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# Acetylation of (*R*,*S*)-propranolol catalyzed by *Candida antarctica* lipase B: An experimental and computational study

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ABSTRACT

The chemo- and enantioselectivity of the *Candida antarctica* lipase B (CalB)-catalyzed acetylation reaction of (*R*,*S*)-propranolol using vinyl acetate as acyl donor and toluene as organic solvent was studied. Because of the poor solubility of propranolol in toluene small quantities of methanol were added as cosolvent. The effects of the propranolol/vinyl acetate ratio, the enzyme purification procedure and the methanol concentration on the reaction were investigated. The reactions occurring in the system were quantitatively investigated using $^{1}H$ and $^{13}C$ NMR spectroscopy. The major reactions were the hydrolysis and alcoholysis of vinyl acetate, as a consequence of the presence of residual water and methanol in the reaction medium. Furthermore, the NMR analysis confirmed that *O*-acetyl-propranolol was formed exclusively. The reaction was also found to be enantioselective favoring the faster transformation of the *R*-propranolol. In addition to the experiments, molecular modeling was used to study the formation of the reactive Michaelis complexes between propranolol and acetylated CalB, using a combined molecular docking and molecular dynamics (MD) procedure. Only for the *O*-acetylation we found binding modes of the substrate leading to formation of the product, which explains the experimentally observed chemoselectivity of CalB.



## 1. Introduction

Propranolol (1-iso-propylamino-3-(1-naphthoxy)-2-propanol), a beta-adrenergic blocking agent used for treatment of arterial hypertension and other cardiovascular disorders [1–3], is still commercially available as a racemic mixture. However, the desired therapeutic effect is associated with the *S*-enantiomer, and administration of the racemic propranolol mixture causes serious side effects such as bronchoconstriction or diabetes [4–6]. For this reason many researchers have been working on strategies to obtain *S*-propranolol with high enantiomeric purity. Several chemoenzymatic or chemical synthesis routes for obtaining *R*- and *S*-propranolol in enantiomerically pure form have been proposed [7–12]. Several authors have also reported the biocatalytic resolution of (*R*,*S*)-propranolol through lipase catalyzed transesterification and hydrolysis reactions [13–16].

Lipase catalyzed reactions offer a green way for the synthesis of enantiomerically pure chiral drugs in comparison with conventional chemical methods. Many of these reactions are highly selective and can be performed under very mild reaction conditions [17]. Moreover, lipases are well established catalysts in organic synthesis nowadays, because they are highly stable and active in organic solvents [18–20], and they can catalyze a number of biotransformations which involve carboxylic-groups, such as esterification, transesterification and aminolysis [19,21–26].

Among the most frequently studied lipase catalyzed reactions is the enantioselective acylation of racemic amines and secondary alcohols in organic solvents using vinyl or ethyl acetate as acyl donor [27–33]. In contrast, the lipase-catalyzed acylation of amino-alcohols has been less studied and has recently gained attention [34–40]. Particularly, the chemoselectivity of these reactions is still not completely understood. It has been suggested that the lipase-catalyzed chemoselective *N*-acylation of short chain amino-alcohols such as ethanolamine, serine and 3-amino-1-propanol proceeds through initial *O*-acylation followed by spontaneous *O*- to *N*-acyl migration [41,42]. However, recently Le Joubioux et al. [39] have reported experimental results on the lipase-catalyzed acylation of amino-alcohols and related compounds which are not in accordance with the hypothesis of acyl migration as an explanation of the observed chemoselectivity and rate enhancement

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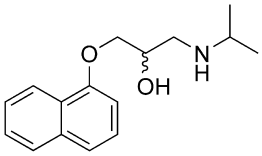


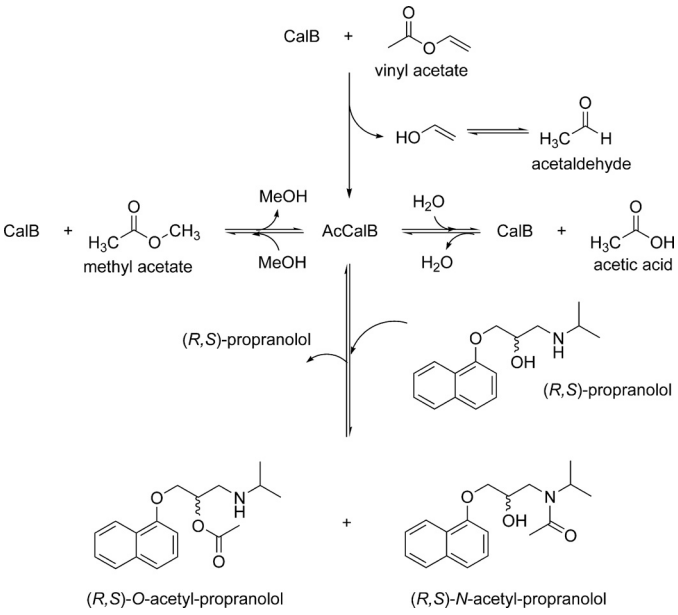
**Fig. 1.** (*R*,*S*)-propranolol.

for *N*-acylation. Therefore, a new reaction mechanism has been proposed [39,43]. This mechanism suggests an *N*-acylation reaction catalyzed by the enzyme and involves a proton shuttling in the transition state mediated by the nucleophilic groups present in the amino-alcohols linked together through an intramolecular hydrogen bond and by the availability of a suitable base (His 224) to act as a proton acceptor in the active site of the enzyme. This proton shuttle is concomitant with a nucleophilic attack of the nitrogen atom of the amino group at the acyl enzyme. This new mechanism explains the observed chemoselectivity in case of amino-alcohols exhibiting a variable carbon chain length between the amino and alcohol groups, such as alaninol, isopropranolamine, 4-amino-1-pentanol and 6-amino-1-hexanol [39]. It also explains the *N*-acylation observed in case of bifunctional compounds which are structurally related to alaninol but exhibit no alcohol group in their structure (1-methoxy-2-propylamine and 1,2-diaminopropane) and thus cannot possibly be *O*-acylated, ruling out the hypothesis of an *O*- to *N*-acyl migration.

In general, lipases catalyze *N*- or *O*-acylation of amino-alcohols with a chemoselectivity which depends on the amino-alcohol structure, the solvent, the acyl donor and the type of lipase used as biocatalyst [35,39,41,44–46]. *Pseudomonas* sp. lipase, for example, has been shown to catalyze the *O*-selective acylation of 2-amino-1-phenylethanols in ether solvents using vinyl acetate as acyl donor [45]. The lipase-catalyzed *N*- and *O*-acylation of propranolol has also been investigated using different lipases (but not CalB) and solvents (chloroform, toluene and diisopropylether), using different divinyl carboxylates as acyl donors [46]. It was found that usually *N*-acylation is favored over *O*-acylation, but the selectivity depends on the type of lipase and the solvent in which the reaction is performed.

