ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 15 (2001) 213–222

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Substrate spectrum of mandelate racemase Part 2. (Hetero)-aryl-substituted mandelate derivatives and modulation of activity

Ulfried Felfer, Ulrike T. Strauss, Wolfgang Kroutil, Walter M.F. Fabian, Kurt Faber*

Department of Chemistry, Organic and Bioorganic Chemistry, Heinrichstrasse 28, A-8010 Graz, Austria

Received 9 March 2001; accepted 15 May 2001

Abstract

Efficient enzymatic racemization of 2-hydroxy-2-heteroaryl-acetic acid derivatives by mandelate racemase under mild conditions is reported for the first time. (i) Steric limitations for aryl-substituted mandelate derivatives were elucidated to be particularly striking for *o*-substituents, whereas *m*- and *p*-analogues were freely accepted, as well as heteroaryl- and naphthyl-analogs. (ii) The electronic character of substituents was found to play an important role: whereas electron-withdrawing substituents dramatically enhanced the racemization rates, electron-donating analogs caused a depletion. This effect could be ascribed to an α-carbanion-stabilization in accordance with the known enzyme mechanism. The latter was modeled by comparison of gas phase deprotonation energies as a useful parameter to describe resonance stabilization. The calculated data nicely correlate with the experimentally observed activities for a specific substrate as long as other parameters, such as steric restrictions, are absent or play a minor role.

Keywords: Mandelate racemase [EC 5.1.2.2]; Enzymatic racemization; *Pseudomonas putida* ATCC 12633; α-Hydroxy heteroarylacetic acid; Mandelic acid

1. Introduction

The controlled racemization of organic compounds [1] constitutes the key to the transformation of racemates into a single stereoisomer in 100% theoretical yield by employing dynamic (kinetic) resolution [2,3] or stepwise deracemization techniques [4]. Recently, mandelate racemase [EC 5.1.2.2] was recognized as a valuable catalyst for the racemization of stereochemically stable α-hydroxycarboxylic acids, such as mandelate, at neutral pH and ambient temperature. These properties allowed it to be combined with a lipase to furnish a two-enzyme deracemization process of its natural substrate, (±)-mandelate [5]. Despite of the detailed mechanistic knowledge on mandelate racemase [6], very little is known on its substrate tolerance [7–9]. Recently, we have shown that the 2-hydroxycarboxylic acid moiety, which for a long time was thought to be an absolute requirement, could be replaced by a 2-hydroxyamide moiety [10].

In this paper, we wish to present possible variations of the aryl moiety of mandelate as well as the electronic influence of different substituents on the stabilization of the α-carbanion intermediate combined with steric aspects. Structural modifications of the natural substrate — mandelate — considered here include (Scheme 2): (i) replacement of the phenyl ring of mandelate by a heteroaryl moiety (substrates **2**–**4**);

* Corresponding author. Tel.: +43-316-380-5332; fax: +43-316-380-9840.
E-mail address: kurt.faber@kfunigraz.ac.at (K. Faber).

1381-1177/01/$ – see front matter
PII: S1381-1177(01)00035-2

(ii) extension of the conjugated π-system by introduction of a 2-naphthyl moiety (substrate **5**); (iii) substitution of the phenyl ring by electron-donating or -withdrawing substituents (substrates **6**–**12**).[1] In order to estimate the importance of resonance stabilization of the α-carbanion-intermediate, molecular orbital calculations of the relative deprotonation energies at carbon C-2 (with respect to mandelate) were performed in addition to experimentally determined relative activities.

2. Experimental

2.1. General

(*R*)-Mandelic acid **1** (e.e. 98%) and thiophene-2-carbaldehyde were purchased from Fluka, furfural, 3-furaldehyde, 2-naphthaldehyde, (*R*)-*o*-chloromandelic acid (**6**) and (*R*)-*m*-chloromandelic acid (**7**) were purchased from Aldrich, *p*-fluorobenzaldehyde was obtained from Merck. Column chromatography was performed using Merck 60 silica gel (40–63 μm). ^{1}H 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.

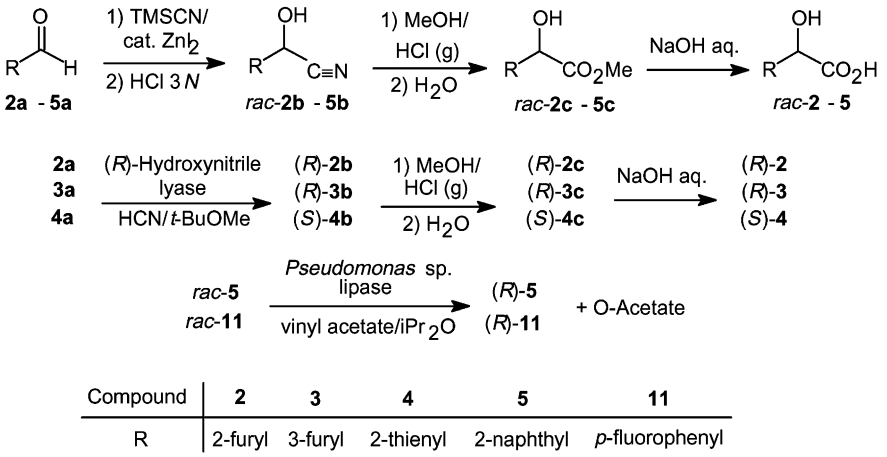
A semi-purified enzyme preparation of mandelate racemase was prepared as previously described, the specific activity was 107 μmol mg^{-1} min^{-1} [11].[2] Relative rates of racemization were determined using an assay based on the online measurement of the decline of optical rotation versus time [12]. In experiments, where the substrate was not optically pure, the e.e. of mandelate (used as standard) was adjusted to that of the substrate in order to obtain comparable data for the 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 under the reaction conditions employed was verified for all substrates (i) in the absence of biocatalyst and (ii) in the presence of heat-denatured enzyme and was proven to be <0.5% within 48 h for all substrates (Scheme 1).

