Dynamic kinetic resolution (DKR) reactions in which a stereoselective enzyme and a racemization step are coupled in one pot would represent powerful tools for the production of enantiopure amines through enantioconvergence of racemates. The exploitation of DKR strategies is currently hampered by the lack of effective, enzyme-compatible and scalable racemization strategies for amines. In the present work, the proof of concept of a fully biocatalytic method for amine racemization is presented. Both enantiomers of the model compound 1-methyl-3-phenylpropylamine could be racemized in water and at room temperature using a couple of wild-type, non-proprietary, enantiocomplementary amine transaminases and a minimum amount of pyruvate/alanine as a co-substrate couple. The biocatalytic simultaneous parallel cascade reaction presented here poses itself as a customizable amine racemization system with potential for the chemical industry in competition with traditional transition-metal catalysis.

Introduction

Enantiomers of a drug are known to exert different physiological effects. Regulatory agencies have therefore progressively tightened the requirements for the approval and the commercialization of chiral drugs,[1] and this trend is likely to continue in the future. For this reason, chiral amines used as resolving agents, chiral auxiliaries or synthons in the synthesis of chiral drugs, and this trend is likely to continue in the future. For this reason, chiral amines used as resolving agents, chiral auxiliaries or synthons in the synthesis of pharmaceutical compounds are particularly interesting from the commercial point of view.[2–3] Over the last 20 years, several biocatalytic routes for the synthesis of valuable chiral amines have been investigated with the purpose of establishing more environmentally friendly and affordable routes than the traditional racemate resolution via formation of diastereomeric salts.[3–5] Several biocatalytic approaches for the synthesis of enantiopure chiral amines have also been reported, mostly involving hydrolases, oxygenases, dehydrogenases and transferases in kinetic resolution (KR), dynamic kinetic resolution (DKR) and asymmetric synthesis (AS) reactions.[3–6] Among these methods, DKR, in which a highly enantioselective enzyme is combined in one pot with a compatible racemization system, is of particular interest since it would allow the synthesis of valuable enantiopure amines with yields close to 100% from more readily accessible racemates.

The applicability of DKR to amines is limited by the scarcity of reported efficient enzyme-compatible racemization methods,[7] which usually require expensive transition-metal chemo-catalysts[7–9] (mostly Raney-Ni,[8,10] Shvo-type,[11–15] palladium,[10,15–19] or ruthenium based[20]), expensive non-selective reducing agents such as ammonia borane,[21] and/or harsh conditions[11,22] that are not suitable for large-scale application or in combination with enzymatic steps. The best-known example of a successful DKR on a laboratory scale was reported by Reetz and Schimossek in 1996. In this work, the substrate (R,S)-1-phenylethylamine was successfully converted into its (R)-acylated form to final 64% yield in 8 days using a lipase as resolving catalyst and Pd on charcoal as racemization catalyst.[23] Extensive research on the combination of lipases and heterogeneous racemization catalysts in DKRs has followed this first report.[24] Amine transaminases (EC 2.6.1.-) are biocatalysts of special interest for the obtainment of chiral amines. This is not only because both (R)- and (S)-selective amine transaminases with varying substrate scopes and operational optima are known.[25–26] Also the reversibility of the reaction they catalyze, i.e. the stereoselective transfer of an amino group from a chiral amino donor substrate to a prochiral carbonyl amino acceptor co-substrate, makes them suitable for both the kinetic resolution and asymmetric synthesis of chiral amines.[27] Limitations such as unfavorable thermodynamic equilibrium in asymmetric synthesis and substrate- and product inhibition phenomena[28,29] have been often overcome by reaction,[30,31,27,32–33] and/or enzyme engineering approaches.[27,29]
One of the most interesting applications of transaminases in biocatalysis consists of a racemization reaction intended for use in DKRs. Koszelewski et al. designed a one-pot cascade involving a pair of known, robust enantiocomplementary amine transaminases coupled via catalytic amounts of a non-readily-available and rather expensive achiral amino donor/acceptor co-substrate system. In this approach, the selection of appropriate pairs of catalysts is complicated by the natural narrow substrate specificity overlap between (R)- and (S)-transaminases and by the need to use accepted achiral co-substrates that must also both be commercially available and show a readily reversible transamination equilibrium with the substrate in order to be used in catalytic amounts. The difficulty to meet all these requirements at once has limited further practical applications of the system.

