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Stereocomplementary Asymmetric Reduction of Bulky–Bulky Ketones by Biocatalytic Hydrogen Transfer

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Dedicated to Professor Vicente Gotor on the occasion of his 60th birthday

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Biocatalysts suitable for the reduction of ketones bearing two sterically demanding substituents (bulky–bulky ketones) at elevated substrate concentration (10 g L^{-1}) were identified. The biocatalysts can be employed in a substrate-coupled approach; thus, a simple alcohol such as ethanol or 2-propanol serves as a hydrogen donor. Both enantiomers are accessible by using either *Rhodococcus ruber* DSM 44541 and ethanol or *Ralstonia* sp. DSM 6428 and *Sphingobium yanoikuyae* DSM 6900 and ethanol or 2-propanol as the hydrogen source. For *Rhodococcus ruber* DSM 44541, it was found that bulky–bulky ketones were only transformed when ethanol was used as a hydrogen source, whereas no conversion was observed when 2-propanol was employed. From the substrate spectrum, as well as from the cosubstrate preference, it became clear that a different alcohol dehydrogenase than the previously described ADH-"A" is active in the presence of ethanol in *Rhodococcus ruber*.

Introduction

Stereoselective reduction of ketones is one of the most employed methods for the synthesis of nonracemic chiral alcohols in organic synthesis.[1] In this context, biocatalytic approaches employing reductases or alcohol dehydrogenases (ADHs) have very recently gained increasing importance due to the high chemo-, regio-, and stereoselectivities obtained in these processes.[2] The preferred substrate pattern of most highly stereoselective ADHs possessing a broad substrate spectrum resembles small–bulky ketones; thus, one substituent of the ketone, the "small" one, is limited to a sterically nondemanding group such as methyl, ethyl, or chloromethyl.[2d] Only a few examples have been reported for the reduction of ketones bearing two bulky groups (bulky–bulky ketones) by using either (fermenting) whole cells[3] or purified enzymes.[4] In most cases where organisms or enzymes have been searched for the reduction of bulky–bulky ketones, it was not the aim to identify a broad applicable catalyst, but to focus on the synthesis of a specific alcohol as the target precursor for bioactive compounds.[3c] Only a few intensive studies on ADHs for reduction of bulky–bulky ketones have been performed.[4] Besides the limited knowledge about these reductases for bulky–bulky ketones, another limitation is the low ketone concentrations used and the commonly moderate stereoselectivities obtained. Furthermore, processes catalyzed by ADHs require cofactors such as pyridine nucleotides NAD(P)H or $NAD(P)^+$, which need to be recycled by employing a regeneration system. Various chemical, electrochemical, photochemical, and enzymatic methods have been established in order to regenerate nicotinamide cofactors.[2a,5] The method exploited probably most often is the so-called "enzyme-coupled" approach, where a second (and preferably irreversible) enzymatic reaction is employed to shift the equilibrium to the desired product. For instance, formate dehydrogenase (FDH) from *Candida boidinii*,[6] glucose dehydrogenase,[7] or phosphite dehydrogenase[8] have been successfully applied. Recently, "designer bug" whole cells containing the overexpressed genes of the desired enzymes (ADH plus enzyme for the recycling system) have been developed with very promising results.[7,9] Nevertheless, due to the usage of an additional enzyme, the complexity of the system is increased, which, to a certain stage, inhibits the general acceptance of biocatalytic reductions by

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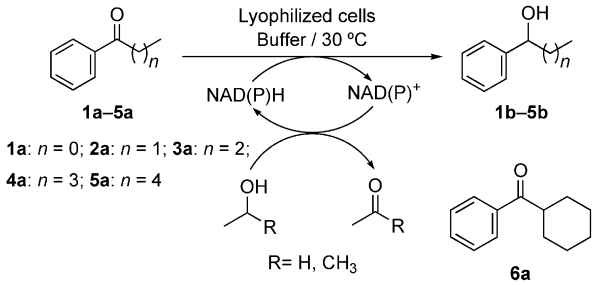
synthetic chemists. A simpler approach makes use of a single enzyme; thus, the desired reaction, as well as the recycling of the cofactor, is achieved by a single catalyst (Scheme 1), generally known as the "substrate-coupled" approach.[4d,10] In this case, a small alcohol such as 2-propanol or ethanol can be used as a hydrogen source, which must be applied in excess to drive the equilibrium to the product side, implying that the enzymes involved must possess a high stability to tolerate the increased concentration of cosubstrate.