Among different lipases catalyzing acylation reactions of amines and secondary alcohols, *Candida antarctica* lipase B (CalB) has proven to be one of the most efficient [33,47,48]. This enzyme has recently been used in immobilized form to carry out the acetylation of (*R*,*S*)-propranolol with vinyl acetate in toluene, displaying moderate enantioselectivity (maximum *E* value of 57), which allowed to obtain *S*-propranolol with an enantiomeric purity of 96% at a relatively low conversion rate of 30%. Nevertheless this *E* value is higher or comparable than those observed in the kinetic resolution of propranolol either via esterification or hydrolysis reactions using other lipases such as *Pseudomonas cepacia*, *Rhizopus Niveus* and *Pseudomonas fluorescens* [13]. However, a detailed analysis of all reactions occurring in this system has not been reported. Such analysis is expected to give valuable insights into the reaction, allowing to improve the enantioselective synthesis of *S*-propranolol. In principle, propranolol can be acetylated at the hydroxyl or the amino group (Fig. 1). Therefore, one or both acetylation reactions could be responsible for the experimentally observed enantioselectivity.

Complementary to experiments, computational modeling has become a useful tool to improve the understanding of lipase-catalyzed reactions, giving insight into these reactions at a level of detail which is experimentally not accessible. This often helps to improve the rate and enantioselectivity of these reactions through a more rational design of the reaction conditions [49–52]. The methodologies applied in the computational studies range




**Scheme 1.** Scheme of the reactions occurring in the CalB-catalyzed acetylation of propranolol using vinyl acetate as acyl donor and a solution of toluene/methanol as solvent. AcCalB denotes acetylated CalB or the acetyl-enzyme complex, an acyl-active intermediate in the reaction which can react with any of the nucleophiles participating of the reaction (methanol, water or propranolol). The competing reactions are the alcoholysis and hydrolysis of vinyl acetate, and the acetylation of propranolol.

from studies on small model systems over quantum mechanical/molecular mechanical (QM/MM) studies and docking studies to molecular dynamics (MD) studies [53–60].

In this work, the acetylation reaction of propranolol catalyzed by CalB (the free enzyme) using vinyl acetate as acyl donor, and a mixture of toluene/methanol (96:4 or 93:7, v/v) as solvent was carried out. Using methanol in the reaction medium increased the solubility of propranolol, but at the same time increased the complexity of the reaction system, due to competing reaction of methanol in addition to hydrolysis of vinyl acetate (Scheme 1).

$^{1}$H and $^{13}$C NMR spectroscopy was used for monitoring all reactions occurring in the system and for determining the chemoselectivity of the acetylation of propranolol. For a full assignment of the chemical shifts corresponding to each chemical compound in the reaction mixture, DEPT135° as well as HMBC and HSQC heteronuclear correlation techniques were used. In addition, the enantioselectivity of the reaction was determined.

To gain a deeper understanding of the experimentally observed chemoselectivity, a molecular modeling study of the formation of the Michaelis complexes between propranolol and acetylated CalB (AcCalB) was performed. This allowed us to test the accessibility of the nucleophilic groups of propranolol in the CalB catalyzed acetylation reaction.

## 2. Experimental

### 2.1. Experimental methods

#### 2.1.1. Materials

*Candida antarctica* lipase B (CalB) was obtained from Novozymes (Parana, Brazil). Racemic mixtures and pure enantiomers of (*R*,*S*)-propranolol-HCl, p-Nitrophenylbutyrate (pNPB), Triton X-100 and other reagents were purchased from Sigma–Aldrich (St. Louis, USA). All reagents and solvents used were of analytical or high-performance liquid chromatography grade.

*2.1.2. Lipase purification*

CalB was purified for being used in the acetylation of (*R*,*S*)-propranolol. The purification was done by chromatography based on the adsorption of the lipase on octyl agarose beads at low ionic strength [61]. A solution of CalB (20 mL, 0.7 mg/mL) in sodium phosphate buffer (5 mM, pH 7) having an activity of 167 U $mg^{-1}$ in pNPB assay (see below) was incubated with octyl agarose beads (4 g) during 2 h. Periodically, the activity of suspensions and supernatants was assayed using the p-nitrophenylbutyrate (p-NPB) assay as described below. The protein concentration in the supernatants was also determined, using Bradford's method [62]. After immobilization, the adsorbed lipase was filtered and washed with distilled water. Then the enzyme was desorbed using sodium phosphate buffer (20 mL, 5 mM, pH 7) containing Triton X-100 (1%, v/v) [61]. This enzyme solution was diluted 5-fold in sodium phosphate buffer (5 mM, pH 7) to dilute the detergent. A fraction (50 mL) of this diluted enzyme solution was dialyzed to remove the detergent. The rest was kept as it was. Finally, both enzyme fractions were lyophilized and used for the acetylation of propranolol. These enzyme preparations are referred here as CalB-I and CalB-II respectively. As Triton X-100 has been shown to promote an increase of the activity of CalB [63], these two enzyme preparations were obtained to evaluate the effect of this detergent on the activity, the chemo- and the enantioselectivity of CalB.

*2.1.3. Hydrolysis of p-NPB*

This assay was performed by measuring the increase in absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of p-NPB (0.4 mM) in sodium phosphate buffer (25 mM, pH 7.0) at 25 °C. To start the reaction, the lipase solution or suspension (0.02 mL) was added to the substrate solution (2.5 mL). One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of p-NPB per minute under the conditions described previously.

*2.1.4. Chemical acetylation of (R,S)-propranolol*

This reaction was carried out using an analogous procedure to the classical methodology of Gatterman [64] for the synthesis of (*R*,*S*)-*O*-butyryl propranolol (*RS*-*O*-BP): (*R*,*S*)-propranolol-HCl (0.2 g; 0.76 mmol) was refluxed with dichloromethane (20 mL) and acetyl chloride (0.08 mL; 0.76 mmol) was very slowly added. After two hours, the reaction mixture was washed successively with equal volumes of saturated aqueous sodium bicarbonate and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to afford *N*-acetyl propranolol (*N*-AP) and *O*-acetyl propranolol (*O*-AP).

*2.1.5. Lipase-catalyzed acetylation of (R,S)-propranolol*

Different experiments were carried out in order to study the effect of the reaction conditions on the velocity, the chemo- and the enantioselectivity of the reaction. As propranolol-HCl is poorly soluble in toluene (<0.5 mg/mL), methanol was added as a cosolvent according to the amount of propranolol to be solvated: (a) (*R*,*S*)-propranolol (39 mM) was dissolved in a solution of toluene/methanol (20 mL, 93/7, v/v) containing vinyl acetate (117 mM). (b) (*R*,*S*)-propranolol (8–28 mM) was dissolved in a solution of toluene/methanol (5 mL, 96/4, v/v) containing vinyl acetate (54 mM). To start these reactions the purified enzyme was added (3 mg). The reaction mixtures were continuously shaken at 200 rpm and 25 °C.

