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Resolution of (±)-5-substituted-6-(5-chloropyridin-2-yl)-7-*oxo*-5,6-dihydropyrrolo[3,4*b*]pyrazine derivatives-precursors of (*S*)-(+)-Zopiclone, catalyzed by immobilized *Candida antarctica B* lipase in aqueous media

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Abstract—The enzymatic resolution of different cyclopyrrolone compounds has been performed in aqueous systems using different immobilized preparations of lipase from *Candida antarctica* (*fraction B*). The relevance of the immobilization protocol in the results has been shown: lipase immobilized on octadecyl-Sepabeads gave the highest stability and enantioselectivity. Commercial Novozym 435 was scarcely enantioselective in the hydrolytic process. On the other hand, the structure of the cyclopyrrolone was also found to be very important to the outcome of the reaction, the best results being achieved with compounds (±)-**1** and (±)-**2**. Thus, using compound (±)-**2**, a ee_s of >95% can be achieved under conditions in which the enzyme preparation can be utilized in 10 recycles without any significant detriment to the enzymatic properties (activity, enantioselectivity). Moreover, this enzyme catalyzes the hydrolytic resolution of chloromethyl carbonate (±)-**5**, a useful intermediate for the synthesis of the hypnotic agent (*S*)-(+)-Zopiclone.

1. Introduction

(±)-Zopiclone (4-methyl-1-piperazinecarboxylic acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-*oxo*-5*H*-pyrrolo-[3,4*b*]pyrazin-5-yl ester)[1] is a hypnotic agent of the cyclopyrrolone class possessing a pharmaceutical profile similar to that of benzodiazepines, with high efficiency and low toxicity. The (*S*)-(+) enantiomer is less toxic and more active than its enantiomer.[2,3]

The preparative resolution of (±)-Zopiclone has been carried out by the formation of the diastereomeric salts with (+)-*O*,*O*′-dibenzoyltartaric acid[3] or (+)- or (−)-malic acid[2] and selective crystallization of the racemic mixture. Moreover, taking into account that some carbonates have been resolved by enzymatic methods,[4] it is possible to use lipases to resolve precursors of this compound via hydrolysis of suitable intermediates in aqueous media or in the presence of water as a co-solvent. Lipases (triacyglycerol acylhydrolases, EC 3.1.1.3) are the most popular enzymes in biocatalysis[5–12] because they combine wide substrate specificity with high regio- and enantioselectivity, and therefore may be used in many different reactions. When using lipases as chemical catalysts, their peculiar mechanism of action (interfacial activation)[13–15] and the dramatic conformational changes involved[16–20] must be considered. The immobilization of the lipase involving different areas of the enzyme, thus giving different rigidity to the enzyme structure or even generating a certain special microenvironment surrounding the enzyme, can alter the shape of the final open form of the lipase (Scheme 1).

This method for modulating the properties of the enzyme has been used successfully to control the catalytic behavior of different enzymes, which suffer drastic

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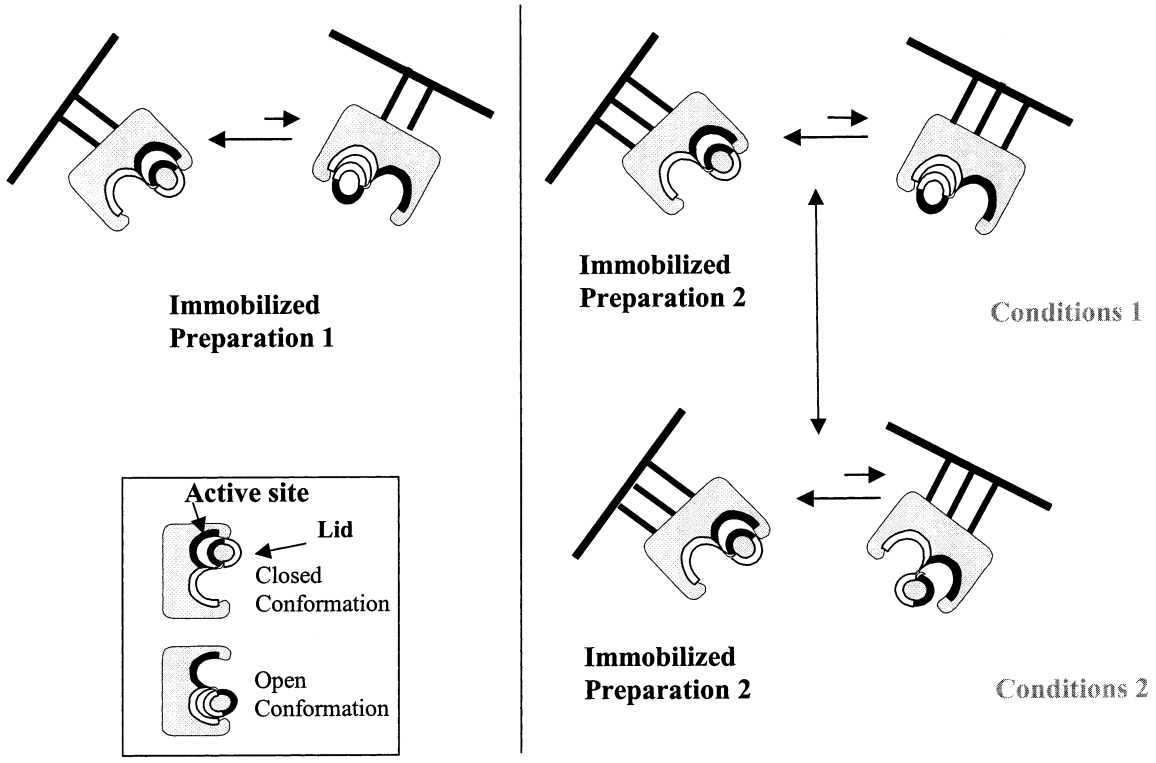


Scheme 1. Conformational engineering of lipases.

conformational changes during catalysis (e.g. penicillin G acylase[16] and lipases[17–20]). In the work reported herein, we have used different immobilization protocols to produce immobilized preparations of lipase from *Candida antarctica* (fraction B) (CAL-B) aiming at finding the optimal immobilized preparation for the hydrolytic resolution of (±)-6-(5-chloropyridin-2-yl)-7-*oxo*-5,6-dihydropyrrolo[3,4*b*]pyrazine derivatives (Scheme 2), intermediates in the production of (*S*)-(+)-Zopiclone.

