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Enzymatic resolution of (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate, an intermediate in the synthesis of (−)-Paroxetine

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Abstract—The enantioselective hydrolysis of (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate using four different microbial lipases was examined. This ester was found to be a poor substrate for most lipases, the activity achieved with the lipase from *Candida antarctica* (fraction B) (CAL-B) being the highest. This enzyme was purified via adsorption on hydrophobic supports and immobilized using different protocols. The enzyme immobilized on octadecyl-Sepabeads was not enantioselective at all ($E=1$). In contrast, glyoxyl-CAL-B preparation gave the unreacted (3*S*,4*R*)-ester in enantiomerically pure form (enantiomeric excess >99%) at 50% conversion. No decrease in the enzyme activity, or alteration in the enantioselectivity were detected, even after ten reaction cycles. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The 4-arylpiperidine moiety is an important structural element in many biologically active compounds, possibly due to its similarity to the aryl alkylamine pharmacophore common to neurotransmitters such as serotonin [5-hydroxy-tryptamine (5-HT)] or dopamine (DA).[1,2]

Drugs that modulate the physiological and pathophysiological actions of 5-HT are useful or potentially useful in the treatment of a variety of human diseases, including depression, anxiety, alcoholism, chronic pain, emesis and eating disorders.[3]

For example, Paroxetine **1** is used in the treatment of depression, obsessive compulsive disorder and panic disorder.[4] This compound is an enantiomerically pure (−)-*trans*-3,4-disubstituted piperidine. Due to its biological activity, efficient methods for the resolution of this compound are very important to the pharmaceutical industry. As a result, several synthetic strategies have been developed for the preparation of this compound as a single enantiomer, including selective recrystallization of diastereomeric salts,[5] chiral auxiliary assisted synthesis,[6–8] biocatalytic resolutions,[9–11] or the asymmetrization of a prochiral diester intermediate.[12]

The retrosynthetic analysis shown in Scheme 1 leads to (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate, (±)-*trans*-**3** as an intermediate in the synthesis of Paroxetine.[13] This compound may be resolved by an enzymatic process (e.g. catalyzed by lipases or esterases) for application in an asymmetric synthesis of Paroxetine.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are perhaps the most frequently used enzymes in organic chemistry because they couple a wide substrate acceptance with a high regio- and enantioselectivity.[14–18] The widespread use of these enzymes in different organisms has contributed to the availability of a broad variety of lipases, some of them commercially accessible, with very different functional and structural features; thus, nature can provide one or several lipases with the desired properties (selectivity, specificity, etc) and this should always be the first step in the search of a suitable biocatalyst.

Moreover, we must bear in mind the dramatic conformational changes of the enzyme molecule during catalysis because lipases may exist in two different structural

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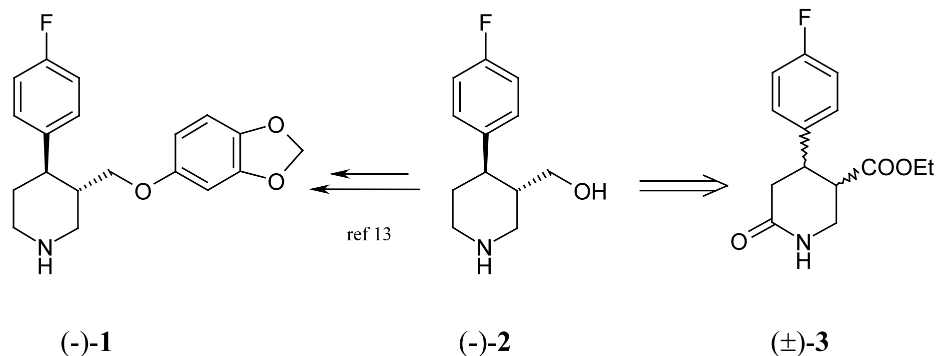


Scheme 1. Retrosynthetic analysis of (−)-Paroxetine.

forms (Scheme 2). One, where the active site of the lipase is secluded from the reaction medium by a helical oligopeptide chain called the 'lid', is considered an inactive (closed) form. The other, which presents the lid displaced and the active site exposed to the reaction medium, is considered to be the lipase in the active (open) form.

