L051 DEVELOPING NOVEL BIOCATALYSTS FOR MANUFACTURING OPTICALLY ACTIVE COMPOUNDS

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Keywords: functional genomic screening, gene directed evolution, alcohol dehydrogenases

Developing novel biochemical processes for the production of active chiral molecules enables pharmaceutical and agrochemical industries to generate novel intellectual property rights and enhance their competitiveness. This approach has now become possible within the required timelines and budgets because of the recent developments in biotechnology. Protéus has developed proprietary technologies for functional genomic screening (Phenomics™, CLIPS-O™) which enables the rapid discovery of novel biocatalysts that are present in natural biodiversity. Biodiversity from extreme environments has proven to be particularly rewarding for industrial biocatalysis. The catalytic properties of these enzymes can then further be improved using L-Shuffling™, a high throughput proprietary directed evolution technology. By combining these two approaches, high performance catalysts can be delivered to the industry within unprecedented accelerated timeframe.

As an example, we will describe the discovery of alcohol dehydrogenases (ADHs). ADHs are of particular interest for catalyzing the reduction of ketones into enantiomerically pure alcohols. The use of proper technologies and strategies enables the rapid screening of the natural biodiversity that results in the isolation and cloning of ADHs covering a broad substrate range. This set of ADHs can then be used as a „toolbox“ to rapidly screen for activity on substrates of industrial interest. However, the performances of natural enzymes might not always reach the industrial standard required for setting up a competitive large-scale industrial manufacturing process. We will discuss L-Shuffling™, a proprietary gene directed evolution developed by Protéus which provides reduced-to-practice means to „breed“ and optimize new, non natural enzymes, allowing to rapidly develop novel biocatalysts and fulfil virtually any industrial requirements.

L052 RATIONAL REDESIGN OF A LIPASE INTO A LYASE

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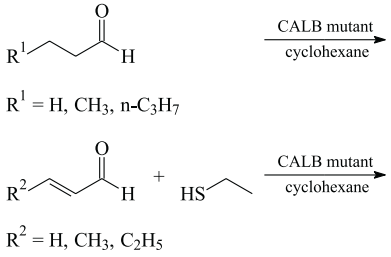
Keywords: rational engineering, aldol additions, Michael additions, *Candida antarctica* lipase B

Enzymes are efficient catalysts in synthetic chemistry and their catalytic activity with unnatural substrates in organic reaction media is an area attracting much attention. Protein engineering has opened the possibility to rationally alter the reaction specificity of enzymes and allow for new reactions to take place in their active sites. We have used this strategy on the well-studied active-site scaffold offered by the serine hydrolase *Candida antarctica* lipase B (CALB, EC 3.1.1.3) to achieve catalytic activity for reactions unnatural to this enzyme, such as aldol additions[1] and Michael additions. The catalytic reactions were first studied in detail by means of quantum chemical calculations in model systems. These calculations showed that the reactions were possible in the active site of a CALB mutant where the nucleophilic active site serine was removed. The predictions from the quantum chemical calculations were then challenged by experiments. Consequently, Ser105 in CALB was targeted by site-directed mutagenesis to create enzyme variants lacking the nucleophilic feature of the active site. The experiments clearly showed an increased reaction rate when the aldol and Michael addition reactions were catalyzed by the mutant enzymes. The reactions were run in cyclohexane with the substrates indicated in Figure. We have shown that the new catalytic activity, harbored in the stable protein scaffold of *Candida antarctica* lipase B, allows aldol and Michael additions of substrates that can not be catalyzed by traditional aldolases.



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L053 CHEMOENZYMATIC SYNTHESIS OF THE CHIRAL SIDE-CHAIN OF ATORVASTATIN

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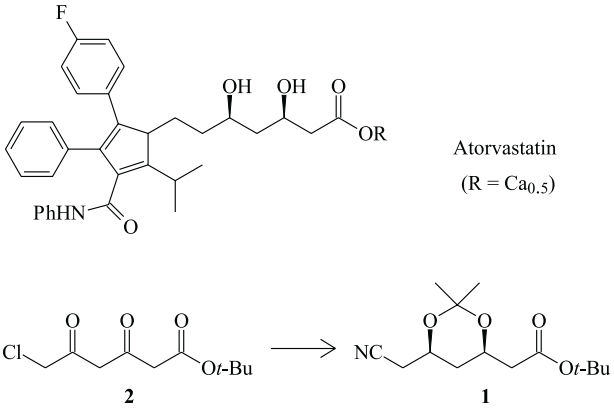
Keywords: atorvastatin, HMG-CoA reductase inhibitors, alcohol dehydrogenase, enantioselective reduction, cyanation

Atorvastatin is an important lipid lowering drug for the treatment of atherosclerosis and other diseases connected to hyperlipidaemia, e.g. coronary heart disease[1]. The isopropylidene-protected dihydroxy ester **1** is the key chiral intermediate of an economical convergent route to this fully synthetic mevinic acid derivative[2].



