BABEŞ-BOLYAI University

CLUJ-NAPOCA

Faculty of Chemistry and Chemical Engineering

Stereoselective aspects in the chemoenzymatic synthesis of (*N*-alkyl-phenothiazin-3-yl) ethanols

Summary

Scientific advisor:

Prof. Dr. Ing. Florin-Dan Irimie

PhD student:

Anca Munceanu

Cluj-Napoca

2012

Table of contents

Keywords: lipase, biotransformation, 1-(10-alkyl-10*H*-phenothiazin-2, 3-yl) ethanols, enzymatic kinetic resolution, immobilization of lipases

1. Introduction

Numerous enantiopure secondary alcohols and their derived amines containing heterocyclic fragments are known for their biological activity[1] others are key intermediaries in the synthesis of a large number of pharmaceutical products[2]

Various chemo- and biocatalytic methodologies based on the enantiomer selective kinetic resolution of racemates and enantiotope selective transformation of prochiral substrates have been developed for the synthesis of optically active compounds[3].

The lipase mediated kinetic resolution of some heteroaryl-1-ethanols has been already successfully performed[4]. Phenothizines based structures are known for their important biological activity covering a plethora of pharmaceutical effects[5].

The simple molecular architecture of 1-substituted-ethanols, which allows subsequent derivatisation and our earlier results for the enzymatic synthesis of highly enantiomerically enriched phenothiazin-3-yl-cyanohydrins[6] and β-hydroxy propanoic acids[7] has attracted our interest to develop an enzymatic procedure for the synthesis of optically pure *N*-alkylated phenothiazin-2,3yl-ethanols.

Encouraged by the recently described enantiotope selective reduction of 2-acetyl-phenothiazine in presence of spiroborate esters as chiral catalysts which provided the enantiopure (*R*)-phenotiazin-2yl-ethanol[8], first the cellular reduction with baker's yeast of *N*-alkylated phenothiazin-3yl-ethanones was investigated. Unfortunately, only low conversion (< 5 %) of the substrates was observed.

In contrast to the cellular biotransformation of the ketones, the lipase mediated kinetic resolution of the racemic *N*-alkylated phenothiazin-3yl-ethanols and of the corresponding esters proved to be efficient methods for the production of optically pure *N*-alkylated phenothiazin-3yl-ethanols and esters.

Moreover, the absolute configuration of the obtained enantiopure compounds was determined by X-ray diffractometry.

With the aim to obtain highly enantiomerically enriched or optically pure heterocyclic ethanols[9], the synthesis of both enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol by enzymatic kinetic resolution of the racemic alcohol or his corresponding acetate was studied.

With the aim to apply these results in industrial processes, the use of an efficient, cheap and stable catalyst is necessarily.

Lipases from *Pseudomonas fluorescens* (PFL) belongs to commonly used commercial lipases. Their immobilization using adsorption[10], cross-linking[11], sol-gel encapsulation[12] combination of the methods[13] or other techniques[14] has been under extensive studies. The most used method for improvement of biocatalysts performance is their immobilization. Many effective immobilization methods have been developed, including binding to a carrier, cross-linking and encapsulation in an organic or inorganic polymeric matrix.

In the present work, PFL encapsulated in sol-gels was prepared and studied for the enzymatic kinetic resolution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol and their acetate, for obtaining both enantiomers of the heterocyclic ethanol, a valuable intermediate for different chiral active compounds. The stability, activity and reusability of enzyme preparations were determined.