2.2. General procedure for the one-pot synthesis of rac-2-hydroxycarboxylic acid methyl esters (*rac*-**2c**–*rac*-**5c**)

Trimethylsilyl cyanide (TMSCN, 24 mmol) was added to freshly distilled aldehyde **2a**–**5a** (20 mmol) in anhydrous CH_2Cl_2 at 0°C. After the addition of a few crystals of zinc iodide, the reaction mixture was stirred at 0°C for 15 min before it was allowed to warm to room temperature. After stirring was resumed for further 45 min, the solvent was evaporated under reduced pressure and the crude TMS cyanohydrin was subjected to acid-hydrolysis (7.5 g of 3 N HCl per gram of the crude mixture) at 0°C. The mixture was stirred for 1 h before it was extracted three times with methyl *tert*-butyl ether (MTBE). The combined organic phases were dried (Na_2SO_4) and the organic solvent was evaporated to yield crude cyanohydrin *rac*-**2b**–**5b**, which was converted to its methyl ester via the corresponding imidate through a modified Pinner reaction [13]. Without purification, the cyanohydrin *rac*-**2b**–**5b** (1 g) 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°C for 2 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°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 the methyl 2-hydroxy carboxylic acid esters *rac*-**2c**–**5c**, which were purified, if necessary, by column chromatography on silica gel using cyclohexane/ethyl acetate (5:1) as eluent. The following compounds were thus obtained.

[1] Possible reduction and modification of the size of the π-system will be published in due course.

[2] Mandelate racemase has been recently made available in large quantities through enzyme induction (23×10^6 U from a 10 l fermentation). This activity allows the racemization of ~1.5 t of mandelate within ~8 h.



Compound	2	3	4	5	11
R	2-furyl	3-furyl	2-thienyl	2-naphthyl	*p*-fluorophenyl

Scheme 1. Synthesis of substrates.

*2.2.1. rac-2-(2-Furyl)-2-hydroxyethanoic acid methyl ester (rac-**2c**)*

Overall yield 2.4 g (77%), yellowish oil. ^{1}H NMR ($CDCl_3$): δ 7.40 (m, 1H), 6.40 (m, 2H), 5.22 (s, 1H), 3.85 (s, 3H), 3.40 (bs, 1H); ^{13}C NMR ($CDCl_3$): δ 172.0 (C), 150.8 (*i*-C), 143.2 (=CH–O), 110.6, 108.8 (2× CH), 66.9 (CHOH), 53.3 (CH_3).

*2.2.2. rac-2-(3-Furyl)-2-hydroxyethanoic acid methyl ester (rac-**3c**)*

Overall yield 0.58 g (34%), yellowish oil. ^{1}H NMR ($CDCl_3$): δ 7.50, 7.40 (2× m, 2× 1H), 6.45 (m, 1H), 5.20 (s, 1H), 3.80 (s, 3H), 3.30 (bs, 1H); ^{13}C NMR ($CDCl_3$): δ 173.7 (C), 143.5, 140.3, (2× CH), 123.5 (*i*-C), 108.7 (CH), 66.3 (CHOH), 53.0 (CH_3).

*2.2.3. rac-2-Hydroxy-2-(2-thienyl)-ethanoic acid methyl ester (rac-**4c**)*

Overall yield 1.85 g (62%), brown oil. ^{1}H NMR ($CDCl_3$): δ 7.30 (d, $J = 5$, 1H), 7.1 (d, $J = 3.5$, 1H), 7.00 (dd, $J = 5.0$ and 3.5, 1H), 5.45 (d, $J = 6.1$, 1H), 3.70 (s, 3H), 3.60 (d, $J = 6.1$, OH); ^{13}C NMR ($CDCl_3$): δ 173.0 (C), 141.4 (*i*-C), 127.0, 125.8, 125.5 (3× CH), 69.1 (CHOH), 53.3 (CH_3).

*2.2.4. rac-2-Hydroxy-2-(2-naphthyl)-ethanoic acid methyl ester (rac-**5c**)*

Overall yield 2.12 g (84%), mp and NMR were identical to reported data [14].

*2.3. General procedure for the hydrolysis of 2-hydroxycarboxylic acid methyl esters rac-**2c**–**5c** to the corresponding acids (rac-**2**–rac-**5**)*

Methyl ester **2c**–**5c** (1 mmol) was stirred in an aqueous NaOH solution (2.5 ml, 2.6 M) at 0°C for 15 min before the mixture was allowed to warm to room temperature. For *rac*-**5**, the reaction was performed in a solvent-mixture of methanol/water (3:1) for better solubility. After 2 h, the mixture was acidified to pH 2.0 (7 M HCl) and extracted with ethyl acetate (4 × 10 ml). The combined organic phases were dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to yield free acid **2**–**5**. The following compounds were thus obtained.