In this presentation, we report a short and highly enantioselective synthesis of the isopropylidene-protected dihydroxy ester **1** (>99 % *ee*, *dr* > 200 : 1). Crucial step of our strategy is an enzymatic reduction of the achiral diketo ester **2** by means of readily available *Lactobacillus brevis* alcohol dehydrogenase[3]. A one-step synthesis of diketo ester **2**, a laboratory scale-up of the NADP-dependent enzymatic reduction (75g scale), and a high-yielding method for performing the unexpected difficult cyanation of the enzymatic reduction product will be presented.

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L054 ENGINEERING AN ENTIRELY ENZYMATIC PROCESS FOR L-ASCORBIC ACID PRODUCTION FROM D-GLUCURONIC ACID

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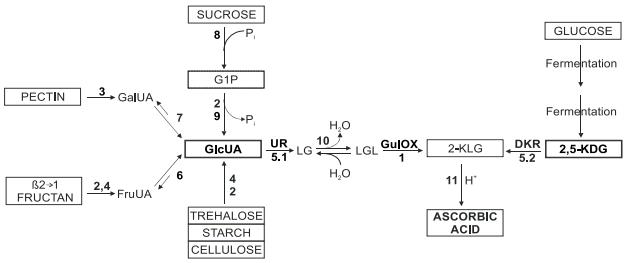
Keywords: L-ascorbic acid, vitamin C, D-uronate reductase, L-gulono-1,4-lactone oxidase

Vitamin C has a very large market share both in the food and drinks industries (more than 50 %) and in the pharmaceutical industries. In addition to the well established chemical process for L-ascorbic acid production[1], a multitude of other chemical or fermentative routes have been developed[2].

Since the Reichstein process is largely a chemical one, there is increasing pressure (e.g. environmental concerns and legislation) to develop efficient and cheap biological alternatives. However, no entirely enzymatic process is documented in the literature to date. Possible routes of this kind are sketched out in Figure 1.



1 L-Gulonolactone oxidase
2 Chemical oxidation
3 Polygalacturonase/Pectin methylesterase
4 Chemical hydrolysis
5.1 Uronate reductase
5.2 2,5-Diketogluconic acid reductase
6 Uronic acid isomerase
7 C_4 epimerase (UDP activated ?)
8 Sucrose phosphorylase
9 Alkaline phosphatase
10 Lactonase
11 Enolisation

Fig. 1**. Reaction scheme for the enzymatic synthesis of L-ascorbic acid *via* D-glucuronic acid (GlcUA) and 2,5-diketo-D-gluconic acid (2,5-KDG), resp. Routes leading to these key intermediates are also integrated;** Fru UA = fructuronic acid, GalUA = galacturonic acid, 2-KLG = 2-keto-L-gulonic acid, LG = L-gulonic acid, LGL = L-gulono-1,4-lactone

By imitating in part the biological pathway, Vitamin C synthesis was started from D-glucuronic acid, which can be prepared chemo-enzymatically from renewable materials, for example from sucrose via glucose 1-phosphate. A three-step process was established:

1. D-Glucuronic acid (0.75 M) was reduced and inverted to L-gulonic acid by NADPH-dependent uronate reductase from *S. cerevisiae* YNN295 cloned into *E. coli* JM109 and expressed under the control of the tac-promotor[3]. The native

coenzyme is regenerated by NAD(P)-dependent glucose dehydrogenase from *Bacillus cereus* in a charged ultrafiltration membrane reactor[4]. Product yield is more than 90 %, the total turnover number of NADP/H was about 10.000.

2. L-Gulono-1,4-lactone was obtained by chemical lactonisation in quantitative yield; a lactonase-catalyzed step is under study.

3. Finally, the lactone is oxidized by a cytosolic fungal oxidase (GulOx) to 2-keto-L-gulonic acid and rearranged to L-ascorbic acid. A tubular enzyme membrane reactor is used to supply oxygen and to regenerate the cofactor effectively. GulOx is now being cloned. In a series of discontinuous experiments (Table I) optimal conditions for maximal substrate transformation (98,6 %) and Vitamin yield (43.1 $g.l^{-1}$) were determined. GulOx stability allows for repeated batch operation. Possible improvements towards an entirely enzymatic production of L-ascorbic acid will be discussed.

