JOC Note

Stereoselective Bioreduction of Bulky-Bulky Ketones by a Novel ADH from *Ralstonia* sp.

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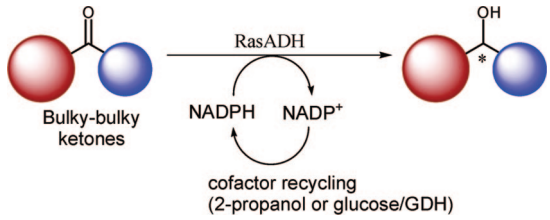

Ketones with two bulky substituents, named bulky-bulky ketones, as well as less sterically demanding ketones were successfully reduced to the corresponding optically highly enriched alcohols using a novel identified recombinant short-chain alcohol dehydrogenase RasADH from *Ralstonia* sp. DSM 6428 overexpressed in *E. coli*.

Stereoselective reduction of ketones using alcohol dehydrogenases (ADHs) has become an important method for industrial preparation of optical pure alcohols.[1] Unfortunately, most of the biocatalytically applicable ADHs show a rather narrow substrate pattern: preferentially ketones are reduced which bear at least one small substituent, thus a sterically nondemanding group like methyl, ethyl, azido-, cyano-, or chloromethyl.[1] Very recently, purified or overexpressed ADHs have been applied for the reduction of bulky-bulky ketones—noncyclic sterically impeded ketones[2]—whereby mainly activated bulky-bulky ketones bearing α- or β-ketoesters have been transformed.[3] However, the stereoselective reduction of bulky aryl alkyl ketones has been scarcely studied.[4] For instance, Zhu and Hua described a NADPH-dependent carbonyl reductase from *Sporobolomyces salmonicolor* able to reduce aryl long-chain alkyl or cyclopropyl derivatives.[5] Besides the limited knowledge about ADHs accepting bulky-bulky ketones another limitation is the low ketone concentration generally applied and the commonly moderate stereoselectivities obtained for these subset of substrates.

In a screening, we identified the wild-type strain *Ralstonia* sp. DSM 6428 capable of transforming sterically hindered ketones. Here, we report the identification of the involved enzyme and a study of its substrate spectrum using recombinant enzyme overexpressed in *Escherichia coli*.

For the identification of the corresponding ADH a gene library was constructed[6] and screened on filter paper for increased NADPH fluorescence[7] during oxidation of 1-phenyl-1-propanol in the presence of $NADP^+$. A putative short-chain dehydrogenase gene was identified (arbitrarily called RasADH), amplified by PCR, and cloned into the plasmid pEamTA.[8] The enzyme RasADH was subsequently overexpressed in *E. coli* DH5α which was applied as lyophilized powder for biocatalytic transformations (denoted as *E. coli*/RasADH).

In biocatalytic reductions, the reducing equivalents can be provided by mainly two approaches: (i) in an "enzyme-coupled" approach a second (and preferably irreversible) enzymatic reaction is employed additionally to the alcohol dehydrogenase to shift the equilibrium to the desired product;[1,9,10] (ii) in the "substrate-coupled" or "biocatalytic hydrogen transfer" approach a single enzyme catalyzes the reduction of the ketone as well as the recycling of the cofactor simultaneously.[1,9,11] Until now, only two ADHs transforming bulky-bulky ketones have been described to work via hydrogen transfer.[3g,l] Testing *E. coli*/RasADH we were very pleased to notice that RasADH could

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(2) Bulky-bulky ketones possess two substituents which are larger than ethyl, azido-, cyano-, or halomethyl groups. ADHs capable of reducing bulky-bulky ketones are not that commonly found in Nature, in contrast to those which reduce ketones with at least one small group (ethyl, azido-, cyano-, or halomethyl groups), the small-bulky ketones.