In homogeneous aqueous media, the lipase molecule is in equilibrium between these two structures, shifted towards the closed form. Upon interaction with a hydrophobic interface such as a lipid droplet, the open form becomes adsorbed to it and this equilibrium shifts towards the open form (interfacial activation).[19,20] This equilibrium between two very dissimilar molecular forms also exists in most immobilized lipases. The alteration of this equilibrium or the exact shape of the open form of the lipase could markedly change the catalytic properties of the enzyme. Thus, we propose the use of immobilization techniques involving different areas of the enzyme surface (and perhaps giving different orientations of the enzyme on the support), conferring different degrees of rigidity to the enzyme structure and even generating microenvironments surrounding

Derivative 1

Derivative 2

Condition 1

Derivative 2

Condition 2

Closed form

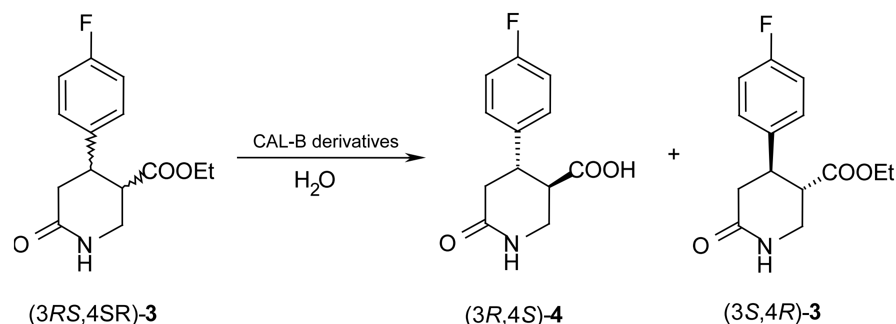
Open form

Active site

Lid

Closed form

Scheme 2. Intended conformational engineering of lipases.



Scheme 3. CAL-B-catalyzed hydrolysis of (±)-*trans*-**3**.

the enzyme to alter the substrate site and the equilibrium between the open and closed forms of the enzyme. This modulation of the enzyme properties can be denominated 'conformational engineering' and it has been used successfully to modulate the behaviour of different enzymes which suffer drastic conformational changes during catalysis, (e.g. penicillin G acylase[21] and lipases[22–25]).

The work reported herein describes the kinetic resolution of (3*RS*,4*SR*)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate **3** through the hydrolysis reaction catalyzed by different immobilized lipases.

2. Results and discussion

2.1. Specific activity of different microbial lipases with (±)-*trans*-3

Lipases from different microbial sources such as *Pseudomonas fluorescens* (PFL), *Humicola lanuginosa* (HLL), *Candida rugosa* (CRL) and *Candida antarctica* (fraction B) (CAL-B) were immobilized on a hydrophobic support via interfacial activation[26] and they were used in the hydrolysis of (±)-*trans*-**3** (Scheme 3). This compound was a poor substrate for these lipases, the specific activities being poor in all cases (Table 1). The highest specific activity was found using CAL-B (the activity was between 9- and almost 30-fold higher than with the other lipases), which was chosen to perform this work.

Table 1. Enzymatic activity of octyl-agarose derivatives from different microbial lipases in the hydrolysis of 2 mM (±)-*trans*-**3** at pH 7 and 45°C

Lipase	Specific activity (μmol h^{-1} mg $lipase^{-1}$)
Pseudomonas fluoresecens (PFL)	0.0027
Candida antarctica B (CAL-B)	0.0257
Candida rugosa (CRL)	0.0009
Humicola lanuginosa (HLL)	0.0082

2.2. Specific activity of the enzyme-immobilized derivatives from CAL-B against (±)-*trans*-3 under different conditions

The initial activity of the different immobilized CAL-B derivatives was analyzed in the hydrolysis of compound **3**, using very low substrate concentrations to ensure complete solubility of the substrate.

When the reaction was carried out at 25°C in aqueous media, the CAL-B immobilized on glutaraldehyde and glyoxyl supports presented the highest specific activity, being around 5-fold higher than that of the octadecyl derivative (Table 2). When the temperature was increased from 25 to 45°C, the specific activity of CAL-B immobilized on octadecyl-Sepabeads increased by a factor of 30, while the specific activity of the other mobilized derivatives increased only by around 10-fold. However, the activity of the glyoxyl derivative was still double that of the interfacially activated immobilized enzyme.