Methanol was chosen as cosolvent based on the solubility of propranolol in this solvent (200 mg/mL). THF, hexane and ethanol were also tested, but the solubility of propranolol in these solvents is 9 mg/mL, 3 mg/mL and 10 mg/mL, respectively.

Blank experiments were carried out in addition, using the same reaction conditions but without addition of the enzyme. No acetylated product of propranolol was detected under these conditions.

*2.1.6. HPLC analysis*

The conversion of the enzyme reaction was determined by HPLC (Agilent 1100) using a Silica gel C-18 column (Zorbax C-18, Agilent Technologies). The mobile phase was composed of acetonitrile and phosphate buffer pH 6 (70:30) and the samples were eluted at 1.0 mL $min^{-1}$. A UV detector (at a wavelength of 289 nm) was used for quantification. The enantiomeric excess of the substrate ($ee_s$) and the product ($ee_p$) was determined using a chiral column (25 cm, ES-OVM, Agilent-Technologies, USA) with methanol/phosphate buffer pH 6 (30:70) at 1.0 mL $min^{-1}$ as mobile phase [13]. The enantioselectivity (*E*) was calculated using the equation reported by Chen et al. [65].

*2.1.7. NMR analysis*

$^{1}H$, $^{13}C$, DEPT135°, HMBC and HSQC NMR spectra were recorded on a Bruker Avance III, 400 MHz spectrometer, using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as internal reference. The experimental conditions were: for $^{1}H$, spectral width (SW) = 4400 Hz (−0.5 to 10.5 ppm), Bruker pulse program zg30 and number of scans = 16; the quantitative $^{13}C$ NMR spectra were obtained in the inverse gated mode for fully decoupled spectra with no Nuclear Overhauser Effect (NOE) with spectral width (SW) = 24,038 Hz (−20 to 219 ppm), Bruker pulse program zgig30 and number of scans = 4096; the two-dimensional phase-sensitive gradient selected edited heteronuclear single quantum coherence – HSQC- spectra were obtained with the Bruker pulse program hsqcedetgp; for the two-dimensional heteronuclear multiple bond correlation – HMBC- spectra the Bruker pulse program hmbcgplpndqf was used; for distortionless enhancement by polarization transfer – DEPT135°- spectra the Bruker pulse program dept135 and 2048 scans were used. The products were identified by performing a full assignment of $^{1}H$ and $^{13}C$ chemical shifts using DEPT135° as well as $^{1}H$–$^{13}C$ HMBC and HSQC heteronuclear correlation techniques (see Supplementary Data for details).

*2.2. Computational methods*

The computational part of this study involved the following stages: preparation of the starting structures corresponding to the protein and the substrate (*R* or *S* propranolol), modeling of the acetylated CalB (the docking target), docking of both enantiomers of propranolol, optimization and structural analysis of the poses with highest interaction free energy from the docking procedure, and finally molecular dynamics simulations to check the reliability of the final models.

*2.2.1. Preparation of the acetylated CalB (AcCalB) structure*

The enzymatic mechanism of lipases (including CalB) involves a catalytic triad consisting of Serine, Histidine and Aspartate (Ser105, His224 and Asp187 in CalB). Lipase catalyzed reactions proceed in two steps (see Scheme S2 of the Supplementary Data). The first step is the addition of an acyl-group to the catalytic serine of the enzyme, yielding the acyl-enzyme. In the second step, the acyl-group can react with several nucleophiles, such as water, alcohols, amines or peroxides [19]. According to the general mechanism of lipase catalyzed acylations, acylation as well as deacylation, proceed via a tetrahedral intermediate (TI), which is stabilized by NH and OH functions in the so-called oxyanion hole of the enzyme, constituted by the residues Thr40 and Gln106 in CalB. In the CalB-catalyzed acetylation of (*R*,*S*)-propranolol, the deacylation part of the reaction mechanism (reaction **2** in Scheme S2) may be chemo- (*N*- or *O*-acetylation) and stereoselective (acetylation of *R*- or

*S*-propranolol). Therefore, the first part of our molecular modeling was to study acetyl–CalB, in order to generate structures to be used as targets in the docking of both enantiomers of propranolol.

The initial coordinates of CalB were taken from the crystal structure with entry number 1TCA in the Protein Data Bank [66]. This structure contains two units of *N*-acetyl-glucosamine (NAG) which are far from the active site and are not expected to have any influence on the selectivity. Therefore these NAGs were removed. The PDB2PQR software package was used to add the missing hydrogen atoms, check the CalB structure together with its crystal waters by PROPKA in order to generate proposals for protonation states of amino acid side chains, and to correct flipped side chains of asparagine, histidine or glutamine residues [67–71]. We rechecked all proposed protonation states and the side chains of asparagine, histidine or glutamine residues for plausibility. Based on this procedure no flips of any residue were applied and all the polar and potentially charged amino acids were used in their ionized form, except Asp-134 which was protonated.

Once the structure of CalB was protonated, the hydroxyl group of the Ser105 side chain was replaced by acetate in order to mimic the acylenzyme complex, and the new residue corresponding to the acetylated catalytic serine was labeled SEA. The acetate oxygen was placed in three different conformations in the oxyanion hole, ensuring that it can form hydrogen bonds with the residues of the oxyanion hole, and mimicking the oxyanion orientation in the second tetrahedral intermediate. The structures obtained for the acylenzyme were named ACE01, ACE02 and ACE03 according to the order in which they were created. Each of the three structures was then saved and transferred to the CHARMM program [72] (version 35b5) to be used as initial structures for a first series of MD simulations in explicit toluene molecules. CHARMM force field parameters for the acetylated serine (SEA) were adopted from serine and methyl acetate.

#### 2.2.2. Molecular modeling of the Michaelis complexes of acetyl–CalB propranolol

To build the Michaelis complexes (MCCs), *R* and *S* propranolol were non-covalently docked against each acetyl–CalB structure using the Autodock Vina software [73,74]. This approach allowed exploring different orientations of these substrates within the catalytic pocket. The best conformers were chosen on the basis of the binding affinity as well as on the basis of geometrical criteria, namely the distance of the amino and hydroxyl groups of propranolol from the acetylated serine. As the applied docking procedure treats the protein as a rigid body, the acetyl enzyme–substrate complexes selected were submitted to a careful post-docking optimization, in order to take into account a potential induced fit effect (little displacements in the protein structure due to the presence of the ligand) [75]. From the post-docking optimization procedure possible productive binding modes (binding modes of the substrate which lead to formation of the product) for *R* and *S* propranolol were identified. These complexes were then submitted to 100 ps of MD simulation in explicit toluene in order to evaluate their dynamic behavior. The results of all MD simulations were analyzed using the program VMD [76].

We refer the reader to Section 1 of the Supplementary Data for a complete description of the computational procedures used.