*2.3.1. rac-2-(2-Furyl)-2-hydroxyethanoic acid (rac-**2**)*

Yield 0.38 g (83%), yellowish crystals, mp: 108–111°C (MeOH), lit. [15] 115°C (EtOH). ^{1}H NMR (acetone-d_6): δ 7.60 (d, $J = 0.8$, 1H), 7.50 (d, $J = 1.8$, 1H), 6.40 (dd, $J = 1.8$ and 0.8, 1H), 5.25 (s, 1H); ^{13}C NMR (acetone-d_6): δ 172.6 (C), 153.5 (*i*-C), 143.6, 111.4, 109.0 (3× CH), 67.6 (CHOH).

*2.3.2. rac-2-(3-Furyl)-2-hydroxyethanoic acid (rac-**3**)*

Yield 0.09 g (66%), white-yellowish crystals, mp: 70–72°C (petroleum ether/acetone). ^{1}H NMR

(acetone-d_6): δ 7.60, 7.50, 6.50 (3× m, 3× 1H), 5.20 (s, 1H); ^{13}C NMR (acetone-d_6): δ 174.3 (C), 144.2, 141.2 (2× CH), 125.8 (*i*-C), 110.2 (CH), 66.8 (CHOH).

2.3.3. *rac*-2-Hydroxy-2-(2-thienyl)-ethanoic acid (*rac*-**4**)

Yield 0.03 g (66%), brown oil. ^{1}H NMR (acetone-d_6): δ 7.40, 7.20, 7.00 (3× m, 3× 1H), 5.50 (s, 1H); ^{13}C NMR (acetone-d_6): δ 173.6 (C), 143.9 (*i*-C), 127.6, 126.3, 126.1 (3× CH), 69.9 (CHOH).

2.3.4. *rac*-2-Hydroxy-2-(2-naphthyl)-ethanoic acid (*rac*-**5**)

Yield 1.77 g (68%), yellow crystals, mp: 153°C, lit. [14] 155°C (benzene). NMR-data were identical to literature values [16].

rac-*p*-Fluoromandelic acid (*rac*-**11**) was synthesized according to a known procedure [17].

Yield 1.2 g (77%), white crystals, mp: 134–135°C (Et_2O), lit. [17] 138–139°C (benzene). ^{1}H NMR (DMSO-d_6): δ 7.42 (m, 2H), 7.15 (m, 2H), 5.04 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 173.9 (C), 161.7 (d, J = 243, C), 136.5 (d, J = 3.0, C), 128.6 (d, J = 8.2, 2× CH), 114.9 (d, J = 21.2, 2× CH), 71.7 (CHOH).

2.4. General procedure for the preparation of nonracemic 2-hydroxycarboxylic acid methyl esters (*R*)-**2c**, (*R*)-**3c** and (*S*)-**4c** from the corresponding aldehyde 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 literature [18]. 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 dry (*R*)-hydroxynitrile lyase preparation was rehydrated with citrate buffer (9 ml, 20 mM, pH 5.5) for 30 min. The freshly prepared MTBE-HCN solution (~40 mmol HCN) was added at once at +3°C before freshly distilled aldehyde **2a**–**4a** (20 mmol) was added via a syringe. The reaction was stirred at +3°C for 24 h before the biocatalyst was separated by filtration. The filtrate was extracted with MTBE (2× 50 ml), the combined organic layer was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to yield the crude nonracemic cyanohydrin [(*R*)-**2b**, (*R*)-**3b**, (*S*)-**4b**] which was immediately transformed to the corresponding nonracemic 2-hydroxycarboxylic acid methyl ester [(*R*)-**2c**, (*R*)-**3c**, (*S*)-**4c**] via the Pinner-reaction as described above. The following compounds were thus obtained.

2.4.1. (*R*)-2-(2-Furyl)-2-hydroxyethanoic acid methyl ester [(*R*)-**2c**]

Overall yield 0.71 g (23%), yellowish oil. e.e. 96% (GC); $[\alpha]_D^{20}$ −124.1° (*c* 2.53, $CHCl_3$), lit. [19] −128.5° (*c* 1, $CHCl_3$). NMR-data were identical to that of *rac*-**2c**.

2.4.2. (*R*)-2-(3-Furyl)-2-hydroxyethanoic acid methyl ester [(*R*)-**3c**]

Overall yield 0.62 g (34%), colorless oil. e.e. 96% (GC); $[\alpha]_D^{20}$ −73.1° (*c* 1.15, CH_2Cl_2). NMR-data were identical to that of *rac*-**3c**.

2.4.3. (*S*)-2-Hydroxy-2-(2-thienyl)-ethanoic acid methyl ester [(*S*)-**4c**]

Overall yield 0.254 g (14%), colorless oil. e.e. 96% (GC); NMR-data were identical to that of *rac*-**4c**.

2.5. General procedure for the hydrolysis of nonracemic 2-hydroxycarboxylic acid methyl esters [(*R*)-**2c**, (*R*)-**3c**, (*S*)-**4c**] to the corresponding chiral acids [(*R*)-**2**, (*R*)-**3**, (*S*)-**4**]

The identical procedure was used as described for the hydrolysis of racemic methyl esters. The following compounds were thus obtained.

