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Rapid identification of new bacterial alcohol dehydrogenases for (*R*)- and (*S*)-enantioselective reduction of ß-ketoesters†

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New bacterial alcohol dehydrogenases with high and complementary enantioselectivity for the reduction of ethyl 3-keto-4,4,4-trifluorobutyrate 1 and methyl 3-keto-3-(3′-pyridyl)-propionate 3 have been rapidly identified by use of a new methodology consisting of preselection of micro-organisms based on degradation ability and high-throughput screening with a miniaturized system coupled with fast analysis of enantioselectivities.

Asymmetric reduction of carbonyl groups provides a simple route to optically active alcohols that are useful synthons and pharmaceutical intermediates. Despite the effectiveness of metal catalysts in such transformations,[1] enzymatic reduction[2] remains a useful alternative: it enables not only green but also economically competitive processes through the use of recombinant biocatalysts. Many alcohol dehydrogenase-containing microorganisms, including baker's yeast,[3a] have been found to catalyze enantioselective reduction of carbonyl groups and have been applied in organic synthesis.[3] It is, however, often necessary and a significant challenge to discover new highly enantioselective alcohol dehydrogenases for specific reductions. It is also very difficult to find appropriate enzymes with complementary enantioselectivities.

The discovery of new enantioselective biocatalysts for a given transformation can be achieved by many methods such as the screening of collections of wild type microorganisms or clonal libraries, *in vitro* evolution, and site-directed mutagenesis. While the latter requires knowledge of structure and catalytic mechanism of the enzyme, the first two methods need high-throughput and sensitive enantioselectivity assays. For initial discovery, high-throughput screening of microorganisms is the method of choice. Screening for new enantioselective alcohol dehydrogenases is often performed with randomly collected microorganisms one by one in shaking flasks, which is time-consuming. Fast screening of known microorganisms containing alcohol dehydrogenases was recently reported,[4] which is, however, limited to the known biocatalysts. To quickly discover new biocatalysts, we have developed a practical approach in which we first select microorganisms possibly containing the desired alcohol dehydrogenases based on their degradation ability, and then rapidly screen these strains for high enantioselectivities with a miniaturized system. Here, we describe our success with this approach for the fast identification of new bacterial alcohol dehydrogenases for highly (*R*)- and (*S*)-enantioselective reduction of β-ketoesters.

Reduction of ethyl 3-keto-4,4,4-trifluorobutyrate **1** to optically active ethyl 3-hydroxy-4,4,4-trifluorobutyrate **2** was chosen as the target reaction (Fig. 1). While (*R*)-**2** is a useful intermediate for the preparation of the antidepressant Befloxatone,[5] both enantiomers of **2** are versatile synthons for the preparation of other useful enantiopure fluorine-containing compounds such as glycidic esters and α-amino-β-hydroxyesters.[6] Enzymatic reduction of **1** has been intensively investigated, but the enantioselectivities are unsatisfactory: reduction with baker's yeast yielded (*R*)-**2** in 52% ee without additive,[7a] (*R*)-**2** in 80% ee in the presence of allyl bromide,[7b] and

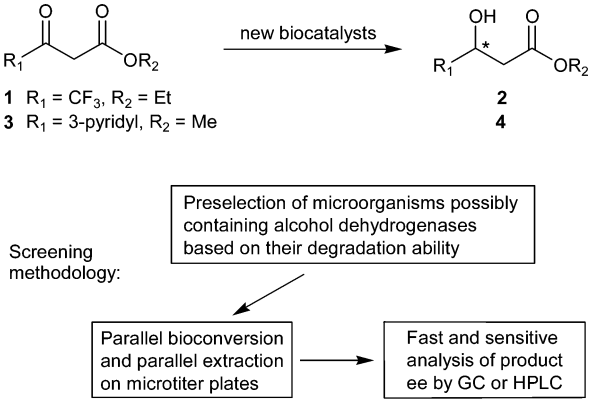

Fig. 1 Asymmetric bioreduction of 3-ketoesters: target reactions and screening methodology.

(*S*)-**2** in 65% ee in the presence of allyl alcohol,[7b] respectively; and reduction with *Candida utilis* gave (*S*)-**2** in 59% ee.[7c]

To discover new alcohol dehydrogenases with better enantioselectivity for the target reduction, we preselected a collection of amino acid-degrading microorganisms as a source of biocatalysts for screening, since alcohol dehydrogenases are often involved in their degradation pathways. 162 phenylalanine- and tyrosine-degrading bacteria were isolated in our laboratory from soil samples and used for the screening. Screening experiments were carried out in 96 deep wells of a microtiter plate which allows for parallel inoculation, growth, and bioconversion.[8] Biotransformations of compound **1** (2 mM) were performed in 0.55 ml 50 mM potassium phosphate buffer (pH = 8.0) at 25 °C and 300 rpm for 2 h. The reaction mixtures were extracted in parallel into $CHCl_3$ (0.45 ml) in microtiter wells, which quickly produces samples in sufficient volume for analysis. The product ee was quantified by GC on a chiral column (Lipodex-A) with an autosampler. The analysis is fast with a retention time of 11.9 and 12.2 min for (*S*)- and (*R*)-**2**, respectively. The analysis is also very sensitive and allows for product ee determinations in samples with only 2% conversion. 106 out of 162 phenylalanine- and tyrosine-degrading strains were found to catalyze the reduction of **1** to **2**. High enantioselectivity was observed with 13 strains, as shown in Table 1: 8 strains generated (*S*)-**2** in > 80% ee, whereas 5 strains produced (*R*)-**2** in > 80% ee. Among positive strains, *Klebsiella pneumomiae* Phe-E4 and *Bacillus pumilus* Phe-C3 catalyzed the reduction of **1** giving (*S*)-**2** in 90% ee and (*R*)-**2** in 86% ee, respectively. The former one demonstrated anti-Prelog's selectivity,[9] which is rather rare among alcohol dehydrogenases.

