The Substrate Spectrum of Mandelate Racemase: Minimum Structural Requirements for Substrates and Substrate Model

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Abstract: Mandelate racemase (EC 5.1.2.2) is one of the few biochemically well-characterized racemases. The remarkable stability of this cofactor-independent enzyme and its broad substrate tolerance make it an ideal candidate for the racemization of non-natural α-hydroxycarboxylic acids under physiological reaction conditions to be applied in deracemization protocols in connection with a kinetic resolution step. This review summarizes all aspects of mandelate racemase relevant for the application of this enzyme in preparative-scale biotransformations with special emphasis on its substrate tolerance. Collection and evaluation of substrate structure-activity data led to a set of general guidelines, which were used as basis for the construction of a general substrate model, which allows a quick estimation of the expected activity for a given substrate.

Keywords: deracemization; enzymatic racemization; α-hydroxycarboxylic acid; mandelate racemase; racemase-lipase two-enzyme process; substrate spectrum

1 Introduction

The interconversion of enantiomers – racemization – goes in hand with a loss of "chiral value"[1] and thus has been generally regarded as an undesired side reaction rather than a synthetically useful transformation. As a consequence, the controlled racemization of organic compounds has been scarcely studied and the major body of data available to date originates from industrial research, which was mainly driven by the demand to improve the economic balance of kinetic resolution processes.[2] It was only recently that the importance of synthetic protocols for the controlled racemization of organic compounds under mild reaction conditions for the development of so-called "deracemization processes" has been recognized.[3] In this context, *enzymatic* racemization[4,5] holds great potential, not only in view of the mild (physiological) reaction conditions, but also due to the high compatibility of biocatalysts with each other to be applied in dynamic kinetic resolution.

Due to the fact that the vast majority of biochemical processes are stereospecific, Nature has faced little need for racemization and, as a consequence, "racemases" are a small group of enzymes, which have been biochemically classified as subgroup EC 5.1.X.X among the diverse and heterogeneous group of isomerases. Despite their rare occurrence in Nature, their importance in synthetic organic chemistry lies in the fact that they can catalyze "chemically impossible" isomerization reactions. Besides numerous racemases acting on α-amino acids, the best-studied racemase so far is mandelate racemase (EC 5.1.2.2).

Mandelate racemase is produced by the soil bacterium *Pseudomonas putida* ATCC 12633 presumably to allow the strain to funnel D-mandelate (*via* racemization) into the L-specific mandelate degradation pathway[6,7] and to use it as a carbon- and energy-source.[8] The biochemical characteristics of this enzyme,[9] its structure,[10] (PDB entry 1MDR) and mechanism of action were studied in great detail by Kenyon and Hegeman.[11,12] The inducible octameric enzyme (subunit 39 kDa) has been made available in large amounts by fermentation of *Pseudomonas putida* ATCC 12633 using *rac*-mandelate as inducer[13] and it has been cloned.[14] Enzyme immobilization onto the cationic carrier DEAE-cellulose leads to enhanced activity and facilitates recovery.[15] Simple assays for mandelate-racemase activity based on circular dichroism[16] or on the decline of optical rotation over time[17] have been developed.

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From left to right: Kurt Faber, Ulrike Wagner, Ulfried Felfer, Barbara Larissegger-Schnell, Wolfgang Kroutil.

Ulfried Felfer, born 1970 in Oberzeiring (Styria/Austria), did his M.Sc. on "man-made" enzymes with Prof. H. Griengl (Graz/Austria) and Prof. S. M. Roberts at the University of Exeter (UK). After graduating from Graz University of Technology in 1996 he continued his research with Prof. K. Faber on dynamic kinetic resolution, where he received his Ph. D. in 1998. In 1999 he moved to DSM Fine Chemicals Austria. Since 2002 he is responsible for scale-up into (pilot) production scale.

Marian Goriup, born 1975 in Leoben/Austria, studied technical chemistry at the University of Technology in Graz and wrote his Diploma Thesis with Prof. Faber on mandelate racemase. Under the supervision of Prof. Saf he got his Ph. D. at the Institute for Chemistry and Technology of Organic Materials/Graz in 2001. Afterwards he went to industry and spent one year in Germany at Multek before he changed in 2003 to Borealis in Vienna.

Marion Koegl, born 1978 in Graz/Austria, finished her undergraduate training with a Diploma Thesis at the University of Graz under the supervision of Prof. Faber in 2003. During her undergraduate studies she participated at a student exchange program with Montreal/Canada (September 1994 – February 1995). She started her Ph. D. under the supervision of Prof. Mulzer at the University of Vienna in 2003.

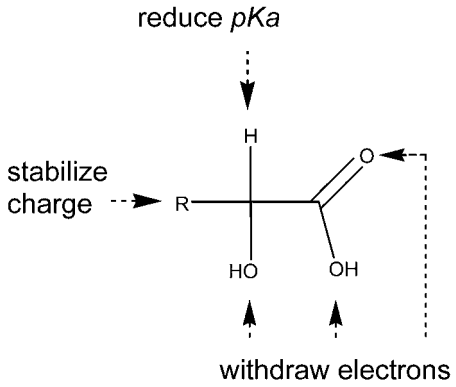
Ulrike G. Wagner (born 1960) received her undergraduate training in chemistry at the University of Graz. After her Ph. D. in Graz in crystallography she went for a post-doc to the Department of Structural Chemistry at Weizmann Institute of Science (Rehovot, Israel). She collected one year of industrial experience at the Sandoz Research Institute in Vienna (Austria) before she became an assistant professor at the University of Graz in 1991, where she was promoted to associate professor in 1997. In 1999 she did a sabbatical at the ESRF (European synchrotron radiation facilities) in Grenoble, France.

Barbara Larissegger-Schnell, born 1966 in Altenmarkt (Salzburg/Austria), studied chemistry at the University of Graz, where she received her Ph. D. in 1994. Since 1993 she is part-time assistant (with maternity breaks 1995–1996 and 2002–2003) at the Department of Chemistry. Until 1998, she was a co-worker in the research group of Prof. Thomas Kappe and in 1998 she joined the research group of Prof. Kurt Faber.