2.5.1. (*R*)-2-(2-Furyl-)2-hydroxyethanoic acid [(*R*)-**2**]

Yield 0.18 g (78%), brown oil. e.e. 97% (GC, analyzed as the methyl ester); $[\alpha]_D^{20}$ −60.6° (*c* 0.058, MeOH). NMR-data were identical to that of *rac*-**2**.

*2.5.2. (R)-2-(3-Furyl-)2-hydroxyethanoic acid [(R)-**3**]*

Yield 0.09 g (66%), yellowish crystals, mp: 70–72°C (petroleum ether/acetone). e.e. 95% (GC, analyzed as the methyl ester); $[\alpha]_D^{20}$ −51.0° (*c* 0.75, MeOH). NMR-data were identical to that of *rac*-**3**.

*2.5.3. (S)-2-Hydroxy-2-(2-thienyl)-ethanoic acid [(S)-**4**]*

Yield 0.14 g (99%), brown crystals, mp: 58–60°C (petroleum ether/acetone), lit. [19] 83–84°C, benzene; e.e. 72% (HPLC); $[\alpha]_D^{20}$ −89.8° (*c* 1.09, acetone) and −58.0° (*c* 0.12,[3] H_2O), lit. [19] −99.6° (*c* 1, H_2O). NMR-data were identical to that of *rac*-**4**.

*2.6. General procedure for the preparation of nonracemic 2-hydroxy acids [(R)-**5**, (R)-**11**] from rac-2-hydroxy acids [**5**,**11**] employing lipase-catalyzed acyl transfer [21]*

rac-2-Hydroxycarboxylic acid **5**, **11** (10 mmol) was dissolved in diisopropyl ether (60 ml). Vinyl acetate (200 mmol) and *Pseudomonas* sp. lipase (Amano P, 1 g) were added and the suspension was shaken at room temperature at 150 rpm. The reaction was monitored by HPLC and at a conversion of 50% it was stopped by filtration of the enzyme. The lipase was washed with diisopropyl ether, and the combined organic phases were evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, petroleum ether/ethyl acetate (2:1) containing 0.1% glacial acid. The following compounds were thus obtained.

*2.6.1. (R)-2-Hydroxy-2-(2-naphthyl)-ethanoic acid [(R)-**5**]*

Yield 0.1 g (14%), yellowish crystals, mp was identical to literature [16] 162.0–163.5°C. e.e. 82% (HPLC, analyzed as methyl ester); $[\alpha]_D^{20}$ −108° (*c* 0.18, H_2O), −144° (*c* 0.98, EtOH) [16]. NMR-data were identical to literature [16].

*2.6.2. (R)-p-Fluoromandelic acid [(R)-**11**]*

Yield 0.2 g (19%), white crystals, mp: 148.7–150.5°C (petroleum ether/ethyl acetate), lit. [22] 153–156°C. e.e. >99% (HPLC); $[\alpha]_D^{20}$ −128.1° (*c* 0.3, EtOH), lit. [22] −134° (*c* 0.3, EtOH). NMR-data were identical to that of *rac*-**11**.

*2.7. General procedure for the derivatization of carboxylic acids **2**–**4** to the corresponding methyl esters for GC-analysis*

A diazomethane solution (3 M eq. in diisopropyl ether, ∼0.2 M) was added to a solution of the 2-hydroxy acid (1 eq.) in diisopropyl ether. The reaction was monitored by TLC and the solution was left until it became colorless. This solution was directly used for GC analysis.

2.8. Determination of absolute configuration

Compounds (*S*)-**4**, (*R*)-**5** and (*R*)-**11** were elucidated by comparison of optical rotation values with literature data. For (*R*)-**2** and (*R*)-**3** it was assumed to be (*R*) since (*R*)-hydroxynitrilase is known to produce exclusively the (*R*)-enantiomer [23]. This assumption is consistent with the fact that the sign of optical rotation for all (*R*)-enantiomers [(*S*) in case of substrate **4** due to a switch in the CIP-priorities] was found to be negative for all 2-hydroxy acids throughout this study.

2.9. Determination of enantiomeric purity

Enantiomeric compositions were determined via GC or HPLC on a chiral stationary phase.

2.9.1. HPLC

Jasco HPLC-system (pumps PU-980, multi-wavelength-detector MD-910, autosampler AS-950, degasser CMA/260). Two columns were used for HPLC analysis [Chiralpak AD (Daicel, 0.46 cm × 25 cm), Chiracel OD (Daicel, 0.46 cm × 25 cm)]; *rac*-**1**: flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic acid (90:10:0.1); T_{ret} 23.6 min (*S*), 27.7 min (*R*), column AD; *rac*-**4**: flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic acid (95:5:1); T_{ret} 16.0 min (*S*), 17.3 min (*R*), column AD; *rac*-**5** (analyzed as its methyl ester): flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic acid (80:20:1); T_{ret} 14.5 min (*S*), 16.2 min (*R*), column OD-H; *rac*-**6**: flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic

[3] At a concentration of $c = 1$ the turbidity of the solution impedes accurate measurements.

acid (90:10:0.1); T_{ret} 31.0 min (*S*), 34.7 min (*R*), column AD; *rac*-**7**: flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic acid (90:10:0.1); T_{ret} 19.8 min (*S*), 23.6 min (*R*), column AD; *rac*-**11**: flow 0.5 ml min^{-1}, eluent heptane/*i*-propanol/trifluoroacetic acid (90:10:0.1); 25.4 min (*S*), 27.5 min (*R*), column AD.

