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Enantioselective acylation of α-aminonitriles catalysed by *Candida antarctica* lipase. An unexpected turnover-related racemisation

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Abstract—*Candida antarctica* lipase B (Novozyme 435) catalysed the enantioselective acylation of 2-amino-2-phenylacetonitrile **1** with ethyl phenylacetate affording a near enantiopure product in 47% yield. Acylation of **1** and 2-amino-4-phenylbutyronitrile with ethyl acetate yielded an unexpected partially racemised final product. The racemisation was shown to be turnover related and is ascribed to the increased acidity of the α-proton in the formation of the tetrahedral intermediate in the active site of the enzyme.

1. Introduction

α-Aminonitriles are important intermediates in the synthesis of amino acids and amino acid amides. They can be hydrolysed chemically or enzymatically, using nitrilases and nitrile hydratases, to the corresponding amino acids or amino acid amides.[1–4] Enantioselective enzymatic hydrolysis, however, is not always feasible, especially to the amides, since nitrile hydratases (biocatalysts for amide formation) are rarely enantioselective.[5] Hence, an efficient resolution of α-aminonitriles would be of interest, because the enantiopure nitriles can subsequently be converted in a stereoretentive reaction.[6]

Handling α-aminonitriles is difficult due to their tendency to decompose via a retro-Strecker reaction at basic or neutral conditions. The liberated aldehyde may in turn cause racemisation via the formation of a Schiff base. Additionally, deprotonation at the α-carbon atom, which readily takes place under very mild basic conditions due to the electron-withdrawing effect of the cyano group, also results in racemisation.

The resolution of α-aminonitriles has generally been accomplished by crystallisation techniques,[7] which are unpredictable and, hence, time consuming to optimise. Moreover, in the case of α-aminonitriles special care is required to avoid decomposition during such procedures. Enzyme mediated kinetic resolution in organic medium is therefore an attractive option. Lipases, in particular, tolerate organic reaction media well. Subsequent to the first lipase mediated resolution of a chiral amine,[8] the methodology has been widely applied.[9] The lipase B from *Candida antarctica*, for example, catalyses the acylation of (*R*)-1-phenylethylamine with near absolute enantioselectivity.[10,11]

The lipase mediated resolution of α-aminonitriles has, with a single exception, been disregarded; only Nakai et al.[12] have described the resolution of 2-amino-2-phenylacetonitrile and some of its derivatives with fairly good stereoselectivity using lipases from *Pseudomonas* and a lipase from *Chromobacterium viscosum* as catalysts.

In this paper, we report a study of the stereoselective *C. antarctica* lipase B (Novozym 435) catalysed acylation of three different α-aminonitriles with ethyl acetate and ethyl phenylacetate. An unexpected racemisation of the final product was observed, which prompted us to investigate the mechanism in more detail.

2. Results

2.1. Synthesis of α-aminonitriles and standards

Three reactant nucleophiles were used in this study, 2-amino-2-phenylacetonitrile **1**, 2-amino-4-phenylbutyronitrile **2** and 2-amino-3,3-dimethylpropionitrile **3**.

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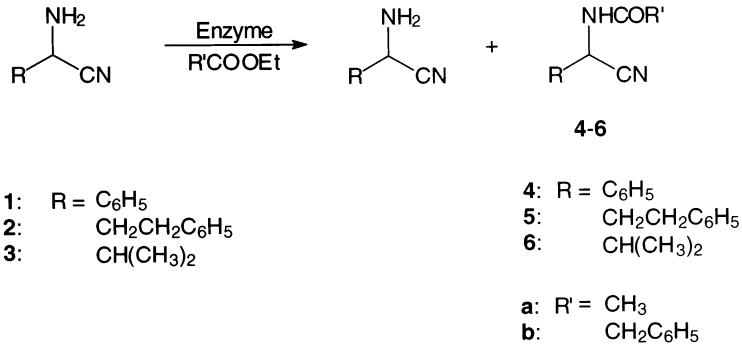

Figure 1. Acylation of α-aminonitriles.

Compounds **2** and **3** are not commercially available and were synthesised following a procedure based on the Strecker synthesis (see Section 5).[1]

The enantiopure α-aminonitriles **1**–**3** were obtained by crystallisation using (+)- or (−)-tartaric acid as the resolving agent and methanol/toluene mixtures as solvent (see Section 5 for details). After repeated crystallisations an e.e. higher than 97% was achieved, except for (*S*)-**3**, which could not be obtained in higher purity than 76% e.e. Standards of all the racemic acylated aminonitriles were synthesised chemically by treatment of the α-aminonitrile with the corresponding acyl chloride.

2.2. Enzymatic reactions

The racemic nitriles **1**–**3** (25 mM in anhydrous diisopropyl ether) were acylated in the presence of a number of lipases (Fig. 1). Ethyl acetate (1 M) and ethyl phenylacetate (0.1 M) were used as acyl donors, chosen because of their easy subsequent removal, using a suitable acylase. Zeolite NaA was added in order to minimise hydrolytic side reactions.

From a range of enzymes tested, only Novozym 435 showed a significant conversion when using ethyl acetate as the acyl donor (Table 1). Only **1** was acylated by ethyl phenylacetate in the presence of this enzyme.

The reaction of **1** with ethyl acetate in the absence of zeolite NaA was very slow, only 7% conversion in 24 h, compared with 50% conversion in the presence of zeolite NaA in the same reaction time.

2.3. Racemisation of α-aminonitriles

Racemisation of the enantiopure α-aminonitriles was to be expected, even under the mild conditions used in the enzymatic reactions, considering their stereochemical lability. The extent of racemisation was studied by incubating the pure enantiomers obtained by crystallisation of their tartrates (Section 2.1), as the free base under different conditions for 24 h.

Solutions of enantiopure **1** and **2** in diisopropyl ether or ethyl acetate did not racemise, but the addition of zeolite NaA to the solution caused significant racemisation to 38 and 64% final e.e. for (*R*)-**1** and (*R*)-**2**, respectively, in 24 h. When (*R*)-**1** was incubated in diisopropyl ether in the presence of the catalyst, Novozym 435, some racemisation (70% final e.e.) was also detected. The same effect was observed when (*R*)-**1** was incubated with SP525 (a *C. antarctica* lipase B preparation) immobilised on Accurel EP100.[13] (*R*)-**2** and (*S*)-**2** also racemised (approx. 88% e.e.) in the presence of Novozym 435. The racemisation is presumably caused by alkaline sites in the zeolite and buffer salts in the lipase preparations.