2. Teoretical part (literature review)

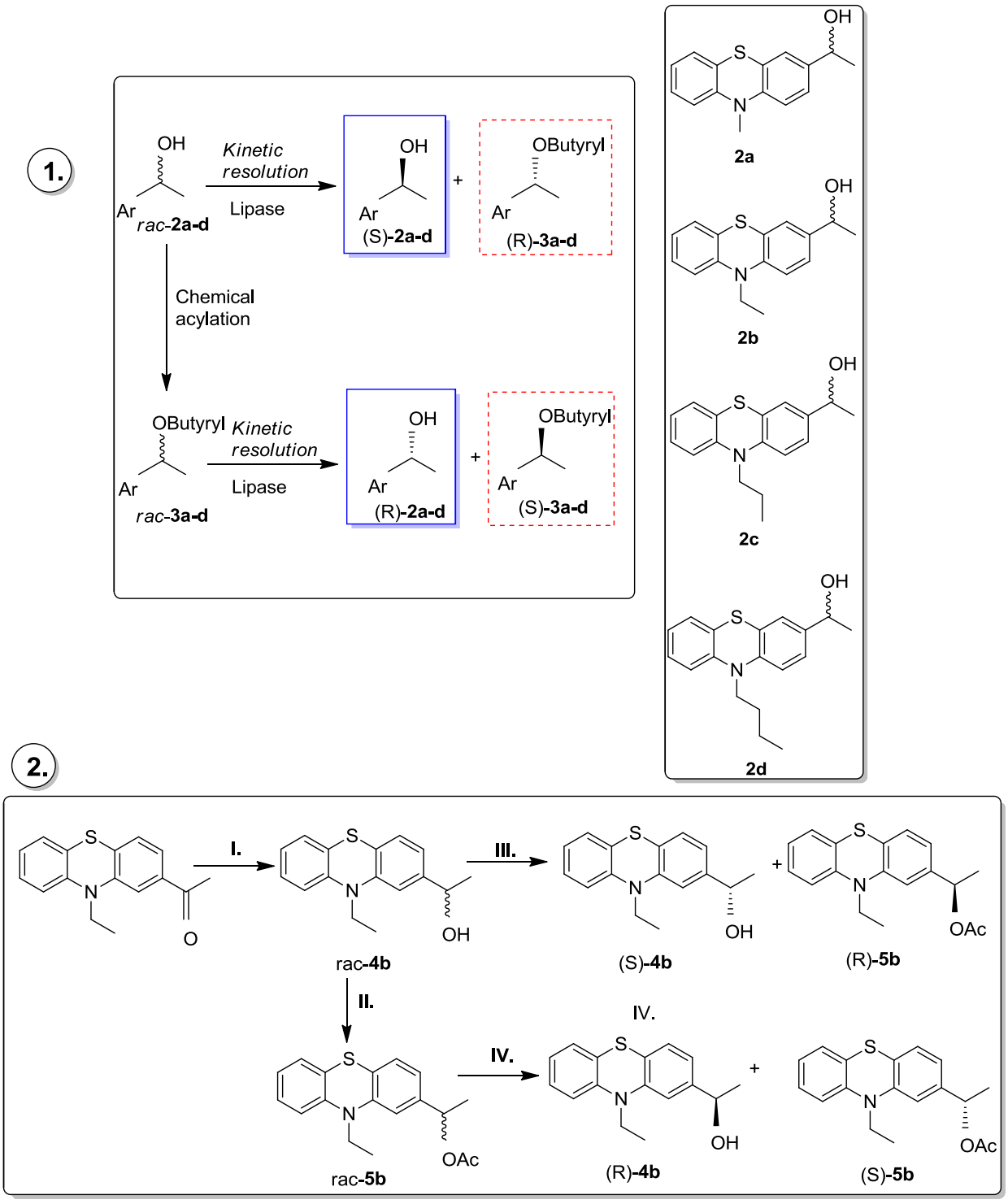
3. Aim of the study

1a. Development of an efficient and new stereoselective procedure for synthesis of both enantiomers of 1-(10-alkyl-10*H*-phenothiazin-3-yl) ethanols (**2a-d**) and their butanoates **3a-d** by enantiomer selective kinetic resolution lipase mediated of alcoholic and esteric racemates. It is known that lipases usually retain their enantiomer preference in acylation and in hydrolysis or alcoholysis[15]. Consequently, such reactions should result the opposite enantiomeric forms of the reaction counterparts. A good result was obtained earlier for the enzymatic synthesis of highly enantiomerically enriched phenothiazin-3-yl- cyanohydrins[6] and β-hydroxy propanoic acids[7].

1b. Increasing of enantiomer selectivity and activity of enzyme by selecting the most favourable solvent and nucleophil agents.

2a. The synthesis of opposite enantiomeric forms**,** (*R*) and (*S*) of 1-(10-ethyl-10*H*-phenothiazin-2-yl) ethanol **4b** and its corresponding acetate **5b** by kinetic resolution of the racemic *rac*-**4b** şi *rac*-**5b** in the pressence of PFL. Determination of optimal conditions for enzymatic acylation and enzymatic alcoholysis.

2b. Improving the catalytic performance of PFL by sol-gel encapsulation. Evaluation of stability, activity and reusability of the enzyme preparation in enzymatic kinetic resolution of heteroaryl-1-ethanols, *rac*-**4b.**



Scheme 1 **1. Biotransformation of *N*-alkylated phenothiazin-3yl-ethanols and their esters**

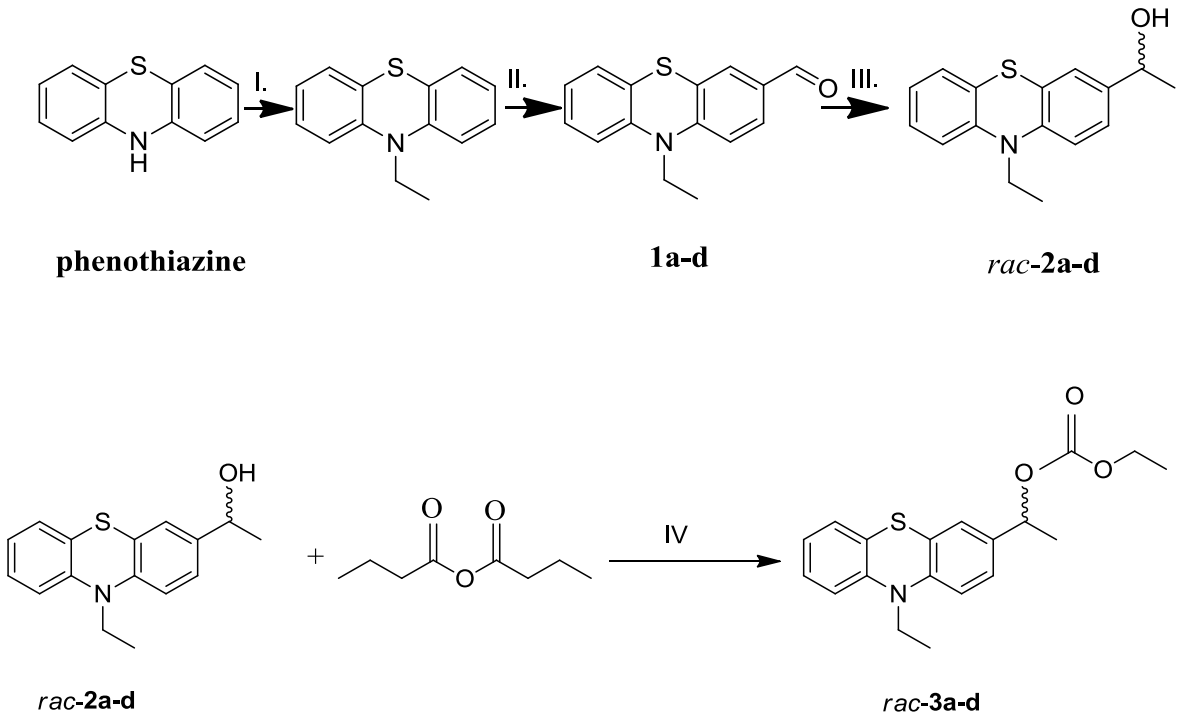
2. The synthesis of (*R*) şi (*S*) enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanols and his corresponding acetate by*Pseudomonas fluorescens* lipase

4. Results and discussion

4.1. Synthesis and biotransformation of *N*-alkylated phenothiazin-3yl-ethanols and of the corresponding esters

4.1.1. Chemical synthesis

Racemic 1-(10-alkyl-10*H*-phenothiazin-3-yl) ethanols (*rac*-**2a-d**) were prepared by the Grignard reaction starting from (10-alkyl-10*H*-phenothiazin-3-yl) carbaldehydes **1a-d** synthesized as previously described by prof Irimie team[16]. During the work-up and purification procedures the instability of *N*-substituted phenothiazin-3-yl-ethanols (*rac*-**2a-d**) was observed. While at room temperature and neutral pH they are relatively stable, slowly decomposing compounds, at higher temperature or at lower pH the easy water elimination could be the explanation for the high instability of *rac*-**2a-d**. This phenomenon is in good concordance with the earlier reported[17] water elimination even at low temperature from various phenylethanols in presence of zeolites (solid-state acids) or silica gel, forming the corresponding unsaturated compounds. During the isolation by vacuum distillation of *rac*-**2a-d** from solutions the stability of the main compounds was significantly increased by adding aliphatic alcohols into the media. *Rac*-**2a-d** served for the preparation of the racemic esters *rac*-**3a-d** by the chemical acylation with various acid anhydrides in dry dichloromethane, in presence of triethylamine and a catalytic amount of DMAP (Scheme 22). In contrast to *rac*-**2a-d** the esteric compounds *rac*-**3a-d** were stable at higher temperature (t <80 ºC), but they easily hydrolyzed even in presence of weak acids or bases.



