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New biocatalytic procedures for the enantioselective synthesis of 3-aryl-3-hydroxy propanoates

Ph.D. Thesis Abstract

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1. Introduction

In the last decades there was an almost exponential increase in the requisite of high quantities of various optically pure substances. The main reason for this need is the necessity for increasing the life quality of human civilization. The people need new medicines of better quality (agrochemicals and pharmaceutically important substances) and other fine chemical products (detergents, perfumes, insecticides, agrochemicals, etc.); all of them are based on different chemical building-blocks. The optically pure β-hydroxy acids and their derivatives are just some of the most desired classes of substances.

These compounds are used as chiral intermediates because they have at least two functional groups (the hydroxy and the carboxy group) which can be chemically transformed into various other functional groups (e.g. aldehyde, amine, amide). Several papers reported the synthesis of: (i) antibiotics (based on β-lactam rings, such as penems, carbapenems), (ii) naturally compounds (based on γ- lactonic acid, such as isomuronic and neuropogolic acid), (iii) selective serotonin and norepinephrine reuptake inhibitors (fluoxetine, duloxetine, etc.) and (iv) HMG-CoA reductase inhibitors (so called statins) using β-hydroxy acids and their derivatives as starting materials. For their synthesis the chemical industry should employ environmental friendly methods (green chemistry). Consenquently eco-friendly biocatalytic (enzymatic) procedures will become more usefull in our century.

Once the relation between the chirality and the biological activity was understood, the importance of chirality increased and focused the attention of many researchers in the field. By using single enatiomeric drugs (the so called eutomer) the therapeutic profile can be improved and a more selective pharmacodynamic profile can be created. Paracelsus quote: "Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy" is a very good description for the importance of the quantities used in medicines. If racemates are used, a higher quantity of medicines has to be administrated to the patient due to the fact that in general only one of the enantiomers is responsible for their positive effect.

The production of single enatiomeric drugs promotes the synthesis of optically pure substances. Among others, optically pure 3-aryl-3-hydroxy propanoates are an important group of chemicals that need to be produced. Using enzymes as biocatalysts, optically pure (*R*)- and (*S*)-3-aryl-3-hydroxy propanoates were successfully synthesized.

Lipases

Due to their high enantio- and regioselectivity, enzymes, especially lipases, are commonly used as catalysts in the synthetic organic chemistry. Beside natural substrates (triglycerides), they are able to catalyze the transformation of a wide range of aliphatic, cyclic, aromatic and organometallic compounds.[1] Furthermore, lipases are stable in non-aqueous organic solvents because they naturally work at the lipids/water interface. Fixing the reaction conditions appropriately lipases can be used either for esters synthesis or esters hydrolysis. Due to the fact that they are produced in hundreds of tons per year as detergents additives and recently were obtained also by genetic engineering techniques, they are now available at accessible price. By gene expression in an appropriate microorganism such as a fungi, yeast or bacteria (e.g., *Escherichia coli*), large scale production of lipases has been realized. *Via* genetic engineering techniques these enzymes can also be easily modified to improve properties such as selectivity, specificity and resistance. However, native lipases can be used efficiently only once. This drawback can be overcome by immobilization, when their reuse becomes possible. Moreover, by an adequate immobilization procedure it is also possible to improve and moderate some of their properties like thermostability and selectivity.[2]

The main applications of lipases in catalytic asymmetric synthesis involve the kinetic resolution of racemates. The success of a kinetic resolution process depends on the fact that the two enantiomers react at different rates with a chiral entity (enzyme). The main drawbacks of traditional kinetic resolution (maximum 50% theoretical yield and the decrease of enantiomeric excess (e.e.) with the conversion) can be overcome in the dynamic kinetic resolution (DKR), when a racemic mixture is transformed into one of the enantiomers by the *in situ* racemization of the less reactive enantiomer during the process (Figure 1.).[3]

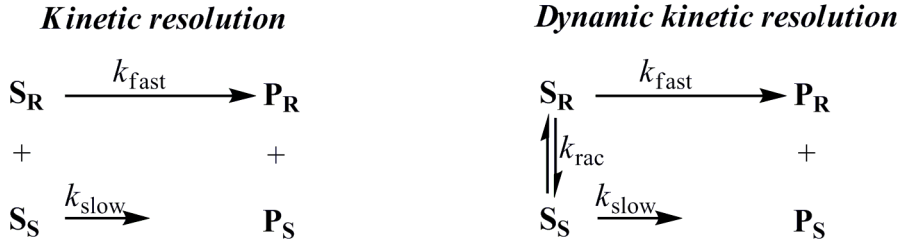


Figure 1. Kinetic resolution versus dynamic kinetic resolution

2. Heterocyclic β-hydroxy acids as building blocks for synthesis of pharmaceuticals and other fine chemicals

2.1. β-hydroxycarboxylic acids and their esters as chiral synthons and starting materials

Optically active β-hydroxycarboxylic acids and their esters are versatile chiral synthons, due to their dual functionality. Thus, enantiopure β-hydroxycarboxylic acids and their derivatives have been used as starting materials for the synthesis of optically pure bioactive compounds such as vitamins, antibiotics, pheromones and flavor compounds.[4] Some of these are presented in Scheme 2.

The pharmaceuticals depicted in Scheme 2 having various functions are used in the treatment of various diseases. Some of their applications are described hereinafter:

1) Duloxetine, Atomoxetine and Fluoxetine are inhibitors of serotonin-norepinephrine reuptake and are used in the treatment of diabetic neuropathy, depression and anxiety.[5]

2) Simvastatin, Pravastatin and Atorvastatin belong to the class of drugs known as *statins*. They inhibit the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), which catalyzes the reductive conversion of HMG-CoA into mevalonate, which is important in an early rate limiting step of cholesterol biosynthesis. By inhibiting the mevalonate synthesis, this medicine decreases the level of LDL cholesterol (LDL=low-density lipoprotein). The clinical tests of statins show a direct relationship between the level of LDL cholesterol and coronary heart diseases, thus a high level of LDL increases the mortality caused by these diseases.[6]

3) Natural substances as building blocks of several medicines derived from γ-lactonic acid as: Lichesterolic acid, Neuropogolic acid, Isomuronic acid.[7]

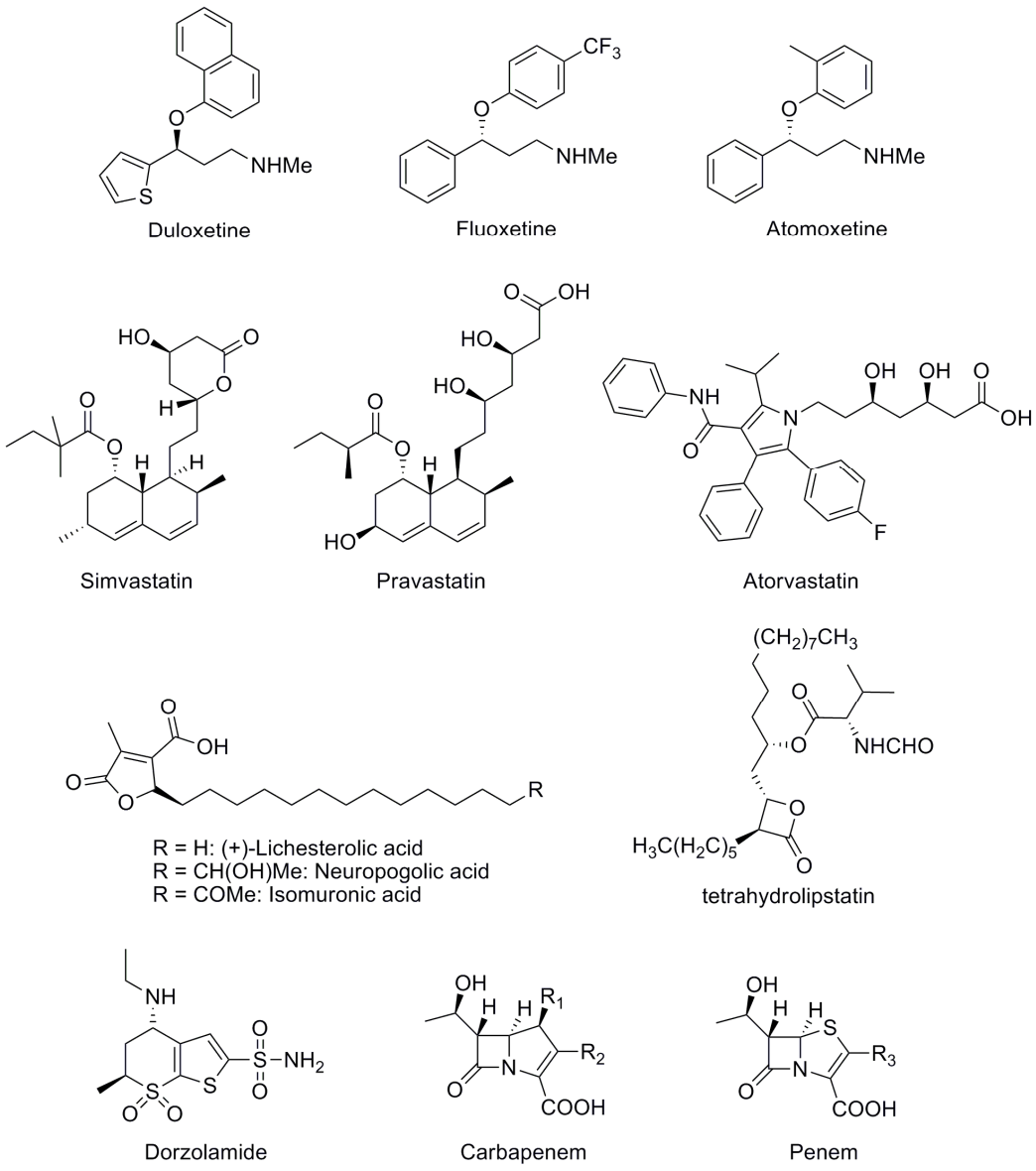
4) (-)-Tetrahydrolipstatin (comercial name Orlistat), a derivative of lipstatin, isolated from *Streptomyces toxytricini* in 1987, is an irreversible and selective pancreatic lipase inhibitor.[8]

5) Dorzolamide (comercial name Trusopt) is used in ophthalmic solutions, reducing the elevated intraocular pressure in open-angle glaucoma and ocular hypertension.[9]

6) Penem, Carbapenem are a relativly new class of antibiotics with broad antimicrobial spectra, some of them still being in clinical phases. Penems and carbanems are used for hospital and community patogens, when other drugs are inefficient.[10]

β-hydroxycarboxylic acids and esters are also proper starting materials for other important chemical substances such as: lactames, amides, amino acids, 1-3 diols, amino alcohols etc. (Scheme 3).

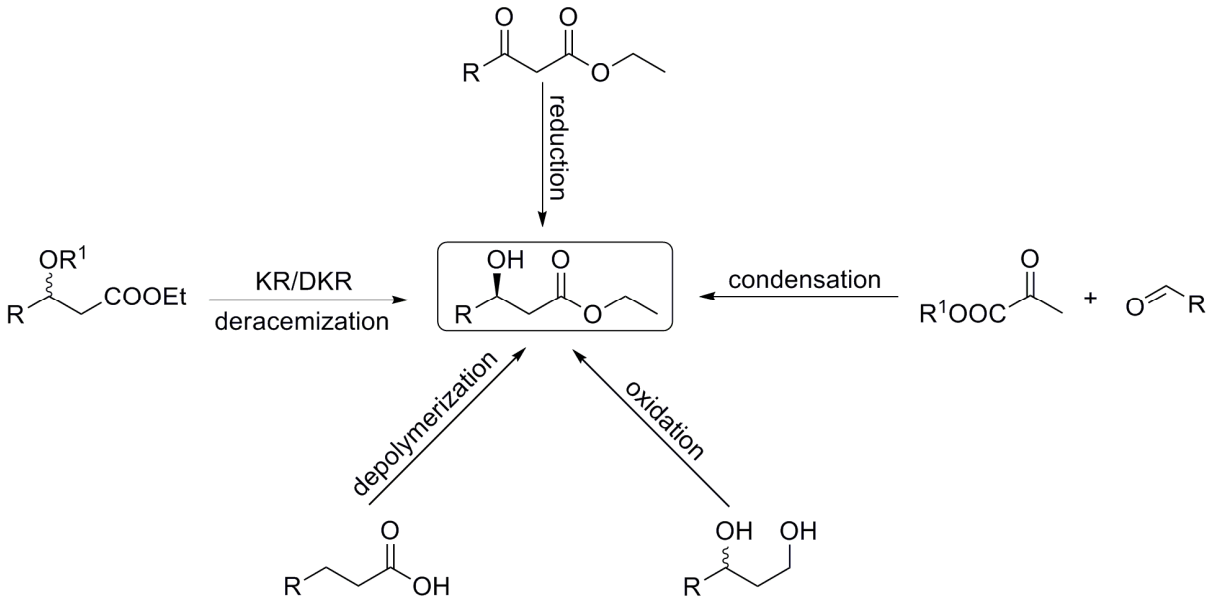
As a prerequisite of valuable substances belonging to these groups, various synthetic routes were developed for the chemical transformation of β-hydroxycarboxylic acids or esters.



Scheme 2. Pharmaceutically important substances which can be synthesized from β-hydroxy acids and their derivatives

3. Literature review. Biocatalytic approaches toward β-hydroxy acids and their esters

In this section, the previously reported biocatalytic preparation methods of optically active 3-alkyl and -aryl-3-hydroxy propanoates is rewieved. The main methodologies are presented in Scheme 6.



Scheme 6. General methodologies to obtain 3-alkyl and -aryl-3-hydroxy propanoates

4. Aims of the study

In the last decades there was an almost exponential increase in the requisite of high quantities of various optically pure substances. The main purpose for this need is the necessity of increasing the life quality of our civilization. The people need new medicines of better quality (agrochemicals and pharmaceutically important substances) and other fine chemical products (detergents, perfumes, insecticides, etc.) and all of them are based on different chemical building-blocks. The optically pure β-hydroxy-acids and esters are just some of the most desired classes of substances. Their industrial synthesis by eco-friendly biocatalytic (enzymatic) procedures will become more practicable in our century. Due to their high enantio- and regioselectivity, enzymes, especially lipases, are commonly used as catalysts in the synthetic organic chemistry. Beside natural substrates (triglycerides), they are able to catalyze the transformation of a wide range of aliphatic, cyclic, aromatic and organometallic compounds. Lipases can be used either for esters synthesis or esters hydrolysis by fixing the reaction conditions appropriately. The properties of these lipases are widely discussed in several papers.[11] Since in our study the lipases were used just for synthetic and immobilization purposes, only some of their characteristics are presented in Table 4.

The aim of this work has been to study and to develop a new general and sustenable (reusability of the biocatalyst) method for the lipase-catalyzed regio- and stereoselective acylation and hydrolysis of the ethyl 3-aryl-3-hydroxy propanoates bearing various aryl moieties: phenyl, substituted phenyl, phenyl-furanyl, substituted phenyl-furanyl, thiophen-2 and 3-yl, furan-2 and -3-yl, benzofuran-2 and 3-yl and *N*-alkyl phenothiazin-yl (Figure 2).

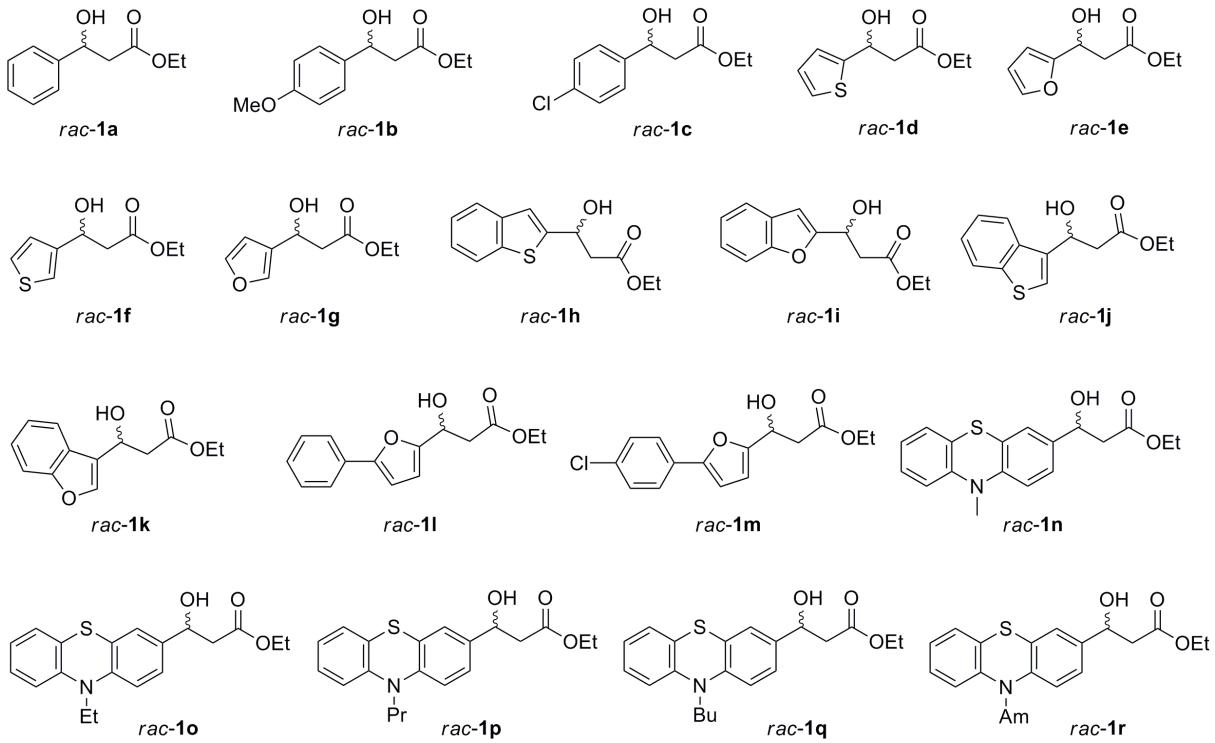
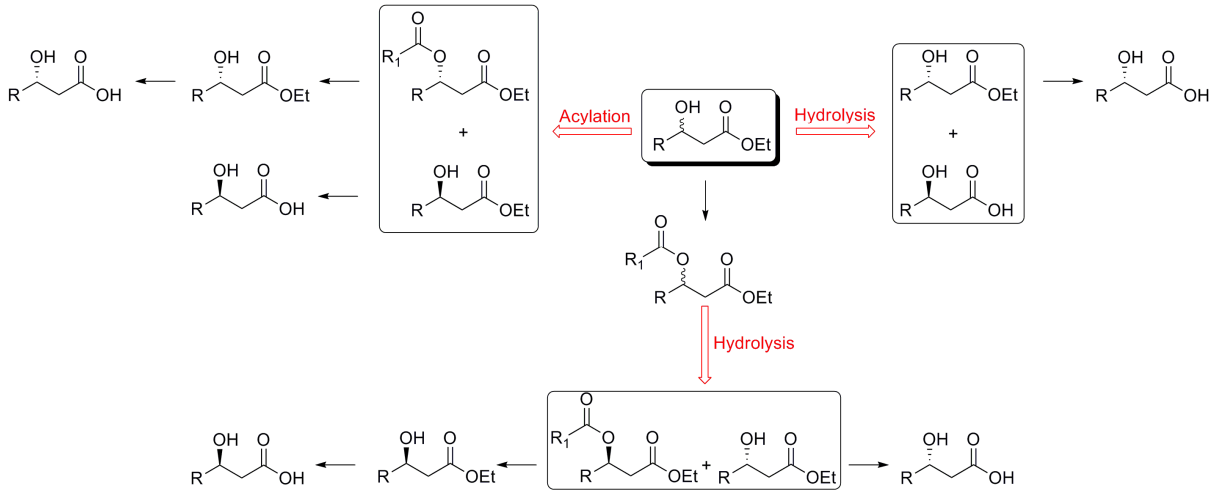


Figure 2. Racemic ethyl β-hydroxy propanoates (*rac*-**1a-r**) used as substrates in the selective enzymatic kinetic resolutions

Table 4. Characteristics of lipases

Characteristic	**CaL-A**	**CaL-B**	**AK**	**CRL**
Origin	fungal	fungal	bacterial	fungal
Molecular mass	45 kDa	33 kDa	33 kDa	64 kDa
Number amino acid	431	317		534
Catalytic triad	Ser 184	Ser 105		Ser 209
	Asp 334	Asp 187		Asp 341
	His 366	His 224		His 449
pH range		3.5-9.5	4-10	5.5-9.5
pH optimum	7		8	7
Optimal temperature			40-50	30
Denaturation temperature	>90°C	50-60 °C	>70°C	85°C
Interfacial activation	yes	no	yes	yes
Isoelectric point (p*I*)	7.5	6	4	4.3

Using the dual functionality of the target compounds, the development of new regio- and enantioselective biocatalytic methods (Scheme 13) and the improvement of the existing ones for the synthesis of highly enantiomerically enriched β-hydroxy acids and esters is described.



