New enzymatic strategies for the preparation of pharmaceutically important enantiomeric β-amino acid derivatives

Ph.D. Thesis

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2022

*“If I have seen further, it is by standing on the shoulders of Gaints (**Prof. Ferenc Fülöp**)”*

Isaac Newton

PUBLICATIONS

Papers related to the thesis

1. **(I) Shahmohammadi, S.**; Fülöp, F.; Forró, E.
   Efficient synthesis of new fluorinated β-amino acid enantiomers through lipase-catalyzed hydrolysis.
   Molecules. **2020**, *25*, 5990, DOI: 10.3390/molecules25245990 **IF.:4.412**

2. **(II) Shahmohammadi, S.**; Faragó, T.; Palkó, M.; Forró, E.
   Green strategies for the preparation of enantiomeric 5-8-membered carbocyclic amino acid derivatives through CALB-catalyzed hydrolysis.
   Molecules. **2022,** *27*, 2600, DOI: 10.3390/molecules27082600 **IF.:4.412**

Other

3. **(III)** Németi, G.; Berkecz, R.; **Shahmohammadi, S.**; Forró, E.; Lindner, W.; Péter, A.; Ilisz, I.
   Enantioselective high-performance liquid chromatographic separation of fluorinated β-phenylalanines utilizing *Cinchonane* alkaloid-based ion exchanger chiral stationary phases.
   J. Chromatogr. A. **2022,** *1670*, 462974, DOI: 10.1016/j.chroma.2022.462974 **IF.:4.759**

Conference lectures

4. **Sayeh Shahmohammadi**, Ferenc Fülöp, and Enikő Forró
   Synthesis of new fluorine-substituted β-amino acid enantiomers through lipase catalyzed hydrolysis;
   25th International Symposium on Analytical and Environmental Problems
   October 7-8. 2019. Szeged, Hungary, Poster Presentation

5. **Sayeh Shahmohammadi**, Ferenc Fülöp, and Enikő Forró
   Lipase catalyzed hydrolysis of fluorine-substituted β-amino esters;
   Virtual Conference Held by Hungarian Foundation to Support Selectively the Organic Chemists
   May 20. 2020. Szeged, Hungary, Poster Presentation

6. **Sayeh Shahmohammadi**, Tünde Faragó, Márta Palkó, and Enikő Forró
   Green strategies to prepare amino acid enantiomers by CALB catalyzed hydrolysis of carbocyclic amino esters;
   II. FKF Szimpozium
   June 16-18. 2021. Virtual, Hungary, Oral Communication & Poster Presentation

7. **Sayeh Shahmohammadi**, Tünde Faragó, Márta Palkó, and Enikő Forró
   Green enzymatic strategies for the preparation of enantiomeric carbocyclic β-amino acid derivatives;
   4th International Green Catalysis Symposium
   April 19-22. 2022. Rennes, France, Poster Presentation

Abbreviations

acetone ----------Me_2CO

acetonitrile ----------MeCN

ammonium acetate ----------NH_4OAc

acetyl ----------Ac

area ----------A

Burkholderia cepacia ----------PS

Burkholderia cepacia immobilized ----------PSIM

benzyl ----------Bn

1-butyl-3-methyl-imidazolium tetrafluoroborate ----------$BMIM.BF_4$

Candida antarctica lipase B ----------CALB

Candida antarctica lipase A ----------CALA

Candida rugosa lipase ----------CRL

Candida cylindracea lipase ----------CCL

conversion ----------conv.

concentration ----------C

chloroform ----------$CHCl_3$

chlorosulfonyl isocyanate ----------CSI

diisopropyl ether ----------iPr_2O

disodium phosphate ----------Na_2HPO_4

dipotassium phosphate ----------K_2HPO_4

dichloro methane ----------CH_2Cl_2

diethyl ether ----------Et_2O

enantiomeric excess ----------*ee*

enantiomeric excess of substrate ----------ee_s

enantiomeric excess of product ----------ee_p

enantioselectivity ----------*E*

equivalent ----------equiv.

ethanol----------EtOH

ethyl ----------Et

ethyl acetate ----------EtOAc

gas chromatography----------GC

high performance liquid chromatography----------HPLC

high-speed ball milling ----------HSBM

high resolution mass spectroscopy----------HRMS

initial rate ----------V_0

kinetic resolution ----------KR

lipase from *Pseudomonas flourescens*----------AK

lipase from *Candida rugosa* ----------AY

lipase from *Aspergillus niger* ----------AS

liquid-assisted grinding----------LAG

monosodium phosphate----------NaH_2PO_4

2-methyl-2-butanol ----------2M2B

methyl ----------Me

2-methyltetrahydrofuran----------2-Me-THF

methoxy----------OMe

methanol ----------MeOH

Mucor miehei lipase----------MML

normal-butyl----------*n*-Bu

normal-propyl ----------*n*-Pr

normal-hexyl ----------*n*-Hex

nuclear magnetic resonance----------NMR

Porcine pancreatic lipase ----------PPL

Pseudomonas cepacia lipase----------PCL

potassium bicarbonate ----------$KHCO_3$

revolutions per minute ----------rpm

racemic----------*rac*

reaction time----------Rt

sodium bicarbonate----------$NaHCO_3$

sodium sulfite ----------Na_2SO_3

sodium sulfate----------$NaSO_4$

supercritical carbon dioxide----------$scCO_2$

temperature ----------Temp

triethylamine ----------Et_3N

tert-butyl methyl ether ----------*t*BuOMe

tert-butyl ----------*t*Bu

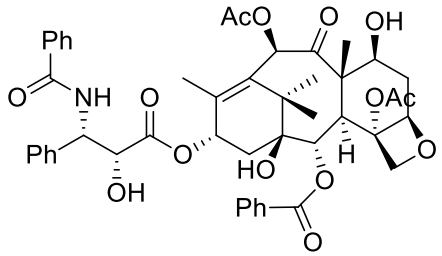
tetrahydrofuran----------THF

thionyl chloride ----------$SOCl_2$

valine ----------Val

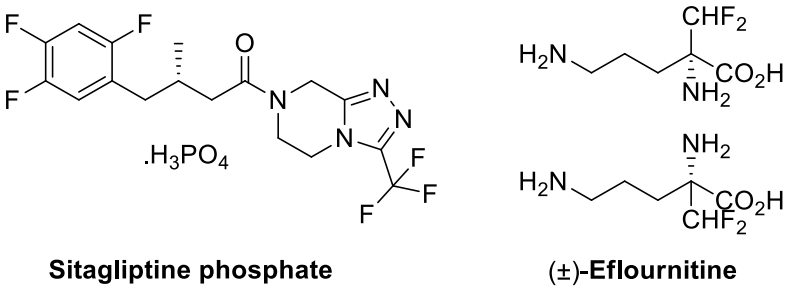
1. Introduction

In recent years, optically pure β-aryl-substituted β-amino acids have acquired considerable interest due to their pharmacological importance, unique and remarkable biological activity [1–4], their utility in synthetic chemistry [5–7] and drug research [8–10]. Therefore, this class of compounds has been described as a crucial scaffold in the design and synthesis of feasible pharmaceutical drugs. For instance, (*S*)-β-phenylalanine can find application as a fundamental component in the synthesis of novel antibiotics [11]. 3-Amino-3-phenylpropionic acid, a key pharmaceutical building block, is present in anticancer agents such as **Taxol** [12] (Scheme 1).



Scheme 1.

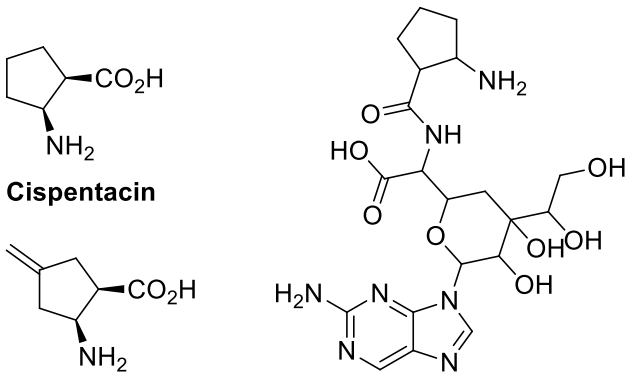
Enormous attainments in the development of fluorinated β-amino acid drugs proved the high significance of this group of compounds in pharmaceutical chemistry. The continuous rise is associated with the unique properties of the fluorine atom with respect to its high electronegativity and the polarity of the C–F bond [13,14]. Additionally, the pK_a, affinity, dipole moment, stability, lipophilicity, and bioavailability of groups adjacent to fluorine can be altered [15–17]. Therefore, fluorine-containing compounds are providing stronger activity and stability, longer half-life, and better bioabsorbability [18–20], especially in the fields of pharmaceutical intermediates [21–24], cancer treatment [25], antiviral agents [26], photovoltaics, diagnostic probes, and bioinspired materials [18]. In addition, natural proteins incorporating fluorinated amino acids also showed many unique characteristics. These compounds are employed in the biotherapeutics protein–protein interaction and in the synthesis of chemicals with better quality [27–36]. For example, (±)-**Eflornitine**, a fluorine-incorporated β-amino acid drug was used for the treatment of trypanomiasis [37] and against facial hirsutism in women [38]. Januvia™ (**Sitagliptin phosphate**) an antidiabetic agent contains an (*R*)-3-amino-4-(2,4,5-trifluorophenyl)butanoic acid subunit [39] (Scheme 2).



Sitagliptine phosphate (±)-**Eflournitine**

Scheme 2.

Another class of molecules with valuble therapeutic effects are carbocyclic β-amino acids. The most obvious examples are **Cispentacin** [(1*R*,2*S*)-2-aminocyclopentanecarboxylic acid] and **Icofungipen** [(1*R*,2*S*)-2-amino-4-methylenecyclopentanecarboxylic acid] both exhibiting antifungal activity [2,3,41–44]. For instance, **Amipurimycin**, a peptidyl nucleoside antibiotic, bears a cispentacin subunit (Scheme 3) [45]. Carbocyclic β-amino acids can be employed as building blocks for the synthesis of modified peptides and self-organizing foldameric structures with increased activity and stability [3,46]. They can also be applicable in heterocyclic [47–49] and combinatorial [50,51] chemistry.



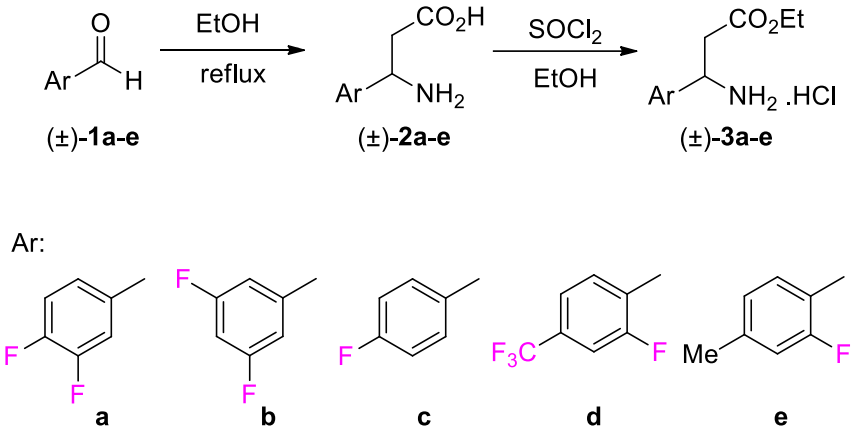
Cispentacin

Icofungipen **Amipurimycin**

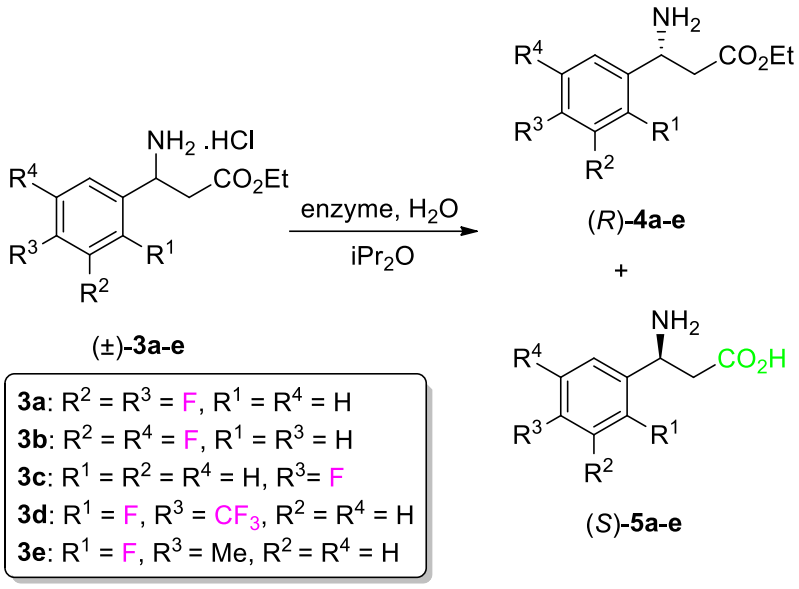
Scheme 3.

Besides, different approaches for the synthesis of β-aryl-β-amino acid enantiomers [52–54] and numerous enzymatic procedures were developed for the synthesis of enantiomerically pure β-amino acids through enantioselective lipase-catalyzed hydrolysis of β-amino esters [55–57] or ring opening of β-lactams [58,59] in an organic medium. In recent years, due to stringent requirements for the development of environmentally benign strategies, green approaches like solvent-free systems [60–62] and application of mechanochemical forces with the recovery and reusability of catalysts [63–69] have gained increasing attention.

The present Ph.D. work has been planned to accomplish two major goals. In view of the significance of fluorine-substituted compounds, the first aim was to synthesize a selection of (±)-β-amino carboxylic ester hydrochloride salts **3a–e** (Scheme 4), then to generate an appropriate lipase-catalyzed method for their resolution through hydrolysis, furnishing enantiopure new β-fluorophenyl-substituted β-amino acids (*S*)-**5a–e** and unreacted β-amino esters (*R*)-**4a–e** (Scheme 5).

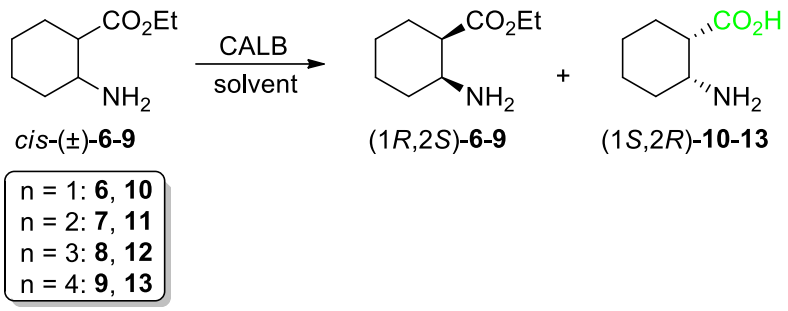


Scheme 4.



Scheme 5.

The second objective of my work was a comparative investigation of different green strategies and then to build an environmentally benign CALB-catalyzed hydrolysis of *cis* carbocyclic amino esters **6–9** (Scheme 6).



Scheme 6.

The fluorinated and carbocyclic amino ester substrates were prepared on the basis of known literature methods [70–75]. Adequate analytical methods were devised to follow the enzymatic reactions and calculate enantiomeric excess values (*ee*), conversions, and enantioselectivities (*E*). In the frame of preliminary experiments, preparative-scale resolutions were performed under the optimized reaction conditions.

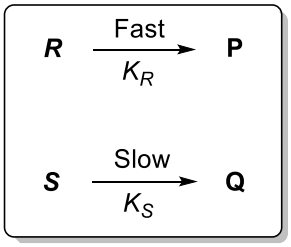
2. Literature background

The augmented reality of the significance of chirality associated with its biological activity created an extensive need for the development of enantiomerically enriched intermediates at a reduced cost. A number of methods to access enantiomerically pure compounds including synthesis from chiral pool, asymmetric synthesis from prochiral substrates, and resolution of racemic mixtures have been published in recent years [76]. Indeed, enzyme is one of the key ingredients that helps to perform green chemistry. In particular, there has been interest in the use of lipases in hydrolytic reactions to obtain enantiopure products, largely because of commercial availability of enzymes and their applicability on an industrial scale [77,78]. Last but not least, the use of enzymes contributes to battle the disadvantages of undesired by-products, toxic effluents, and poor substrate selectivity [79].

