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Biocatalytic racemization of α-hydroxycarboxylic acids using a stereo-complementary pair of α-hydroxycarboxylic acid dehydrogenases

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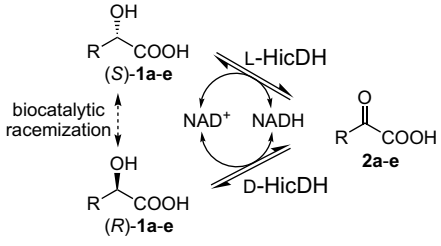
ABSTRACT

Biocatalytic racemization of aliphatic, (aryl)aliphatic and aromatic α-hydroxycarboxylic acids was achieved via a reversible oxidation-reduction sequence using a pair of stereo-complementary Prelog- and anti-Prelog D- and L-α-hydroxyisocaproate dehydrogenases from *Lactobacillus confusus* DSM 20196 and *Lactobacillus paracasei* DSM 20008, resp., overexpressed in *Escherichia coli*. The mild reaction conditions ensured essential 'clean' isomerization, undesired 'over-oxidation' of the substrate forming the α-ketoacid could be suppressed by exclusion of O_2 and adjustment of the NAD^+/NADH-ratio.

1. Introduction

Racemization is an important (but often overlooked) tool for the recycling of unwanted stereoisomers derived from kinetic resolutions and the key to dynamic (kinetic) resolution processes (DKR).[1] The majority of DKRs published so far either rely on (transition) metal-catalysis,[1a–c,e,2] on free radicals[3] or on (zeolite-type) acid-base catalyzed isomerization.[4–6] Although enzymatic racemization plays a somewhat minor role in this context, it shows great potential in view of the mild reaction conditions and the excellent compatibility of enzymes with each other.[7] In search for clean and efficient enzymatic racemization of stereochemically stable α-hydroxycarboxylic acids we found that the desired activity was widespread among *Lactobacillus* spp.,[8] in particular *Lactobacillus paracasei* DSM 20008, DSM 20207 and *Lactobacillus delbrueckii* DSM 20074, which allowed the isomerization of a wide range of aliphatic, (hetero)aryl- and aryl-aliphatic α-hydroxycarboxylic acids. Subsequent inhibitor-studies using a cell-free extract of *L. paracasei* DSM 20207 proved that the involvement of a putative racemase could be ruled out and suggested that the racemization proceeded through a reversible oxidation–reduction sequence via the corresponding α-ketoacid as intermediate catalyzed by NAD(P)H-dependent dehydrogenases (Scheme 1).[9] Kinetic analysis of a model system employing a set of stereo-complementary alcohol dehydrogenases possessing Prelog- and anti-Prelog selectivity revealed that clean isomerization of *sec*-alcohols was indeed feasible.[10]



HicDH = α-Hydroxyisocaproate Dehydrogenase

Substrate **1**	**a**	**b**	**c**	**d**	**e**
R	$(CH_3)_2CH\text{-}CH_2$-	*n*-C_4H_9-	*n*-C_6H_{13}-	Ph-CH_2-	Ph-

Scheme 1. Enzymatic racemization of α-hydroxycarboxylic acids using D- and L-α-hydroxyisocaproate dehydrogenases (HicDH).

In order to extend this system to α-hydroxycarboxylic acids, we conducted a search for a suitable set of stereo-complementary α-hydroxycarboxylic acid dehydrogenases (α-ketoacid reductases).

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Detailed analysis of the literature suggested two candidates: L-[11] and D-2-hydroxyisocaproate dehydrogenase[12] (HicDH) from *Lactobacillus confusus* DSM 20196 and *L. paracasei* DSM 20008, respectively, which were investigated for the stereoselective reduction of α-ketoacids.[10,11] Moreover, their substrate profile (in the reduction mode) nicely matched that of the racemization-activity of *Lactobacillus* spp.[8] This unexpected observation prompted us to use D- and L-HicDH as stereo-complementary components for the design of a redox-based racemization system for α-hydroxycarboxylic acids by mimicking a 'racemase'. D- and L-HicDH from *Lactobacillus* DSM 20008 and DSM 20196 were cloned and overexpressed into *E. coli*[13] (details will be published in a forthcoming paper).