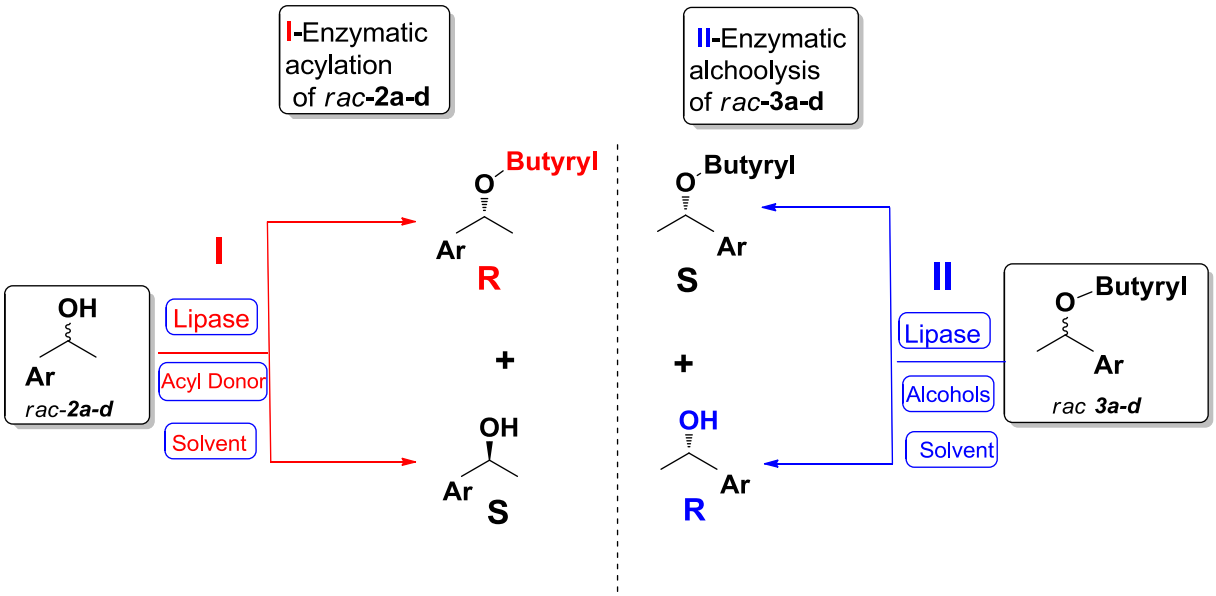
Scheme 22. Chemical synthesis of *rac*-**2a-d** and *rac*-**3a-d**

4.1.2. Enzymatic synthesis

To investigate the stereoselectivity of the reactions involving chiral 1-(10-alkyl-10*H*-phenothiazin-3-yl) ethanols (*rac*-**2a-d**) and their esters (*rac*-**3a-d**), the chromatographic separation of the enantiomers was first established.

The base-line separation of all the enantiomers of *rac*-**2,3a-d** was performed using various HPLC chiral columns.

4.1.2.1. Analytical scale biotransformations



Acyl donor : vinyl acetate, vinyl propionate
vinyl butyrate,vinyl pyvalate;

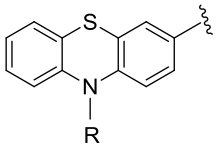
Solvent : dichlormethane, chloroform, THF, *t*-MBE, toluene, acetonitrile;

Lipase : CaL-A, CaL-B, L-AK, Lypozyme TL IM,F, PPL, *Mucor miehei,BCL;*

Alcohols: MeOH,EtOH,*n*-PrOH, *n*-BuOH;

Solvent: *n*-hexan, acetonitrile, dioxane;

Lipase : CaL-B;

Ar: 

Scheme 24. Analitical scale biotransformation of *rac*-**2a-d** and *rac*-**3a-d**

Analytical scale enzymatic acylation of *rac*-2a-d

The analytical scale enantiomer selective enzyme catalyzed acylation of racemic 1-(10-alkyl-10*H*-phenothiazin-3-yl)-ethanols (*rac*-**2a-d**) was first studied. Using the racemic 1-(10-ethyl-10*H*-phenothiazin-3-yl) ethanol (*rac*-**2b**) as model compound, a wide selection of commercial lipases was screened for the enzyme-catalyzed acetylations with vinyl acetate as acylating agent and solvent (**Scheme 24**, **Table 1**).

Several commercial lipases such as lipase B from *Candida antarctica* (CaL-B, formulated as Novozym 435), lipase L-AK, lipase from *Burkoholderia cepacia* (BCL), Lipozyme TL IM and lipase

from *Mucor miehei* exhibited high enantiomer selectivity and activity (E>200, c= 24-50% after 6 h, **Table 1**, entries 2-6), while lipase A from *Candida antarctica* (CaL-A) was showed high activity but moderate selectivity (**Table 1**, entry 1). Lipase F and lipase from porcine pancreas (PPL) exhibited only moderate enantiomer selectivities and low activities (E 13 and 14 respectively, see **Tables 1**, entries 7-8), while lipases from *Candida rugosa*, *Candida cyclindracae*, *Rhizopus arrhizus*, *Mucor javanicus* and *Candida lypolitica* were inactive and nonselective enzymes (c<11%, E<3). As it was expected, almost all enzymes followed the so-called Kazlauskas rule predicting the (*R*)-enantiomer preference of lipases. Only lipase F exhibited opposite enantiomer preference and resulted in the formation of the (*S*)-**3b** (**Table 1**, entry 7).