2.1. Direct lipase-catalyzed hydrolytic reactions

2.1.1. Kinetic resolution of amino esters

An enzymatic process, in which the transformations of the enantiomers (***R*** and ***S***) of a racemic substrate into products (**P** and **Q**) is taking place with different reaction rates, is called kinetic resolution (KR) (Scheme 7). If the procedure is highly enantioselective (*E* shows as how many times faster one enantiomer is transformed into the product than the other one), the ratio of the reaction rates $k_R/k_S \rightarrow \infty$ only ***R*** will be transformed into product **P**, while the opposite enantiomer ***S*** will be recovered unchanged at 50% conversion [79–82]. In a less enantioselective KR, in addition to the desired **P** and ***S*** enantiomers, ***R*** and **Q** will also be present in the reaction mixture. Therefore, determination of enantiomeric excess values (*ee*) is needed.

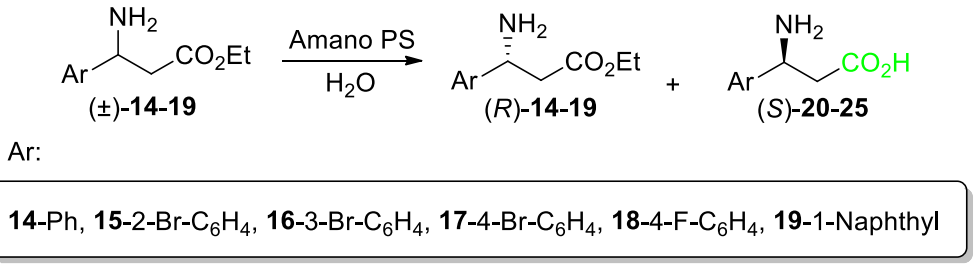


Scheme 7.

2.1.1.1. Lipase-catalyzed hydrolysis of acyclic β-amino carboxylic esters

Based on the principles of green chemistry, biocatalysis can be defined as a sustainable technology [83]. Preparation of high-value pharmaceuticals and chemicals by using of enzymes due to their intrinsic chirality [84] and high enantioselectivity [85] has been gaining extraordinary attention over the past decade. Among them, lipases have been extensively used in kinetic resolution of carboxylic acids and their derivatives through hydrolysis processes. Enantioselective lipase-catalyzed synthesis of β-amino acids through hydrolytic reaction of both cyclic and acyclic β-amino esters [55–57,86,87] has been reported by several groups. In 2011, Busto *et al.* published a comperhensive overview of the enzymatic hydrolyses of *N*-heterocyclic β-amino esters [88].

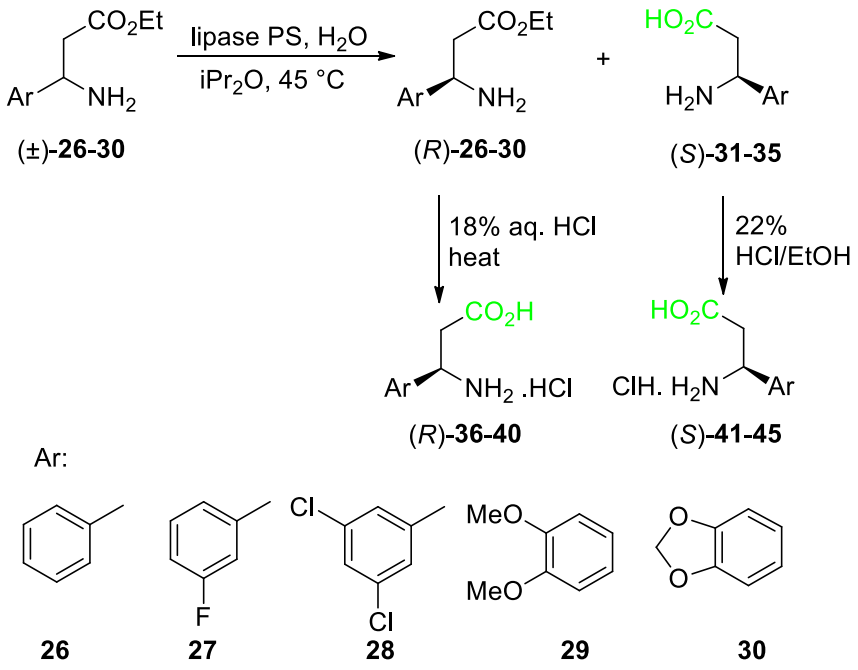
A simple enzymatic resolution of β-amino esters (±)-**14–19** was disclosed, wherein the nitrogen atom was not protected (Scheme 8). The enantiomerically enriched aromatic β-amino acids (*S*)-**20–25** with (*ee* ≥ 77%) and β-amino esters (*R*)-**14–19** (*ee* ≥ 74%) at 50% conversion were summerized [89]. The influence of pH on selectivity was examined. With pH 8 being preferred, racemic β-phenylalanine ethyl ester served the amino acid with an (*ee* > 99%), while an (*ee* ≥ 73%) was measured at pH 7 at the same conversion. The correlation of optical rotation of the isolated β-phenylalanine with that existed in the literature indicated that the (*S*)-ester is preferentially hydrolyzed.



Scheme 8.

Lipase PS-catalyzed enantioselective hydrolysis of racemic ethyl esters (±)-**26–30** in iPr_2O with H_2O (0.5 equiv.) at 45 °C was performed (Scheme 9) [90]. β-Aryl-β-amino acid enantiomers (*S*)-**31–35** were obtained with high enantioselectivities (*E* ≥ 110), excellent enantiomeric excesses (*ee* > 99%) at conversions close to 50% and in good yields (≥40%). Easy separation of the products was achieveable. Transformations of unreacted β-aryl-β-amino ester enantiomers (*R*)-**26–30** with 18% aqueous HCl resulted in the formation of enantiomers of β-aryl-β-amino acid.HCl

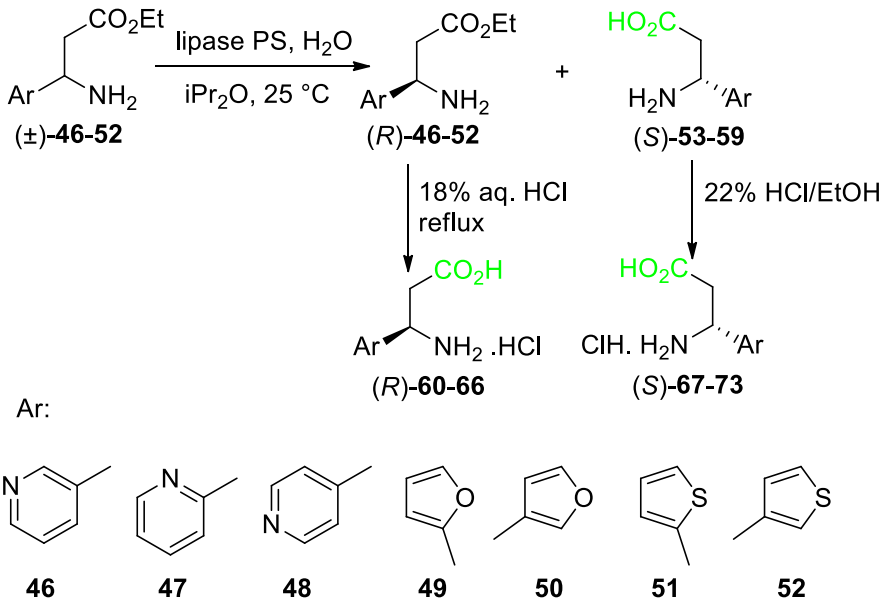
salts (*R*)-**36**–**40** (*ee* ≥ 97%). In the frame of preliminary experiments, PPL and lipase AK catalyzed the hydrolysis of **26** in iPr_2O with H_2O (0.5 equiv.) at 45 °C with moderate enantioselectivities, but no selectivity was observed with lipase AY and Chirazyme L-5. Lipase PS exhibited excellent enantioselectivity (*E* > 200). A slight drop was observed, when the hydrolysis of **26** was carried out at 25 °C with lipase PS. The enantioselectivity and reaction rate were not affected by the amount of added H_2O, but a dramatic decrease was observed when iPr_2O/H_2O 1/1 (v/v) was used (*E* = 30). The slowest hydrolysis was observed in $CHCl_3$, while the highest reaction rate was measured in iPr_2O, *t*BuOMe, and *n*-hexane with excellent enantioselectivity (*E* > 200) with all solvents tested. Expectedly, by increasing the amount of enzyme, the hydrolysis rate of **26** increased.



Scheme 9.

Tasnadi *et al*. described the enantioselective (*E* > 200) lipase PS-catalyzed hydrolysis of β-heteroaryl-β-amino esters (±)-**46**–**52** [91] (Scheme 10). The reactions were carried out with H_2O (0.5 equiv.) in diisopropyl ether at 25 °C. The β-heteroaryl-substituted β-amino acid enantiomers (*S*)-**53**–**59** were isolated with excellent enantiomeric excess (*ee* ≥ 97%) at conversion ≥49% and in good yield (40%). On the basis of enzyme screening data using **46** as the model compound, lipolase and chirazyme L-5 did not exhibit any selectivity in the hydrolysis of **46** in iPr_2O with H_2O (0.5 equiv.) at 45 °C. Moderate enantioselectivity (*E* ≤ 6) was achieved when PPL and lipase AK were tested. High enantioselectivity (*E* = 99) was afforded by using lipase PS. Decreasing the

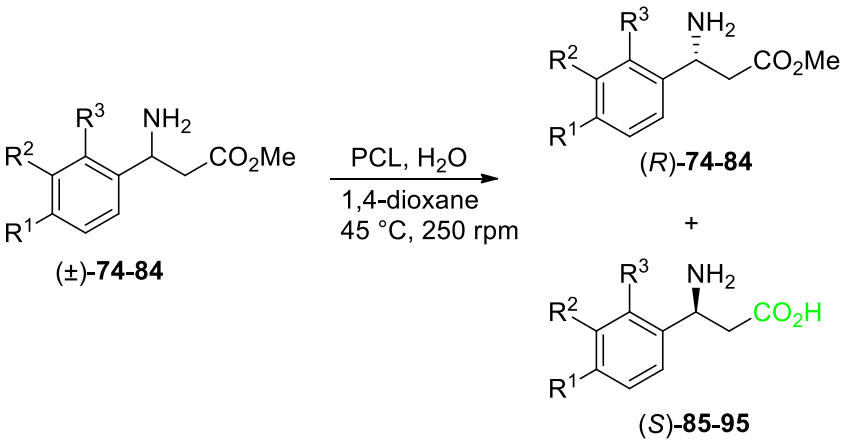
temperature to 25 °C served excellent enantioselectivity (E > 200). Among the tested solvents, the reaction rate in Me_2CO was the lowest and it was the highest in iPr_2O, *t*BuOMe, and *n*-hexane, while the enantioselectivities were high (E > 100) in all cases except in *tert*-amyl alcohol (E = 16). It was shown that by increasing the amount of added water, the enantioselectivity decreased and higher hydrolysis rate was observed when more enzyme was added to the reaction media.



Scheme 10.

A stereoselective chemoenzymatic route was reported to prepare a wide range of optically active 3-amino-3-arylpropanoic acid derivatives (±)-**74–84** [92] (Scheme 11). The resolution of synthesized racemic amino esters in 1,4-dioxane, H_2O (5 equiv.) with PCL at 45 °C supplied mostly enantiopure (*S*)-3-amino-3-arylpropanoic acids **85–95** and methyl (*R*)-esters **74–84** with excellent enantiodescrimination at conversions close to 50%. The effect of support, including ceramics (PCL-C I, PCL-C II) and diatomite (PCL-D) used to immobilize PCL enzyme, was explored with compound **74** selected as substrate and 1,4-dioxane chosen as solvent, H_2O (5 equiv.) at 30 °C. PCL immoblized on diatomite showed a high enantioselectivity value (E > 200). The enantioselectivity remained unchanged, when the hydrolysis reaction was performed in THF and MeCN. Increasing the temperature to 45 °C resulted in an increase of the enantiomeric ratio, while a slight deactivation of the enzyme was observed at 60 °C. Moreover, the reactivity was not affected with an increase in the quantity of H_2O, but the use of twice as much enzyme sped up reaching conversions close to 50%. Performing the hydrolytic reaction of racemate **74** under optimized conditions with PCL-C Amano (a preparation from Amano Pharmaceutical Co.)

afforded an excellent enantiopereference and activity in a shorter reaction. The (*S*)-enantiopereference of PCL was demonstrated by comparison of the optical rotation values described in the literature. Additionally, methyl (±)-3-amino-3-phenylpropanoate with an (*S*)-configuration reacted in a rapid raction. It is due to the optimum binding location and conformation of a phenyl ring and the β-amino propanoate core confirming the enantiopereference of the transformation.

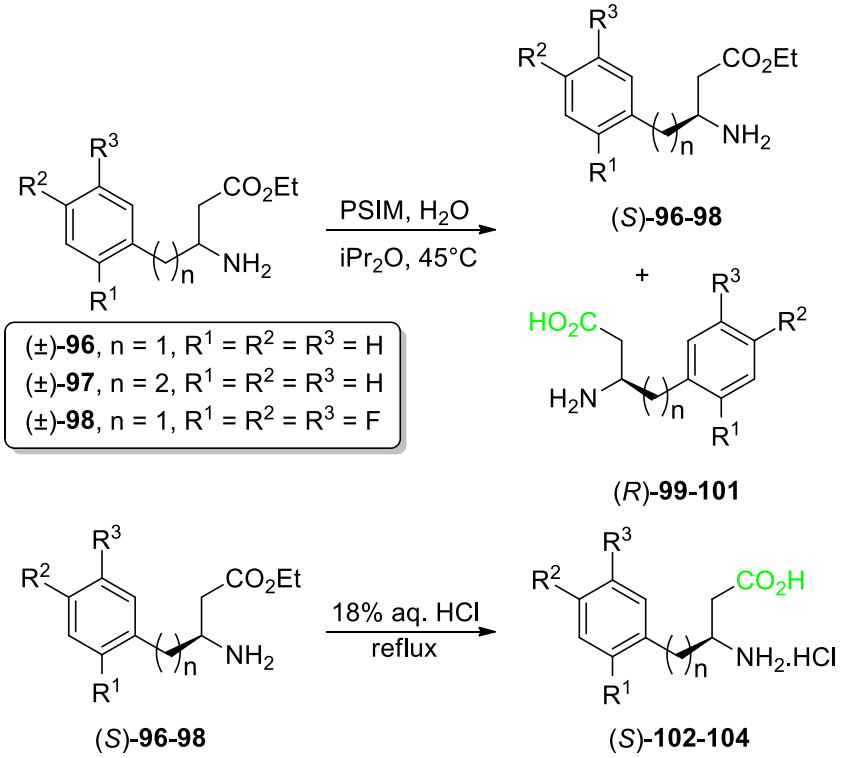


74 $R^1 = R^2 = R^3 = H$
75 $R^1 = Me$, $R^2 = R^3 = H$
76 $R^1 = OMe$, $R^2 = R^3 = H$
77 $R^1 = OPh$, $R^2 = R^3 = H$
78 $R^1 = CF_3$, $R^2 = R^3 = H$
79 $R^1 = Ph$, $R^2 = R^3 = H$
80 $R^1 = Br$, $R^2 = R^3 = H$
81 $R^1 = Cl$, $R^2 = R^3 = H$
82 $R^1 = F$, $R^2 = R^3 = H$
83 $R^1 = H$, $R^2 = F$, $R^3 = H$
84 $R^1 = R^2 = H$, $R^3 = F$

Scheme 11.