Table 2. Enzymatic activity of different CAL-B derivative-catalyzed hydrolysis of 2 mM (±)-*trans*-**3** at different temperatures

Immobilized derivative	Activity[a] (25°C)	Activity[a] (45°C)
Glutaraldehyde	0.005	0.057
Glyoxyl	0.0065	0.066
Octadecyl	0.001	0.03

[a] Specific hydrolytic activity (μmol mg_{prot}^{-1} h^{-1}). The relative error was estimated to be ±3%.

To perform an industrially viable reaction, it was necessary to use higher substrate concentrations (e.g. 50 mM). To achieve this goal was necessary to add some organic co-solvent. The solubility of (±)-*trans*-**3** in several solvents at different concentrations was analyzed (Fig. 1). The best results were found when using acetonitrile and 1,4-dioxane. However, we also have to consider the likely inhibition produced by the co-solvent on the activity of the immobilized enzyme. Fig. 2 shows that the deleterious effect of the solvent on the initial rate of the immobilized derivative could be correlated with the Log P of the co-solvent ($r=58\%$). Diglyme presented the lowest inhibitory effect. At this organic solvent concentration, all immobilized derivatives were completely stable during the reactions.

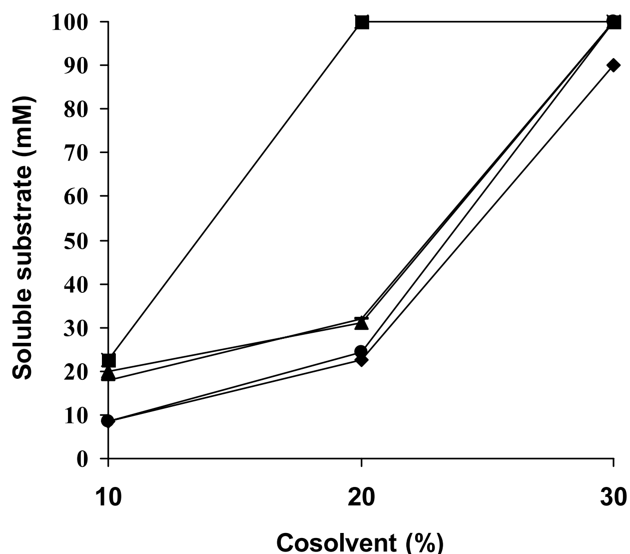


Figure 1. Solubility of (±)-*trans*-**3** in a variety of solvents. Acetonitrile (squares), DMF (triangles), diglyme (rhombus), dioxane (line), cellosolve (circles). Experiments were carried out using 100 mM of substrate and the analysis was performed by HPLC.

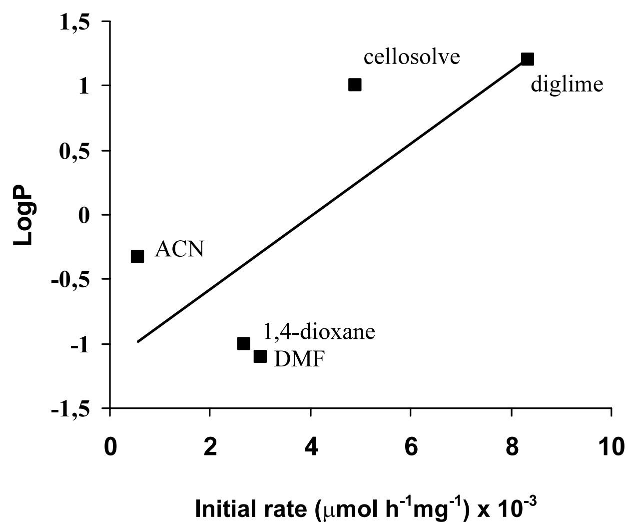


Figure 2. Hydrolysis of (±)-*trans*-**3** catalyzed by octadecyl-Sepabeads: relationship of initial rate and log. Abbreviations: ACN, acetonitrile; DMF, dimethylformamide; cellosolve, 2-ethoxyethanol; diglyme, 2-methoxyethyl ether. Experiments were performed using 2 mmol of substrate and 20% of co-solvent, as described in Section 4.