Table 1. Lipase-catalyzed acetylation of *rac*-**2b** with vinyl acetate

Entry	Enzyme	Time (h)	C (%)	ee $_{(R)\text{-}\mathbf{3b}}$ (%)	ee $_{(S)\text{-}\mathbf{2b}}$ (%)	E
1	CaL-A	4	40,5	63,5	88,2	30
2	CaL-B	6	50,4	98,4	>99,5	>>200
3	L-AK	6	50,3	98,8	>99,5	>>200
4	BCL	6	40	99,5	66,9	>200
5	Lypozime TL IM	6	24,5	99,0	32,1	>200
6	Lipase from Mucor miehei	6	30,9	98,6	44,1	200
7	Lipase F[a]	48	4,8	85,7	4,3	13
8	PPL	6	1,7	86,6	1,5	14

[a] inverse selectivity

It is known that the nature of the solvent and the acyl donor could significantly influence the selectivity of the enantiomer selective acylation. The acylation of *rac*-**2b** with vinyl acetate in presence of L-AK in several solvents was tested (**Table 2**). Chloroform proved to be the most appropriate solvent for the acetylation (E >> 200, c= 49.8% after 9 hours, **Table 2**, entry 2).

Table 2. Lipase catalyzed acetylations of *rac*-**2b** with L-AK in different solvents

Entry	Solvent	C[a](%)	$ee_{(R)\text{-}\mathbf{3b}}$ (%)	$ee_{(S)\text{-}\mathbf{2b}}$(%)	*E*
1	Dichlormethane	44,3	99,3	78,9	>200
2	Chloroform	49,8	99,3	98,5	>200
3	THF	41,9	97,8	70,5	194
4	*t*-BME	50,4	97,8	99,4	>200
5	Toluene	50,1	98,4	98,7	>200
6	Acetonitrile	49,9	98,2	97,6	>200

The acylation of *rac*-**2b** was also performed with other three acyl donors: vinyl propionate, vinyl butyrate and vinyl pivalate in chloroform. It was found that the highest selectivity and reactivity for the L-AK catalyzed acylation of *rac*-**2b** was performed with vinyl butanoate (E >> 200). Similar good results were found using the same conditions for the enzymatic acylation of other *rac*-**2a, c, d** (**Table 3**, entries 5-8).

Table 3. Acylations of *rac*-**2a-d** in presence of L-AK in chloroform

Entry	Acilating agent	Compound	Time(h)	C(%)	$ee^{c}_{(R)\text{-}\mathbf{3}}$(%)	$ee^{c}_{(S)\text{-}\mathbf{2}}$(%)	*E*
1	Vinyl acetate[a]	**2b**	9	49,8	99,3	98,5	>200
2	Vinyl propionate[a]	**2b**	10	44,9	99,5	81,1	>200
3	Vinyl butyrate[a]	**2b**	10	42	>99,8	72,6	>>200
4	Vinyl pyvalate[a]	**2b**	50	1	>99,8	1	>200
5	Vinyl butyrate[b]	**2a**	24	50	>99,8	>99,8	>>200
6	Vinyl butyrate[b]	**2b**	27	50	>99,8	>99,8	>>200
7	Vinyl butyrate[b]	**2c**	28	50	>99,8	>99,8	>>200
8	Vinyl butyrate[b]	**2d**	30	50	>99,8	>99,8	>>200

[a] substrate-L-AK ratio 1:2

[b] substrate-L-AK ratio 1:1

[c] the detection limit on chiral HPLC analysis was 0.1%

Analytical scale enzymatic alcoholysis of *rac*-3a-d

It is known that lipases usually retain their enantiomer preference in acylation and in hydrolysis or alcoholysis[15]. Consequently, such reactions should result the opposite enantiomeric forms of the reaction counterparts.

A wide selection of commercial hydrolases was screened to investigate the enantioselectivity of the analytical scale enzymatic lysis of *rac*-**3b**. The reactions were performed in neat anhydrous methanol, ethanol, propanol and butanol, followed by experiments in various solvents such as halogenated derivatives, hydrocarbons, ethers, saturated linear, cyclic and aromatic hydrocarbons, acetonitrile and dioxane using 10 equiv. of each of the above mentioned alcohols when the substrate–enzyme ratio was 1:1, w/w. For the preparation of the opposite enantiomeric forms of the optically active products (*S*)-**3b** and (*R*)-**2b** best results were obtained for the CaL-B mediated alcoholysis in acetonitrile. It is important to note that the stereoselectivity was high for all of the tested nucleophiles (**Table 4**, entries 1-4), but the reaction rate of the enzymatic methanolysis was highest. These conditions were found to be optimal also for the enzymatic alcoholysis of the other three substrates *rac*-**3a, c, d**. (**Table** 4, entries 5, 7, 8).

Table 4. CaL-B mediated alcoholysis of *rac*-**3a-d** with various alcohols in acetonitrile

Entry	Alcohol	Compound	Time(h)	C(%)	$ee^{c}_{(R)\text{-}\mathbf{2}}$(%)	$ee^{c}_{(S)\text{-}\mathbf{3}}$(%)	*E*
1	Methanol	**3b**	24	49,4	>99,8	97,6	>200
2	Ethanol	**3b**	24	46,6	>99,8	87,3	>200
3	*n*-Propanol	**3b**	24	48,2	>99,8	92,9	>>200
4	*n*-Butanol	**3b**	24	43,1	>99,8	75,8	>200
5	Methanol	**3a**	30	50	>99,8	>98,8	>>200
6	Methanol	**3b**	32	50	>99,8	>98,8	>>200
7	Methanol	**3c**	31	50	>99,8	>98,8	>>200
8	Methanol	**3d**	32	50	>99,8	>98,8	>>200

4.1.2.2. Preparative scale synthesis of both (*R*) - and (*S*)-2,3a-d

Using the optimal conditions found for the analytical scale enzymatic acylation of 1-(10-alkyl-10*H*-phenothiazin-3-yl)ethanols (*rac*-**2a-d**) and enzymatic methanolysis of the corresponding racemic butanoates (*rac*-**3a-d**) the preparative scale enzymatic synthesis of both ((*S*)- and (*R*)-**2,3a-d** was performed. To demonstrate the usefulness of these enzymatic procedures 1 g of the subtrate was used for each enzymatic kinetic resolution

Due to the high enantiomer selectivity of the used biocatalysts (L-AK for acylation and CaL-B for methanolysis) the synthesis of both enantiopure (*S*)- and (*R*)-1-(10-alkyl-10*H*-phenothiazin-3-yl)ethanols ((*S*)- and (*R*)-**2a-d**) and corresponding butanoates (*R*)- and (*S*)-**3a-d** was performed (ee > 99.8%, E >>200 for each compound**; Table 5**).