An efficient, direct *Burkholderia cepacia*-catalyzed hydrolysis of β-amino esters (±)-**96–98** with H_2O (0.5 equiv.) as a nucleophile in iPr_2O at 45 °C was deviced [93] (Scheme 12). The pharmacologically important β-arylalkyl-substituted β-amino acid enantiomers (*R*)-**99–101** and, in particular, (*R*)-3-amino-3-(2,4,5-trifluorophenyl)butanoic acid, the intermediate of the antidiabetic drug sitagliptine, were prepared with excellent enantioselectivity (*E* ~ 200) and high enantiomeric excesses (*ee* ≥ 96%), and they were isolated in good yields (≥42%), wherein the ester enantiomers were transformed into β-amino acid.HCl (*S*)-**102–104** with 18% aqueous HCl. On the basis of preliminary experiments, hydrolysis of model compound (±)-**96** was tested with a number of lipases in *t*BuOMe with H_2O (0.5 equiv.) at 25 °C. CALA and Lipolase displayed low *E* values, while PPL and lipase AK catalyzed the reaction with moderate *E* values. Lipase PSIM proved to be the best enzyme to hydrolyze racemate **96**. Increasing the temperature from 25 to 45 °C served

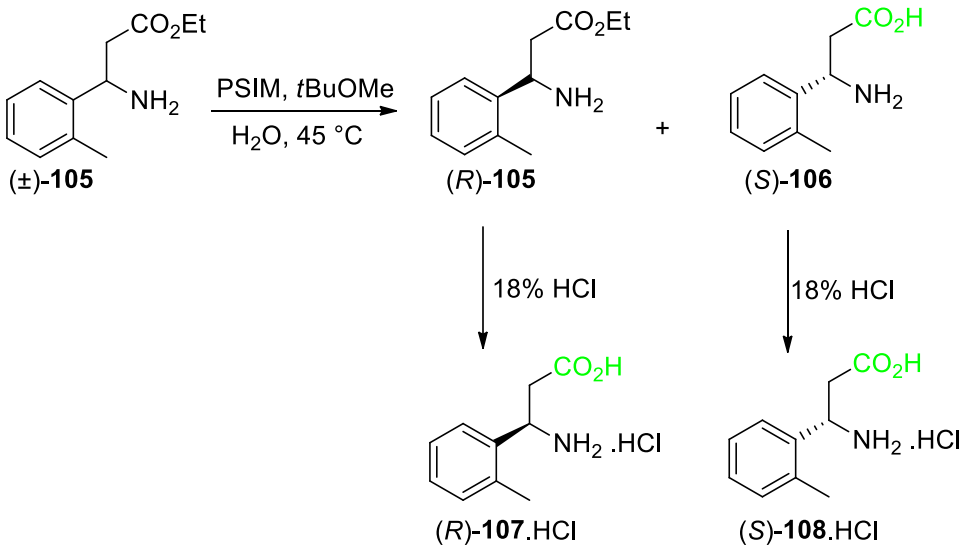
a considerable elevation in conversion. Furthermore, the reaction rate increased when a large amount of enzyme was used, but the enantioselectivity apparently did not change. Hydrolysis of **96** in iPr_2O and *t*BuOMe exhibited the highest *E* values and conversions, whereas carrying out the hydrolytic reaction without solvent caused an increase in reaction rate, but a drop in *E*.



Scheme 12.

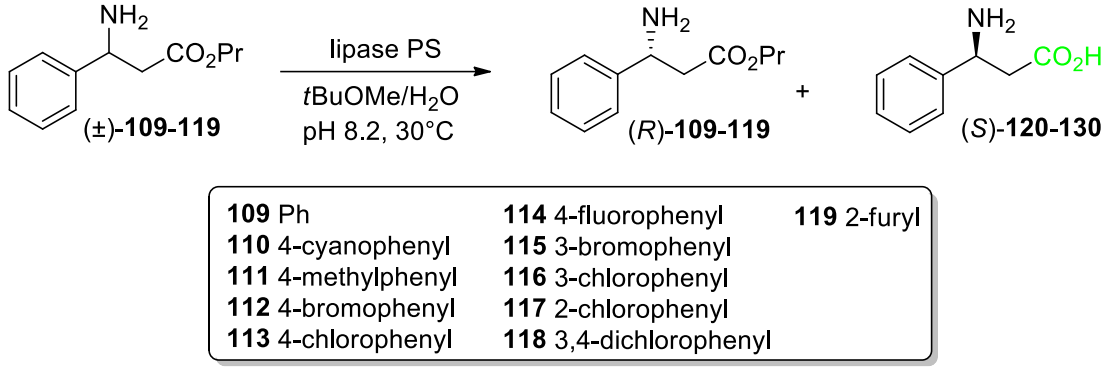
Forro *et al.* performed the resolution of racemic ethyl 3-amino-3-(*o*-tolyl)propanoate (±)-**105** in *t*BuOMe with added H_2O at 45 °C [94] (Scheme 13). The enantiomers of product amino acid (*S*)-**106** (ee_p = 98%) and unreacted amino ester (*R*)-**105** (ee_s > 99%) were isolated. Enantiomer (*R*)-**105** was hydrolyzed to (*R*)-**107**.HCl and the isolated enantiomer (*S*)-**106** was treated with 18% HCl to form the (*S*)-**108**.HCl product. Various lipases were tested in the hydrolysis of (±)-**105** in *t*BuOMe with 0.5 equiv. of H_2O at 25 or 60 °C. Chirazyme L-5 or lipase AY did not exhibit any enantioselectivity, while moderate results were detected with Lipolase and PPL ($6 \leq E \leq 16$). Lipase AK and Lipase PSIM proved to be the best enzymes (conversion 43% after 63 h, $E \geq 87$). Elevation of the temperature from 25 to 45 °C in the hydrolysis catalyzed by lipase AK resulted in an increase in both reaction rate and *E*. When the reaction was performed with lipase PSIM at 45 °C, the reaction became at least 10 times faster with excellent *E* (>200). The hydrolytic reaction in EtOAc, EtOH, Me_2CO at 25 °C with lipase AK proceeded considerably slower, although all

afforded high *E* (>200). Increasing the amount of lipase PSIM from 30 to 50 and then to 75 mg mL^{-1}, rose the reaction rate. Various quantities of added H_2O (from 0 to 10 equiv.) were tested in a lipase PSIM-catalyzed reaction at 45 °C. As the H_2O amount was increased, the reaction became significantly faster.



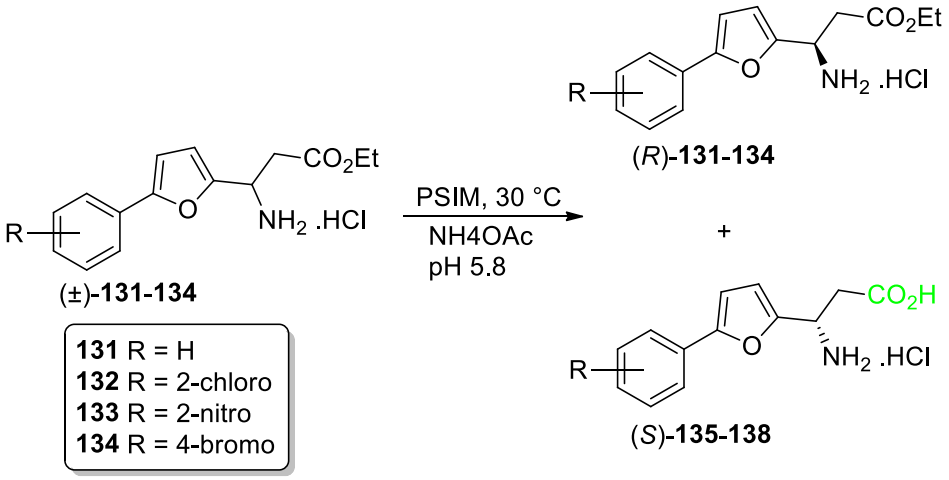
Scheme 13.

Grayson *et al.* described Amano lipase PS-catalyzed hydrolysis of a wide range of aromatic and heteroaromatic propyl esters (±)-**109–119** in *t*BuOMe/H_2O, pH ~8.2 at 30 °C [95] (Scheme 14). The (*S*)-**120**–**130** product amino acid enantiomers, including (*S*)-β-phenylalanine **120**, a key pharmaceutical building block, were prepared with (*ee* ≥ 98%) and yields ranging from 16% to 50%. Sterically hindered aromatics and a derivative with the furyl group showed low activity performed in hydrolytic reactions, while high enantioselectivity was obtained when the propyl ester was used. The improvement of reaction rate and selectivity was acheivable by changing from the ethyl ester to propyl or butyl esters.



Scheme 14.

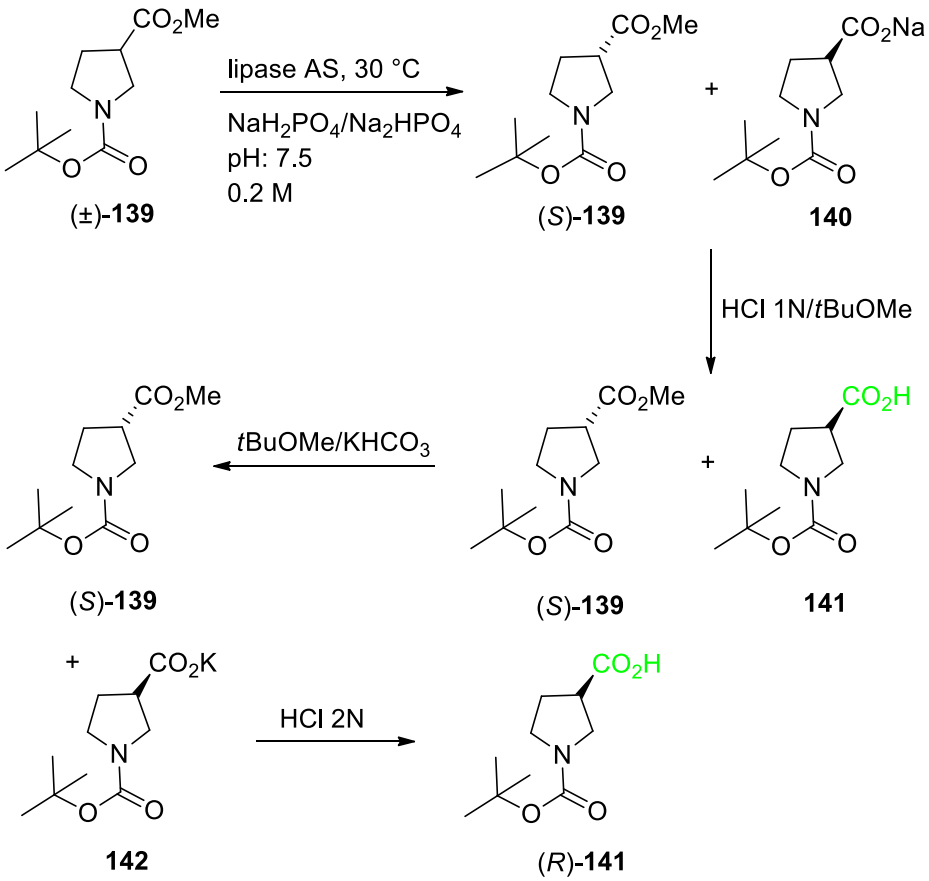
Nagy *et al.* devised lipase-mediated biotransformation of racemic phenylfuran-2-yl-β-alanine hydrochlorides *rac*-**131–134**·HCl salts [96] (Scheme 15). The hydrolytic reactions were carried out with lipase *Burkholderia cepacia* (PSIM) for compound **131** and lipase *Pseudomonas fluorescens* (AKIM) for compounds **132–134** in NH_4OAc pH 5.8 at 30 °C. The acid (*S*)-**135–138** (ee_p 86–95%) products and unreacted ester enantiomers (*R*)-**132–134** (ee_s 80–94%) were separated at conversions close to 50%. As an exception, the purification of (*R*)-**131** failed. For stability reasons, (*R*)-**131–134**·HCl salt esters were hydrolyzed and characterized as amino acid hydrochloride salts.



Scheme 15.

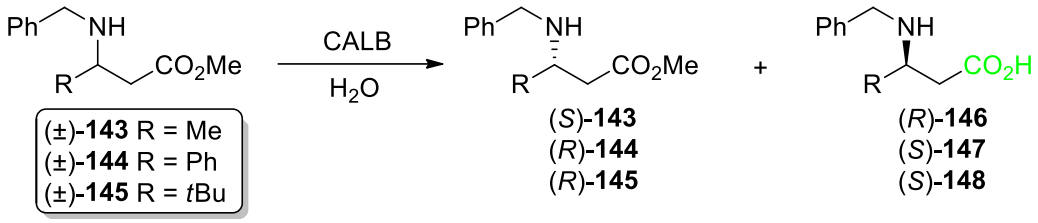
2.1.1.2. Lipase-catalyzed hydrolysis of N-heterocyclic-based amino carboxylic esters

A successful enzymatic resolution of *N*-substituted-β-proline was designed [97]. The resolution of 1-*tert*-butyl-3-methylpyrrolidine-1,3-dicarboxylate (±)-**139** was catalyzed with Lipase AS in NaH_2PO_4/Na_2HPO_4 buffer pH 7.5 (C 0.2 M) at 30 °C for 24 h (Scheme 16). The progress of reaction was monitored by chiral HPLC. After the separation of the organic and aqueous layers at 55% conversion, (*S*)-1-*tert*-butyl-3-methylpyrrolidine-1,3-dicarboxylate (*S*)-**139** (*ee* = 98%) as an oil and solid (*R*)-1-(*tert*-butoxy-carbonyl)pyrrolidine-3-carboxylic acid (*R*)-**141** (*ee* = 85%) were isolated. Screening results clearly exhibited the hydrolytic suitability of CALA to perform the resolution (conv. 69%, *ee* = 98%). Since the aim was to maintain the conversion close to 50%, an excellent optical purity in a reaction time of about 24 h could be achieved. The configuration was determined to be (*S*) for the isolated ester enantiomer and (*R*) for the acid, by comparison of the elution profile of commercially available materials under the same chiral HPLC conditions.



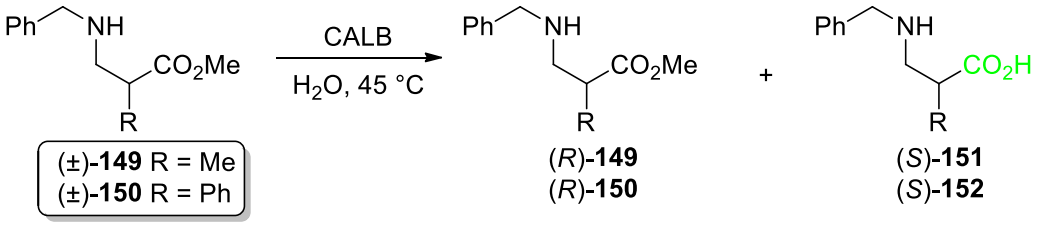
Scheme 16.

Rangel *et al*. examined the ability of CALB to resolve racemic *N*-benzylated-β^2, -β^3, and -$\beta^{2,3}$ amino acid methyl esters through hydrolytic reactions [98]. CALB-catalyzed hydrolysis of compound (±)-**143** was carried out in *n*-hexane at 45 °C with H_2O (0.5 equiv.) (Scheme 17). The product (**146**) was obtained with good enantiopurity (*ee* = 85%, *E* = 25) at 45% conversion. The hydrolysis of racemate **144** proceeded more slowly, even though the reaction was performed at a higher temperature of 60 °C and with a higher equiv. of H_2O (0.75 equiv.). Nevertheless, product **147** was isolated in enantiopure form (*ee* > 99%, *E* > 200, at conv. 23%). No reaction took place when compound (±)-**145** was used as a substrate in *n*-hexane at 60 °C. Upon switching to 2M2B as a more polar solvent, the hydrolysis was completed at 50% conversion with excellent enantioselectivity (*E* > 200) and product **148** was obtained in an enantiomerically pure form (*ee* > 99%).



Scheme 17.