2. Results and discussion

2.1. Characterization of the different enzyme preparations: use of low substrate concentration

First, the initial activity displayed by different immobilized preparations from CAL-B in the hydrolysis reaction in fully aqueous media was studied. However, even at low substrate concentrations (only 0.05 mM) some co-solvent (10%) was necessary to fully dissolve it. Table 1 summarizes the most significant results.

The reaction was performed at 25°C and pH 7 using dioxane as co-solvent. In this case, due to the high spontaneous chemical hydrolysis of the substrate, special care was needed to design rapid experiments (e.g. in 2 h less than 1% of the substrate was chemically hydrolyzed). The glutaraldehyde and CNBr preparations showed the higher values of activity, being almost two-fold more active than the octadecyl, the glyoxyl or the commercial Novozym 435 preparations. This shows how different immobilization protocols may yield enzymes with very different specificity for different substrates.

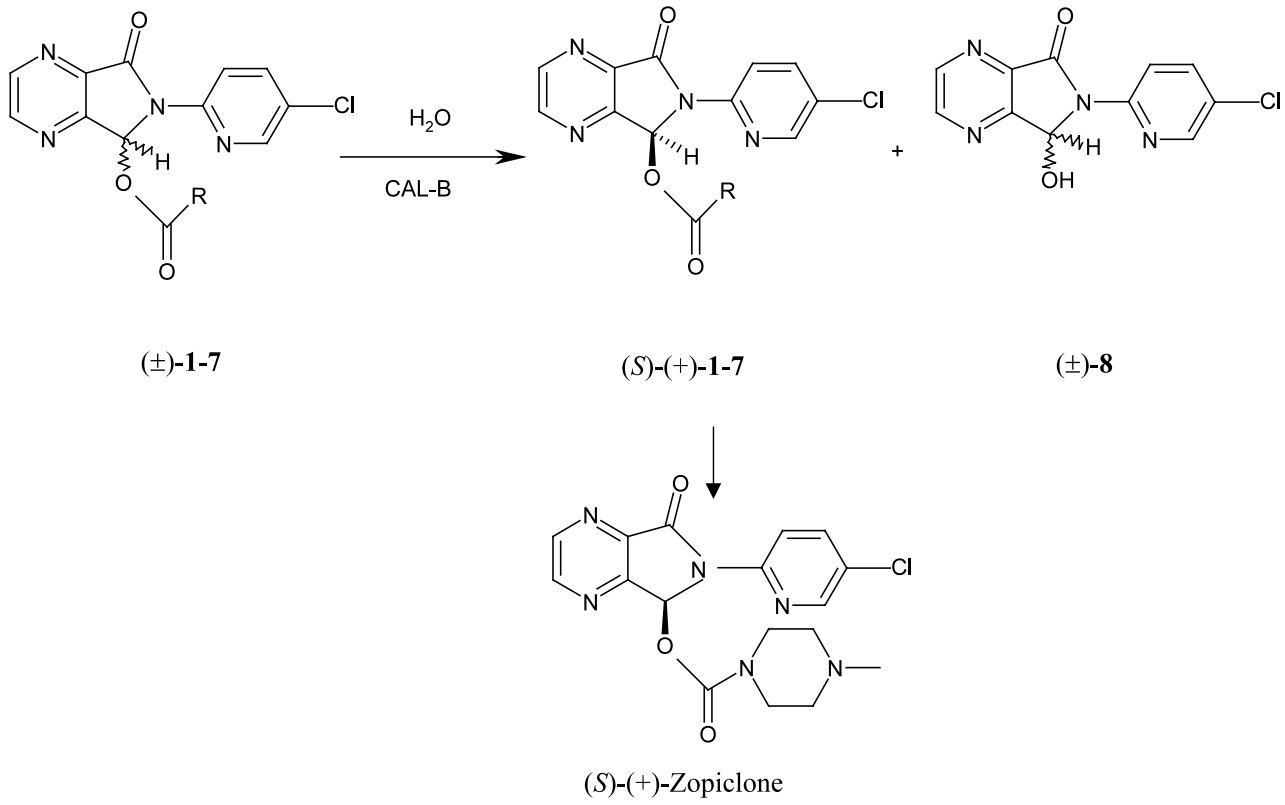
The enantioselectivity was very high (E >100), with a stereochemical preference for hydrolysis of the R isomer, for our lipase-immobilized preparations while Novozym 435 preparation did not show any selectivity.

2.2. Use of higher substrate concentrations

From an industrial point of view, it is convenient to work at higher substrate concentrations. In order to achieve that, higher concentrations of co-solvent are needed. Moreover, it is also necessary to have a biocatalyst resistant to these drastic conditions. Thus, it is important to study the stability of these immobilized preparations under these conditions.

All immobilized preparations remained fully active at 25°C and different pH values (7 or 5) after 150 h of incubation, while the activity of the soluble lipase decreased slightly. However, at 50°C (Table 2) only the octadecyl-Sepabeads preparation retained 100% of activity after this time, while the covalent preparations suffered a decrease in the activity of at least ca. 50% (the glyoxyl preparation being slightly more stable than the glutaraldehyde one). The CNBr preparation retained only 3% of activity and the PEI preparation was completely deactivated after 35 h.

In the presence of 50% (v/v) 1,4-dioxane, only the octadecyl and glyoxyl preparations retained 100% of



Scheme 2. Enantioselective hydrolysis of precursors of Zopiclone catalyzed by different CAL-B immobilized preparations.