Scheme 13. Used methodologies for lipase mediated kinetic resolution

The selection of the useful enzymes for the present purposes was performed following a well established screening procedure. The active and selective enzymes, lipases from *Pseudomonas fluorescens* (lipase AK „Amano" 20, free and immobilized form), *Candida rugosa* (CRL, AYS Amano), *Candida antarctica* lipase A (CAL-A free adsorbed on Celite in the presence of sucrose) and *Candida antarctica* lipase B (CAL-B as the commercial Novozyme 435 preparation) were further used for preparative scale and immobilization purposes.

The prerequsite for an efficient DKR which is providing both enantiomeric forms of o chiral compound is to own two ditsinct biocatalytic procedure which are working with opposite stereopreference. It is known that CaL-A and CaL-B in some cases show opposite enantiopreference using the same substrate. Maintaining their high stereoselectivity, the influence of the reaction conditions (solvent, acyl donor, temperature etc) upon the sense of the stereochemical outcome of these two known lipases was investigated.

In order to improve the selectivity and activity of the investigated lipases and to enhance their usability and reusability in batch, but mostly in continuous flow processes, their

immobilization was performed. Several immobilization techniques like adsorption, cross-linking and sol-gel techniques were examined and optimized.

Last, but not least the development of a more echo-friendly biocatalytic procedure using fatty acids and esters as reaction counterparts for the enzymatic acylation as well the lipase mediated stereo- and regioselective hydrolysis of the "natural-like" fatty acyl-β-hydroxy propanoates was performed.

5. Results and discussion

5.1. General description of the work

First, the chemical synthesis of the investigated substrates (different β-hydroxy -acids, -esters, and -diesters) was realized.

In order to track the selectivity and the activity of the enzymatic reactions the analytical chiral separation of the racemic compounds was performed using various chiral columns.

With all these data in our hands the analytical scale enzymatic reactions (screening) were performed which allowed finding the optimal conditions for each individual biotransformation. Using the found optimal conditions, the preparative scale biotransformations yielded enantiopure products which were analysed and characterized by various analytical methods (various chromatographic methods, NMR, MS and IR spectrometry) and their absolute configuration was determined using VCD.

Based on the similitude of the aryl moieties from ethyl 3-aryl-3-hydroxy propanoates, first the enzyme mediated biotransformations were investigated for three different series of variously substituted β-hydroxy esters: a) thiophen-2- and -3-yl, furan-2- and -3-yl and unsubstituted phenyl moieties; b) benzo[*b*]thiophen-2- and -3-yl and benzofuran-2- and -3-yl moieties and c) *N*-substituted phenothiazine based substrates.

After the lipase mediated selective *O*-acylation and hydrolysis of the β-hydroxy esters as well as the hydrolysis of the corresponding diacetates were both studied, the selective *O*-acylation was found as the most suitable method for the highly enantiomer selective enzymatic transformation.

In order to develop a general methodology for the lipase catalyzed kinetic resolution of β-hydroxy esters, beside the previously mentioned substrates, also others such as phenyl, variously substituted phenyl and phenyl-furan-2-yl ones were included in the investigation. To improve the catalytic performance of the selected lipase (L-AK) the immobilization of this enzyme was also performed. The tailor made immobilization managed to improve some important properties of lipase AK for the selective *O*-acylation of various β-hydroxy esters, but did not give a better substrate tolerance and therefore the method could not be yet generalized. Finally the general lipase mediated selective acylation method of this broad range of substrates was finally obtained using long chain fatty acids and their esters as acyl donors.

5.2. Enzymatic synthesis with commercial and home-made enzymes

The obtained β-hydroxy esters bearing various aryl moieties were used as substrates for the enzyme mediated stereoselective reactions using almost exclusively lipases as chiral catalysts. Both procedures the stereoselective *O*-acylation and the regio- and enantioselective hydrolysis of the racemic diesters were investigated. In order to found the optimal conditions the analytical scale selective *O*-acylation was realized in anhydrous conditions using various acylating agents in several organic solvents in the presence of a wide range of lipases. The same optimization procedure was investigated also for the lipase catalyzed enantioselective hydrolysis of the racemic diesters in organic solvents in presence of various amounts of water or buffers. The found optimal conditions were helped us to perform the preparative scale reactions for the synthesis of highly enantiomerically enriched resolution products.

Since the involved substances used in our studies showed structural instability (water elimination, polycondensation, etc.) and enantiolability (thermal or acido-basic racemization) their further chemical transformations into the desired β-hydroxy acids was impaired. This observation encouraged us to develop a multi-step and multi-enzymatic preparative-scale useful procedure for catalyze efficiently the transformation of the starting racemic β-hydroxy esters into both, (*R*)- and (*S*)-β-hydroxy acids.

The reusability of the biocatalyst is one of the key factors to set-up a more practical procedures. If the lipases are immobilized their reusability becomes reliable. For this purphose several methodologies were tested. Besides reusability by immobilization increased enzyme stability and selectivity is also expected, providing increased chemical applicability of lipases.

In order to develop a greener lipase catalysed procedure, finally the enzymatic resolution in presence of long chain fatty acids and their esters as reactants were investigated.

The detailed description of all mentioned topics is further given.

5.2.1. CaL-A and CaL-B showing opposite enantiopreference in the kinetic resolution of thiophenyl- and furanyl-β-hydroxy esters

(*S*)-3-hydroxy-3-(thiophen-2-yl)propanoate (*S*)-**1d** is a precursor for the synthesis of duloxetine (a serotonin-norepinephrine reuptake inhibitor). For this reason the preparative scale kinetic resolution of racemic ethyl 2- and 3-furyl- and 2- and 3-thienyl-3-hydroxypropanoates has been studied.

Enzyme screening. Extensive screening of various enzyme preparations for the *O*-acylation of *rac*-**1d** as model compounds in neat vinyl acetate started the analytical scale studies (Table 6). The formation of the acyl-enzyme intermediate, an ester intermediate between the serine hydroxyl from the active site of the lipase and an acyl donor, is pivotal for lipase catalysis. This means that a β-hydroxy ester as an added alcohol nucleophile enantioselectively binds in the nucleophile binding site before reacting with the intermediate, whereas it enantioselectively binds in the acyl binding site as an acyl donor and forms the intermediate by itself. Accordingly, care is needed when choosing an achiral acyl donor for enzymatic *O*-acylation due to the dual nature of the present substrates. For CAL-B catalysis competitive interesterification may be an especially serious side reaction,[12] the type of reaction also exploitable in various applications.[13] To prevent interesterification as a side reaction with CAL-B, vinyl acetate was chosen as commonly used irreversible acyl donor. Vinyl acetate as more reactive acyl donor than the substrate ester *rac*-**1d** also prevents another possible side reaction: the enzymatic dimerization of the substrate. With CAL-A, interesterification and dimerization are less likely due to the restricted acyl binding pocket, as already mentioned. The obtained results for the model substrate show two interesting features.

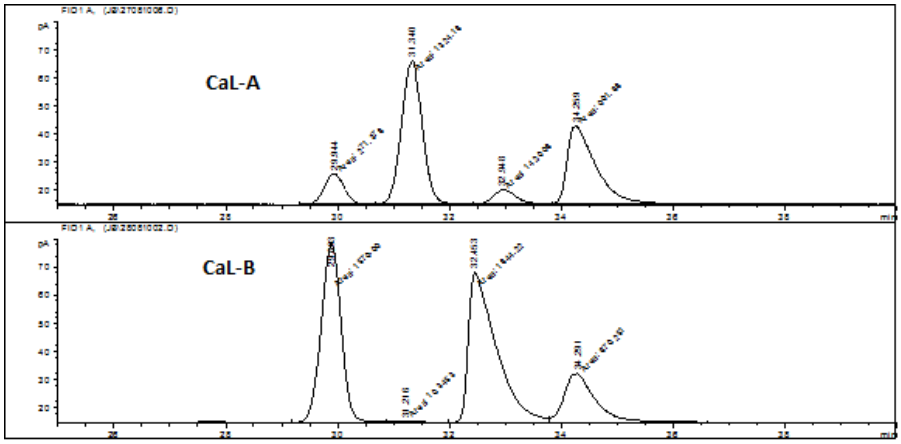


Figure 3. Chromatograms for CaL-A and CaL-B mediated selective acylation of *rac*-**2d** in neat vinyl acetate

Firstly, the *S*-enantiomers reacted with CAL-A and *Rhizopus oryzae* lipase (Table 6, entries 1-3 and 10-11), whereas the other lipases indicated *R*-enantioselectivity (entries 4-9 and 12-17, Figure 3.). Clearly, CAL-B (entry 4) along with various CAL-A preparations, especially when adsorbed on celite in the presence of sucrose (entries 2),[14] gave faster and most enantioselective reactions with *rac*-**1d**. In addition to the lipases shown in Table 6, lipases from porcine pancreas, *Mucor miehei*, *Rhizopus arrhizus*, *Mucor javanicus*, *Aspergillus niger*, *Candida lipolytica* and *Penicillium camemberti* were practically inactive under the screening conditions.

Table 6. Acylation of *rac*-**1d** (0.050 M) with enzymes (50 mg/mL) in neat $CH_3CO_2CH{=}CH_2$ at r.t.

Entry	Enzyme	Time(h)	*c*(%)	ee_{2d}(%)	Config.	ee_{1d}(%)	Config.	*E*
1	CaL-A (lyoph.)	1	25	75	(*S*)	20	(*R*)	7
2	CaL-A on Celite	1	49	78	(*S*)	93	(*R*)	54
3	CaL-A CLEA	1	38	75	(*S*)	39	(*R*)	10
4	CaL-B*	20	7	94	(*R*)	8	(*S*)	40
5	L-AK (lyoph.)	20	25	80	26	(*R*)	12	20
6	L-AK on Celite	20	20	70	18	(*R*)	7	20
7	CRL	20	35	94	(*R*)	38	(*S*)	71
8	CRL sol-gel	20	29	91	(*R*)	39	(*S*)	50
9	Lipase TL-IM	20	2	94	(*R*)	2	(*S*)	36
10	Lipase F	20	7	99	(*S*)	6	(*R*)	124
11	Lipase F immob.	20	6	23	(*S*)	2	(*R*)	2
12	PPL	20	2	41	(*R*)	1	(*S*)	2
13	LPS	20	4	34	(*R*)	3	(*S*)	2
14	PS-C II	20	7	35	(*R*)	85	(*S*)	5
15	PS-D	20	55	61	(*R*)	77	(*S*)	10
16	Alcalase CLEA	20	24	88	(*R*)	28	(*S*)	20

* Novozyme 435

As shown in Table 6, CRL and Lipase-F seem to be appropriate for the kinetic resolution of *rac*-**1d** with the *E* values 71 and 124 respectively (entries 17 and 20). On the other hand, in the case of lipase from *Candida rugosa* the optimization studies did not give satisfactory results (Table 7). A possible explanation is the presence of the isoenzymes in the enzyme, which has been already reported.

Several papers describe the inverse selectivity of lipase F for secondary alcohols, but a detailed study for lipase F catalyzed acylation of β-hydroxy esters is not known by us. The effect of solvent and acylating agent was further studied.

Table 7. CRL (50 mg/mL) catalyzed acetylation of ethyl 3-hydroxy-3-(thiophen-2-yl)propanoate (*rac*-**1d**, 50 mg/mL) with different type of acyl donor (5 eq.) and solvent, at room temperature in presence of molecular sieves

Entry	Solvent	Acyl donor	Time	*c* (%)	ee_P (%)	ee_S (%)	*E*
1	Diethyl ether	VA	6	27	88	40	24
2	Cyclohexane	VA	6	16	91	21	25
3	Toluene	VA	6	18	89	23	22
4	*n*-octane	VA	6	24	93	36	42
5	*n*-octane	VP	16	45	92	90	79
6	*n*-octane	VB	16	50	84	97	47

The selectivity and activity of the enzyme decreased when acyl donors with higher chain length were used (Table 8, entries 1-3). The best solvent was toluene (Table 8, Entry 6). Non-polar solvents (entries 4-7) gave better results, while polar solvent were not appropriate as reaction media (entries 8-12). The main drawback of lipase F in the preparative scale kinetic resolution is their low activity, despite its high enantiomer selectivity ((*S*)-selective enzyme). A possible explanation is the low protein content of the commercial available enzyme preparation.

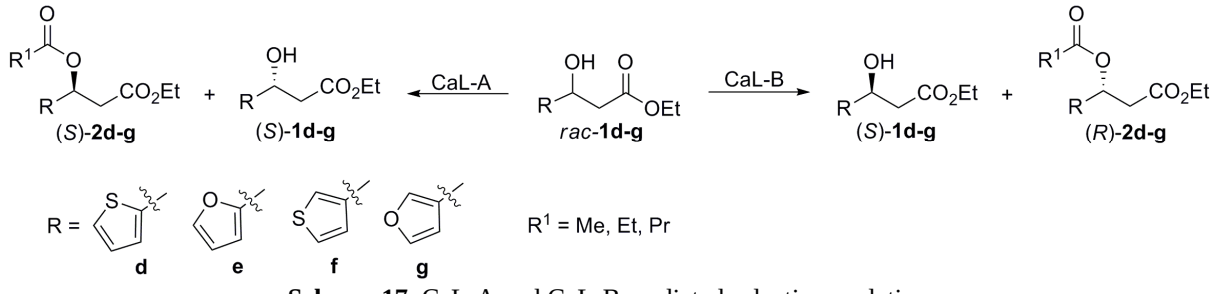
Table 8. Lipase F (50mg/mL) catalyzed acylation of ethyl 3-hydroxy-3-(thiophen-2-yl)propanoate (*rac*-**1d**, 50 mg/mL) with different types of acyl donor (5 eq.) in various solvents at room temperature in the presence of molecular sieves

Entry	Solvent	Acyl donor	*c*(%)	ee_P(%)	ee_S(%)	*E*
1	VA	VA	18	98	22	124
2	VP	VP	14	94	19	84
3	VB	VB	7	85	10	71
4	*n*-Octane	VA	13	96	14	50
5	*n*-Hexane	VA	15	96	17	63
6	Toluene	VA	14	97	16	70
7	Benzene	VA	6	96	6	50
8	Acetonitrile	VA	6	92	6	25
9	Dioxane	VA	0.1	77	0.1	8
10	Chloroform	VA	6	91	6	22
11	Dichloromethane	VA	8	91	7	24
12	THF	VA	6	93	5	29

For this reason, CRL and Lipase F were not further studied.

Accordingly, attention was turned to CAL-A and CAL-B, two fascinating lipases which were previously exploited, even in nonconventional ways, for instance in the *N*-acylation of β-amino esters.[15] The observed reverse enantioselectivity, although being moderate, encouraged us to continue with these lipases.

The acylation of *rac*-**1d** in neat vinyl acetate, propanoate and butanoate (used as acyl donors and solvents) revealed opposite effects of CAL-A and CAL-B on *E* values (Table 9, Scheme 17).



Scheme 17. CaL-A and CaL-B mediated selective acylation

Table 9. CaL-A and CaL-B (50mg/mL) catalyzed acylation of ethyl 3-hydroxy-3-(thiophen-2-yl)propanoate (*rac*-**1d**, 50mg/mL) in neat different types of acyl donor at room temperature

Entry	Acyl donor/solvent	CaL-A[a]				CaL-B[b]			
		c(%)	ee_P(%)	ee_S(%)	*E*	*c*(%)	ee_P(%)	ee_S(%)	*E*
1	VA	50	78	93	54	5	71	3	6
2	VP	62	61	99	19	15	97	17	74
3	VB	51	71	73	13	45	>99	79	»200

[a] after 1h for on Celite immob.CaL-A; [b] after 60 h for CaL-B

Using CAL-A, the highest enantioselctivity (*E*=54, Entry 1) was in neat vinyl acetate, whereas CAL-B gave excellent enantioselectivity (*E*>200, Entry 3) in vinyl butanoate.

Further the effect of the solvent was studied for the acylation of *rac*-**1d** with CAL-A and vinyl acetate (VA) as acyl donor or with CAL-B and vinyl butanoate (VB) respectively (Table 10).

Table 10. CaL-A and CaL-B (50mg/mL) catalyzed acylation of ethyl 3-hydroxy-3-(thiophen-2-yl)propanoate (*rac*-**1d**, 50mg/mL) with CaL-A and VA or CaL-B and VB as acyl donor (5 eq.) in different solvent at room temperature

Entry	**Solvent**	**CaL-A[a]**				**CaL-B[b]**			
		c(%)	ee_P(%)	ee_S(%)	*E*(%)	*c*(%)	ee_S(%)	ee_P(%)	*E*(%)
1	*n*-Octane	21	74	20	8	44	95	75	94
2	*n*-Hexane	20	74	18	8	46	98	85	»200
3	Toluene	14	72	12	7	49	98	93	»200
4	Benzene	12	81	11	10	46	99	85	»200
5	MTBE	19	77	18	10	45	98	81	>200
6	Diethylether	31	73	33	9	50	98	97	»200
7	Acetonitrile	21	86	22	16	8	93	8	27
8	Dioxane	1	52	1	3	3	>99	3	»200
9	Chloroform	3	67	2	5	6	>99	6	»200
10	Dichloromethane	7	87	6	15	9	>99	10	»200
11	THF	9	86	9	15	8	>99	9	»200
12	net acyl donor	50	78	93	54	45	>99	7	»200

[a] after 1h for CaL-A on Celite immob.; [b] after 60h for CaL-B

The reaction in neat vinyl ester was acceptable for both lipases (Table 10, entry 12). In addition, reactivity in ethers and hydrophobic solvents was good in presence of CAL-B and the reactions proceeded with excellent enantioselectivities (*E*>200) (Entries 1-6 and 9) in most cases. Since vinyl butanoate is relatively expensive and its boiling point is high, the experiments with CAL-B were continued using vinyl butanoate in *tert*-butyl methyl ether (MTBE) as a low-boiling solvent.