2.9.2. GC

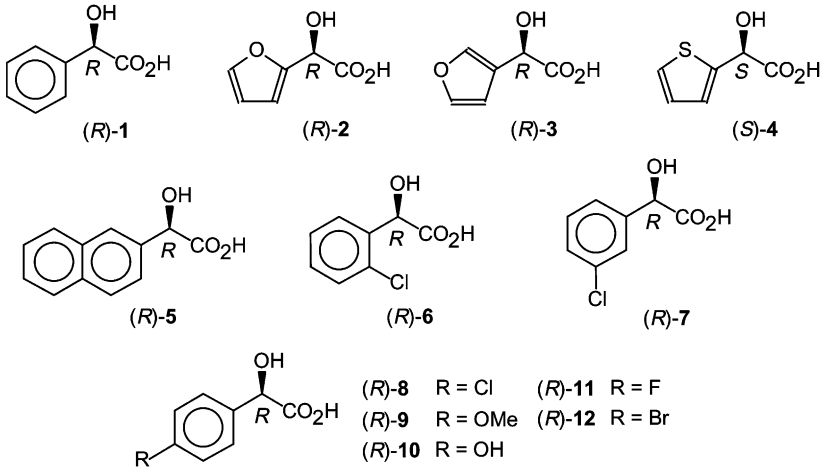
Analyses were performed on a Shimadzu GC-14A equipped with FID on a Chirasil-DEX CB column (25 m × 0.25 μm) with hydrogen as carrier gas (1 bar): *rac*-**2** (analyzed as methyl ester): 100°C isotherm, T_{ret} 3.7 min (*R*), 4.7 min (*S*); *rac*-**3** (analyzed as methyl ester): 110°C isotherm, T_{ret} 4.3 min (*R*), 4.8 min (*S*); *rac*-**4** (analyzed as methyl ester): 110°C isotherm, T_{ret} 7.9 min (*R*), 8.4 min (*S*).

2.10. Computational details

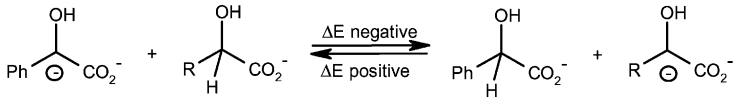
All calculations were done by using the Gaussian 98 program suite [24]. Geometries were completely optimized using ab initio Hartree-Fock and the Becke's three-parameter hybrid HF/density functional method [25] with the Lee et al. correlation function [26]. All stationary points were characterized as minima by frequency calculations at the same level of theory as geometry optimizations were performed. Zero point energies are unscaled. The 6-31+G* basis set was used throughout. In addition, single point calculations (B3LYP/6-31+G* geometry) at the MP2/6-31+G* and, in selected cases, B3LYP/6-311++G**, B3LYP/6-311++G(2df, p) and MP2/6-311++G** levels of theory were performed. For the semiempirical calculations the AMPAC program package [27] with the AM1 [28] and PM3 Hamiltonians were used [29].

3. Results and discussion

A series of non-natural substrate-analogues to mandelate were synthesized in non-racemic form through biocatalytic protocols based on (i) the asymmetric cyanohydrin formation using (*R*)-hydroxynitrile lyase from almond or via (ii) kinetic resolution employing lipase catalyzed acyl transfer. For all racemization experiments, the enantiomer corresponding to (*R*)-mandelate was used. It should be noted that the apparent (*S*)-configuration of substrate **4** is only due to a switch in CIP-priorities, but it is *homochiral* to (*R*)-mandelate in a strict sense. The substrates were subjected to the action of a crude enzyme preparation [11] in aqueous buffer (HEPES, 50 mM, pH 7.5, 3.3 mM $MgCl_2 \cdot 6H_2O$) at room temperature. The rate of racemization was measured using a previously described assay procedure based on the decline of the optical rotation [12]. Activities are denoted in % relative to mandelate as the natural substrate (Scheme 2).



Scheme 2. Investigated α-hydroxy acids.



Scheme 3. Model for the calculation of gas-phase deprotonation energies.

Since experimentally determined activities were measured relative to mandelate (**1**), deprotonation energies of the α-carbon were modeled as the corresponding reaction energies relative to mandelate (Scheme 3). Gas phase deprotonation energies at the α-carbon as obtained by semiempirical (AM1, PM3), ab initio (HF, MP2) as well as density functional theory methods (B3LYP, for details of the calculations see the Section 2) are listed in Table 1. The energy difference (ΔE) is negative, if the carbanion intermediate of the synthetic substrate is more stable than that of mandelate and vice versa. Consequently, the equilibrium is shifted to the right or left, respectively. It has to be emphasized that these calculations yield gas phase deprotonation energies and, as a consequence, neither steric, nor electronic or 'environment'-effects, induced by the enzyme surface or the solvent, etc. are therefore taken into account.