Scheme 1. "Substrate-coupled" approach for the stereoselective reduction of ketones by using 2-propanol or ethanol.

In this study our aim was twofold: (i) to identify organisms that possess stereoselective reductases/alcohol dehydrogenases for the reduction of bulky–bulky ketones and (ii) to identify organisms that can use an alcohol such as 2-propanol or ethanol as a hydrogen source in a "substrate-coupled" approach.

Results and Discussion

We previously identified a highly solvent-stable ADH from the bacterial strain *Rhodococcus ruber* DSM 44541 named ADH-"A" that has been used for the bioreduction of small–bulky ketones with excellent activities and Prelog stereoselectivities in a "substrate-coupled" approach by using 2-propanol as a hydrogen source.[10f,11] However, when testing bulky–bulky ketones such as 1-phenyl-1-alkanones (**2a**–**5a**) with the wild type cells of *Rhodococcus ruber* with 2-propanol (14% v/v) as hydrogen donor the activity dropped significantly as soon as the small substituent of the ketone was larger than an ethyl moiety and no activity was found for the even more sterically demanding analogues valerophenone (**4a**) or hexanophenone (**5a**) (Table 1).

Despite these first discouraging results, ethanol (8% v/v) was tested as a hydrogen source with the same substrates. To our surprise, we did not only observe a change in activities, but also a change in stereoselectivity and in the scope of the accepted substrates. Now the bulky–bulky substrates butyrophenone (**3a**), valerophenone (**4a**), and hexanophenone (**5a**) were reduced to the corresponding alcohols by using ethanol as a cosubstrate (Table 1). Activities were clearly better for bulky–bulky substrates than for acetophenone (**1a**), which is a typical example of a small–bulky

Table 1. Reduction of 1-phenyl-1-alkanones (10 g L^{-1}) with lyophilized cells of *Rhodococcus ruber* DSM 44541 by employing 2-propanol or ethanol as cosubstrate.

Substrate	2-PrOH		EtOH	
	App. activity [μmol h^{-1} g^{-1}]	*ee* [%][a]	App. activity [μmol h^{-1} g^{-1}]	*ee* [%][a]
1a	103[b]	99 (*S*)	17.3	99 (*S*)
2a	29.0	99 (*S*)	30.7	40 (*S*)
3a	3.4	n.d.	30.0	40 (*R*)
4a	0	n.d.	30.8	53 (*R*)
5a	0	n.d.	19.4	49 (*R*)

[a] Determined by GC or HPLC on a chiral stationary phase. n.d. = not determined. [b] See ref.[11b]

ketone. Additionally, it was remarkable that the stereoselectivity changed from a perfect Prelog (*S*) preference for **1a** to an anti-Prelog (*R*) preference when the acyl chain became sterically more demanding. No activity was found when the previously identified recombinant ADH-"A" from this strain[11a] was tested with **3a**–**5a**. Furthermore, ADH-"A" showed no activity for known substrates when ethanol instead of 2-propanol was used as hydrogen source. This proves that by applying ethanol as a cosubstrate, ADH-"A" is not active on bulky–bulky-ketones; therefore, a different enzyme reduces the investigated bulky–bulky ketones by exploiting ethanol for cofactor recycling. Therefore, when employing *Rhodococcus ruber*, bulky ketones can only be reduced in the presence of ethanol. Obviously, a small change in the reaction conditions (i.e. different cosubstrate) affected dramatically the activity and substrate spectrum of the whole-cell catalyst.

Only few examples can be found in the literature for the usage of ethanol as a cosubstrate in the "substrate-coupled" approach.[12] Although ethanol is supposed to be more biocompatible than 2-propanol, the formation of more harmful and reactive acetaldehyde as byproducts limits its use.