Finally, the acylation of *rac*-**1d-g** with CAL-A in neat vinyl acetate and with CAL-B with vinyl butanoate in MTBE was tested (Table 11).

Table 11. Kinetic resolution of *rac*-**1d-g** (50mg/mL) with lipases from *Candida antarctica* (50mg/mL)

Entry	Substrate	CaL-A[a]				CaL-B[b]			
		c(%)	ee_P(%)	ee_S(%)	*E*	*c*(%)	ee_S(%)	ee_P(%)	*E*
1	*rac*-**1d**	48	93	80	54	50	96	95	>200
2	*rac*-**1e**	49	85	86	33	51	>99	94	>200
3	*rac*-**1f**	50	73	72	14	50	>99	98	»200
4	*rac*-**1g**	46	85	80	22	48	99	91	»200

[a] after 1h for CaL-A on Celite immob. in net VA; [b] after 70h for CaL-B in MTBE and 5 eq. VB as acyl donor

The obtained results clearly indicated excellent *R*-enantioselectivity for the CAL-B-catalyzed *O*-acylations (*E*>200), whereas CAL-A displayed moderate *S*-selectivity (*E*=14-54).

Finally, preparative scale kinetic resolutions of *rac*-**1d-g** were successfully performed using CAL-B in MTBE, allowing the preparation of the (*R*)-esters **2d-g** (R^1=Pr) and the unreacted (*S*)-**1d-g** in highly enantiomerically enriched forms (ee=95-99%, Table 11).

In spite of low enantioselectivities (*E* = 14-54), the preparative scale kinetic resolutions were performed also with CAL-A in neat vinyl acetate. In accordance with the opposite enantiodiscriminations of CAL-A and CAL-B, the signs of the specific rotations $[\alpha]_D$ of the starting materials **1** are opposite, as are those of the products **2**. The absolute configurations of the resolution products are based on the $[\alpha]_D$ values in Table 12 and on the literature values +19 (*c* 1.7, $CHCl_3$, ee=84%)[16] for (*R*)-**2g**, +18 (*c* 1.6, $CHCl_3$, ee=54)[16] and +15 (*c* 0.9, $CHCl_3$, ee=75%)[17] for (*R*)-**1e** and -94.2 (*c* 1.3, $CHCl_3$, ee=90%)[17] for (*R*)-**2e** (R^1=Pr).

Table 12. Kinetic resolution of *rac*-**1d-g** at preparative scale.

	CAL-B(Novozym 435)				**CAL-A on Celite**		
Product	yield(%)/ ee(%)/$[\alpha]_D$[a]	Product (R^1=Pr)	yield (%)/ ee(%)/$[\alpha]_D$[a]	Product	yield(%)/ ee(%)/$[\alpha]_D$[a]	Product (R^1=Me)	yield (%)/ ee(%)/$[\alpha]_D$[a]
(*S*)-**1d**	46/99/-17	(*R*)-**2d**	48/96/+88	(*R*)-**1d**	45/68/+11	(*S*)-**2d**	44/94/-63
(*S*)-**1e**	47/99/-16	(*R*)-**2e**	48/97/+85	(*R*)-**1e**	47/63/+10	(*S*)-**2e**	45/90/-60
(*S*)-**1f**	46/99/-44	(*R*)-**2f**	48/99/+49	(*R*)-**1f**	47/51/+21	(*S*)-**2f**	40/81/-47
(*S*)-**1g**	46/97/-29	(*R*)-**2g**	47/99/+52	(*R*)-**1g**	46/60/+16	(*S*)-**2g**	41/88/-58

[a] 10^{-1} deg cm^2 g^{-1}; *c* = 1.0, $CHCl_3$, T = 25°C. Isolated yields based on the maximum theoretical recovery from the racemic starting material.

Conclusions

An enantioselective *O*-acylation method was developed to resolve the racemic ethyl 3-hydroxy-3-(thiophenyl/furanyl)propanoates (*rac*-**1d-g**) with vinyl butanoate in MTBE and CAL-B (Novozym 435) as enantioselective catalyst. The highly enantioselective reactions (*E*>200) allowed the resolution of the racemates in almost theoretical yields. Interestingly, CAL-A (adsorbed on celite) shown opposite enantioprefence to that obtained with CAL-B. Although the enantioselectivities with CAL-A were more or less moderate (*E* = 14-54), the resolution products (*R*)-**1d-g** and (*S*)-**2d-g** (R^1=Me) were separated and the specific rotations were determined to witness the opposite optical rotations of the (*S*)-**1d-g** and their butanoate esters (*R*)-**2d-g** (R^1=Pr) obtained in CAL-B catalysis.

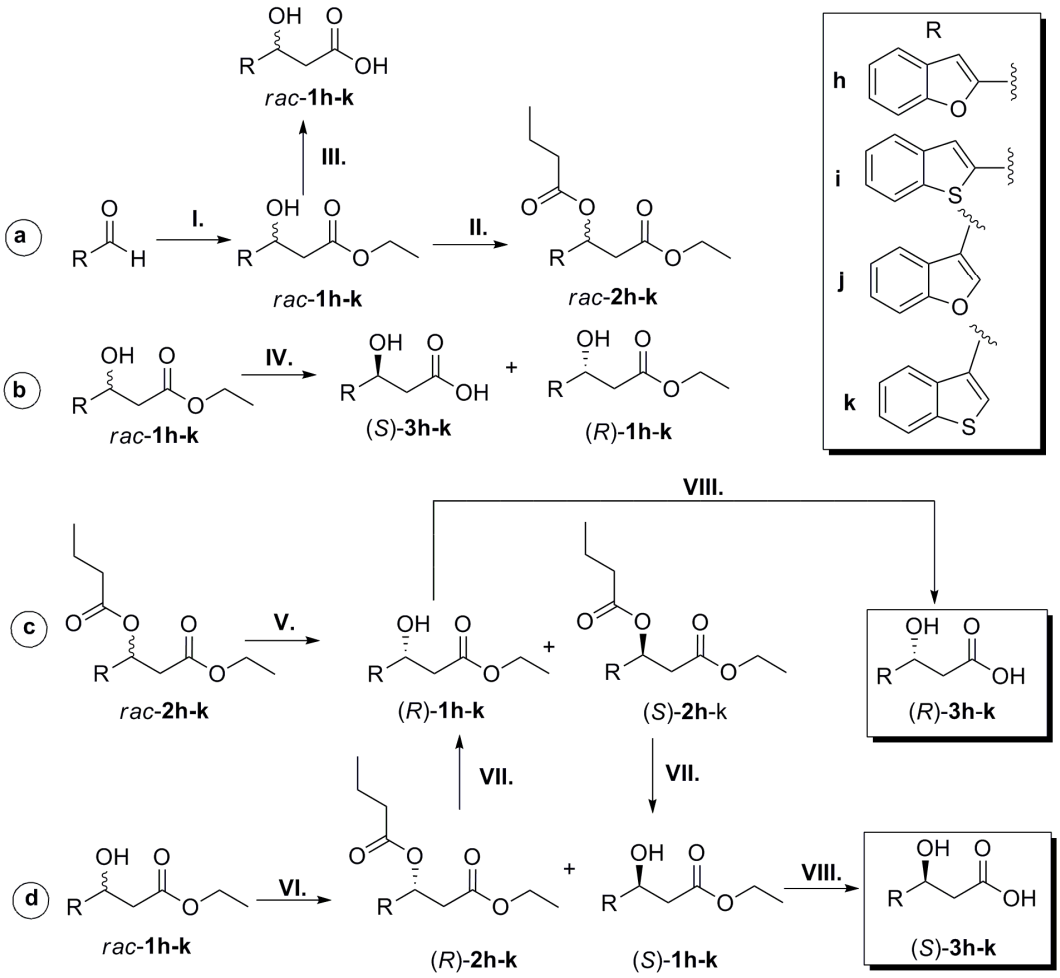
5.2.2. Lipase catalysed synthesis of (*R*)- abd (*S*)-benzofuranyl- and benzo[*b*]thiophenyl-3-hydroxy-propanoic acids

As far we know, the synthesis of optically active 3-benzofuranyl- and benzo[*b*]thiophenyl-3-hydroxypropanoates by enzymatic kinetic resolutions was not yet described.

As a part of our interest in development of enzymatic stereoselective methods for the preparation of optically active heteroaromatic compounds, the enzyme mediated enantioselective synthesis of various 3-heteroaryl-3-hydroxy-propanoic acids **3h-k** and their derivatives **1,2h-k** was developed (Scheme 18).

The absolute configuration of the synthesized enantiomerically enriched compounds was assigned by vibrational circular dichroism (VCD) measurements combined with quantum

chemical calculations at *ab initio* (DFT) level of theory which is a well-established technique for determination of the absolute configuration and conformation of small to medium-sized chiral molecules in solution.[18]



I. Ethyl 2-bromoacetate, Zn, THF, reflux; **II.** $CoCl_2$, $(CH_3\text{-}CH_2\text{-}CH_2\text{-}COO)_2O$, CH_3CN, reflux;
III. PLE in water or K_2CO_3 in water and reflux; **IV.** BCL, H_2O, organic solvent; **V.** CRL, water, organic solvent;
VI. $CH_3\text{-}CH_2\text{-}CH_2\text{-}COO\text{-}CH{=}CH_2$, lipase, solvent; **VII.** BCL, H_2O, organic solvent; **VIII**. PLE, water.

Scheme 18. Lipase mediated enantioselective synthesis of various 3-heteroaryl-3-hydroxy-propanoic acids **3h-k** and their derivatives **1,2h-k**

Enzymatic synthesis

Analytical scale enzymatic hydrolysis of *rac*-1h-k

A wide selection of commercial hydrolases was screened for the analytical scale selective hydrolysis of racemic esteric compounds *rac*-**1h-k**. The bioprocess was monitored chromatographycally with HPLC. Most of the tested enzymes like lipase A, AK, AY, F, G, M, R and Lypozyme TL IM were catalytically inactive. Lipase A and lipase B from *Candida antarctica* (CaL-A and CaL-B formulated as Novozym 435), lipase from *Candida rugosa* (CRL) and PLE exhibited high enzymatic activity but low enantiomer selectivity towards all *rac*-**1h-k**. The complete hydrolysis of all substrates occured in 2-4 hours. Only BCL (former name LPS) proved to be an efficient catalyst for synthesis of the highly enantiomerically enriched (*R*)-**1h-k** (ee=97-99%), while the enantiomeric purity of the formed (*S*)-**3h-k** were found to be low (ee<50%) at conversions closed to 50% (yields between 45-48% for the isolated (*R*)-**1h-k** and (*S*)-**3h-k** from the analytical scale reaction mixtures (Table 13)). This apparent contradiction could be explained only by the slow racemization of the 3-heteroaryl-

3-hydroxy-propanoic acids in the presence of water. The rate of racemization was even higher when instead of water different buffers were used in the pH range 3-8.

Table 13. The enantioselective hydrolysis of *rac*-**1h-k** mediated by lipase from *Burkholderia cepacia* (BCL) in distilled water

Substrate	Time(h)	$Yield_{(R)\text{-}1h\text{-}k}$ (%)	$ee_{(R)\text{-}1h\text{-}k}$ (%)	$Yield_{(S)\text{-}3h\text{-}k}$ (%)	$ee_{(S)\text{-}3h\text{-}k}$(%)
rac-**1h**	24	46	97	47	48
rac-**1i**	24	45	98	47	46
rac-**1j**	32	48	99	49	42
rac-**1k**	32	47	98	48	41

Analytical scale enzymatic hydrolysis of *rac*-2h-k

Next, the small scale biotransformation of the double esteric compounds *rac*-**2h-k** was studied with the same enzymes. Because the enzymatic alcoholysis with several anhydrous alcohols proved to be totally inefficient (yields < 5% after 7 days), further the enzymatic hydrolysis of *rac*-**2h-k** was studied. While most of the tested enzymes were catalytically inactive for this purpose, BCL hydrolyzed *rac*-**2h-k** rapidly and regiospecific, but in a nonstereoselective manner into *rac*-**1h-k** in cyclohexane-THF solvents mixture (1:1, v/v) using 10 equiv. of water. The presence of *rac*-**3h-k** in the reaction mixture was not detected even after 7 days reaction time. Only CRL proved to be a proper biocatalyst for the enantiomer selective hydrolysis of *rac*-**2h-k** (Table 14, Scheme 18, route c). After 24-26 hours, at approx. 50% conversion for the resolution of *rac*-**2i-k**, both products were obtained with high enantiomeric excess (E > 200), but only with moderate enantiomeric excess (E =36 for *rac*-**2h,** Table 14).

Table 14. The enantioselective hydrolysis of *rac*-**2h-k** in presence of CRL

Substrate	Time(h)	*c*(%)	$ee_{(S)\text{-}2h\text{-}k}$	$ee_{(R)\text{-}1h\text{-}k}$	*E*
***rac*-2h**	24	49	95	95	>200
***rac*-2i**	24	47	79	87	36
***rac*-2j**	26	50	95	95	>200
***rac*-2k**	26	49	94	94	>200

Analytical scale enzymatic acylation of *rac*-1h-k

Another approach for the stereoselective synthesis of 3-heteroaryl-3-hydroxy-propanoic acid derivatives was based on the enantiomer selective acylation of the 3-heteroaryl-3-hydroxypropanoates *rac*-**1h-k**. For this, first the analytical scale enantiomer selective enzyme-catalyzed acylations were tested in vinyl acetate in presence of several enzymes.

In case of *rac*-**1h,i** most of the tested enzymes were catalytically inactive. Lipozyme from *Mucor miehei*, Lipase AK and Lipozym TL IM have shown low reactivity and moderate selectivity. Only with Novozym 435 the conversion was closed to 50%after 96 h. However, the selectivity of the reaction was poor (E<7).

It is known that the nature of the solvent and the nucleophile could significantly influence the selectivity of the enantiomer selective acylation. The acylation of *rac*-**1i** with vinyl acetate in presence of Novozym 435 was tested in several solvents, as saturated and aromatic hydrocarbons, cyclic saturated ethers. Cyclohexane proved to be the most appropriate solvent (E= 22 and 46% conversion after 4 days). Further the acylation of *rac*-**1i** was performed with other two acyl donors (vinyl propanoate and vinyl butanoate) in cyclohexane. It was found that the highest selectivity and reactivity was obtained with vinyl butanoate (E>200). Similar results were found using the same conditions for the enzymatic resolution of *rac*-**1h** (Table 15).

For the enzymatic acylation of *rac*-**1j,k** only CaL-A proved to be catalytically active. After performing the same optimization procedure as described above, the highest selectivity

for the CaL-A mediated enantiomer selective acylation of *rac*-**1j,k** was obtained in benzene as solvent and with vinyl butanoate as acyl donor (Scheme 18, route d).

Table 15. The influence of the type of acyl donor upon the selectivity of the enzymatic acylation of *rac*-**1h-k**

Substrate	Reactant	Time(h)	c(%)	$ee_{(S)-1}$	$ee_{(R)-2}$	*E*
rac-**1h**[a]	Vinyl acetate	65	17	14	72	7
	Vinyl propanoate	72	32	37	60	12
	Vinyl butanoate	48	50	99	99	»200
rac-**1i**[a]	Vinyl acetate	106	46	42	49	4
	Vinyl propanoate	160	41	64	94	66
	Vinyl butanoate	60	50	98	98	>200
rac-**1j**[b]	Vinyl acetate	24	45	60	72	11
	Vinyl propanoate	24	50	67	67	10
	Vinyl butanoate	24	48	85	92	65
rac-**1k**[b]	Vinyl acetate	16	44	38	49	4
	Vinyl propanoate	16	40	60	90	35
	Vinyl butanoate	16	49	91	93	87

[a] cyclohexane, Novozym 435; [b] benzene, CaL-A

Analytical scale enzymatic synthesis of both (*R*)- and (*S*)-3h-k

Based on our earlier observation, that BCL and PLE are highly active and nonstereoselective catalyst for the hydrolysis of *rac*-**2h-k** into *rac*-**1h-k** and *rac*-**1h-k** into *rac*-**3h-k** respectively, these two enzymes were further used for the analytical scale synthesis of (*S*)- and (*R*)-**3h-k** without altering the enantiomeric composition of the final isolated products (Scheme 18, route c,d). First by the BCL mediated quantitative hydrolysis of (*S*)-**2h-k** and (*R*)-**2h-k** obtained with the procedures described, respectively, both (*S*)- and (*R*)-**1h-k** were synthesized. Further using a 1:1 substrate-enzyme *ratio* both (*S*)- and (*R*)-**1h-k** were rapidly transformed into the corresponding enantiomerically enriched 3-heteroaryl-3-propanoic acids ((*S*)- and (*R*)-**3h-k**).

Preparative scale synthesis of both (*R*)- and (*S*)-3h-k

Following the sequence depicted in Scheme 18 route c and d, the preparative scale synthesis of both (*R*)- and (*S*)-**3h-k** was setup. All the dilutions, substrate–biocatalyst *ratio* and reaction conditions were the same as in the case of the analytical scale reactions.

First the enantiomer selective hydrolysis of *rac*-**2j,k** and the enantiomer selective acylation of *rac*-**1h,i** were performed. The reactions were monitored by HPLC and TLC and were stopped at an approx. 50% conversion, removing the enzyme by filtration. Further, using BCL as catalyst, the full amount of the previously isolated optically active (*S*)- and (*R*)-**2h-k,** were quantitatively transformed into (*S*)- and (*R*)-**1h-k**. Finally, by a fast PLE mediated hydrolysis, both (*R*)- and (*S*)-**1h-k** were transformed into the corresponding hydroxypropanoic acids. The target compounds, (*R*)- and (*S*)-**3h-k**, were isolated with good yields without altering their enantiomeric composition (Table 16).

Table 16. Yields and specific rotations for the isolated enantiomerically enriched **1-3h-k**

	(*S*)-1h-k			(*R*)-1h-k			(*R*)-2h,i and (*S*)-2j,k			(*S*)-3h-i			(*R*)-3j-k		
	Yield[a]	*ee*	$[\alpha]_D^{25b}$	Yield[a]	*ee*	$[\alpha]_D^{25b}$	Yield[a]	*ee*	$[\alpha]_D^{25b}$	Yield[a]	*ee*	$[\alpha]_D^{25b}$	Yield[a]	*ee*	$[\alpha]_D^{25b}$
h	48.5	99	-13.5	48.5	99	+13.5	49.0	99	+28.5	47.5	99	-5.8	48.0	98	+5.7
i	49.0	98	-24.3	48.5	98	+24.3	49.0	98	+38.5	48.0	98	-19.6	48.0	98	+19.6
j	48.5	94	-42.5	49.0	94	+42.3	49.5	94	-67.5	48.0	94	-25.1	48.5	94	+25.1
k	49.0	95	-24.5	49.5	95	+24.7	49.5	95	-44.5	48.5	95	-10.0	49.0	94	+9.9

[a] yields are given for the isolated compounds based on the maximum theoretical recovery from the racemic starting compounds; [b] measured in $CHCl_3$, at 10 $mg \times mL^{-1}$

The absolute configuration of optically active 1h-k synthesized by the enantiomer selective acylation of *rac*-1h-k

VCD spectra of (-)-**1h-k** obtained by the enantiomer selective acylation of *rac*-**1h-k** with unknown absolute configuration, recorded in $CDCl_3$ solution, are shown in Figure 1. The spectra, dominated by the negative $\nu_{C=O}$ band of the ester carbonyl group at ~1715 cm^{-1} have a rather similar pattern between 1800-1300 cm^{-1}. This indicates that the nature of the heteroatom (O or S) or the position of the side-chain on the achiral heterocyclic moiety has only little influence on the overall shape of the VCD spectrum. This can be explained by the fact that molecules with closely related structures typically have regions of similar VCD spectra, particularly those originating from vibrations of structurally identical parts of the molecules which are not strongly coupled with vibrations of the structurally different parts[1]
The determination of the absolute configuration was based on comparison of the computed and measured spectra for compound (-)-**1h** (Figure 4).