## 3. Results and discussion

### 3.1. Chemical acetylation of (R,S)-propranolol

The chemical acetylation of (*R*,*S*)-propranolol was carried out as a first attempt to identify the possible acetylated products of propranolol directly from a mixture of these compounds using NMR spectroscopy. This was done in order to explore the feasibility of quantifying the chemoselectivity of any kind of acetylation reaction of propranolol, chemical or enzymatic, without any previous purification or separation of the reaction mixture. The NMR analysis of the reaction mixture revealed that a mixture of the mono-acetylated compounds *O*-AP and *N*-AP was formed. The di-acetylated product (*N*,*O*-AP: *N*,*O*-acetyl-propranolol) was not detected under our experimental conditions. The $^{13}$C NMR data of propranolol and its acetylated products are shown in Table 1. The $^{13}$C and $^{1}$H NMR signals corresponding to the carbon and hydrogen atoms of the acetate group (—**C**O**C**H$_3$ in Table 1) were chosen to determine the chemoselectivity of the reaction. We found by integration of any of these peaks that the chemical acetylation of (*R*,*S*)-propranolol with acetyl chloride leads to the formation of both *N*-AP and *O*-AP, with a ratio of 3:1 in favor of *N*-AP (Table 2). This is consistent with the higher nucleophilicity of the amino compared to the hydroxyl group. A similar reactivity of the amino group has been observed in the chemical acetylation of other amino-alcohols [77,78].

**Table 1**
$^{13}$C NMR chemical shifts (in ppm) of propranolol and its acetylated products in the reaction mixture of the chemical acetylation reaction.

| Carbon[a] | Propranolol | Acetyl propranolol | |
|---|---|---|---|
| | | *O*-AP[b] | *N*-AP[c] |
| Ar—**C** | 154.33 | 154.26 | 154.06 |
| | 134.49 | 134.47 | 134.51 |
| | 127.50 | 127.56 | 127.68 |
| | 126.46 | 126.42 | 126.42 |
| | 125.89 | 125.87 | 125.98 |
| | 125.53 | 125.38 | 125.28 |
| | 121.87 | 121.85 | 121.85 |
| | 121.57 | 121.57 | 121.57 |
| | 120.62 | 120.66 | 120.66 |
| | 104.88 | 104.81 | 104.81 |
| Ar—O**C**H$_2$— | 70.65 | 68.20 | 69.78 |
| Ar—OCH$_2$**C**H— | 68.40 | 71.63 | 72.41 |
| —CH**C**H$_2$N— | 49.46 | 45.40 | 46.15 |
| —**C**H(CH$_3$)$_2$ | 49.03 | 49.56 | 50.29 |
| —CH(**C**H$_3$)$_2$ | 23.04 | 22.21 | 21.38 |
| | 22.92 | 21.78 | 21.23 |
| **C**=O[d] | | 171.38 | 173.92 |
| —CO**C**H$_3$[d] | | 21.02 | 20.64 |

[a] Chemical shifts correspond to the underlined carbon atoms in bold.
[b] *O*-acetyl-propranolol.
[c] *N*-acetyl-propranolol.
[d] Chemical shifts used for quantification of the chemoselectivity of the reaction. The $^{1}$H NMR chemical shift of the methyl of the acetate group (—COC**H$_3$**), with value of 2.16 and 2.22 ppm for *O*-AP and *N*-AP respectively was used in addition.

### 3.2. CalB-catalyzed acetylation of (R,S)-propranolol

A first experiment was performed using 39 mM (*R*,*S*)-propranolol and 117 mM vinyl acetate in 20 mL of toluene/methanol (93:7, v/v). The reaction was very slow and was stopped after 18 days when the conversion rates reached 22% and 25% for CalB-I and CalB-II respectively. The higher conversion obtained with CalB-II may be attributed to hyperactivation of the enzyme due to the presence of Triton X-100 during its lyophilization. When CalB is exposed to Triton X-100, the formation of bimolecular lipase aggregates is avoided and the active site of the enzyme is expected to be more exposed to the reaction medium and the substrate, which results in a faster transformation of the substrate [63].

From the NMR analysis of the reaction mixtures the following reaction products were identified: acetaldehyde (aldehyde proton resonance at 9.38 ppm), acetic acid (carboxylic proton resonance at 9.98 ppm), methyl acetate (acetyl protons resonance at 2.04 ppm) and *O*-AP (acetyl protons resonance at 2.18 ppm). The amount of

**Table 2**
Chemo- and enantioselectivity of the chemical and enzymatic acetylation of propranolol.

| Enzyme preparation | Solvent | c[a] (%) | t (h) | Ratio[b] N-AP/O-AP | $ee_s$ (%) | $ee_p$ (%) | E | Sp[c] |
|---|---|---|---|---|---|---|---|---|
| Chemical acetylation[d] | Dichloromethane | 85 | 2 | 75/25 | nd[g] | nd[g] | nd[g] | nd[g] |
| CalB-I[e] | Toluene/methanol (93:7) | 22 | 432 | <3/>97 | nd[g] | nd[g] | nd[g] | nd[g] |
| CalB-II[e] | Toluene/methanol (93:7) | 25 | 432 | <3/>97 | nd[g] | nd[g] | nd[g] | nd[g] |
| CalB-I[f] | Toluene/methanol (96:4) | 21 | 12 | <3/>97 | 26 | 96 | 63 | *R* |
| CalB-I[f] | Toluene/methanol (96:4) | 62 | 99.7 | <3/>97 | >99 | 61 | 63 | *R* |
| CalB-I[f] | Toluene/methanol (96:4) | 70 | 140 | <3/>97 | >99 | 42 | 63 | *R* |
| CalB-II[f] | Toluene/methanol (96:4) | 33 | 12 | <3/>97 | 46 | 95 | 61 | *R* |
| CalB-II[f] | Toluene/methanol (96:4) | 73 | 99.7 | <3/>97 | >99 | 37 | 61 | *R* |
| CalB-II[f] | Toluene/methanol (96:4) | 82 | 140 | <3/>97 | >99 | 22 | 61 | *R* |

[a] Conversion.
[b] Determined by NMR spectroscopy. Assuming an error of 3%.
[c] Stereochemical preference.
[d] Reaction conditions: propranolol (38 mM), acetyl chloride (38 mM) and 20 mL of solvent.
[e] Reaction conditions: propranolol (39 mM), vinyl acetate (117 mM), CalB (3 mg) and 20 mL of solvent.
[f] Reaction conditions: propranolol (28 mM), vinyl acetate (54 mM), CalB (3 mg) and 5 mL of solvent. The enantioselectivity of CalB-I and CalB-II was determined at a conversion of 21% and 33%, respectively.
[g] Not determined.

acetaldehyde, acetic acid and methyl acetate increased faster than the amount of *O*-AP. This indicates that the main reactions were the alcoholysis and hydrolysis of vinyl acetate and not the desired acetylation of (*R*,*S*)-propranolol. $^{13}C$ or $^{1}H$ NMR chemical shifts for *N*-AP and *N*,*O*-AP were not observed. The absence of *N*-AP and *N*,*O*-AP may be due to the inaccessibility of the amino group of propranolol for the acyl transfer by the AcCalB complex or its unavailability for an intramolecular *O*- to *N*-acyl transfer in the structure of *O*-AP as a consequence of the preferred conformation of propranolol in the active site of CalB (see Sections 3.3.1 and 3.3.2).