3.1. Hetero-aryl derivatives

It was anticipated that the replacement of the phenyl moiety of the natural substrate — mandelate — by a five membered hetero-aromatic system should not cause any steric restrictions in such a way that these substrates would not be accepted any more. However, the presence of a hetero-atom, such as O or S, in the five-membered ring reduces the resonance energies relative to benzene and thus makes these analogs 'less aromatic'. As a consequence, the aryl-moiety becomes more polar and is less suited to delocalize and thus stabilize π-electrons. Indeed, (*R*)-2-hydroxy-2-(2-furyl)-acetic acid (*R*)-**2** and (*R*)-2-hydroxy-2-(3-furyl)-acetic acid (*R*)-**3** were racemized with moderate activity (14 and 23%, respectively) compared to that of (*R*)-mandelic acid (*R*)-**1** (100%, Table 1). Substrates (*R*)-**2** and (*R*)-**3** are

Table 1
Relative activities and calculated deprotonation energies ΔE (density functional theory, ab initio HF, and semiempirical AM1 and PM3) of substrates[a]

Substrate	Relative activity (%)[b]	ΔE (kcal mol^{-1})			
		B3LYP	HF	AM1	PM3
(*R*)-**1**	100	0.0	0.0	0.0	0.0
(*R*)-**2**	14	6.0, 6.7[c], 4.4[d]	4.9	2.2	3.2
(*R*)-**3**	23	13.7, 14.4[c], 12.3[d]	14.0	3.5	15.0
(*S*)-**4**	27	−1.5, −0.4[c], −1.3[d], 0.6[e]	−3.5	−4.7	−11.8
(*R*)-**5**	26	–	–	−16.7	−15.3
(*R*)-**6**	<1	−3.5, −2.1[c]	−3.4	−5.1	−6.2
(*R*)-**7**	61	−9.4, −9.0[c]	−9.8	−8.4	−8.5
(*R*)-**8**	326[f]	−7.3, −6.7[c], −7.3[d]	−7.7	−9.5	−8.8
(*R*)-**9**	17[f]	−1.4, −0.3[c]	1.9	−1.8	−1.1
(*R*)-**10**	45[f]	2.4, 2.2[c], −0.7[d], 1.3[e], −0.4[g]	4.6, 17.2[h]	−1.1	−8.8
(*R*)-**11**	96	−0.6, −0.5[c], −0.7[d], 0.3[e], −0.1[g]	1.4	−6.5	−6.8
(*R*)-**12**	376[f]	–	–	−12.4	−10.6

[a] B3LYP: B3LYP/6-31+G*//B3LYP/6-31+G*+ΔZPE(B3LYP/6-31+G*); HF: HF/6-31+G*//HF/6-31+G*+ΔZPE(B3LYP/6-31+G*).
[b] Relative activities are expressed as % relative to the natural substrate (mandelate).
[c] MP2/6-31+G*//B3LYP/6-31+G*+ΔZPE(HF/6-31+G*).
[d] B3LYP/6-311++G**//B3LYP/6-31+G*+ΔZPE(B3LYP/6-31+G*).
[e] MP2/6-311++G**//B3LYP/6-31+G*+ΔZPE(B3LYP/6-31+G*).
[f] Data from [7].
[g] B3LYP/6-311++G(2df, p)//B3LYP/6-31+G*+ΔZPE(B3LYP/6-31+G*).
[h] Planarity of the OH group enforced.

typical electron-excess heteroaromatic compounds and hence cannot stabilize the carbanion formed during catalysis as well as mandelate **1**. This is also clearly shown by the calculated deprotonation energies for (*R*)-**2** and (*R*)-**3**, which are positive (Table 1). However, the difference in relative reactivity between (*R*)-**2** and (*R*)-**3** cannot easily be explained, since for substrate (*R*)-**2** at least seven resonance structures for the carbanion intermediate can be drawn theoretically, whereas for substrate (*R*)-**3** only six are possible. In line with this qualitative argument, calculated deprotonation energies are also more positive for (*R*)-**3** than for (*R*)-**2**. Consequently, other factors besides the stability of the carbanion intermediate must exert some influence. The enhanced activity for the thiophene derivative (*S*)-**4** (27%) relative to the furan-analogs presumably results from the comparable electronegativity of sulfur (2.6) to carbon (2.5) and from the 'softness' of the hetero-atom, which leads to enhanced carbanion stabilization in comparison to oxygen. Furthermore, some stabilization of the carbanion by the heteroatom could result from the empty d-orbitals of sulfur acting as electron acceptors. Except at the highest level employed (MP2/6-311++G**) the calculations predict greater stability of the dianion of (*R*)-**4** compared to that of (*R*)-**1**. Raney-Ni reduction of the thiophene moiety of substrate **4** leads to the corresponding aliphatic counterpart — α-hydroxyhexanoic acid — in the absence of racemization [20,30,31]. By using this trick access to saturated aliphatic α-hydroxy acids is possible. After all, it has to be emphasized that the turnover frequency (TOF) of mandelate racemase for its natural substrate is about $1000\,s^{-1}$, thus a relative activity of, e.g. 14% still equals an impressive TOF of $140\,s^{-1}$.

3.2. Extension of the aromatic system and steric hindrance

An extension of the π-system to furnish naphthyl derivative (*R*)-**5** should lead to an enhanced carbanion-stabilization and hence one would expect a considerably increased activity of this substrate. In a qualitative way, six resonance structures of the carbanion intermediate can be drawn; in addition, semiempirical methods also predict a substantial stabilizing effect of the naphthyl moiety as compared to phenyl. However, a relative activity of moderate

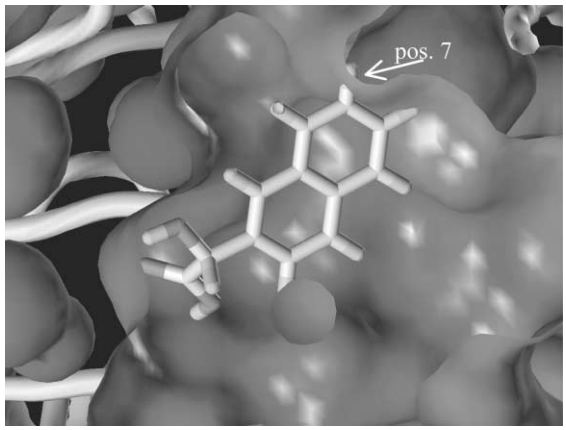


Fig. 1. Substrate (*R*)-**5** modeled into the active site of mandelate racemase.