Table 1. Enzymatic acylation of **1**–**3** by ethyl acetate and ethyl phenylacetate

	Conversion (%)[a]					
Enzyme	**4a**	**5a**	**6a**	**4b**	**5b**	**6b**
Novozym 435	50	56	76	47	–	3
SP 526 (*C. antarctica* lipase A)	<2	–	–	3	–	–
SP 523 (*T. lanuginosus* lipase)	–	–	–	–	–	–
SP 524 (*R. miehei* lipase)	–	5	<2			
IM 20 (*R. miehei* lipase)	<2	–	<2			
Pseudomonas lipoprotein lipase	3	–	<2			
Protease SP 522	–	–	–			

[a] HPLC data with internal standard. Reaction conditions: substrate 25 mM in i-Pr_2O, ethyl acetate 1 M or ethyl phenylacetate 0.1 M, 50 mg/mL enzyme, 50 mg/mL zeolite NaA powder, 40°C, 24 h.

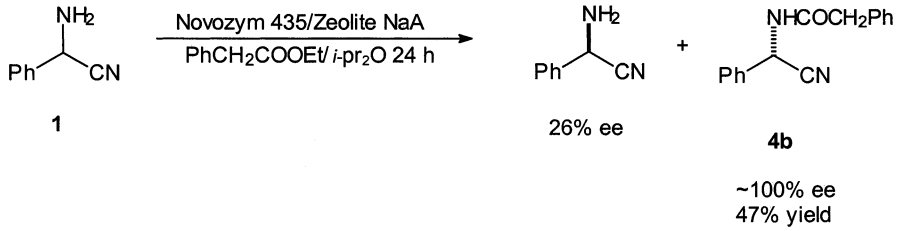

Figure 2. Acylation of **1** with ethyl phenylacetate.

2.4. Acylation with ethyl phenylacetate catalysed by Novozym 435

Compound **1** was acylated by ethyl phenylacetate in the presence of Novozym 435 under the general experimental conditions (Section 2.2). The resulting (*S*)-2-phenylacetamido-2-phenylacetonitrile **4b** was found to be nearly enantiopure at 47% conversion of **1** (Fig. 2). The reaction stopped at this point although, due to the racemisation of the starting material (26% e.e.), (*S*)-**1** was still available. Presumably, small amounts of benzaldehyde in the reaction medium, resulting from some decomposition of the substrate via a retro-Strecker reaction, inhibited the biocatalyst. It was shown in an independent experiment that no acylation took place in the presence of 1% benzaldehyde.

The reaction temperature had, between 4 and 60°C, no effect on the final yield. The addition of pyridoxal as a racemising agent at 4 or 40°C did not improve the yield either. The addition of $NaBH_4$ to the reaction medium as a reducing agent for benzaldehyde proved to be inefficient. The other two α-aminonitriles, **2** and **3**, did not react with ethyl phenylacetate.

2.5. Acylation of 1 with ethyl acetate

Acylation of **1** by ethyl acetate in the presence of Novozym 435 was carried out under standard conditions (Section 2.2). After 24 h a conversion of 51% was obtained (Table 2); the product was almost racemic, with e.e. of 10% in favour of the (*S*)-enantiomer. In contrast, the unconverted **1** showed a considerable enantiomeric excess, 50%, of the (*R*)-enantiomer. This indicated that the enzyme was selective for the (*S*)-enantiomer, but the product was unexpectedly partially racemised.

Calculation of the enantiomeric balance[†] at this conversion revealed that a 19% excess of the (*R*)-enantiomer was present, whereas zero or, considering the likelihood of substrate racemisation, an excess of (*S*)- would be expected, as was indeed observed in the acylation of **1** with ethyl phenylacetate (see above).

We checked whether product racemisation was responsible by incubating an enantiomerically enriched sample of (*S*)-**4a** (60% e.e.) under the reaction conditions for 24 h, but no racemisation resulted. These results suggest that during the acylation with ethyl acetate of **1**, a turnover-related racemisation is taking place.

A more detailed study of this reaction (Fig. 3) showed a gradual increase in the e.e. of the substrate over time, indicating that **1** was acylated more rapidly than it racemised. During the first 5 h the turnover-related racemisation detected in the product exceeded that of the substrate, i.e. the enantiomeric balance showed an increasing bias towards the (*R*)-enantiomer instead of the (*S*)-enantiomer. After 5 h the enantiomeric balance seemed to stabilise.

[†] We define the enantiomeric balance (EB) at a given conversion as the difference of the total (*R*)- and (*S*)-enantiomers of reactant and product according to the following equation:

$$EB = [(S)\text{-reactant} + (S)\text{-product}] - [(R)\text{-reactant} + (R)\text{-product}]$$
$$= [(S)\text{-reactant} - (R)\text{-reactant}] + [(S)\text{-product} - (R)\text{-product}]$$
$$= (1-c)\,\text{e.e.}_s + c\ \text{e.e.}_p$$

where c = conversion. EB should have the same value before and after the reaction, unless racemisation, inversion or generation of stereocentres takes place. If the starting material is racemic, the EB should be zero.

Table 2. Novozym 435 catalysed acetylation of **1**[a]

Substrate	Conversion (%)	E.e.$_s$ (%)	E.e.$_p$ (%)	EB, 0 h (%)[b]	EB, 24 h (%)[b]
1	51	50 (*R*)	10 (*S*)	0	19 (*R*)
(*R*)-**1**	14	72 (*R*)	59 (*R*)	~100 (*R*)	70 (*R*)
(*S*)-**1**	49	24 (*S*)	3 (*S*)	~100 (*S*)	48 (*S*)

[a] HPLC data. Reaction conditions: substrate 25 mM in *i*-Pr_2O, ethyl acetate 1 M, Novozym 435 50 mg/mL, zeolite NaA powder 50 mg/mL, 40°C, 24 h.

[b] Calculated for each case as EB = (%(*R*)-substrate+%(*R*)-acylated product)–(%(*S*)-substrate+%(*S*)-acylated product).