In order to verify the chemoselectivity of CalB in favor of *O*-AP at higher conversions, the effect of the propranolol concentration on the synthesis of acetyl propranolol was evaluated with the aim of decreasing the reaction time. Using CalB-II as biocatalyst, the reaction was carried out with 8–28 mM of (*R*,*S*)-propranolol and 54 mM vinyl acetate in 5 mL of toluene/methanol (96:4, v/v). Under these reaction conditions the alcoholysis of vinyl acetate is notably decreased and the transformation of propranolol occurs faster.

As shown in Fig. 2, the reaction rate increases as the concentration of propranolol increases. The conversion rate of the substrate increased from 38% to 82% after 140 h by increasing the concentration of the substrate from 8 mM to 28 mM. The almost linear dependence of the reaction rate on the propranolol concentration suggests that CalB has not a very high affinity for this substrate. The same behavior has previously been observed in the acetylation of propranolol and atenolol (a similar compound) catalyzed by immobilized derivatives of CalB [13,24]. It is also observed that the competitive effect of both the hydrolysis and alcoholysis of vinyl acetate decreases as the concentration of propranolol is increased. The hydrolysis and alcoholysis of vinyl acetate are 2.6 and 4.4 times faster than the acetylation reaction, respectively, when 8 mM of propranolol is used. With 28 mM of propranolol the alcoholysis of vinyl acetate is 1.2 times faster than the propranolol acetylation and the hydrolysis of vinyl acetate is not detected.

Because a reasonable conversion rate was obtained using 28 mM propranolol, the reaction was carried out under the same reaction conditions with the enzyme preparation CalB-I as biocatalyst. As we found previously, the reaction occurred faster with CalB-II (Fig. 3). The chemoselectivity of the reaction was then determined in the conversion range 21–82%. Interestingly, as shown in Table 2, with all enzyme preparations of CalB the *O*-acetylated product is formed exclusively, even at high conversion of the substrate. This suggests the same acylation mechanism for both propranolol enantiomers.

The chemoselectivity of the CalB-catalyzed acetylation of propranolol mainly depends on the ability of the nucleophilic groups of propranolol to be transformed to the corresponding product in the active site of CalB. This suggests that the structure of the substrate is

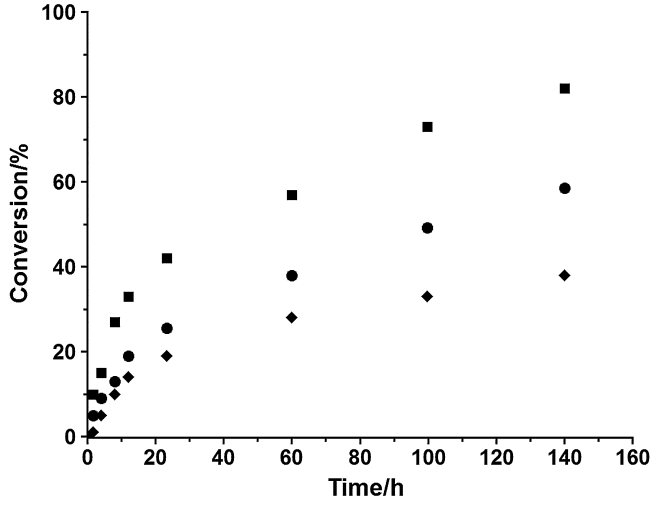



**Fig. 2.** Effect of the propranolol concentration on the rate of the CalB-catalyzed acetylation of (*R*,*S*)-propranolol. The biocatalyst was CalB-II. Experiments were performed as described in Section 2.1.5. (*R*,*S*)-propranolol: (♦) 8 mM (●) 18 mM (■) 28 mM.

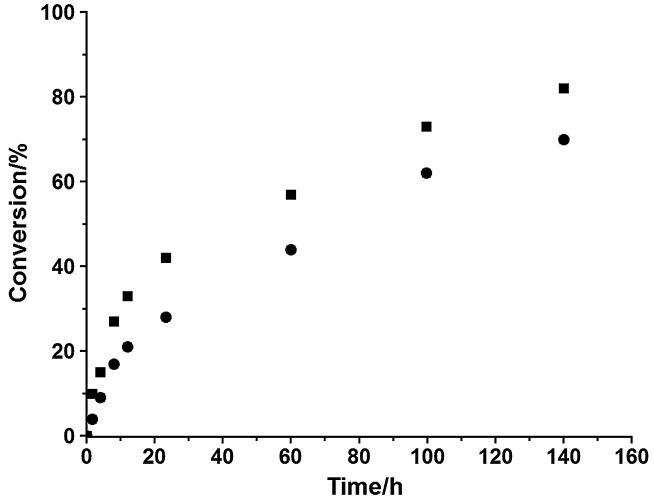



**Fig. 3.** Effect of the enzyme purification procedure on the rate of the CalB-catalyzed acetylation of (*R*,*S*)-propranolol. Experiments were performed as described in Section 2.1.5 using 28 mM of (*R*,*S*)-propranolol. Biocatalyst: (■) CalB-II (●) CalB-I.

a determining factor for the chemoselectivity of CalB and not only the nucleophilicity of the hydroxyl and amino groups of propranolol. This conclusion is in agreement with our experimental results and with recent results of Le Joubioux et al., who found that the chemoselectivity of the CalB catalyzed acylation of amino-alcohols depends on the structure of the substrate [39].

The enantioselectivities of CalB-I and CalB-II were also investigated. The *E* values are 63 and 61, respectively, and the same enantiopreference in favor of the transformation of the *R*-propranolol (Table 2). The *E* values obtained for CalB-I and CalB-II are quite similar, which means that the presence of Triton X-100 during the purification procedure did not affect significantly the enantioselectivity of CalB. These *E* values are also similar (although slightly higher) to those obtained with immobilized CalB on Eupergit C ($E = 57$) [13].

Thus the reaction conditions affect mainly the reaction rate of the acetylation of propranolol and of the competing alcoholysis and hydrolysis of vinyl acetate, and not the chemo- and enantioselectivity of the reaction.

### 3.3. Computational results

In the three structures obtained for the AcCalB complex the catalytic triad is stable and well conserved after the MD simulations. Therefore these structures are reactive. These structures differ in how the carbonyl carbon is exposed to the nucleophilic attack. Therefore, *R*- and *S*-propranolol were non-covalently docked against each of these structures. See Section 4 of the Supplementary Data for details on the AcCalB complex.