Table I
Comparative results of discontinuous transformation of L-gulono-1,4-lactone to L-ascorbic acid by fungal GulOx under different experimental conditions

Expt. No	L-Gulono--1,4-lactone	GulOx	Catalase	L-Ascorbic acid		GulOx act remaining
	pH	[mM]	[U.ml^{-1}]	[kU.ml^{-1}]	[%] (h)	[g.l^{-1}]	[%]
1	4.0	20	1	0	0	0	0
2	4.0	200	1	0.5	0	0	0
3	4.0	200	1	1.0	18.4 (6)	4.8	18
4	5.0	200	1	1.0	56.6 (22)	14.2	96
5	5.0	200	2.5	1.0	79.6 (22)	22.0	95
6	6.0	200	2.5	1.0	98.6 (23)	24.1	98
7	6.0	300	2.5	1.0	96.4 (21)	33.6	92
8	6.0	500	2.5	1.0	83.9 (24)	43.1	96
9	4.0	200	1	1.0	46.1 (23)	12.4	0
10	5.0	200	1	1.0	62.6 (22)	16.3	78
11	6.0	200	2.5	1.0	97.4 (22)	22.2	97

Optimal conditions for maximal substrate conversion (98,6 %) were 200 mM of L-gulono-1,4-lactone; pH 6.0 (experiment 6). At 500 mM substrate a final L-ascorbic acid concentration of 43.1 $g.l^{-1}$ (≡ 84 % conversion) was possible (expt. 8). Loss of GulOx activity was 2 and 4 %, resp.

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L055 NEW BIOCATALYSTS FROM THE METAGENOME: ALPHA/BETA HYDROLASE FOLD ENZYMES

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Keywords: metagenome, enzyme libraries, screening

The alpha/beta-hydrolase family comprises many different enzyme classes which share the same tertiary protein fold. Some of these enzymatic activities are of major interest to biotechnological industries: Lipases and esterases are today the most often used enzymes in organic synthesis of fine chemicals[1]. Epoxide hydrolases are of high value because of the possible production of enantiopure epoxides or their corresponding vicinal diols[2]. Haloperoxidases have received increasing attention due to their ability to halogenate a variety of commercially important compounds. Additionally, these enzyme might be interesting for their sulfoxidation and epoxidation capabilities[3]. However, the availability of appropriate biocatalysts of many of these enzyme classes for changing industrial applications is still limited.

Our aim is therefore to provide novel enzyme libraries by accessing the functional sequence space of biodiversity in order to facilitate the identification of suitable biocatalysts for specialized applications. Limited to screening strain collections biotechnology has missed to evaluate up to 99 % of existing microbial resources. This was due to the inability to cultivate most microorganisms. Novel strategies of directly cloning „metagenome DNA“ as the genetic blueprints of entire microbial consortia are becoming increasingly applicable to circumvent this restriction[4].

Screening of metagenomic libraries by both a sequence homology based approach and an activity based approach has led to the identification of several new and highly diverse enzymes of the alpha/ beta hydrolase family.

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P001 FLEXIBILITY OF LIPASE BROUGHT ABOUT BY REACTION TEMPERATURE AND ADDITIVES CONTROLS ITS ENANTIOSELECTIVITY IN ORGANIC SOLVENTS: A RATIONAL APPROACH FOR OPTIMIZATION OF ENANTIOSELECTIVITY FOR GIVEN ENZYMATIC REACTION

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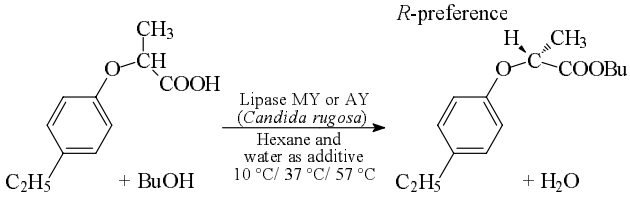
Keywords: chiral resolution, enantioselectivity, lipase, conformational flexibility, EPR spectroscopy

Since the basic discovery showing enzymatic activity even in organic solvents by Klibanov et. al.[1], a number of strategies for enhancing the enantioselectivity and activity of enzymes have appeared. An ultimate goal, especially for organic chemists is to control rationally the enantioselectivity of enzyme as a function of experimental conditions. It is generally accepted that the flexibility of enzymes plays an important role in the discrimination between the enantiomers of the substrate used[2]. Further developments, however, are waited a model showing how to control the enzyme's enantioselectivity by its flexibility.

In our recent studies, we have reported a relationship between the flexibility of enzyme brought about by the additive such as DMSO (ref.[3]) or the solvent nature[4] and its enantioselectivity for enzyme-catalyzed reactions, the flexibility of which was estimated by EPR spectroscopy. Here, our discussion is focused mainly on the effects of the reaction temperature on the flexibility of lipase, because the reaction temperature is also anticipated to strongly affect flexibility of enzyme, resulting in altering its enantioselectivity.