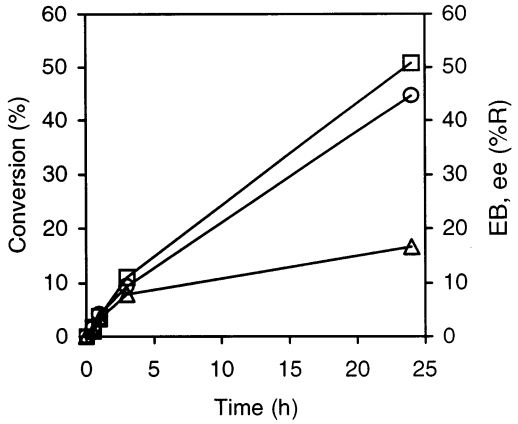

Figure 3. Acetylation of **1**: conversion, e.e.$_s$ and EB as function of time. Conversion (□), e.e.$_s$ (○), EB (△).

When starting from enantiomerically pure (*R*)-**1** (Table 2), the reaction afforded a product with an e.e. of 59% (*R*)-enantiomer after 24 h, with 14% conversion. This could possibly be explained by racemisation of the substrate, producing (*S*)-substrate, which would be preferably acylated by the enzyme ((*S*)-selective), thus reducing the e.e.$_p$. On the other hand, when starting from (*S*)-**1** a nearly racemic product (e.e.$_p$ 3% (*S*)-) was obtained after 24 h with 49% conversion. The same results were obtained with SP 525 immobilised on Accurel EP100.

These observations led us to the following conclusions: the acylation of **1** with ethyl acetate catalysed by *C. antartica* lipase B is (*S*)-selective but affords a partially racemised product. Separate experiments showed that the product does not racemise under the experimental conditions, suggesting a turnover-related racemisation. In addition, the (*S*)-enantiomer shows a higher degree of racemisation than the (*R*)-enantiomer.

2.6. Acylation of 2 and 3 with ethyl acetate catalysed by Novozym 435

The acylation of **2** with ethyl acetate (Table 3) gave similar results to that of **1**. The enzyme was also (*S*)-selective; the e.e. of the unconverted substrate was 46% (*R*)- at 56% conversion. Again, the 5% e.e. of (*S*)-product was in contradiction with the calculated value, 36%, assuming EB = 0, or higher in the case of racemisation of the substrate alone. Hence, the enantiomeric balance showed an excess of the (*R*)-enantiomer (18%), whereas an opposing enantiomeric excess was anticipated.

When using enantiopure (*S*)-**2** as starting material, an (*S*)-configurated product with an e.e. of 9% formed, whereas the acylation of (*R*)-**2** resulted in the formation of an (*R*)-configurated product with an e.e. of 67%. Hence, in this case also, the (*S*)-enantiomer underwent racemisation more than the (*R*)-enantiomer.

In the case of the acylation of **3** with ethyl acetate, the e.e.$_s$ could not be accurately determined. (*R*)-**3**, however, was converted for 48% in 24 h to an (*R*)-enantiomeric product with e.e. of 91%. The acylation of 76% e.e. (*S*)-**3** was faster (72% conversion in 24 h) and the product had an (*S*)-configuration and an e.e. of 85%. This shows again the preference of the enzyme for (*S*)-configured substrates. When starting from the equivalent racemic substrate, however, a product with an e.e. of zero was obtained, which again indicates that turnover-related racemisation occurs. Hence, in this case the racemisation is not so severe and the enantiomeric bias is less than that observed with **1** and **2**.

2.7. Acylation of 1-phenylethylamine

1-Phenylethylamine presents a structure which is similar to that of **1**, the difference being the cyano group in **1** instead of the methyl group in 1-phenylethylamine. Although their chemical properties and nucleophilicity are different, it could be interesting to compare the behaviour of these two substrates in the acylation catalysed by the lipase B of *C. antarctica*.

Parallel reactions were carried out with **1** and 1-phenylethylamine under the standard experimental conditions using ethyl acetate and ethyl phenylacetate as acyl donors. The results are summarised in Table 4.

Table 3. Novozym 435 catalysed acetylation of **2** and **3**[a]

Substrate	Conversion (%)	E.e.$_s$ (%)[b]	E.e.$_p$ (%)	EB, 0 h (%)[d]	EB, 24 h (%)[d]
2	56	46 (*R*)	5 (*S*)	0	18 (*R*)
3	76		~0[c]	0	
(*R*)-**2**	34	90 (*R*)	67 (*R*)	~100 (*R*)	82 (*R*)
(*R*)-**3**	48		91 (*R*)[c]	~100 (*R*)	
(*S*)-**2**	47	82 (*S*)	9 (*S*)	~100 (*S*)	14 (*S*)
(*S*)-**3**	72		85 (*S*)[c]	76 (*S*)	

[a] HPLC data. Reaction conditions: substrate 25 mM in *i*-Pr_2O, ethyl acetate 1 M, Novozym 435 50 mg/mL, zeolite NaA powder 50 mg/mL, 40°C, 24 h.

[b] The e.e.$_s$ of **3** could not be determined accurately.

[c] Determined by ^{1}H NMR in the presence of Eu(hfc)$_3$.

[d] Calculated as EB = (%(*R*)-substrate+%(*R*)-acylated product)–(%(*S*)-substrate+%(*S*)-acylated product).

Table 4. Novozym 435 catalysed acylation of **1** and 1-phenylethylamine[a]

Substrate	Acyl donor	Time (min)	Conversion (%)	E.e.$_p$ (%)
1	Ethyl acetate	30	<2	
1-Phenylethylamine	Ethyl acetate	30	43	~100
1	Ethyl phenylacetate	60	25	~100
1-Phenylethylamine	Ethyl phenylacetate	60	24	~100

[a] HPLC data. Conditions: substrate 25 mM in *i*-Pr_2O, ethyl acetate 1 M or ethyl phenyl acetate 0.1 M, Novozym 435 50 mg/mL, 50 mg/mL zeolite NaA powder, 40°C.

1-Phenylethylamine reacted, in the presence of Novozym 435, rapidly with ethyl acetate as well as with ethyl phenylacetate. The conversions were 43% in 30 min and 25% in 1 h, respectively. Both reactions were completely enantioselective. In contrast, **1** reacted sluggishly with ethyl acetate (2% in 30 min), whereas its reaction with ethyl phenylacetate took place at the same rate as that of 1-phenylethylamine and was completely enantioselective.

These results show that **1** and 1-phenylethylamine react in a similar fashion when acylated by ethyl phenylacetate, giving the expected results for an acylation catalysed by a lipase enzyme. The lower nucleophility of **1** and the chemical differences between a cyano group and a methyl group exert only a limited effect on the reaction rate. However, when the acyl donor is ethyl acetate, these two substrates react in a very different way. The reaction of **1** is unexpectedly slow, when it could be expected to be of the same order as the reaction of 1-phenylethylamine.