2. Results and discussion

The starting α-hydroxyacid enantiomer **1** was oxidized by the corresponding HicDH at the expense of NAD^+ to furnish the corresponding prochiral α-ketoacid **2** as transient species, which in turn was stereoselectively reduced by the second stereo-complementary HicDH to produce the corresponding mirror-image enantiomer (Scheme 1). Since the cofactor is recycled internally, only catalytic amounts are required and external recycling is not required. Due to the reversibility of both redox reactions, the overall result is an equilibrium between both α-hydroxyacid enantiomers (*R*)- and (*S*)-**1** via α-ketoacid **2**. Moreover, the amount of α-ketoacid **2** can be kept at a minimum by exclusion of O_2 and by adjusting the ratio of NADH/NAD^+ in favor of NADH in order to shift the equilibriums towards the α-hydroxycarboxylic acid **1**.[10] Overall, the racemization proved to be essentially 'clean' and no by-products were formed, except for traces of the intermediate α-ketoacid **2** (≤3%).

In order to analyze the proper functioning of the system, racemization of (*R*)- and (*S*)-mandelic acid **1e** was investigated using both HicDH clones separately and in tandem using a 1:1 mixture of D- and L-HicDH (Fig. 1). As expected, only negligible isomerization occurred in presence of a single HicDH, while good racemization rates were obtained with the tandem-system (Fig. 1).

α-Hydroxycarboxylic acids are widespread structural building blocks of natural products and are versatile chiral synthons for the synthesis of pharmaceuticals.[14] 3-Phenyllactate **1d** is used as chiral building block for the synthesis of bioactive compounds, such as natural antibiotic agents.[15] Mandelate **1e** serves as building block for the production of semisynthetic penicillins, cephalosporins[16] and anti-obesity agents.[17] In order to prove the applicability of the method, HicDH-catalyzed racemization of a set of structurally diverse substrates was investigated by using a series of (linear and branched) aliphatic **1a**–**c**, aryl-aliphatic **1d** and aromatic **1e** α-hydroxycarboxylic acids.

The difference in racemization rates of enantiomers is a common phenomenon in enzymatic racemization and is a result of the difference in the individual K_M- and k_{cat}-values for enantiomers for a given enzyme.[18] Excellent racemization rates (50–96% relative to the natural substrate **1a**) were obtained for the aliphatic (entries 2–5, Table 1) and aryl-aliphatic α-hydroxycarboxylic acids (entries 6–7). Mandelate (entries 8–9) was racemized more slowly (average relative rate of both enantiomers ca. 25%), which illustrates the flexibility of this system.

Table 1
Relative racemization rates of substrates **1a**–**e**

Entry	Substrate	R	Relative rate[a] [%]
1	(*S*)-**1a**	$(CH_3)_2CH–CH_2–$	100
2	(*S*)-**1b**	*n*-C_4H_9–	74
3	(*R*)-**1b**	*n*-C_4H_9–	65
4	(*S*)-**1c**	*n*-C_6H_{13}–	81
5	(*R*)-**1c**	*n*-C_6H_{13}–	50
6	(*S*)-**1d**	Ph–CH_2–	82
7	(*R*)-**1d**	Ph–CH_2–	96
8	(*S*)-**1e**	Ph–	32
9	(*R*)-**1e**	Ph–	15

[a] Relative rates were measured from the slope of the decline of ee versus time at the onset of the reaction (conversion <5%), values are expressed as % relative to the natural substrate **1a**, which was set as standard (100%).