The enantioselectivity for two kinds of *Candida rugosa* lipases-catalyzed reactions was investigated under the various temperature conditions (see scheme). The behavior of the obtained enantioselectivity as a function of the reaction temperature showed an opposite trend for two kinds of lipases in the different preparations form *Candida rugosa*, high temperature-induced high enantioselectivity and low tempertureinduced high enantioselectivity[5]. The opposite trend can be ascribed to the experimental results that the lipases have the individual optimum flexibilities to produce their maximal enantioselectivities.

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P002 DIFFERENT STRATEGIES FOR THE BIOCATALYTICAL CHARACTERIZATION OF *Candida rugosa* LIPASES IN ORGANIC SOLVENTS

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Keywords: isoenzymes, characterization, *C. rugosa* lipase, acyl transfer, organic solvents

It is well known that the different composition of *Candida rugosa* lipases (CRL), depending on the origin of the sample (concentration and isoenzymes percentage) leads to some reproducibility problems described when performing asymmetric synthesis in organic solvents[1,2]. Thus, for rationalizing such processes, a previous synthetical characterization of the catalyst behaviour in organic media should be performed.

In the present work we report several new and easy-to-perform methodologies with the aim of carrying out a complete characterization of *C. rugosa* lipases for biocatalytical purposes. Thus, as a quick first test, the heptyl oleate synthesis turns out to be a useful tool for discriminating between lipases and esterases in non-aqueous media[3]. For that process, while initial rate is directly related to the concentration of lipases in the crude sample, the final yield is mainly dependent on both the water produced and the capability of the

crude biocatalyst for distributing such water molecules. This influence can be qualitatively predicted through water sorption isotherms and thermogravimetric and differential thermal analysis (TGA/DTA).

However, in the measurement of the concentration of lipases by kinetical methods, under heterogeneus biocatalysis, some other parameters (such as solvent, temperature, stirring speed or water activity) play an important role in the initial rate. In that sense, it is necessary to find a test reaction where the final yield could be dependent (mainly) on the concentration of lipases. This is possible by using the transesterification of 1-heptanol with vinyl acetate in *n*-hexane; in fact, the acetaldehyde produced as secondary product is toxic for *C. rugosa* lipases[4], and thus the different final yield obtained by using variable amounts of CRL shows a linear dependence on the concentration of lipases. Furthermore, by simply changing the alcohol employed (using cyclohexanol) the proportion of the isoenzyme Lip3 (also described as a cholesterol esterase[5]) can be semi-quantitatively estimated by comparison of the final yields obtained in the transesterification of both alcohols. As an additional confirmation, those reactions catalyzed by the pure recombinant Lip1 isoenzyme show a much lower rate for the transesterification of cyclohexanol *versus* that obtained for 1-heptanol. Therefore, we must conclude that cyclic secondary alcohols are mainly acylated by Lip3, and thus, being Lip3 a cholesterol esterase[5], the different catalytical rates obtained for the acylation of several cyclic alcohols should be rationalize by comparison of the cholesterol molecule with different cycloalkanols tested. In addition, this fact could explain why the Kazlauskas' rule[6] is valid, in *C. rugosa* lipases, only for cyclic alcohols, and not for acyclic secondary ones.

Finally, the strategy of characterization is succesfully tested in the transesterification of 3-methyl-2-butanol with vinylacetate, where it is shown that final yield obtained is once again mainly dependent on the relative proportion of Lip3 in the crude *C. rugosa* biocatalyst.

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P003 INFLUENCE OF ALKYL-SUBSTITUTED SILANE PRECURSORS OF SILICA GELS ON THE ENZYMATIC ACTIVITY OF IMMOBILIZED MICROBIAL LIPASE FROM *Candida rugosa*

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Keywords: lipase, sol-gel, immobilized lipase, *Candida rugosa*

Investigations are being carried out to optimize the procedure for lipase encapsulation for application in the modification of vegetable oils. In this study, we present a method to immobilize lipase by entrapment in chemically inert hydrophobic silica gels, which are prepared by the hydrolysis of alkyl-substituted silanes like tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS), and polydimethysiloxane (PDMS) in the presence of enzyme and additives such as polyethyleneglycol (PEG, PM-1450) or polyvinylalcohol (PVA). For the precursor TEOS a novel methodology was established under nitrogen inert atmosphere and used an aqueous solution of lipase, hydrochloric acid, ultra-pure water, ammonium hydroxide, ethanol, and stabilizing additive (PEG 1450). For the gel precursors MTMS and PDMS, a typical immobilization procedure uses: an aqueous solution of lipase, sodium fluoride as a catalyst, alkoxysilane derivative precursors, and additives (PEG-1450 and PVA).

