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Biocatalytic Racemization of (Hetero)Aryl-aliphatic α-Hydroxycarboxylic Acids by *Lactobacillus* spp. Proceeds via an Oxidation–Reduction Sequence

Bettina M. Nestl,[a,b] Silvia M. Glueck,[a,b] Melanie Hall,[a] Wolfgang Kroutil,[a] Rainer Stuermer,[c] Bernhard Hauer,[c] and Kurt Faber*[a]

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The biocatalytic racemization of a range of (hetero)aryl- and (di)aryl-aliphatic α-hydroxycarboxylic acids has been achieved by using whole resting cells of *Lactobacillus* spp. The essentially mild (physiological) reaction conditions ensure the suppression of undesired side reactions, such as elimination, decomposition or condensation. Cofactor/inhibitor studies using a cell-free extract of *Lactobacillus paracasei* DSM 20207 reveal that the addition of redox cofactors (NAD^+/NADH) leads to a distinct increase in the racemization rate, while strong inhibition is observed in the presence of Thio-NAD^+, which suggests that the racemization proceeds by an oxidation–reduction sequence rather than involvement of a "racemase" enzyme.

Introduction

Racemization, in general, is an energetically "downhill" reaction due to an increase of entropy[1] and thus has been considered more often as an undesired side-reaction rather than a synthetically useful transformation. As a consequence, the controlled racemization of organic compounds has been scarcely studied deliberately and a significant part of the data available to date stems from industrial research predominantly reported in the patent literature. However, recent developments aiming at the quantitative transformation of racemates into a single stereoisomeric product without the occurrence of an unwanted stereoisomer − processes that are generally referred to as "deracemization"[2] − have highlighted the key role of racemization in synthetic organic chemistry. A detailed analysis of the data available to date reveals that chemical racemization techniques largely depend on harsh reaction conditions, such as thermal racemization as well as acid/base catalysis,[3] and only recently have milder methods based on Meerwein–Ponndorf–Verley–Oppenauer catalysts been reported.[4] As a consequence, the possibility of process control over chemical racemization is very limited and undesired side-reactions, such as elimination, condensation and/or rearrangement and decomposition, set a low ceiling on the synthetic utility of deracemization processes involving racemization. In this context, enzymatic racemization holds great potential, since it takes place at essentially mild reaction conditions − typically at room temperature, atmospheric pressure and neutral pH − which largely avoids the formation of by-products and thus allows "clean" racemization.[5]

We have recently shown that mandelate racemase [EC 5.1.2.2] from *Pseudomonas putida* ATCC 12633 is an excellent biocatalyst for the racemization of a wide spectrum of β,γ-unsaturated α-hydroxycarboxylic acids.[6] The substrate spectrum of mandelate racemase has been found to be remarkably wide and encompasses various types of β,γ-unsaturated α-hydroxycarboxylic acids, such as substituted (hetero)aryl mandelic acid[7] (or amide)[8] analogues and even cyclic and open-chain 2-hydroxy-3-butenoic acid derivatives. However, aliphatic and aryl-aliphatic α-hydroxycarboxylic acids, which are unable to stabilize the α-carbanion intermediate formed during enzymatic racemization, turned out to be non-substrates.[6]

In order to circumvent this limitation of mandelate racemase regarding its inability to interconvert the enantiomers of aliphatic and aryl-aliphatic α-hydroxycarboxylic acids, a matching α-hydroxyacid racemase activity was sought. Based on early reports on the lactate racemase activity of halophilic Archaea, anaerobic rumen bacteria and *Lactobacillus* spp., a screening recently provided a set of lactic acid bacteria that are able to racemize a broad spectrum of aliphatic and aryl-aliphatic α-hydroxycarboxylic acids at fair rates.[9] Whereas the racemization of aliphatic α-hydroxycarboxylic acids bearing structurally demanding branched side-chains proceeded at moderate rates, straight-chain analogues and aryl-aliphatic derivatives, such as