26% for (*R*)-**5** was found, which can be attributed to steric hindrance within the active site of the enzyme. Based on the X-ray structure of the enzyme [32], this is exemplified in Fig. 1: Substrate (*R*)-**5** was modeled [4] into the active site of mandelate racemase [32] and it can easily be seen that the hydrogen on C-7 of the naphthyl ring (top position) literally protrudes through the surface of the enzyme's active site, thus the binding of the substrate is hampered and additional conformational changes of the enzyme are required to effect racemization. A similar impeding steric effect was found in the series of positional isomers of chloro-substituted mandelates (see below).

3.3. Electronic substituent effects

Provided that resonance stabilization of the α-carbanion intermediate is a crucial factor governing the activity of mandelate racemase for a given substrate, one would anticipate an increased activity for derivatives bearing electron-withdrawing substituents, e.g. (*R*)-**8**. On the other hand, analogs bearing electron-donating groups should exert lower activity than the natural substrate. In the series of *p*-substituted compounds (*R*)-**8**–(*R*)-**12** this expectation was nicely confirmed by

[4] An X-ray structure containing (*S*)-atrolactic acid as irreversible inhibitor was used for the modelling. The α-hydroxycarboxylic acid moiety of (*R*)-**5** was aligned in the same way as the inhibitor by using the respective salt bridges (for Mg^{2+}) and hydrogen bonds (for Lys164 and Glu 317) as 'electronic anchors'.

the experimental results. *p*-Fluoromandelate [(*R*)-**11**] represents an interesting case inasmuch there is essentially no effect on the relative activity (96% of mandelate). In line with this experimental finding, calculated deprotonation energies are close to 0 indicating little if any stabilizing or destabilizing effect of this substituent. In contrast, for the *p*-Cl-[(*R*)-**8**] as well as the *p*-Br-derivative [(*R*)-**12**], a dramatic carbanion-stabilization was computed.

3.4. Steric substituent effects

According to the calculations, all positional isomers of chloro-mandelic acid derivatives have a better resonance stabilization and should thus be more reactive than mandelate: In particular the *m*-isomer should be the most reactive followed by the *p*- and the *o*-isomer (Table 1). However, the experiment showed that the sterically less hindered *p*-chloro substrate (*R*)-**8** is the most reactive followed by mandelate. The *m*-chloro-derivative (*R*)-**7** displayed 61% of relative activity, while the *o*-isomer (*R*)-**6** was not transformed at all. Going in line with the observations above, steric hindrance within the active site of the enzyme seems to be a critical parameter. Specifically, modeling the *o*-isomer (*R*)-**6** into the active site of mandelate racemase indicates a very close contact between the chlorine atom and the carboxylate group of Glu-247. [5]

For substrates bearing electron-donating substituents, such as *p*-methoxy [(*R*)-**9**] and *p*-hydroxy mandelate [(*R*)-**10**], reduced activities are expected due to decreased carbanion stabilization, while steric hindrance of the *p*-substituents can largely be neglected. For these compounds, semiempirical methods turn out to yield unreliable deprotonation energies since for both derivatives a greater stability of the carbanion intermediate is predicted. Furthermore, density functional theory methods also appear to underestimate the destabilizing effect of electron-donating substituents as compared to results obtained by second-order Møller-Plesset perturbation theory. For both compounds the calculated structures of the C-2 deprotonated carbanions are characterized by a perpendicular orientation of the *p*-hydroxy- and *p*-methoxy-group with respect to the aromatic ring, thereby minimizing the electron-donating ability of the *p*-substituent. If coplanarity is enforced in the computations, the relative deprotonation energy of (*R*)-**10** is increased from 4.6 to 17.2 kcal mol^{-1} at the HF/6-31+G** level of theory. Any restriction within the active site towards the possibility to adopt this optimal conformation of the substituent would greatly decrease the stability of the intermediate.

As a compromise between accuracy of the calculated deprotonation energies and cost effectiveness the use of the B3LYP/6-31+G* level of theory is recommended, accept for compounds containing third row elements. Here a larger basis set should be used.

In summary, we have shown for the first time that a range of 2-hydroxy-2-heteroaryl acetic acid derivatives as well as 2-hydroxy-2-naphthyl acetic acid are well-accepted non-natural substrates for mandelate racemase. The data reveal that (i) the stabilization of the α-carbanion intermediate occurring during racemization [6] by electron-withdrawing substituents at the aromatic moiety significantly contributes to enhance the relative activity of racemization. (ii) Steric restrictions with respect to the spatial requirements of the size of the aryl-moiety and the position of aryl substituents apply to naphthyl-derivatives and *o*-substituted mandelates. It was shown that gas-phase deprotonation energy of the α-carbon atom is a suitable parameter to describe the extent of resonance stabilization, which correlates well with experimentally obtained values for the relative activity non-natural mandelate derivatives, as long as other parameters, such as steric hindrance are not dominant.