During the preparative scale enzymatic acylations the formation of several byproducts was observed, consequently the yields for the isolated products were lower with 3-4% in comparison to those of the enzymatic methanolyis. Due to the significant structural instability of the 1-(10-alkyl-10*H*-phenothiazin-3-yl)ethanols in presence of mineral or organic acids, the appearance of the byproducts can be explained with the inherent formation of a minimal amount of butyric acid liberating from vinyl butyrate through the reaction with the water present in the enzyme preparation. After the completion of the reaction during the solvent evaporation it was important to avoid the heating of the solution because the produced optically pure ethanols showed thermal instability, which leads to decomposition. The stability of the main compounds was increased by adding into the reaction mixture 5-10 mL of ethanol before the solvent was removed in vacuo at low temperatures (t < 10 ºC). Due to its weak acidic nature, silica gel proved to be useless for the purification of the target compounds. For this reason after the preparative scale reaction the separation and purification of the reaction products was performed with vacuum chromatography using a minimal amount of neutral aluminium oxide.

Table 5. Preparative scale synthesis of enantiomerically pure 1-(10-alkyl-10*H*-phenothiazin-3-yl)ethanols ((*S*)- and (*R*)-**2a-d**) and their butanoaste esters ((*S*)- and (*R*)-**3a-d**)

L-AK mediated acylation of *rac*-**2a-d** in chloroform			CaL-B mediated methanolysis of *rac*-**3a-d** in acetonitrile		
Product	Yield[a] [%])	$[\alpha]_D$[b]	Product	Yield[a] [%])	$[\alpha]_D$[b]
(*S*)-**2a**	45	-52,9	(*R*)-**2a**	49	+52,7
(*S*)-**2b**	45	-94,4	(*R*)-**2b**	49	+94,5
(*S*)-**2c**	46	-112,4	(*R*)-**2c**	49	+112,5
(*S*)-**2d**	45	-95,0	(*R*)-**2d**	49	+94,9
(*R*)-**3a**	47	+172,2	(*S*)-**3a**	49	-172,6
(*R*)-**3b**	48	+242,3	(*S*)-**3b**	49	-242,5
(*R*)-**3c**	46	+266,0	(*S*)-**3c**	49	-266,4
(*R*)-**3d**	48	+249,6	(*S*)-**3d**	49	-249,2

4.1.3. The absolute configuration of optically active (*R*)-2b synthesized by the enantiomer selective alcoholysis of *rac*-2b

The X-ray crystal structure gave unambiguously the *R* enantiomer for (+)-**2b**. Interestingly, in the crystal structure two independent molecules are present in the asymmetric unit exhibiting two different chiralities of the tertiary *N* atom (**Figure 8**). The heterocyclic rings in both molecules have the boat conformation and the phenothiazine moieties are related as conformational enantiomers.

The absolute configuration of the rest of the compounds was established comparing their signs of the specific rotation with (+)-(*R*)-**2b**.

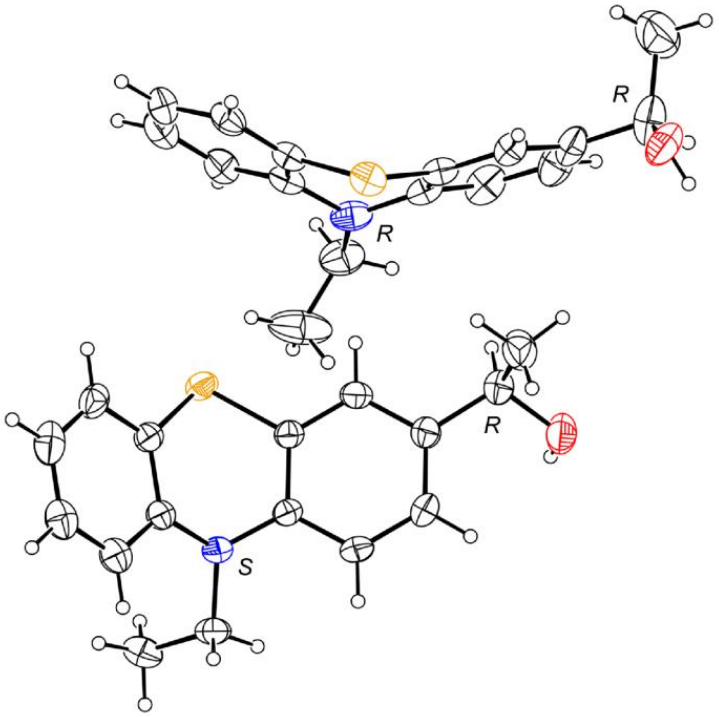


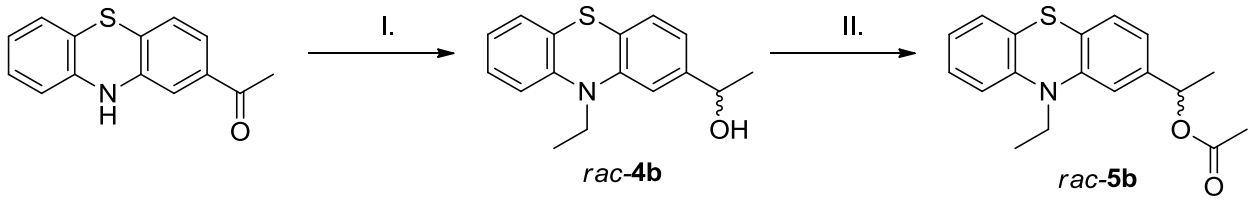
Figura 8. Absolute configuration of optically active of two independent molecules (*R*)-**2b.**

4.2. The synthesis of (*R*) şi (*S*) enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanols by enzymatic kinetic resolution of the racemic alcohol or his corresponding acetate with *Pseudomonas fluorescens* lipase

4.2.1. Chemical synthesis

The (*N*-ethyl-phenothiazin-2-yl)-1-ethanol *rac*-**4b** was chosen as model compound for this study, due to their availability by simple reaction. The racemic substrate was prepared using a modified previously described method[18], as shown in **Scheme 25**. As starting compound the commercially available 1-(10*H*-phenothiazin-2-yl) ethanone was used. By alkylation with ethyl iodide in presence of sodium hydride, followed by chemical reduction with sodium borohydride of the carbonyl group or by reduction followed by *N*-alkylation in the same conditions, the racemic ethanol can be obtained in high yield. Chemical acetylation of the alcohol with acetyl chloride in presence of triethylamine and catalytic amount of DMAP gave the corresponding racemic acetate *rac*-**4b.**