[a] Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz,
Heinrichstrasse 28, 8010 Graz, Austria
Fax: +43-316-380-9840
E-mail: Kurt.Faber@Uni-Graz.at
[b] Research Centre Applied Biocatalysis,
Petersgasse 14, 8010 Graz, Austria
[c] BASF AG, GVF/E-B9,
67056 Ludwigshafen, Germany

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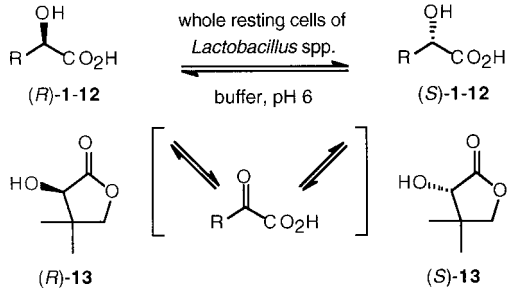
3-phenyllactic acid and 2-hydroxy-4-phenylbutyric acid, proved to be excellent substrates. The latter compounds possess interesting bioactivity themselves or serve as chiral building blocks for the synthesis of numerous pharmaceutical products, such as viral protease and ACE inhibitors.[10]

Results and Discussion

In order to elucidate the full potential of this mild biocatalytic racemization, we investigated the substrate spectrum of various *Lactobacillus* spp., which turned out to be the top candidates from our initial screening of a range of functionalized (hetero)aryl- and (di)aryl-aliphatic α-hydroxycarboxylic acids, all of which serve as important intermediates for the synthesis of pharmacologically active compounds. In order to provide a basis for scale-up studies, the mechanism and type of enzyme(s) responsible for this biocatalytic racemization were studied.

The racemization of substrates **1**–**13** was tested using rehydrated lyophilized cells of *Lactobacillus* spp. (Scheme 1, Table 1) in aqueous buffer at pH 6. In order to obtain comparable activities, relative rates of racemization were calculated from the slope of initial progress curves of the decline of *ee* vs. time at a conversion of <5%. The relative rates were calculated by setting the racemization rate of the unfunctionalized substrate (*R*)-3-phenyl lactate (**1**) as standard (100%).



Substrate	R	Substrate	R
(*R*)-**1**	Ph-CH_2-	(*R*)-**8**	2-thiophenyl-
(*R*)-**2**	*p*-Me-C_6H_4-CH_2-	(*S*)-**9**	$Ph_2(CH_3O)C$-
(*R*)-**3**	*p*-Et-C_6H_4-CH_2-	(*R*)-**10**	Ph-$(CH_2)_2$-
(*S*)-**4**	*o*-Cl-C_6H_4-CH_2-	(*S*)-**11**	*p*-F-C_6H_4-$(CH_2)_2$-
(*S*)-**5**	*o*-Br-C_6H_4-CH_2-	(*S*)-**12**	*p*-Cl-C_6H_4-$(CH_2)_2$-
(*S*)-**6**	*p*-NO_2-C_6H_4-CH_2-	(*R*)-**13**	n. a.
(*S*)-7	2-naphthyl-		

Scheme 1. Biocatalytic racemization of (hetero)aryl- and (di)aryl-aliphatic α-hydroxycarboxylic acids **1**–**13**.

Approximately half of the strains investigated showed excellent racemization rates with unfunctionalized aryl-alkyl derivatives **1** and **10**, with the former substrate being slightly superior.[9] 3-Phenyllactic acid (**1**) and several aryl-substituted derivatives thereof are frequently used chiral components of pharmaceuticals, such as rhinovirus protease inhibitors[11] and natural antibiotic agents.[10] The latter compounds are particularly difficult to racemize using conventional methods due to the ease of elimination of H_2O to form cinnamic acid.