In the present paper, the proof of concept of a more accessible transaminase-catalyzed racemization reaction is presented, in which the use of the broadly accepted alanine/pyruvate co-substrate system increases the chances to identify a couple of non-proprietary enantiocomplementary catalysts and limits the goal of engineering, when necessary, to only one of the two substrates participating in the reaction. Our results show that both enantiomers of the model compound 1-methyl-3-phenylpropylamine (1a,b) could be racemized to a final <10% ee using two non-proprietary enantiocomplementary amine transaminases selected from a panel of only 10 enzymes (7 (R)-selective and 3 (S)-selective) and using a variant of the simple and inexpensive UV-based acetophenone assay. Purified enzymes (50 mU) were used in water, at pH 7.5, at atmospheric pressure, in the presence of the enantiopure substrate 1a, (S)-enantiomer, or 1b, (R)-enantiomer, (15 mM) and minimal amounts of enantiopure alanine of opposite configuration than that of the target amine (3a, L-alanine, or 3b, D-alanine) (Scheme 1). In this setup the catalytic activity of the two enzymes is coupled via continuous production and consumption of the amino acceptor pyruvate 4, which is added to the reaction in limited amounts. Although distant from the metrics necessary for industrial application, the approach presented here represents a promising novel, tunable and optimizable enzyme-compatible racemization system for amines with potential for DKR processes.

**Results and Discussion**

**Selection and Production of Catalysts and Choice of the Operational pH**

Suitable catalysts were selected among a collection of enzymes including those commercially offered in the ACS-ATA-KIT (Enzymicals AG, Table S1) and the *Chromobacterium violaceum* (S)-ATA [Cv-(S)-ATA] in its wild-type and W60C variants. The enzymes were screened for the acceptance of ketone 2 in the variant of the acetophenone assay shown in Figure S1. The *Aspergillus oryzae* (R)-ATA [Ao-(R)-ATA] and the Cv-(S)-ATA showed the highest initial catalytic rates and were therefore selected for use in the racemization reaction, which was performed at pH 7.5 to allow for sufficient enzyme activity and better (S)-ATA stability. Both the Ao-(R)-ATA and the Cv-(S)-ATA were then recombinantly expressed in *E. coli* BL21 (DE3) and used in the racemization of 1a and 1b in their pure form to avoid the occurrence of side reactions and to have a tight control over the relative amounts of the two catalysts, which is a key parameter in the early-stage optimization of any multi-enzymatic reaction. While the Cv-(S)-ATA was expressed and purified from the strain previously described in the literature following established procedures, the catalytically active C-terminal His-tagged Ao-(R)-ATA was successfully over-expressed (Figures S3 and S4, Supporting Information) and purified by IMAC for the first time with yields around 70 mg/L. Removal of imidazole resulted in fast and irreversible protein precipitation, suggesting a stabilizing role of imidazole for Ao-(R)-ATA. Flash-freezing in liquid nitrogen and storage at −80 °C proved optimal for the long-term storage of both enzymes as it prevented loss of activity over repeated cycles of freeze-thawing. The concentration of catalytically active protein in the final enzyme preparations was assessed using a standard UV-based activity assay in the presence of a known amount of purified protein. The calculated specific activity values, corresponding to 103 mU/mg for Cv-(S)-ATA and 169 mU/mg for Ao-(R)-ATA (Figure S1, Supporting Information), account for...
the presence of inactive forms of the enzymes in the protein preparation and enable a tight control over the actual amount of catalysts added to the reaction.