3. Discussion

3.1. Racemisation mechanism

We propose here a reaction mechanism that would explain the observed turnover-related racemisation of the product when using ethyl acetate as the acyl donor. From the increase in the e.e.$_s$ and the results of blank reactions, it is clear that an enantioselective enzyme catalysed acylation is taking place. At short reaction times, hardly any racemisation of the substrate is detected, indicating that the observed racemisation of the product takes place during or following the formation of a complex between the enzyme, the acyl donor and the aminonitrile (a tetrahedral intermediate). An amide bond as well as an ester bond are then formed, the amide bond being stronger. The irreversible formation of product then takes place.

The mechanism of lipase catalysed acylation (Fig. 4), involves nucleophilic attack by the aminonitrile on the acyl-enzyme species (**A**, see Fig. 4) to give a tetrahedral intermediate (**B** in Fig. 4) in which the oxyanion is stabilised by hydrogen bonding with the residues in the oxyanion hole (Thr40 and Gln106).[14,15] In α-aminonitriles the α-proton is somewhat acidic due to the electron-withdrawing effect of the cyano group. The formation of the tetrahedral intermediate would enhance the acidity of this α-proton even more by the inductive effect of the partially positively charged adjacent nitrogen that is interacting with the catalytic histidine.

Considering first the electronic effects, this acidic α-proton in the tetrahedral intermediate could be interacting with the oxyanion via a stable five-membered ring structure (**B** in Fig. 4). The reaction could then proceed following two different paths, formation of **C** or formation of **D**, depending on the relative electrophilicity of the α-proton and the carbon directly bound to the catalytic serine, which would be competing for the negative charge of the oxygen. The carbanion intermediate (**D**) is presumably in equilibrium with its planar tautomer **E**, and proton transfer from the hydroxyl group to **E** would give rise to racemisation, being a possible means of the formation of either **D** or **D′**.

It could be thought that a basic residue in the active site of the enzyme would catalyse this racemisation process; however, preliminary molecular modelling studies (data not shown) showed that no basic residues in the active site of *C. antarctica* lipase B were located close enough to the α-proton of the substrate to be involved in this process. This indicated the oxyanion itself to be a good candidate to catalyse the proton transfer process.

When the acyl donor is ethyl phenylacetate (R = $PhCH_2$) the electrophilicity of the carbon atom bound to the serine (derived from the carbonyl group) is enhanced, favouring the formation of product (**C** in Fig. 4). On the other hand, with acyl donors such as ethyl acetate containing electron-donating substituents, this route is less favoured, and **D** could be preferentially formed. Steric factors such as the orientation and size of the substrate could also play an important role. Substituents in the α-aminonitrile could also influence this equilibrium by enhancing the acidity of the α-proton to different degrees. Therefore, electron donor substituents such as the isopropyl group in **3** would favour less the formation of **D** than a phenyl group, as in **1**. This is in agreement with the lower degree of racemisation observed in the formation of **6**.

The existence of the planar intermediate **E** from resonance stabilisation of the anion would be crucial for racemisation. Substrates with electronegative substituents, such as fluorine, that are unable to form

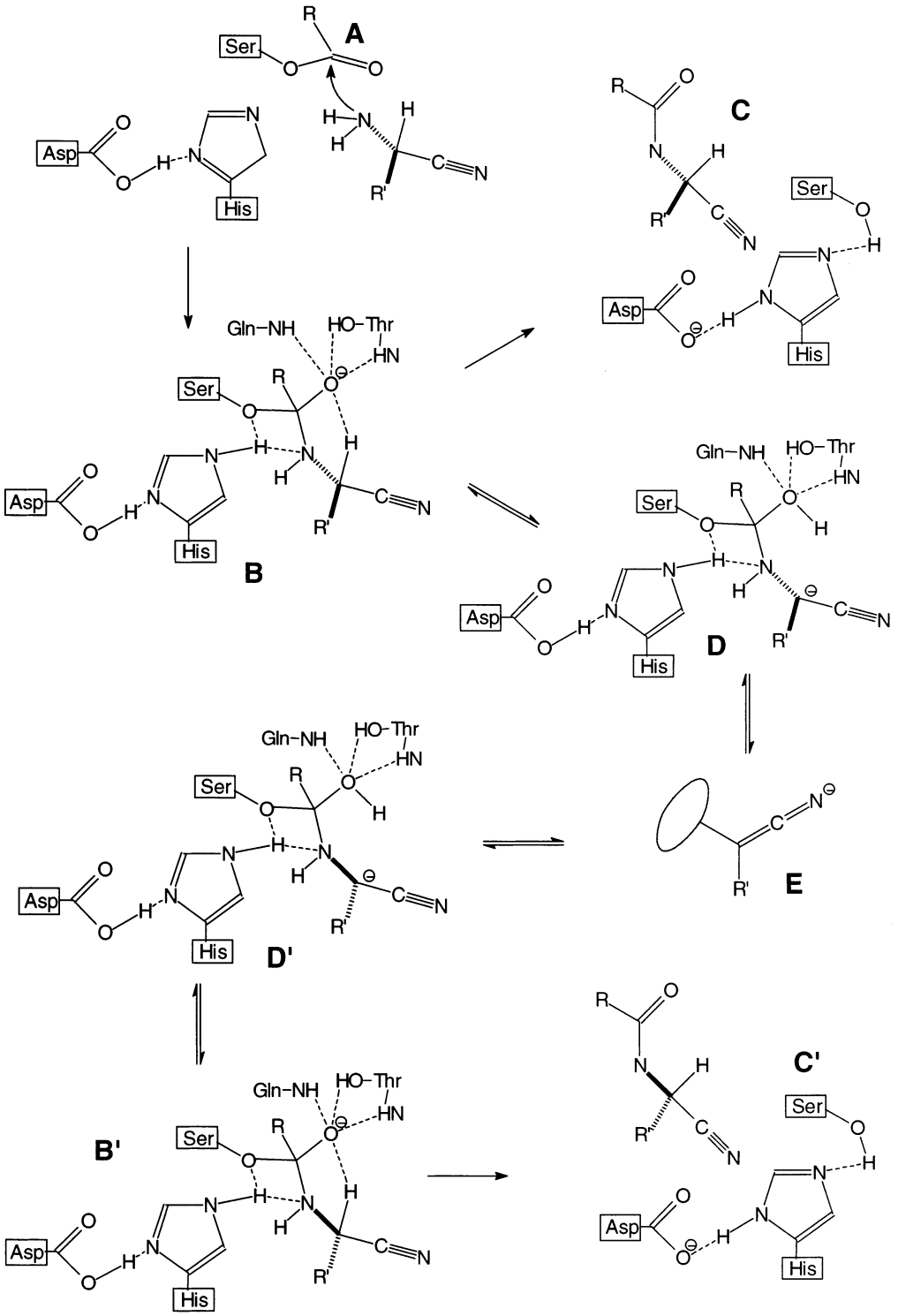

Figure 4. Possible mechanism to explain racemisation of α-aminonitriles in the moment of reaction.

planar intermediates analogous to **E**, do not undergo racemisation.[16] This resonance effect confers a relatively high stability on this intermediate, favouring its accumulation, which could account for the low rate of the process.