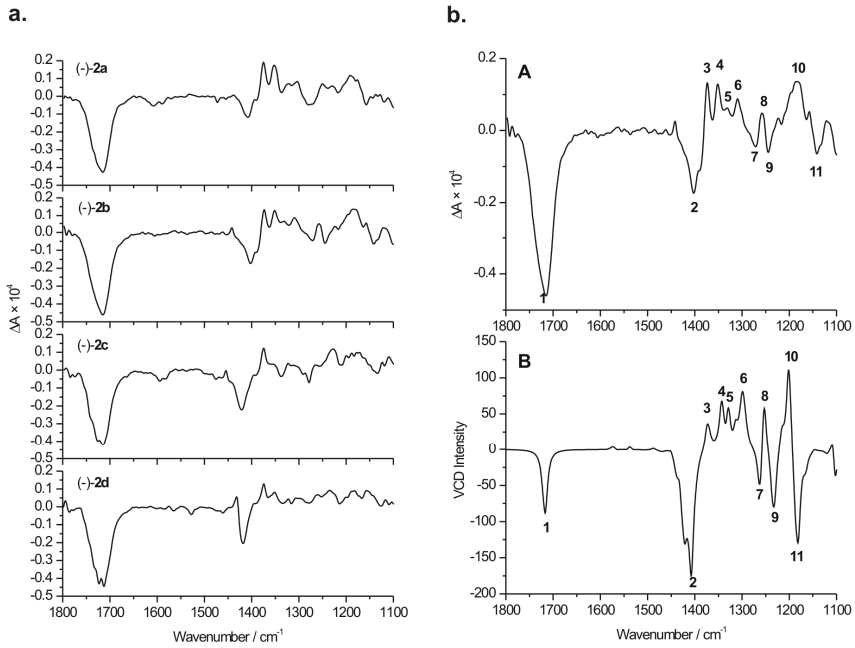


Figure 4. a. VCD spectra of compounds (-)-**1h-k** measured in $CDCl_3$; b. VCD spectrum of (-)-**1h** measured in $CDCl_3$ solution (**A**) in comparison with the simulated VCD spectrum of (*S*)-**1h** (**B**), obtained as a population-weighted sum of the calculated spectra of individual conformers. Corresponding bands are labeled with identical numbers.

The calculations were performed for the (*S*)-**1h** enantiomer and the four lowest-energy conformers, shown in Figure 5, with a total estimated population of 96% were considered for the simulation of the theoretical VCD spectrum. All of these low-energy conformers contain an intramolecular H-bond between the ester carbonyl and the OH group in the β position, in accordance with the relatively low wavenumber value (~1715 cm^{-1}) of the ester $\nu_{C=O}$ band in the experimental spectrum (a typical, non H-bonded aliphatic ester would absorb at ~1740 cm^{-1}).

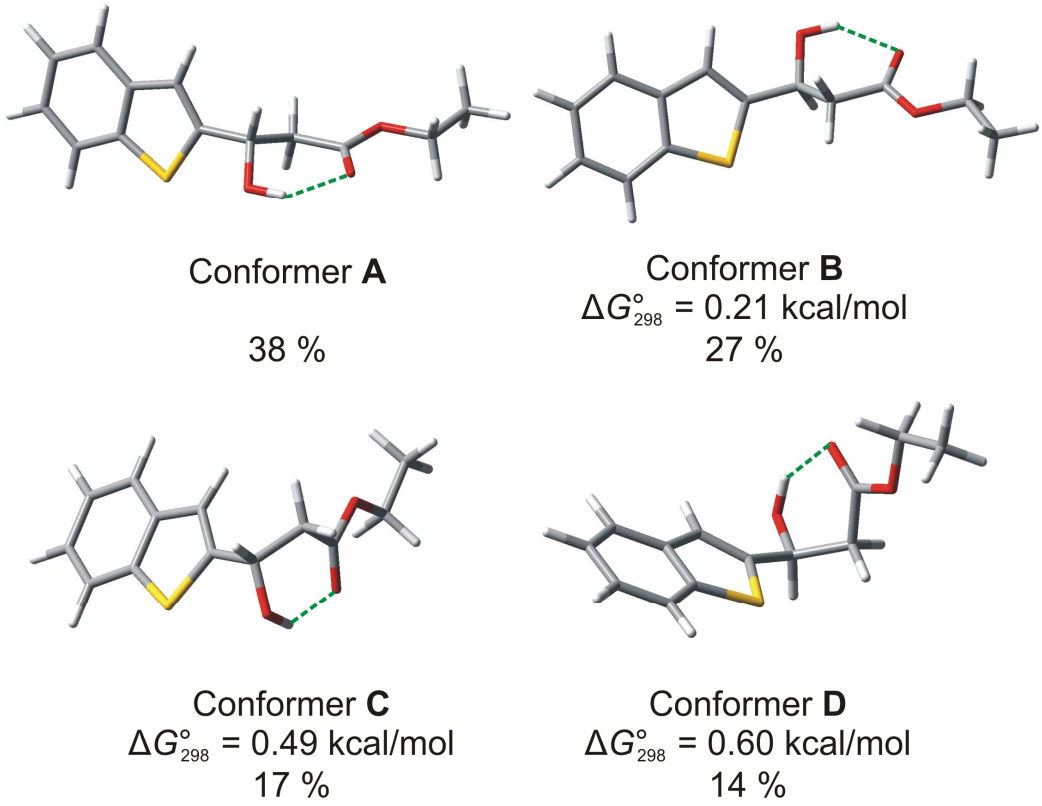


Figure 5. Computed structures of the most abundant equilibrium conformers of (*S*)-**1h** with the indication of their relative Gibbs free energies and estimated populations

The agreement between the calculated and measured VCD spectra is reasonably good, both in terms of wavenumber values and the signs of the VCD bands (the matching pairs are labeled with corresponding numbers in Figure 4) which permits to unambiguously assign the absolute configuration to *S*. Beside the carbonyl vibration (1), bands 3-5 are of particular diagnostic value, being contributed from the coupled bending vibrations of the CH_2, CH, OH and CH_3 groups. For example, the negative band 2 at 1402 cm^{-1} can be assigned to a $\beta_s CH_2$+δCH+βOH coupled vibration, the positive band 3 at 1374 cm^{-1} is contributed from a $\delta_s CH_3$+$\gamma_s CH_2$(ethyl)+ δCH+$\gamma_s CH_2$ coupled vibration.

Conclusions

The enzyme catalyzed enantiomer selective hydrolysis of the racemic 3-heteroaryl-3-hydroxypropanoic acid ethyl esters *rac*-**1h-k** and 2-(ethoxycarbonyl)-1-heteroaryl-ethyl butyrates *rac*-**2h-k** respectively and the enantiomer selective acylation of *rac*-**1h-k** were studied. After the optimal conditions of the enzymatic kinetic resolutions were found, the preparative scale multienzymatic synthesis of both highly enantiomerically enriched enantiomers of the 3-heteroaryl-3-hydroxypropanoic acids was performed.

From the high similarity of the experimental VCD spectra of the investigated enantiomeric (-)-**1h-k**, especially in the 1800-1300 cm^{-1} spectral region, dominated by vibrations of groups directly attached or close to the chiral centre and not strongly coupled with vibrations of the heterocyclic moiety, it is reasonable to suppose that all have the same absolute configuration, notably *S*, as confirmed by quantum chemical calculations in the case of (-)-**1h**

5.2.3. Enzymatic kinetic resolution of *N*-alkylated *rac*-phenothiazin-3-yl-β-hydroxy-esters

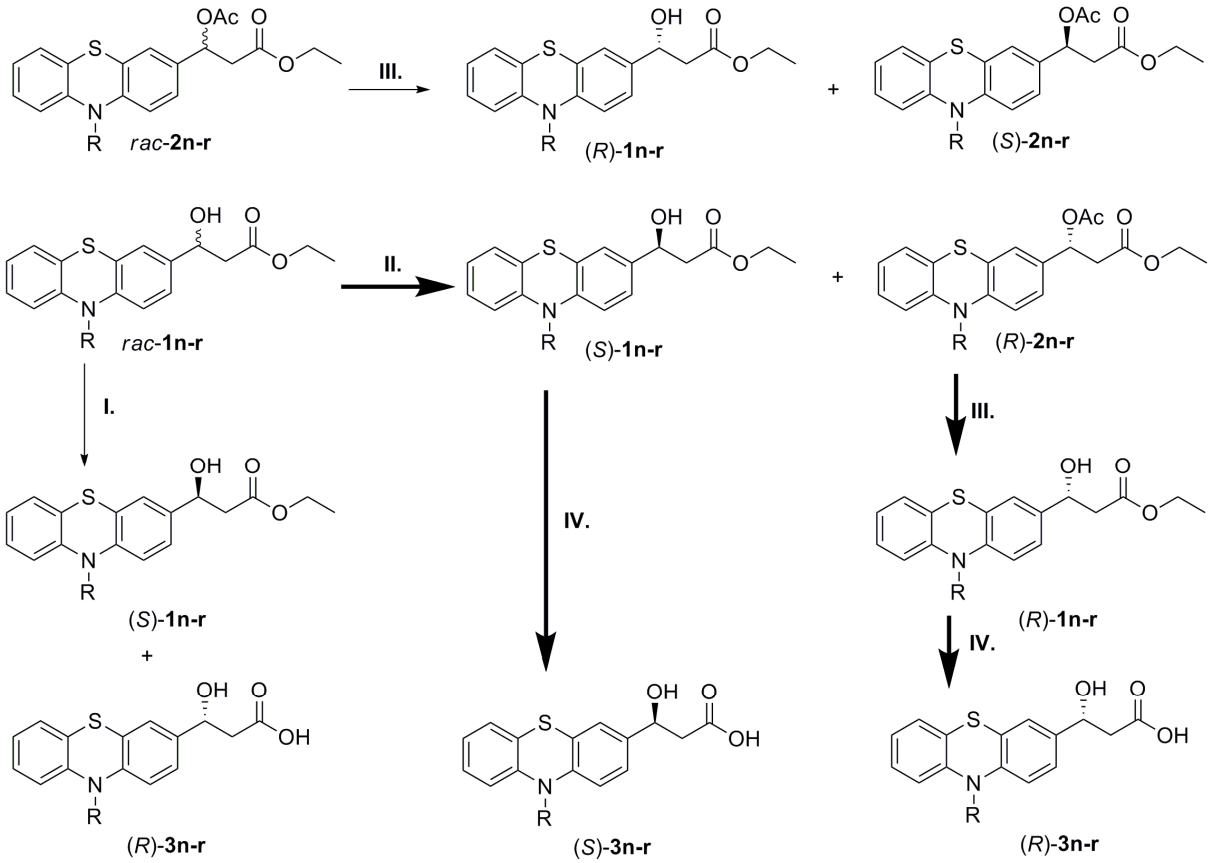
Phenothiazines are one of the oldest and largest groups of neuroleptic drugs[19] and represent a valuable template for the synthesis of different structures, able to interact with a large variety of biological processes, leading to clinically used drugs like tranquilizers,[20] anti-inflammatory,[21] antimalarial[22] and antimicrobial[23] agents. While some phenothiazines are stimulating the penetration of anticancer agents *via* the blood-brain barrier,[24] others inhibit human plasmatic leucine-enkephalin aminopeptidases, enzymes known to regulate the turnover rate of a wide range of bioactive substances. Recently some of them have been considered as potential drugs in the management of Creutzfeldt-Jacob diseases,[25] others with phenothiazin-3-yl-pyrazolo[3,4-*d*]pyrimidine structure have been evaluated for their antitubercular activity against *Mycobacterium tuberculosis* H37 Rv.[26] It has been reported[27] that some phenothiazines inhibit intracellular replication of viruses including human immunodeficiency viruses (HIV). Furthermore, some of these derivatives exhibit significant citostatic effect.[28] For these reasons a great interest has arisen for the design and synthesis of new phenothiazines to explore their anticancer activities.

By their multiple derivatisation possibilities, highly enantiomerically enriched 3-(phenothiazin-3-yl)-3-hydroxy-propanoic acids are good candidates for the synthesis of biologically active compounds.

Optically active β-hydroxy-β-aryl-propanoic acids and their derivatives are highly functionalized chiral synthons, of which the chiral β-hydroxy-β-arylpropionates are precursors for the synthesis of enantiopure pharmaceuticals covering a plethora of actions.

As a part of our interest in development of enzymatic stereoselective methods for the preparation of optically active heteroaromatic compounds, the enzyme mediated enantioselective synthesis of both enantiomers of five optically enriched *N*-substituted (phenothiazin-3-yl)-3-hydroxypropanoic acids **3n**-**r** and their derivatives **1-2n**-**r** was developed (Scheme 19).

The absolute configuration of the synthesized enantiomerically enriched compounds was assigned by vibrational circular dichroism (VCD) measurements combined with quantum chemical calculations at *ab initio* (DFT) level of theory which is a well-established technique for determination of the absolute configuration and conformation of small to medium-sized chiral molecules in solution.[29]



I. PPL, THF, water; II. CaL-A, CH_3-COO-CH=CH_2, acetonitrile; III. BcL, THF, water; IV. Lipase F, THF, water.

Scheme 19. Biotransformations of phenothiazine-based derivatives

Enzymatic synthesis

Analytical scale biotransformations

Analytical scale enzymatic hydrolysis of esteric compounds *rac*-1n-r

In our first approach the enzymatic hydrolysis of *rac*-**1n**-r in presence of various water miscible organic solvents was studied. Experiments were performed in mixtures of water with acetonitrile, dioxane and tetrahydrofuran (water-organic solvent 25:1, v/v) using a 1:1 (w/w) substrate-enzyme *ratio*. Enzymes like lipase from *Burkoholderia cepacia* (BCL), *Penicillium camemberti*, *Pseudomonas fluorescens* (AK), *Aspergillus niger*, *Candida antarctica*, *Candida lypolitica* and *Mucor miehei* were catalytically inactive or shown small activity; others like lipases from *Thermomyces lanuginosus* (TL IM), *Candida rugosa* (CRL) shown poor activity and selectivity. Lipases from *Rhizopus oryzae* (Lipase F), *Rhizopus arrhizus* or *Mucor javanicus*, were highly active but non-selective catalysts, the complete hydrolysis of *rac*-**1n-r** occurring in 2-3 hours. Only lipase from hog pancreas (PPL) exhibited good enzymatic activity and enantiomer selectivity toward all *rac*-**1n-r**. All the results of the enzymatic hydrolysis of *rac*-**1n-r** shown that tetrahydrofuran (THF) is the most proper cosolvent for these reactions. It is important to note that the amount of the organic solvent in the reaction media influenced significantly the results of these reactions. Using various mixtures of water-THF, the activity and selectivity of the selected enzyme (PPL) was determined. It was found that with small amounts of THF (water-THF from 100:1 to 20:1, v/v) the reaction rate was generally lower (conversions between 19-29 % after 24 hours), while the selectivity increase

(ee ~99 % for (*S*)-**3n**-**r**). After an extra 24 h reaction time the conversions increased, but a considerable loss of the enantiomeric excess for both products was observed. When the THF content of the reaction media was higher (water-THF from 15:1 to 5:1), the rate and the selectivity of reaction strongly decreased. Moreover, during the enzymatic hydrolysis the occurrence of *cis*- and *trans*-acrylic acids as by-products was observed (chromatographically detected, showing the same retention times as the external standards). This fact can be explained by the enantiolability and structural instability of the produced (*S*)-3-(phenothiazin-3-yl)-3-hydroxypropanoic acids ((*S*)-**3n**-**r**) at lower pH (6.5-6.8). When instead of water, phosphate or Tris buffer was used (pH 6-8 with increments of 0.5, 20 mM both), the racemization and decomposition rate was similar to those found in water. All these results show that the large scale enzymatic hydrolysis of *rac*-**1n**-**r** could not lead the highly enantiomerically enriched resolution products ((*R*)-**1n**-**r** and (*S*)-**3n**-**r**) with good yields.

Analytical scale enzymatic acylation of *rac*-1n-r

The analytical scale enantiomer selective enzyme catalyzed acylation of racemic 3-hydroxypropanoates *rac*-**1n**-**r** was further studied. *rac*-**1p** was used as model compound for the optimization of the procedure. First the acetylation of *rac*-**1p** in presence of several enzymes in pure vinyl acetate was tested. Most of the tested enzymes like CaL-B, lipase from *Penicililum camemberti*, lipase from *Mucor javanicus*, lipase F, PPL, lipase from *Rhizopus arrhizus*, lipase from *Aspergillus niger*, lipase from *Candida lipolytica* and *Mucor miehei* were catalytically inactive. CrL, L-AK and TL IM showed good enantioselectivities but their activities were low (ee > 90% for (*R*)-**2p**, c < 15 % after 5 days). Only immobilized lipase A from *Candida antarctica* (CaL-A on celite) proved to be highly active and selective, however the catalytic performance of this enzyme was strongly influenced by the used immobilization procedure. If the enzyme was immobilized by reticulation with glutaraldehyde (CLEA) or covalently attached to dry acrylic beads (IMM-CALA), moderate enantiomeric excess of the products was observed even at low conversion. Using lipase A from *Candida antarctica* immobilized by adsorption on Celite (CaL-A) as catalyst the reaction underwent faster and the selectivity was considerably improved (ee>90% for both reaction products at ~50 % conversion).

It is known that the nature of the solvent and the nucleophile could significantly influence the activity and selectivity of the enantiomer selective enzymatic acylation. The CaL-A mediated acylation of *rac*-**1p** with vinyl acetate in several solvents was tested (Table 17).