A total number of 163 (*R*-propranolol) and 149 (*S*-propranolol) complexes met the conditions described for the docking procedure. These complexes could be grouped according to two binding modes: one in which the naphthyl group of propranolol is oriented toward the top of the binding pocket of CalB and one in which the naphthyl group is oriented toward the bottom of the binding pocket, referred here as Binding Mode I and Binding Mode II, respectively (Fig. 4; Figs. S17 and S18 of the Supplementary Data). The binding free energies of both enantiomers are similar and in the range from −4.7 to −7.1 kcal mol$^{-1}$. In case of *R*-propranolol the conformers present a binding free energy between −4.9 and −7.0 kcal mol$^{-1}$, and those of *S*-propranolol between −4.7 to −7.1 kcal mol$^{-1}$. These values are in good agreement with the expected low affinity of CalB for propranolol according to the reaction rates observed in our experiments.

According to the general mechanism of lipase catalyzed acylations, for *N*- or *O*-acetylation of propranolol, the amino or hydroxyl group of propranolol has to be positioned simultaneously close to the His224:N$\varepsilon$ and SEA:C atoms of AcCalB, respectively. Only complexes satisfying this criterion for the hydroxyl group were found, which gives an explanation for the exclusive formation of *O*-acetylpropranolol observed in our experiments. This can be understood taking into account that a proper position of the amino group in order to be acetylated by any of the AcCalB, requires that a large side chain with a naphthyl group is positioned in the binding pocket of CalB without unfavorable steric contacts. In contrast to this, in case of the acetylation of the hydroxyl group, two side chains of medium size have to be positioned in the binding pocket, resulting in more favorable structures. This also explains why complexes in which the amino group of propranolol is able to be acetylated by AcCalB via the mechanism proposed by Le Joubioux et al. [43] were not found.

In the next step we only considered complexes with a binding affinity stronger than −5.5 kcal mol$^{-1}$ and selected those for optimization in which the reaction could take place easily according to the proximity of the atoms involved in the reaction. A total number of 12 and 7 complexes were selected for the *R*- and *S*-enantiomer, respectively. These complexes are representative for the two binding modes found for *R*- and *S*-propranolol. We refer the reader to the supplementary data for details about these complexes (see Table S2 and Figs. S12–S16). Once the complexes were optimized, they were analyzed to determine if they correspond to productive binding modes according to three criteria used in previous studies

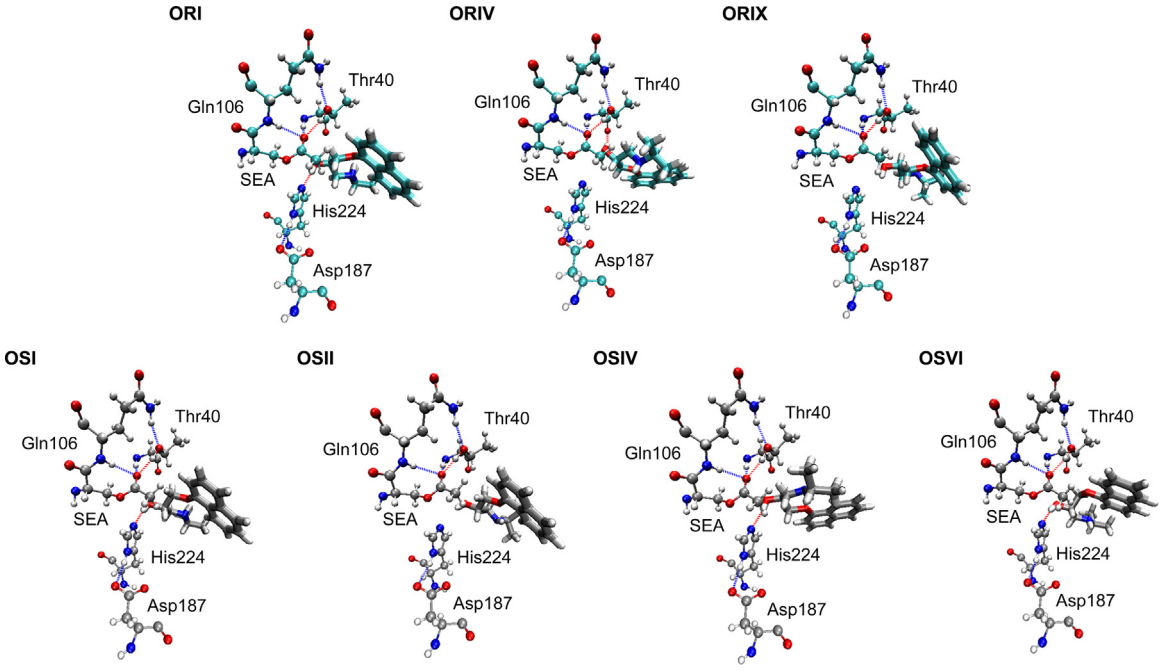



**Fig. 4.** Top view of the best complexes obtained for *R*-propranolol (ORI, ORIV and ORIX) and *S*-propranolol (OSI, OSII, OSIV and OSVI) after optimization. The catalytic triad and the oxyanion hole are shown in CPK representation. The substrate is shown in licorice representation. Hydrogen bonds are shown as blue and red dashed lines, indicating whether the proton donor is a nitrogen or oxygen atom, respectively.

**Table 3**
Relevant interatomic distances, bond lengths and angles corresponding to the best AcCalB-propranolol complexes after optimization[a].

| Distance (Å) | *R*-propranolol | | | *S*-propranolol | | | |
|---|---|---|---|---|---|---|---|
| | ORI | ORIV | ORIX | OSI | OSII | OSIV | OSVI |
| His224:Nε-Sub:**H** | 1.98 (173) | 4.78 | 3.18 (135) | 1.96 (169) | 2.78 (162) | 2.09 (167) | 1.92 (170) |
| SEA:C-Sub:**O** | 3.99 (98) | 3.50 (120) | 4.65 (108) | 3.90 (101) | 4.67 (102) | 3.68 (105) | 4.03 (99) |
| His224:Nε-Sub:*H* | 5.89 | 4.71 | 7.85 | 6.20 | 5.83 | 5.66 | 5.14 |
| SEA:C-Sub:*N* | 6.59 | 4.88 | 7.90 | 6.73 | 7.33 | 4.87 | 6.73 |
| Asp187:$O_D$-His224:$H_{ND}$ | 1.87 (168) | 1.78 (171) | 1.88 (165) | 1.85 (168) | 1.93 (164) | 1.89 (168) | 1.83 (166) |
| SEA:O-Gln106:NH | 2.09 (149) | 2.03 (151) | 2.31 (138) | 2.11 (152) | 2.29 (137) | 2.08 (147) | 2.15 (152) |
| SEA:O-Thr40:NH | 2.05 (161) | 1.97 (160) | 1.86 (165) | 1.96 (162) | 1.90 (164) | 2.12 (160) | 1.96 (163) |
| SEA:O-Thr40:OH | 1.86 (169) | 1.83 (167) | 1.66 (172) | 1.85 (169) | 1.65 (173) | 1.89 (166) | 1.87 (166) |
| Gln106:H2N-Thr40:Oγ | 1.98 (165) | 1.99 (165) | 2.04 (159) | 1.98 (165) | 2.06 (158) | 1.99 (164) | 1.98 (165) |
| Sub:*H*-Sub:O | 2.45 (95) | 3.10 | 3.86 | 2.28 (99) | 2.28 (123) | 2.01 (133) | 3.77 |
| Sub:**H**-Thr40:O | 4.44 | 1.86 (154) | 4.52 | 4.49 | 4.18 | 4.33 | 4.67 |