Encouraged by our results mentioned above by using ethanol, we tested more than 70 different lyophilized bacteria, yeasts, and fungi in the "substrate-coupled" approach by employing ethanol (8% v/v) as well as 2-propanol (14% v/v) as the cosubstrate for the reduction of cyclohexyl phenyl ketone (**6a**) as a model substrate for bulky–bulky ketones. Two bacterial strains, namely, *Ralstonia* sp. DSM 6428 and *Sphingobium yanoikuyae* DSM 6900, were identified to perform the desired transformation and to accept 2-propanol as well as ethanol as cosubstrate (Table 2).

Table 2. Strains identified for the reduction of cyclohexyl phenyl ketone (**6a**, 10 g L^{-1}) in the "substrate-coupled" approach.

Microorganism	Cosubstrate	App. activity [μmol h^{-1} g^{-1}]
Ralstonia sp. DSM 6428	2-propanol	6.6
Sphingobium yanoikuyae DSM 6900	2-propanol	9.3
Ralstonia sp. DSM 6428	ethanol	11.3
Sphingobium yanoikuyae DSM 6900	ethanol	12.6

Having identified suitable catalysts for the conversion of bulky–bulky ketones, an optimization of the reaction conditions (pH cosubstrate concentration) for each catalyst was

performed. The optimum pH value was investigated by employing 2-propanol, as well as ethanol, as the cosubstrate (Figure 1). Interestingly, depending on the cosubstrate used, different pH optima were obtained. For instance, for *Ralstonia* sp., the optimum pH was 7.0 in the case of 2-propanol and 7.5 in the case of ethanol. By employing *Sphingobium yanoikuyae*, the highest activity was found at pH 7.0 for 2-propanol and at pH 8.0 for ethanol.

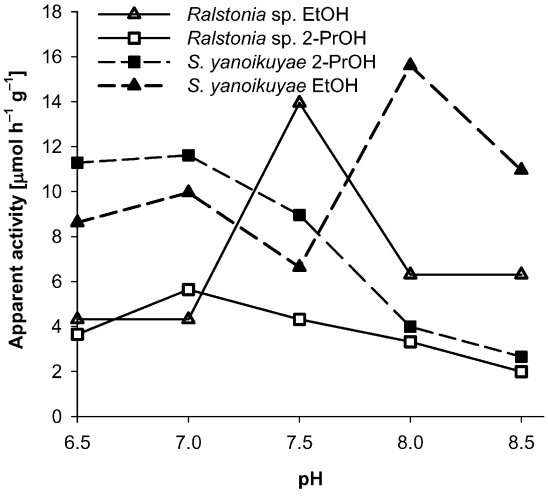


Figure 1. Profile of activity/pH for the reduction of **6a** (10 g L^{-1}, t = 24 h) by hydrogen transfer with the use of 2-propanol (14% v/v) or ethanol (8% v/v) as cosubstrate.

Optimization of the concentration of the cosubstrate showed that 5% v/v cosubstrate led to the highest apparent activity for *Sphingobium* for both cosubstrates and for *Ralstonia* by employing 2-propanol (Figure 2). An exception was *Ralstonia* sp. when employing ethanol: in this case, 10% v/v led to the highest apparent activity. As can be deduced from Figure 2, the usage of cosubstrate led to increased conversion, before activity diminished probably due to inhibition or deactivation. In the case where no cosubstrate was used, also no activity (*Ralstonia* sp.) or significantly reduced activity (*Sphingobium yanoikuyae*) was observed.

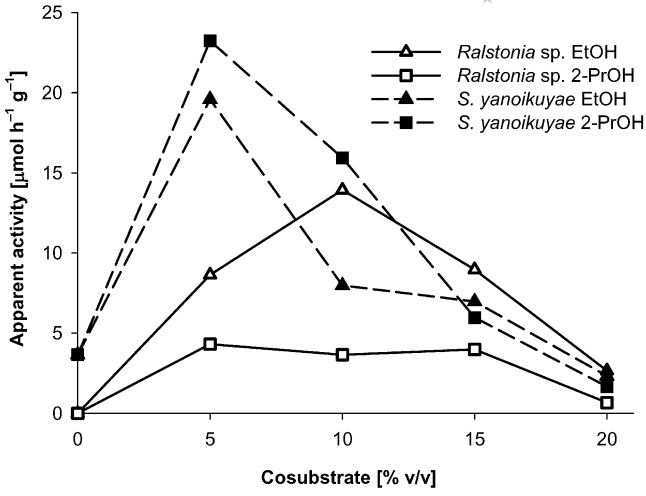


Figure 2. Apparent activity at varied concentration of cosubstrate 2-propanol or ethanol for the reduction of **6a** (10 g L^{-1}, t = 24 h, Tris-HCl buffer 50 mM, pH 7.5).