Table 1. Enzymatic activity of immobilized preparations of CAL-B in the hydrolysis of (±)-**1**, 0.05 mM and pH 7, 10% dioxane and 25°C

Immobilized preparation	Specific activity (μmol mg lipase^{-1} min^{-1})	Enzyme activity (μmol min^{-1} g biocatalyst)	E (R/S)
CNBr	0.19	2.24	>100
Octadecyl	0.10	1.16	>100
PEI	0.15	1.80	>100
Glyoxyl	0.09	1.08	>100
Glutaraldehyde	0.16	1.87	>100
Novozym sp. 435	–	1.00	1.00

The relative error was estimated to be ±5.

Table 2. Stability of CAL-B immobilized preparations under different conditions. (The numbers refer to the residual activity after 150 h of incubation)

Immobilized preparation	Residual activity (%)		
	50°C	25°C, 50% dioxane	37°C, 40% dioxane
Octadecyl	100	100	100
Glyoxyl	50	100	100
Glutaraldehyde	40	93	57
CNBr	3	80	40
PEI	0 (35 h)	65	30

the activity. The glutaraldehyde preparation lost 7% of the initial activity after 150 h. On the other hand, the activities of the PEI or CNBr preparations decreased by 35 or 20% of the initial activity in the same time period, respectively (Table 2). Under these conditions, the octadecyl-Sepabeads preparation no desorption of the adsorbed enzyme was seen, while with Novozym 435 the enzyme was partially desorbed.

At 37°C and in the presence of 40% (v/v) dioxane (Table 2), again octadecyl-Sepabeads and glyoxyl preparations retained 100% of the initial activity while the glutaraldehyde preparation lost 43% of the activity. In the cases of the CNBr and PEI preparations, these maintained 40 and 30% of the initial activity, respectively.

These results suggested that glyoxyl, glutaraldehyde and octadecyl preparations could be suitable biocatalysts for this biotransformation.

We next performed a study to optimize the concentration of co-solvent in the reaction medium to allow higher substrate ((±)-**1**) concentration. To this end, we used several solvents with different grades of hydrophobicity, trying to reach a substrate concentration of 10 mM (Table 3).

Table 3. Solubility of substrate in a variety of co-solvent in aqueous media

Co-solvent	% (v/v)	Maximum soluble concentration (mM)
1,4-Dioxane	44	<10
	48	10
	52	10
Acetonitrile	44	<10
	48	<10
	52	10
Acetone	44	<10
	48	<10
	52	<10

The best results were found for 1,4-dioxane with which it was possible to dissolve the desired amount of compound (±)-**1** using 48% of co-solvent.

The activity of the different immobilized preparations in the presence of dioxane was analyzed (Table 4). All immobilized preparations suffered a dramatic loss of activity in the presence of the organic solvent (by a factor of 100). However, the activities were still significant.

2.3. Reactions using a concentration of 10 mM of substrate

Next, after the conditions with respect to concentration and co-solvent were optimised, the activity of the immobilized preparations in the hydrolysis reaction using 10 mM of (±)-**1** was examined (Table 5). A comparison of the activities in Tables 2, 4 and 5 suggested that the decrease in enzyme activity in the presence of organic solvents may be due mainly to competitive inhibition by the solvent.

Table 4. Activity of the different immobilized preparations in the hydrolysis of compound (±)-**1** at 0.05 mM, pH 7 and 25°C with 50% (v/v) dioxane

Immobilized preparation	Specific activity ($\times 10^{-3}$)	Enzyme activity ($\times 10^{-2}$)
Octadecyl	0.59	0.71
Glutaraldehyde	0.70	0.85
Glyoxyl	0.75	0.90

Specific activity: μmol/(mg lipase min); enzyme activity: μmol/(g of catalyst min).
The relative error was estimated to be ±5.

The highest activities were obtained with the octadecyl and covalently immobilized enzyme preparations. Moreover, it was possible to observe that, although all preparations presented a very high enantioselectivity, the enzyme interfacially activated[21] on octadecyl Sepabeads preparation was the one with the highest E value, allowing us to achieve the highest enantiomeric excess ee_s >99%, even though 0.5% spontaneous chemical hydrolysis took place (Table 5).

Taking into account all the parameters (activity, stability and enantioselectivity), the octadecyl-Sepabeads preparation seemed the best for this reaction. The reaction rate is very rapid and 45% conversion was reached in 50 min. The reaction became slower at near to 50% conversion and after 48 h the conversion did not surpass 51% hydrolysis, showing the high enantioselectivity of the enzyme.

2.4. Enzymatic resolution of other precursors of Zopiclone

In the previous section it was shown that the enzymatic resolution of substrate (±)-**1** was effective, especially with lipase immobilized on octadecyl-Sepabeads support. Unfortunately, the high cost of the vinyl chloroformate required for the synthesis of compound (±)-**1** is

Table 5. Resolution of substrate (±)-**1** catalyzed by different immobilized CAL-B preparations. The hydrolysis was performed using 10 mM substrate concentration at 25°C, pH 7 and 50% dioxane

Immobilized preparation	Specific activity[a]	Enzyme activity[b]	Enantiomeric preference	Ee_s[c]	E[d]
Octadecyl	0.10	1.20	*R*	>99	>500
Glutaraldehyde	0.12	1.44	*R*	97	277
Glyoxyl	0.13	1.56	*R*	94	115

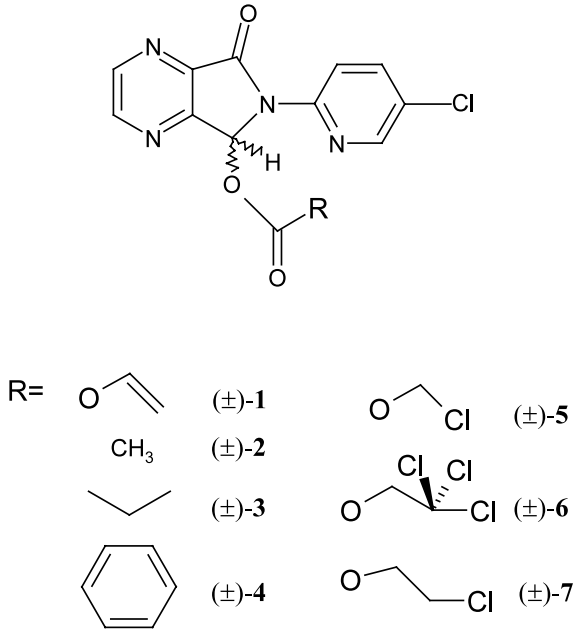
[a] Specific activity: μmol/(mg lipase min).
[b] Enzyme activity: μmol/(g of catalyst min). The relative error was estimated to be ±5.
[c] Ee_s = enantiomeric excess of substrate at 50% of conversion.
[d] E = enantioselectivity.