To explain why the (*S*)-enantiomer is more racemised than the (*R*)-enantiomer, we should consider the three-dimensional structure of *C. antarctica* lipase B and its active site.[14,15,17] Molecular modelling studies of the acylation of 1-phenylethanol have been published.[17] Using this model and considering the structural analogy with **1**, it can be seen how the preferred (*S*)-enantiomer forms a stabilising hydrogen bond with a catalytic histidine residue, thus enhancing the acidity of its α-proton (Fig. 5). The opposite (*R*)-enantiomer

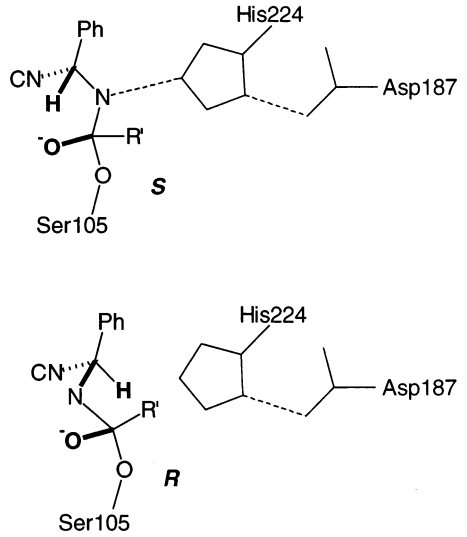

Figure 5. Simplified model of the interaction of (*S*)-**1** and (*R*)-**1** with the active site of the lipase B of *C. antarctica*. Based on the structural analogy of **1** and 1-phenyl ethanol and its interaction with this same enzyme (Ref. 17).

would not form this hydrogen bond so efficiently, resulting in a lower acidity of the α-proton. In addition, in the (*S*)-enantiomer, the oxyanion and the α-proton are in a favourable orientation for interaction, while in the case of the (*R*)-enantiomer, this interaction would be more restricted. These two reasons would determine that the racemisation route (formation of **D** in Fig. 4) is more favoured for the (*S*)-enantiomer.

The fact that **2** and **3** do not react with ethyl phenylacetate seems to be due to steric restrictions in the active site of the enzyme. Compound **1** reacts slower with ethyl phenylacetate than with ethyl acetate, even if the phenylacetyl–enzyme complex is more activated. This suggests that the phenyl group introduces restrictions for the approach of the nucleophile, making, somehow, the reaction with **2** and **3** not feasible.

In short, we have observed a novel turnover-related racemisation in the lipase-catalysed acylation of α-aminonitriles, for which we propose a plausible mechanism. Presumably, this reaction mechanism could also be possible for other lipases, and research is now in progress to clarify this aspect.

4. Conclusion

Candida antarctica lipase B mediates the acylation of 2-amino-2-phenylacetonitrile by phenylacetic acid ethyl ester with a near-absolute preference for the (*S*)-enantiomer.

The reaction of 2-amino-2-phenylacetonitrile, as well as related α-aminonitriles, with ethyl acetate was likewise (*S*)-selective, but the reaction was accompanied by an unexpected turnover-related racemisation of the product, which affected the (*S*)-enantiomer more than the (*R*)-enantiomer.

5. Experimental

5.1. General methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution (unless otherwise specified) with TMS as internal standard using a 400 MHz Varian-VXR 400S spectrometer. Optical rotations were measured on a Perkin–Elmer 241-C polarimeter at 580 nm (Na lamp). The e.e. of the substrates was determined by chiral HPLC on a Daicel Chemical Industries Ltd 4.6×150 mm 5 μ Crownpak CR (+) column using a Waters 625 pump and a Waters 486 UV detector operating at 215 nm. For **3** a Shodex RI SE-61 RI detector was used. The eluent was aqueous $HClO_4$ at a flow of 0.6 mL/min. Details about pH and temperature are specified for each case. Two different chiral columns were employed for measuring the e.e. of the products, a Chiralcel OD 0.46×30 cm and a Chiralcel OD-RH 0.46×15 cm, both from Daicel Chemical Industries Ltd at a flow of 0.6 mL/min. Conversions were measured on a custom-packed Waters 7 μ Symmetry C18 8×150 mm Radial Pak cartridge, contained in a Waters KCM 8×10 module. The eluent was methanol–water at a flow of 1 mL/min. Details are specified for each case. Solvents were dried over zeolite CaA (Uetikon, activated at 400°C for 24 h). Enantiopure (*R*)-2-amino-2-phenylacetonitrile was supplied by DSM (Geleen, The Netherlands). All other chemicals were obtained from common commercial sources.

5.2. Enzymes

Novozym 435 (*C. antarctica* lipase B on Lewatit E), SP525 (*C. antarctica* lipase B), SP 526 (*C. antarctica* lipase A), protease SP 522, SP 523 (*Thermomyces lanuginosus* lipase), SP 524 and lipozym IM 20 (*Rhizomucor miehei* lipase on Duolite A 568) were a gift from Novo Nordisk A/S, (Bagsværd, Denmark). *Pseudomonas* lipoprotein lipase (PSL) was a gift from Boehringer Mannheim. SP 525 was immobilised on Accurel EP100 according to a published procedure.[13]

5.3. Synthesis of α-aminonitriles

5.3.1. 2-Amino-4-phenylbutyronitrile 2. A modification of the Strecker reaction was used.[1] Water (32 mL) and NH_4OH concentrated aqueous solution (20 mL) were added to a mixture of NH_4Cl (5.3 g) and NaCN (2.9 g) and the mixture was cooled in an ice bath. To this solution phenylpropionaldehyde (19.8 mL) was added dropwise in half an hour, and the reaction mixture was vigorously stirred for 3 h at room temperature. The reaction mixture was extracted with diethyl ether (3×10 mL); the ether layer was extracted with 1N HCl (3×10 mL), and the aqueous layer was washed again with ether (2×10 mL). Water was evaporated under vacuum and the final product was dried at low pressure in an oven overnight. A white solid (57% yield) was obtained, identified as the **2** hydrochloride salt. ^{1}H NMR in (D_2O solution): 7.33 (m, 5H, Ar); 4.43 (m, 1H, α-CH); 2.84 (m, 2H, β-CH_2); 2.31 (m, 2H, γ-CH_2). ^{13}C NMR: 140.50, 130.50, 130.11, 128.49 (Ar); 117.20 (CN); 42.63 (α-CH); 33.33 (β-CH_2); 32.07 (γ-CH_2).