After optimization of the reaction parameters, several 1-phenyl-1-alkanones **1a**–**5a** differing in the length of the alkyl chain were chosen as test substrates and reduced to the corresponding alcohols by employing 2-propanol, as well as ethanol, as cosubstrates (Table 3).

A clear effect of the size of the alkyl chain could be observed for both strains as well as for both cosubstrates: substrates bearing a more bulky alkyl group (**2a**–**5a**) were transformed faster than the small–bulky ketone acetophenone (**1a**), indicating the expected preference of these strains to reduce bulky–bulky ketones. Especially, *Ralstonia* sp. transformed the more bulky–bulky ketone **2a** 30 times faster than small–bulky ketone **1a** when employing ethanol. In general, apparent activities were slightly lower with ethanol relative to those with 2-propanol. To our delight, the stereopreference of the reduction of ketones **3a**–**5a** led to the (*S*) enantiomer, which was complementary to the results obtained with *Rhodococcus* strain/ethanol; thus, the identified biocatalysts allowed the synthesis of both enantiomers in enantioenriched form. Comparison of the *ee* values

Table 3. Reduction of 1-phenyl-1-alkanones by using lyophilized cells of *Ralstonia* sp. and *Sphingobium yanoikuyae* with the use of 2-propanol or ethanol as cosubstrate.[a]

Microorganism	Substrate	2-PrOH		EtOH	
		App. activity [µmol h^{-1} g^{-1}]	*ee* [%][b]	App. activity [µmol h^{-1} g^{-1}]	*ee* [%][b]
Ralstonia sp. DSM 6428	**1a**	6.9	40 (*S*)	0.3	n. d.
Ralstonia sp. DSM 6428	**2a**	13.3	53 (*S*)	10.2	60 (*S*)
Ralstonia sp. DSM 6428	**3a**	15.7	84 (*S*)	14.7	87 (*S*)
Ralstonia sp. DSM 6428	**4a**	13.4	77 (*S*)	11.6	90 (*S*)
Ralstonia sp. DSM 6428	**5a**	17.4	75 (*S*)	11.8	95 (*S*)
S. yanoikuyae DSM 6900	**1a**	5.3	n.d.	9.1	49 (*S*)
S. yanoikuyae DSM 6900	**2a**	18.1	66 (*S*)	15.2	59 (*S*)
S. yanoikuyae DSM 6900	**3a**	17.9	84 (*S*)	17.7	87 (*S*)
S. yanoikuyae DSM 6900	**4a**	16.9	86 (*S*)	18.4	79 (*S*)
S. yanoikuyae DSM 6900	**5a**	18.6	97 (*S*)	15.6	89 (*S*)

[a] See Experimental Section for details. [b] Measured by GC or HPLC on chiral stationary phases, see details in Supporting Information. n.d. = not determined.

obtained by employing ethanol or 2-propanol, *Ralstonia* sp. showed higher stereoselectivities with ethanol than with 2-propanol, whereas the opposite was observed in the case of *Sphingobium yanoikuyae*. Thus, by employing *Ralstonia* sp. and ethanol, the most bulky ketones **4a** and **5a** were transformed with *ee* values ≥90% to the corresponding alcohol, whereas by employing *Sphingobium yanoikuyae* and 2-propanol the (*S*)-alcohol **5b** was obtained with 97% *ee* The varying *ee* values under the different conditions could be attributed either to the presence of different ADHs with opposite stereoselectivities and cosubstrate preferences or structural changes in the involved enzymes under varied reaction conditions.