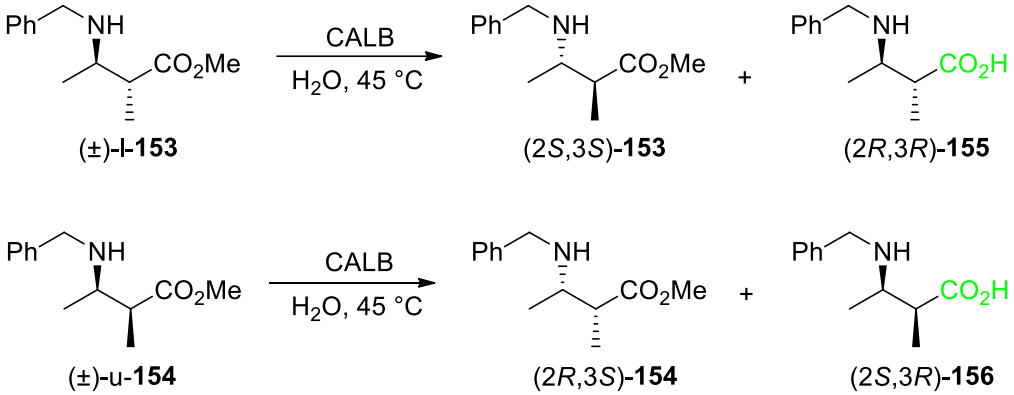
The hydrolysis of racemate **149** in *n*-hexane at 45 °C with H_2O (0.5 equiv.) took place smoothly with 40% conversion after 2 h, while there was no enantiodiscremination (*E* = 1.4) [98] (Scheme 18). The attempt to increase the *E* by employing 2M2B was unsuccessful. No hydrolysis was found for compound (±)-**150** in *n*-hexane, whereas 22% conversion was observed in 2M2B. Practically, compound **152** was obtained in racemic form.



Scheme 18.

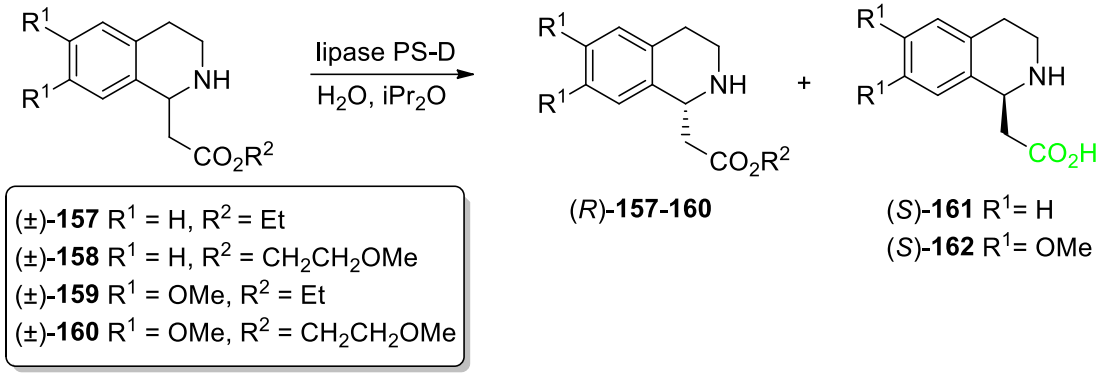
The hydrolysis of (±)-l-**153** in *n*-hexane at 45 °C was slow (36% conversion after 48 h) and very similar to that observed for (±)-**143** [98] (Scheme 19). Even using a higher amount of enzyme

did not make a significant improvement in the conversion value. Both *E* and conversion (*E* = 9, conv. 29%) decreased, when the hydrolysis was performed in 2M2B. By contrast, CALB exhibited complete enantiodiscrimination (*ee* > 99%) toward (±)-u-**154** at 45 °C, when *n*-hexane and 2M2B were used as solvents.



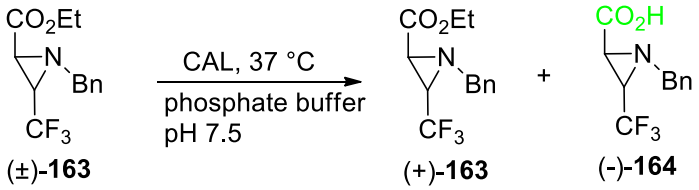
Scheme 19.

Kinetic resolution of ethyl and 2-methoxyethyl esters (±)-**157–160** catalyzed by *Burkholderia cepacia* lipase (lipase PS-D) was performed with added H_2O in iPr_2O at room temperature [99] (Scheme 20). The enantiomers of 1,2,3,4-tetrahydroisoquinoline-1-acetic acid and 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-1-acetic acid [(*S*)-**161** and (*S*)-**162**, respectively] were isolated with excellent enantiomeric excess values (*ee* > 99% and 98%). In the frame of enzyme sreening, lipase PS-D (Amano lipase PS on Celite) and lipase PS-C II (Amano preparation on Toyonite 200M) with 0.5, 2, and 4 equiv. of added H_2O were tested for the hydrolysis of model compound (±)-**157**. Reactivities with both enzymes were low. However, with increasing H_2O content, the reactivity increased. Being more pronounced with lipase PS-D, excellent enantioselectivity (*E* > 200) was achieved. The best reactivity and enantioselectivity values were found in iPr_2O (*E* > 200 after 3 days) in comparison to all other tested solvents including Et_2O, *t*BuOMe, toluene, and 2-Me-THF. Performing the reaction at elevated temperature (47 °C) resulted in a drop in enantioselectivity (*E* = 91).



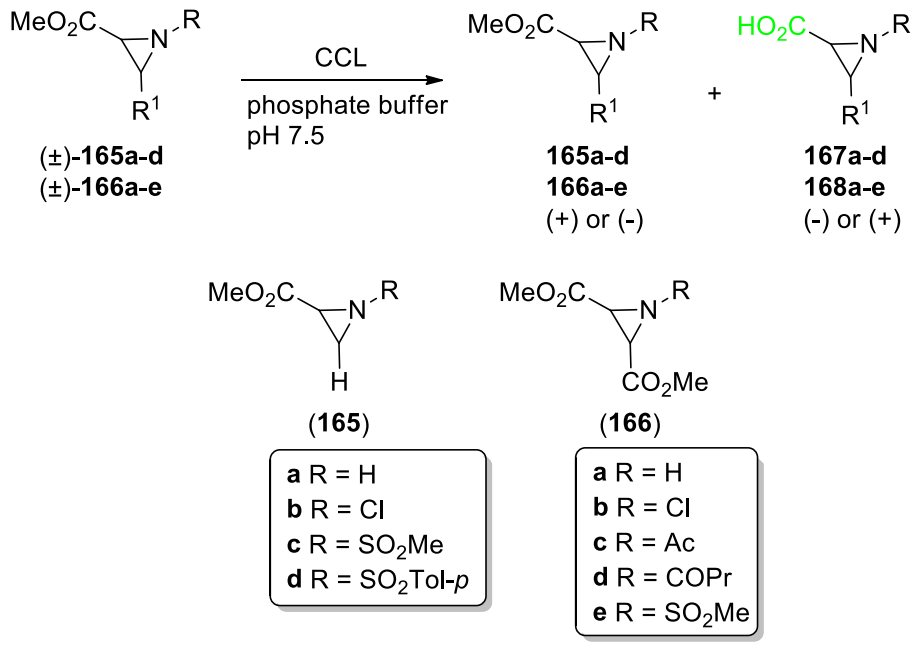
Scheme 20.

Candida antarctica-catalyzed hydrolysis of racemic *trans*-1-benzyl-3-trifluoromethyl-2-ethoxycarbonylaziridine (±)-**163** was described by Davoli *et. al*. [100] (Scheme 21). The reaction was carried out in phosphate buffer pH 7.5 (0.1 mol dm^{-3} and NaCl 0.1 mol dm^{-3}) at 37 °C and with an enzyme/aziridine ratio (w/w) of 1:50. The reaction (in a suspension) was stopped at a conversion of 45% after 30 min with an enantioselectivity value of > 100. The unreacted ester (+)-**163** was isolated from the reaction mixture by extraction with diethyl ether followed by column chromatography with a high enantiomeric excess value (*ee* = 83%). The product aziridinecarboxylic acid (−)-**164** (*ee* = 98%) was isolated from the aqueous phase by acidification with 10% HCl until pH 1 and extraction with ethyl acetate.



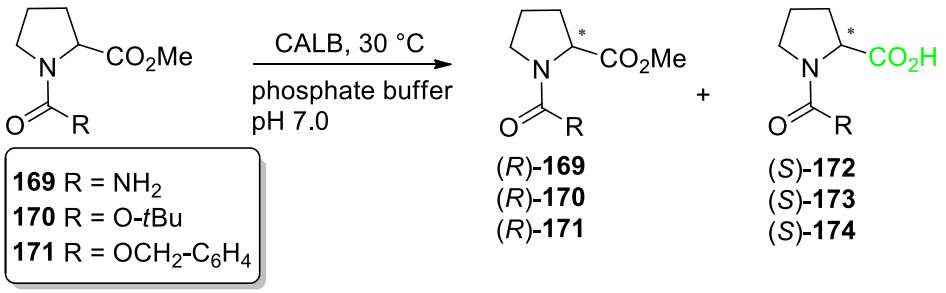
Scheme 21.

Resolution of *N*-substituted aziridine-2-carboxylates (±)-**165a–d** and -2,3-dicarboxylates (±)-**166a–e** with lipase *Candida cylindracea* was developed in phosphate buffer pH 7.5 at room temperature [101] (Scheme 22). The hydrolysis of racemates afforded the unhydrolyzed esters and product acids. The unreacted aziridines (**165a–d** ee_s 14–72%, **166a–e** ee_s 13–95%) and hydrolyzed aziridine enantiomers (**167a–d** ee_p 0–64%, **168a–e** ee_p 4–60%) were obtained in conversions of 50–80%.



Scheme 22.

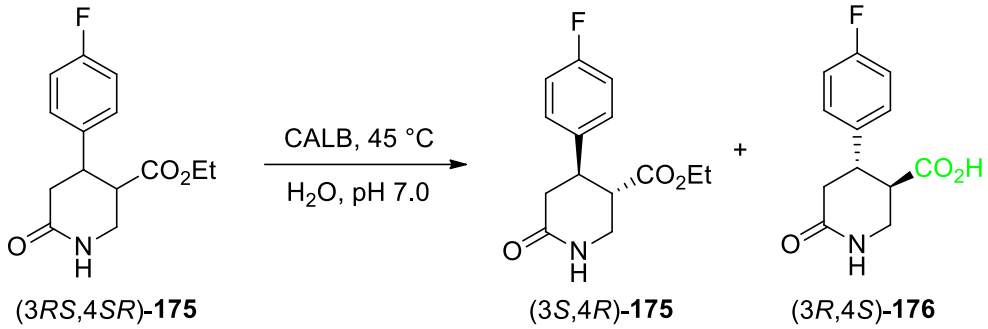
In an attempt to examine the hydrolytic activity of *Candida antarctica* lipase B (Chirazyme L-2) for the hydrolysis of *N*-carbamoyl (±)-**169–171** derivatives, the reactions were performed in phosphate buffer pH 7.0 (0.1 M for **169**, **171** and 0.02 M for **170**) at 30 °C [102] (Scheme 23). Monitoring the reaction of (±)-**169** for 72 h showed no trace of **172**. The hydrolysis of (±)-**170** after 25 h resulted in product (*S*)-**173** (with *ee* > 99.9%) and unreacted ester (*R*)-**170** (with *ee* 98.7%) at nearly 50% conversion with high enantioselectivity (*E* > 100). The resolution of (±)-**171** was completed after 38 h yielding product (*S*)-**174** (with *ee* > 99.8%) and unreacted ester (*R*)-**171** (with *ee* = 97.8%) at a conversion close to 50% with high enantioselectivity (*E* > 100).



Scheme 23.

The enantioselective hydrolysis of *trans*-4-(4'-fluorophenyl)-6-oxo-piperidin-3-ethyl carboxylate (±)-**175** was performed using a glyoxyl-*Candida antarctica* (CALB) preparation with 20% co-solvent (5% dioxane, 15% diglyme) at pH 7.0 and 45 °C [103] (Scheme 24). The

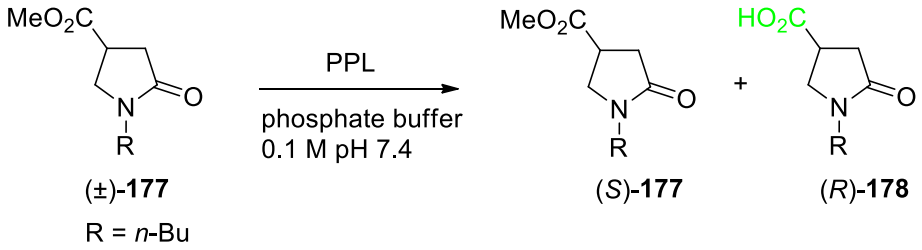
unreacted ester (3*S*,4*R*)-**175** was isolated (extraction with cyclohexane) in enantiomerically pure form (*ee* > 99%) at 50% conversion after 300 h.



(3*RS*,4*SR*)-**175** (3*S*,4*R*)-**175** (3*R*,4*S*)-**176**

Scheme 24.

PPL-catalyzed resolution of methyl 1-(1-butyl)-5-oxo-3-pyrrolidinecarboxylate (±)-**177** in phosphate buffer pH 7.4 (0.1 M) at room temperature was described by Felluga *et al*. [104] (Scheme 25). At low conversion (conv. 23%, *E* = 9), enantiomeric acid product (*R*)-**178** was isolated with a moderate enantiomeric excess value (*ee* = 75%). When the conversion reached 86%, the enantiomer of unreacted ester (*S*)-**177** with a high enantiomeric excess value (*ee* = 96%) was obtained. The reaction was not enantioselective at all, when enymes *Candida rugosa* lipase (CRL) and *Mucor miehei* lipase (MML) were used.

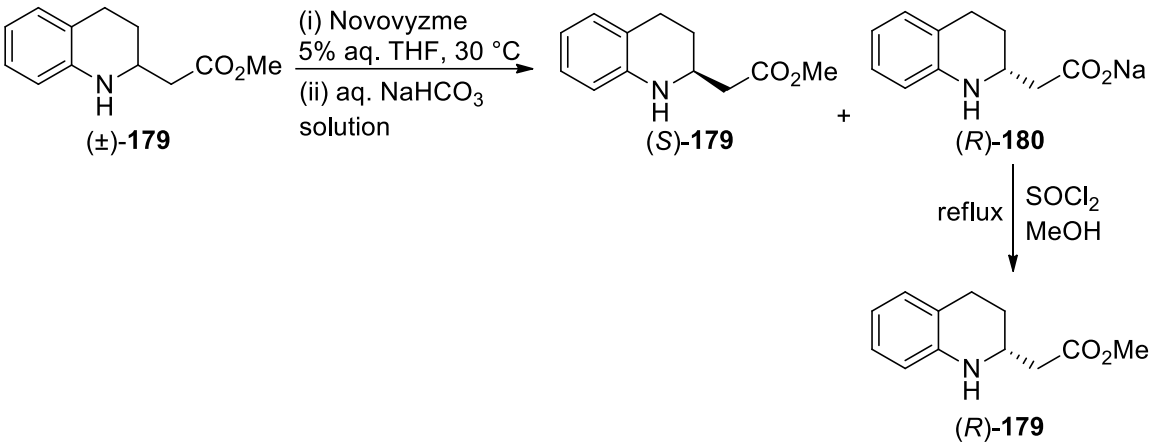


(±)-**177**
R = *n*-Bu

(*S*)-**177** (*R*)-**178**

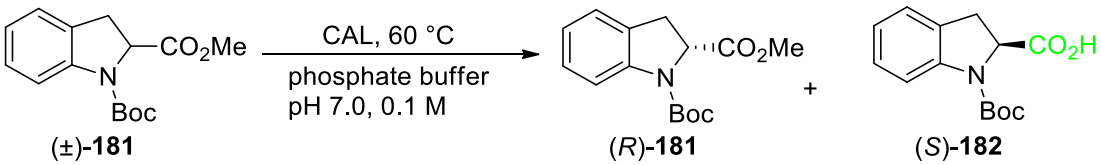
Scheme 25.

The kinetic resolution of racemic methyl 2-(1,2,3,4-tetrahydroquinolin-2-yl) acetate ester (±)-**179** was performed with Novozym® 435 in a 5% aqueous solution of THF under stirring at 30 °C for 3 days [105] (Scheme 26). The (*S*)-β-amino ester **179** (*ee* = 91%) was obtained by adding an aqueous $NaHCO_3$ solution to remove (*R*)-sodium carboxylate **180**. The (*R*)-β-amino ester **179** (*ee* = 96%) was obtained from the aqueous layer containing the sodium carboxylate in methanol and thionyl chloride after treatment at reflux trremperature.