Table 6. Enantioselective hydrolysis of the different compounds under different reaction conditions catalyzed by octadecyl-Sepabeads preparation

Compound	Substrate concentration (mM)	Dioxane (%)	Temperature (°C)	Activity[a] (μmol mg^{-1} min^{-1})	Relation (w/v)	Ee_s[b]	E[d]
(±)-**1**	10	50	25	0.10	1/30	>99	>500
(±)-**2**	10	30	37	0.66	1/40	>95[c]	>300
(±)-**3**	10	50	25	0.04	1/40	60	7.1
(±)-**4**	0.5	40	25	0	1/30	Nd	Nd
(±)-**5**	10	50	25	1.25×10^{-2}	1/40	>99	>100
(±)-**6**	0.1	40	25	0.50×10^{-5}	1/40	Nd	Nd
(±)-**7**	4	50	25	1.40×10^{-3}	1/40	>99	>100
(±)-**7**	6	50	37	1.67×10^{-3}	1/40	>99	>100

[a] Specific activity: μmol/(mg lipase min). The relative error was estimated to be ±5.
[b] Enantiomeric excess calculated by ^1H NMR.
[c] Ee_s = enantiomeric excess of substrate at 50% of conversion.
[d] E = enantioselectivity.

a drawback for the scale-up of the process. Hence, carbonates (±)-**5**, (±)-**6**, and (±)-**7**, that are also precursors of Zopiclone, were tested under similar conditions. Several ester derivatives [(±)-**2**, (±)-**3**, and (±)-**4**] were also studied in order to evaluate the performance of the octadecyl-Sepabeads preparation (Table 6). Because of the high stability of the enzyme preparation, the limit for the reaction conditions was fixed by the substrate stability (conditions where less than 0.5% of the substrate was chemically hydrolyzed) (Scheme 3).



Scheme 3. (±)-5-Substituted-6-(5-chloropyridin-2-yl)-7-*oxo*-5,6-dihydro pyrrolo[3,4*b*]pyrazine derivatives, precursors of (*S*)-(+)-Zopiclone.

The highest activity for the octadecyl-Sepabeads catalyst was observed in the hydrolysis of the acetyl ester (±)-**2**, a substrate significantly more stable than **1**. The hydrolysis of (±)-**2** was six times faster than that of the reference compound (±)-**1** (increasing almost by a factor of 2 as a result of the higher temperature and around a factor of 3 due to the enhanced enzyme activity against this substrate). For compound (±)-**3** a three-fold decrease in activity relative to (±)-**1** was observed.

Compounds (±)-**4** and (±)-**6** were very water-insoluble and only 0.5 or 0.1 mM concentrations, respectively, could be achieved with 40% dioxane. The immobilized preparation seemed to be inactive towards substrate (±)-**4**, while it was possible to detect some activity with substrate (±)-**6**, although it was very low. On the other hand, compound (±)-**5** presented similar solubility to (±)-**1** although the activity was almost 10-fold lower. Substrate (±)-**7** was less soluble than (±)-**5**, and only 4 mM (at 25°C) and 6 mM (at 37°C) solutions could be prepared using the maximum concentration of dioxane studied (50%). Activity of the octadecyl preparation at both temperatures was lower than that with (±)-**1**.

Regarding the enantioselectivity of these reactions, the immobilized preparation presented a very low value of E in the hydrolysis of compound (±)-**3** affording a 60% ee_s at 50% of conversion.

By contrast, using the other substrates, high enantiomeric excesses after purification were obtained for ester (±)-**2** (ee >95% by ^1H NMR analysis) and carbonates (±)-**5** and (±)-**7** (ee >99% by chiral HPLC analysis).

In the hydrolysis of compound (±)-**2** and octadecyl-Sepabeads preparation as biocatalyst, 50% conversion was obtained in less than 45 min using a ratio of 1:37 grams of biocatalyst/reaction volume (Fig. 1). The specific activity and enantioselectivity of the preparation remained the same over 10 reaction cycles. Fig. 2 shows that the reaction times required to reach a 50% hydrolysis were identical in each cycle, and the ee achieved was in all cases higher than 99%.

From the results shown in Table 6, it can be concluded that the size of the side chain (R) of the carbonate has a remarkable effect on the activity of the catalyst. Good reaction rates can be obtained only when the R group

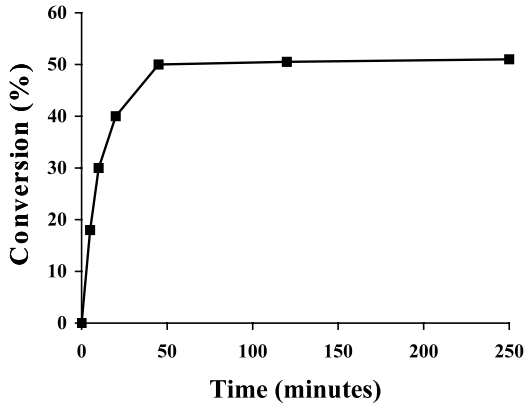


Figure 1. Reaction course of the enzymatic hydrolysis of (±)-**3** catalyzed by octadecyl-CAL-B preparation. Experiments were performed using a concentration of substrate of 10 mM with 30% (v/v) 1,4-dioxane and pH 7, 37°C, as described in Section 4.

is sufficiently small; either with ester (**2** and **3**) or with carbonate (**1** and **5**) derivatives. The best results for the enzymatic resolution were obtained with ester (±)-**2**, which possesses the smallest R group (methyl). Unfortunately, this compound cannot be transformed into enantiopure Zopiclone due to racemization at the stereogenic center in the subsequent steps.[22,23] Nevertheless, the values of activity and enantioselectivity for the resolution of vinyl carbonate (±)-**1** and chloromethyl carbonate (±)-**5** are also quite good, thus this procedure can be employed for the preparation of (*S*)-Zopiclone.