[a] The atoms corresponding to the hydroxyl and amino group of the substrate (Sub) are marked in bold and italic respectively. The Oxygen of the naphthoxy group of propranolol is indicated as Sub:O. Angles in degrees for both the corresponding hydrogen bonds and the nucleophilic attack (Sub:**O**···SEA:C═O) are given in brackets. SEA is the acetylated serine of CalB.

dealing with molecular modeling of lipase-catalyzed reactions: (a) protein distortion (b) conservation of the hydrogen bond interactions between the acetate oxygen of SEA and the residues of the oxyanion hole (c) a short distance of the nucleophile groups of propranolol to the His224:Nε and SEA:C atoms [47,79,80].

Although some conformational rearrangements of the protein could improve the binding affinity, a high distortion of the CalB structure would result in a loss of activity if some essential residues for the catalytic activity such as the residues of the oxyanion hole are disturbed. Therefore the RMSD value between the initial CalB crystal structure and the final optimized complex must be small. Vallikivi et al. [80] found that in case of complexes between AcCalB and prostaglandins, RMSD values higher than 3.0 Å were experimentally well correlated with non-productive complexes. Therefore this RMSD value was chosen as a limit. This RMSD value has also been successfully used as a limit in other studies on lipase-catalyzed reactions [47,79]. In our work, the superimposition of all heavy atoms of the optimized AcCalB-propranolol complexes to the CalB crystal structure resulted in RMSD values of only about 0.55 Å, even though the side chains were finally free to move, which may be explained by the rigidity and high conservation of the CalB structure in toluene.

To facilitate the acetylation reaction it is necessary that the AcCalB-propranolol complexes form hydrogen bonds with amino acids of the oxyanion hole, namely with the backbone (—NH) of the residues Thr40 and Gln106 and the side chain (—OH) of Thr40. We considered a complex as productive if at least two of these three hydrogen bonds are conserved after the post-docking optimization. Hydrogen bond distances and angles involving the substrate in the optimized complexes are given in Table S3 of the Supplementary Data.

Finally, the third condition for considering a complex as productive is the proximity of the hydroxyl or amino group of propranolol to the catalytic residues. As mentioned above, any of these groups has to be positioned simultaneously close to the His224:Nε and SEA:C atoms. The proton transfer to the catalytic histidine enhances the nucleophilicity of the amino or hydroxyl group attacking the SEA:C atom. We decided to use a maximal value of 4 Å for these distances, following other authors studying lipase-catalyzed acetylation reactions [47,79,81]. The distances involved in the nucleophilic attack corresponding to each complex after optimization are given in Table S4 of the Supplementary Data.

All complexes selected from the docking procedure satisfy the first two criteria after optimization. However, not all of them satisfy the third criterion. The best complexes obtained for *R*- and *S*-propranolol after the post-docking optimization (according to whether the nucleophile groups of propranolol have distances and angles suitable for the nucleophilic attack) are shown in Fig. 4. All of these complexes correspond to possible reactive complexes for the *O*-acetylation of propranolol.

### 3.3.1. *Reactive complexes between R-propranolol and AcCalB*

Three complexes were retained for *R*-propranolol after optimization, namely ORI, ORIV and ORIX (Fig. 4). In ORI and ORIX the substrate is oriented in Binding Mode I. In these complexes the naphthyl group of propranolol is positioned in the same plane, but toward the exterior of the catalytic cavity in ORI and toward the enzyme core in ORIX. The isopropylamine group is oriented toward the exterior of the catalytic cavity in both ORI and ORIX. In the complex ORIV the substrate is positioned in Binding Mode II, with the naphthyl group pointing to the exterior of the catalytic cavity and the isopropylamine group toward the enzyme core.

The relevant interatomic distances corresponding to these complexes, including those used as criteria to consider a complex as productive, are given in Table 3. ORI is the only complex which satisfies the distance criteria to react, as the hydroxyl group of propranolol is positioned simultaneously at 3.99 Å from SEA:C and 1.98 Å from His224:Nε. Furthermore, the angle for the nucleophilic attack is 98°, which is just slightly smaller than the ideal angle (107°) for a nucleophilic addition to a carbonyl group as described by Burgi et al. [82]. Thus, ORI may be considered as a productive complex leading to formation of the *O*-acetylated product. In contrast, the *N*-acetylation of propranolol cannot occur via ORI as the amino group of propranolol is positioned at a distance larger than 4 Å from the catalytic residues.

In ORIV and ORIX the amino group of propranolol is also positioned far from the catalytic residues, while its hydroxyl group is well positioned. However, these complexes do not satisfy completely the distance criteria to react. In ORIV the oxygen atom of the hydroxyl group of propranolol is positioned to a distance of 3.50 Å from SEA:C, but the hydrogen atom of the hydroxyl group is forming a hydrogen bond with the backbone of Thr40 (with a distance of 1.86 Å), such that the interaction with the His224:Nε atom is quite weak (4.78 Å). In contrast, in ORIX the hydrogen atom of the hydroxyl group is positioned at a distance of 3.18 Å from the His224:Nε atom, but the distance for the nucleophilic attack is 0.65 Å larger than required, although the angle of attack is 108°, which is very close to the ideal angle.

In all complexes for *R*-propranolol, the naphthyl and isopropylamine side chains of propranolol are stabilized in their positions in the binding pocket by the respective non-polar residues surrounding: Leu140, Ala141, Leu144, Val154, Ile189, Ile285, Ala281, Ala282, Leu278 and Trp104 (see Fig. S17 of the Supplementary Data). Additionally, in ORI and ORIV there is an intramolecular hydrogen bond between the amino group and the oxygen atom of the naphthoxy group of *R*-propranolol, which also contributes to stabilize these

complexes. This hydrogen bond is stronger in case of ORI with a distance of 2.45 Å.