Therefore, combining the best features of the solvents, we determined that a mixture of diglyme (lower enzyme inhibition) and dioxane (good substrate solubilization) could be the best option, and this mixture, in a proportion of 1:3 dioxane:diglyme, was investigated.

The activity of different immobilized derivatives was examined in the hydrolysis reaction using 10 mmol of substrate (Table 3). Co-solvent was present at a level of 5% (3.75% diglyme, 1.25% dioxane, 95% water), which was necessary to fully dissolve 10 mmol of compound **3**. The activity of the glyoxyl derivative was almost 4 times more active than that of the glutaraldehyde- or octadecyl-immobilized derivatives. The commercially available Novozym Sp-435 preparation presented the highest activity, being almost twice as active as the glyoxyl-CAL-B derivative. It is possible that the higher activity of the commercial preparation can derived from the higher specific activity of the immobilized lipase (e.g. by a better orientation) or from higher loading of the commercial derivative. In fact, in other reactions, the octadecyl-CAL-B derivative was more active than the commercial preparation for example in the hydrolysis of (±)-ethyl mandelate, (±)-methyl phenylacetate, (±)-ethyl butyrate (results not shown).

2.3. Enantioselective resolution of (±)-*trans*-3 at different conditions using CAL-B-immobilized derivatives

The E values in the hydrolysis of (±)-*trans*-**3** at pH 7 and 45°C are shown in Table 3 for the different CAL-B-immobilized derivatives.

Table 3. Enantioselective hydrolysis of (±)-*trans*-**3** at 10 mM and 5% co-solvent (3.75% diglyme, 1.25% dioxane) at pH 7 and 45°C catalyzed by different immobilized derivatives of CAL-B

Immobilized derivative	Reaction time (h)	Conversion (%)	Enzyme activity (μmol h^{-1} g support^{-1})	Specific activity (μmol h^{-1} mg lipase^{-1})	Preferred enantiomer	ee_s	E
Octadecyl	178	20	0.113	0.009	3*R*,4*S*	1	1
	540	50				1	1
Glutaraldehyde	100	18	0.18	0.015	3*R*,4*S*	20.2	29
	350	50				84	30
Glyoxyl	44	21	0.48	0.04	3*R*,4*S*	>26	>100
	300	50				>99	>100
Novozym	18	15	0.85	nd	3*R*,4*S*	11.5	5.3
SP-435	68	50				52	5.2

ee_s = enantiomeric excess of substrate; E = enantioselectivity.

The preferred isomer for all of the immobilized derivatives studied was the (3*R*,4*S*) isomer. The CAL-B-glyoxyl derivative showed the highest *E* value (>100), while the octadecyl derivative was not enantioselective at all ($E=1$). Novozym sp.435 also gave very low enantioselectivity ($E=5$). Finally, when the lipase was immobilized on glutaraldehyde support the enantioselectivity obtained reached a value around 30.

Thus, depending on the derivative used; the enzyme presented higher or lower enantioselectivity in the resolution of compound **3**. Bearing in mind the activity and enantioselectivity, the CAL-B-glyoxyl preparation was chosen as the optimal one.

2.4. Preparative resolution of (±)-*trans*-3

Taking into account the fact that when the glyoxyl derivative was used none of the (3*S*,4*R*)-product was detected at 50% conversion as previously described (even when the reaction time was doubled), we carried out the hydrolysis at a concentration of 50 mM using 20% co-solvent (15% diglyme/5% dioxane) (Table 4).

Hydrolyses were carried out up to 52% conversion (over 400 h when using 1 g of immobilized CAL-B and 10 mL of substrate) and pure unreacted (3*S*,4*R*)-**3** (enantiomeric excess (ee) >99) was isolated after extraction with cyclohexane.

Ten cycles could be performed with identical results with respect to the catalytic properties of the derivative (activity, enantioselectivity).

3. Conclusion

It has been possible to achieve the enzymatic resolution of (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate (±)-*trans*-**3** via a hydrolysis reaction. This was possible by selecting the best available lipase (CAL-B) and the best immobilized lipase preparation (glyoxyl support).