3. Conclusions

The effect of enzyme immobilization on the catalytic properties of different CAL-B preparations in the resolution of several cyclopyrrolone compounds in aqueous media has been studied. This work has demonstrated the strong influence of the enzyme preparation and the nature of the substrate.

The octadecyl-Sepabeads preparation was the most efficient among those examined. This preparation presented the highest enantioselectivity, good activity, and high stability under the harsh reaction conditions. Moreover, it presents an additional advantage, which is the possibility of re-use of the support, thanks to the reversible adsorption of the enzyme on the support. In the presence of surfactant (Triton X-100 4%) it is possible to desorbe all enzyme and to immobilize fresh enzyme.[21]

With this improved catalyst, it has been possible to resolve vinyl carbonate (±)-**1** and chloromethyl carbonate (±)-**5** with high enantioselectivity, thus obtaining the remaining (*S*)-(+) isomers of **1** and **5** with high ee_s. Besides, the acetyl derivative (±)-**2** was the best substrate for the enzyme, giving enantiomeric excess higher than 95% and E >100.

Therefore, the enzymatic resolution of carbonates (±)-**1** and (±)-**5** with octadecyl-Sepabeads immobilized preparation in aqueous media is a comparable alternative to the resolution in organic media with commercial enzyme preparations.[22,23]

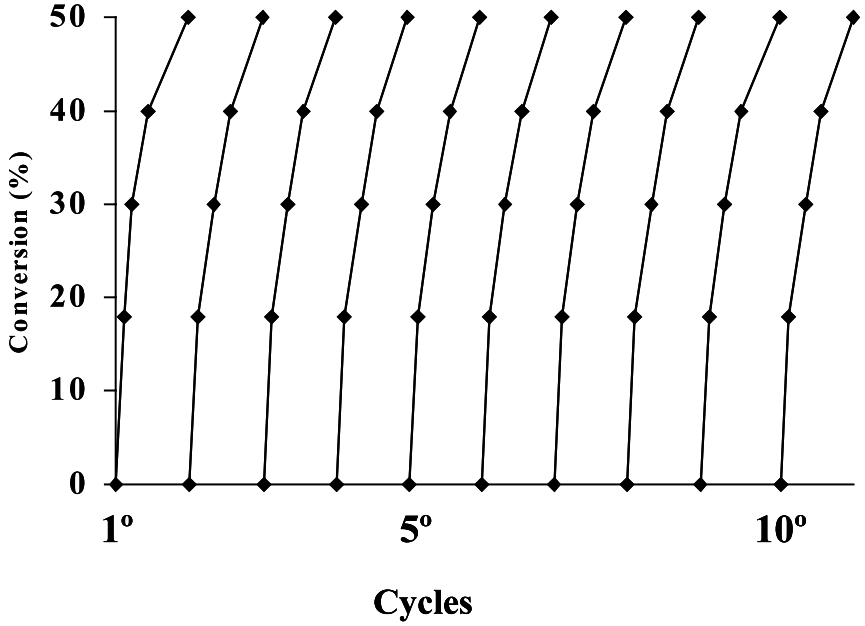


Figure 2. Different cycles of hydrolysis reaction of compound (±)-**3** catalyzed by octadecyl-Sepabeads CAL-B-preparation. Experiments were performed using a substrate concentration of 10 mM with 30% (v/v) 1,4-dioxane and pH 7, 37°C and 50% of conversion was obtained after 45 min of reaction and it was maintained during 10 cycles.

4. Experimental

4.1. Materials

The lipase from *Candida antarctica* (*fraction B*) (Novozym 525 L) (CAL-B) was from Novo Nordisk (Denmark). Octyl-agarose 4BCL and cyanogen bromide (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Octadecyl-Sepabeads was generously donated by Resindion Srl (Mitsubishi Chem. Coorp) (Milan, Italy). Agarose 10 BCL was kindly donated by Hispanagar (Burgos, Spain). Glyoxyl-agarose,[24] glutaraldehyde-agarose[25] and PEI-agarose[26] were prepared as previously described. Polyethyleneimine (PEI) (Mr 25000), glutaraldehyde, Triton X-100, *p*-nitrophenyl propionate (*p*-NPP) were from Sigma. Other reagents and solvents used were of analytical or HPLC grade.

4.2. Purification of CAL-B

In order to purify the lipase, the enzyme preparation was incubated in the presence of octyl-agarose at low ionic strength, following the previously described procedure.[27] Periodically, activity of suspensions and supernatants was assayed by using the *p*-NPP assay as described below. Final immobilization yield was over 95%. After that, adsorbed lipase preparation was abundantly washed with distilled water. To desorbe the enzyme, the adsorbed lipase was washed with Triton X-100 1% in 5 mM sodium phosphate buffer solution at pH 7 and 25°C.

4.3. Immobilization of CAL-B on different supports

Different preparations were produced following the procedures previously described.

(i) Interfacial adsorption on hydrophobic support (octadecyl-Sepabeads[21]). To immobilize the purified lipase in octadecyl support, the enzyme solution (containing 1% triton) was diluted 500–fold with 5 mM sodium phosphate buffer at pH 7.

(ii) Ionically adsorbed lipase on solid supports coated with PEI[25] (ionic microenvironment surrounding large areas of the protein). The immobilization was carried out in 5 mM sodium phosphate buffer pH 7 at 25°C. After immobilization the preparations were washed with distilled water.

(iii) Multipoint covalent immobilization on glyoxyl-agarose beads (through areas with the highest density of lysine (Lys) groups).[24] The immobilization was carried out in 25 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 preparation was washed with an excess of distilled water.