Replacement of the phenyl moiety by a bulky naphthyl group (**7**) or by a heterocyclic thiophene unit (**8**) gave good racemization rates of up to two thirds of the relative activity of **1**. Compound **8** is an important intermediate for the synthesis of the platelet antiaggregant and antithrombotic drug Clopidogrel;[12] the naphthyl derivative **7** is a key element of an α^2-macroglobulin inhibitor.[13]

p-Alkyl-substituted 3-phenyllactic acid derivatives bearing electron-donating substituents (**2**, **3**) showed very low racemization rates (2–3% relative to **1**), whereas electron-withdrawing groups, such as *o*-Cl (**4**), *o*-Br (**5**) or *p*-NO_2 (**6**) were tolerated rather well, with relative rates of 18–51%. Compound **6** is used in the preparation of pharmaceutically important benzoxazine- and benzothiazine-containing β-aryl-α-oxypropionic acid derivatives.[14] In an analogous fashion, 4-(*p*-halophenyl)-2-hydroxybutanoates (**11**, **12**) were well accepted and were racemized at about two thirds of the relative rate of the unsubstituted analogue **10**. Both compounds are components of HIV-protease inhibitors[15] and inhibitors of factor Xa, which has emerged as an attractive target for the treatment of thrombosis.[16]

The remarkable flexibility of this biocatalytic racemization system was demonstrated by the fact that an extremely bulky substrate [2-hydroxy-3-methoxy-3,3-diphenylpropionic acid (**9**)] was also accepted at a fair rate (relative rate 14%). The (*S*)-enantiomer of the latter compound is a key intermediate for the synthesis of ET receptor antagonists BSF 420627, BSF 302146 and LU 135252 (Darusentan).[17]

Table 1. Relative activities of *Lactobacillus* spp. for the racemization of substrates **1**–**13** (significant values are printed in **bold**).

Microorganism	Relative rate [%][a]												
	(*R*)-**1**	(*R*)-**2**	(*R*)-**3**	(*S*)-**4**	(*S*)-**5**	(*S*)-**6**	(*S*)-**7**	(*R*)-**8**	(*S*)-**9**	(*R*)-**10**	(*S*)-**11**	(*S*)-**12**	(*R*)-**13**
Lactobacillus oris DSM 4864	n.d.	0.2	0.3	**14.2**	0.0	4.4	0.0	0.5	0.3	n.d.	1.5	0.0	0.3
Lactobacillus confusus DSM 20196	n.d.	0.2	0.3	0.0	0.0	4.6	11.5	0.2	0.8	n.d.	7.3	**19.0**	0.3
Lactobacillus halotolerans DSM 20190	0.3	3.0	0.3	0.0	0.0	11.5	**24.7**	0.2	1.2	**36.7**	6.8	10.3	0.3
Lactobacillus paracasei DSM 2649	**31.1**	2.2	0.3	13.9	**50.9**	3.4	3.4	9.1	0.5	**61.1**	0.2	3.2	0.3
Lactobacillus paracasei DSM 20008	**60.2**	2.0	0.3	21.0	**36.5**	3.9	1.9	2.2	0.3	**65.3**	**49.9**	22.8	0.3
Lactobacillus sakei DSM 20017	7.6	0.2	0.3	3.0	**45.9**	10.3	**29.6**	**21.3**	5.6	9.3	**30.6**	**57.0**	0.3
Lactobacillus delbrueckii DSM 20074	**50.3**	0.2	0.3	**26.4**	**55.2**	7.6	10.5	**65.8**	3.6	**59.9**	5.9	**52.8**	0.3
Lactobacillus paracasei DSM 20207	**100.0**	1.7	1.9	**33.3**	**26.2**	**18.1**	**66.5**	**27.6**	**14.0**	**79.9**	**54.0**	**54.0**	1.5

[a] Relative racemization rates were determined from the steady slope of the decline of enantiomeric composition vs. time during the initial stage of the reaction. The activity of (*R*)-2-phenyllactate (**1**) using *Lactobacillus paracasei* DSM 20207 was arbitrarily set as standard (100%); n.d. = not determined.

Since the large-scale industrial synthesis of (*S*)-**9** is based on resolution of the racemate by crystallization,[18] recycling of the undesired (*R*)-stereoisomer by racemization is highly desirable. However, chemical racemization methods based on acid- or base-catalysis failed: depending on the reaction conditions, elimination of methanol (to form 3,3-diphenylpyruvic acid) or C–C bond cleavage to form benzophenone and a C2 fragment (presumably glyoxylic or hydroxyacetic acid) are the main degradation pathways.