5.3.2. 2-Amino-3,3-dimethylpropionitrile 3. The same procedure described for **2** was followed. From 2-methylpropionaldehyde (19.8 mL), NaCN (4.6 g), NH_4Cl (4.9 g), aqueous NH_4OH (20 mL) and H_2O (30 mL), the hydrochloric salt of **3** was obtained as a white solid (7.01 g, 55% yield). ^1H NMR: 4.75 (s, 3H, NH_3^+); 4.41 (d, 1H, α-CH); 2.29 (m, 1H, β-CH); 1.12 (d, 3H, CH_3); 1.10 (d, 3H, CH_3). ^{13}C NMR: 116.51 (CN); 48.91 (α-CH); 31.20 (β-CH); 19.49 (CH_3); 17.10 (CH_3).

5.4. Resolution of α-aminonitriles by crystallisation with tartaric acid

5.4.1. General procedure. A procedure described for amino acid esters[18] was followed. The free aminonitrile was dissolved in methanol or a methanol–toluene mixture. An equivalent of tartaric acid (D- or L- depending on the desired enantiomer) was added dissolved in the same solvent mixture. The solution was allowed to stand for 24 h, then the precipitated salt was filtered, and washed with methanol. The e.e. was determined by chiral HPLC analysis using a Crownpak CR (+) column and aqueous $HClO_4$ solution as eluent (pH 2 and 19°C for **1**, pH 2 and 49°C for **2**, and pH 1 and 0°C for **3**). For e.e.s below 97%, the salt was suspended in new solvent for a further 24 h. This procedure was repeated until the desired e.e. was achieved. The method was not optimised, as only enantiopurity and not yield was important for this study.

5.4.2. (*S*)-2-Amino-2-phenylacetonitrile (*S*)-1. Following the general procedure, **1** (555 mg) was crystallised with D-tartaric acid (552 mg) in methanol–toluene (10:13, v/v 1.3 mL). The crystallisation process was repeated three times and a final product with an e.e. of 97% was obtained (28% yield). Free base $[\alpha]_D$: −27.5 (*c* 1, CH_2Cl_2).

5.4.3. (*R*)-2-Amino-4-phenylbutyronitrile (*R*)-2. Following the general procedure, **2** (1.84 g) was crystallised with L-tartaric acid (1.82 g) in methanol (10 mL). The crystallisation process was repeated three times and a final salt with an e.e. of 98% was obtained (11% yield). Free base $[\alpha]_D$: −11.5 (*c* 1, CH_2Cl_2).

5.4.4. (*S*)-2-Amino-4-phenylbutyronitrile (*S*)-2. From **2** (857 mg) and D-tartaric acid (856 mg), following the same procedure as for (*R*)-**2**, a salt with an e.e. greater than 97% was obtained (106 mg, 6% yield). Free base $[\alpha]_D$: +10.8 (*c* 1, CH_2Cl_2).

5.4.5. (*R*)-2-Amino-3,3-dimethylpropionitrile (*R*)-3. Following the general procedure, **3** (3.88 g) in methanol–toluene (7:3, v/v, 155 mL) was crystallised with D-tartaric acid. The procedure was repeated three times and the salt was obtained with an e.e. greater than 97% (26% yield). Free base $[\alpha]_D$: +13.3 (*c* 1, $CHCl_2$).

5.4.6. (*S*)-2-Amino-3,3-dimethylpropionitrile (*S*)-3. Following the same procedure as for (*R*)-**3**, from **3** (560 mg) and L-tartaric acid, after 4 crystallisations (*S*)-**3** (e.e. = 76%) was obtained in 19% yield.

5.5. Synthesis of standards

5.5.1. Acetylation of α-aminonitriles. General procedure: A mixture of the aminonitrile (free base, one equivalent), pyridine (two equivalents) and the same volume of water was cooled in an ice-water bath. Acetyl chloride (1.3 equivalents) was added dropwise under vigorous stirring. After 3 h, CH_2Cl_2 (30 mL) and 1N aqueous HCl (20 mL) were added to the reaction mixture. The organic layer was further washed with 1N HCl (20 mL), then with $NaHCO_3$ solution (20 mL), dried over Na_2SO_4, and the solvent evaporated under pressure.

5.5.2. 2-Acetamido-2-phenylacetonitrile 4a. Following the general procedure, from **1** (140 mg), **4a** (139 mg, 75% yield) was obtained as a yellowish solid. ^1H NMR: 7.44 (m, 5H, Ar); 6.54 (d, 1H, NH); 6.08 (d, 1H, CH); 2.04 (s, 3H, CH_3). ^{13}C NMR: 169.37 (CO); 133.20, 129.59, 129.40, 127.05 (Ar); 117.48 (CN); 44.13 (CH); 22.73 (CH_3). Mp 101°C.

5.5.3. 2-Acetamido-4-phenylbutyronitrile 5a. Following the general procedure, from **2** (482 mg), **5a** (575 mg, 95% yield) was obtained as a yellowish solid. ^1H NMR: 7.24 (m, 5H, Ar); 6.36 (d, 1H, NH); 4.83 (m, 1H, CH); 2.81 (m, 2H, γ-CH_2); 2.13 (m, 2H, β-CH_2); 1.97 (s, 3H, CH_3). ^{13}C NMR: 169.72 (CO); 139.20, 128.84, 128.37, 126.76 (Ar); 118.48 (CN); 40.15 (CH); 34.40 (γ-CH_2); 31.61 (β-CH_2); 22.74 (CH_3). Mp 61°C.