Kurt Faber, born 1953 in Klagenfurt (Carinthia/Austria), studied chemistry at the University of Graz, where he received his Ph. D. in 1982. From 1982–1983 he moved to St. John's (Canada) for a post-doc and continued his career at the University of Technology (Graz), where he became associate professor in 1997. The following year he was appointed full professor at the University of Graz, where he is heading his research group devoted to the use of biocatalysts for the synthetic transformation of non-natural compounds. He was a visiting scientist at University of Tokyo (1987/1988), Exeter University (1990), University of Trondheim (1994) and Stockholm University (2001).

Wolfgang Kroutil (born 1972 in Graz, Austria) received his undergraduate training in chemistry at the University of Technology in Graz and completed his graduate studies in Exeter (UK) and Graz. After his Ph. D. he collected two years of industrial experience in the biocatalysis research group at Syngenta (formerly Novartis CP) in Basel (Switzerland) and in the R&D department of Krems Chemie Chemical Services (Austria). In 2000 he became assistant professor and was promoted to associate professor in 2004 at the University of Graz.

The catalytic mechanism of mandelate racemase is a masterpiece of chemical catalysis, where evolution has managed to catalyze a "chemically impossible" reaction, made feasible through the combined synergistic effects of a number of chemical operators on the substrate within the active site of the enzyme.[18–22] The major cat-



Scheme 1. Schematic representation of mandelate racemase catalysis.

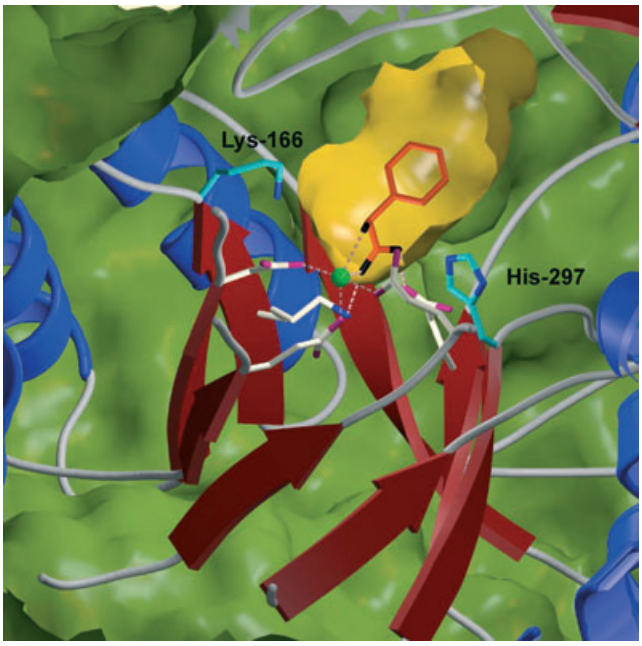
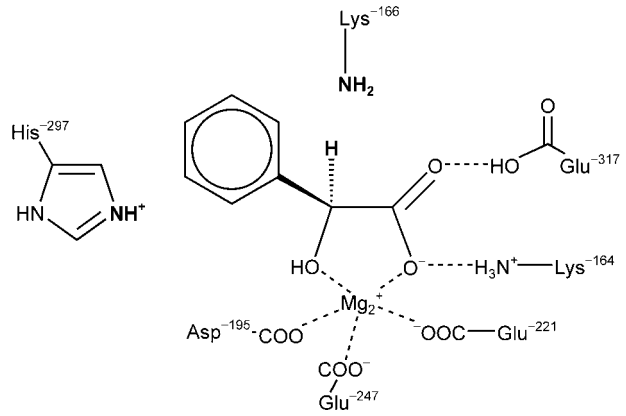


Figure 1. Active site of mandelate racemase containing (*S*)-mandelate. The main structural element (so-called TIM-barrel) consisting of a circular arrangement of eight parallel β-sheets is shown in dark red. On top of it, the catalytically active Mg^{2+} ion (green) is positioned through a network of H-bonds and salt bridges (dashed) within an array of Asp, Glu and Lys residues. The polar α-hydroxycarboxylic acid moiety of the substrate (orange) is docked onto this same array, while the non-polar aryl moiety is accommodated in the lipophilic binding pocket (yellow). Catalytically active bases for proton abstraction juxtaposed on each side of the substrate (Lys – 166, His – 297, turquoise) are annotated.

alytic hurdle during the interconversion of mandelate enantiomers is the abstraction of the α-proton of mandelate possessing a (formal) pK_a value of 29[23] to generate the corresponding achiral enolate intermediate (Scheme 1), which is facilitated through resonance stabilization by the phenyl-substituent R.[24]



Scheme 2. Schematic docking of (*S*)-L-mandelate onto the chemical operators within the active site of mandelate racemase. The pair of catalytically active bases and the α-H are emphasized in bold.

The problem is overcome by the binding of both enantiomers within the active site through a tight network of salt bridges and hydrogen bonds involving two Brønsted acids (Glu 317 and Lys 164) and a Lewis acid (Mg^{2+} in the native enzyme[25]) (Scheme 2). This intimate arrangement of the α-hydroxycarboxylic acid moiety of the substrate within the electron-withdrawing ligands within the active site diminishes the electron density at the α-position to such an extent that the α-H abstraction by an adjacent base becomes feasible through a "two-base-mechanism".[26] Racemization takes place *via* deprotonation by two enantiomer-specific bases juxtaposed on either side of the chiral α-carbon atom, i.e., His-297 and Lys-166 for (*R*)- and (*S*)-mandelate, respectively; while one base acts as base, the other functions as the corresponding acid for proton delivery from the opposite side. This "molecular ping-pong game" makes mandelate racemase an extremely efficient catalyst with a turnover frequency of 1000 s^{-1},[27] meaning that 1.0 g of the enzyme racemizes approximately 1.7 kg of mandelic acid per hour.

2 Substrate Spectrum

The value of an enzyme for the selective biotransformation of non-natural organic compounds on a preparative scale is predominantly characterized by its substrate tolerance, i.e., how many substrates of more or less related structure can be transformed at acceptable rates. Although first hints on the relaxed substrate specificity of mandelate racemase were published quite early,[14] it was the availability of this enzyme by fermentation[13] that facilitated the exploration of its substrate tolerance considerably.[15,28] Moreover, molecular modeling showed that the hydrophobic binding pocket within the active site (which accommodates the phenyl moiety of the natural substrate, mandelate, see Fig. 1) shows a

Table 1. Substrate tolerance of mandelate racemase.