Using the CAL-B glyoxyl derivative, high enantioselectivity (*E* >100) was achieved, to obtain pure (3*S*,4*R*)-**3** (unreacted ester) which is the intermediate with the correct configuration to allow the synthesis of (−)-**2**, an immediate precursor of (−)-Paroxetine.

The results obtained on using other derivatives of the same enzyme showed moderate enantioselectivities, from $E=1$ for octadecyl-Sepabeads derivative to $E=30$ for the glutaraldehyde-CAL-B derivative. These results suggest that the properties of this lipase in this reaction may be strongly modulated by so-called conformational engineering–directed immobilization (altering the rigidity and environment of the enzyme).[22–25]

4. Experimental

4.1. General

The lipases from *C. antarctica* (fraction B) (CAL-B) (Novozym 525 L) and immobilized (Novozym Sp-435) and *Humicola lanuginosa* (Novozym 871) were purchased from Novo Nordisk (Denmark). Lipase from *Candida rugosa* (Type VII) (specific activity 875 U/mg solid), glutaraldehyde, Triton X-100, *p*-nitrophenyl propionate (*p*NPP) were purchased from Sigma Chemical Co. Lipase from *Pseudomonas fluorescens*, PS ‘Amano’ lot. LPSA21250457 was purchased from Amano Enzyme Inc. (NAGOYA, Japan). Octyl-agarose 4BCL was purchased from Pharmacia Biotech (Uppsala, Sweden). Octadecyl-Sepabeads was generously donated by Resindion Srl (Mitsubishi Chem. Coorp.) (Milan, Italy). Glyoxyl-agarose 10 BCL was kindly donated by the company Hispanagar SA (Burgos, Spain). Glyoxyl-agarose[27] and glutaraldehyde-agarose[28] were prepared as previously described. (3*R*,4*S*), (3*S*,4*R*) and (3*RS*,4*SR*)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate **3** were kindly donated by Vita Invest S.A. (Barcelona, Spain). Protein concentration was measured using Bradford’s method.[29] Other reagents and solvents used were of analytical or HPLC grade.

4.2. Immobilization of different lipases on octyl-agarose support

The five microbial lipases were immobilized on octyl-agarose support by interfacial adsorption at low ionic strength (5 mM) and 25°C and pH 7. Periodically, activity of suspensions and supernatants was analyzed by using the *p*NPP assay as described below. In all cases the enzymatic load of the immobilized derivatives was around 10 mg/ml of support, with a yield higher than 95%.

Table 4. Hydrolytic resolution of (±)-*trans*-**3** catalyzed by glyoxyl-CAL-B derivatives using 50 mM of substrate and 20% co-solvent (5% dioxane, 15% diglyme) at pH 7 and 45°C

Reaction time (h)[a]	Enzyme activity (μmol h^{-1} g support^{-1})	Specific activity (μmol h^{-1} mg lipase^{-1})	Preferred enantiomer	ee_s	*E*
300 h	1.92	0.16	3*R*,4*S*	>99	>100

[a] Time taken to reach 50% of conversion

ee_s = enantiomeric excess of substrate; *E* = enantioselectivity.

4.3. Preparation of the enzyme

To purify the lipase from any other contaminant proteins (e.g. esterases), the enzyme preparation was incubated in the presence of octyl-agarose at low ionic strength following the previously described procedure.[26] Periodically, activity of suspensions and supernatants was analyzed by using the *p*NPP assay as described below. After immobilization, adsorbed lipase derivative was abundantly washed with distilled water. To desorb the enzyme, the adsorbed lipase was washed with Triton X-100 1% in 5 mM sodium phosphate buffer at pH 7 and 25°C.

Following these protocols, quantitative immobilization of lipase activity was observed and the SDS-PAGE analysis of the adsorbed protein derivative only showed a single band with a molecular weight corresponding to that of the native lipases. Final yield was near 100%.

4.4. Immobilization of lipases on different supports

Different derivatives were prepared following the procedures previously described:

(i) Interfacial adsorption on a hydrophobic support, Sepabeads resin with the surface covered by octadecyl groups.[30] To immobilize the purified protein in octadecyl support, the Triton was diluted 500-fold with sodium phosphate buffer 5 mM at pH 7.