(iv) Covalent immobilization on glutaraldehyde-agarose beads.[24] The immobilization was carried out in 25 mM sodium phosphate buffer pH 7 at 25°C. In order 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 preparation was washed with an excess of distilled water.

(v) Immobilization on CNBr-activated Sepharose 4B using the protocol from Amershan Biosciences[28] (at pH 7, the immobilization should proceed by the most reactive group: the terminal amino).

Activities of suspensions and supernatants were assayed by using the *p*-NPP assay as described below. Protein concentration was determined by the Bradford method.[29] Enzyme load was 12 mg lipase/mL of support and in all cases more than 95% of the lipase activity became immobilized on the different supports.

4.4. Hydrolysis of *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 *p*-NPP (0.4 mM) in 25 mM sodium phosphate buffer at pH 7 and 25°C. To start 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.

4.5. Enzymatic hydrolysis of (±)-1–7

The activities of different immobilized preparations of CAL-B were analyzed in the hydrolysis reaction of compounds (±)-**1**–**7** at different conditions.

Procedure A: substrate (±)-**1** was dissolved at 25°C in a mixture of 10 mM sodium phosphate buffer (pH 7, 10 mL) with 10 or 50% (v/v) 1,4-dioxane to a concentration of 0.05 mM and 300 mg of enzymatic preparation were added to this solution.

Procedure B: substrate (±)-**1** was dissolved at 25°C in a mixture of 10 mM sodium phosphate buffer solution (pH 7, 3.5 mL) with 50% (v/v) 1,4-dioxane to a concentration of 10 mM and enzymatic preparation (120 mg) was added to this solution.

The enzymatic activity of the octadecyl-Sepabeads preparation in the hydrolysis of compounds (±)-**2**–**7** was determined under the following conditions.

Substrate (±)-**2** was dissolved at 37°C in 10 mM sodium phosphate buffer solution (pH 7, 8 mL) at with 30% (v/v) dioxane to a concentration of 10 mM and octadecyl-Sepabeads (200 mg) were added.

Substrate (±)-**3** was dissolved at 25°C in a mixture of 10 mM sodium phosphate buffer solution (pH 7, 8 mL) with 50% (v/v) dioxane to a concentration of 10 mM and octadecyl-Sepabeads (200 mg) were added.

Substrate (±)-**4** was dissolved at 25°C in a solution of 10 mM sodium phosphate buffer (pH 7, 8 mL) with 40% (v/v) dioxane to a concentration of 0.5 mM and the enzymatic preparation (250 mg) was added.

Substrate (±)-**5** was dissolved at 25°C in 10 mM buffer solution (4 mL) at pH 7 with 50% (v/v) dioxane to a concentration of 10 mM and biocatalyst (100 mg) was added.

Substrate (±)-**6** was dissolved at 25°C in 10 mM sodium phosphate buffer solution (pH 7, 4 mL) at with 40% (v/v) dioxane to a concentration of 0.1 mM of substrate and immobilized preparation (100 mg) was added.

Finally, substrate (±)-**7** was dissolved at 25°C in solution of 10 mM sodium phosphate buffer (8 mL) at pH 7 with 50% (v/v) dioxane to a concentration of 4 mM of substrate and the biocatalyst (200 mg) was added. Moreover, it was possible to dissolve up substrate to a concentration of 6 mM at 37°C using the same conditions in the activity analyses.

During the course of the reactions, the pH of the mixture was kept constant by automatic titration using a pH-stat Mettler Toledo DL50 graphic. The specific and enzymatic activities were defined as μmol of substrate hydrolyzed per minute per mg of immobilized protein or per gram of immobilized preparation in each case.

The degrees of hydrolysis were quantified by reverse-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). The elution was isocratic with a mobile phase of acetonitrile (50%) and 10 mM ammonium phosphate buffer (50%) at pH 6.9, a flow rate of 1.5 mL/min, the adsorbance at 270 nm and at 20°C. The retention times (t_r) were: (±)-**8** (2.98 min), (±)-**1** (11.3 min), (±)-**2** (6.33 min), (±)-**3** (14.75 min), (±)-**4** (18.5 min), (±)-**5** (11 min), (±)-**6** (27.5 min), (±)-**7** (9.9 min).

4.6. Determination of enantiomeric excess and *E* value

With substrate (±)-**1**, the enantiomeric excess (ee_s) of the remaining ester was analyzed by Chiral HPLC at different conversion degrees. The column was a Chiracel OD, the mobile phase was an isocratic mixture of 40% hexane, 60% ethanol and 0.1% triethylamine at a flow rate 0.6 mL/min; detection at 254 nm. Optical purity analyses of compound (±)-**2** was performed by ^{1}H NMR using europium tris[3-(heptafluoropropylhydroxy methylene)-(+)-camphorate]) as a chiral shift reagent in $CDCl_3$.

With chloro carbonates (±)-**5** and (±)-**7** the enantiomeric excesses (ee_s) were determined by chiral HPLC analysis using a CHIRALPAK AS column (4.6×250 mm). Two peaks were obtained for (±)-**5** with a mobile phase of 60% hexane, 35% ethanol and 5% *i*-propanol, at a flow rate of 1 mL/min, at 254 nm. (*S*)-(+)-**5**, t_R 10.34 min; (*R*)-(−)-5, t_R 15.28 min. In the case of substrate (±)-**7**, the mobile phase was a mixture of 60% hexane, 40% *i*-propanol, at a flow rate of 0.8 mL/min, 254 nm. (*S*)-(+)-**7**, t_R 26 min; (*R*)-(−)-**7**, t_R 32.8 min.

The enantiomeric ratio was expressed as the *E* value calculated from the enantiomeric excess (ee_s) of the remaining ester and the conversion degrees (c) according to method previously reported by Chen et al.[30]

4.7. Synthesis of compounds

Optical rotations were measured using a Perkin–Elmer 241 polarimeter and are quoted in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H and ^{13}C NMR spectra were obtained with TMS (tetreamethylsilane) as internal standard using a Bruker AC-300 (^{1}H-300 MHz and ^{13}C-75.5 MHz) spectrometer. Mass spectra were recorded on a Hewlett–Packard 1100 LC/MSD.