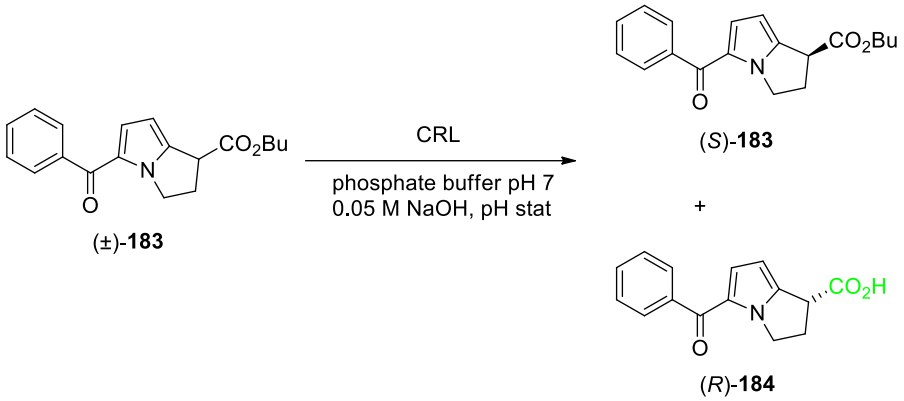
Scheme 26.

Methyl (±)-1-*t*-butoxycarbonyl-indoline-2-carboxylate **181** was resolved using *Candida antarctica* lipase (Chirazyme L-2) in phosphate buffer solution pH 7.0 (0.1 M) at 60 °C [106] (Scheme 27). The reaction proceeded efficiently at a conversion of 49.9% with excellent enantioselectivity (*E* > 1000). The hydrolyzed product (*S*)-**182** carboxylic acid (*ee* > 99.9%) and the unreacted ester (*R*)-**181** (*ee* > 99.6%) were isolated.



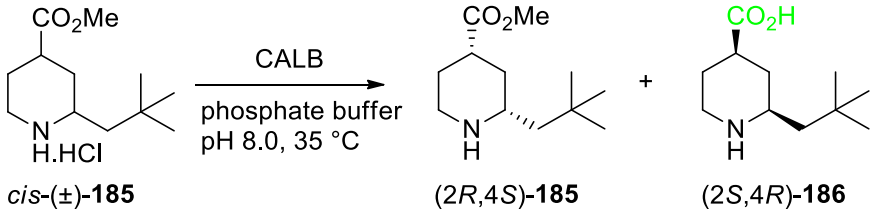
Scheme 27.

Kim *et al.* performed the enzymatic resolution of the butyl ester of ketorolac (±)-**183** in phosphate buffer pH 7.0 and NaOH (0.05 M) pH stat [107] (Scheme 28). Several lipases were tested. Lipase *Candida rugosa* afforded acid enantiomer (*R*)-**184** (*ee* = 91%, conv. 47% after 7 h) and unreacted ester enantiomer (*S*)-**183** (*ee* = 46%, conv. 84% after 28 h). CALB resulted in the enantiomers of acid (*ee* = 100%, conv. 19% after 0.5 h) and the ester (*ee* > 99%, conv. 60% after 3 h). Lastly, *Mucor meihei* yielded the acid enantiomer (*ee* = 71%, conv. 26% after 0.5 h) and the unchanged ester enantiomer (*ee* = 92%, conv. 96% after 2.5 h).



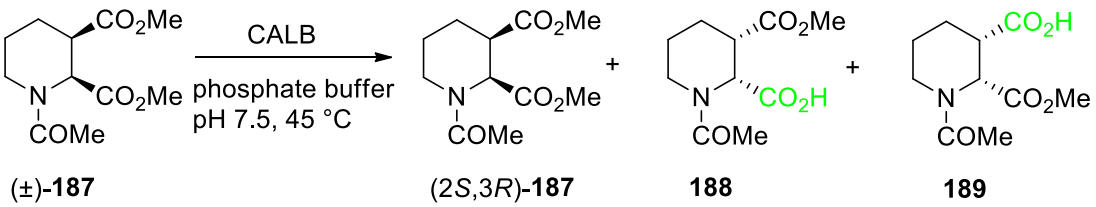
Scheme 28.

AZD6564, an oral fibrinolysis inhibitor [108], was preparaed in multikilogram-scale including an enzymatic resolution of (±)-*cis*-methyl 2-neopentylpiperidine-4-carboxylate hydrochloride (±)-**185** [109] (Scheme 29). The CALB-catalyzed hydrolysis was performed in H_2O/K_2HPO_4 buffer pH 8.0 at 35 °C. The unreacted ester enantiomer (2*R*,4*S*)-**185** was isolated with an ee_s = 97% and in a yield of 35%.



Scheme 29.

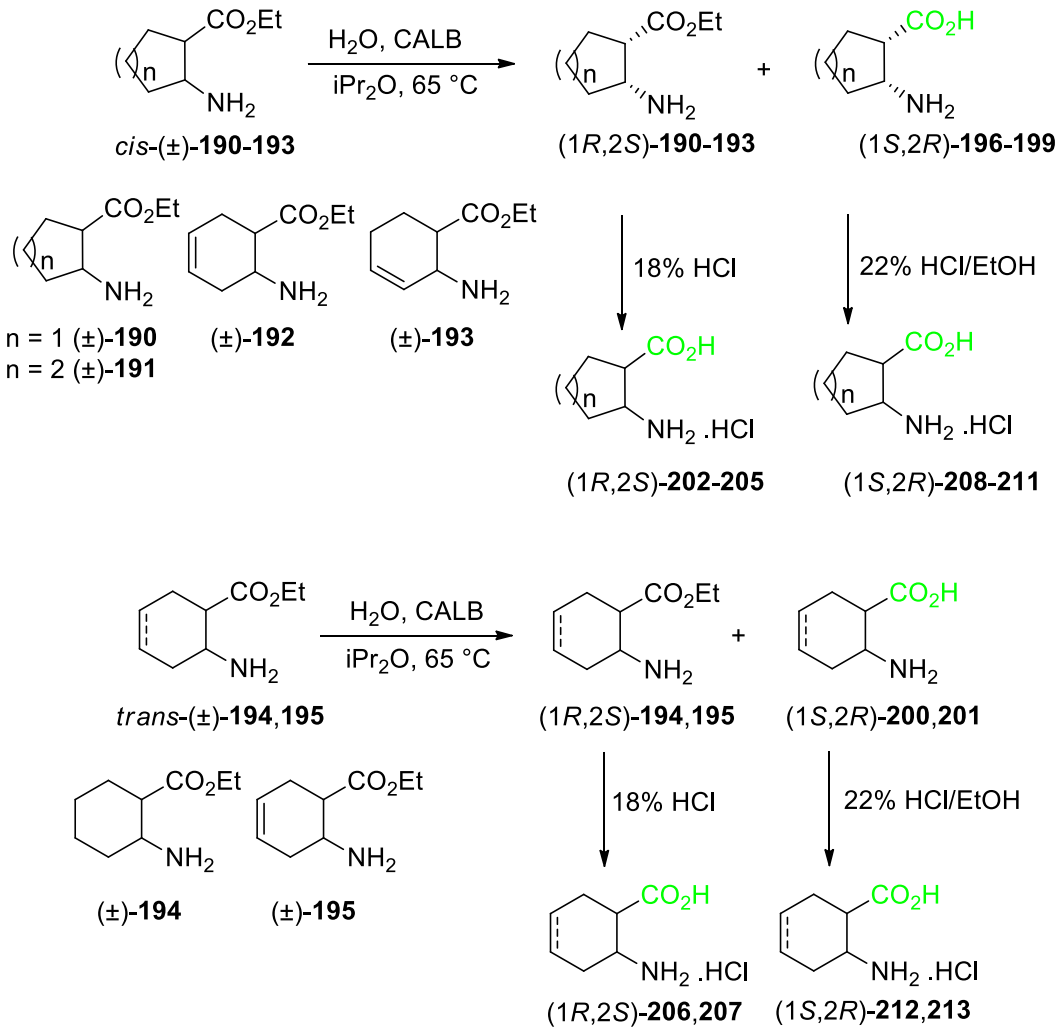
Moxifloxacin, an antibacterial agent against gram-negetive microorganisms [110] has been prepared with various synthetic pathways. For the hydrolysis of diester (±)-**187**, an efficient soluble CALB (Addyzme)-catalyzed method in sodium phosphate buffer pH 7.5 (0.01 M) at 45 °C was described [111] (Scheme 30). The reaction proceeded in a fully stereo- and regioselective manner. The unreacted diester (2*S*,3*R*)-**187** was isolated with (*ee* > 99%) and in a yield of 46%.



Scheme 30.

2.1.1.3. Lipase-catalyzed hydrolysis of carbocyclic β-amino carboxylic esters

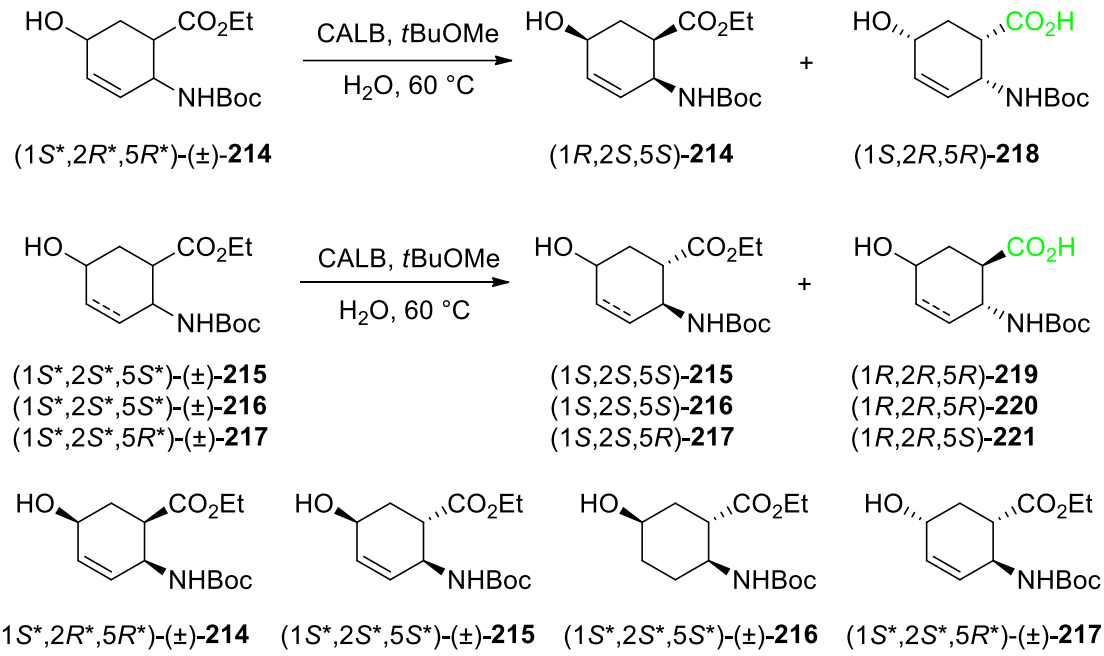
The first direct CALB-catalyzed enantioselective hydrolysis of carbocyclic β-amino esters *cis*-(±)-**190–193** and *trans*-(±)-**194**, **195** in iPr_2O was developed by Forro *et al.* for the synthesis of *cis* and *trans* β-amino acid enantiomers, when H_2O (0.5 equiv.) was used at 65 °C [57] (Scheme 31). Besides the easy separation of products, high enantioselectivities (*E* usually > 100) were observed. The β-amino acid enantiomers *cis*-(1*S*,2*R*)-**196–199** and *trans*-(I*S*,2*R*)-**200**,**201** were isolated with (ee_p 96–99%) and in good yields ≥42%.



Scheme 31.

Direct CALB-catalyzed enantioselective hydrolysis of hydroxy-substituted β-amino esters (±)-**214–217** with added H_2O (0.5 equiv.) in *t*BuOMe at 60 °C was reported by Forro *et al.* [112]

(Scheme 32). They performed enzyme screening experiments relating to the hydrolysis of (±)-**217** in iPr_2O with H_2O (0.5 equiv.). While there was no selectivity with lipase AK (*Pseudomonas flurescens*), all other lipases including CALA (*Candida antarctica* lipase A), CALB (*Candida antarctica* lipase B), lipase PS (*Burkholderia cepacia*), AY (*Candida rugosa*), and PPL (*Porcine pancreactic* lipase) showed catalytic activity, but moderate enantioselectivity (ee_s = 48% after 48 h) was observed using CALB at 60 °C. Several other solvents were also tested. The results revealed much slower and less enantioselective reactions in *n*-hexane and 1,4-dioxane. Better enantioselectivity in toluene and considerably higher *E* and reaction rate in *t*BuOMe were experienced. It was noteworthy to summerize that in preparative-scale resolution, racemates of β-amino acid ethyl esters underwent polymerization.

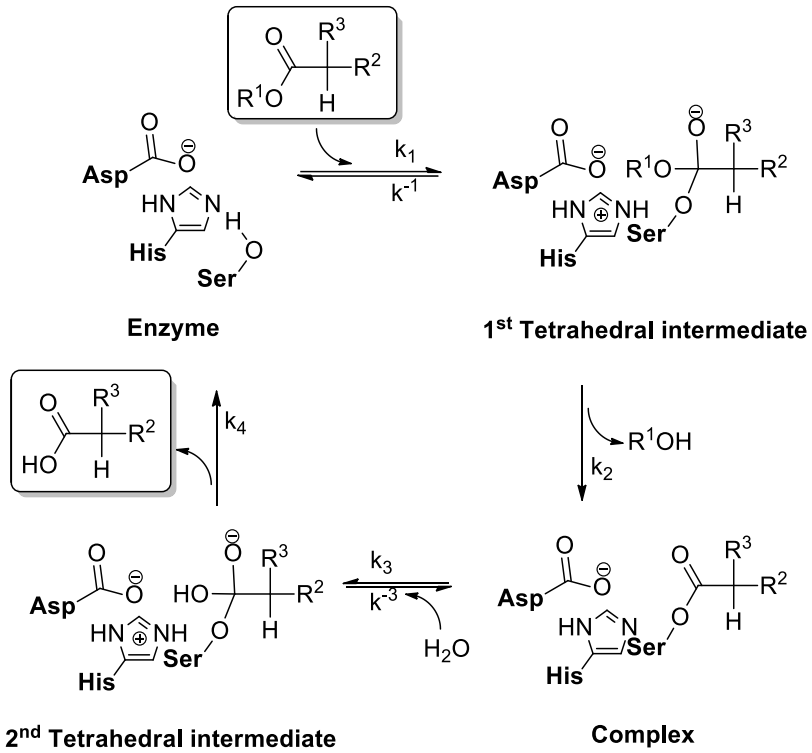


Scheme 32.

2.1.2. Mechanism of lipase-catalyzed ester bond hydrolysis

Lipases perform the catalytic activity with acylation–deacylation displacement mechanism. The hydrolytic mechanism starts with an acylation step by abstracting a proton of serine by the imidazole moiety of histidine, which results in the nucleophilic attack on the carbonyl group of the substrate amino carboxylic ester [113] (Scheme 33). The resultant first tetrahedral intermediate looses alcohol R^1OH to form acyl–enzyme complex. In a deacylation step by the nucleophilic

attack of H_2O, the second tetrahedral intermediate forms. In the end, elimination of the product amino carboxylic acid regenerates the enzyme.

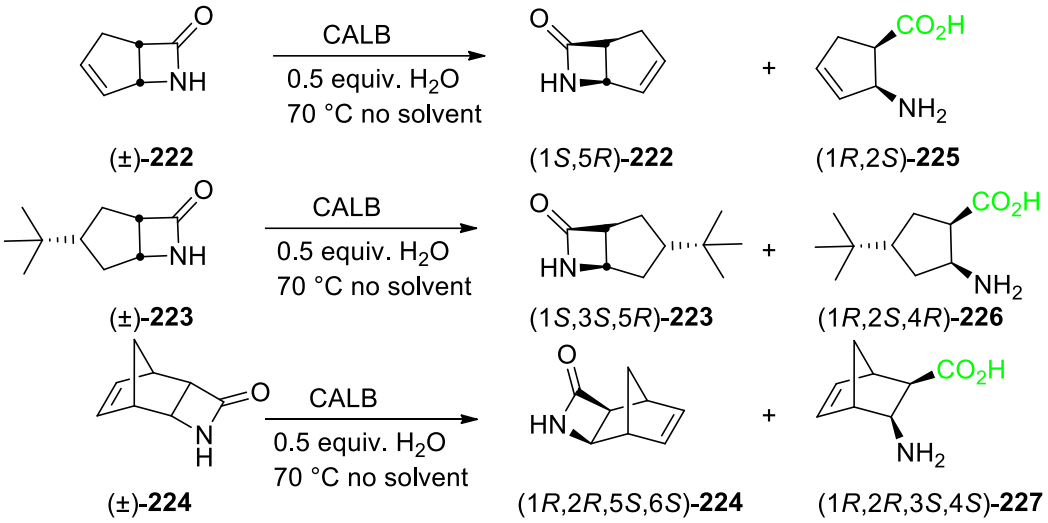


Scheme 33.