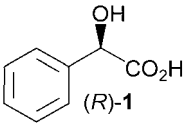
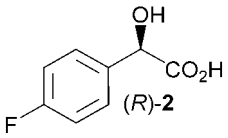
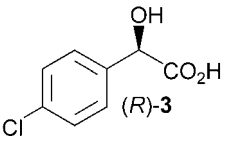
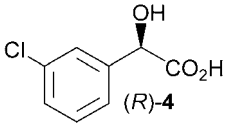
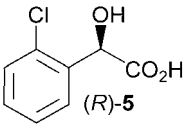
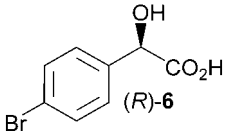
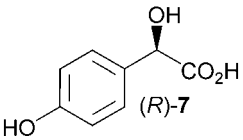
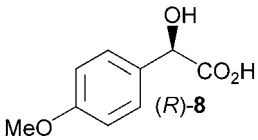
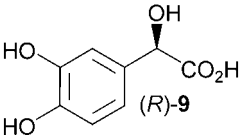
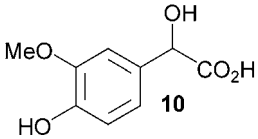
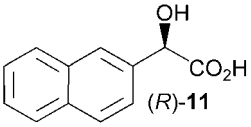
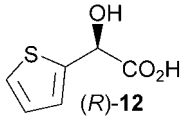
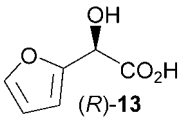
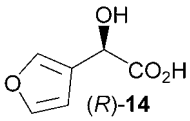
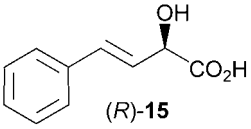
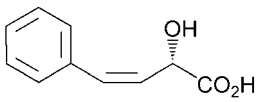
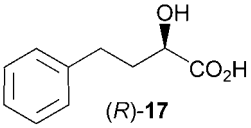
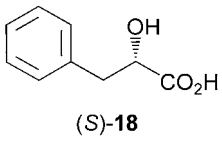
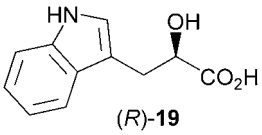
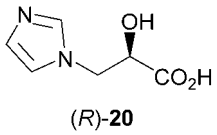
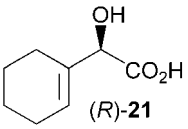
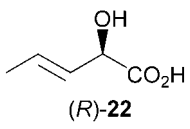
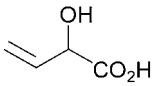
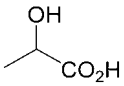
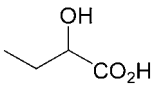
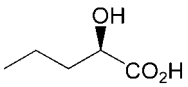
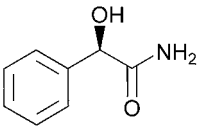
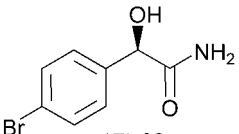
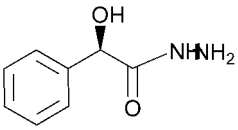
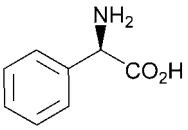
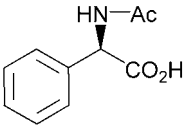
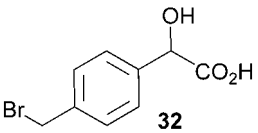
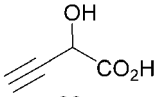
Substrate	Relative Activity [%][a]	Ref.	Substrate	Relative Activity [%][a]	Ref.
 (*R*)-**1**	100	n. a.	 (*R*)-**2**	96	[32]
 (*R*)-**3**	326	[31]	 (*R*)-**4**	61	[32]
 (*R*)-**5**	⩽1[b]	[32]	 (*R*)-**6**	376	[31]
 (*R*)-**7**	45	[31]	 (*R*)-**8**	17	[31]
 (*R*)-**9**	0.03[b]	[31]	 **10**	⩽0.02[b]	[31]
 (*R*)-**11**	26	[32]	 (*R*)-**12**	59[c]	[32]
 (*R*)-**13**	24[c]	[32]	 (*R*)-**14**	38[c]	[32]
 (*R*)-**15**	53	this study	 (*S*)-**16**	⩽1	this study
 (*R*)-**17**	⩽1[b]	this study	 (*S*)-**18**	⩽0.01[b]	[31]
 (*R*)-**19**	⩽0.01[b]	[31]	 (*R*)-**20**	5.4	[31]
 (*R*)-**21**	50	this study	 (*R*)-**22**	36	this study

Table 1 (cont.)

Substrate	Relative Activity [%][a]	Ref.	Substrate	Relative Activity [%][a]	Ref.
 **23**	35	[33]	 **24**	$\leqslant 0.01$[b]	[31]
 **25**	$\leqslant 0.01$[b]	[33]	 (*R*)-**26**	1	this study
 (*R*)-**27**	15	[28]	 (*R*)-**28**	22[b]	[28]
 (*R*)-**29**	$\leqslant 1$	[28]	 (*R*)-**30**	$\leqslant 1$[b]	[28]
 (*R*)-**31**	$\leqslant 1$[b]	[28]	 **32**	n. a.[e]	[34]
 **33**	8.5[d]	[35]			

n. a. not applicable.
[a] Relative activities are expressed as % relative to the natural substrate mandelate (*R*)-**1** (100%).
[b] Limit of sensitivity of the assay procedure used.
[c] Previously published values were corrected mathematically by the initial ratio of the specific optical rotations of substrate to reference (mandelate).
[d] Substrate **32** acts as strong inhibitor.
[e] Not applicable, substrate **33** undergoes elimination of HBr to form *p*-methylbenzoyl formate.