4.7.1. (±)-6-(5-Chloropyridin-2-yl)-7-*oxo*-5-(vinyloxycarboyloxy)-5,6-dihydropyrrolo[3,4*b*]pyrazine, (±)-1. Vinyl chloroformate (0.40 mL, 4.38 mmol, 1.15 equiv.) was added to a suspension of (±)-**8** (1.0 g, 3.81 mmol) and anhydrous pyridine (1.2 mL) in anhydrous dichloromethane (8 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 10 h (it was monitored by TLC until the initial product was consumed). The mixture was extracted with water and dichloromethane. The organic phase was dried over $NaSO_4$, filtered and concentrated under reduce pressure. Yield: 75%, white powder, mp = 130–132°C; IR (KBr): 1770, 1736 cm^{-1}, ^{1}H NMR ($CDCl_3$), δ (ppm): 8.91 (dd, 2H, 2CH), 8.52 (d, 1H, CH, $^3J_{HH}$ = 8.98 Hz), 8.40 (d, 1H, CH, $^4J_{HH}$ = 2.58 Hz), 8.02 (s, 1H, CH), 7.82 (dd, 1H, CH, $^4J_{HH}$ = 2.58 Hz, $^3J_{HH}$ = 8.98 Hz), 7.20 (dd, 1H, CH, $^3J_{trans}$ = 6.16 Hz, $^2J_{gem}$ = 13.84 Hz), 4.95 (dd, 1H, CH, $^3J_{cis}$ = 2.30 Hz, $^2J_{gem}$ = 13.84 Hz), 4.69 (dd, 1H, CH, $^3J_{cis}$ = 2.30 Hz, $^3J_{trans}$ = 6.14 Hz). ^{13}C NMR ($CDCl_3$): 162.3 (C=O), 153.9 (C=O), 151.0 (C), 148.3 (CH), 148.2 (CH), 147.2 (C), 146.5 (CH), 143.7 (C), 142.2 (CH), 138.1 (CH), 128.3 (C), 115.6 (CH), 98.8 (CH_2), 80.3 (CH). EM (ESI$^+$, m/z): 334 (M+H)$^+$, 356 (M+Na)$^+$.

4.7.2. 6-(5-Chloropyridin-2-yl)-5-(*O*-acetyl)-7-*oxo*-5,6-dihydropyrrolo[3,4*b*]pyrazine, (±)-2. Acetyl chloride (0.33 mL, 4.57 mmol, 2 equiv.) was added to a suspension of (±)-**8** (600 mg, 2.29 mmol) and anhydrous pyridine (0.74 mL) in anhydrous dichloromethane (10 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 5 h (it was monitored by TLC until the initial product was consumed). The mixture was extracted with water and dichloromethane. The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure. Yield: 85%; mp 170–173°C; IR (KBr): 1735 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 8.87 (dd, 2H, 2 CH), 8.51 (d, 1H, CH, $^3J_{HH}$ = 8.70 Hz), 8.37 (d, 1H, CH, $^4J_{HH}$ = 2.56 Hz), 8.13 (s, 1H, CH), 7.80 (dd, 1H, CH, $^4J_{HH}$ = 2.56 Hz, $^3J_{HH}$ = 8.70 Hz), 2.11 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 170.0 (C=O), 163.5 (C), 155.7 (C=O), 149.0 (CH), 148.6 (CH), 148.2 (C), 147.4 (CH), 144.6 (C), 138.8 (CH), 129.0 (C), 116.7 (CH), 77.9 (CH), 21.4 (CH_3). MS (ESI$^+$) m/z (%): 327 [(M+Na)$^+$, 100%].

4.7.3. 6-(5-Chloropyridin-2-yl)-5-(*O*-butyryl)-7-*oxo*-5,6-dihydropirrolo[3,4*b*]pyrazine, (±)-3. Butyryl chloride (0.47 mL, 4.57 mmol, 2 equiv.) was added to a suspension of (±)-**8** (600 mg, 2.29 mmol) and anhydrous pyridine (0.74 mL) in anhydrous dichloromethane (10 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 5 h (it was monitored by TLC until the initial product disappeared). The mixture was extracted with water and dichloromethane. The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure. Yield: 87%; mp 139–142°C; IR (KBr): 1724 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.85 (dd, 2H, 2CH), 8.49 (d, 1H, CH, $^3J_{HH}=8.76$ Hz), 8.33 (d, 1H, CH, $^4J_{HH}=2.55$ Hz), 8.13 (s, 1H, CH), 7.78 (dd, 1H, CH, $^4J_{HH}=2.55$ Hz, $^3J_{HH}=8.76$ Hz), 2.30 (t, 2H, CH_2), 1.62 (q, 2H, CH_2), 0.97 (t, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 172.6 (C=O), 163.5 (C), 155.8 (C=O), 149.0 (CH), 148.5 (CH), 148.2 (C), 147.3 (CH), 144.6 (C), 138.8 (CH), 129.0 (C), 116.6 (CH), 77.7 (CH), 36.5 (CH_2), 18.8 (CH_2), 14.1 (CH_3). MS (ESI^+) m/z (%): 411 [$(M+Na)^+$, 100%], 355.