5.5.4. 2-Acetamido-3,3-dimethylpropionitrile 6a. Following the general procedure, from **3** (441 mg), **6a** (488 mg, 75% yield) was obtained as a yellowish oil. ^1H NMR: 7.20 (d, 1H, NH); 4.75 (m, 1H, α-CH); 2.06 (s, 3H, $COCH_3$); 2.04 (m, 1H, β-CH); 1.11 (d, 3H, CH_3); 1.07 (d, 3H, CH_3). ^{13}C NMR: 170.34 (CO); 118.01 (CN); 46.70 (α-CH); 31.47 (β-CH); 22.67 ($CO\mathit{CH_3}$); 18.69 (CH_3); 18.09 (CH_3).

5.5.5. *N*-Acetyl-phenylethylamine. Following the same general procedure as for aminonitriles, from 1-phenylethylamine (1.26 g), *N*-acetyl-phenylethylamine (812 mg, 48% yield) was obtained as a white solid. ^1H NMR: 7.32 (m, 5H, Ar); 5.92 (d, 1H, NH); 5.12 (q, 1H, CH); 1.96 (s, 3H, CH_3); 1.47 (d, 3H, CH_3). ^{13}C NMR: 169.13 (CO); 143.21, 128.66, 127.36, 126.20 (Ar); 48.78 (CH); 23.42 ($\mathit{CH_3}$-CO); 21.73 ($\mathit{CH_3}$-CH). Mp 60°C.

5.5.6. (*S*)-2-Acetamido-2-phenylacetonitrile (*S*)-4a. Free base (*S*)-**1** (65 mg) was dissolved in acetic anhydride (3 mL). One drop of H_2SO_4 was added and the solution was refluxed for 30 min. Water (20 mL) was added and the resulting mixture was boiled for 30 min in order to hydrolyse the remaining acetic anhydride. The reaction mixture was then extracted with dichloromethane (2×20 mL), washed with aqueous $NaHCO_3$ solution (15 mL), then with 1N HCl (15 mL) and finally with H_2O (15 mL). The organic solvent was dried over Na_2SO_4, and evaporated under vacuum, yielding (*S*)-**4a** with an e.e. of 60% (72 mg, 84% yield).

5.5.7. Phenylacetylation of α-aminonitriles. General procedure: Free aminonitrile (1 mmol) was dissolved in a mixture of acetone/water (1:1 v/v 2 mL) and cooled in an ice-water bath. Phenylacetyl chloride (1.5 equivalents), dissolved in diethyl ether (1 mL), was added dropwise under stirring. After 3 h, the solvent was evaporated and the resulting residue was dissolved in dichloromethane (20 mL). The solution was washed with 1N HCl (2×10 mL), then with $NaHCO_3$ aqueous solution, dried over Na_2SO_4, and finally the solvent was evaporated under pressure.

5.5.8. 2-Phenylacetamido-2-phenylacetonitrile 4b. Following the general procedure, from **1** (300 mg), **4b** (439 mg, 77% yield) was obtained as a yellowish solid. ^{1}H NMR: 7.37 (m, 10H, Ar); 6.20 (d, 1H, NH); 6.10 (d, 1H, CH); 3.62 (d, 2H, CH_2). ^{13}C NMR: 170.24 (CO); 133.52–126.76 (Ar); 117.25 (CN); 44.05 (CH); 43.09 (CH_2). Mp 121°C.

5.5.9. 2-Phenylacetamido-4-phenylbutyronitrile 5b. Following the general procedure, from **2** (281 mg), **5b** (330 mg, 67% yield) was obtained as a yellowish solid. ^{1}H NMR: 7.25 (m, 10H, Ar); 5.64 (d, 1H, NH); 4.84 (m, 1H, CH); 3.56 (s, 2H, $COCH_2$); 2.71 (m, 2H, γ-CH_2); 2.03 (m, 2H, β-CH_2).^{13}C NMR: 170.23 (CO); 139.04–126.78 (Ar); 118.15 (CN); 43.27 (CH); 40.14 (CO*CH*$_2$); 34.54 (γ-CH_2); 31.52 (β-CH_2). Mp 102°C.

5.5.10. 2-Phenylacetamido-3,3-dimethylpropionitrile 6b. Following the general procedure, from **3** (131 mg), **6b** (211 mg, 73% yield) was obtained as a white solid. ^{1}H NMR: 7.31 (m, 5H, Ar); 6.22 (d, 1H, NH); 4.74 (m, 1H, α-CH); 3.59 (s, 2H, CH_2); 1.93 (m, 1H, β-CH); 0.97 (d, 3H, CH_3); 0.95 (d, 3H, CH_3). ^{13}C NMR: 170.59 (CO); 133.94, 129.29, 129.18, 127.70 (Ar); 117.72 (CN); 46.53 (α-CH); 43.17 (CH_2); 31.55 (β-CH); 18.43 (CH_3); 17.91 (CH_3). Mp 70°C.

5.5.11. *N*-Phenylacetyl-1-phenylethylamine. Following the same general procedure as for aminonitriles, from phenylethylamine (1.0 g), *N*-phenylacetyl-1-phenylethylamine (1.12 g, 57% yield) was obtained as a white solid. ^{1}H NMR: 7.25 (m, 10H, Ar); 8.38 (d, 1H, NH); 5.90 (q, 1H, CH); 3.46 (d, 2H, CH_2); 1.35 (d, 3H, CH_3). ^{13}C NMR: 169.04 (CO); 144.58–125.82 (Ar); 47.79 (CH); 42.20 (CH_2); 22.45 (CH_3). Mp 111°C.

5.6. Enzymatic reactions

5.6.1. General procedure. The reactions were carried out at 40°C in 2 mL vials at 1 mL scale unless otherwise specified. To a solution of the substrate, 25 mM, and ethyl phenylacetate, 0.1 M, or ethyl acetate, 1 M, in anhydrous diisopropyl ether, 50 mg enzyme and 50 mg zeolite NaA were added. Three independent parallel reactions were performed to measure conversion, e.e.$_p$ and e.e.$_s$. For measuring the extent of conversion, an internal standard was added to the reaction mixture. To determine the e.e.$_s$, an independent reaction was performed and an aliquot (50 μL) was withdrawn at the specified time. The solvent was evaporated, the residue redissolved in 200 μL $HClO_4$ aqueous solution (pH 1) and centrifuged. The supernatant was analysed by chiral HPLC using a Crownpak CR (+) column and the analytical conditions described in Section 5.4.1.