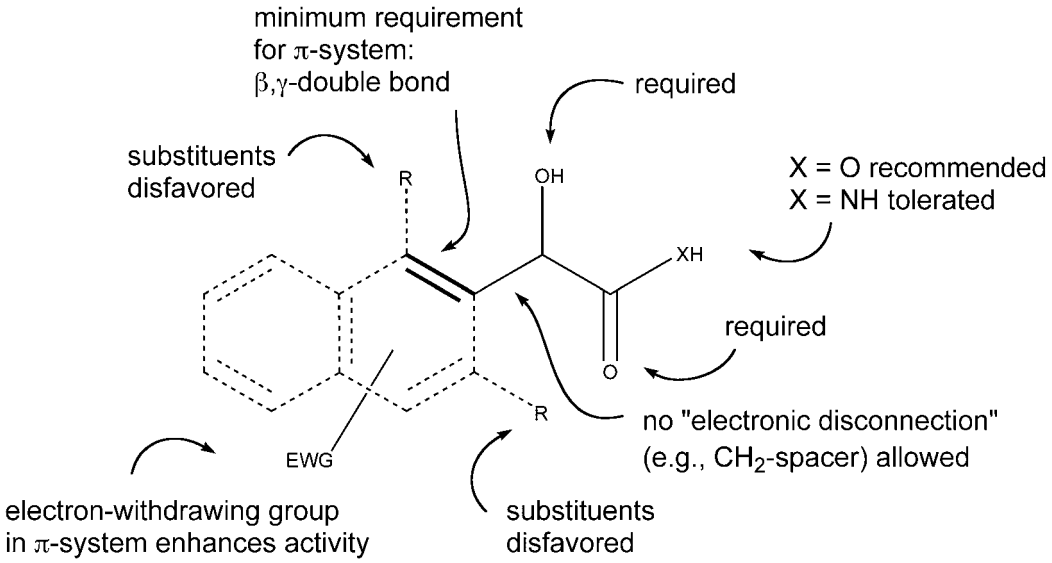
remarkable plasticity;[29] particularly the latter feature makes this enzyme a highly desired "broad substrate spectrum" catalyst.

Careful analysis of the substrate structure-activity data reported so far together with six novel data sets allowed us to deduce a set of rules which facilitate the prediction of the relative activity of a given substrate for mandelate racemase (Table 1).

(1) Variation of the aryl moiety: Substituents on the aryl moiety of mandelate are well-accepted, in particular in the (sterically less stringent) *p*-position, such as halogen (**2**, **3**, **6**), hydroxy or alkoxy (**7**, **8**). While some reduction in activity is observed with *m*-substituents (**4**), *o*-substituted (**5**) and *m, p*-disubstituted mandelate derivatives (**9, 10**) are not accepted, presumably due to steric hindrance, which is most striking for the *o*-derivative. Total replacement of the phenyl moiety by a bulky naphthyl group (**11**) or by a heterocycle, such as furan (**13**, **14**) or thiophene (**12**) gave moderate to good racemization rates.

(2) Non-aromatic substrates: A remarkable feature of the flexibility of mandelate racemase lies in the fact that the aromatic system can be drastically reduced to a minimal π-system consisting of a single β,γ-C=C moiety while retaining a good part of its catalytic activity (35–50%), which is valid not only for cyclic derivatives (**21**), but also for straight-chain analogues (**22**, **23**).

(3) Aliphatic and aryl-aliphatic substrates: Aliphatic (**24**–**26**) and aryl-aliphatic α-hydroxycarboxylic acids (**17**–**20**), which have no (or an "electronically disconnected") aryl moiety cannot contribute to the resonance stabilization of the α-carbanion intermediate[24] and are



Scheme 3. Substrate model for mandelate racemase.

therefore non-substrates. However, when the aryl moiety is linked through a conjugated π-system (*E*-**15**), carbanion stabilization is feasible and, as a consequence, good activity is observed. The inactivity of the corresponding *Z*-isomer (**16**) is most likely caused by steric restrictions.

(4) Variation of the α-hydroxycarboxylic acid moiety: Bearing in mind that the α-hydroxycarboxylic acid moiety has a critical function as docking group though multipoint attachment within the active site of the enzyme, any alterations in this region have to be regarded with caution. All attempts to alter the α-hydroxy group failed so far: α-phenylglycine (**30**) and the corresponding *N*-acetyl derivative (**31**) were totally inactive, most likely due to the inability of the amino/ammonium or aminoacetyl group to form a tight salt bridge with the catalytically active Mg^{2+} ion. Most remarkably, variation of the carboxylate moiety to the corresponding carboxamide was tolerated to some extent: mandeloamide (**27**) and its *p*-bromo analogue (**28**) were racemized with acceptable rates (15 and 22%, respectively).

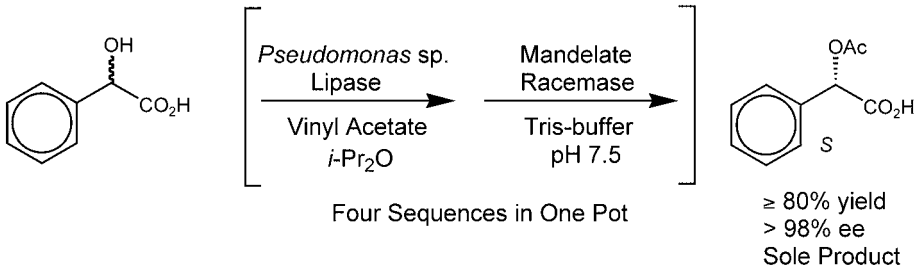
(5) Electronic effects: Overall, the requirement for resonance stabilization of the α-carbanion intermediate during catalysis appears to be a striking prerequisite for catalysis: Whereas mandelate derivatives bearing electron-donating substituents (**7**, **8**) show markedly reduced racemization rates, electron-withdrawing groups, such as Cl (**3**) or Br (**6**) lead to significantly (more than three-fold) enhanced racemization rates. A similar rate-enhancing effect by a *p*-Br substituent was observed for the corresponding mandeloamides (**27**) and (**28**). For the fluoro analogue (**2**), the electron-withdrawing effect is cancelled out by the well-known "back-donation" effect of F.[30] The ability of heterocycles to stabilize the α-carbanion intermediate is nicely paralleled with their ability for resonance stabilization through their aromaticity (expressed as the resonance energy): Whereas furan analogues (**13**) and (**14**) (resonance energy of furan *ca.* 56 kJ/mol) showed relative rates of 24–38%, the corresponding thiophene analogue (**12**) gave an enhanced rate of 59% (resonance energy of thiophene *ca.* 84 kJ/mol, *cf.* resonance energy of benzene 124 kJ/mol).

(6) Steric effects: Negative effects of steric hindrance clearly appear to dominate in close vicinity of the α-center, i.e., *o*-substituted mandelates (**5**) are not racemized at all, and some hindrance seems to be true also for the *m*-position (**4**, **9**, **10**). This is in good agreement with the inactivity of the "bent" *Z*-configurated substrate (**16**). In contrast, there is apparently quite some space available within the active site beyond the aryl moiety to accommodate a bulky naphthyl group (**11**) (*cf.* Fig. 1).