To evaluate these immobilization procedures, lipase from *Candida rugosa* immobilized in different gel matrixes was fully characterized with respect to their morphological properties (i. e., particle size, surface area and pore size distribution) and used in ester hydrolysis and synthesis. The coupling yields for the lipase immobilized by encapsulation using MTMS and MTMS/PDMS as precursors, was very poor (9.0 %). These data suggest that these precursors may cause conformational changes of the enzyme chain upon its adhesion to the support surface. The latter probably caused steric hindrance which may render certain regions of the enzyme molecule inaccessible to the substrate (olive oil). In this case, the addition of an additive did not produce beneficial effect. However, the positive effect of the PEG-1450 was noticed for the derivatives prepared by the encapsulation technique using TEOS as precursor. The highest coupling yield (31.98 %) was observed for the immobilized derivative obtained by lipase encapsulation in the presence of the PEG-1450; confirming the efficiency of this kind of additive[1, 2].

Further information on the catalytic activity was obtained by testing the derivatives prepared in synthetic applications, that is, in esterification reactions with *n*-butanol and butyric acid, and a different activity dependence was found. Better performance was achieved by derivatives resulting from the encapsulation of *Candida rugosa* lipase in the gels prepared with MTMS as precursor, in the presence of PEG (PM-1450). This lipase preparation exhibits an increased esterification activity (154.85 $\mu mol.g^{-1}.min^{-1}$) that is 3 times greater than that prepared with TEOS (51.98 $\mu mol.g^{-1}.min^{-1}$), and 1.74 times greater than that prepared with MTMS/PDMS (88.79 $\mu mol.g^{-1}.min^{-1}$) as precursor.

The results presented here allowed a deeper understanding of the process of encapsulation of commercial *Candida rugosa* lipase in the presence of an additive, using TEOS and MTMS as precursors.

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P004 SUBSTRATE-ASSISTED CATALYSIS IN A REDESIGNED HYDROLASE TO INCREASE ENANTIOSELECTIVITY

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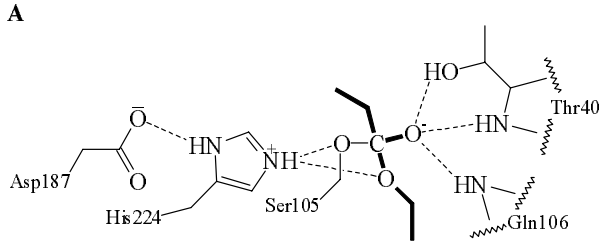
Keywords: substrate-assisted catalysis, rational design, enantioselectivity, *Candida antarctica* lipase B, isothermal titration calorimetry

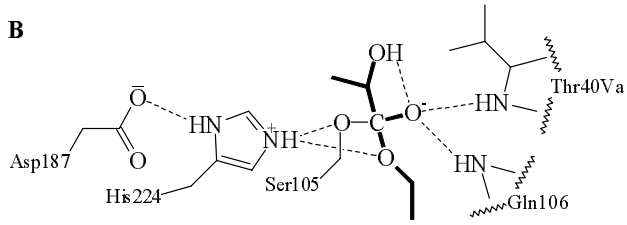
We have changed the enantioselectivity of a hydrolase towards substrates carrying a hydrogen-bond donor. A mutation in the oxyanion hole allows the hydrogen-bond donor of the substrate to stabilize the transition state. By this means the enantioselectivity was improved compared to that of the wild type enzyme.

In our experiments we used lipase B from *Candida antarctica*. It has a catalytic machinery that consists of the triad Ser-His-Asp (ref.[1]). In the active site is also the oxyanion hole, a spatial arrangement of hydrogen-bond donors, which plays a major role in the stabilisation of the transition state conformation[2]. The oxyanion is stabilized by two backbone amide hydrogen atoms and the side chain hydroxyl group of Thr40 (ref.[3]) **(A)**. By changing threonine 40, we have removed one of the hydrogen-bond donors capable of stabilizing the transition state. With substrates carrying a hydroxyl group we have performed substrate-assisted catalysis in the modified enzyme and increased the enantioselectivity greatly[4] **(B)**.

A



B



To further understand what determines the enzyme specificity we now measure how the point mutation affects transition state stabilization (k_{cat}) and substrate binding (K_M). We do this with substrates carrying either a hydroxyl or an amino group as potential hydrogen-bond donor. The catalytic constants are determined with isothermal titration calorimetry measurements.