3. Conclusion

In summary, efficient enzymatic racemization of a set of aliphatic, aryl-aliphatic and aromatic α-hydroxycarboxylic acids was achieved via a reversible oxidation-reduction sequence using a pair of stereo-complementary α-hydroxycarboxylic acid dehydrogenases, i.e. D- and L-α-hydroxyisocaproate dehydrogenases from *L. confusus* DSM 20196 and *L. paracasei* DSM 20008, resp., which were cloned and overexpressed in *E. coli*. Exclusion of molecular oxygen and careful adjustment of the NAD^+/NADH-ratio prevented undesired 'over-oxidation' of the substrate and reduced the formation of the non-chiral α-ketoacid intermediate (≤3%).

4. Experimental

4.1. General

The following commercially available chemicals were used as received: *rac*- and (*S*)-2-hydroxyisocaproic acid **1a**, *rac*-2-hydroxyhexanoic acid **1b**, *rac*-2-hydroxyoctanoic acid **1c** (TCI), *rac*-, (*R*)- and (*S*)-phenyllactic acid **1d** (Sigma Aldrich), *rac*-, (*R*)- and (*S*)-mandelic acid **1e** (Fluka), *Candida antarctica* lipase immobilized on acrylic resin was purchased from Sigma (L 4777), and *C. antarctica* lipase B

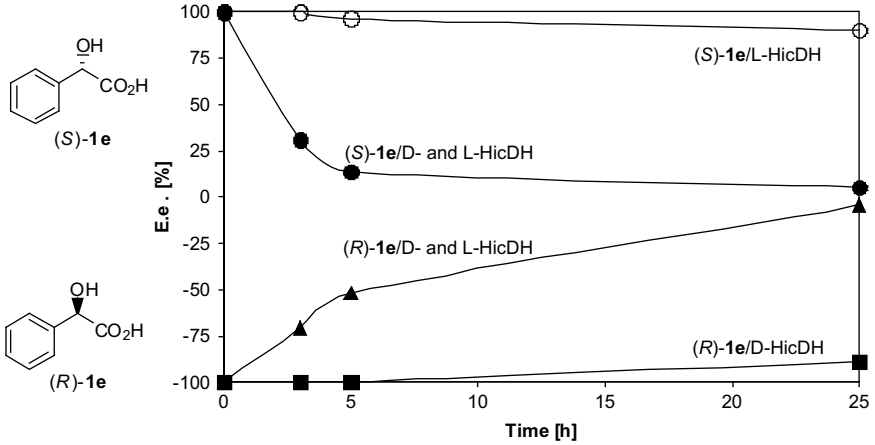

Figure 1. Time course of the racemization of (*R*)- and (*S*)-mandelic acid **1e** in presence of a single (D- or L-) or tandem (D- and L-) HicDH system.

(Novozym 435) was from Novozymes. The cloning and overexpression of D-hydroxyisocaproate dehydrogenase (HicDH) from *L. paracasei* DSM 20008 and L-HicDH from *L. confusus* DSM 20196 in *E. coli* will be published in a forthcoming paper. NMR spectra were measured in $CDCl_3$ using a Bruker AMX spectrometer at 360 (^1H) and 90 (^{13}C) MHz. Chemical shifts are reported relative to TMS (δ 0.00) and coupling constants (J) are given in Hz. TLC plates were run on silica gel Merck 60 (F_{254}) and compounds were visualized by spraying with Mo-reagent [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (100 g/L), $Ce(SO_4)_2\cdot 4H_2O$ (4 g/L) in H_2SO_4 (10%)]. Conversion and enantiomeric excess were determined via GC or HPLC on a chiral stationary phase. GC analyses were carried out on a Varian 3800 gas chromatograph equipped with a FID detector using H_2 as carrier gas (14 psi) and an Astec Chiraldex B-TA column (column A, 40 m, 0.25 mm, 0.12 μm film). Temperature of the injector and detector were 180 and 200 °C, respectively, using a split ratio of 90:1. HPLC analyses were carried out on a Shimadzu HPLC system equipped with a Chiralpak AD column (column B, 25 cm, 0.46 cm). Optical rotation values ($[\alpha]_D^{20}$) were measured on a Perkin–Elmer polarimeter 341 at 589 nm (Na-line) in a 1 dm cuvette and are given in units of [(deg×mL)/(g×dm)].