Considering the feasibility of a spontaneous *O*- to *N*-acyl migration, this hydrogen bond as well as the orientation of the amino group of propranolol would be responsible for the chemoselectivity observed in the reaction. In all complexes obtained for *R*-propranolol it is clearly observed that the nitrogen atom of the substrate is not well positioned for a nucleophilic attack at the *O*-acyl group. In addition, in the complexes where the proton of the amino group of propranolol is forming an intramolecular hydrogen bond with the naphthoxy oxygen, such *O*- to *N*-acyl migration is more difficult, as this interaction would avoid the proton transfer from the amino group of propranolol to the carbonyl oxygen.

### 3.3.2. Reactive complexes between S-propranolol and AcCalB

Four complexes were retained for *S*-propranolol after optimization, namely OSI, OSII, OSIV and OSVI (Fig. 4). The substrate is positioned in Binding Mode I in OSI, OSII and OSVI. In these complexes the isopropylamine side chain of the substrate is oriented toward the exterior of the binding pocket, while its naphthyl group is oriented toward the exterior of the pocket in OSI and OSII, and toward the pocket in OSVI, but in the same plane. OSI and OSII are very similar; the main difference is the orientation of the isopropylamine group of propranolol in the binding pocket. In the complex OSIV, the substrate is oriented in Binding Mode II, with the naphthyl group positioned toward the exterior of the catalytic cavity and the isopropylamine group toward the interior.

The relevant interatomic distances involving the *S*-propranolol complexes are also given in Table 3. Similar to the *R*-propranolol complexes, the hydroxyl group of propranolol is positioned close to the catalytic residues while its amino group is far from these residues. OSI and OSIV fulfill the distance criteria to react. The hydroxyl group of *S*-propranolol is positioned toward the SEA:C and His224:N$\varepsilon$ atoms with distances of less than 4 Å in these complexes. Furthermore the angle of attack is 101° and 105° respectively, which is close to the Burgi angle. Therefore these complexes may be considered as productive complexes for *S*-propranolol.

OSVI and OSII do not fulfill the distance criteria strictly. The hydrogen atom of the hydroxyl group of *S*-propranolol is positioned at a distance less than 4 Å from the His224:N$\varepsilon$ atom, but the distance for the nucleophilic attack is 4.03 Å and 4.67 Å for OSVI and OSII, respectively. It can be noted that in case of OSVI this distance is only slightly larger than the threshold. In case of OSII this distance is larger but the angle of attack is 102°, which is close to the ideal angle.

As in the case of *R*-propranolol, in the *S*-propranolol complexes, the naphthyl and isopropylamine groups of propranolol are also stabilized by interactions with non-polar residues of the binding pocket surrounding (see Fig. S18 of the Supplementary Data). Furthermore, the intramolecular hydrogen bond between the amino and naphthoxy groups is also observed in the complexes OSI, OSII and OSIV, and is stronger than in the *R*-propranolol complexes. This hydrogen bond has a distance of 2.28 Å in OSI and OSII, and 2.01 Å in OSIV. Thus, the *S*-propranolol complexes exhibit a similar behavior as the *R*-propranolol complexes. Because *S*-propranolol is the slowly reacting enantiomer, this intramolecular hydrogen bond and the orientation of the amino group of propranolol explains why no *N*-acetylated product resulting from a possible *O*- to *N*-acyl migration was found even at high conversion of the substrate. This corroborates our suggestion that the acylation mechanism is the same for both propranolol enantiomers.

### 3.3.3. MD simulations of the reactive complexes

In order to complement our computational study, we carried out 100 ps of molecular dynamics simulations on the complexes retained after optimization. Essentially the stability of both the hydrogen bond interactions and the position of the hydroxyl group and the dynamic behavior of the docked substrates were revised. The results showed that the protein did not undergo significant conformational changes; the maximal RMSD values considering all the heavy atoms were about 1.0–1.2 Å. The visual analysis of trajectories showed that the hydrogen bonds between the carbonyl oxygen (SEA:O) and the residues of the oxyanion hole (Thr40 and Gln106) were stable during the MD simulations. The stability of the position of the hydroxyl group was evaluated by monitoring the acetylation distances (SEA:C–Sub:O) along the trajectories. The minimal and maximal values for the distance between the hydroxyl oxygen atom of propranolol and the carbonyl carbon of SEA are given in Table 4. All complexes were able to form reactive complexes during the MD simulation except ORIX. In particular, in the MD simulation of ORIV it was observed that the hydrogen atom of the hydroxyl group of propranolol flips to form a hydrogen bond with the His224:N$\varepsilon$ atom without affecting the distance for the nucleophilic attack, leading therefore to a productive complex. A different temporal stability was observed for each complex as revealed by the value of the maximal fluctuation in the acetylation distance. ORI, ORIV, OSIV and OSVI were the most stable complexes. ORIV and OSVI presented the lower maximal fluctuation of this distance, with a value of 3.7 Å and 1.6 Å, respectively. The amino group of propranolol was never found at a distance less than 4 Å from SEA in the MD simulations, which reinforces our previous conclusion about the chemoselectivity of the reaction.

**Table 4**
Minimal and maximal values of the interatomic distance between the propranolol hydroxyl oxygen and the carbonyl carbon of SEA[a] in the MD simulations of the reactive complexes found after the post-docking optimization.

| SEA:C-Sub:O distance (Å)[a] | *R*-propranolol | | | *S*-propranolol | | | |
|---|---|---|---|---|---|---|---|
| | ORI | ORIV | ORIX | OSI | OSII | OSIV | OSVI |
| Minimum | 3.1 | 2.9 | 4.2 | 2.9 | 3.4 | 3.3 | 2.8 |
| Maximum | 6.8 | 7.2 | 13.5 | 9.5 | 6.4 | 6.5 | 4.4 |

[a] SEA is the acetylated serine of CalB.

## 4. Summary and conclusions

The chemo- and enantioselectivity of the *Candida antarctica* Lipase B (CalB)-catalyzed acetylation of (*R*,*S*)-propranolol using vinyl acetate as acyl donor and a mixture of toluene/methanol as solvent were studied. A competing hydrolysis and alcoholysis of vinyl acetate occurred in addition to the acetylation of propranolol. However, these reactions could be controlled by increasing the propranolol concentration, allowing us to suppress completely the hydrolysis and the alcoholysis of vinyl acetate to the extent that it was only 1.2 times faster than the acetylation of propranolol. The reaction conditions affected mainly the acetylation reaction rate but not the chemo- and enantioselectivity. Exclusive formation of the *O*-acetyl-propranolol was observed under all reaction conditions. CalB was also found to be enantioselective favoring the transformation of *R*-propranolol.

Molecular modeling of the Michaelis complexes between propranolol and acetylated CalB (AcCalB) was also carried out. Only reactive AcCalB-propranolol complexes leading to the formation of *R*- or *S*-*O*-acetyl-propranolol were identified, which is consistent with our experimental results. In these reactive complexes, both propranolol enantiomers fit into the CalB binding pocket in two binding modes, with the naphthyl or amino group positioned either at the bottom or the top of the binding pocket.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.09.019.

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