Transaminase-Catalyzed Racemization of 1a and 1b

Racemization of the model amine compounds 1a and 1b was achieved employing Cv-(S)-ATA and Ao-(R)-ATA (50 mU each) in a one-step reaction in the absence of excess amino donor or additional equilibrium-displacement systems (Scheme 1). The two enantiomers 1a and 1b of the target 1-methyl-3-phenylpropylamine (15 mM) were independently racemized in aqueous solution in the presence of minimal amounts of amino acceptor pyruvate (4.5 mM, 0.3 eq.), amino donor alanine 3a or 3b (10 mM, 0.7 eq.) and PLP (0.1 mM) over a reaction time of 4 days, which corresponds to the chemocatalyzed racemization time for this compound reported in previous work.3,17 The chosen quantities ensure that racemization can only be the outcome of the catalytic coupling of the two transaminases, since only the enzyme-catalyzed replenishment of the otherwise limiting amino-acceptor pyruvate (0.3 eq.) can sustain the racemization cycle. For each reaction mixture, opposite enantiomers of 1-methyl-3-phenylpropylamine (1a,b) and alanine (3a,b) were used as substrate and amino donor co-substrate, respectively. Although the addition of an enantiopure amino donor cannot be avoided due to the strict enantioselectivities of the enzymes, our results show that, in contrast to other reported transaminase-based systems, only a minimal amount of amino donor (0.67 eq.) suffices to sustain the asymmetric synthesis half-reaction, with a positive impact on the overall atom economy of the racemization reaction (Table 1). The obtained data demonstrate that racemization of 1a,b could be achieved to final ee < 10% in the described bi-enzymatic reaction, regardless of the starting enantiomer of 1, with absolute concentrations of ketone 2 (4-phenyl-2-butaneone) above 20% of the initial substrate concentration (> 3 mM). The presence of trapped intermediate ketone, i.e. ketone accumulated during the course of the reaction and not further converted to its corresponding amine, is inherent to this type of racemization reaction44 to an extent that depends on the position of the equilibrium. The coupling to a kinetic resolution step in a dynamic kinetic resolution system is expected to contribute to a reduction of the amount of trapped ketone. PLP (0.4 μmol) was supplemented daily to the reaction to ensure continuous cofactor availability. This cofactor addition pattern proved both unnecessary for the purpose of racemization and undesirable with respect to both product ee and amount of trapped ketone intermediate. The control reaction including substrate 1b supplemented with a minimal amount of PLP only at time 0 (0.6 μmol) shows both better ee value and a lower concentration of ketone. We suspect that PLP could interfere stoichiometrically in the reaction by removing amino groups from the system in the form of PMP and that this phenomenon might be relevant for the accumulation of ketone when excess PLP is used in combination with enzymes that, like Cv-(S)-ATA, show low affinity for the cofactor moiety.34

Based on the presented results, we believe that the described approach should be further investigated for different types of substrate amines for DKR purposes. Appropriate system- and substrate-dependent optimizations are expected to be needed. The attractiveness of a fully enzyme-compatible and renewable system for racemization of primary amines justifies, in the context of what is currently available, further efforts in this direction.

| Table 1. Transaminase-catalyzed one-step racemization of 1a and 1b under tested conditions. |
| Starting amine | Catalytic system[a] | Total amount of PLP [μmol (mol%)] | ee of amine after 4 days[b] | Absolute concentration of ketone after 4 days[c] [% (μmol)] |
|----------------|------------------|-----------------------------------|--------------------------|-----------------------------|
| 1a             | No enzymes       | 1.7 (11)                          | > 99 % (S)               | 3 (0.5)                     |
| 1a             | Ao-(R)-ATA Cv-(S)-ATA | 1.7 (11)                          | 9 % (S)                  | 36 (5.4)                    |
| 1b             | No enzymes       | 1.7 (11)                          | > 99 % (R)               | 4 (0.6)                     |
| 1b             | Ao-(R)-ATA Cv-(S)-ATA | 1.7 (11)                          | 5 % (R)                  | 42 (6.3)                    |
| 1b[d]          | No enzymes       | 0.10 (0.7)                        | > 99 % (R)               | 4 (0.6)                     |
| 1b[d]          | Ao-(R)-ATA Cv-(S)-ATA | 0.10 (0.7)                        | 3.8 % (R)                | 28 (4.2)                    |

[a] Substrate amine 1a or 1b (15 mM), amino donor of opposite chirality 3b or 3a (10 mM) and amino acceptor 4 (4.5 mM) in HEPES pH 7.5 (50 mM) supplemented with PLP (0.1 mM). PLP (0.4 μmol) was supplemented daily unless otherwise specified. Equal amounts of catalysts (50 mU) was used, when present; [b] Values obtained from chiral HPLC analysis; [c] Values obtained from quantitative achiral GC; [d] PLP was not supplemented daily after the start of the reaction.