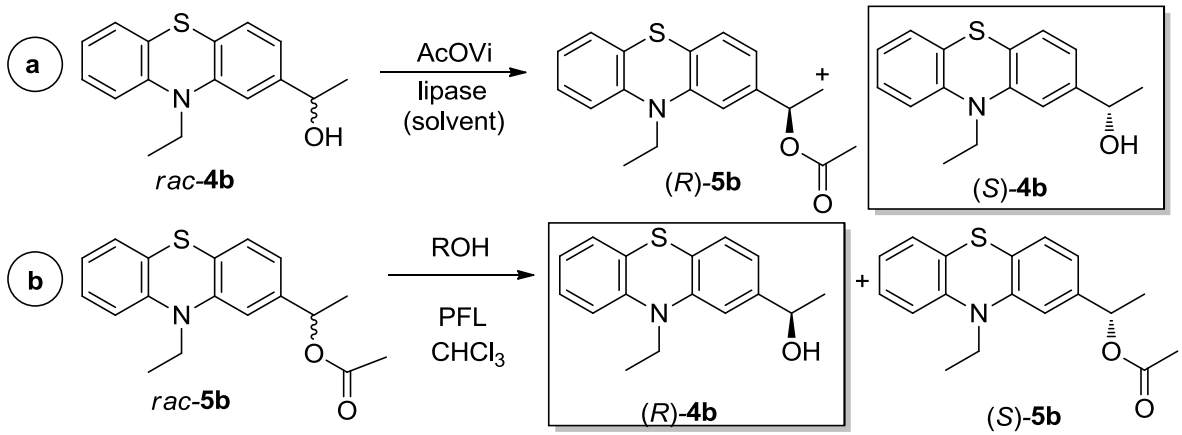
To investigate the stereoselectivity of the enzymatic reactions, the chromatographic separation of the enantiomers of the racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol (*rac*-**4b**) and their acetate (*rac*-**5b**), was first established. The base-line separation was performed using an appropriate chiral HPLC column.



I. 1. NaH, EtI, DMF; 2. $NaBH_4$, NaOH/methanol or 1. $NaBH_4$/methanol; 2. NaH, EtI, DMF;
II. AcCl, NEt_3, DMPA, CH_2Cl_2

Scheme 25. Chemical synthesis of the racemic substrates.

4.2.2. Biotransformations with lipases



Scheme26. Lipase mediated enzymatic kinetic resolution processes .

4.2.2.1. Analytical scale biotransformations

Analytical scale enzymatic acylation of *rac*-4b

First, the analytical scale enantiomer selective lipase catalyzed acetylation of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol (*rac*-**4b**) was studied in neat vinyl acetate using some commercial available lipases (**Scheme 26**)

Most of them exhibited good enantiomer selectivities and activities. The best results were obtained with immobilized lipases B from *Candida antarctica* (CaL-B), commercialized as Novozym 435 and with lipase from *Pseudomonas fluorescens* (PFL) (**Table 6**, entry 1-2, *E* >>200, at 50% conversion). Lipase A from *Candida antarctica* (CaL-A) was also selective and active (*E* = 198 at 48% conversion), while lipases from *Candida rugosa* (CrL), *Candida cilindracea* (CcL) and from *Thermomyces lanuginosa* (TLIM) were selective but less active (only 15-34 % conversion after 24 h). Other commercialy lipases such as lipase from *Mucor javanicus* and pancreas pig lipase were only moderate active and selective (*E*=30-70, at 10-25% conversion, data not presente in **Table 6**).

Table 6. Lipase catalyzed acetylations of *rac*-**4b** in neat vinyl acetate in presence of some lipases after 24 h

Entry	Lipase	ee $_{(R)\text{-}\mathbf{5b}}$ (%)	ee $_{(S)\text{-}\mathbf{4b}}$(%)	*c*(%)	*E*
1	CaL-B	>99	>99	50	>>200
2	PFL	>99	98,4	50	>>200
3	CaL-A	89	98,8	48	198
4	CcL	>99	99,5	15	>200
5	CrL	>99	99,0	25	>200
6	TLIM	>99	98,6	34	>200

It is known that the nature of the solvent could significantly influence the selectivity of the enantiomer selective acylation, the acylation of *rac*-**4b** with vinyl acetate in presence of PFL selected as catalyst, was tested in several organic solvents (**Table 7**). Chloroform proved to be the most appropriate solvent for the acetylation (*E*> 200, *c* = 50% after 24 hours, **Table 7**, entry 1).

Table 7. Lipase catalyzed acetylations of *rac*-**4b** (5 mg) with vinyl acetate (10 µL) and PFL (5 mg) in different solvents (200 µL) after 24 h

Entry.	Solvent	ee$_{(R)\text{-}\mathbf{5b}}$ (%)	ee$_{(S)\text{-}\mathbf{4b}}$ (%)	*c*(%)	*E*
1	Chloroform	>99	96.3	50	>200
2	*n*-Hexane	>99	77.1	56	44
3	Toluene	>99	72.7	58	36
4	Tetrahydrofuran	>99	87.1	53	85
5	Dichlorometane	>99	45.9	68	14
6	Acetonitrile	95.2	94	50	122

Analytical scale enzymatic alcoholysis of *rac*-5b

It is known that lipases usually retain their enantiomer preference in hydrolysis or alcoholysis[15]. Consequently, such reactions should result in the opposite enantiomeric forms of the reaction counterparts (**Scheme 26b**). The degree of enantioselectivity in alcoholysis reactions of the racemic acetate *rac*-**5b** was tested in four alcohols (methanol, ethanol, propan-1-ol and butan-1-ol) with PFL as catalyst. The highest selectivity was achieved for ethanolysis (**Table 8**, entry 2), yielding the highly enantiomerically enriched opposite form: (*R*)- **4b** and (*S*)-**5b.**

Table 8. Lipase catalyzed alcoholysis of *rac*-**5b** with PFL in chloroform after 24 h

Entry	Alcohol	ee (S)-**5b** (%)	*ee (R)-**4b** (%)*	*c*(%)	*E*
1	Methanol	95	79	45	94
2	Ethanol	99	99	50	››200
3	*n*-Propanol	91	85	48	58
4	*n*-Butanol	85	75	47	28

4.2.2.2. Improving of biocatalyst performance by immobilization

Preparation of immobilized PFL catalysts

Using a combination of methyltrimethoxysilane ($MeSi(OMe)_3$, MTMS) and tetramethoxysilane ($Si(OMe)_3$, TMOS), a hydrophobic matrix, suitable to enhance the activity of the entrapped lipase, was prepared using an optimized[19] Reetz method[20] based on the base-catalyzed *in situ* polymerization of the silanes. The previously reported optimal conditions (TMOS/MTMS=1/5; water/gel ratio 9/1 and fluoride ion-catalyzed hydrolysis of silane precursors)[19] were used. The influence of celite and sucrose as additives on the performance and stability of biocatalyst in the studied EKR processes was also studied.