(7) Substrate inhibitors: In rare cases, unexpected results were obtained with α-hydroxycarboxylic acids. The acetylenic analogue propargyl glycolate (**33**) was slowly racemized, but acted as a strong inhibitor.[35] *p*-Bromomethyl mandelate (**32**) underwent unexpected elimination of hydrogen bromide to form *p*-methylbenzoyl formate. The latter reaction could be sufficiently explained by expulsion of bromide anion from the α-carbanion intermediate, followed by rapid tautomerization of the formed *exo*-methylene enol to furnish the corresponding α-carbonyl compound.[34]

3 Substrate Model for Mandelate Racemase

The data on the substrate-activity pattern of mandelate racemase available so far allow us to construct a simple predictive "substrate model"[36] (Scheme 3). The "ideal" substrate consists of an α-hydroxycarboxylic acid. While



Scheme 4. Deracemization of (±)-mandelate using a stepwise two-enzyme process.

carboxamides are tolerated to some extent, replacement of the α-hydroxy group is forbidden. The requirement for a π-system attached to the α-carbon is stringent, thus no "electronic disconnecting" spacers, such as CH_2 are allowed. Ideally, the π-system should be fully aromatic, although the minimum requirements consist of a single C=C bond. Electron-withdrawing groups on this system dramatically enhance the activity up to several-fold. Steric restrictions apply for the *o*-aryl position, while ample space is available in the *p*-position, at the far side of the substrate (*cf.* Fig. 1).

4 Application of Mandelate Racemase in Deracemization Processes

The first application of mandelate racemase for the deracemization of a *rac*-α-hydroxycarboxylic acid on a preparative scale was demonstrated recently.[37] Thus, when *rac*-mandelate was subjected to kinetic resolution *via Pseudomonas* sp. Lipase-catalyzed acyl transfer in diisopropyl ether using vinyl acetate as acyl donor, (*S*)-*O*-acetyl mandelate was formed while the (*R*)-mandelate remained untouched. Since the formed product is not a substrate for mandelate racemase, the non-converted substrate enantiomer could be racemized by mandelate racemase *in situ*, i.e., without substrate-product separation. Although mandelate racemase is remarkable stable towards deactivation by organic solvents, it requires high water activity for catalytic activity and, as a consequence, the enzyme is *inactive*, but not *deactivated* in organic solvents, such as diisopropyl ether. Thus, the reaction medium had to be switched from organic to aqueous.[38,39] After four cycles, (*S*)-*O*-acetyl mandelate was obtained in $\geqslant$80% yield and $>$98% ee as the sole product.

In summary, mandelate racemase constitutes a biochemically well-characterized and remarkably stable biocatalyst for the racemization of α-hydroxycarboxylic acids under mild (physiological) conditions for application in the deracemization of (±)-α-hydroxycarboxylic acids. The enzyme shows a broad substrate tolerance within certain well-defined structural limitations, which can be deduced by a general substrate model proposed in this paper.

5 Experimental Section

Modeling

Figure 1 was created using Pdb coordinates 1MRA by replacing (*S*)-atrolactic acid (used as irreversible inhibitor) with (*S*)-mandelate. Rendering was accomplished by render (Raster3d);[40] the picture was created using Molscript[41] and surfaces were generated as reported.[42]

Determination of Activity

A semi-purified enzyme preparation of mandelate racemase was prepared as previously described, the specific activity was 107 μmol mg^{-1} min^{-1}.[13] Relative rates of racemization were determined using an assay based on the online measurement of the decline of optical rotation *versus* time with the corresponding corrections.[43] In experiments, where the substrate was not optically pure, the ee of mandelate (used as standard) was adjusted to the ee of the substrate in order to obtain comparable data for relative activity. Optical rotations were measured on a Perkin-Elmer Polarimeter 341 in a 1-mL cuvette of 10 cm length. The absence of spontaneous racemization and undesired side-reactions under the reaction conditions was verified for all substrates (i) in the absence of biocatalyst and (ii) in the presence of heat-denatured enzyme; it was proven to be $<$0.5% within 48 h for all substrates. For non-substrates the negative result was verified for a period of 24 hours and then analyzing the mixtures by HPLC.

Crotonaldehyde, ethyl glyoxylate, ethynylbenzene, 1-cyclohexene-1-carboxaldehyde, (*S*)-2-hydroxy-4-phenylbutyric acid ethyl ester and (*R*)-2-hydroxy-4-phenylbutyric acid were commercially available. Column chromatography was performed using Merck 60 silica gel (0.040–0.063 mm). ^1H and ^{13}C NMR were recorded on a Bruker 200 MHz spectrometer at 200 and 50.3 MHz, respectively, using TMS as internal standard. Chemical shifts are reported in ppm (δ) and coupling constants (*J*) are given in Hz.

Synthesis of (*R*)-(*E*)-2-Hydroxy-3-pentenoic Acid [(*R*)-22] from Crotonaldehyde Employing (*R*)-Hydroxynitrile Lyase from Almond Meal

Caution: Due to the handling of solutions containing hydrogen cyanide, the following procedure was performed in a well-ventilated hood. A crude enzyme preparation of (*R*)-hydroxynitrile lyase [(*R*)-HNL] from almonds was prepared according