Attempts to racemize the α-hydroxylactone pantolactone **13**, which is an important intermediate for the production of D-panthothenic acid (vitamin B_5), however, revealed the limits of the method by showing exceedingly low rates.

In order to enable scale-up of this useful method, investigations into the type of enzyme responsible for the racemizing activity biochemical studies were initiated using *Lactobacillus paracasei* DSM 20207 and (*S*)-3-phenyllactate (**1**) as substrate. While the majority of the activity was retained in a crude cell-free extract prepared by ultrasonication, no active fractions containing an expected "racemase" could be obtained by protein purification using hydrophobic interaction, anion- or cation-exchange chromatography.[19] However, detailed analysis of the reaction mixture revealed the formation of traces of phenylpyruvic acid, phenylacetic acid, benzoic acid and benzaldehyde, which are presumably formed by enzymatic oxidation and decarboxylation.[20] In order to gain a quick insight into the complex enzymatic pathways, inhibitor and cofactor studies were performed: The involvement of a "lactate racemase" possessing a relaxed substrate specificity could be excluded by inhibitor experiments.[21] A conceivable racemization by the reversible action of thiamine pyrophosphate-dependent C–C lyases and decarboxylases or flavin-dependent dehydratases was excluded since addition of the corresponding cofactors did not show significant effects.

In contrast, the addition of nicotinamide redox cofactors gave conclusive results (Figure 1): whereas NADPH or NADP$^+$ had little effect, the addition of NAD$^+$, NADH or a mixture of both significantly increased the racemization rates (+61%), as determined by initial progress curves. The corresponding inverse proof was drawn by addition of Thio-NAD$^+$ (thionicotinamide adenine dinucleotide, oxidized form), which acts as a strong inhibitor of nicotinamide-depending redox enzymes, and which suppressed racemization of (*S*)-**1** to a large extent (–66%).

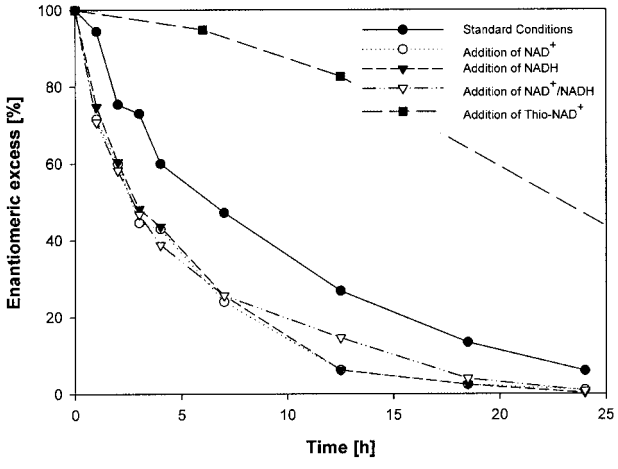


Figure 1. Rate enhancement and inhibition of the racemization of (*S*)-**1** (expressed as decrease of enatiomeric excess over time) using *Lactobacillus paracasei* DSM 20207 in the presence of NAD$^+$, NADH, NAD$^+$/NADH (1:1) and Thio-NAD$^+$ as cofactor or inhibitor, respectively.

These results strongly support the assumption that the mechanism of this biocatalytic racemization predominantly proceeds through the sequential action of enantio-complementary NADH-dependent α-keto acid reductases via the corresponding (observed) α-keto acid as achiral intermediate and that the involvement of a lactate racemase[22] possessing a broad substrate-spectrum can be excluded. Analysis of the biochemical literature for stereochemically matching pairs of α-keto acid reductases suggest L-[23] and D-2-hydroxy-4-methylpentanoate dehydrogenase[24] from *Lactobacillus confusus* (DSM, 20196) and *Lactobacillus paracasei* (DSM, 20008), respectively, and D-mandelate dehydrogenases from *Lactobacillus curvatus*[25] and *Streptococcus faecalis*.[26] The substrate spectrum of these enzymes, which was elucidated for the asymmetric bioreduction of α-keto carboxylic acids,[27] would nicely fit to the relative activities observed for the racemization of substrates from this study.