Racemization Kinetics

The success of dynamic kinetic resolution (DKR) reactions strictly depends on both the selectivity of the enzyme catalyzing the kinetic resolution step and the racemization rate of the slow-reacting enantiomer, which should ideally be at least ten-fold faster than the kinetic resolution step.22,24 The kinetics of the transaminase-catalyzed racemization of 1a and 1b were investigated by monitoring the change in the relative concentrations of the two enantiomers over time (Figure 1) in reaction setups identical to those already described in which PLP (0.1 mM) was exclusively added at time 0. Samples of the reactions were collected and immediately derivatized for analysis at 0.5, 1, 2.5 and 24 hours. Both reactions using either 1a or 1b as substrates show similar progress curves, with a slower increase of the relative concentrations of the two enantiomers as the racemization equilibrium is approached. The initial time points (0.5, 1 and 2.5 hours) describe the systems far from racemization equilibrium, i.e. when one of the two amine enantiomers is present in large excess. This situation resembles the one encountered in dynamic kinetic resolutions, in which the kinetic resolution step selectively and continuously removes one of the enantiomers from the equilibrium. The 24-hours sample, on the other hand, can provide some indication about long-term differences in the two thermody-
in presence of sufficiently stable enzymes is of interest. The racemization times (t\text{rac}) of the systems, herein defined as the time behavior of the system far from the racemization equilibrium and the intended application of the reaction under study, i.e. DKR, the behavior of the system far from the racemization equilibrium and in presence of sufficiently stable enzymes is of interest. The racemization times (t\text{rac}) of the systems, herein defined as the time that would be required to achieve complete racemization of an enantiopure substrate when the initial catalytic rate is held constant, correspond to 13.6 hours for substrate 1\text{b} and 19.8 hours for substrate 1\text{a} (see Supplementary Information). This value could be further reduced by increasing the amount of catalysts. The racemization time t\text{rac} also represents the time that would be required to complete a DKR reaction when the kinetic resolution step is not the rate-limiting reaction and the racemization proceeds at its fastest rate.

Conclusions

In the present paper a novel biocatalytic method for the racemization of primary amines is presented that relies on the use of two wild-type, non-proprietary, enantiocomplementary amine transaminases and that is sustained by internal formation and consumption of the amino acceptor pyruvate. The system enabled the one-pot racemization of the model amine substrate 1-methyl-3-phenylpropylamine starting from either one of its enantiomers and using only minimal amounts of enantiopure D- or L-alanine as amino-donor co-substrate. The system, which shows reduced complexity and better atom economy when compared to previous reports, represents a potentially flexible racemization tool, customizable to meet substrate- and process requirements. We believe that the described racemization system, being it performed under mild reaction conditions that well match the requirements of other biocatalysts, could be exploited in DKR reactions for deracemization applications.

Experimental Section

Materials

All chemicals were purchased from Sigma Aldrich at analytical grade with the exceptions of HEPES (buffer grade), D-alanine (Fluka), imidazole (buffer grade), (R)- and (S)-1-methyl-3-phenylpropylamine (ACROS Organics), isopropyl-ether (stabilized with BHT, TCI), 2-propanol (Roht), n-heptane (Fisher Scientific) and anhydrous sodium sulfate (Merck). All solvents used were of analytical grade. Pyruvate and pyridoxal-5'-phosphate were also purchased from Sigma Aldrich, as sodium salt and monohydrate form, respectively.

For protein purification procedures syringe filters (0.45 and 0.22 \mu m) were purchased from Corning, centrifugal filter units (Amicon Ultra-15, 30 kDa and 50 kDa) from Merck Millipore, Ni-NTA affinity chromatography pre-packed cartridges for affinity chromatography (5 ml) from Nordic Biosite and Sephadex G-25 PD-10 desalting columns (2.5 ml) from GE-Healthcare.

Catalyst Selection

Catalysts were selected among the panel of enzymes commercially offered by Enzymicals AG in the ACS-ATA-KIT and those kindly made available by the KTH biocatalysis group. The variant of the acetophenone assay shown in Figure S1 aided the identification of suitable candidate catalysts by direct comparison of the detected initial catalytic rates. Enzyme solutions of all available catalysts (1 mg/ml, whole-cell lyophilized or purified lyophilized enzyme) were prepared in HEPES pH 7.5 (50 mM) supplemented with PLP (0.1 mM). The substrate solution was prepared dissolving the amino acceptor 4-phenyl-2-butanol (5 mM) and the acceptor methylbenzylamine (5 mM) (either as (R)- or (S)-enantiomer) in HEPES pH 7.5 (50 mM). The assay solutions were prepared mixing buffer (495 \mu l), enzyme solution (5 \mu l) and substrate solution (500 \mu l) in clean quartz cuvettes. Formation of acetophenone was monitored following the increase of A 245 nm over 10 minutes. Initial activities were estimated from the linear regression of the first data points. The enzymes showing highest initial catalytic rates (Aspergillus oryzae (R)-ATA and Chromobacterium violaceum (S)-ATA) were selected for further investigations.