to the literature.[44] Sodium cyanide (2.94 g, 60 mmol) was dissolved in water (75 mL) and the pH was adjusted to 5.5 with glacial acetic acid. The solution was extracted once with methyl *tert*-butyl ether (MTBE, 75 mL). The organic layer was separated and immediately used for enzymic cyanohydrin synthesis. The (*R*)-hydroxynitrile lyase preparation was rehydrated with citrate buffer (9 mL, 20 mM, pH 5.5) for 30 min. Freshly prepared MTBE-HCN solution (~40 mmol HCN) was added at once at $+3\,^{\circ}C$ before freshly distilled crotonaldehyde (1.4 g, 20 mmol) was added *via* a syringe. The reaction was stirred at $+3\,^{\circ}C$ for 24 h after which the biocatalyst was separated by filtration. The filtrate was extracted with MTBE ($2\times$ 50 mL), the combined organic layers were dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to yield the crude, non-racemic cyanohydrin which was immediately transformed to the corresponding (*R*)-*E*-2-hydroxypentenoic acid methyl ester *via* a modified Pinner reaction.[45] Without purification, the cyanohydrin was dissolved in anhydrous diethyl ether (20 mL) and anhydrous methanol (2 mL) was added. Dry HCl gas was bubbled through the solution for about 15 min to precipitate the hydrochloride salt of the corresponding imidate, which was then left at $4\,^{\circ}C$ for two days to complete crystallization. The salt was filtered and washed with cold anhydrous diethyl ether (10 mL). The ice-cold hydrochloride salt was suspended in diethyl ether (5 mL/g salt) and distilled water (same amount as ether) was added dropwise. The solution was stirred for 15 min at $0\,^{\circ}C$ before it was allowed to warm to room temperature. The organic phase was separated and the aqueous phase was extracted with diethyl ether. The combined organic layers were washed with water until the pH was neutral, dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to yield (*R*)-*E*-2-hydroxypentenoic acid methyl ester, which was directly hydrolyzed with sodium hydroxide solution [20 mL, 10% (w/v)]. After completion (2 h), the reaction mixture was extracted with cyclohexane (4×30 mL), acidified to pH 2.0 (1 M aqueous HCl) and extracted with ethyl acetate (5×70 mL). The organic layers were combined, dried (Na_2SO_4) and concentrated to afford (*R*)-*E*-2-hydroxy-3-pentenoic acid (*R*)-*E*-**22** with 94% ee; overall yield: 0.71 g (42%), $[\alpha]_D^{20}$: -60.2 (c 1.32, acetone). ^1H NMR (acetone-d_6): $\delta=1.7$ (3H, d, $J=7$ Hz, CH_3), 4.6 (1H, d, $J=6$ Hz, CH-2), 5.6 (1H, dd, $J=6$, 15 Hz, =CH-3), 5.8 (1H, m, =CH-4); ^{13}C-NMR: (acetone-d_6): $\delta=17.6$ (CH_3), 71.8 (CHOH), 128.3 (C-3), 129.5 (C-4), 174.4 (COOH).

Synthesis of *rac*-(*E*)-2-Hydroxy-3-pentenoic Acid (*rac*-22) from Crotonaldehyde

Trimethylsilyl cyanide (TMSCN; 2.38 g, 3.0 ml, 24 mmol) was added to a solution of freshly distilled crotonaldehyde (1.4 g, 1.66 ml, 20 mmol) in absolute dichloromethane (5 ml). Afterwards ZnI_2 (5 mg) was added, the flask was sealed and the mixture was stirred at $0\,^{\circ}C$ for 15 min before it was allowed to warm to room temperature and stirred for another 45 min. The organic solvent was evaporated under reduced pressure to give the crude TMS cyanohydrin. Without further purification the crude intermediate was suspended in aqueous HCl (3 N, 20 mL) on an ice bath. After stirring for about 1 h at $0\,^{\circ}C$ the mixture was allowed to warm to room temperature. Methyl *tert*-butyl ether (MTBE, 20 mL) was added and the mixture was vigorously stirred for 15 min, before the organic phase was separated. The aqueous layer was extracted with MTBE (3×20 mL). The combined organic phase was dried (Na_2SO_4) and the organic solvent evaporated under reduced pressure to yield the crude cyanohydrin, which was further transformed into *rac*-(*E*)-2-hydroxy-3-pentenoic acid *via* the modified Pinner reaction followed by ester hydrolysis as described above for the (*R*)-(*E*)-2-hydroxy-3-pentenoic acid (*R*)-**22**; overall yield of pure *rac*-(*E*)-2-hydroxy-3-pentenoic acid (*rac*-**22**): 1.4 g (54%). The NMR spectra were identical to those of the non-racemic material.

Synthesis of (*R*)-(*E*)-2-Hydroxy-4-phenyl-3-butenoic Acid [(*R*)-15] from Cinnamaldehyde Employing (*R*)-Hydroxynitrile Lyase from Almond Meal

The reaction was performed as described for (*R*)-(*E*)-2-hydroxy-3-pentenoic acid [(*R*)-**22**] starting from cinnamaldehyde. The product (*R*)-(*E*)-2-hydroxy-4-phenyl-3-butenoic acid was obtained as yellowish crystals; overall yield: 0.43 g (30%); mp 114–117 °C (petroleum ether/acetone) {ref.[46] 139 °C}; $[\alpha]_D^{20}$: -81 (*c* 0.29, MeOH) {ref.[46] -98 (*R*) (*c* 1, MeOH)}; ee 63% (HPLC). ^{13}C and ^1H NMR matched with reported data.[47]

Synthesis of *rac*-(*E*)-2-Hydroxy-4-phenyl-3-butenoic Acid (*rac*-15) from Cinnamaldehyde

The reaction was performed as described for *rac*-(*E*)-2-hydroxy-3-pentenoic acid (*rac*-**22**) starting from cinnamaldehyde (3.32 g, 25 mmol) and TMSCN (2.97 g, 30 mmol) to afford *rac*-(*E*)-2-hydroxy-4-phenyl-3-butenoic acid (*rac*-**15**); overall yield: 2.6 g (62%). The structure was verified as described for the non-racemic product.

Synthesis of (*R*)-2-Hydroxypentanoic Acid [(*R*)-26] from (*R*)-(*E*)-2-Hydroxy-3-pentenoic Acid [(*R*)-22]

(*R*)-(*E*)-2-Hydroxy-3-pentenoic acid [(*R*)-**22**; 0.25 g, 2.15 mmol) was dissolved in absolute methanol (20 mL) before Pd/C (10%, 5 mg) was added. The flask was evacuated twice and flushed with hydrogen. The reaction mixture was stirred at room temperature for 2 h, the catalyst was removed by filtration and the organic solvent was removed under reduced pressure to give (*R*)-2-hydroxypentanoic acid [(*R*)-**26**]; yield: 0.227 g (89%); 94% ee; $[\alpha]_D^{20}$: $+1$ (*c* 1.05, MeOH). The structure was confirmed by comparison of the ^1H NMR spectrum with literature data.[48]

Racemic 2-hydroxy pentanoic acid (*rac*-**26**) was synthesized by analogy starting from *rac*-2-hydroxy-3-pentenoic acid (*rac*-**22**).

Synthesis of (*S*)-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic Acid [(S)-16]

Substrate (*S*)-**16** was obtained *via* the following reaction sequence starting from ethynylbenzene and ethyl glyoxylate with *rac*-2-hydroxy-4-phenyl-3-butynoic acid ethyl ester as the first intermediate.