Table 17. The enantioselective acylation of *rac*-**1n**-**r** with vinyl acetate and CaL-A in different solvents

Entry	1	Solvent	Time (h)	*c* (%)	$ee_{(S)\text{-}1n\text{-}r}$ (%)	$ee_{(R)\text{-}2n\text{-}r}$ (%)	*E*
1	**n**	Acetonitrile	32	53	95	83	42
2	**o**	Acetonitrile	40	53	98	86	64
3	**p**	*t*-BME	48	31.9	43.6	93.2	43
4	**p**	Acetonitrile	48	50	97	95	157
5	**p**	Chloroform	48	11.3	2.4	18.7	1
6	**p**	CH_2Cl_2	48	5	4.2	79.5	9
7	**p**	Toluene	48	34.1	50.5	97.4	127
8	**p**	THF	48	4.2	2.9	67.3	2
9	**q**	Acetonitrile	60	50	97	97	>200
10	**m**	Acetonitrile	72	50	98	98	»200

Acetonitrile and toluene (Table 17, entries 4 and 7) proved to be the most appropriate solvents for the acetylation of *rac*-**1p** (*E*= 157 and 127 at 50 and 34.5 % conversion after 48 hours). In *tert*-butyl-methyl-ether (*t*-BME) the selectivity was moderate (entry 3), while in

polar solvents as chloroform, dichloromethane and THF the catalyst was inefficient (4.2-11.3 % conversion after 48 h, *E* = 1-9; Table 17, entries 5, 6, 8). The CaL-A mediated acylation of *rac*-**1p** with vinyl propanoate and vinyl butyrate in acetonitrile was also studied and no considerable changes for the selectivity of reaction were found as compared to those obtained by using vinyl acetate (data not shown). In accordance with the optimal conditions found for the biotransformation of *rac*-**1p** the CaL-A mediated analytical scale acetylation with vinyl acetate of *rac*-**1n,o,q,r** in acetonitrile was also performed (entries 1, 2, 9, 10) with good reactivity and selectivity. For the CaL-A mediated acetylation an increasing of the selectivity with the size of the alkyl chain was observed, the stereoselectivity number *E* rose from 42 for *rac*-**1n** to 462 for *rac*-**1r**.

Analytical scale enzymatic hydrolysis of *rac*-2n-r and (*R*)-2n-r

A wide selection of commercial hydrolases was screened to investigate the enantio- and regioselectivity of the analytical scale enzymatic lysis of *rac*-**2n-r**.

The analytical scale reactions were performed in neat anhydrous methanol, ethanol, propanol and butanol, followed by experiments in various solvents as halogenated derivatives, hydrocarbons, ethers, saturated linear, cyclic and aromatic hydrocarbons, acetonitrile and dioxane using 5 equiv. of each of the above mentioned alcohols when the substrate-enzyme *ratio* was 1:1, w/w. All these experiments proved to be totally inefficient (yields <5 % after 2 days).

The enzymatic hydrolysis of *rac*-**2p** in several organic solvents was further investigated. The same strategy and optimization methodology as described was used. While most of the tested enzymes were catalytically inactive for this purpose, only BCL and lipase AK hydrolyze *rac*-**2p** with good enantio- and regioselectivity in water-THF mixture (20:3, v/v) (*E*= 183 and c= 45 % after 22 h with BcL and *E*= 50 and c= 22 % after 22 h with lipase AK) using a high enzyme-substrate *ratio* (2:1, w/w). During these reactions the carboxyethylic moiety of the substrates was not affected, the presence of 3-acetoxy-3-(10-alkyl-10*H*-phenothiazin-3-yl)propanoic acids and 3-hydroxy-3-(10-alkyl-10*H*-phenothiazin-3-yl)propanoic acids (**3n-r**) in the reaction mixture was not detected. Using less water content or a smaller enzyme-substrate *ratio*, the reaction time increased and a considerable amount of degradation products appeared, lowering the yields of the useful products. Using the previously described reaction conditions the BCL mediated hydrolysis of *rac*-**2n-r** was further tested (Table 18).

Table 18. The enantioselective hydrolysis of *rac*-**2p** in water-THF mixture (20:3, v/v) with BCL

Substrate	*c* (%)	$ee_{(S)\text{-}2}$ (%)	$ee_{(R)\text{-}1}$ (%)	*E*
2n	43.4	84.1	96.7	46
2o	43.5	83.6	96.3	44
2p	41.9	88.7	95.8	64
2q	40.7	82.4	95.1	95
2r	45.2	80.3	97.3	183

The same procedure also proved to be efficient for the hydrolysis of the enantiomerically enriched ethyl (*R*)-3-acetoxy-3-(10-alkyl-10*H*-phenothiazin-3-yl)propanoates (*R*)-**2n-r,** synthe-sized as previously described, into (*R*)-ethyl 3-hydroxy-3-(10-methyl-10*H*-phenothiazin-3-yl)propanoates (*R*)-**1n-r**. The products were isolated with good yields enhancing their enantiopurities as compared to those of the starting compounds (Table 19).

Table 19. The enantioselective hydrolysis of (*R*)-**2n-r** in water-THF mixture (20:3, v/v) with BCL

Substrate	$ee_{(S)\text{-}2n\text{-}r}$ (%)	Product	Yield[a] (%)	$ee_{(R)\text{-}1n\text{-}r}$ (%)
(*R*)-**2n**	83	(*R*)-**1n**	75	95
(*R*)-**2o**	86	(*R*)-**1o**	77	97
(*R*)-**2p**	95	(*R*)-**1p**	85	98
(*R*)-**2q**	97	(*R*)-**1q**	86	99
(*R*)-**2r**	98	(*R*)-**1r**	85	98

[a] after 24 hours

Preparative scale synthesis of both (*R*)- and (*S*)-3n-r

Following the sequence depicted in Scheme 19, the preparative scale synthesis of both (*R*)- and (*S*)-**1-3n-r** was set up. All the dilutions, substrate–biocatalyst *ratio* and reaction conditions were the same as in the case of the analytical scale reactions.

The enantiomeric excesses and optically rotatory power of the obtained optically enriched compounds are presented in Table 20. First the enantiomer selective *O*-acetylation of *rac*-**1n-r** with vinyl acetate in presence of CaL-A in acetonitrile was performed, affording quantitatively (*S*)-**1n-r** and (*R*)-**2n-r**. The reactions were monitored by HPLC and TLC and were stopped at an approx. 50% conversion, removing the enzyme by filtration. Further, using BCL as catalyst, the previously isolated optically active (*R*)-**2n-r** was hydrolyzed into (*R*)-**1n-r**. Finally, by a fast and nonselective hydrolysis mediated by lipase F, both (*S*)- and (*R*)-**1n-r** were transformed into the corresponding hydroxyl-propanoic acids (*S*)-**3n-r** and (*R*)-**3n-r**. The target compounds were isolated with good yields without altering their enantiomeric composition (Table 20).

Table 20. Enantiomeric excesses and specific rotations for the isolated enantiomerically enriched **1-3n-r**

Comp.	Yield[a] (%)	ee [%]	$[\alpha]_D$ [b]	Comp.	Yield[a] (%)	ee [%]	$[\alpha]_D$ [b]	Comp.	Yield[a] (%)	ee [%]	$[\alpha]_D$ [b]
(*S*)-**1n**	49.0	95.7	-72.9	(*R*)-**2n**	49.0	80	+97.6	(*S*)-**3n**	48.5	95.5	-23.6
(*S*)-**1o**	49.0	98.5	-67.5	(*R*)-**2o**	48.5	82	+157.0	(*S*)-**3o**	48.0	98	-19.5
(*S*)-**1p**	48.5	92.5	-57.6	(*R*)-**2p**	48.5	94.5	+164.2	(*S*)-**3p**	48.0	92	-28.0
(*S*)-**1q**	48.0	96.3	-78.7	(*R*)-**2q**	48.0	96.4	+179.1	(*S*)-**3q**	48.5	96	-27.1
(*S*)-**1r**	49.0	95.7	-80.7	(*R*)-**2r**	49.0	95.7	+151.3	(*S*)-**3r**	49.0	95	-27.2
(*R*)-**1n**	40.0	95	+73.1					(*R*)-**3n**	37.5	95	+23.4
(*R*)-**1o**	39.0	97	+66.5					(*R*)-**3o**	38.0	96	+19.1
(*R*)-**1p**	42.0	98	+61.0					(*R*)-**3p**	42.5	97	+29.6
(*R*)-**1q**	43.0	99	+80.9					(*R*)-**3q**	43.0	98	+27.7
(*R*)-**1r**	42.0	98	+82.6					(*R*)-**3r**	42.0	97	+27.8

[a] based on *rac*-**1n-r** as starting compounds; [b] c = 1.0, $CHCl_3$ for **1-2n-r** and DMF for **3n-r** (at 23-24 ºC).

The absolute configuration of optically active 1n-r synthesized by the enantiomer selective acylation of *rac*-1n-r

The VCD spectra of (-)-**1n-r** obtained by enantiomer selective acylation of *rac*-**1n-r** with unknown absolute configuration, recorded in $CDCl_3$ solution, are shown in Figure 6. The spectra, dominated by the negative $\nu_{C=O}$ band of the ester carbonyl group at ~1712 cm^{-1} have a rather similar pattern in the represented 1800-1100 cm^{-1}. This indicates that the size of the alkyl substituent on the N atom of the achiral phenothiazine moiety has only very little influence on the overall shape of the VCD spectrum. This can be explained by the fact that molecules with closely related structures typically have regions of similar VCD spectra, particularly those originating from vibrations of structurally identical parts of the molecules which are not strongly coupled with vibrations of the structurally different parts.

The determination of the absolute configuration was based on comparison of the measured and computed spectra for compound (-)-**1n** (Figure 6).

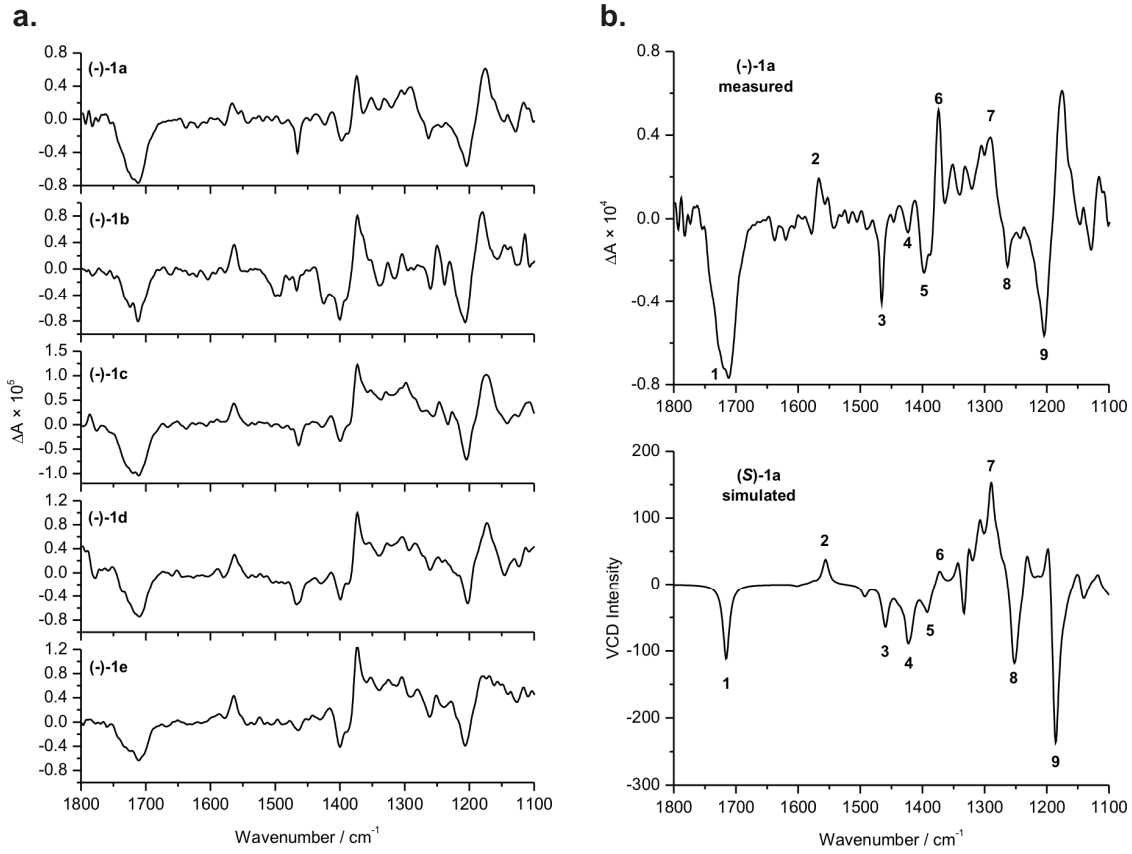


Fig 6. a. VCD spectra of compounds (-)-**1n**-**r** measured in $CDCl_3$; b. VCD spectrum of (-)-**1n** measured in $CDCl_3$ solution (top) in comparison with the simulated VCD spectrum of (*S*)-**1n** (bottom), obtained as a population-weighted sum of the calculated spectra of individual conformers. Corresponding bands are labelled with identical numbers

The calculations were performed for the (*S*)-**1n** enantiomer and the eight lowest-energy conformers were considered for the simulation of the theoretical VCD spectrum, out of which the most abundant four, with a total estimated population of 81% are shown in Figure 7.

All of these low-energy conformers contain an intramolecular H-bond between the ester carbonyl and the OH group in the β position, in accordance with the relatively low wavenumber value (~1712 cm^{-1}) of the ester $\nu_{C=O}$ band in the experimental spectrum (a typical, non H-bonded aliphatic ester would absorb at ~1740 cm^{-1}).

The agreement between the calculated and measured VCD spectra is reasonably good, both in terms of wavenumber values and the signs of the VCD bands (the matching pairs are labelled with corresponding numbers in Figure 6b) which permits to unambiguously assign the absolute configuration to *S*. Besides the carbonyl vibration (1), bands 2-9 are of particular diagnostic value, being contributed from the coupled bending vibrations of the CH_2, CH, and OH groups attached to the chiral centre, as well as ring vibrations of the heteroaromatic moiety. For example, the positive band 2 at 1567 cm^{-1} can be assigned to a coupled δCH + heteroaromatic ring vibration βOH; the negative band 9 at 1204 cm^{-1} is contributed from a βOH+γasCH2+ δCH+βCH(aromatic) coupled vibration.

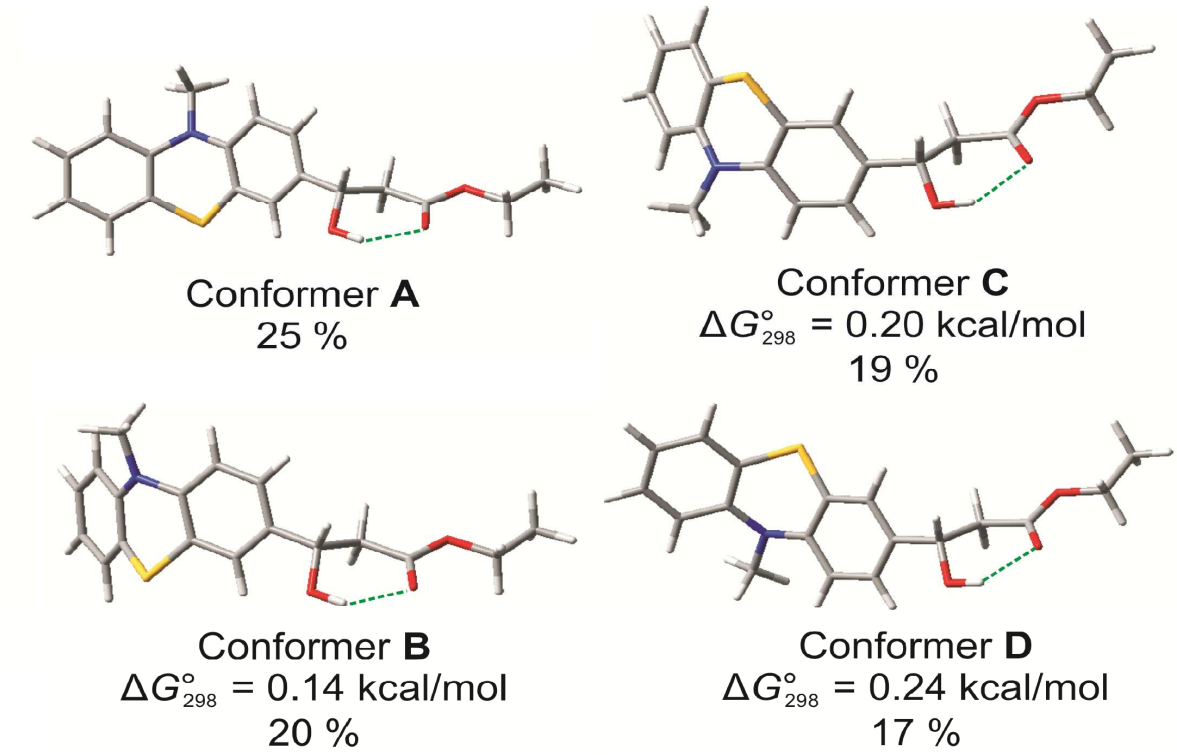


Figure 7. Computed structures of the most abundant equilibrium conformers of (*S*)-**1n** with the indication of their relative Gibbs free energies and estimated populations

Conclusions

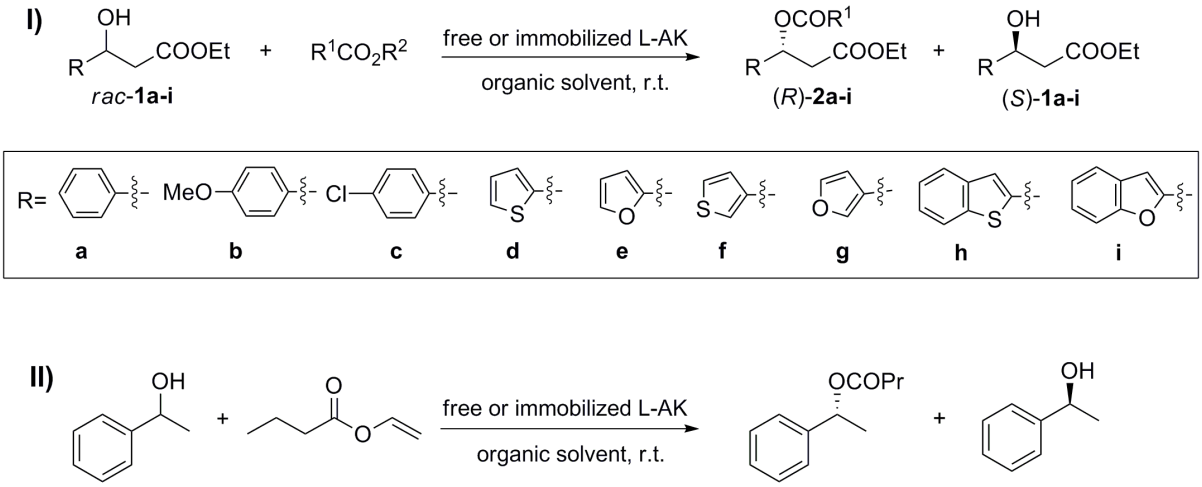
The synthesis of both enantiomers of five new optically enriched *N*-substituted (phenothiazin-3-yl)-3-hydroxypropanoic acids by a multienzymatic procedure was setup. First the CaL-A mediated acetylation of the racemic ethyl 3-hydroxy-3-(10-alkyl-10*H*-phenothiazin-3-yl)propanoates was performed. The isolated diesteric compounds (*R*)-**2n-r** were selectively hydrolysed by BCL into (*R*)-**1n-r**. Finally by a fast and nonstereoselective enzymatic hydrolysis catalysed by lipase F both (*R*)- and (*S*)-**1n-r** were transformed into the desired products. From the high similarity of the experimental VCD spectra of the investigated enantiomeric derivatives (-)-**1n-r** in the 1800-1100 cm^{-1} spectral region and the expected little to no influence of the alkyl substituent of the N atom of the phenothiazine ring on the vibrations of groups surrounding the chiral centre, it is reasonable to suppose that all have the same *S* absolute configuration, as confirmed by quantum chemical calculations n the case of (-)-**1n**.