Acylation of *rac*-4b and ethanolysis of *rac*-5b with sol-gel immobilized PFL

PFL sol-gel catalysts were tested for the acylation of *rac*-**4b** (0.1 M) with vinyl acetate and ethanolysis of *rac*-**5b** (0.1 M) in chloroform. The obtained results indicate excellent applicability of the catalyst obtained in presence of both celite and sucrose in the studied enzymatic resolution processes (*E*>> 200 with all enzyme preparations).

Reuse of immobilized enzymes

One of the most important features of immobilized enzymes in synthetic applications is their reusability. For testing the recycling capacity, the acylation of *rac*-**4b** (0.1 M) with vinyl acetate (0.2 M) in chloroform was repeated with the same enzyme preparation up to 4 times. Every reaction was allowed to proceed to approx. 50% conversion before the catalyst was subjected to the next cycle. Between the cycles the catalyst was washed with dry chloroform and used without drying. The obtained results (enantiomeric excesses of the resulted compounds and enantiomeric *ratio E*) are presented in **Table 9**.

Table 9. Reuse of the sol-gel PFL enzyme preparations (1.5 mg protein/mL) for the *O*-acylation of *rac*-**4b** (0.1 M) with vinyl acetate (0.2 M) in chloroform (1 mL) at room temperature

	PFL cycle				
	powder	1	2	3	4
Time (h)	24	20	20	22	22
c (%)	50	49	48	50	49
$ee_{(S)\text{-}\mathbf{4b}}$	96	97	97	96	94
$ee_{(R)\text{-}\mathbf{5b}}$	99	98	96	95	93
E	>>200	>200	>200	183	115

The enzymatic activity decreased slowly (5%) after 3 cycles and significantly (10%) after 4 times, respectively. The reuse had no significant effect on enzymatic enantioselectivity,

allowing the preparation of (*S*)-**4b** and (*R*)-**5b** from *rac*-**4b** in highly enantiomerically enriched forms (ee>95%) still on the 4th reuse cycle.

4.2.2.3.Preparative scale synthesis of both (*R*)- and (*S*)-4b,5b

The resulted optimum for the analytical scale reactions was succesfully applied for the preparative scale synthesis of both highly enantiomerically enriched stereoisomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl) ethanol and his acetate. To demonstrate the usefulness of these enzymatic procedures 500 mg of the racemic substrate was used for each EKR (acetylation and alcoholysis reaction). Whereas the enzymatic acetylation afforded the acetate (*R*)-**5b** and the alcohol (*S*)-**4b** in high enantiomeric excess, alcoholysis of the racemic acetate *rac*-**5b**, yielded the opposite enantiomeric forms (*R*)-**4b** and (*S*)-**5b.**

Data on yield and enantiomeric excess of the products [(*S*)-**5b** and (*R*)-**4b**] are shown in **Table 10** (entry 2).

Table 10. Preparative scale enzymatic resolution of *rac*-**4b, 5b** with PFL in chloroform

Entry	Substrate	ee (%)		η^*	*E*	ee (%)		η^*	*E*
		(*R*)-**5b**	(*S*)-**4b**	*(%)*		(*R*)-**4b**	(*S*)-**5b**	*(%)*	
1	*rac*-**4b**	99	96	47	>>200				
2	*rac*-**5b**					99	99	48	>>200

The absolute configuration of the obtained enantiopure products was assigned using the earlier published data for optically pure enantiomers of **4b**[8].

5. Conclusions

1. An efficient enzymatic procedure for synthesis of optically pure *N*-alkyl-phenothiazin-3-yl-ethanols and their butanoates has been described. Due to the low stability of these compounds the optimal conditions for the preparative scale biotransformation followed by the isolation and purification of the products was setup.

By enzymatic kinetic resolutions, both optically pure enantiomers of four 1-(10-alkyl-10*H*-phenothiazin-3-yl) ethanols (**2a-d**) and four butanoates (**3a-d**) were synthesized with high yields.

Whereas the L-AK mediated acylation afforded the enantiopure (*R*)-butanoates (*R*)-**3a-d** and (*S*)-alcohols (*S*)-**2a-d**, the CaL-B catalysed methanolysis of the racemic butanoates *rac*-**3a-d** yielded the opposite enantiomeric forms of the target compounds ((*R*)-**2a-d** and (*S*)-**3a-d**).

The absolute configuration was determined by X-ray diffractometry for the optically active (*R*)-**2b**.

2. The synthesis of both enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl) ethanol **4b** and his acetate **5b** has been achieved by enzymatic kinetic resolution with PFL as efficient biocatalyst.

Whereas the enzymatic acetylation of the racemic ethanol **4b** afforded the (*R*) enantiomer of acetate [(*R*)-**5b**] and the (*S*) enantiomer of the alcohol [(*S*)-**4b**] in high enantiomeric excess, the ethanolysis of the racemic acetate *rac*-**5b** yielded the opposite enantiomeric form [(*R*)-**4b** and (*S*)-**5b**].

The stability, activity and reusability of the sol-gel encapsulated PFL were determined. The immobilized biocatalyst provides to be efficient in both studied resolution processes.

References

[1](a)Hull,K.G.;Visnic,M.;Tautz,W.;Sheffron,A.,Tetrahedron,**1997**,53,12405-2414;(b)Truppo,M.D. Pollard,D.;Devine,P.*Org.Lett.*,**2006**,9,335–338;(c)DeMartino,G.;LaRegina,G.;DiPasquali,A.; Ragno,R.;Bergamini,A.;Ciaprini,C.;Sinistro,A.;Maga,G.;Crespan,E.;Artico,M.;Silvestrini,R. *J.Med.Chem*.,**2005**,48,4378–4388;(d)Challenger,C.A.,*ChiralDrugs*;JohnWiley&Sons:NewYork **2001**;(e)Tanis,S.P.;Evans,B.R.;Nieman,J.A.;Parker,T.T.;Taylor,W.D.;Heasley,S.E.;Herrington,P.M.; Perrault,W.R.;Hohler,R.A.;DoAK,L.A.;Hester,M.R.;Seest,E.P.,*Tetrahedron:Asymmetry*,**2006**, 17,21–54.