2.2. Green strategies for amino ester hydrolysis

2.2.1. Solvent-free system

A solvent-free system, which is a simple mixture of reactants, has the advantages of the absence of solvents, higher substrate concentration, notable cost reduction, and being environmentally benign [61]. In accordance with efforts to generate green non-conventional media, Forro *et al.* described, that since H_2O is present at the surface of the enzyme, it is sufficient to cleave the β-lactam ring, performed under solvent-free conditions [62]. Based on preliminary results, the preparative-scale resolutions for the enantioselective CALB-catalyzed ring cleavage of (±)-**222–224** were carried out with H_2O (0.5 equiv.) at 70 °C (Scheme 34). β-Amino acid enantiomers (1*R*,2*S*)-**225**, (1*R*,2*S*,4*R*)-**226**, and (1*R*,2*R*,3*S*,4*S*)-**227** were isolated with excellent enantiomeric excess values (>99%) and in good yields (≥41%).

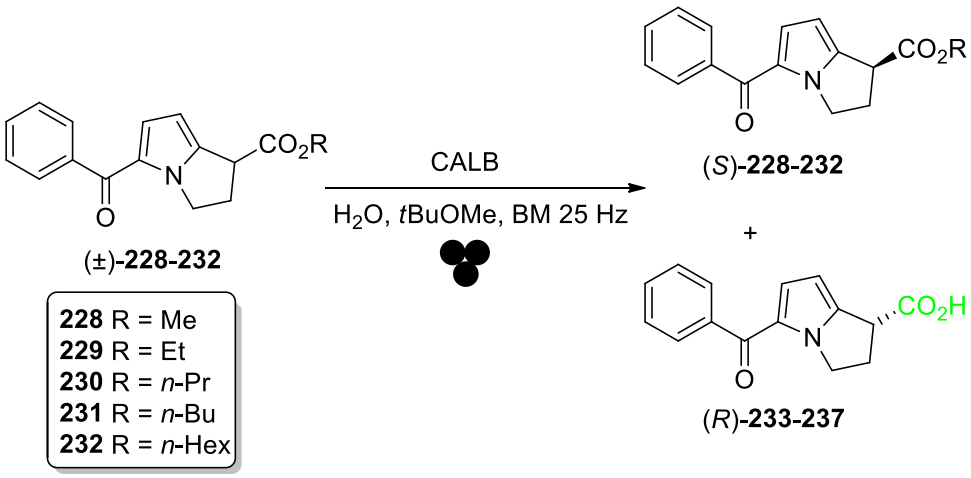


Scheme 34.

2.2.2. Ball milling

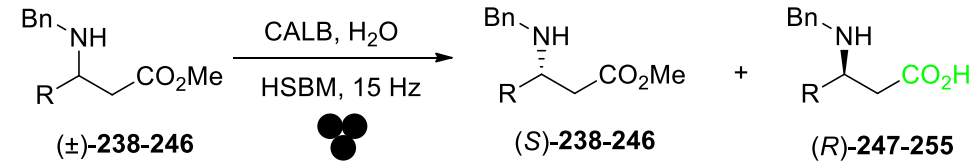
On the basis of sustainable biocatalysis combined with mechanochemical forces, enantioselective synthesis of biologically active molecules through mechanoenzymatic kinetic resolution of racemic compounds has been developed [114–118].

In this regard, a mixture of racemic Ketorolac esters (±)-**228–232**, CALB, H_2O (1 equiv.), and *t*BuOMe was placed in an agate milling jar (12 mm of diameter, 4.6 mL capacity) with an agate ball (6 mm of diameter, 480 mg of weight) [119] (Scheme 35). The resulting mixtures were milled at 25 Hz frequency and the reactions proceeded with exceptional enantioselectivity ($E \geq 500$). The (*S*) ester and (*R*) acid enantiomers were obtained with high *ee* at a conversion of 46%.



Scheme 35.

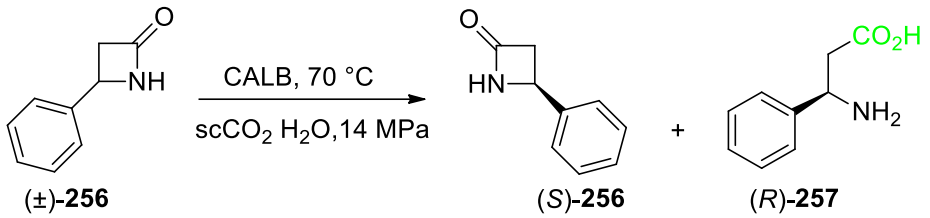
In a noteworthy study, Perez-Venegas *et al.* demonstrated the employment of ball milling for liquid-assisted grinding (LAG) enzymatic resolution of *N*-benzylated-β^3-amino esters (±)-**238–246** yielding enantioenriched *N*-benzylated-β^3-amino acids [120] (Scheme 36). The reaction was performed employing CALB, H_2O (0.5 equiv.), and 2M2B as a LAG at 15 Hz. The hydrolytic reaction of racemates **238–240** proceeded with excellent enantioselectivity (E > 200), while there was a drop in E with other substrates. The *N*-benzylated-β^3-amino acid (*R*)-**247–255** enantiomers were isolated with high *ee* values (ee_p 80–98%) and yields from 4% to 49%.



Scheme 36.

2.2.3. Supercritical fluid

Enzymes, and especially lipases, can be stable in supercritical carbon dioxide ($scCO_2$) [121–124]. Their reaction rates and enantioselectivity can be optimized via solvent pressure and temperature [125–127]. The use of $scCO_2$ has certain advantages over other non-aqueous media, *e.g.*, increased mass transfer and solvent-free products obtained after depressurization. Thus, considering the advantages, Utczás *et al.* investigated CALB-catalyzed kinetic resolution of *rac*-4-phenyl-2-azetidinone **256** in $scCO_2$ with H_2O (0.5 equiv.) at 14 MPa and 70 °C. [128] (Scheme 37). The resulting (*R*)-β-phenylalanine **257** (*ee* ≥ 98%) and (*S*)-4-phenyl-2-azetidinone **256** (*ee* ≥ 99%) could be easily obtained at 50% conversion with high enantioselectivity (E ≥ 100).

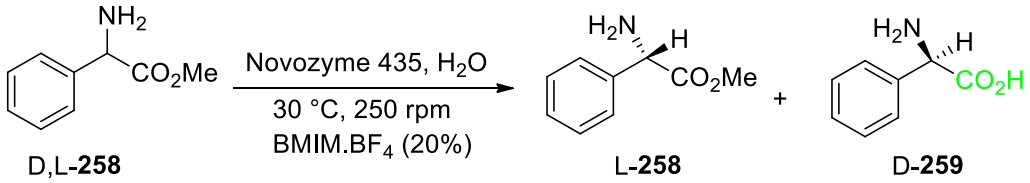


Scheme 37.

2.2.4. Ionic liquid

The feasibility of emplying ionic liquids [129–133] as solvents in enzymatic catalysis [134–142] has been explored. Ionic liquids as environmentally benign alternatives to organic solvents are extensively acknowledged for nonaqueous biocatalytic reactions [143]. The enhancement of enzyme stabilities, selectivities, and activities in transformations in ionic liquids was demonstrated by several groups [144–147].

In the light of these faverouble features, Novozyme 435-catalyzed asymmetric hydrolysis of racemic D,L-phenylglycine methyl ester **258** was carried out using 20% ionic liquid 1-butyl-3-methyl-imidazolium tetrafluoroborate (BMIM.BF_4) at 30 °C and 250 rpm by Liu *et. al*. [148] (Scheme 38). The enantiomeric excess of the residual substrate (L-**258)** of 93% was achieved at a conversion of 53.1% in 30 min. The initial rate of enzyme-mediated hydrolysis was V_0 = 3.07 mM min^{-1} with enantioselectivity of (E = 34).



Scheme 38.

3. Materials and Methods

3.1. Materials and instruments

Lipase PSIM (*Burkholderia cepacia* immobilized on diatomaceous earth, N: 509) and lipase AK (*Pseudomonas flourescens* immobilized on immobead 150, 90678) were from Amano Pharmaceuticals, whereas lipase AY (*Candida rugosa* immobilized in Sol-Gel-AK, 62279) was from Fluka. PPL (*Porcine pancreatic* lipase, L3126) and CALB (lipase B from *Candida antarctica* immobilized on macroporous acrylic resin, Cat. N.: L4777) were purchased from Sigma. Substituted benzaldehydes, cycloalkenes, most of the solvents of the highest analytical grade, and anhydrous sodium sulfate (a.r.) used as drying agent, were from Sigma-Aldrich. Triethylamine was from Merck. 2-Methyl-2-butanol (98%) was from TCI, whereas ethyl acetate, chloroform, and acetone (a.r.) were from Novochem. Diethyl ether (a.r.) was from Molar Chemicals Kft.

The ball-milling apparatus was Retsch 400 (Retsch GmbH, Haar, Germany). Melting points were determined with Kofler and Hinotek X-4 apparatus (Hinotek, Ningbo, China) and they are uncorrected. Optical rotations were measured with a Perkin-Elmer 341 and a Jasco P 2000 polarimeter. ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker Avance (Bruker Biospin, Karlsruhe, Germany) DRX 500, 125, and 471 MHz spectrometer. HRMS flow injection analysis was performed with Thermo Scientific Q Exactive Plus Hybrid Quadrupole-Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer coupled to a Waters Acquity I-Class UPLCTM (Waters Manchester, UK). Batch reactions were performed in New Brunswick Scientific Innova 4000 incubator shaker with shaking speed of 185 rpm. The enantiomeric excess *ee* values for the unreacted β-amino carboxylic ester and the β-amino acid enantiomers produced were determined by GC equipped with a Chirasil-L-Val column.

3.2. Enzymatic experiments

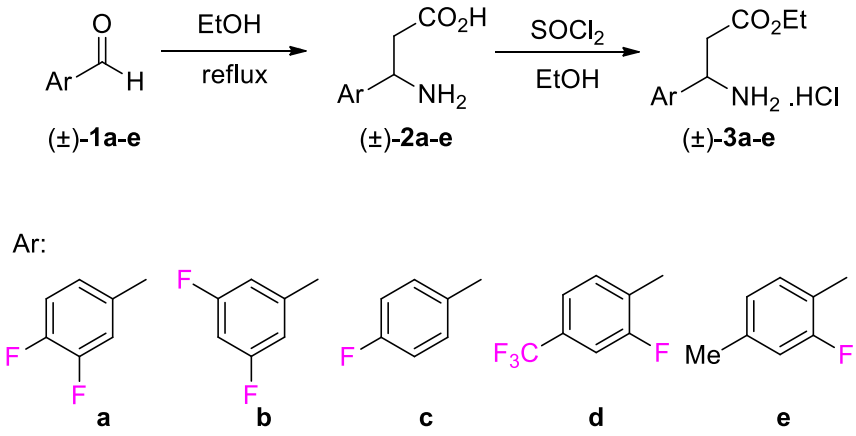
In a typical batch reaction, the enzyme was added to the solution of the racemic compound followed by adding other required additives and the mixture was shaken in the incubator shaker at given temperatures. Preparative-scale resolutions were performed under the optimized reaction conditions. The product enantiomers were characterized with ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy, melting point, MS analysis, and optical rotation.

In ball milling reactions, the substrate was mixed with LAG and H_2O, then the enzyme was added to the mixture. The progress of reactions was followed by collecting samples at a determined frequency. Preparative-scale resolution of ethyl *cis*-2-aminocyclohexanecarboxylate (±)**-7** was carried out under optimized frequency and substrate/enzyme ratio. The product enantiomers were characterized by ^{1}H NMR spectroscopy and determining the melting point.

3.3. Synthesis of starting materials

Compounds **2a–e** were synthesized based on the modified Rodionov synthesis [70,71] through condensation of the corresponding aldehydes **1a–e** (2 g) with malonic acid (1 equiv.) in the presence of NH_4OAc (2 equiv.) in EtOH under reflux conditions for 8 h [90]. The resulting white crystals were filtered off, washed with acetone and then recrystallized from H_2O and acetone (Scheme 4).

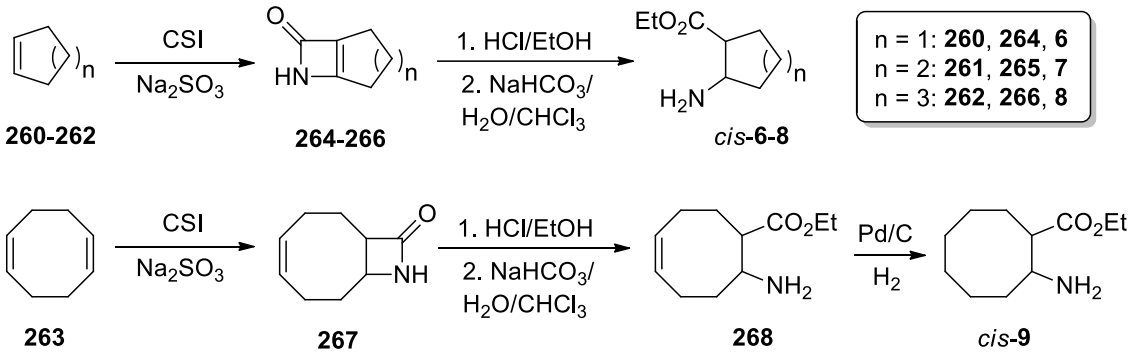
$SOCl_2$ (1.05 equiv.) was added to 30 mL of EtOH kept under –15°C with saline ice. To this solution, **2a–e** (1 g) were added at once. The mixture was stirred at 0 °C for 30 min, then at room temperature for 3 h and, finally, heated under reflux conditions for 1 h. The solvent was evaporated off under reduced pressure and the resulting white **3a–e**.HCl salts were recrystallized from EtOH and Et_2O (Scheme 4).



Scheme 4.

The synthesis of racemic ethyl 2-aminocycloalkanecarboxylates **6–9** was carried out based on literature data, starting from 50 mmol cycloalkene [72–74,149] (Scheme 39). The 1,2-dipolar cycloaddition of chlorosulfonyl isocyanate (CSI) to cycloalkenes took place regioselectively, in accordance with Markovnikov orientation [149], resulting in racemic *cis* β-lactams. The desired

amino esters **6–8** with *cis*-cyclopentane, -cyclohexane, and -cycloheptane skeletons and unsaturated ethyl *cis*-2-aminocyclooct-5-ene carboxylate **267** were obtained by treating *cis*-lactams with 22% ethanolic HCl as a result of ring cleavage. Then compound **268** was reduced catalytically under H_2 [75] to give saturated ethyl *cis*-2-aminocyclooctanecarboxylate **9**. A slightly modified literature procedure was used to synthesize seven-membered β-lactam **266.** The procedure started by dropwise addition of CSI (4.42 g, 31 mmol, 1.0 equiv.) to neat cycloheptene (3.0 g, 31 mmol, 1.06 equiv.) at 78 °C over 60 min (keeping the reaction temperature as close to 78 °C as possible). Afterwards, the mixture was cooled to room temperature over a period of 60 min and then kept stirring at that temperature for 18 h. Next, the reaction mixture was added dropwise to a stirred suspension of ice water (170 mL), Na_2SO_3 (17 g), and $NaHCO_3$ (51 g) over a period of 20 min. It was followed by warming up the mixture to 23 °C, stirring at this temperature for 20 min, and then adding CH_2Cl_2 (50 mL) and stirring for an additional 5 min. The solids collected by vacuum filtration were rinsed sequentially with water (2 × 10 mL) and CH_2Cl_2 (2 × 100 mL) and then discarded. The organic layer was separated from the filtrate and the aqueous layer was extracted with CH_2Cl_2 (3 × 25 mL). The combined organic phase was dried over Na_2SO_4, filtered, and the solvent was evaporated off under vaccum to afford **266** as a pale solid.