5.2.4. Biotransformation with taylor-immobilized L-AK

As motivation of the present work, L-AK belongs to commonly used commercial lipases and the acylations of *rac*-**1a** and *rac*-**1d-i** with free L-AK powder previously showed low reactivity and only moderate to good enantioselectivities.

The immobilization of lipases from *Pseudomonas* sp. using adsorption[30] cross-linking methods,[31] sol-gel encapsulation,[32] combinations of these methods[33] and using other techniques[34] have been thoroughly described. Most of the studies have been performed independently using different amounts of the enzyme, making comparison of the methods impossible.

In the present work, lipase AK from *Pseudomonas fluorescens* (L-AK) adsorbed on Celite (diatomaceous earth, diatomite) in the presence of sucrose, L-AK cross-linked as CLEAs and L-AK encapsulated in sol-gels was studied. Free L-AK powder and the immobilized preparations were characterized and optimized in the terms of effects on relative activities (a measure of short term stability), reuse (a measure of long term stability) and enantiomeric ratio *E* (a measure of enantioselectivity) using constant protein contents (1.5 mg protein in mL) for the acylation of *rac*-**1a** as a model reaction in organic solvents (Scheme 20). For substrate scope, the optimized catalysts were subjected to the enantioselective acylation of aryl- and heteroaryl-β-hydroxy esters (*rac*-**1a-i**). For characterization of the new L-AK preparations, 1-phenylethanol was also included in the activity and enantioselectivity studies.



Scheme 20. Acylation of β-hydroxy esters and 1-phenylethanol with L-AK.

Generalities about immobilization

With increasing of the chemical applications of lipases, the need to stabilize the enzyme and to improve enzymatic activity and selectivity has become crucial. Accordingly, effective immobilization methods have been developed, including binding to a carrier, cross-linking and encapsulation in an organic or inorganic polymeric matrix. For lipase immobilization, adsorption on various solid supports as a simple, inexpensive and easy to perform method has been traditionally most commonly used. The possibility for enzyme leaching from the immobilizate, especially in aqueous solutions, is the disadvantage of this method. Covalent immobilization involves the multipoint covalent attachment of an enzyme directly or through a spacer on a carrier. One of the benefits of this method is that enzyme leaching is not possible. From several cross-linking methods, CLEC (cross-linked enzyme crystals) technology was commercialized in 1990s. However, CLEA (cross-linked enzyme aggregates)

technology is practically replacing this expensive and time consuming method since the early 2000s. A suitable dialdehyde, such glutaraldehyde, is commonly used to perform the cross-linking between protein crystals (CLEC) and physical enzyme aggregates (CLEA) in these carrier-free technologies. The advantage of CLEAs over CLECs is based on enzyme precipitation by inorganic salts or organic solvents rather than on the crystallisation of the purified enzyme. Encapsulation is a mild and especially useful method for enzymes which are easily deactivated, for instance by covalent immobilization. Sol-gel encapsulation, in particular, has been proven to be favorable for lipase immobilization as sol-gel formation easily proceeds by the acid- or base-catalyzed hydrolysis of silanes, the hydrolysis of the mixture of tetraalkoxysilanes and alkyltrialkoxysilanes being commonly used. The methodology leads to inert glasses with high porosity and high thermal and mechanical resistance, with varying hydrophobicity depending on the structure and amount of the alkyltrialkoxysilane component

Free L-AK powder in solvent screening

Ethyl 3-hydroxy-3-phenylpropanoate (*rac*-**1a**) was chosen as model compound to investigate the effect of various immobilization techniques on the catalytic performance of L-AK (Scheme 20). *Rac*-**1a** was first subjected to acylation with vinyl acetate and free L-AK powder (1.5 mg/mL protein in the reaction) in toluene, diisopropyl ether (DIPE) and methyl *tert*-butyl ether (MTBE) (Fig. 8). While the reactions in DIPE (E = 111) and MTBE (E = 61) were slightly faster than those performed in toluene, best enantioselectivity (E = 135) was obtained in toluene, yielding both the product (*R*)-**2a** (R^1=Me) and the unreacted substrate (*S*)-**1a** with 95 % ee at 50 % conversion after 15 h. In order to prevent or at least to minimize the proportion of the enzymatic hydrolysis of the ester substrate and product by the residual water of the seemingly dry enzyme powder, the solvent screening was performed in the presence of molecular sieves (4 Å). Although acylation with vinyl butanoate was found to give higher enantioselectivity than with vinyl acetate, the optimizations of the L-AK immobilizates were performed using vinyl acetate as acyl donor in toluene, since the less enantioselective case is more revealing for the fine tuning of enzymatic enantioselectivity, in particular.

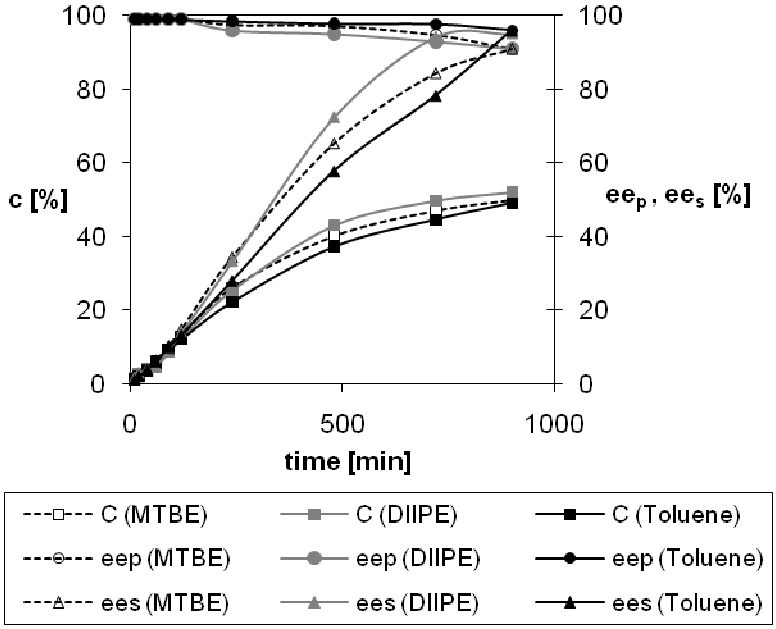


Fig. 8. Solvent screening for the *O*-acetylation of *rac*-**2a** (0.1 M) with L-AK powder (25 mg/mL) and vinyl acetate (0.2 M) in organic solvents.

Immobilized L-AK catalysts in the *O*-acylation of *rac*-2a in toluene

I) Adsorbtion on celite.

Adsorption on hydrophilic Celite is a common lipase immobilization method. In the present work, L-AK was adsorbed on Celite in presence of sucrose as previously described[14] and the obtained preparation is called herein L-AK on Celite. According to spectroscopic evidence, sugars may improve a biocatalyst for instance by reducing protein/protein interactions and thus protein aggregation. The presence of a sugar may also even favor the distribution of the adsorbed or residual water over the dry enzyme preparation and reduce the ability of a solvent to stripe the water, thus preventing the enzymatic hydrolysis of hydrolyzable substrates and/or products. As expected, both relative activity and enantioselectivity in the *O*-acylation of *rac*-**1a** with vinyl acetate in toluene were improved by catalysis with L-AK on Celite compared to catalysis with free L-AK powder (Table 21). Moreover, molecular sieves were not needed to prevent the enzymatic ester hydrolysis.

Table 21. Relative activity (x) and enantioselectivity of L-AK (1.5 mg protein/mL) for the *O*-acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) in toluene at room temperature.

Entry	Enzyme preparation	x[a] (%)	*E*
1	L-AK powder	100	145
2	L-AK on Celite	127	>200

[a] Relative activity x is defined as *v*(immobilized enzyme)/*v*(free enzyme)∗100, where *v* is the initial rate of the reaction in μmol∗ (h∗mg lipase)$^{-1}$.

II) CLEA.

The preparation of CLEAs is a two-step procedure where the precipitation of the enzyme from an aqueous solution is followed by the *in situ* cross-linking, mostly with glutaraldehyde. Additives or co-precipitants can be used in order to force the lipase to adopt a more active conformation. In the present work, precipitation (type A) and co-precipitation (type B) methods were used for preparation L-AK CLEAs. The obtained catalysts were then studied for the *O*-acylation of *rac*-**1a** with vinyl acetate in toluene (Table 22).

With Type A catalysts, precipitation with saturated ammonium sulfate, DME and acetone resulted in preparations with dramatically reduced activity and enantioselectivity (Table 22, entries 1-5) as compared to the result obtained with free L-AK powder (Table 22, entry 1; *E* = 135 with 100% relative activity). Moreover, the reactions stopped before reaching completion, making these enzyme preparations useless for successful kinetic resolutions. Further the enzymatic activity deceased after the reduction with $NaBH_4$ of imine linkages between glutaraldehyde and the free amino groups of the enzyme, forming amine linkages (Table 22, compare entry 1 with 2 and entry 4 with 5). Efforts to optimize type A precipitations with respect to time, temperature and precipitation agents were not successful either. Evidently, glutaraldehyde was harmful to L-AK and/or the enzyme precipitated in an unfavorable conformation. It was previously shown that the presence of co-precipitants help the enzyme to reach a more active conformation. For this reason, the negligible reactivity in the acylation of *rac*-**1a** with L-AK CLEA prepared with 18-crown-6 as a co-precipitant in DME (type B method, entry 6) was a disappointment. On the other hand, *N*-hexadecylacetamide (CeAmide) or hexadecan-1-ol (CeOH) as co-precipitation agent in acetone gave L-AK CLEAs with considerably improved activity and enantioselectivity as compared to type A cases (entries 7 and 8 compared to 1-5). It is important to note that both reactions proceeded also to 50% conversion, allowing the enantioseparation by kinetic resolution. Although less active than L-AK powder, the L-AK CLEA obtained by acetone/CeAmide precipitation was used in further studies.

Table 22. Relative activity (x) and enantioselectivity for the *O*-acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) and L-AK CLEA preparations (1.5 mg protein/mL) in toluene at room temperature.

Entry	Type	Precipitation agent	x (%)	*E*
1	A	sat. $(NH_4)_2SO_4$	32	80
2	A[a]	sat. $(NH_4)_2SO_4$	26	64
3	A	DME	30	52
4	A	DME with 18-crown-6	1	-[b]
5	A	Acetone	22	67
6	A[a]	Acetone	15	81
7	B	CeAMiD	75	162
8	B	CeOH	65	160

[a] After reduction of the Schiff base with $NaBH_4$; [b] not determinate, $c < 5\%$.

III) Sol-gel.

There are several ways to encapsulate enzymes in a sol-gel matrix. Herein, the combination of methyltrimethoxysilane [$MeSi(OMe)_3$, MTMS] with tetramethoxysilane [$Si(OMe)_3$, TMOS] was chosen to generate a hydrophobic matrix which is expected to enhance the interfacial activation and the enzymatic activity of the entrapped lipase. Two different methods, type A and B, were also used. For the type A sol-gel method (called also a xerogel), the sol precursor was separately prepared in acidic water followed by evaporation of the formed methanol before to proceed to enzyme encapsulation. For the sol-gel type B, the Reetz method, based on the base-catalyzed *in situ* polymerization of the silanes during the encapsulation process, was optimized.[32]

For the acylation of *rac*-**1a** with vinyl acetate and a type A L-AK sol-gel preparation in toluene, considerably reduced relative activities were observed (Table 23). Activity was highest when a 3.8/1 TMOS/MTMS *ratio* was used (entries 1-4). Ipa as additive had not effect on the enzymatic activity (entries 1 and 2), whereas by washing the encapsulated enzyme with Ipa the relative activity decrease (entry 3). The addition of Celite into the aqueous L-AK solution before encapsulation improved the enantioselectivity (entry 4), while the addition of L-AK on Celite catalyst gave an immobilizate with lower activity (entry 5). It is worth to note that mixing L-AK on Celite with the sol precursor is difficult and may lead to the observed lower activity. Encapsulation with a 1/5 [TMOS]/[MTMS] *ratio*, previously found optimal for the fluoride ion-catalyzed hydrolysis of the silane mixture, resulted in considerable decrease of the relative activities (entries 6-8). The work was not continued with the type A L-AK sol-gel preparations.

Table 23. Relative activity (x) and enantioselectivity for the *O*-acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) and Type A L-AK sol-gel preparations (1.5 mg protein/mL) in toluene at room temperature.

Entry	TMOS/MTMS (mmol/mmol)	Ipa gel/wash	Additives/enzyme form	x (%)	*E*
1	3.8/1	no/no	no/L-AK powder	46	113
2	3.8/1	yes/no	no/L-AK powder	45	118
3	3.8/1	yes/yes	no/L-AK powder	17	144
4	3.8/1	no/no	Celite[a]/L-AK powder	35	>200
5	1/5	no/no	no/L-AK powder	3[b]	>200
6	1/5	yes/no	no/L-AK powder	4[b]	>200
7	3.8/1	no/yes	no/AK on Celite	7[b]	>200
8	1/5	no/yes	no/AK on Celite	3[b]	>200

[a] 75 mg of Celite.
[b] Reaction stopped before 50% conversion.

The type B method in preparing L-AK sol-gel without additives showed the optimal 9/1 water/gel (Figure 9A) and 5/1 [MTMS]/[TMOS] ratios (Figure 9B) for the acylation of *rac*-**1a** with vinyl acetate in toluene. The reduction of activity at low water contents can be explained

by enzyme aggregation, while at higher water contents only a partial encapsulation of the enzyme is the possible explanation, part of the lipase being adsorbed on the surface of the gel or in a free form. The optimal ratio [TMOS][MTMS]=1/5 for the type B catalysts indicates different hydrophobicity demands compared to the type A L-AK sol-gels with the 3.8/1 optimal ratio. The type B L-AK sol-gel preparations were always washed with Ipa after encapsulation, since this procedure was previously shown to be favorable for achievable enzymatic activity.[35,32]

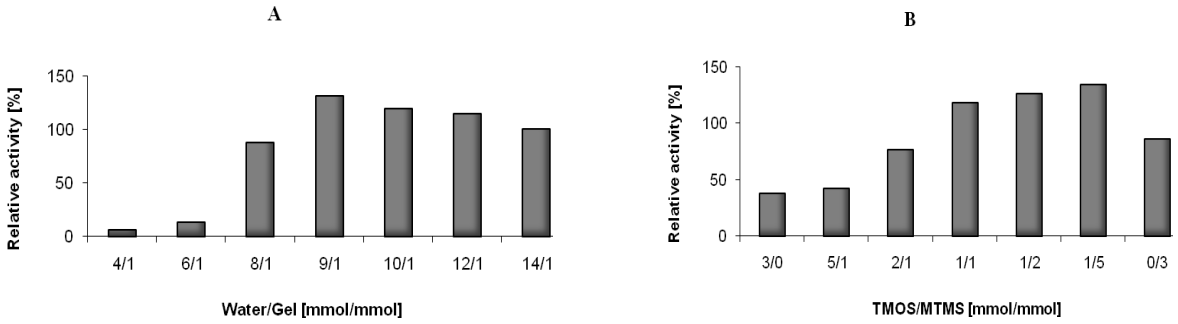


Figure 9. Effect of (A) the amount of water in gel (TMOS/MTMS 1:5) and (B) the ratio (TMOS/MTMS) of a silane precursor on the enzyme activity for the acylation of *rac*-**2a** (0.1 M) with vinyl acetate (0 .2 M) in toluene.

Based on the above information, type B L-AK sol-gel catalysts with or without additives were prepared using the [TMOS]/[MTMS]=1/5 and water/gel=9/1 ratio followed by sol-gel washing with Ipa. The obtained biocatalysts were subjected to the acylation of *rac*-**1a** with vinyl acetate in toluene (Table 24). The L-AK sol-gel preparations obtained without additives (entry 3) or with Ipa, Celite and sucrose as additives (entry 6) turned to be of highest activity and of excellent enantioselectivity. Otherwise, enantioselectivities considerably varied from case to case, and the additives tended to decrease the relative activity of the L-AK sol-gels (entries 4,5, 7-12). Interestingly, the presence of Celite and sucrose in aqueous L-AK solution in immobilization (entry 6) increased enzymatic activity while the addition of Celite alone decreased activity (entry 4). This result confirms the positive effect of sucrose for the outcome of the catalyst prepared. On the other hand, the encapsulation of L-AK on Celite (L-AK adsorbed on Celite in the presence of sucrose) caused a drop in enzymatic activity (entries 13 and 14) resulting in a mixture which was not homogenous and indicated relatively strong adsoption forces in the L-AK on Celite preparation under the gelation conditions.The addition of PEG-400 improved the activity (entry 2) while the addition of sucrose and/or Celite together with PEG-400 lowered it (entries 9 and 10) with simultaneous negative effects on enantioselectivities. The sol-gels prepared without additives (relative activity 134 and E>200, entry 3) and in the presence of Celite and sucrose (relative activity 122 and E>200, entry 6), were used for further experiments.

Table 24. Relative activity (x) and enantioselectivity for the *O*-acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) and sol-gel preparations (Type B; TMOS/MTMS 1:5;1.5 mg protein/mL) with additives in toluene (1 mL) at room temperature.

Entry	Ipa content gel[a]/wash	Additives/enzyme form	x (%)	*E*
1	yes/yes	no/ L-AK powder	97	>200
2	yes/yes	PEG 400/ L-AK powder	110	165
3	no/yes	no/ L-AK powder	134	>200
4	no/yes	Celite[b]/ L-AK powder	44	97
5	yes/yes	Celite[b]/ L-AK powder	52	150
6	yes/yes	Celite[b], sucrose[c]/ L-AK powder	122	>200
7	yes/yes	methyl-β-CD[d]/ L-AK powder	36	63
8	yes/yes	KCl[e]/ L-AK powder	19	41
9	yes/yes	PEG 400, sucrose[c]/ L-AK powder	47	160
10	yes/yes	PEG 400, sucrose[c], Celite[b]/ L-AK powder	55	130
11	yes/no	PEG 400, sucrose[c], Celite[b]/ L-AK powder	25	162
12	yes/yes	hexadecyl amine[f]/ L-AK powder	28	45
13	no/yes	hexadecyl amine[f]/ L-AK powder	56	134
14	yes/yes	no/AK on Celite	26	>200
15	no/yes	no/AK on Celite	25	>200

[a] 100 μL; [b] 50 mg; [c] 100 mg; [d] 100 mg; [e] 2 mmol; [f] 20 mg.