Figure 1. Reaction course for the racemization reactions performed using enantiopure 1\text{a} or 1\text{b} (15 mM) as substrates in presence of the opposite alanine enantiomer 3\text{b} or 3\text{a} (10 mM), amino acceptor 4 (4.5 mM) and purified catalysts (50 mU each). The reactions (1 ml) were run in HEPES pH 7.5 (50 mM) at room temperature and in the dark under mild agitation. *Data referring to the first experiment where fresh PLP (0.4 \mu mol) was added every 24 hours.
Design of Expression Plasmids and Production of Catalysts-Producing Bacterial Strains

Aspergillus Oryzae (R)-ATA (Ao-(R)-ATA)

The Ao-(R)-ATA coding sequence was retrieved from the results of a default BLAST search in which the Aspergillus fumigatus (R)-ATA sequence (UniProt KB ID: Q4WNL4) was used as query. Two sequences from Aspergillus oryzae were retrieved, both sharing 73% identity with the query and differing by only 4 amino acid residues (LV/L 7, M/I 53, A/V 248 and K/E 293). The variant LMAK (GenBank: EITB92926.1) was reverse-translated using the tool available on www.bioinformatics.org/sms2. The nucleotide sequence was then codon-optimized for E. coli and supplemented with Ndel and Xhol terminal restriction sites. Gene synthesis, restriction and ligation in PET-29b were purchased from GenScript®. The final Ao-(R)-ATA construct included a C-terminal LE insertion between the enzyme sequence and the hexahistidine tag. The plasmid was transformed into electrocompetent E. coli BL21 (DE3) cells using a Bio-Rad Gene Pulser II. A single colony growing on LB agar plates supplemented with kanamycin was inoculated into sterile liquid LB medium (15 ml) supplemented with kanamycin (50 mg/L) and incubated overnight at 37 °C and 200 rpm agitation. This preculture (5 ml) was subsequently inoculated in sterile LB medium (50 ml) supplemented with antibiotic. Growth was carried out at 37 °C and 200 rpm agitation until OD600 = 0.56. Glycerol stocks were prepared in 40% v/v glycerol, frozen in liquid nitrogen and kept at −80 °C for long term storage.

Chromobacterium Violaceum (S)-ATA (Cv-(S)-ATA)

The E. coli BL21 (DE3) expressing strain was made available by the BL21 (DE3) expressing strain was made available by the International Bacillus Lab. (IBL). Chromobacterium violaceum (S)-ATA (Cv-(S)-ATA) protein was flash frozen and stored at −80 °C.

Activity Estimation

Enzyme activity was estimated in the variant of the acetophenone assay shown in Figure S1 (Supporting Information) using UV-clear disposable cuvettes and a Cary 3000 spectrophotometer. The final assay solution was obtained mixing 1 ml substrate solution (5 mM 4-phenyl-2-butanone, 5 mM (R)- or (S)-methylbenzylamine) and 50 μg purified enzyme, added as 5 μl of a 10 mg/ml enzyme dilution prepared from the frozen stocks, whose real enzyme content was estimated on a NanoDrop spectrophotometer (ThermoFisher Scientific) using the molar extinction coefficients calculated in ProtParam® (E 0.1%=1.560 and 1.239 for Cv-(S)-ATA and Ao-(R)-ATA, respectively). The increase of A245nm, corresponding to the target was eluted in a linear gradient to 500 mM imidazole in 30 minutes, flow rate 2 ml/min. The protein eluted as a single symmetric peak at 350 mM imidazole. The fractions corresponding to the peak were collected, merged and concentrated by ultrafiltration using Amicon Ultra centrifugal filter units (cut-off 50 kDa). The imidazole was removed by desalting using a PD-10 column (GE Healthcare). After a final concentration step to 37.5 mg/ml the protein was flash frozen and stored at −80 °C.

Chromobacterium Violaceum (S)-ATA

Cell pellets were resuspended and lysed in 50 mM HEPES pH 7.5, 10 mM imidazole following the same protocol described for Ao-(R)-ATA. Clarification and loading onto the affinity column (Nordic Biosite, 5 ml) also followed the same procedures reported for Ao-(R)-ATA. After washing with five column volumes of buffer, the target was eluted in a linear gradient to 500 mM imidazole in 30 minutes, flow rate 2 ml/min. The protein eluted as a single symmetric peak at 350 mM imidazole. The fraction corresponding to the peak was collected, merged and concentrated by ultrafiltration using Amicon Ultra centrifugal filter units (cut-off 50 kDa). The imidazole was removed by desalting using a PD-10 column (GE Healthcare). After a final concentration step to 37.5 mg/ml the protein was flash frozen and stored at −80 °C.