5.6.2. Reaction of 1 and ethyl phenylacetate. The conversion was monitored using **5a** (25 mM) as internal standard. After 24 h, MeOH (0.8 mL) was added to dissolve the precipitates; an aliquot was taken and analysed by HPLC: Symmetry column, MeOH–H_2O (60:40, v/v). The e.e.$_p$ was measured using the same procedure and the HPLC analysis was performed on an OD-RH column, AcN–H_2O (25:75, v/v).

5.6.3. Reaction of 1 and ethyl acetate. The conversion was monitored using **5a** (25 mM) as internal standard. After 24 h an aliquot was taken and analysed by HPLC: Symmetry column, MeOH–H_2O (40:60, v/v). To determine the e.e.$_p$ the same procedure was used and the HPLC analysis was performed on an OD-RH column, AcN–H_2O (10:90, v/v).

5.6.4. Reaction of 2 and ethyl phenylacetate. The conversion was determined following the same procedure described for the reaction of **1** and ethyl phenylacetate.

5.6.5. Reaction of 2 and ethyl acetate. The conversion was monitored using **4a** (25 mM) as internal standard. After 24 h an aliquot was taken and analysed by HPLC: Symmetry column, MeOH–H_2O (4:6 v/v). To determine the e.e.$_p$ a reaction was performed. After 24 h, 1N HCl (0.8 mL) was added and the mixture shaken for 3 min. An aliquot (50 μL) was withdrawn from the upper organic layer, and analysed by HPLC using an OD column and hexane–isopropanol (95:5, v/v) as eluent.

5.6.6. Reaction of 3 and ethyl phenylacetate. The conversion was determined following the same procedure described for the reaction of **1** and ethyl phenylacetate.

5.6.7. Reaction of 3 and ethyl acetate. The conversion was monitored using **5a** (25 mM) as internal standard. After 24 h, MeOH (0.8 mL) was added to dissolve the precipitates, an aliquot was taken and analysed by HPLC: Symmetry column, MeOH–H_2O (4:6, v/v). To determine the e.e.$_p$, a preparative scale reaction was performed. The enzyme and zeolite NaA were filtered off and washed with methanol. The organic solvent was evaporated and the residue was dissolved in dichloromethane. The solution was washed with 1N HCl (3×10 mL), dried over Na_2SO_4, filtered and evaporated. The resulting residue was analysed by ^{1}H NMR in the presence of Eu(hfc)$_3$. From **3** (99 mg), **6** (76 mg, 54% yield) with an e.e. of almost zero was obtained. From 11 mg (*S*)-**3** (76% e.e.), (*S*)-**6** (7.7 mg, 49% yield) was obtained with an e.e. of 85%. From 47 mg (*R*)-**3**, (*R*)-**6** (22 mg, 33% yield) was obtained with an e.e. of 91%.

5.6.8. Reaction of 1-phenylethylamine and ethyl phenylacetate. Ethyl phenylacetamide (25 mmol) was used as internal standard. For each data point three aliquots of 100 μL were withdrawn and evaporated to dryness. One

aliquot was dissolved in MeOH to determine conversion. The second aliquot was treated with 1N HCl and extracted with ethyl acetate, a new aliquot was withdrawn from the organic layer, evaporated, and dissolved in isopropanol to determine the $e.e._p$. The third aliquot was dissolved in $HClO_4$ aqueous solution (pH 1) to determine the $e.e._s$. The conversion was measured by HPLC using a Symmetry column and MeOH–H_2O (3:7, v/v) as eluent. The $e.e._p$ was determined using an OD column and hexane–isopropanol (95:5, v/v) as eluent. The $e.e._s$, was measured using a Crownpak CR (+) column at 28°C, and $HClO_4$ aqueous solution (pH 1) as eluent.

5.6.9. Reaction of 1-phenylethylamine and ethyl acetate. The same procedure and analysis methodology was used for this reaction as the ones previously described for the reaction of 1-phenylethylamine with ethyl phenylacetate.

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References

1. Fukuda, Y.; Fukui, H.; Harada, T.; Izumi, Y. *J. Ferment. Technol.* **1971**, *49*, 1011–1016.
2. Blah, T. C.; Miura, A.; Wakamoto, A.; Ohka, Y.; Furuhashi, D. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 184–190.
3. Yagasaki, M.; Ozaki, A. *J. Mol. Catal. B: Enzym.* **1998**, *4*, 1–11.
4. Wegman, M. A.; Heinemann, U.; Van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 251–255.
5. Stolz, A.; Trott, S.; Binder, M.; Reinhard, B.; Hirrlinger, B.; Layh, N.; Knackmuss, H.-J. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 137–141.
6. Wegman, M. A.; Heinemann, U.; Stolz, A.; Van Rantwijk, F.; Sheldon, R. A. *Org. Proc. Res. Dev.* **2000**, *4*, 318–322.
7. Gastrock, W. H.; Wepplo, P. J. US Patent 4,683,324 A, 1987.
8. Puertas, S.; Brieva, R.; Rebolledo, F.; Gotor, V. *Tetrahedron* **1993**, *49*, 4007–4014.
9. Van Rantwijk, F.; Hacking, M. A. P. J.; Sheldon, R. A. *Monatsh. Chem.* **2000**, *131*, 549–569.
10. De Castro, M. S.; Sinisterra, V. *Tetrahedron* **1998**, *54*, 2877–2892.
11. Conde, S.; López-Serrano, P.; Martínez, A. *J. Mol. Catal. B: Enzym.* **1999**, *7*, 299–306.
12. Nakai, K.; Hiratake, J.; Jun'ichi, O. *Bull. Inst. Chem. Res.* **1992**, *70*, 333–337.
13. Pedersen, S.; Eigtved, P. PCT Int. Appl. WO 90/15868, 1990.
14. Orrenius, F.; Haeffner, F.; Rotticci, D.; Ohrner, N.; Norin, T.; Hult, K. *Biocatal. Biotransform.* **1998**, *16*, 1–15.
15. Uppenberg, J.; Hansen, M. T.; Patkar, S.; Alwyn Jones, T. *Structure* **1994**, *2*, 293–308.
16. Hult, K., personal communication.
17. Uppenberg, J.; Ohrner, N.; Norin, M.; Hult, K.; Kleywegt, G. J.; Patkar, S.; Waagen, V.; Anthonsen, T.; Alwyn Jones, T. *Biochemistry* **1995**, *34*, 16838–16851.
18. Clark, J. C.; Phillips, G. H.; Steer, H. R. *J. Chem. Soc., Perkin Trans. 1* **1976**, 475–481.