[2]Silverman,R.B.*TheOrganicChemistryofDrugDesignandDrugAction*;ElsevierAcademicPress:Burlington,MA,**2004**

[3] Turner,N.J. *Curr.Opin.Chem.Biol.,* **2004**, 8,114–119

[4] Ghanem,A.; Aboul-Enenin,H.Y.,*Chirality,***2005**,17,1–15

[5] Lin,G.;Midha,K.K.;Hawes,E.M. *J.Heterocycl.Chem*., **1991**, 28,215–219;El-Said,M.K.,*Pharmazi*e **1981,**36,678679;Dominguez,J.N.;Lopez,S.;Charris,J.;Iarruso,L.;Lobo,G.;Semenow,A.;Olson,J.E.; Rosenthal,P.J., *J.Med.Chem*.**1997**, 40,2726–2732;Raval,J.P.;Desai,K.K**.,** *ARKIVOC***, 200**5,21–28; Motohashi,N.;Kawase,M.;Saito,S.;Sakagami,H.,*Curr.DrugTargets*,**2000**,1,237–246;Mosnaim,A.D. Ranade,V.V.;Wolf,M.E.;Puente,J.;Valenzuela,A.M.*,Am.J.Ther*.,**2006**,13,261273;Trivedi,A.R.;Siddiqui,A.B.;Shah,V.H.,*ARKIVOC,* **2008**,210–217;Aszalos,A.,*Acta Microbiol*. Immunol.Hung., 2003, 50,43–53;Kurihara,T.; Motohashi,N.;Sakagami,H.H.;Molnar,J. *AnticancerRes,* **1999**, 19,4081–4083

[6]Paizs,C.,Tahtinen,P.;Tosa,M.;Majdik,C.;Irimie;F.D.;Kanerva,L.T.;Tetrahedron**,2004,**60, 10533–10540

[7] Brem,J.;Tosa,M.I.;Paizs,C.;Vass,E.;Irimie,F.D.,*Tetrahedron: Asymmetry,***2010**,21 365–373

[8]Stepanenko,V.;DeJesus,M.;Correa,W.;Guzman,I.;Vazquez,C.;Ortiz,L.;Ortiz-Marciales,M. Tetrahedron:Asymmetry **2007**, 18,2738–2745.

[9] Brem,J.,Tosa,M.I.;Paizs,C.;Munceanu,A.;Calogovic,D.M.;Irimie,F.D., *Tetrahedron: Asymmetr*y**, 2010**;21,1993–98

[10] Vänttinen,E.;Kanerva,L.T., *J. Chem. Soc. Perkin Trans 1* 1**994**, *1*, 3459-3463; Långvik, O.; Saloranta,T.;Kirilin, A.;Liljeblad, A.;Mäki-Arvela,P.;Kanerva,L.T.;Murzin,D.Y.;Leino,R.*ChemCat Chem* **2010**, *2*, 1615-1621.

[11] Devi, B.L.A.P.; Guo, Z.; Xu, X. *J. Am. Oil. Chem. Soc.* **2009**, *86*, 637-64

[12] Zarcula,C.;Corîci,L.;Croitoru,R.;Ursoiu,A.;Peter,,. *J. Mol. Catal. B: Enzym.* **2010**, *65,* 79-86; Tomin,A,.;Weiser,D.;Hellner,G.;Bata,Zs.;Corici,L.;Péter,F.; Koczka,B. Poppe,L. *Process Biochem.* **2011**, *46*, 52-58.

[13] Santos,J.C.;Paula,A.V.;Nunes,G.F.M.; de Castro, H.F. *J. Mol. Catal. B: Enzym.* **2008**, *52-53*, 49-57.

[14] Gorokhova,I.V.;Ivanov, A.E.; Zubov, V.P. *Russ. J. Bioorg. Chem.* **2002**, *28*, 38-43; Mendes, A.A.; Giordano, R.C.; de L.C. Giordano, R.; de Castro, H.F. *J. Mol. Catal. B: Enzym.* **2011**, *68*, 109-115; Gorokhova, I.V.; Ivanov, A.E.; Zubov, V.P. *Russ. Chem. B, Int. Ed.* **2001**, *50*, 152-154.

[15] Bornscheuar,U.T;Kazlauskas,R.J.;*Hydrolases in Organic Synthesis*, 2nd Ed.,Wiley VCH,**200**6,61-

[16]Tosa,M.;Paisz,C.;Moldovan,P.;Novak,L.;Kolonits,P.;Szabo,E.;Poppe,L.;Irimie,F.D.,*Journal of Molecular Catalysis B:Enzymatic*,**2002,**17,241-248;

[17]Zhu,Y.;Fow,K.L.;Chuah,G.K.;Jaenicke,S.,*Chem.Eur.J.*,**2007**,13,541-547;Costa,L.;Coehlo,A.; Lemos,F.;Ribeiro,F.R.;Cabral,A.,*J.Appl.Catal.*,**2009**,354,33-37,Nishiguchi,T., Kamio,I., *C.J.Chem. Soc.,Perkin Trans*,**1989**,707-710

[18]J.Brem,Pilbák,S.;Paizs,C.;BánócziG.;IrimieF.D.;Toşa,M.I.;Poppe*,L.;Tetrahedron:Asymmetry* **2011**, 22,916-923

[19] Brem,J.;Turcu,M.C.;Paizs,C.; Lundell, K.; Tosa, M.I. Irimie, F.D.; Kanerva, L. *Process Biochemistry* **2012**, *47,* 119–126

[20]Reetz,M.T;Zonta,A.;Simpelkamp,J.*Angew.Chem.Int.Ed.Engl.,***1995**,*34*,301-303;Reetz,M.T.; Zonta,A.;Simpelkamp,*J.Biotechnol.Bioeng.***1996**,*49*,527-34;Reetz,M.T.;Tielmann,P; Wiesenhöfer, W.;Könen,W.; Zonta, A.*Adv.Synth.Catal.***2003**,*345*,717-728.