***rac*-2-Hydroxy-4-phenyl-3-butynoic acid ethyl ester:**[49,50] *n*-Butyllithium (18.5 mL, 2.5 M in hexane; 46 mmol) was added dropwise to ethynylbenzene (5 g, 49 mmol) in THF (50 mL) at −78 °C under argon atmosphere. The reaction mixture was stirred for 30 min at −78 °C before ethyl glyoxylate (9.7 mL, 43% in toluene)[51] was added within 15 min. After 6 h of stirring at −78 °C the reaction was stopped by addition of glacial acetic acid (5 mL) and the mixture was warmed to 0 °C. After addition of brine (40 mL) the mixture was extracted with ethyl acetate (3 × 50 mL), the combined organic phases were dried (Na_2SO_4), the solvent was evaporated and the product was purified using silica gel chromatography (petroleum ether/ethyl acetate = 10:1) to give pure *rac*-2-hydroxy-4-phenyl-3-butynoic acid ethyl ester as a yellow oil; yield: 200 mg (0.98 mmol, 2%). ^{1}H NMR ($CDCl_3$): δ = 1.4 (3H, t, *J* = 7 Hz, CH_3), 3.4–3.6 (1H, br, OH), 4.3 (2H, q, *J* = 7 Hz, CH_2), 5.1 (1H, s, −C<u>H</u>OH−), 7.3–7.4 (3H, m, Ar), 7.4–7.5 (2H, m, Ar); ^{13}C NMR: ($CDCl_3$): δ = 14.0 (CH_3), 61.9, 62.8 (CH_2-O, −CHOH), 84.3, 85.4 (Ar-<u>C</u>≡<u>C</u>), 121.9–131.9 (6C, Ar), 170.4 (C=O).

***rac*-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid ethyl ester:** *rac*-2-Hydroxy-4-phenyl-3-butynoic acid ethyl ester (200 mg, 0.98 mmol) was reduced to the corresponding *Z*-alkene using quinoline (208 mg, 1.36 mmol) and Lindlar catalyst (80 mg, 5% wt Pd on $CaCO_3$ with Pb, Sigma) in ethanol (2 mL). The mixture was stirred under a hydrogen atmosphere (1 bar) for 6 h. The catalyst was filtered off and Amberlite IR-120 (240 mg, H^+ form) was added to remove the remaining quinoline. The mixture was stirred at room temperature for 2 h, filtered and the organic solvent was evaporated under reduced pressure to give *rac*-(*Z*)-2-hydroxy-4-phenyl-3-butenoic acid ethyl ester as a yellow oil; yield: 158 mg (79%). ^{1}H NMR ($CDCl_3$): δ = 1.4 (3H, t, *J* = 7 Hz, -CH_3), 4.3 (2H, q, *J* = 7 Hz, CH_2), 5.1 (1H, d, *J* = 10 Hz, −CH=CH−C<u>H</u>OH−), 5.7 (1H, t, *J* = 10 Hz, −CH=C<u>H</u>−CHOH−), 6.8 (1H, d, *J* = 11 Hz, −C<u>H</u>=CH−CHOH−), 7.2–7.5 (5H, m, Ar); ^{13}C NMR ($CDCl_3$): δ = 14.0 (CH_3), 62.3, 67.3 (CH_2-O, −CHOH−), 127.3, 127.8, 128.3, 128.9, 135.8, 134.7 (<u>Ar-C</u>H=<u>C</u>H), 173.9 (C=O).

***rac*-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid:** *rac*-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid ethyl ester (158 mg, 0.767 mmol) was hydrolyzed in 1 M aqueous LiOH (1.8 mL) and THF (0.2 mL) at room temperature for 24 h. The reaction mixture was acidified with 1 M HCl to pH 2 and extracted with ethyl acetate (4 × 4 mL), the combined organic phase was dried (Na_2SO_4) and the solvent evaporated under reduced pressure to give *rac*-(*Z*)-2-hydroxy-4-phenyl-3-butenoic acid as yellow crystals; yield: 120 mg (0.67 mmol, 87%); mp 102.2–104 °C. ^{1}H NMR ($CDCl_3$): δ = 5.1 (1H, d, *J* = 10 Hz, −C<u>H</u>OH−), 5.7 (1H, t, *J* = 10 Hz, −CH=C<u>H</u>−CHOH−), 6.87 (1H, d, *J* = 10 Hz, −C<u>H</u>=CH−CHOH−), 7.2–7.5 (5H, m, Ar); ^{13}C-NMR ($CDCl_3$): δ = 67.1 (-CHOH), 126.6, 127.9, 128.4, 128.9, 135.5; 134.7 (<u>Ar-C</u>H=<u>C</u>H−), 176.7 (C=O).

***(S)*-(*Z*)-2-Acetoxy-4-phenyl-3-butenoic acid:** *rac*-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid (10 mg, 0.056 mmol) was dissolved in diisopropyl ether (1.3 mL) before 260 μL vinyl acetate and lipase from *Candida rugosa* (11 mg, Amano AY-30) were added. The suspension was shaken at 130 rpm at 28 °C in Eppendorf tubes (1.5 mL). Samples were taken regularly to measure ee and conversion. The (*Z*)-isomer proved to be a very bad substrate for the lipase-mediated kinetic resolution. After 28 days the lipase was filtered off, the organic solvent was evaporated and the product mixture was purified by silica gel chromatography [petroleum ether/ethyl acetate/glacial acetic acid 2:1:(50 μL/L)] to give (*S*)-(*Z*)-2-acetoxy-4-phenyl-3-butenoic acid; yield: 5 mg (0.022 mmol, 39%); ee (product): > 97% (HPLC), enantioselectivity *E* > 100. ^{1}H NMR ($CDCl_3$): δ = 2.12 (3H, s, CH_3−COO−), 5.78 (1H, t, *J* = 11 Hz, −CH=C<u>H</u>−CHOCOCH$_3$), 5.86 (1H, d, *J* = 10.5 Hz, −CH=CH−C<u>H</u>OH−), 6.94 (1H, d, *J* = 11.2 Hz, −C<u>H</u>=CH−CHOH−); 7.27–7.45 (5H, m, Ar).

