotransformation reactions were performed under non-sterile conditions on a 750 mL scale in a stirred tank reactor with two Rushton turbine impellers, four baffles and a total volume of 3 L. The reactor, containing 250 mL buffer and 500 mL octane (2 mM dodecane), was heated to 60 °C and degassed by purging for 15 min with nitrogen. After supplementation with 5 g TADH–Eupergit (corresponding to approximately 60 mg TADH), the reactions were started by addition of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (aqueous concentration: 5 μM). $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was added continuously (1.2 ml h^{-1} of a 5 mM stock solution). After 24 h, the phases were separated by settling and the organic phase was removed under reduced pressure. The product was purified chromatographically on silica using chloroform as the eluent (R_f (3-methylcyclohexanol) = 0.3, R_f (2-decalol) = 0.35).

4.2.1. Characterization of the product from preparative-scale chemoenzymatic reduction of 1c. ^1H NMR (250 MHz, $CDCl_3$), δ (ppm): 4.0–3.94 (m, 1H), 1.83–1.69 (m, 1H), 1.62–1.50 (m, 4H), 1.48–1.35 (m, 2H), 1.21–1.10 (m, 1H), 0.97–0.85 (m, 1H), 0.81 (d, J = 6.8 Hz, 3H); $[\alpha]^{589} = +2.2$ (neat).

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