4.2. HicDH-clone maintenance

E. coli clones (L-HicDH and D-HicDH) were maintained on LB-plates (16 g/L agar) containing ampicillin (100 mg/L), spectinomycin (20 mg/L) and chloramphenicol (100 mg/L), resp. Sub-culturing was performed every week, the plates were left in an incubator for 7–9 h at 37 °C, long-term storage was at +4 °C.

4.3. Growth of microorganisms

E. coli clones (D- and L-HicDH) were grown in a LB-medium which was prepared by sterilising a 1L solution of the following components in 3 baffled 1L-flasks: Trypton (10 g/L), NaCl (5 g/L), yeast extract (5 g/L). A pre-culture was prepared by inoculating 125 mL of LB-medium containing the same concentrations of antibiotics as described above for the agar plates. The pre-culture was shaken at 120 rpm and 37 °C until an OD-value of 1 (600 nm) was reached. Afterwards the pre-culture was transferred into three baffled 1 L-flasks containing a total amount of 1 L of sterile LB-medium containing the antibiotics and inducers as follows: 100 mg/L ampicillin, 20 mg/L spectinomycin, 100 mg/L chloramphenicol, 0.5 g/L rhamnose and 100 μM IPTG. The cultures were shaken at 120 rpm and 37 °C overnight, then the cells were harvested by centrifugation (8000 rpm, 20 min), washed with potassium phosphate buffer (100 mM, pH 7), shock-frozen in liquid nitrogen, and lyophilized. Cells were stored at 4 °C and used as such for biotransformations.

4.4. General screening-procedure for the biocatalytic racemization

All racemization experiments were performed in glass vials capped with septums. For degassing, potassium phosphate buffer (100 mM, pH 7) was filtered using a Millipore filter (0.22 μm) attached to a glass bottle by applying a vaccum using a water-jet pump. The buffer solution was vigorously stirred for ca. 0.5 h during degassing, until no more bubbles were visible. The system was ventilated and maintained under an argon atmosphere. Lyophilized *E. coli* cells (D- and L-HicDH, 60 mg each; 120 mg D- or L-HicDH) were rehydrated in degassed potassium phosphate buffer (2.3 mL, 100 mM, pH 7) for 1 h at 30 °C on an orbit shaker at 120 rpm under an argon atmosphere. Aliquots of cofactor solution (100 μL, NADH/NAD^+ 5 mg/3 mg in 1 mL buffer) and substrate solution (15 mg **1a–e** in 150 μL buffer) were added. The mixture was shaken at 30 °C and 120 rpm under an argon atmosphere. Samples of 850 μL were withdrawn at intervals of 0.5–3 h, 4–6 h and 20–24 h depending on the substrate.

4.4.1. General work up

The reaction mixture was acidified with 6 M HCl (1 drop) and the cells were removed by centrifugation. The supernatant was extracted with ethyl acetate and the organic phase was dried over Na_2SO_4. In case of the aryl-alkyl **1d** and the aromatic substrate **1e** the determination of the conversion and enantiomeric excess was carried out by HPLC on a chiral stationary phase. The alkyl substrates **1a–c** were derivatized to their corresponding 2-acetoxyacids derivatives or methyl carboxylates, resp.

4.5. Synthesis of substrates

4.5.1. General procedure for lipase mediated kinetic resolution

(*R*)- and (*S*)-2-Hydroxyhexanoic acid **1b** and (*R*)- and (*S*)-2-hydroxyoctanoic acid **1c** were synthesized by lipase kinetic resolution in analogy to a known procedure[19] with the following modifications: To a solution of *rac*-2-hydroxyacid in *tert*-butyl methyl ether, were added vinyl acetate and lipase and the mixture was vigorously stirred at rt until a conversion of 50% was reached. The enzyme was removed by filtration and the solvent was evaporated under reduced pressure. Flash chromatography on silica gel Merck 60 (230–400 mesh) using CH_2Cl_2/MeOH/AcOH (gradient 95:5:0.1 to 70:30:0.1) resulted in optically pure (*S*)-2-acetoxyacid and non-reacted (*R*)-2-hydroxy acid **1b**,**c**.