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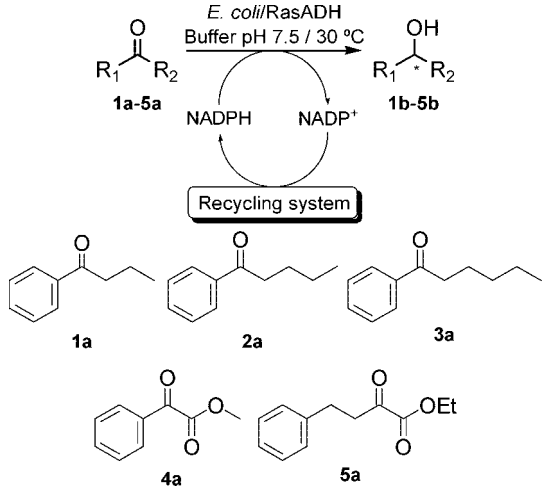
TABLE 1. Comparison of the Recycling System for the *E. coli*/RasADH-Catalyzed Reduction of Bulky-Bulky Ketones[a]



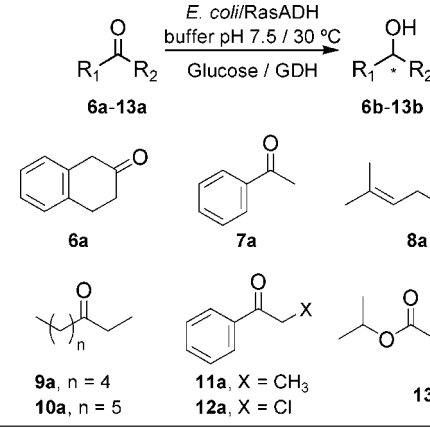
substrate	cofactor recycling systems: glucose/GDH rel rate (%)[b]	cofactor recycling systems: 2-propanol 10% v v^{-1} rel rate (%)[b]	ee[c] (%)
1a	49	21	>99 (*S*)
2a	8	1.8	>99 (*S*)
3a	7	7	>99 (*S*)
4a	100	9	96 (*S*)[d]
5a	40	3.4	64 (*S*)[d]

[a] Substrate concentration: 10 g L^{-1}. [b] 100% corresponds to 32.4 nmol substrate converted per min per mg *E. coli*/RasADH and were calculated from initial progress curves. [c] Measured by chiral HPLC or GC. [d] Change in CIP priority.

transform bulky-bulky ketones at the expense of 2-propanol, thus working via hydrogen transfer. Therefore, both cofactor recycling approaches can be applied for reduction with this enzyme.

Subsequently, we compared the two recycling systems, namely (i) the "enzyme-coupled" approach using glucose with glucose dehydrogenase (GDH) and (ii) the hydrogen transfer approach employing just 2-propanol as hydrogen source for the reduction of various bulky-bulky ketones (Table 1). It became clear that glucose/GDH was more efficient, since in all cases the measured activities were higher with GDH than with 2-propanol: for instance, in the case of **4a** and **5a** a 10-fold acceleration was observed using glucose/GDH. In contrast to the activity, the choice of the recycling system did not have any influence on the optical purity; thus, ee's were the same with both recycling systems. The bulky-bulky aryl alkyl ketones **1a**−**3a** were reduced to optically pure (*S*)-**1b**−**3b** (ee >99%). For substrates **2a** and **3a**, this is the highest ee ever obtained via biocatalytic reduction. For substrates **4a** and **5a**, a switch in stereopreference was observed compared to **1a**−**3a**. Obviously, the α-keto esters **4a** and **5a** bind in a different mode in the active site of the enzyme; thus, the aromatic moiety is not on the same side as in the case of substrates **1a**−**3a**.

TABLE 2. *E. coli*/RasADH-Catalyzed Reductions of Several Small-Bulky Ketones[a]



substrate	rel rate[b] (%)	ee[c] (%)
6a	38	73 (*R*)
7a	30	>99 (*S*)
8a	10	82 (*S*)
9a	26	98 (*S*)
10a	11	98 (*S*)
11a	64	>99 (*S*)
12a	98	>99 (*R*)[d]
13a	51	37 (*S*)[d]

[a] Substrate concentration: 10 g L^{-1}. [b] 100% corresponds to 32.4 nmol substrate converted per min per mg *E. coli*/RasADH and were calculated from initial progress curves. [c] Measured by chiral GC. [d] Change in CIP priority.