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P005 STABILIZATION OF AN INTRACELLULAR *Mucor circinelloides* LIPASE FOR APPLICATION IN NON-AQUEOUS MEDIA

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Keywords: immobilization, membrane-bound lipase, *Mucor circinelloides*

The membrane-bound *M. circinelloides* lipase displays very high synthetic activity and can be used for synthesis of various esters, as was described elsewhere[1–3]. Different methods of this enzyme stabilization, facilitating its application in a milieu of organic solvents for synthesis of esters of higher fatty acids and aliphatic alcohols, glycerol and saccharides, were tested. Within the scope of these investigations, the lipase was isolated from the mycelium, purified and immobilized on octyl- and palmityl-cellulose, diatomaceous earth, porous glass and silica aerogel[4], and immobilized *in situ* in the mycelium (by means of de-fatting and de-hydration of the mycelium with acetone). The membrane-bound lipase was also entrapped in carragenan[4], polyvinyl alcohol cryogel and chitosan. Preparations of the immobilized lipase, in a form of mycelium pellets stabilized by treatment with glutaraldehyde, polyvinyl alcohol and alginate, were also obtained. The problems related to an application of the lipase entrapped in the PVA cryogel in organic solvents have been already presented[5]. In this work, the results of esters (sucrose, glucose, butyl and propyl oleates and caprylates) synthesis, carried out in petroleum and di-*n*-pentyl ethers, and catalyzed by various preparations of the immobilized lipase, have been compared. The lipase isolated from the cells (solubilization with sodium cholate) and immobilized on solid carriers showed a weak catalytic activity. An effective method of *M. circinelloides* lipase stabilization was found to be an entrapment of the membrane-bound enzyme in poly(vinyl pyrrolidone)-containing chitosan granules, prepared by using the phase inversion method. This method ensured the high mechanical and chemical resistance of the biocatalyst beads. Chitosan beads do not shrink in organic solvents, in contrast to PVA-gel beads. Moreover, an activation of the chitosan-biocatalyst beads during the initial batches of their iterative use for esters synthesis was observed. The maximum yields of butyl oleate and sucrose caprylate synthesis were 90 % and 75 %, respectively. The lipase preparations with high synthetic activity can be also produced by cross-linking of the *M. circinelloides* mycelium pellets (harvested from optimal medium inoculated with the suspension of sporangiospors in 0.1 % Triton X-100) with glutaraldehyde. Cross-linking of the lipase-containing pellets with PVA and glutaraldehyde, provides both high activity (approximately 3.3 $\mu kat.g^{-1}$) and operational stability of the catalyst for 120 hrs of sucrose caprylate synthesis. Water released during esters synthesis should be removed from the beads in order to provide the satisfactory yield of their repeated usage. The synthesis yield can be significantly enhanced by optimization of the reaction conditions.

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P006 SUGAR ESTERS SYNTHESIS BY A MEMBRANE-BOUND *M. circinelloides* LIPASE IN MICROREACTOR EQUIPPED WITH THE WATER ACTIVITY – MONITORING SENSOR

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Keywords: saccharide esters, *Mucor circinelloides* lipase, mathematical modelling

Esters of saccharides and fatty acids belong to a class of natural, “green”, nonionic surfactants. They are synthesized from natural components of foods and show prebiotic properties, enabling their application in food production as dietetic fats, emulsifiers, plasticizers and as antimicrobial and protective coatings for fruits[1]. The mycelium bound lipase was prepared by dehydration of *Mucor circinelloides* mycelium with acetone[2,3]. The lipase displays high stability in the medium of apolar organic solvents at 100 °C and shows high activity in hydrolysis and synthesis of esters[4]. The reactions of the sugar fatty acid esters synthesis were carried out at 50 °C in 37-ml microreactor equipped with a stirrer (120 rpm), containing 2 mmol of sugar, 2 mmol of fatty acid and 0.2 g of the lipase suspended in 10 ml of the mixture of di-*n*-pentyl ether and petroleum ether, and modified with astaxanthin or carotene, acting as activator or inhibitor of the enzyme depen-

dently on the concentration in the reaction mixtures. Free fatty acids were titrated with 0.05 M NaOH up to pH 10.0 using TitroLine (Schott) titrator. The esters concentration was determined using HPLC (ODS: 250 × 5.6 mm column, the eluent: acetonitrile-water (8 : 2), refractometric detector). Changes in thermodynamic water activity and temperature of reaction medium throughout ester synthesis were monitored using the water activity digital sensor AwVC-DIO (Rotronik) coupled with a computer (Fig. 1).