Scheme 39.

3.4. Analytical method

Based on GC chromatograms, enantiomeric excess, enantioselectivity, and conversion values were calculated by using equations 1–4 where A_1, A_2, A_3, A_4 are peak area of the enantiomers and $A_2 > A_1$, $A_3 > A_4$:

1. $ee_s = (A_2 - A_1) / (A_1 + A_2)$

2. $ee_p = (A_3 - A_4) / (A_3 + A_4)$

3. **Conv. = $ee_s/(ee_s + ee_p)$** [150]

4. **$E = \{ln[(1 - c) \times (1 + ee_p)]/ln[(1 - c) \times (1 - ee_p)]\}$** [151]

The *ee* values for the unreacted β-amino carboxylic esters and the β-amino acid enantiomers produced were determined by GC equipped with a Chirasil-L-Val column after double derivatization [152,153], with (*i*) diazomethane [Caution! the derivatization with diazomethane should be performed under a well-ventilated hood] and (*ii*) acetic anhydride in the presence of 4-dimethylaminopyridine and pyridine.

Conditions of the measurements in detail, such as the rate of temperature rise and retention times, can be found in the original papers. [I,II]

4. Results and Discussion

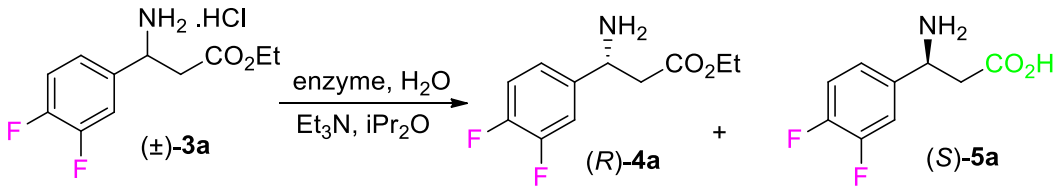
4.1. Lipase-catalyzed hydrolysis of flourinated β-amino carboxylic ethyl ester hydrochloride salts [I]

4.1.1. Preliminary experiments

A wide variety of (*S*)-β-amino carboxylic acid enantiomers was prepared earlier through hydrolysis of the corresponding racemic β-amino carboxylic esters. For instance, lipase PS-catalyzed (*S*)-selective hydrolysis of (±)-**26–30** with H_2O (0.5 equiv.) in iPr_2O at 45 °C afforded (*S*)-**31–35** (*ee* ≥ 97%) [90], lipase PS-catalyzed *S*-selective hydrolysis of (±)-**46–52** with H_2O (0.5 equiv.) in iPr_2O at 25 °C furnished the desired pharmacologically important β-heteroaryl-substituted β-amino acids (*S*)-**53–59** (*ee* ≥ 97%) [91], and lipase PCL-mediated (*S*)-selective hydrolysis of (±)-**74–84** with H_2O (0.5 equiv.) in 1,4-dioxane at 45 °C yielded β-amino acids (*S*)-**85–95** (*ee* ≥ 97%) with excellent enantioselectivity [92].

4.1.1.1. Enzyme screening

Encouraged by these results achieved in enzyme-mediated enantioselective hydrolysis, the hydrolysis of model compound (±)-**3a** (Scheme 5) was conducted with Et_3N (5 equiv.) and H_2O (0.5 equiv.) in the presence of 30 mg mL^{-1} PSIM in iPr_2O at 45 °C (Table 1, entry 1). The lipase PSIM enzyme provided an *E* value of 108 (48% conv. in 10 min). In the frame of enzyme screening, lipase AY (*Candida rugosa*), lipase AK (*Pseudomonas flourescens*), PPL (*Porcine pancreatic*) lipase, and CALB (Table 1, entries 2–5) were tested and all showed hydrolytic activity. However, with the exception of lipase AK affording an ee_p value of 75% and a moderate *E* (8) (19% conv. in 10 min, entry 3), low reactivities and low enantioselectivites were achieved (entries 2, 4, and 5). Based on GC chromatograms, it is worth mentioning that PPL catalyzed the reaction with opposite enantioselectivity. Consequently, lipase PSIM proved to be the best in catalyzing of (±)-**3a**.



Scheme 5.

Table 1. Enzyme screening in the hydrolysis of (±)-**3a**[a].

Entry	Enzyme (mg mL^{-1})	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	Lipase PSIM	88	95	48	108
2	Lipase AY	2	9	18	1
3	Lipase AK	18	75	19	8
4	PPL	2	29	5	2
5	CALB	2	5	30	1

[a]0.025 M substrate, 30 mg mL^{-1} lipase, 1 mL iPr_2O, 5 equiv. Et_3N, 0.5 equiv. H_2O, at 45 °C after 10 min. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.1.1.2. Solvent screening

Since enzymatic activities strongly depend on the type of solvent in the reaction media, it is a necessity to examine the effect of solvent on enantioselectivity and reaction rate. For this reason, we selected a range of green solvents. Very different *E* and reaction rate values were observed in the tested solvents (Table 2). The hydrolytic reactions of **3a** in *t*BuOMe, 2-Me-THF and in propylene carbonate were rapid (conv. > 51% after 10 min, *E* = 59, 113, and 27, entries 1, 2, and 4), while in EtOAc the reaction proceeded relatively slowly with low enantioselectivity (conv. 11% after 10 min, *E* = 3, entry 3). Even though the highest *E* (113) was obtained with 2-Me-THF the most expensive solvent (entry 2), we concluded that iPr_2O was the most suitable and cost-effective solvent (Table 1, entry 1).

In an attempt to perform greener chemistry based on earlier results [62], the reaction was also carried out under solvent-free conditions. A reasonable enantioselectivity (74) was observed in addition to a rapid transformation (conv. 49% after 10 min, entry 5).

Table 2. Solvent screening in the hydrolysis of (±)-**3a**[a].

Entry	Solvent (1 mL)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	*t*BuOMe	95	88	52	59
2	2-Me-THF	97	93	51	113
3	EtOAc	6	52	11	3
4	Propylene carbonate	92	79	54	27
5	no solvent	90	92	49	74

[a]0.025 M substrate, 30 mg mL^{-1} lipase PSIM, 5 equiv. Et_3N, 0.5 equiv. H_2O, at 45 °C after 10 min. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.1.1.3. Temperature screening

In order to follow the progress of the reaction while maintaining high enantioselectivity, it was wise to slow down the reaction. When the reaction temperature

was decreased from 45 °C to 25 °C, both the reaction rate and the enantioselectivity for the hydrolysis of **3a** clearly decreased (30% conv. in 10 min, *E* = 48, Table 3, entry 1 *vs* 48% conv. in 10 min; *E* = 108, Table 1, entry 1). Lowering the temperature to 3 °C provided the highest degree of conversion (50% in 10 min, Table 3, entry 2) and enantioselectivity (*E* = 134).

Table 3. Effect of temperature in the hydrolysis of (±)-**3a**[a].

Entry	Temp (°C)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	25	40	94	30	48
2	3	94	95	50	134

[a]0.025 M substrate, 30 mg mL^{-1} lipase PSIM, 1 mL iPr_2O, 5 equiv. Et_3N, 0.5 equiv. H_2O, at 3 °C after 10 min. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.1.1.4. PSIM concentration screening at 3 °C

In order to explore this unexpected outcome further, we carried out the reaction with different enzyme concentrations at 3 °C. No significant difference was observed in the reaction rates (Table 4), when the enzyme concentration decreased from 10 to 5 and then to 2 mg mL^{-1} (~50% conv. in 10 min, entries 1–3). However, the enantioselectivity dropped significantly (*E* = 63), when the reaction was performed with 2 mg mL^{-1} of enzyme (entry 3). Taking into account both high enantioselectivity and reaction rate, 45 °C was determined as an optimal temperature.

Table 4. Effect of enzyme concentration in the hydrolysis of (±)-**3a**[a].

Entry	PSIM (mg mL^{-1})	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	10	97	97	50	>200
2	5	95	98	49	>200
3	2	85	92	48	63

[a]0.025 M substrate, 1 mL iPr_2O, 5 equiv. Et_3N, 0.5 equiv. H_2O, at 3 °C after 10 min. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.1.1.5. Enzyme concentration screening at 45 °C

As a metter of fact, reaction rates are affected by the enzyme concentration. The more enzyme added to the reaction medium, the higher reaction rate would be observed [92]. To ascertain the fact, a set of preliminary experiments was performed to determine the optimal enzyme concentration (Table 5). The reaction rate for the hydrolysis of (±)-**3a** clearly increased as the concentration of enzyme was increased. Considering *E*, reaction times (the

time needed to reach 50% conversion) (Table 1, entry 1), the use of 30 mg mL^{-1} enzyme was selected for preparative-scale resolutions.

Table 5. Effect of enzyme concentration at 45 °C in the hydrolysis of (±)-**3a**[a].

Entry	PSIM (mg mL^{-1})	ee_s (%) [b]	ee_p (%) [c]	Conv. (%)	*E*
1	2	4	81	5	10
2	5	15	85	15	14
3	10	21	86	20	17
4	20	46	92	33	38
5	40	97	89	52	74

[a]0.025 M substrate, 1 mL iPr_2O, 5 equiv. Et_3N, 0.5 equiv. H_2O, after 10 min. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.1.2. Preparative-scale resolutions [I, III]

Preparative-scale resolution of (±)-**3a–e** were performed under the optimized conditions [30 mg mL^{-1} lipase PSIM, Et_3N (5 equiv.), H_2O (0.5 equiv.) in 10 mL of iPr_2O at 45 °C] (Table 6). Reactions were stopped by filtering off the enzyme at conversions close to 50% after reaction times of 8 h **3a**, 72 h **3b**, 18 h **3c**, 26 h **3d**, and 23 h **3e** with excellent enantioselectivity (>200). The unreacted β-amino carboxylic ester enantiomers (*R*)-**4a–e** with high *ee* (≥94%) and good yields (≥38%) and product β-amino acids (*S*)-**5a–e** with excellent *ee* (>99%) and yields of ≥48% were isolated.

Table 6. Lipase PSIM-catalyzed hydrolysis of (±)-**3a–e**[a].

(±)	Rt (h)	Conv. (%)	*E*	β-Amino acid (5a–e)				β-Amino ester (4a–e)			
				Yield (%)	Isomer	*ee*[b] (%)	$[\alpha]_D^{25}$ (H_2O)	Yield (%)	Isomer	*ee*[c] (%)	$[\alpha]_D^{25}$ ($CHCl_3$)
3a	8	50	>200	48	(*S*)	>99	–3.1[d]	49	(*R*)	97	+17.9[e]
3b	72	49	>200	48	(*S*)	>99	–5[f]	38	(*R*)	94	+9[g]
3c	18	50	>200	49	(*S*)	>99	–3[h]	49	(*R*)	>99	+18.9[i]
3d	26	49	>200	49	(*S*)	>99	–11[j]	48	(*R*)	>99	+20.3[k]
3e	23	50	>200	48	(*S*)	>99	–13[l]	47	(*R*)	>99	+16[m]

[a]30 mg mL^{-1} PSIM in iPr_2O, 5 equiv. Et_3N, 0.5 equiv. H_2O, at 45 °C. [b]According to GC after derivatization. [c]According to GC after double derivatization. [d]*c* = 0.28. [e]*c* = 0.44. [f]*c* = 0.26. [g]*c* = 0.29. [h]*c* = 0.28. [i]*c* = 0.41. [j]*c* = 0.19 (MeOH). [k]*c* = 0.53. [l]*c* = 0.21 (MeOH). [m]*c* = 0.13.

4.1.3. Determination of absolute configurations

The absolute configurations were assigned by comparing the [α] values with literature data [58,92,154] as well as the same enantiopreference for the (*S*)-selective hydrolysis by lipase PSIM.

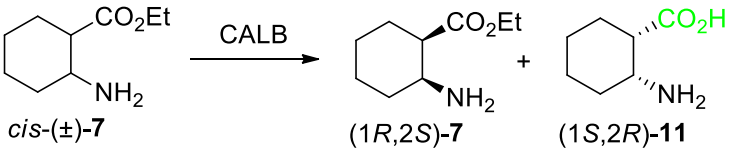
4.2. CALB-catalyzed hydrolysis of carbocyclic 5–8-membered *cis* β-amino ethyl esters [II]

4.2.1. Preliminary experiments

The first direct enantioselective hydrolysis of carbocyclic *cis* and *trans* β-amino esters (±)-**190**–**193** and (±)-**194**, **195** using CALB in iPr_2O, H_2O (0.5 equiv.) at 65 °C [57] afforded β-amino acid enantiomers *cis*-(1*S*,2*R*)-**196–199** and *trans*-(1*S*,2*R*)-**200**,**201** with (ee_p 96–99%). The method was utilized as an efficient route to synthesize the pharmacologically valuable cispentacin (1*S*,2*R*)-**196**.

4.2.1.1. Solvent screening

In view of these results, a set of preliminary experiments was planned to find a sustainable method to preparae carbocyclic β-amino acid enantiomers. The hydrolysis of model compound ethyl *cis*-2-aminocyclohexanecarboxylate **7** (Scheme 6) was performed in iPr_2O without added H_2O. The reaction was completed, since H_2O present in the reaction medium (<0.1%) or in the enzyme preparation (<5%) was sufficient for the hydrolysis at 65 °C (Table 7, entry 1). In order to enable green chemistry, various green solvents were tested (entries 2–6). The reaction in *t*BuOMe gave better enantioselectivity than that found in iPr_2O (conv. 39% in both cases, E = 66 and 133 after 8 h, entries 1, 2). Solvents propylene carbonate, 2-Me-THF, and 2-methyl-2-butanol (2M2B) provided more modest results in terms of conversion and enantioselectivity (conv. 23%, 12%, 6%, and E = 73, 74, 65 respectively, after 8 h, entries 4–6), while no reaction took place in EtOAc (entry 3). In the end, *t*BuOMe was confirmed to be the best environmentally benign solvent.



Scheme 6.

Table 7. Green solvent screening in the hydrolysis of ethyl *cis* **7**[a]

Entry	Solvent (1 mL)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	iPr_2O	60	95	39	66
2	*t*BuOMe	63	>99	39	133
3	EtOAc	-	-	-	-
4	Propylene carbonate	30	>99	23	73
5	2-Me-THF	14	>99	12	74
6	2M2B	6	>99	6	65

[a]0.025 M substrate, 30 mg mL^{-1} CALB, (S/E, 1:7), at 65 °C after 8 h. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.2.1.2. Enzyme reusability in tBuOMe

Definitely, recyclability and recovery of the enzyme as a catalyst are two key criteria in green concept. Therefore, in order to determine the catalytic activity of reused enzyme and find the impact on progress of a reaction, the hydrolysis of ethyl *cis*-2-aminocyclohexanecarboxylate **7** was carried out with CALB in three cycles (Table 8). Whereas the reaction rate decreased gradually, the enantiomeric excess of the product appeared not to be affected ($ee_p \geq 96\%$), which was in harmony with earlier results described in literature [62]. In contrast, enantioselectivity dropped upon reusing the enzyme.

Table 8. Catalytic activity of recycled enzyme in the hydrolysis of ethyl *cis* **7**[a]

CALB (mg mL^{-1})	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
First use	78	97	45	180
Second use	58	96	37	82
Third use	43	96	31	71

[a]0.025 M substrate, 30 mg mL^{-1} CALB, (S/E, 1:7), 1 mL of *t*BuOMe, at 65 °C after 12 h. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.2.1.3. Temperature screening in solvent-free reactions

Performing the reactions under solvent-free conditions is one of the best choices, when considering eco-friendly techniques. In this respect, β-lactam ring opening of (±)-**222–224** was performed with CALB, H_2O (0.5 equiv.) at 70 °C [62] with remarkable

results achieved in terms of enantioselectivity (>200) and *ee* values (>99%) of the isolated β-amino acid enantiomers. In the light of these results, we performed the hydrolysis of 6-membered *cis* amino ester **7** in the presence of 30 mg CALB without added H_2O, considering that the H_2O present in the enzyme preparation (<5%) was sufficient for the hydrolysis at 65 °C (Table 9, entry 2). Since temperature is a key factor and it certainly affects both *E* and the rate of enzymatic reactions, results found at different temperatures were analyzed. When the reaction was carried out at room temperature (23 °C) (*E* = 45, entry 1) or at higher temperatures of 70 °C and 80 °C (*E* = 13, 11, entries 3, 4), a significant decrease in *E* was observed. The enantiomeric excess values of the product remained high (≥94%) at 23 and 65 °C, with a moderate drop at 70 °C and with a notable decline at 80 °C apparently due to overrun conditions (conv. > 50%). On the basis of these observations, 65 °C was selected as the optimum temperature.