4.7.4. 6-(5-Chloropyridin-2-yl)-5-(*O*-benzoyl)-7-*oxo*-5,6-dihydropyrrolo[3,4*b*]pyrazine, (±)-4. Benzoyl chloride (0.44 mL, 3.81 mmol, 2 equiv.) was added to a suspension of (±)-**8** (500 mg, 1.90 mmol) and anhydrous pyridine (0.62 mL) in anhydrous dichloromethane (10 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 4 h (it was monitored by TLC until the initial product disappeared). The mixture was extracted with water and dichloromethane. The organic phase was treated with sodium sulfate, filtered and concentrated until completely dry. Yield: 89%; mp 212–216°C; IR (KBr): 1732 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.86 (dd, 2H, 2CH), 8.56 (d, 1H, CH, $^3J_{HH}=8.72$ Hz), 8.41 (s, 1H, CH), 8.28 (d, 1H, CH, $^4J_{HH}=2.52$ Hz), 7.96 (dd, 1H, CH, $^4J_{HH}=2.52$ Hz, $^3J_{HH}=8.72$ Hz), 7.79 (dd, 2H, 2CH), 7.55 (d, 1H, CH), 7.42 (t, 2H, 2CH); ^{13}C NMR ($CDCl_3$) δ (ppm): 166.1 (C=O), 163.6 (C), 155.9 (C=O), 149.1 (CH), 148.6 (CH), 148.1 (C), 147.5 (CH), 144.7 (C), 138.8 (CH), 134.4 (CH), 130.6 (CH), 129.3 (C), 129.1 (CH), 129.0 (C), 116.5 (CH). MS (ESI^+) m/z (%): 389 [$(M+Na)^+$, 60%], 405 [$(M+K)^+$, 100%].

4.7.5. 5-(Chloromethyloxycarbonyloxy)-6-(5-chloropyridin-2-yl)-7-*oxo*-5,6-dihydropyrrolo[3,4*b*]pyrazine, (±)-5. Chloromethyl chloroformate (0.8 mL, 7.61 mmol) was added to a solution of the (±)-**8** (1 g) and anhydrous pyridine (1.2 mL) in anhydrous dichloromethane (10 mL) under N_2 at 0°C.

The mixture was stirred at 25°C for 17 h (it was monitored by TLC until the initial product disappeared). After that, it was extracted with water and dichloromethane. The organic phase was treated with sodium sulfate, filtered and evaporated under reduced pressure. Yield: 86%, mp 135–137°C; IR (cm^{-1}): 1748, 1804, ^1H NMR ($CDCl_3$), δ (ppm): 8.89 (dd, 2H, 2CH), 8.50 (d, 1H, CH, $^2J_{HH}=8.85$ Hz), 8.37 (d, 1H, CH, $^3J_{HH}=8.85$ Hz), 7.97 (s, 1H, CH), 7.80 (dd, 1H, CH, $^2J_{HH}=2.52$ Hz, $^3J_{HH}=8.88$ Hz), 5.81 (dd, 2H, CH_2), ^{13}C NMR ($CDCl_3$), δ (ppm): 163.0 (C=O), 154.5 (C=O), 152.8 (C), 149.1 (CH), 148.0 (C), 147.4 (CH), 144.5 (C), 139.0 (CH), 129.2 (C), 116.3 (CH), 81.6 (CH), 73.2 (CH_2). EM-ESI+: [M+Na] = 376.9, [M+H] = 355.0.

4.7.6. 6-(5-Chloropyridin-2-yl)-7-*oxo*-5-(2,2,2-trychloroethyloxycarbonyloxy)-5,6-dihydropyrrolo-[3,4*b*]pyrazine, (±)-6. 2,2,2-Trichloroethyl chloroformate (0.8 mL) was added to a solution of (±)-**8** (1 g) and anhydrous pyridine (1.2 mL) in anhydrous dichloromethane (30 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 5 h (it was monitored by TLC until the initial product disappeared). After that, it was extracted with water and dichloromethane. The organic phase was treated with sodium sulfate, filtered and evaporated under reduced pressure. Yield: 98%, mp 201–203°C; IR (cm^{-1}): 1788, 1745, ^1H NMR ($CDCl_3$), δ (ppm): 8.91 (dd, 2H, 2CH), 8.52 (d, 1H, CH, $^2J_{HH}=8.77$ Hz), 8.34 (d, 1H, CH, $^3J_{HH}=2.31$ Hz), 8.03 (s, 1H, CH), 7.82 (dd, 1H, CH, $^2J_{HH}=8.72$ Hz, $^3J_{HH}=2.56$ Hz), 4.91 (m, 2H, CH_2), ^{13}C NMR ($CDCl_3$), δ (ppm): 162.4 (C=O), 153.9 (C=O), 152.5 (C), 148.3 (CH), 147.3 (C), 146.6 (CH), 143.8 (C), 138.3 (CH), 128.6 (C), 115.8 (CH), 93.8 (C), 80.8 (CH), 76.4 (CH_2). EM-ESI+: [M+Na] = 458.9.

4.7.7. 5-(2-Chloroethyloxycarbonyloxy)-6-(5-chloropyridin-2-yl)-7-*oxo*-5,6-dihydropyrrolo-[3,4*b*]pyrazine, (±)-7. 2-Chloroethyl chloroformate (0.8 mL) was added to a suspension of (±)-**8** (1 g) and anhydrous pyridine (1.2 mL), in anhydrous dichloromethane (20 mL) under N_2 at 0°C. The mixture was stirred at 25°C for 7 h (it was monitored by TLC until the initial product disappeared). After that, it was extracted with water and dichloromethane. The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure. Yield: 98%, mp 177–178°C; IR (cm^{-1}): 1766, 1741, ^1H NMR ($CDCl_3$), δ (ppm): 8.86 (dd, 2H, 2CH), 8.52 (d, 1H, CH, $^2J_{HH}=8.98$ Hz), 8.39 (d, 1H, CH, $^3J_{HH}=2.58$ Hz), 7.99 (s, 1H, CH), 7.82 (dd, 1H, CH, $^2J_{HH}=8.98$ Hz, $^3J_{HH}=2.58$), 4.53 (m, 2H, CH_2), 3.74 (t, 2H, CH_2), ^{13}C NMR ($CDCl_3$), δ (ppm): 162.8 (C=O), 154.6 (C=O), 153.6 (C), 148.6 (CH), 148.6 (CH), 147.8 (C), 147.1 (CH), 144.3 (C), 138.6 (CH), 128.9 (C), 116.3 (CH), 80.8 (CH), 68.5 (CH_2), 41.3 (CH_2). EM-ESI+: [M+Na] = 391.0.

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