***(S)*-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid [(*S*)-16]:** (*S*)-(*Z*)-2-Acetoxy-4-phenyl-3-butenoic acid (5 mg, 0.022 mmol) was dispersed in Mg-HEPES buffer (3 mL, 50 mM HEPES, 3.3 mM $MgCl_2 \cdot 6\ H_2O$, pH 7.6) in a ultrasonic bath. After addition of lipase from *Candida rugosa* (40 mg), the mixture was shaken at 30 °C at 130 rpm for 20 days. The mixture was centrifuged and the pH was adjusted to pH 1 using 1 N aqueous HCl. Extraction with ethyl acetate (4 × 5 mL), drying (Na_2SO_4) and evaporation of the organic solvent under reduced pressure afforded (*S*)-(*Z*)-2-hydroxy-4-phenyl-3-butenoic acid; yield: 3.5 mg (0.02 mmol, 91%), $[\alpha]_D^{20}$: +59.8 (*c* 0.225, MeOH), ee 91% (HPLC). NMR data were identical to those of the racemic compound. The absolute configuration was deduced using three different indicators: (i) Kazlauskas rule,[52] ii) comparison of the sense of the optical rotation with other homochiral β,γ-unsaturated-α-hydroxy acids, and (iii) comparison of elution order on chiral HPLC with other β,γ-unsaturated-α-hydroxy acids.

rac-(*Z*)-2-Acetoxy-4-phenyl-3-butenoic Acid

A mixture of *p*-dimethylaminopyridine (10 mg) and acetic anhydride (38.8 mg, 0.38 mmol) was stirred at 4 °C for 15 min before *rac*-(*Z*)-2-hydroxy-4-phenyl-3-butenoic acid (50 mg, 0.28 mmol) was added. Stirring was continued at room temperature for 16 h. Dichloromethane (11 mL) and ice water (6 mL) were added and the mixture was stirred for 30 min, the organic phase was separated, the aqueous phase was extracted with dichloromethane (5 mL), the combined organic phases were dried (Na_2SO_4) and the organic solvent was evaporated under reduced pressure. Purification using silica gel column chromatography [petroleum ether (ethyl acetate/glacial acid = 2:1:(50 μL/L)] afforded the pure product as a colorless oil; yield: 30 mg (0.18 mmol, 64%). The NMR data were identical to those of (*S*)-(*Z*)-2-acetoxy-4-phenyl-3-butenoic acid.

Synthesis of *rac*-2-Cyclohexenyl-2-hydroxyethanoic Acid (*rac*-21)

Starting from 1-cyclohexene-1-carboxaldehyde (0.59 g, 5.4 mmol) the synthesis was performed as described for *rac*-(*E*)-2-hydroxy-3-pentenoic acid (*rac*-**22**) to afford *rac*-2-cyclohexenyl-2-hydroxyethanoic acid (*rac*-**21**); overall yield: 0.46 g (75.4%). ^{1}H NMR ($CDCl_3$): δ = 1.53 (4H, m), 2.0 (4H, m), 4.3 (1H, s, C<u>H</u>OH-), 5.7 (1H); ^{13}C-NMR ($CDCl_3$): δ = 21.8, 21.9, 23.6, 24.4 (aliphatic C, cyclohexenyl), 74.5 (<u>C</u>HOH), 124.3, 135.9 (C=C, cyclohexenyl), 173.9 (C=O).

Synthesis of (*R*)-2-Cyclohexenyl-2-hydroxyethanoic Acid [(*R*)-21] Starting from *rac*-21 using Lipase "Amano P"

rac-2-Cyclohexenyl-2-hydroxyethanoic acid (*rac*-**21**; 0.398 g, 2.55 mmol), was dissolved in diisopropyl ether (50 mL), vinyl acetate (10 mL, 108 mmol) and Lipase "Amano P" (0.5 g) were added and the mixture was shaken at room temperature at 180 rpm for 2 days and afterwards again for 2 days at 40 °C. The lipase was removed by filtration and the organic solvent was removed by evaporation under reduced pressure. (*R*)-2-Cyclohexenyl-2-hydroxyethanoic acid [(*R*)-**21**] was purified by silica gel chromatography (petroleum ether/ethyl acetate = 1:1 with 50 mL AcOH per liter solvent) giving the enantiopure product; yield: 0.179 g (corresponding to 90% of 50% theoretical yield); ee > 99%; $[\alpha]_D^{20}$: −68 (*c* 0.2, H_2O).

The absolute configuration was deduced as described for (*S*)-(*Z*)-2-hydroxy-4-phenyl-3-butenoic acid but as third indicator the elution order on chiral GC.

GC Analysis

rac-(*E*)-2-Hydroxy-3-pentenoic acid (*rac*-**22**) was analyzed as the corresponding methyl ester; column: Chrompack Chirasil Dex (25 m × 0.32 mm × 0.25 μm, 0.75 bar N_2), isotherm 100 °C, 3.3 min (*R*), 3.8 min (*S*).

rac-2-Hydroxy-pentanoic acid (*rac*-**26**) was analyzed as the corresponding methyl ester; column: Chrompack Chirasil Dex (25 m × 0.32 mm × 0.25 μm, 1.0 bar N_2), isotherm 100 °C, 2.3 min (*R*), 2.6 min (*S*).

rac-2-Cyclohexenyl-2-hydroxyethanoic acid (*rac*-**21**) was analyzed as the corresponding methyl ester; column: Chiraldex B-TA, Astec (30 m × 0.25 mm, 0.55 bar H_2), isotherm 150 °C, 34.4 min (*R*), 37.0 min (*S*).

HPLC analysis

Jasco HPLC system (pumps PU-980, multi-wavelength-detector MD-910, autosampler AS-950, degasser CMA/260), solvent: *n*-heptane/2-propanol/TFA = 90:10:1, flow 0.65 mL/min.

rac-(*Z*)-2-Hydroxy-4-phenyl-3-butenoic acid (*rac*-**16**), Chiracel OD-H (Daicel, Ø 0.46 × 25 cm), 11.4 min (*R*), 14.8 min (*S*).

rac-(*E*)-2-Hydroxy-4-phenyl-3-butenoic acid (*rac*-**15**), Chiralpak AD (Daicel, Ø 0.46 cm × 25 cm), 16.8 min (*R*), 20.8 min (*S*).

rac-2-Hydroxy-4-phenylbutyric acid (*rac*-**17**), Chiralpak AD (Daicel, Ø 0.46 cm × 25 cm), 14.9 min (*R*), 17.6 min (*S*).

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