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Racemization Reaction Setup

All reactions and controls (1 ml) were performed in HEPES pH 7.5 (50 mM) supplemented with glycerol (20% v/v) in sealed 1.5 ml glass vials kept in the dark under mild agitation at room temperature for 4 days. The racemization of the model compound 1-methyl-3-phenylpropylamine (15 mM) was tested starting from each one of its pure enantiomers 1a and 1b (purchased from
ACROS organics) in the presence of the opposite alanine enantiomer 3b or 3a (10 mM) as amino donor for the asymmetric synthesis half-reaction and pyruvate 4 (4.5 mM) as amino acceptor in the kinetic resolution half-reaction (Figure 1). PLP (0.1 mM) was also added to reactions and controls at time zero. Additional cofactor (0.4 μmol) was supplemented daily to all mixtures with the exception of one control racemization reaction (containing 1b as substrate) in which no additional PLP was added after time zero. The reactions were started upon simultaneous addition of Ao-(R)-ATA and Cv-(S)-ATA (50 nM each) to the reaction mixtures. The corresponding volume in buffer was added to each control mixture.

Reaction Workup and Analytics

Samples for analysis (200 μl) were transferred to clean tubes and basified adding 1 M NaOH (20 μl). Ethyl acetate (220 μl) was then used to perform extraction. Phase separation was achieved by centrifugation.

GC Quantitative Analysis of 4-phenyl-2-butano ne

GC quantitative analysis of 4-phenyl-2-butano ne was performed on a Varian Star 3400 CX equipped with an Agilent DB-5 ms Ultra Inert column (30 m × 0.25 mm × 0.25 mm) and a FID detector using N₂ as carrier. A calibration curve for the target 4-phenyl-2-butano ne (R² = 0.985 %) was obtained in the concentration range 2–8 mM using acetonophenone (15 mM in anhydrous ethyl acetate) as external standard. For the sample analysis, the acetonophenone phase obtained from the extraction (90 μl) was dried over anhydrous sodium sulfate and a small amount (38 μl) was mixed with the external standard (10 μl) in a clean tube. After a 1:2 dilution in anhydrous ethyl acetate, 1 μl of sample was manually injected for analysis. The GC method consisted in a linear temperature gradient (10 °C/min) from 50 °C (initial time 2 min) to 250 °C. Retention times were 9.5 min for the external standard acetonophenone and 11.79 min for the target compound 4-phenyl-2-butano ne. The values of the peak integrals have been automatically assigned by the program Varian Workstation Version 6.41 Multi Instrument using default integration parameters.

HPLC Qualitative Analysis of N-acetyl-(R,S)-1-methyl-3-phenylpropylamine and Estimation of V_R and t_max

Qualitative chiral analysis of the final 1-methyl-3-phenylpropylamine mixture was performed after acetylation of the primary amino group. Derivatization was performed adding acetic anhydride (5 μl) to the ethyl acetate phase obtained from the reaction workup (100 μl). The organic phase was washed with 0.1 M NaOH (100 μl) and dried over anhydrous sodium sulfate. The ethyl acetate was then evaporated. In the case of the racemization samples (Table 1), 5 μl of the final amide mixture residue dissolved in hexane/isopropanol (90:10) was used for analysis. Separation of the two amide enantiomers was achieved on a Chiracel OD–H column using isotropic elution in hexane/isopropanol (90:10) at a flow rate of 1 ml/min. Retention times were 12.2 min and 14.5 min for the (R)- and (S)-enantiomers, respectively. In the case of the samples used for the determination of the racemization kinetics (Figure 1), the final amide mixture residue dissolved in isopropyl-ether (10 μl) was injected into a Chiralpak AD–H column (250 mm, analytical scale). Isocratic elution in n-heptane:2-propanol (98:2) at a flow-rate of 0.75 μl/min gave good separation, with retention times 28 and 30 minutes for the (R)- and (S)-enantiomers, respectively. Peak integrals were calculated using OpenChrom (Alder, Community Edition v 1.2.0). The ratios of the enantiomers in the mixtures were calculated by dividing the integral of the newly formed enantiomer by the integral of the starting enantiomer. The values obtained were plotted in Excel and the slopes of the linear interpolations of the first two points were defined as the V_R of the racemization reactions. t_max was then defined as the abscissa value of the intersection point between the linear interpolation and y=1, corresponding to a ratio of the enantiomers of 1, i.e. a racemate (Figure S5 and S6).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Chiral amines · Enzyme catalysis · Racemization · Transaminase

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