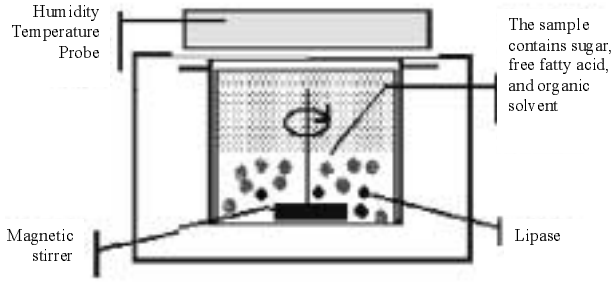

Fig. 1. **The scheme of enzymatic reactor equipped with an on line water activity (a_w) measuring probe**

The effect of molar ratio of the substrates in the reaction mixtures on the lipase activity was studied. The optimal molar ratio of caprylic acid and sucrose was found to be 9 : 5 and the corresponding degree of the sucrose conversion in di-*n*-pentyl ether medium exceeded 90 %. The dependence of water activity of bi-phasic systems di-*n*-pentyl ether – water on the phase volume coefficient $A = V_{org}/V_{water}$ (were V_{org} – organic phase volume; V_{water} – water phase volume) and an effect of water activity on the saccharide esters synthesis yield were also examined. The invented mathematical model of esters synthesis in a biphasic system was experimentally verified by sucrose and glucose esters synthesis[5]. Modelling was done by using the Mathematica 4.2 program.

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P007 NEW EFFICIENT ENZYMATIC PROCEDURES CATALYSED BY LIPASES FOR THE SYNTHESIS OF VALUABLE REGIOPROTECTED PRECURSORS OF D-FRUCTOSE IN PRODUCTION OF SUGAR DERIVATIVES

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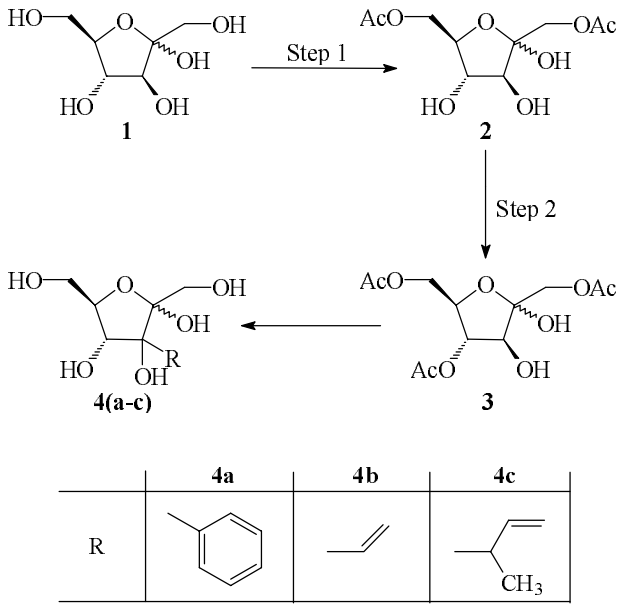
Keywords: fructose, lipase, regioselectivity, enzyme inhibitors, sugar derivatives

Carbohydrates have long been and still are important sources of chirality in the synthesis of many biological active compounds. Furthermore, many of them are components of different natural products which display powerful biological properties[1]. For these reasons many studies have been reported about chemical transformations, especially at anomeric carbons. Regioprotected derivatives of carbohydrates are important intermediates towards the synthesis of very valuable compounds. Conventional chemical methods often are not able to achieve this task and can require the use of toxic solvents and inorganic catalysts that leave residual traces in the final product; furthermore they often require difficult and expensive purification procedures. Conversely enzymatic processes offer important alternatives for the synthesis of these molecules. In particular lipases and proteases have been exploited with success in the preparation of regioprotected sugar derivatives[2].

D-Fructose **(1)** is a very sweet sugar and it is greatly abundant in the natural saccharides that exhibit interesting biological activities. We propose in this study new biocatalysed routes towards the synthesis of different regioprotected fructose derivatives. A two step enzymatic process for the synthesis of 1,4,6-triacetyl fructofuranose **(3)** has been developed: in a first step D-fructose was acetylated in tetrahydrofurane using lipase from *Candida antarctica* (Novozyme) as the catalyst to give the regioselectively protected 1,6-diacetyl fructofuranose **(2)** as sole product. In a second step the recovered diester **2** was selectively acetylated at the C-4 hydroxy group using lipase from *Candida rugosa* in *tert*-butylmethylether to give compound **3** that**,** bearing a free OH function at C-3, is a valuable starting material for further selective chemical transformations. For example oxidation of C-3 hydroxy group, followed by coupling via the Grignard reaction allows to synthesise interesting sugar derivatives **(4a-c)** of potential application as enzyme inhibitors.

Subsequently the C-4-OH preference observed in the recognition of *C. rugosa* lipase was exploited, *via* alcoholysis reaction, for the synthesis of rare fructose isomers.