In summary, we have shown that the substrate tolerance of various *Lactobacillus* spp. for the biocatalytic racemization of (hetero)aryl- and (di)aryl-aliphatic α-hydroxycarboxylic acids under mild (physiological) conditions encompasses a wide range of compounds that are currently used for the manufacture of pharmaceuticals in nonracemic form. Cofactor and inhibitor studies suggest that the racemization proceeds predominantly by an equilibrium-controlled enzymatic oxidation–reduction sequence through the corresponding α-keto carboxylic acid as the (nonchiral) intermediate, catalyzed by stereo-complementary NADH-dependent α-keto acid dehydrogenases. The application of this clean racemization in combination with a biocatalytic kinetic resolution step[28] to furnish a single enantiomer from the racemate in 100% theoretical yield by avoiding the occurrence of an unwanted stereoisomer is currently being studied.

Experimental Section

General: The following chemicals were purchased and used as received: (Sigma Aldrich) D-(+)-3-phenyllactic acid, *rac*- and L-(–)-4-nitrophenylalanine, *rac*- and L-(–)-2-chloro- and *rac*- and L-(–)-2-bromophenylalanine; (Lancaster) L-(–)-3-phenyllactic acid; (Bachem) *rac*- and L-(–)-2-naphthylalanine, *rac*- and D-(+)-thienylalanine. (*S*)-2-Hydroxy-3-methoxy-3,3-diphenylpropionic acid (*S*)-**9** and (*R*)-pantolactone [(*R*)-**13**] were obtained from BASF AG, Ludwigshafen.

For general analytical and synthetic methods, the synthesis of substrates (*R*)-**2**–**8** and (*S*)-**11**,**12** and their spectroscopic and physical data see the Supporting Information.

Biocatalytic Procedures

Bacterial Strains: All strains were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany, http://www.dsmz.de/).

Medium for Active Strains: *Lactobacillus paracasei* DSM 20008, DSM 20207, DSM 2649, *Lactobacillus sakei* DSM 20017, *Lactobacillus halotolerans* DSM 20190, *Lactobacillus delbrueckii* DSM 20074, *Lactobacillus confusus* DSM 20196 and *Lactobacillus oris* DSM 4864 were grown on medium #11 as suggested by DSMZ (http://www.dsmz.de/). The following components of the medium were sterilized in five separate groups: group I: Pepticase (10 g L^{-1}, Sigma), bacteriological peptone (10 g L^{-1}, Oxoid), yeast extract (5 g L^{-1}, Oxoid); group II: Glucose (20 g L^{-1}, Fluka); group III: Tween 80 (polyoxyethylene-sorbitan-monooleate, 1 g L^{-1}, Aldrich); group IV: K_2HPO_4 (2 g L^{-1}, Merck); group V: sodium acetate trihydrate (8.3 g L^{-1}, Fluka), ammonium citrate (2 g L^{-1}, Fluka), $MgSO_4 \cdot 7H_2O$ (0.20 g L^{-1}, Fluka), $MnSO_4$ (0.05 g L^{-1}, Fluka).

Strain Maintenance: Lactobacilli were maintained on agar plates using the above described medium with the addition of agar. The following components of the medium were sterilised in five separate groups: group I: Pepticase (10 g L^{-1}, Sigma), bacteriological peptone (10 g L^{-1}, Oxoid), yeast extract (5 g L^{-1}, Oxoid); group II: glucose (20 g L^{-1}, Fluka); group III: Tween 80 (polyoxyethylene-sorbitan-monooleate, 1 g L^{-1}, Aldrich); group IV: K_2HPO_4 (2 g L^{-1}, Merck), agar (18 g L^{-1}, Oxoid); group V: $NaOAc \cdot 3H_2O$ (8.3 g L^{-1}, Fluka), ammonium citrate (2 g L^{-1}, Fluka), $MgSO_4 \cdot 7H_2O$ (0.20 g L^{-1}, Fluka), $MnSO_4$ (0.05 g L^{-1}, Fluka). The pH was adjusted to 6.2–6.5. Agar plates (40–45 plates out of 1 L medium) were prepared using the warm sterilised medium. The plates were kept in an incubator for 48 h at 30 °C (*Lactobacillus paracasei* DSM 20008, DSM 20207, DSM 2649, *Lactobacillus sakei* DSM 20017, *Lactobacillus halotolerans* DSM 20190, *Lactobacillus confusus* DSM 20196) and at 37 °C (*Lactobacillus delbrueckii* DSM 20074, *Lactobacillus oris* DSM 4864); long-term storage was at +4 °C.