Since the novel catalyst showed excellent stereoselectivity for bulky-bulky ketones, we wondered how the stereoselectivity would change for the reduction of small-bulky ketones, ketones bearing a methyl, ethyl, or chloromethyl substituent. Since bulky-bulky ketones like **3a** were transformed, we expected that this catalyst should possess a rather spacious active site allowing small-bulky ketones to bind in various modes and conformations. Therefore, we anticipated that smaller ketones would be reduced with lower stereoselectivity. Although this proved to be true for various substrates like alkyl methyl ketone **8a** or chloromethyl-substituted ketone **13a** (Table 2), we were astonished to notice that other small-bulky substrates like acetophenone **7a** or ω-chloroacetophenone **12a** were reduced with perfect stereoselectivity. As an example, the reduction of **12a** was performed on 60 mg scale, yielding the corresponding optically pure (*R*)-alcohol (ee > 99%) at >99% GC conversion with 91% isolated yield. Ketones bearing a bulky group and an ethyl moiety (**9a**−**11a**) were also reduced with excellent stereoselectivity. The absolute configuration obtained for the open-chain

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alcohols **7b**−**12b** correlated in all cases with Prelog's rule,[12] except for the β-ketoester **13a**. For this type of substrate, again a switch in stereopreference was observed as already noticed for **4a** and **5a**. These results showed that the novel alcohol dehydrogenase RasADH transforms different types of substrates with high stereoselectivity including bulky-bulky as well as small-bulky ketones.

In summary, we have reported the identification and overexpression of a novel short chain dehydrogenase originated from *Ralstonia* sp. DSM 6428 which accepts bulky-bulky α-keto ester derivatives as well as small-bulky ketones, and is one of the first examples that transforms bulky-bulky alkyl aryl ketones showing very high stereoselectivities in most of the cases studied. Thus, for the reduction of such sterically demanding substrates like **2a** and **3a** perfect ee's (>99%) were achieved, which was never obtained before via biocatalytic means.[5,13] The obtained chiral alcohols are precursors for valuable products: e.g., enantiopure halohydrin **12b** has been used as a precursor of several pharmaceutical compounds[14] like for the synthesis of (*R*)-salmeterol,[15] fluoxetine, or nisoxetine.[16]

Experimental Section

General methods. *Ralstonia* sp. DSM 6428 is commercially available from the German culture collection DSMZ. Ketones **1a**−**13a** and racemic alcohols **4b**, **6b**−**11b**, (*R*)-**1b**, (*S*)-**1b**, (*R*)-**5b**, (*S*)-**5b**, and 2-propanol were commercially available. Racemic compounds **2b**, **3b**, **12b**, and **13b** were synthesized by conventional reduction from the corresponding ketones ($NaBH_4$, MeOH, room temperature).[17] GDH from *Bacillus megaterium* was purchased from Jülich Fine Chemicals. All other reagents and solvents were of the highest quality available.

General Protocol for the Biocatalytic Reduction of Ketones Employing Lyophilized Cells of *E. coli*/RasADH Using GDH. Lyophilized cells of *E. coli*/RasADH (20 mg) were rehydrated in Tris-HCl buffer (600 μL, 50 mM, pH 7.5, 1 mM NADPH) for 30 min at 30 °C and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, the corresponding ketone (10 g L^{-1}), glucose (5 equiv), and GDH (1 U) 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 a chiral stationary phase (see the Supporting Information).

General Protocol for the Biocatalytic Reduction of Ketones Employing Lyophilized Cells of *E. coli*/RasADH Using 2-Propanol. Lyophilized cells of *E. coli*/RasADH (20 mg) were rehydrated in Tris-HCl buffer (600 μL, 50 mM, pH 7.5, 1 mM NADPH) for 30 min at 30 °C and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, 2-propanol (67 μL, 10% v v^{-1}) and the corresponding ketone (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 a chiral stationary phase (see the Supporting Information).

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Supporting Information Available: Procedures of molecular biology, analytics, and experimental procedures are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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