Table 9. Temperature screening in the hydrolysis of ethyl *cis* **7**[a] under solvent-free conditions

Entry	Temp (°C)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	23	11	95	11	45
2	65	66	94	41	70
3	70	72	72	50	13
4	80	91	57	62	11

[a]5 mg substrate, 30 mg CALB, (S/E, 1:6) after 8 h. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.2.1.4. CALB quantity screening in solvent-free system

In order to improve the enantioselectivity, the reaction was performed utilizing various quantities of CALB. When the enzyme quantity was increased from 30 mg (conv. 41% after 8 h, *E* = 70, Table 9, entry 2) to 50 mg (conv. 48% after 8 h, *E* = 177, Table 10, entry 1) and 70 mg (conv. 50% after 8 h, *E* = 73, Table 10, entry 2) in small-scale reactions, a positive response in *E* especially with 50 mg enzyme (substrate/enzyme ratio 1:10) was observed. It should be noted that even the substrate/enzyme ratio of 1:10 could be considered high but due to better result, it was determined to be the best ratio in the solvent-free system.

Table 10. Enzyme quantity screening in the hydrolysis of ethyl *cis* **7**[a] in solvent-free system

Entry	CALB (mg)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	*E*
1	50	88	97	48	177
2	70	91	92	50	73

[a]5 mg substrate, 8 h under solvent-free conditions. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.2.1.5. Frequency screening using ball milling

Conducting reactions based on mechanochemical approaches is another possible strategy to practice green chemistry. In this context, employment of ball mills has left its mark on the road to sustainable enzymatic resolution. As mentioned earlier, enzymatic hydrolysis of *N*-benzylated-β^3-amino esters (±)-**238–246** were performed with H_2O (0.5 equiv.), 2M2B as a LAG at 15 Hz using ball milling [120]. The hydrolytic reaction gave *N*-benzylated-β^3-amino acid enantiomers **247–255** with high and excellent *ee* values (83–98%). Inspired by this result, we performed a hydrolytic reaction of ethyl *cis*-2-aminocyclohexanecarboxylate **7** by using an agate jar (10 mL volume) with three agate balls (5 mm of diameter), 0.5 equiv. of added H_2O, *t*BuOMe as a LAG (η = V (liquid; μL)/m (reagents; mg) [155] η = 2.4) at 25 Hz (Table 11, entry 1), since it was found that an enantiomeric excess of 80% at 55% conversion was achievable for (*R*)-*N*-benzylated-β^3-amino acid **247** (Scheme 36) with operating frequency of 25 Hz. Unfortunately, very low conversion and enantioselectivity values were observed (conv. 3% after 6 h, *E* = 6), which was possibly resulting from enzyme destruction. Therefore, the operating frequency was analyzed and found that enantioselectivities started to increase by lowering the frequency (conv. 3%, 5%, 5%, 14% and *E* = 19, 16, 21, 147, respectively after 6 h, entries 2–5). Finally, the enzyme could remain intact and the best combination of conversion and *E* was observed at 3 Hz, which was considered to be optimum frequency. The catalytic acticty of the enzyme was not affected (*E* = 89, entry 6), when the reaction was performed with *t*BuOMe-assisted grinding without H_2O at the optimized frequency. This could be explained by the H_2O content of solvent and H_2O present in enzyme preparation. This quantity was sufficient to carry out the reaction.

Table 11. Frequency screening in the hydrolysis of ethyl *cis* **7**[a] throughout milling

Entry	Frequency (Hz)	ee_s (%)[c]	ee_p (%)[d]	Conv. (%)	*E*
1	25	2	69	3	6
2	15	3	90	3	19
3	10	5	87	5	16
4	8	5	91	5	21
5	3	15	98	14	147
6[b]	3	16	97	14	89

[a]10 mg substrate, 20 mg CALB, (S/E, 1:2), 0.5 equiv H_2O, 24 μL of LAG, after 6 h using ball mills. [b]Without added H_2O, [c]According to GC after derivatization. [d]According to GC after double derivatization.

4.2.1.6. CALB quantity screening using ball milling

In another attempt to improve the enantioselectivity and reaction rate, the hydrolysis of **7** was screened with different enzyme quantities. When the amount of enzyme was increaded from 20 to 30 mg, both the conversion and the enantioselectivity increased considerably (conv. 20%, E > 200, Table 12, entry 1), while reducing to 10 mg was accompanied with a drop in both conversion and E (conv. 14%, E = 11, Table 12, entry 2). As a result, the substrate/enzyme ratio of 1:3 is the most appropriate ratio.

Table 12. Enzyme quantity screening in the hydrolysis of ethyl *cis* **7**[a] throughout milling

Entry	CALB (mg)	ee_s (%)[b]	ee_p (%)[c]	Conv. (%)	E
1	30	24	>99	20	>200
2	10	13	81	14	11

[a]10 mg substrate, 0.5 equiv H_2O, 24 μL of LAG, at 3 Hz after 6 h using ball mills. [b]According to GC after derivatization. [c]According to GC after double derivatization.

4.2.2. Preparative-scale resolutions

The preparative-scale resolution of compound ethyl *cis* **7** under the optimized conditions of the investigated strategies was performed (Table 13). The resolution in *t*BuOMe was carried out in a single step. When attempting this approach at a larger scale, for the reason of economy, a low substrate/enzyme ratio of 1:4.5 was employed, which maintained excellent enantioselectivity achieved in reasonable reaction time. The reaction was stopped at 50% conversion by filtering off the enzyme (after 23 hours, entry 1). The unreacted β-amino ester (1*R*,2*S*)-**7** (ee_s = 96%) was isolated and next, the product β-amino acid (1*S*,2*R*)-**11** (ee_p > 99%) was obtained. Then, resolutions under solvent-free conditions (entry 2) and using ball milling (entry 3) were performed in two steps. Reactions were stopped at conv. < 50% (underrun step) (conv. 27%, ee_p = 96%, entry 2; conv. 14%, ee_p > 99%, entry 3) by adding *t*BuOMe to the reaction mixtures and filtering off the enzyme, to yield the crystalline product β-amino acid (1*S*,2*R*)-**11**. The repeated enzymatic reactions were stopped at conv. > 50% **(**overrun step) (data in parenthesis: conv. 59%, ee_s > 99%, entry 2; conv. 67%, ee_s = 98%, entry 3), yielding the unreacted β-amino ester (1*R*,2*S*)-**7**.

Table 13. Preparative-scale resolution of ethyl *cis* **7** [a]in *t*BuOMe, [b]under solvent-free conditions and [c]throughout milling

Entry	Rt (h)	ee_s (%)[d]	ee_p (%)[e]	Conv. (%)	*E*
1[a]	23	96	>99	50	>200
2[b]	2(22)	35(>99)	96(69)	27(59)	58(27)
3[c]	8(67)	20(98)	>99(48)	14(67)	163(11)

[a]100 mg substrate, 30 mg mL^{-1} CALB, (S/E, 1:4.5), 15 mL *t*BuOMe, at 65 °C, in organic media (one-step resolution). [b]100 mg substrate, 1000 mg CALB, (S/E, 1:10), at 65 °C, under solvent-free conditions (two steps resolution). [c]100 mg substrate, 300 mg CALB, (S/E, 1:3), 0.5 equiv H_2O, 244 µL of *t*BuOMe, at 3 Hz, throughout milling (two-step resolution). [d]According to GC after derivatization. [e]According to GC after double derivatization.

The best combination of conversion and enantioselectivity was observed in the reaction carried out in *t*BuOMe (conv. 50%, *E* > 200, after 23 h, entry 1). Therefore, preparative-scale hydrolysis of ethyl *cis*-2-aminocyclopentaneecarboxylate **6**, ethyl *cis*-2-aminocycloheptanecarboxylate **8**, and ethyl *cis*-2-aminocyclooctanecarboxylate **9** was performed in *t*BuOMe in the presence of CALB at 65 °C (Table 14). It is noteworthy that the same substrate/enzyme ratio (1:4.5) was applicable in the large-scale hydrolysis of **6**. However, due to the slow reaction observed in small-scale reactions, resolution of substrates with larger cycles **8** and **9** necessitated a higher substrate/enzyme ratio (1:7.5). As the reactions progressed, the ee_p values of product amino acid enantiomers **10, 12,** and **13** started to decrease, while the ee_s values of unreacted esters **6, 8,** and **9** increased. In order to obtain enantiopure amino acid products, the hydrolysis was performed in two steps; namely, once underrun (conv. < 50%) then overrun (conv. > 50%) conditions.

Reactions were stopped after 4 hours at conv. 36% (ee_p = 96%, substrate **6**), after 23 hours at conv. 20% (ee_p = 98%, substrate **8**), and after 23 hours at conv. 20% (ee_p > 99%, substrate **9**) by adding *t*BuOMe to the reaction mixtures and filtering off the enzyme, resulting in the crystalline product β-amino acids (1*S*,2*R*)-**10, 12,** and **13** with of yields ≥25%. The repeated enzymatic reactions were stopped after 24 hours at conv.75% (ee_s = 98%, substrate **6**), 3 days at conv. 69% (ee_s = 91%, substrate **8**), and 20 days at conv. 62% (ee_s = 62%, substrate **9**), yieldeding the unreacted β-amino esters (1*R*,2*S*)-**6, 8,** and **9** with yields of ≥27%.

Table 14. CALB-catalyzed preparative-scale hydrolysis of carbocyclic *cis* β-amino esters **6–9**

(±)	Rt (h)	Conv. (%)	β-Amino acid (10–13)				β-Amino ester (6–9)			
			Yield (%)	Isomer	*ee*[b] (%)	$[\alpha]_D^{25}$ (H_2O)	Yield (%)	Isomer	*ee*[c] (%)	$[\alpha]_D^{25}$ (EtOH)
6	4(24)	36(75)	25	(1*S*,2*R*)	96	+9.41[g]	31	(1*R*,2*S*)	98	–6.94[g]
7	23	50	33	(1*S*,2*R*)	>99	+19.84[h]	27	(1*R*,2*S*)	96	–11.13[g]
8	23(3d)	20(69)	32	(1*S*,2*R*)	98	+6.54[h]	30	(1*R*,2*S*)	91	–4.09[i]
9	23(20d)	20(62)	28	(1*S*,2*R*)	>99	–19.15[k]	27	(1*R*,2*S*)	62	+20.92[j]

[a]100 mg substrate, 30 mg mL^{-1} CALB, (S/E, 1:4.5), in 15 mL *t*BuOMe, at 65 °C. [b]100 mg substrate, 30 mg mL^{-1} CALB, (S/E, 1:4.5), in 15 mL *t*BuOMe, at 65 °C. [c]100 mg substrate, 50 mg mL^{-1} CALB, (S/E, 1:7.5), in 15 mL *t*BuOMe, at 65 °C. [d]100 mg substrate, 50 mg mL^{-1} CALB, (S/E, 1:7.5), in 15 mL *t*BuOMe, at 65 °C. [e]According to GC after derivatization. [f]According to GC after double derivatization. [g]*c* = 0.20. [h]*c* = 0.25. [i]*c* = 0.23. [j]*c* = 0.19. [k]*c* = 0.22

4.2.3. Determination of absolute configurations

The optical rotation values were determined based on literature data [57,156] and considering that CALB exhibits (*S*)-selective hydrolysis for *cis* compounds.

5. Summary

1. Based on the modified Rodionov sysnthesis, racemic fluorophenyl-substituted β-amino acids (±)-**2a–e** were synthesized through the reaction of the corresponding aldehydes **1a–e** in the presence of NH_4OAc with malonic acid in EtOH at reflux temperature. The white crystals formed were filtered off and washed with acetone and then they were recrystallized from H_2O and acetone with yields ranging from 15% to 51%.

2. β-Amino carboxylic esters (±)-**3a–e**.HCl were prepared in the presence of $SOCl_2$, EtOH, and the corresponding (±)-**2a–e** by esterification. The resulting white salts were recrystallized from EtOH and Et_2O with yields ranging from 76% to 98%.

3. In order to determine the optimized conditions for the resolution of (±)-**3a–e**.HCl, a set of preliminary screenings including enzyme, solvent, temperature, and concentration of enzyme were conducted.

4. The preparative-scale hydrolysis of racemic β-amino carboxylic ester hydrochloride salts **3a–e** was carried out under optimized conditions: in iPr_2O with lipase PSIM, in the presence of Et_3N with H_2O as a nucleophile at 45 °C. Reactions were stopped by filtering off the enzyme at a conversion close to 50% with excellent enantioselectvities (E > 200). The enantiomers could be easily separated by organic solvent/H_2O extraction. Both unreacted amino carboxylic esters (*R*)-**4a–e** with high *ee* (≥94%) and good yields (≥38%) as well as product amino acids (*S*)-**5a–e** with excellent *ee* (>99%) and yields (≥48%) were isolated.

5. In accordance with the Markovnikov orientation, the regioselective 1,2-dipolar cycloaddition of chlorosulfonyl isocyanate (CSI) to cyclopentene **260**, cyclohexene **261**, cycloheptene **262,** and 1,5-cyclooctadiene **263** delivered racemic *cis* β-lactams **264**, **265**, **266**, and **267**. Ring cleavage of *cis* lactams **264**–**266** and **267** with 22% ethanolic HCl yielded the desired *cis* carbocyclic amino esters **6**–**8** and unsaturated ethyl *cis*-2-aminocyclooct-5-ene carboxylate **268**. Then the latter was subjected to catalytic reduction to give saturated ethyl *cis*-2 aminocyclooctanecarboxylate **9**.

6. Preliminary experiments including green solvent, enzyme quantity, temperature and frequency screening, as well as enzyme recyclability and reusability testing were revealed the best reaction conditions.

7. Sustainable CALB-catalyzed strategies have been demonstrated for the resolution of 5–8-membered (±)-*cis*-**6**–**9** carbocyclic β-amino esters through hydrolysis in green organic solvent *t*BuOMe, under solvent-free conditions and using ball milling for compound. In the frame of preliminary experiments, the best combination of enantioselectivity and reaction rate was observed in green organic solvent, therefore preparative-scale resolutions were performed with CALB in *t*BuOMe as a green solvent at 65 °C resulting in the desired enantiomeric unreacted β-amino esters (1*R*,2*S*)-**6**–**9** with yields ranging from 27% to 31% and ee_s values of ≥91%, and product β-amino acids (1*S*,2*R*)-**10**–**13** with yields ranging from 25% to 33% and high ee_p values (≥96%).

8. It is worth mentioning that, the lipase-catalyzed hydrolysis of 7- and 8-membered carbocyclic β-amino esters was described for the first time.

9. The resulted enantiomers were characterized by GC measurements, optical rotations, ^{1}H, ^{13}C, and ^{19}F NMR, HRMS analysis and melting point values.

Acknowledgements

I would like to express my deepest thanks to my supervisor, Professor Ferenc Fülöp for being an ideal teacher and a mentor offering advice and encouragement with a perfect blend of insight and humor. I am grateful for my time working with professor Fülöp and I am truly indepted to him.

My warmest thanks are due to my supervisor Professor. Enikő Forró, for her beautiful heart and continuous support. I have benefited from her wealth of knowledge and her help has been invaluable during all stages of my work.

I would like to thank my dearest officemate Dániel Gombkötő and all members at the Institute of Pharmaceutical Chemistry especiallay the nicest Prof. István Szatmári for their help and friendship. I feel very fortunate to be able to work in such a collaborative environment.

Finally, I would like to give the sweetest thanks to my family and my dear uncle Professor Habib Alipour for their unconditional love and support during my Ph.D. studies.

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ANNEX