Reuse of immobilized enzymes

Reusability is one of the important features of immobilized enzymes in synthetic chemistry. For testing the recycling capacity, the best L-AK preparations [L-AK on Celite (Table 21, entry 2), L-AK CLEA (L-AK precipitated with acetone/CeAMiD, Table 22, entry 7), type B L-AK sol-gels without additives (Table 24, entry 3) and the one prepared in the presence of sucrose and Celite (Table 24, entry 6)] in addition to free L-AK powder were used at constant protein content of 1.5 mg /mL for the acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) in toluene. The reaction was repeated with the same enzyme preparation up to 10 times. Every reaction was allowed to proceed to 50% conversion or as long as the preparation showed activity and enantioselectivity before the catalyst was subjected to the next cycle. Between the cycles the catalyst was washed with dry toluene and thereafter used without drying. The results are shown in Table 25(selectivity) and Figure 10 (relative activity). L-AK powder and L-AK on Celite lost the entire enzymatic activity after the catalysts were used 3 and 4 times, respectively. In the acylation, the L-AK CLEA preparation was not quite satisfactory either as the catalyst showed decreased enzymatic activity (conversion attained after a certain time) and enantioselectivity (E = 162 in the first cycle and 100 after used 10 times). On the other hand, the sol-gel immobilizates both provided excellent catalytic properties. After reused ten times, the relative activity was still 85-87% (134 % initial) for sol-gel without additives and 90-92% (122 % initial) when Ipa, Celite and sucrose were used as additives. At the same time, the reuse had not an effect on enzymatic enantioselectivity, allowing the preparation of (*S*)-**1a** and (*R*)-**2a** from *rac*-**1a** in highly enantiomerically enriched forms (ee>95%) still on the tenth reuse cycle.).

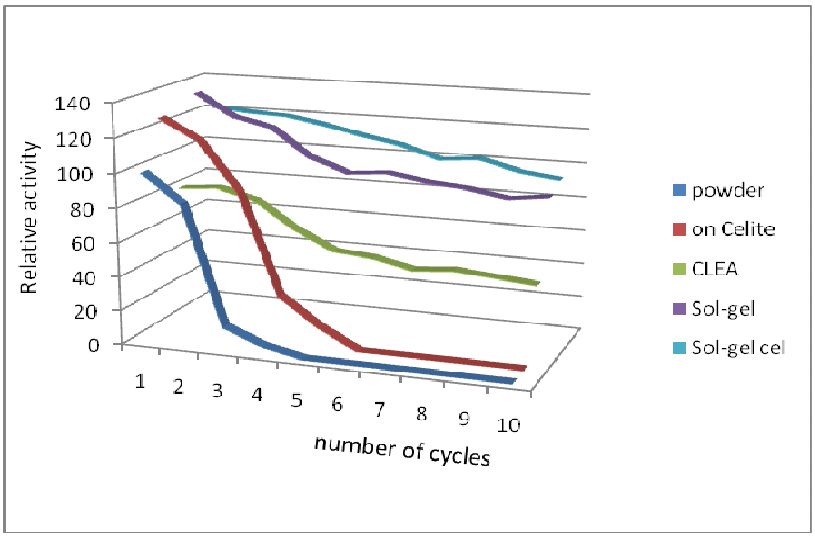


Figure 10. Reuse of different AK-preparations (relative activity)

Table 25. Reuse of different AK-preparations (1.5 mg protein/mL) for the *O*-acylation of *rac*-**1a** (0.1 M) with vinyl acetate (0.2 M) in toluene (1 mL) at room temperature.

L-AK	Cycle 1					Cycle 10				
	powder	**on Celite**	**CLEA**	**Sol-gel[b]**	**Sol-gel[c]**	**powder[a]**	**on celite[a]**	**CLEA**	**Sol-gel[b]**	**Sol-gel[c]**
Time(h)	15	12	20	12	10	-	-	45	15	12
c (%)	50	50	47	50	49	-	-	35	50	50
$ee_{(S)\text{-}\mathbf{2a}}$	95	99	87	96	95	-	-	54	97	98
$ee_{(R)\text{-}\mathbf{3a}}$	95	95	96	95	99	-	-	96	96	97
E	135	>200	162	>200	>200	-	-	100	>200	>200

[a] The preparation inactive.
[b] Type B L-AK sol-gel (TMOS/MTMS=1/5; water/gel ratio 9/1) encapsulating an aqueous L-AK solution.
[c] Type B L-AK sol-gel (TMOS/MTMS=1/5; water/gel ratio 9/1) encapsulating the aqueous solution of L-AK, Ipa, Celite and sucrose.

Immobilized enzymes in the *O*-acylation of *rac*-2a-j

The immobilization studies of the present work indicated interesting new L-AK CLEA and L-AK sol-gel catalysts which were finally tested for the acylation of *rac*-**1a-i** and 1-phenylethanol (0.1 M) with vinyl butanoate in toluene. Vinyl butanoate was chosen due to their higher enantioselectivity compared with those obtained with vinyl acetate for the acylation of *rac*-**1a** with free L-AK powder (Table 26, column 3, entries 1 and 2). The obtained results (Table 26) unambiguously indicate excellent applicabilities of the optimized immobilizates for the *O*-acylations of 3-aryl-3-hydroxypropanoates. From the activity point of view, the immobilized preparations showed nice activity improvements, the acylations of *rac*-**1h** and **1i** with L-AK on Celite and those of *rac*-**1a-c** with L-AK CLEA being exceptions. Considerably enhanced enzymatic activity and *R*-enantioselectivity were observed for the acylation of heteroaromatic substrates *rac*-**1d-g**, when L-AK powder was replaced by L-AK on Celite (column 4) or L-AK sol-gel encapsulating the aquous solution of L-AK, Ipa, Celite and sucrose (column 7, entries 5-8). The results in Table 26 together with those concerning the enzymatic reuse raise the L-AK sol-gel catalyst to the most successful position among our immobilizates. A possible explanation is that Celite and sucrose help in the essential water binding in the enzyme preparation, the sugar working as a lypoprotectant which prevents protein aggregation and drastic protein conformational modifications.[36]

Table 26. Relative activity (x) and enantioselectivity for the *O*-acylation of (hetero)aryl-β-hydroxy esters **1a-i** (0.05 M) and 1-phenyl ethanol (**Ph-OH**; 0.4 M) with vinyl butanoate (2 eq.) and L-AK (1.5 mg protein/mL) in toluene at room temperature.

Entry	Substrate	L-AK Powder (x/*E*)	L-AK on Celite (x/*E*)	L-AK CLEA[a] (x/*E*)	L-AK sol-gel[b] (x/*E*)	L-AK sol-gel[c] (x/*E*)
1	**1a**[d]	100/135	127/>200	81/>200	134/>200	122/>200
2	**1a**	100/>200	135/>200	72/>200	138/>200	125/>200
3	**1b**	100/>200	135/>200	72/>200	138/>200	125/>200
4	**1c**	100/>200	132/>200	76/>200	128/>200	118/>200
5	**1d**	100/68	192/137	118/54	92/55	181/130
6	**1e**	100/81	198/187	122/63	96/74	190/184
7	**1f**	100/57	155/106	116/52	107/63	144/112
8	**1g**	100/66	163/126	121/42	110/68	141/108
9	**1h**	100/>200	91/>200	121/>200	124/>200	116/>200
10	**1i**	100/>200	87/>200	128/>200	127/>200	110/>200
11	**Ph-OH**	100/>200	194/>200	137/>200	139/167	166/>200

[a] L-AK precipitated with acetone/CeAMiD.

[b] Type B L-AK sol-gel (TMOS/MTMS=1/5; water/gel ratio 9/1) encapsulating an aqueous L-AK solution; the gel washed with Ipa.

[c] Type B L-AK sol-gel (TMOS/MTMS=1/5; water/gel ratio 9/1) encapsulating the aqueous solution of L-AK, Ipa, Celite and sucrose.

[d] Vinyl acetate as an acyl donor.

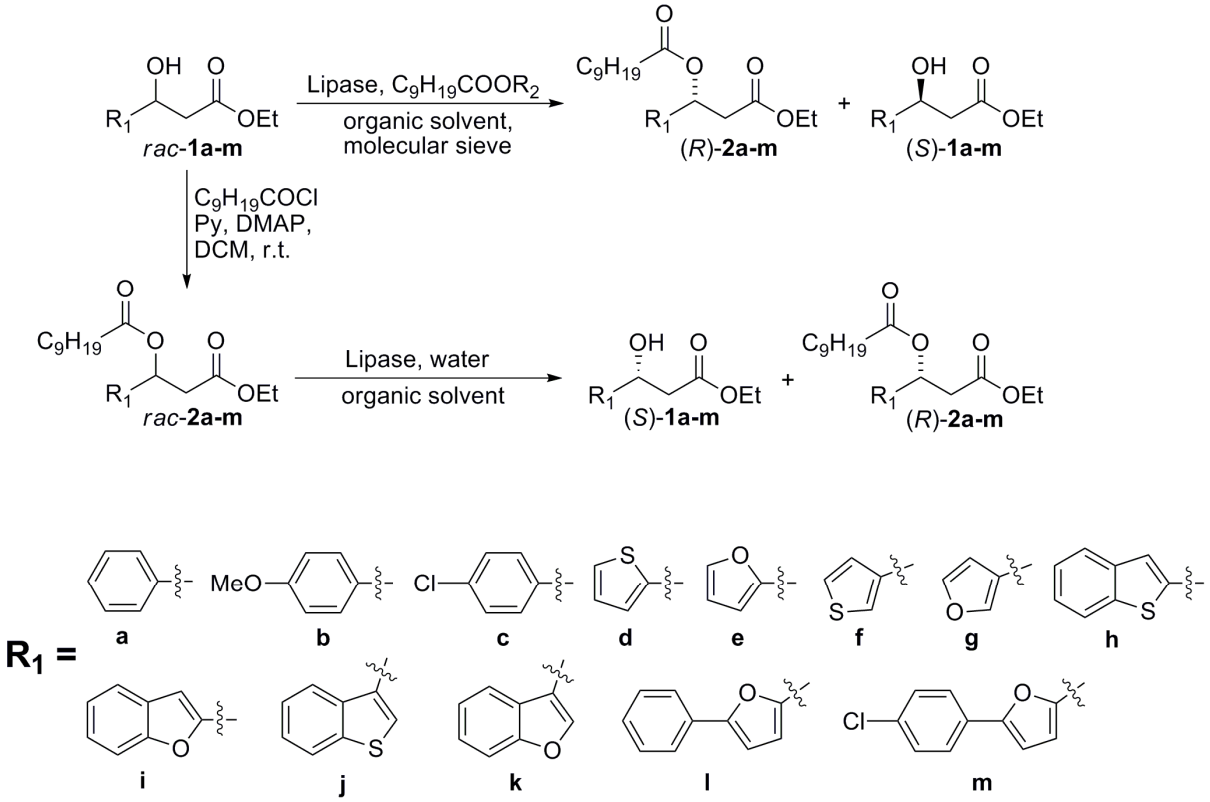
Conclusions

Immobilization of lipase A from *Pseudomonas fluorescens*, lipase AK "Amano"(L-AK) has been studied using adsorption, CLEA technology and sol-gel encapsulation methods. L-AK adsorbed on Celite in the presence of sucrose, L-AK CLEAs using *N*-hexadecylacetamide (CeAmide) or hexadecan-1-ol (CeOH) as co-precipitant in acetone and L-AK sol gel preparations encapsulating L-AK or L-AK together with Celite and sucrose from aqueous solution were the best catalysts in terms of enzymatic activity and enantioselectivity for the acylation of ethyl 3-phenyl-3-hydroxypropanoate (*rac*-**1a**) with vinyl acetate in toluene, used as a model reaction. Considering the relative activities, enantioselectivities and reuse capabilities, the best sol-gel immobilizates were obtained using methyltrimethoxysilane [$MeSi(OMe)_3$, MTMS] and tetramethoxysilane [$Si(OMe)_3$, TMOS] (TMOS/MTMS=1/5; water/gel ratio 9/1) as silanes in fluoride ion-catalyzed hydrolysis when the L-AK and possible additives were encapsulated *in situ*. The study has interestingly shown that L-AK sol-gels obtained when the sol precursor was prepared separately in acidic water and the methanol was evaporated before enzyme encapsulation, the ratio TMOS/MTMS=3.8/1 rather than 1/5 was optimal (although still less active than free L-AK powder), indicating the need for less hydrophobic matrix then.

The work has shown that L-AK sol-gel prepared by type B method (TMOS/MTMS=1/5; water/gel ratio 9/1) encapsulating aqueous solution of L-AK, Celite and sucrose is superior to all other immobilizates in this work also as to the substrate scope (ethyl 3-furanyl-, 3-thiophenyl-, 3-benzofuranyl-, 3-benzo[b]thiophenyl and 3-phenyl-3-hydroxy propanoates as well of 1-phenyl ethanol).

5.2.5. General enzymatic procedure using natur-like substrates and reagents for the resolution of aryl-β-hydroxy-esters

The aim of this work was to study the enantiomer selective lipase-catalyzed O-acylation versus the lipase mediated selective hydrolysis to produce both enantiomeric forms of various β-hydroxy esters and their *O*-acylated diesters with high purity (Scheme 21).



Scheme 21. Kinetic resolution of racemic β-hydroxy esters *rac*-**1a-m** and their diesters *rac*-**2a-m**

Optimization of the enzymatic reactions

Since the enzymatic hydrolysis β-hydroxy esters generally precedes only with moderate stereoselectivity, first the kinetic resolution by enantiomer selective lipase-catalyzed *O*-acylation was investigated.

Lipase-catalyzed kinetic resolution by *O*-acylation

Biocatalyst and solvent screening and the effect of water

To develop a general procedure for the enzymatic kinetic resolution of various aromatic β-hydroxy esters *rac*-**1a-m**, first the stereoselective lipase-catalyzed *O*-acylation of ethyl 3-hydroxy-3-phenylpropanoate *rac*-**1a** was tested under various conditions. Commercial lipases (25 mg/mL, each) were screened for the lipase mediated *O*-acylation of *rac*-**1a** (0.025 M) with decanoic acid as acyl donor (0.1 M) in various organic solvents at room temperature. Free and immobilized forms of Lipases A and B from *Candida antarctica* (CaL-A and CaL-B), lipase from *Burkholderia cepacia* (formerly *Pseudomonas cepacia*; BCL, LPS), lipase from *Pseudomonas fluorescens* (LAK) and lipase from *Candida rugosa* (CrL), were tested as suitable biocatalysts in dried organic solvents. Since even small trace of water could promote

hydrolytic reactions yielding undesired byproducts, all acylation tests were performed in presence of molecular sieves in the reaction mixture.

LAK, LPS and CaL-B were unsatisfactory as biocatalysts for acylation *rac*-**1a** in all of the tested solvents; 3-hydroxy-3-phenylpropanoic acid, formed by enzymatic hydrolysis of the carboxyethyl moiety, was detected as major product. In the control reaction (performed without adding the acyl donor) a quantitative hydrolysis of *rac*-**1a** was observed with all three lipases. Consequently, the high residual water content prohibits the use of these biocatalysts for our main scope.

The similar control reaction indicated no significant hydrolytic activity with CaL-A and CrL, which renders these enzymes as potential biocatalysts in stereoselective acylation of *rac*-**1a**. Therefore, further tests for kinetic resolution of *rac*-**1a** by acylation with decanoic acid were performed only with CaL-A and CrL (Table 27). Among the investigated variations of solvents and enzymes, the best results were achieved in *n*-octane with CrL (AYS Amano) (Entry 7). Therefore, the further tests were performed with this enzyme (CrL, from now on) and solvent.

Table 27. Lipase (25 mg/mL) and solvent (1 mL) screening for the selective *O*-acylation of *rac*-**1a** (0,025 M) with decanoic acid (0,1 M) at room temperature after 15h

Entry	Lipase	Solvent	*c* (%)	$ee_{(R)\text{-}2a}$ (%)	$ee_{(S)\text{-}1a}$ (%)	*E*
1	CaL-A on Celite	DIPE	27.7	8.6	3.3	1.2
2	CaL-A on Celite	Toluene	21.3	12.6	3.4	1.3
3	CaL-A on Celite	*n*-Octane	19.7	12.6	3.1	1.3
4	CrL (free, Type VII.)	DIPE	4.3	82.7	3.7	11
5	CrL (free, Type VII.)	Toluene	6.9	85.5	6.3	13
6	CrL (free, Type VII.)	*n*-Octane	15.8	97.9	18.4	113
7	CrL (free, AYS Amano)[a]	*n*-Octane	17.0	98.5	20.2	161
8	CrL (immob., T2-150)	*n*-Octane	6.0	97.8	6.2	95

[a] Abbreviated as CrL in the further part of this work

The effect of the acyl donor

The fast regio- and enantiomer selective lipase-catalyzed deprotection of various *O*-acylated β-hydroxy esters could provide a practically useful method to perform kinetic resolutions. To achieve this goal, an *O*-acyl moiety with a longer carbon chain is required. For this purpose various fatty acids (decanoic acid, lauric acid and stearic acid) were tested as acyl donors. The preliminary tests indicated that CrL showed catalytic activity only in presence of decanoic acid.

Since the nature of the acyl donor could significantly influence the selectivity and activity of CrL (AYS Amano) in *n*-octane, further the reactions were carried out in presence of various decanoic acid derivatives (Table 28). With ethyl, isopropyl and butyl ester as acyl donors, CrL AYS Amano showed high stereoselectivity but moderate activity (Entries 2-4). When vinyl decanoate as irreversible acyl donor was used, the enantiomer selectivity decreased slightly, however the enzyme activity was considerably enhanced (Entry 5). Accordingly, the further studies were performed with vinyl decanoate as acyl donor.

Table 28. The influence of decanoic acyl donors (0.1 M) on the selectivity of CrL (25 mg/mL) for the *O*-acylation of *rac*-**1a** (0.1M) in *n*-octane at room temperature after 15h

Entry	**Acyl donor**	***c* (%)**	**$ee_{(R)\text{-}2a}$ (%)**	**$ee_{(S)\text{-}1a}$ (%)**	***E***
1	Decanoic acid	17.0	98.5	20.2	161
2	Ethyl decanoate	4.0	98.6	4.1	148
3	Isopropyl decanoate	6.1	>99	6.4	>200
4	Butyl decanoate	2.0	>99	2.1	>200
5	Vinyl decanoate	44.8	97.2	78.9	170

The temperature effect

The dependence of the performance of the CrL in the acylation of *rac*-**1a** with vinyl decanoate in *n*-octane on the reaction temperature was also investigated (Table 29). While the selectivity increased at higher temperatures, a strong irreversible decrease of the activity of CrL was observed due to thermal inactivation. Retesting in the same reaction the already used (at 45 °C) CrL catalyst, resulted in an acylation with quite low conversion (c < 5%). Since the heat treated CrL and the untreated enzyme presented similar enantiomer selectivity (E ~165 and E =170 respectively), we suppose that the enhancement of selectivity at increased temperature (E »200) is determined not by the selective denaturation of a less selective component from the crude enzyme, but by an intrinsic feature of the enzyme. Taking both selectivity and activity into account, the optimal temperature for the acylation of *rac*-**1a** appeared to be 25 °C.

Table 29. The influence of temperature upon selectivity and activity of the CrL (25 mg/mL) mediated selective *O*-acylation of *rac*-**1a** (0.025 M) with vinyl decanoate (0.1 M) in *n*-octane after 15h

Entry	***t* (°C)**	***c* (%)**	**ee$_{(R)\text{-}2a}$ (%)**	**ee$_{(S)\text{-}1a}$ (%)**	***E***
1	15	36.7	95.8	55.6	82
2	25	44.8	97.2	78.9	170
3	35	32.9	>99	48.5	>200
4	45	10.9	>99	12.2	»200

The regio-and enantiomer selective *O*-acylation of racemic aromatic β-hydroxy esters *rac*-**1a-m**

To investigate the biocatalytic behavior of CrL in kinetic resolutions, the *O*-acylation of further racemic aromatic β-hydroxy esters *rac*-**1b-m** were tested under the optimal conditions previously found for *rac*-**1a** (Table 30). Almost in all cases the reaction proceeded with good selectivity and activity, however, the results were considerably influenced by the nature of aromatic ring in the substrates *rac*-**1b-m**.