4.5.2. General procedure for the hydrolysis step

A mixture of (*S*)-2-acetoxy acid (1 mmol) and K_2CO_3 (1 g) in MeOH (8 mL) was stirred at 0 °C until TLC analysis indicated complete hydrolysis. After acidification with HCl (3 M) to pH 2–3, the product was extracted three times with ethyl acetate, the organic layer was dried over Na_2SO_4, the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography on silica gel Merck 60 (230–400 mesh) using CH_2Cl_2/MeOH/AcOH (gradient 95/5/0.1 to 70/30/0.1).

*4.5.2.1. (R)-2-Hydroxyhexanoic acid **1b**.* Kinetic resolution of *rac*-2-hydroxyhexanoic acid (**1b**, 2.22 g, 17 mmol) in *tert*-butyl methyl ether (150 mL) using vinyl acetate (6.2 mL, 67 mmol, 4 equiv) and immobilized *C. antarctica* lipase (Sigma L 4777) (2.33 g) gave (*S*)-2-acetyoxyhexanoic acid (oil, 1.44 g, 48%) and (*R*)-2-hydroxyhexanoic acid **1b** as white solid (mp 62 °C, 844 mg, 38%, 85% ee). $[\alpha]_D^{20}$ −0.62 (*c* 1, $CHCl_3$), $[\alpha]_D^{20}$ −1.46 (*c* 7, EtOH);[20] ^1H NMR (360 MHz, $CDCl_3$): δ=0.93 (3H, t, *J*=7.0 Hz), 1.37–1.46 (4H, m), 1.71 (1H, m, CH_2–CHOH), 1.87 (1H, m, CH_2-CHOH), 4.28 (1H, dd, C*H*OH, *J*=7.4, 4.3 Hz); ^{13}C NMR (90 MHz, $CDCl_3$): δ=13.8, 22.3, 26.8, 33.8, 70.3, 179.7.

*4.5.2.2. (S)-2-Hydroxyhexanoic acid **1b**.* Hydrolysis of (*S*)-2-acetoxyhexanoic acid (1.44 g) with K_2CO_3 (8 g) in MeOH (65 mL) gave (*S*)-2-hydroxyhexanoic acid (**1b**, 1.04 g, 95%, 91% ee); $[\alpha]_D^{20}$ +0.90 (*c* 1, $CHCl_3$); ^1H NMR (360 MHz, $CDCl_3$): δ=0.92 (3H, t, *J*=7.0 Hz), 1.26–1.49 (4H, m), 1.66–1.74 (1H, m, CH_2–CHOH), 1.81–1.88 (1H, m, CH_2–CHOH), 4.27 (1H, dd, C*H*OH, *J*=7.3, 4.2 Hz); ^{13}C NMR (90 MHz, $CDCl_3$): δ=13.8, 22.3, 26.8, 33.8, 70.3, 179.6.

*4.5.2.3. (R)-2-Hydroxyoctanoic acid **1c**.* Kinetic resolution of *rac*-2-hydroxyoctanoic acid (**1c**, 2.01 g, 12.5 mmol) in *tert*-butyl methyl ether (75 mL) using vinyl acetate (5 mL, 54 mmol, 4 equiv) and *C. antarctica* lipase B (Novozym 435, 1.51 g) gave (*S*)-2-acetoxyoctanoic acid (oil, 741 mg) and (*R*)-2-hydroxyoctanoic acid **1c** as white solid (mp 57 °C, 670 mg, 33%, 78% ee); $[\alpha]_D^{20}$−2.36 (*c* 1, $CHCl_3$), $[\alpha]_D^{20}$ −4.3 (*c* 1, $CHCl_3$);[21] ^1H NMR (360 MHz, $CDCl_3$): δ=0.88 (3H, m), 1.22–1.45 (8H, m), 1,72 (1H, m, CH_2–CHOH), 1.85 (1H, m, CH_2–CHOH), 4.28 (1H, m, C*H*OH), 6.2 (2H, s, OH, COOH); ^{13}C NMR (90 MHz, $CDCl_3$): δ=14.0, 22.5, 24.7, 28.9, 31.6, 34.2, 70.3, 179.7.