(ii) Multipoint covalent immobilization on glyoxyl-agarose beads (through areas with the highest density of lysine (Lys) groups).[31] The immobilizations were carried out in 5 mM sodium bicarbonate pH 10 at 25°C. To end the multipoint covalent attachment, sodium borohydride was added to a concentration of 1 mg/ml, after 30 min the derivative was washed with an excess of distilled water.

(iii) Covalent immobilization on glutaraldehyde-agarose beads.[28] The immobilizations were carried out in 25 mM sodium phosphate pH 7 at 25°C. To reduce the reactive groups, a volume of 100 mM sodium bicarbonate pH 10 containing 2 mg/ml of sodium borohydride was added. After 30 min the derivative was washed with an excess of distilled water.

In all cases, the activity of suspensions and supernatants was assayed by using the *p*NPP assay as described below. The enzyme loading was 12 mg of purified lipase /mL of support and in all cases more than 95% of the lipase became immobilized on all different supports offered. Protein concentration was determined by the Bradford method.[29]

4.5. Hydrolysis of *p*-nitrophenylpropionate (*p*NPP)

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM *p*NPP in 25 mM sodium phosphate buffer at pH 7 and 25°C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of *p*NPP activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of *p*NPP per minute (IU) under the conditions described above.

4.6. Enzymatic hydrolysis of (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate, (±)-*trans*-3

The activities of different immobilized derivatives of CAL-B on the hydrolysis reaction were performed in a first experiment by adding the derivative (1 g) to substrate (2 mM, 20 ml) in phosphate sodium buffer (25 mM pH 7) at different temperatures. Next the derivative (1 g) was added to of substrate (10 mM, 10 ml) containing 5% of a the mixed co-solvents (dioxane/2-methoxyethyl ether (diglyme)).

Finally, the activity of the glyoxyl derivative on the hydrolysis reaction was performed by adding the derivative (1 g) to the substrate (50 mM, 10 mL) with 20% cos-olvent (15% diglyme/5% dioxane) at pH 7 and 45°C.

During the reaction, the pH value was kept constant by automatic titration using a pH-stat Mettler Toledo DL50 graphic. The specific activity and enzyme activity were defined as μmol of substrate hydrolyzed per hour per mg of immobilized protein or per g of immobilized derivative in each case.

The degree of hydrolysis was confirmed by reversed-phase HPLC (Spectra Physic SP 100) coupled with an UV detector (Spectra Physic SP 8450) on a Kromasil C18 (25×0.4 cm) column supplied by Analisis Vinicos (Spain). At least triplicates of each assay were made. The elution was isocratic with a mobile phase of acetonitrile (30%) and 10 mM ammonium phosphate buffer (70%) at pH 3.00 and a flow rate of 1 ml/min. The elution was monitored by recording the absorbance at 270 nm. The retention time of acid was 4.67 min while the ester eluted at 19 min.

4.7. Determination of enantiomeric excess

At different conversion degrees, the ee_s of the remaining ester was analyzed by chiral-phase HPLC. The column was a Chiral-AGP (100×4.0 mm), the mobile phase was ammonium phosphate buffer 10 mM at pH 7.00 and the analyses were performed at a flow of 0.5 ml/min by recording the absorbance at 210 nm. The retention time of the (3*R*,4*S*) isomer was 14.73 min and the (3*S*,4*R*) eluted at 18.57 min identified using the pure enantiomers.

4.8. Calculation of *E*-value

Enantiomeric ratio was expressed as an *E* value calculated from the ee_s of the remaining ester and the conversion degree (c) according to Eq. (1) previously reported by Chen et al.[32]

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \quad (1)$$

4.9. Separation protocol of compounds (3*R*,4*S*)-4 and (3*S*,4*R*)-3

10 mL of reaction mixture at 50% of conversion (131 mg of initial substrate) was separated of immobilized preparation by vacuum filtration. The reaction mixture was extracted with cyclohexane (5×10 mL). After that, the aqueous phase did not contain any ester. The solvent of the organic phase was dried over sodium sulfate and evaporated under vacuum. (3*S*,4*R*)-**3** (52.56 mg, 80% yield) was obtained as yellow oil. It was used the chiral HPLC to identified the purity of ester **3** and reversed-phase HPLC to confirm the absence of acid **4**.

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