This screening methodology was further applied to discover new enantioselective enzymes for the reduction of other types of β-ketoesters. Reduction of methyl 3-keto-3-(3′-pyridyl)-propionate **3** to optically active methyl 3-hydroxy-3-(3′-pyridyl)-propionate **4** was selected as the second target, since substrate **3** represents a β-ketoester with a hetero-aromatic substitution and product **4** is a useful intermediate for the preparation of a GPIIb/IIIa antagonist RWJ-53308.[10] Thus far, no enzymatic system has been reported for such reduction. Screening of 162 phenylalanine- and

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Table 1 Screening of amino acid-degrading bacteria for asymmetric reduction of **1** to **2** and of **3** to **4**

		No. of positive strains					
No. of strains screened		**1** → **2**	Producing (*S*)-**2** in > 80% ee	Producing (*R*)-**2** in > 80% ee	**3** → **4**	Producing (−)-**4** in > 80% ee	Producing (+)-**4** in > 80% ee
PA[a]	78	48	2	1	57	3	9
TY[b]	84	58	6	4	66	7	10
Total	162	106	8	5	123	10	19

[a] Phenylalanine-degrading strains. [b] Tyrosine-degrading strains.

Table 2 Preparation of (*S*)-**2**, (*R*)-**2**, (−)-**4**, and (+)-**4** by reduction of **1** and **3** with *K. pneumomiae* Phe-E4, *B. pumilus* Phe-C3, *Pseudomonas* sp. Tyr-F10, and *Acinetobacter* sp. Tyr-B12, respectively

Strain	Substrate	Concentration (mM)	Scale (ml)	Cell density (g l^{-1})	Product	Time (h)	Conversion (%)	Yield (%)	(mg)	Ee (%)
Phe-E4	**1**	10	350	6.0	(*S*)-**2**	28	96	81	514	90
Phe-C3	**1**	20	189	13	(*R*)-**2**	48	98	84	565	90
Tyr-F10	**3**	5.0	130	6.8	(+)-**4**	9	99	82	94	87
Tyr-B12	**3**	15	60	4.5	(−)-**4**	9	96	87	138	98

tyrosine-degrading strains for the reduction of **3** (2 mM) was performed with the same miniaturized system as described above. After parallel extraction, samples were analyzed by HPLC with a chiral column (Chiralcel OB-H), UV detection, and an autosampler, which allows for a sensitive determination of product ee with a retention time of 15.6 and 21.2 min for (+)- and (−)-**4**, respectively. 123 positive strains were found, and many of them showed high and complementary enantioselectivities: 10 strains afforded (−)-**4** in > 80% ee, whereas 19 others gave (+)-**4** in > 80% ee. *Pseudomonas* sp. Tyr-F10 and *Acinetobacter* sp. Tyr-B12 are the best among them, catalyzing the reduction of **3** to give (+)-**4** in 87% ee and (−)-**4** in 94% ee, respectively.

The best four strains, *K. pneumomiae* Phe-E4, *B. pumilus* Phe-C3, *Pseudomonas* sp. Tyr-F10, and *Acinetobacter* sp. Tyr-B12 were selected for further investigation. They were able to grow to a high cell density with tyrosine or phenylalanine as carbon source. Bioconversions with resting cells were examined on a 10-ml scale. The specific activities of *K. pneumomiae* Phe-E4 and *Acinetobacter* sp. Tyro-B12 are quite high: 23 and 25 U/g cdw (U: μmol min^{-1}; cdw: cell dry weight) during the first 30 min, respectively. Relatively low activities were observed for *Pseudomonas* sp. Tyr-F10 (5.1 U/g cdw) and for *B. pumilus* Phe-C3 (2.9 U/g cdw). In the latter case, the activity could be increased to 7.7 U/g cdw by addition of 2% glucose to improve the cofactor regeneration. The enantioselectivities observed in these experiments are nearly the same as those obtained in the screening experiments for all four strains, demonstrating the reliability of the screening methodology.

Frozen/thawed cells of these strains were used for the preparative biotransformation of **1** and **3**. As shown in Table 2, (*S*)-**2**, (*R*)-**2**, (+)-**4**, and (−)-**4** were easily prepared in 90, 90, 87, and 98% ee, respectively, and all in > 80% yield.

Biotransformations with cell-free extracts of these strains suggested that the enzymes responsible for the desired reduction are all soluble alcohol dehydrogenases. The NADPH-dependent alcohol dehydrogenase from *B. pumilus* Phe-C3 demonstrated better enantioselectivity than frozen/thawed cells, giving the product (*R*)-**2** in 97% ee, while the alcohol dehydrogenases from the other three strains showed the same enantioselectivity as the corresponding frozen/thawed cells.

In summary, we have developed a practical methodology for fast identification of new enantioselective alcohol dehydrogenases by: 1) preselection of microorganisms based on their degradation ability; and 2) high-throughput screening with a miniaturized system, coupled with fast and sensitive analysis of enantioselectivities. By use of this method, we have identified four novel bacterial alcohol dehydrogenases from phenylalanine- and tyrosine-degrading strains for the highly (*R*)- and (*S*)-enantioselective reduction of ß-ketoesters **1** and **3**, respectively. The synthetic usefulness of these enzymes has been demonstrated in the preparation of both enantiomers of 3-hydroxyesters **2** and **4** in high ee and high yields.

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