*4.5.2.4. (S)-2-Hydroxyoctanoic acid **1c**.* Hydrolysis of (*S*)-2-acetoxyoctanoic acid (741 mg) using K_2CO_3 (3.67 g) in MeOH (30 mL) gave

(*S*)-2-hydroxyoctanoic acid (**1c**, 460 mg, 78%, 97% ee); $[\alpha]_D^{20}$ +2.23 (*c* 1, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ=0.90 (3H, t, *J*=6.9), 1.31–1.52 (8H, m), 1.66–1.78 (1H, m, C*H*$_2$–CHOH), 1.82–1.92 (1H, m, C*H*$_2$–CHOH), 4.30 (1H, dd, C*H*OH, *J*=7.5, 4.5 Hz); ^{13}C NMR (90 MHz, $CDCl_3$): δ=14.0, 22.5, 24.7, 28.9, 31.6, 34.2, 70.3, 180.1.

4.6. General procedures for derivatization

*4.6.1. Derivatization of substrate **1a** to the corresponding O-acetyl ester for GC analysis*

To the solution of α-hydroxycarboxylic acid **1a** (ca. 5 mg) in ethyl acetate (700 μL) were added acetic anhydride (50 μL) and a catalytic amount of DMAP. The reaction mixture was shaken for 1 h at rt. Water was added (500 μL), the solution was centrifuged and the organic phase was dried over Na_2SO_4.

*4.6.2. Derivatization of substrates **1b**,**c** to the corresponding methyl esters for GC analysis*

To 5 mg of dry α-hydroxycarboxylic acid **1b**,**c**, was added $BF_3\times$MeOH (300 μL). The vials were closed and heated at 100 °C for 1 h. The samples were cooled, water was added (500 μL), products were extracted with hexane (3×300 μL) and the combined organic phases were dried over Na_2SO_4.

4.7. Analytical procedures

See Tables 2 and 3.

Table 2
GC-analyses using a chiral stationary phase

Compound	Column[a]	Conditions[b]	t_R [min]	
			R	*S*
1a	A	A	20.23	20.14
1b	A	B	9.75	10.86
1c	A	C	6.72	6.83

[a] Column: (A) Astec Chiraldex B-TA column (40 m, 0.25 mm, 0.12 μm film).
[b] Conditions: (A) 14 psi H_2 at 80 °C, hold for 5 min, heat rate 2 °C/min to 120 °C, heat rate 15 °C/min to 170 °C, hold for 10 min; (B) 14 psi H_2 at 85 °C, hold for 5 min, heat rate 1 °C/min to 90 °C, heat rate 10 °C/min to 110 °C, heat rate 15 °C/min to 170 °C, hold for 2 min; (C) 14 psi H_2 at 90 °C, heat rate 10 °C/min to 130 °C, heat rate 5 °C/min to 160 °C, heat rate 15 °C/min to 170 °C, hold for 2 min.

Table 3
HPLC-analyses using a chiral stationary phase

Compound	Column[a]	Conditions[b]	t_R [min]	
			R	*S*
1d	B	B	26.2	31.0
1e	B	A	26.3	21.4

[a] Column: Chiralpak AD column (25 cm, 0.46 cm).
[b] Conditions: (A) *n*-heptane/*i*-propanol/trifluoroacetic acid (90:10:0.1), flow 1 mL/min; (B) *n*-heptane/*i*-propanol/trifluoroacetic acid (95:5:0.1), flow 0.8 mL/min.

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