Acknowledgements

We would like to express our cordial thanks to U. Wagner (Graz) for her skillful assistance in structural biology. This study was performed within the Spezialforschungsbereich ‘Biokatalyse’, and financial support by the Fonds zur Förderung der wissenschaftlichen Forschung (Vienna, project no. F115) and the Federal Ministry of Science (Vienna) is gratefully acknowledged.

[5] The modelling was performed by Ulrike Wagner (Department of Chemistry, Structural Chemistry) using the Sybyl molecular modeling package version 6.7.

References

[1] E.J. Ebbers, G.J.A. Ariaans, J.P.M. Houbiers, A. Bruggink, B. Zwanenburg, Tetrahedron 53 (1997) 9417.
[2] R.S. Ward, Tetrahedron: Asymm. 6 (1995) 1475.
[3] R. Noyori, M. Tokunaga, M. Kitamura, Bull. Chem. Soc. Jpn. 68 (1995) 36.
[4] U.T. Strauss, K. Faber, Tetrahedron: Asymm. 10 (1999) 4079.
[5] U.T. Strauss, U. Felfer, K. Faber, Tetrahedron: Asymm. 10 (1999) 107.
[6] G.L. Kenyon, J.A. Gerlt, G.A. Petsko, J.W. Kozarich, Acc. Chem. Res. 28 (1995) 178.
[7] G.D. Hegeman, E.Y. Rosenberg, G.L. Kenyon, Biochemistry 9 (1970) 4029.
[8] D.T. Lin, V.M. Powers, L.J. Reynolds, C.P. Whitman, J.W. Kozarich, G.L. Kenyon, J. Am. Chem. Soc. 110 (1988) 323.
[9] R. Li, V.M. Powers, J.M. Kozarich, G.L. Kenyon, J. Org. Chem. 60 (1995) 3347.
[10] M. Goriup, U.T. Strauss, U. Felfer, W. Kroutil, K. Faber, J. Mol. Catal. B 15 (2001) 211.
[11] H. Stecher, U. Felfer, K. Faber, J. Biotechnol. 56 (1997) 33.
[12] H. Stecher, A. Hermetter, K. Faber, Biotechnol. Tech. 12 (1998) 257.
[13] J. Brusse, W.T. Loos, C.G. Kruse, A. van der Gen, Tetrahedron 46 (1990) 979.
[14] S.K. Latypov, J.M. Seco, E. Quinoá, R. Riguera, J. Org. Chem. 60 (1995) 504.
[15] E.F. Degering, L.G. Boatright, J. Am. Chem. Soc. 72 (1950) 5137.
[16] K. Kinbara, Y. Harada, K. Saigo, Tetrahedron: Asymm. 9 (1998) 2219.
[17] D.G. Lee, T. Chen, J. Am. Chem. Soc. 115 (1993) 11231.
[18] A.M.C.H. van den Nieuwendijk, E.G.J.C. Warmerdam, J. Brussee, A. van der Gen, Tetrahedron: Asymm. 6 (1995) 801.
[19] H. Waldmann, Tetrahedron Lett. 30 (1989) 3057.
[20] Gronowitz, Ark. Kemi 13 (1959) 87.
[21] G.E. Jeromin, M. Alberz, J. Prakt. Chem. 334 (1992) 526.
[22] J.R.E. Hoover, G.L. Dunn, D.R. Jakas, L.L. Lam, J.J. Taggart, J.R. Guarini, L. Philips, J. Med. Chem. 17 (1974) 34.
[23] H. Griengl, H. Schwab, M. Fechter, Trends Biotechnol. 18 (2000) 252.
[24] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, V.G. Zakrzewski, J.A. Montgomery Jr., R.E. Stratmann, J.C. Burant, S. Dapprich, J.M. Millam, A.D. Daniels, K.N. Kudin, M.C. Strain, O. Farkas, J. Tomasi, V. Barone, M. Cossi, R. Cammi, B. Mennucci, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G.A. Petersson, P.Y. Ayala, Q. Cui, K. Morokuma, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J. Cioslowski, J.V. Ortiz, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. Gomperts, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, C. Gonzalez, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, J.L. Andres, C. Gonzalez, M. Head-Gordon, E.S. Replogle, J.A. Pople, Gaussian 98, Revision A.7, Gaussian, Inc., Pittsburgh, PA, 1998.
[25] A.D. Becke, J. Chem. Phys. 98 (1993) 5648.
[26] C. Lee, W. Yang, R.G. Parr, Phys. Rev. B 37 (1988) 785.
[27] AMPAC 6.55© 1999 Semichem, 7128 Summit, Shawnee, KS 66216, USA.
[28] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy, J.J.P. Stewart, J. Am. Chem. Soc. 107 (1985) 3902.
[29] J.J.P. Stewart, J. Comput. Chem. 10 (1989) 209.
[30] S.-K. Kang, J.-H. Jeon, T. Yamaguchi, J.-S. Kim, B.-S. Ko, Tetrahedron: Asymm. 6 (1995) 2139.
[31] K. Nakamura, T. Matsuda, M. Shimizu, T. Fujisawa, Tetrahedron 54 (1998) 8393.
[32] http://www.rcsb.org/pdb/, PDB entry no. 1MDR.