Conclusions

Only few reports deal with the biocatalytic reduction of this type of bulky–bulky aryl alkyl substrates. Salvi and Chattopadhyay performed related reduction reactions by using the fungus *Rhizopus arrhizus*, and although selectivities were good (92% *ee*), the reactions took several days.[13] Homann et al. obtained with different fungi of the genus *Aspergillus* and *Mucor* enantiopure (*S*)-**4b** at moderate substrate concentrations.[3d] Horiuchi and coworkers showed the bioreduction of these ketones by using red algae, but alcohols were obtained after several days of reaction time with moderate stereoselectivities.[14] Recently, Zhu and Hua showed the efficient reduction of bulky–bulky ketones such as isopropyl-, *tert*-butyl-, and cycloalkyl phenyl ketones by employing a carbonyl reductase from *Sporobolomyces salmonicolor*; nevertheless, the catalyst gave nonsatisfactory *ee* values for linear alkyl–phenyl ketones, for example, (*S*)-**5b** with 34% *ee*[4c] None of the above reports involve a "substrate-coupled" approach. We have successfully identified whole-cell biocatalysts that can accept bulky ketones at elevated substrate concentrations (10 g L^{-1}) under the "substrate-coupled" conditions by using 2-propanol or ethanol as cosubstrates. For alcohols **3b**–**5b**, both enantiomers are accessible by choosing the appropriate biocatalyst.

Experimental Section

General Methods: Ketones **1a**–**6a**, racemic alcohols **1b** and **2b**, and (*R*)- and (*S*)-**3b** were commercially available either from Sigma–Aldrich–Fluka (Vienna, Austria) or Lancaster (Frankfurt am Main, Germany). Racemic compounds **4b**, **5b**, and **6b** were synthesized by conventional reduction from the corresponding ketones ($NaBH_4$, MeOH, room temperature).[10e] All other reagents and solvents were of the highest quality available.

Lyophilized cells of *Ralstonia* sp. DSM 6428, *Sphingobium yanoikuyae* DSM 6900, and *Rhodococcus ruber* DSM 44541 were obtained by cultivation in 250 mL of a complex medium [10 g L^{-1} yeast extract (Oxoid L21), 10 g L^{-1} bacteriological peptone (Oxoid L37), 10 g L^{-1} glucose (Fluka 49150), 2 g L^{-1} NaCl (Roth 9265.1), 0.15 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Fluka 63140), 1.3 g L^{-1} NaH_2PO_4 (Fluka 71496), 4.4 g L^{-1} K_2HPO_4 (Merck 5101), and distilled water] in one liter baffled shake flasks at 30 °C at 120 rpm. After 3 d, the cells were harvested by centrifugation (8000 rpm, 20 min), washed with phosphate buffer (50 mM, pH 7.5), shock frozen in liquid nitrogen and lyophilized.

General Method for the Biocatalytic Reduction of Ketones by Employing Lyophilized Cells of *Ralstonia sp.* DSM 6428: Lyophilized cells of *Ralstonia* sp. (20 mg), stored at 4 °C, were rehydrated in Tris/HCl buffer (600 μL, 50 mM, pH 7 with 2-propanol or pH 7.5 with ethanol) for 30 min at 30 °C and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, 2-propanol (32 μL, 5% v/v) or ethanol (67 μL, 10% v/v) and the corresponding ketone (**1a**–**6a**, 10 g L^{-1}) were added. Reactions were shaken at 30 °C and 120 rpm for 24 h and stopped by extraction with ethyl acetate (2 × 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried (Na_2SO_4). Conversions and enantiomeric excesses of the corresponding alcohols were determined by GC or HPLC analysis by employing a chiral stationary phase.

General Method for the Biocatalytic Reduction of Ketones by Employing Lyophilized Cells of *Sphingobium yanoikuyae* DSM 6900: Lyophilized cells of *Sphingobium yanoikuyae* (20 mg), stored at 4 °C, were rehydrated in Tris/HCl buffer (600 μL, 50 mM, pH 7 with 2-propanol or pH 8 with ethanol) for 30 min at 30 °C and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Cosubstrate 2-propanol or ethanol (32 μL, 5% v/v) and the corresponding ketone (**1a**–**6a**, 10 g L^{-1}) were added. Reactions were shaken at 30 °C and 120 rpm for 24 h and stopped by extraction with ethyl acetate (2 × 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried (Na_2SO_4). Conversions and enantiomeric excesses of the corresponding alcohols were determined by GC or HPLC analysis on chiral stationary phases.

Supporting Information (see footnote on the first page of this article): Further protocols for the biotransformations and GC and HPLC data are shown.

Acknowledgments

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