Growth of Microorganisms: Strains were grown in flask cultures without shaking at 30 °C (*Lactobacillus paracasei* DSM 20008, DSM 20207, DSM 2649, *Lactobacillus sakei* DSM 20017, *Lactobacillus halotolerans* DSM 20190, *Lactobacillus confusus* DSM 20196) and at 37 °C (*Lactobacillus delbrueckii* DSM 20074, *Lactobacillus oris* DSM 4864). After transfer from agar plates, the microorganisms were grown for 3 d. Then, the cells were harvested by centrifugation (18000 *g*), washed twice with BIS-TRIS buffer (50 mM, 10^{-2} M $MgCl_2$, pH 6), lyophilized, and stored at +4 °C. Approximately 1.5–2 g of lyophilized cells was obtained from 1 L of medium.

General Procedure for the Biocatalytic Racemization: Lyophilized cells (50 mg) were rehydrated in aqueous BIS-TRIS buffer (50 mM, 10^{-2} M $MgCl_2$, 0.5 mL, pH 6) for 1 h at 42 °C with shaking at 150 rpm. Substrates **1**–**13** (5 mg) were added, followed by shaking of the reaction mixture at 150 rpm and 42 °C for 24 h. Then, the reaction mixture was acidified with 2 M HCl (1 drop) and the cells were removed by centrifugation. The supernatant was extracted with ethyl acetate and the organic phase was dried with anhydrous sodium sulfate. The determination of racemization was carried out by analysis of the enantiomeric excess by HPLC or GC on a chiral stationary phase. For HPLC determination, the organic phase was evaporated under reduced pressure and the residue was dissolved in HPLC eluent (without trifluoroacetic acid and formic acid). For details see the Supporting Information.

Cell Disruption: Cell disruption was carried out using a digital ultrasonifier (Branson, 250 W). For cell breakage a portion of wet cells of *Lactobacillus paracasei* DSM 20207, which was obtained from 0.33 L of culture (about 2.5 g wet cell paste) was suspended in 6 mL of BIS-TRIS buffer (50 mM, 10^{-2} M $MgCl_2$, pH 6). The cells were broken by treatment with energy for 20 min (1 s pulse followed by 2 s rest period for cooling) with an amplitude of 30% (corresponding to 60 W). During cell disruption the suspension was externally cooled with ice. Afterwards the crude cell lysate was centrifuged at +4 °C and 18000 rpm (38.000 *g*) for 30 min for the removal of cell debris.

Assay Procedure for Racemization Activity: Cell-free extract of *Lactobacillus paracasei* DSM 20207 (400 μL) was placed into an Eppendorff vial (1 mL) followed by addition of (*S*)-3-phenyllactic acid (**1**; 3 mg), which was dissolved in BIS-TRIS buffer (50 mM, 10^{-2} M $MgCl_2$, pH 6, 50 μL) and the pH was adjusted to 6. A defined amount of stock solution of the cofactor or inhibitor, respectively, dissolved in BIS-TRIS buffer (50 mM, 10^{-2} M $MgCl_2$, pH 6) was added, to reach a final concentration of 2 and 5 mM. For reasons of comparison, parallel test reactions were performed under standard conditions in the absence of additive. The mixtures were shaken at 42 °C and 130 rpm on a rotary shaker for a defined time. For work-up, the samples were acidified with 3 M HCl (1 drop), the products were extracted with diethyl ether, and, after centrifugation, the organic phase was dried with anhydrous sodium sulfate. The organic phase was evaporated under reduced pressure and the residue was dissolved in the HPLC eluent (without trifluoroacetic acid). The determination of racemization activity was carried out by HPLC analysis. For details see the Supporting Information.

Acknowledgments

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