Table 30. Selective *O*-acylation of different aromatic β-hydroxy esters *rac*-**1b-m** (0.025 M) with CrL (25 mg/mL) in *n*-octane with vinyl decanoate (0,1 M) at room temperature after 15 h

Entry	Substrate	*c* (%)	ee$_{(R)\text{-}2a}$ (%)	ee$_{(S)\text{-}1a}$ (%)	*E*
1	*rac*-**1b**	49.7	98.3	97.2	>200
2	*rac*-**1c**	22.0	98.5	27.8	174
3	*rac*-**1d**	52.0	92.1	>99	182
4	*rac*-**1e**	50.7	87.3	89.9	45
5	*rac*-**1f**	51.6	91.1	97.1	91
6	*rac*-**1g**	35.2	94.7	51.5	61
7	*rac*-**1h**	47.8	97.2	89.1	>200
8	*rac*-**1i**	41.9	>99	72.1	»200
9	*rac*-**1j**	20.3	87.7	22.3	19
10	*rac*-**1k**	21.9	97.5	27.4	103
11	*rac*-**1l**	40.7	96.6	66.2	115
12	*rac*-**1m**	51.7	93.3	>99	»200

The outcome of the acylation was obviously dependent on several factors such the size and charge distribution of the aromatic moiety or the linear or bent nature of the substrate. In general, the polar and small substituents were less beneficial in terms of both activity and selectivity (Entries 2 and 6). Whereas the extension of the bulkiness in linear direction was tolerated and beneficial (Entries 1, 7, 8), the bulky but bent substrates were less tolerated and acylated at lower rate and selectivity (Entries 9, 10). The slightly bent and polarized phenylfurane compound resulted in acylation with moderate rate and selectivity (Entry 11). Interestingly, further extension of the substituent [the 5-(4-chlorophenyl)furan-2-yl moiety] resulted in excellent substrate *rac*-**1m** (Entry 12). An additional effect influencing the overall

rate of the acylation might be the low solubility of certain substrates which was quite pronounced for benzo[*b*]thiophen-3-yl-hydroxy-propanoate *rac*-**1j**.

Lipase-catalyzed regio-and enantiomer selective hydrolysis

It is already known that lipases usually retain their enantiomer preference in hydrolysis or alcoholysis. Consequently, by reactions of opposite directions the opposite enantiomeric forms of the alcohol and ester fractions should result. It was previously reported that CrL is a highly enantioselective catalyst for the hydrolysis of various types of racemic aromatic β-hydroxy-β-arylpropanoates or of their *O*-acylated derivatives.[37] Therefore, the catalytic performance of CrL for the selective hydrolysis of the decanoate esters *rac*-**2a-m** was also investigated.

Solvent screening for lipase-catalyzed regio- and enantiomer selective hydrolysis

In order to found the optimal reaction condition, various organic solvents were used as reaction media for the CrL mediated selective hydrolysis of racemic diester *rac*-**2a** (0.025 M). In case of water miscible solvents (acetonitrile, THF) and polar solvents of high water solubility (DCM) the tests with water content of 10μL/mL indicated non-optimal results (data not shown). The best results in terms of enzyme activity and selectivity were obtained in water-saturated nonpolar solvents (Table 31). In *n*-octane or toluene (Table 31, entries 3, 4) higher selectivity and lower activity was detected in comparison with those obtained in ethers (Table 31, entries 1, 2). Due to the better solubility of the substrates and to the higher activity of the enzyme in DIPE for our further study this solvent was used as reaction media.

Table 31. Solvent screening for the hydrolysis of the racemic diacetate, *rac*-**2a** (0.025 M) in presence of CrL (25 mg/mL) at room temperature after 15h

Entry	Solvent	*c* (%)	$ee_{(R)\text{-}\mathbf{1a}}$ (%)	$ee_{(S)\text{-}\mathbf{2a}}$ (%)	*E*
1	DIPE	17.2	98.8	20.5	>200
2	MTBE	16.4	98.6	19.4	171
3	Toluene	4.3	>99	4.5	»200
4	*n*-Octane	12.8	99.1	14.6	>200

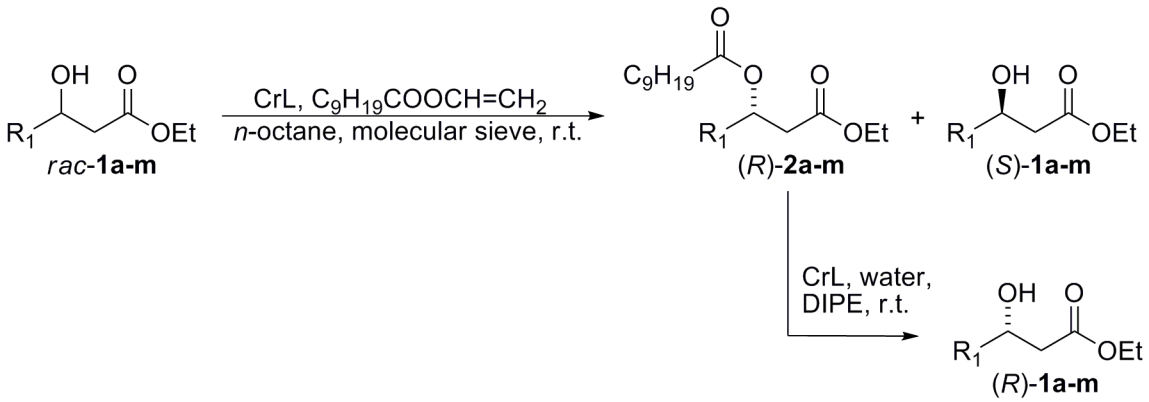
Selective hydrolysis of aromatic diesters *rac*-2a-m

The CrL-catalyzed hydrolysis of the further substrates *rac*-**2b-m** (Table 32) were investigated under the optimal conditions found for the selective hydrolysis of *rac*-**2a** (Table 31, Entry 1). Importantly, no traces of byproducts due to the hydrolysis of the ethoxycarbonylic group were detected in any case. Only the highly hydrophobic phenyl-furan substituted diester *rac*-**2l** was hydrolyzed with higher selectivity (Table 32, Entry 11) than that found for the *O*-acylation of its parent alcohol *rac*-**2l** (Table 30, Entry 11). In all other cases, lower selectivities were detected compared to those found for the enzymatic selective *O*-acylation. The substituent effects in the selective CrL-catalyzed hydrolysis were almost opposite to those obtained for the enzymatic *O*-acylation. Lower selectivity was obtained for the methoxylated *rac*-**2b** (Table 32, Entry 1) compared to the substrates bearing the stronger electron-withdrawing chlorine (Table 32, Entries 2 and 12). The kinetic resolution results for the *O*- and *S*-containing heteroaryl diesters *rac*-**2b-k**, indicated that the aromatic character had the highest impact upon the enantiomer selectivity of the enzymatic hydrolysis (Table 32, Entries 3-10).

Table 32. CrL (25 mg/mL) mediated regio- and enantiomer selective hydrolysis of various diesters, *rac*-**2b-m** (0,025 M) in DIPE at room temperature after 15 h

Entry	Substrate	*c* (%)	$ee_{(R)\text{-}1}$ (%)	$ee_{(S)\text{-}2}$ (%)	*E*
1	*rac*-**2b**	42.8	90.6	67.8	41
2	*rac*-**2c**	44.9	97.3	79.3	178
3	*rac*-**2d**	51.7	91.5	97.8	101
4	*rac*-**2e**	53.1	86.9	98.5	69
5	*rac*-**2f**	50.3	86.1	87.1	38
6	*rac*-**2g**	57.0	59.0	78.2	9
7	*rac*-**2h**	51.6	91.8	97.8	105
8	*rac*-**2i**	51.0	92.5	96.2	102
9	*rac*-**2j**	23.3	96	29.2	65
10	*rac*-**2k**	26.7	97.5	35.6	112
11	*rac*-**2l**	51.1	95.3	99.6	>200
12	*rac*-**2m**	44.9	95.7	77.9	108

Preparative scale CrL-catalyzed reactions for synthesis of both enantiomers of 1a-m



Scheme 22. Preparative scale sequential kinetic resolution of aromatic β-hydroxy esters *rac*-**1a-m**

While a relatively high 25 mg/mL enzyme concentration was used in the analytical scale tests, first lower enzyme concentrations (from the initial 25 mg/mL to 5 mg/mL) were tested for synthetic purposes. The convenient enzyme concentration was found to be 10 mg/mL, offering similar reaction time and selectivity of the enzymatic acylation as those found for the analytical scale reactions. A sequential kinetic resolution process catalyzed by CrL (Scheme 22) resulted in both enatiomers of aromatic β-hydroxy esters (*R*)- and (*S*)-**1a-m** with good enantiomeric excesses and yields (Table 33). First, the selective *O*-acylation was performed until the conversion slightly exceeded 50%. This first kinetic resolution resulted in the residual substrate fraction (*S*)-**1a-m** in good yield and high enantiomeric excess. Next, the enantiomerically enriched *O*-acylated esters (*R*)-**2a-m** were subjected to the CrL mediated hydrolysis, yielding the (*R*)-enantiomeric form of the aromatic β-hydroxy esters (*R*)-**1a-m** in high enantiopurity. The absolute configurations of the (*R*)-and (*S*)-**1a-k** were determined by comparing the sense of their optical rotations with those existing in literature. The absolute configurations of the novel compounds (*R*)-and (*S*)-**1l-m** were assigned in accordance with the Kazlauskas' rule.

Conclusion

In this paper a new general method for the lipase mediated kinetic resolution of a serie of aromatic β-hydroxy esters was reported. Using lipase from *Candida rugosa* and a long chain fatty acid moiety for the selective acylation and hydrolysis, good yields and stereoselectivities were obtained in organic media under mild conditions. The enantiomeric excess of the formed products were enriched using a sequential kinetic resolution procedure, first by lipase mediated *O*-acylation with vinyl decanoate and second by the enzymatic hydrolysis of the enantiomerically enriched diesters. In this way, both enantiomers of the aromatic β-hydroxy esters were produced with high enantiopurity.

Table 33. Preparative scale synthesis of both enantiomeric forms of aromatic β-hydroxy esters (*R*)- and (*S*)-**1a-m**

Substrate	**Products after acylation**				**Product after hydrolysis**	
	(*S*)-**1a-m**		(*R*)-**2a-m**		(*R*)-**1a-m**	
	Yield[a] (%)	ee (%)	Yield[a] (%)	ee (%)	Yield[a] (%)	ee (%)
rac-**1a**	46	>99	49	90.0	45	>99
rac-**1b**	47	>99	49	98.1	46	>99
rac-**1c**	45	>99	48	90.6	44	>99
rac-**1d**	46	>99	49	90.3	44	>99
rac-**1e**	41	>99	45	71.9	37	>99
rac-**1f**	44	>99	48	83.2	42	>99
rac-**1g**	43	>99	47	77.5	35	81.2
rac-**1h**	49	>99	48	89.5	43	>99
rac-**1i**	48	>99	49	>99	47	>99
rac-**1j**	38	65.9	42	76.6	35	>99
rac-**1k**	44	>99	46	85.4	42	>99
rac-**1l**	43	>99	45	81.7	41	>99
rac-**1m**	48	>99	48	>99	47	>99

[a] Isolated yields based on the racemic starting material *rac*-**1a-m**

6. General conclusions

In conclusion both, selective enzymatic acylation and hydrolysis were tested and compared for the resolution of a large variety of 3-hydroxy-3-aryl-propanoates. It was demonstrated that in therm of stereoselectivity, the enzymatic acylation is superior compared to enzymatic hydrolysis.

Several new procedures were set-up for the enzymatic synthesis of both, optically pure (*R*)- and (*S*)-β-hydroxy acids and esters.

The enantioselective CaL-B (Novozym 435) mediated *O*-acylation with vinyl butanoate of the racemic ethyl 3-hydroxy-3-(thiophenyl/furanyl)propanoates (*rac*-**1d-g**) in MTBE followed the Kazlauskas rule, yielding highly enantiomerically enriched products (*E*>200) at almost theoretical yields. In contrast, CaL-A (adsorbed on Celite) acting as an anti-Kazlauskas enzyme showed opposite enantiopreference to those observed for CaL-B. The **opposite stereopreference** of these two commonly used enzymes (**CaL-A and CaL-B)** in the acylations of β-hydroxy esters was described for the first time by us.

Similar with the *rac*-**1d-g**, the enzymatic kinetic resolution of various 3-heteroaryl-3-hydroxy-propanoates *rac*-**1h-r** was performed with good results and the preparative scale multienzymatic synthesis of both highly enantiomerically enriched (*R*)- and (*S*)-β-hydroxy acids was realized. For the selective *O*-acylation of the racemic starting compounds the same enzymes CaL-A/B were used as chiral catalysts. From the high similarity of the experimental VCD spectra and the one obtained through quantum chemical calculations, the absolute configuration of the new compounds (**1h-r**) was determined.

Tailor made enzyme immobilization for the selective *O*-acylation of the aryl β-hydroxy esters was also successfully realized. Using adsorption, CLEA technology and sol-gel encapsulation methods, the immobilization of lipase AK from *Pseudomonas fluorescens* and lipase AK “Amano” (L-AK) have been studied. It was shown that sol-gel L-AK prepared by the encapsulation of the enzyme from aqueous solution, as well as the celite supported (in presence of sucrose) L-AK are superior in therms of activity and selectivity to all of other immobilized preparations.

A new general methodology for the lipase mediated kinetic resolution of the aryl-β-hydroxy esters (**1a-m**) was developed. Using lipase from *Candida rugosa* (AYS Amano) and a long chain fatty acid moiety for the selective *O*-acylation and hydrolysis, good yields and stereoselectivities were obtained in organic media under mild conditions. The enantiomeric excesses of the formed products were enriched using a sequential resolution combining the lipase mediated *O*-acylation with vinyl decanoate of the racemic β-hydroxy esters and the enzymatic hydrolysis of the previously obtained enantiomerically enriched diesters. In this way, both enantiomers of the aromatic β-hydroxy esters were produced with high enantiopurity.

7. List of publications

I. Scientific publications

1. M.I. Toşa, J. Brem, F.D. Irimie, C. Paizs, J. Rétey: Enantioselective Synthesis of L- and D-Nitrophenylalanines Catalyzed by Mutant (MIO-less) Phenylalanine Ammonia-Lyase, Chemistry A European Journal 2011 (submitted manuscript)

2. J. Brem, M. Naghi, M.I. Toşa, L. Poppe, F.D. Irimie, P. Csaba, *Lipase mediated sequential resolution of aromatic β-hydroxy esters using fatty acid derivatives,* Tetrahedron: Asymmetry 2011, article accepted

3. J. Brem, M.C. Turcu, C. Paizs, K. Lundell, M.I. Toşa, F.D. Irimie, L.T. Kanerva, *Immobilization to improve the properties of Pseudomonas fluorescens lipase for the kinetic resolution of 3-aryl-3-hydroxy esters* , Process Biochemistry 2011 (submitted manuscript)

4. J. Brem, S. Pilbák, C. Paizs, G. Bánóczi, F.D. Irimie, M.I. Toşa, L. Poppe, *Lipases-catalyzed kinetic resolution of racemic 1-(10-ethyl-10H-phenothiazin-1,2, and 4-yl)ethanols and their acetates,* Tetrahedron: Asymmetry 2011, 22, 916-923.

5. C. Paizs, M.I. Toşa, L.C. Bencze, J. Brem, F.D. Irimie, J. Rétey, *2-Amino-3-(5-phenylfuran-2-yl) Propionic Acids and 5-Phenylfuran-2-yl Acrylic Acids are Novel Substrates of Phenylalanine Ammonia-Lyase,* Heterocycles 2011, 82, 1217-1228

6. J. Brem, A. Liljeblad, C. Paizs, M.I. Toşa, F.D. Irimie, L.T. Kanerva *Lipases A and B from Candida antarctica in the enantioselective acylation of ethyl 3-heteroaryl-3-hydroxypropanoates: aspects on preparation and enantiopreference,* Tetrahedron: Asymmetry 2011, 22, 315-322

7. J. Brem, M.I. Toşa, C. Paizs, A. Munceanu, D. Matković-Čalogović, F.D. Irimie, *Lipase-catalyzed kinetic resolution of racemic 1-(10-alkyl-10H-phenothiazin-3-yl)ethanols and their butanoates,* Tetrahedron: Asymmetry 2010, 21, 1993-1998

8. J. Brem, M.I. Toşa, C. Paizs, E. Vass, F.D. Irimie, *Enzyme-catalysed synthesis of (R)- and (S)-3-hydroxy-3-(10-alkyl-10H-phenothiazin-3-yl)propionic acids,* Tetrahedron: Asymmetry 2010, 21, 365-373

9. S. Dalia, I. Lingvay, Sz. Lanyi, D.D. Micu, C.L. Popescu, J. Brem, L.C. Bencze, C. Paizs, *The efec of electromagnetic fields in baker's yeast population dynamics, biocatalytic activity an selectivity,* Studia Universitatis Babes-Bolyai, Chemia 2009, 4 (II), 195-201

10. J. Brem, C. Paizs, M.I. Toşa, E. Vass, F.D. Irimie, *Enzyme-catalysed synthesis of (R)- and (S)-3-heteroaryl-3-hydroxypropanoic acids and their derivatives,* Tetrahedron: Asymmetry 2009, 20, 489-496

II. Conference publications:

1. D. Hapau, J. Brem, F.D. Irimie, V. Zaharia, *Chemoenzimatic synthesis of new optically pure thiazolic derivatives*, 8th International Conference „Students for students, 7-10 April 2011, Cluj Napoca, Romania

2. M. Lăcătuș, M. Naghi, B. Nagy, J. Brem, Florin-Dan Irimie, Biocatalytic synthesis of various optically active (hetero)aryl-β-hydroxy-propanoic acid derivatives, 8th International Conference „Students for students, 7-10 April 2011, Cluj Napoca, Romania

3. A. Varga, B. Nagy, C. Paizs, J. Brem, *Optikailag aktiv (hetero)aril β-hidroxi-propansav szarmazekok biokatalitikus szintezise,* 14th Transsylvanian Students Scientific Conference, 13-15 mai 2011, Cluj-Napoca, Romania

4. D. Hapau, J. Brem, V. Zaharia F.D. Irimie, *Enzymatic synthesis of new optically pure thiazolic derivatives*, The XIVth National Congress of Pharmacy from Romania, 13-16 october 13-16 2010, Targu-Mures, Romania

5. J. Brem, C. Paizs, M.I. Tosa, F.D. Irimie, *Biocatalitic synthesis of optically active heteroaryl-β-hydroxy-propionic acid derivatives*, 11th Central an Eastern European NMR Symposium, 4-6 october 2010, Cluj-Napoca, Romania

6. J. Brem, L.C. Bencze, C. Paizs, M.I. Tosa, F.D. Irimie, *Synthesis and enantiomeric excess analysis with NMR of optically active 3-substituted-10-ethyl-10-phenothiazine-5-xides*, 10th International Symposium and Summer Schol on Bioanalysis, 7-14 iuly, 2010, Zagreb, Croatia

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