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P008 EFFECT OF DIVERSE LIPASES ON THE TRANSESTERIFICATION OF GRAPE SEED OIL WITH SOLKETAL AND GLYCIDOL

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Keywords: *Aspergillus flavus*, *Rhizopus oryzae*, biocatalysts, esterification, solketal, glycidol

The enzymatic transesterification is a potentially attractive route for the modification of the physical and chemical properties of vegetable oils. This reaction can also be used to obtain some compounds that can render monoacylglycerides. These reactions usually take place with high regio and/or enantioselectivity. Moreover, the mild conditions needed to carry out these reactions permits the preparation of products with high yields without the formation of secondary products that sometimes are very difficult to remove.

In this study is described the influence of the biocatalyst on the transesterification of grape seed oil with solketal and glycidol. Two own catalysts, prepared from resting-cells of *Aspergillus flavus* and *Rhizopus oryzae*, and five commercial enzymes, Amano Lipase AYS *(Candida rugosa)* (Aldrich), Novozym 435 (Novo Nordisk), Amano Lipase A *(Aspergillus niger)* (Aldrich), Amano Lipase PS *(Pseudomonas cepacia)* (Aldrich), Lipozyme, immobilized *(Mucor miehei)* (Fluka), were used to carry out this reaction. In a 12 ml vial were added 300 mg of grape seed oil, 135 mg of solketal, 30 mg of biocatalyst and 4 ml of isooctane. The mixture was then magnetically stirred at 50 °C for 196 h. Samples were taken at 24, 48, 72, 96, 144 and 196 h and analyzed by HPLC-RP. Similar experiments were carried out using glycidol instead of solketal as reagent for the transesterifiaction of the vegetable oil.

Among the enzymes assayed, *Rhizopus oryzae* gave the best results. Thus, the solketal derivative yield was 11 $mg.ml^{-1}$ at 72 h reaction. Although the same biocatalyst gave the highest transformation when glycidol was used, the yield was much lower.

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P009 ASSESSMENT OF ACYLGLYCEROL COMPOSITION, OXIDATION PRODUCTS AND FREE FATTY ACIDS IN LIPASE-CATALYSED TRANSESTERIFIED FATS RICH IN OMEGA-3 POLYUNSATURATED FATTY ACIDS

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Keywords: acylglycerols, free fatty acids, immobilized lipase, oxidation products, transesterification

In a previous work[1], a commercial immobilized lipase from *Thermomyces lanuginosa*, Lipozyme™ TL IM, was used

in batch reactors, for the transesterification of fat blends. Mixtures of palm oil stearin (POS), palm kernel oil (PK) and two different commercial concentrates of triglycerides enriched in ω-3 polyunsaturated fatty acids, EPAX 4510TG and EPAX 2050TG, were used for the production of margarines basestocks.

Runs were carried out with values of reaction medium composition, temperature *(T)* and reaction time *(t)* given by a central composite rotatable design (CCRD).

The free fatty acids (FFA) were assayed. These are released in fat hydrolysis and in the first step of lipase-catalysed transesterifications. An increase in FFA was observed and the final values varied between 5.0 % and 9.5 %, as compared to values lower than 0.5 % in the initial blends. FFA formation was independent of the medium composition, and also of reaction temperature and time. This suggests that the production of FFA is mainly a result of the mechanism of lipase-catalysed transesterification. However, the formation of oxidation products was negligible during the time course of the reaction.

The interchange of acyl groups amongst glycerides was followed by non-aqueous reverse-phase high performance liquid chromatography (HPLC) with refractive index detection. "Consumption" of TG was accompanied by the formation of other TG and also mono and diglycerides, depending on the initial medium composition and reaction conditions. When the transesterified fat was treated with alumina, the peaks corresponding to the formation of mono and diglycerides disappeared.

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P010 LIPASE-CATALYZED ESTERIFICATION OF GLYCEROL WITH ALIPHATIC AND SUBSTITED AROMATIC ACID ANHYDRIDES

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Keywords: *Candida antarctica*, esterification, lipase, monoacylglycerol, organic solvent

Due to their emulsifying activity and wide antimicrobial spectrum, monoacylglycerols (MAGs) are by far the most commonly used surfactants[1]. They are also useful chiral synthons and a perspective material for the nanotechnology[2]. Currently, there have been a great interest in the synthesis of optically active monoacylglycerols by enzymatic reactions[3,4]. The use of lipases for this purpose has become an attractive alternative to the bulky chemical asymmetric synthesis.

Recently, we have reported an efficient synthesis of *(R)*-α-monobenzoyl glycerol (MBG) by using carrier-fixed Chirazyme® L-2 *(Candida antarctica)* in 1,4-dioxane as the most suitable solvent and benzoic anhydride as the acyl donor[4]. After optimization of reaction conditions we have synthesized *(R)*-α-MBG with high optical purity in a large scale[5]. Since this reaction does not cause the formation of water or alcohol, it has been completely shifted towards the esterification. By this reason, we have found benzoic anhydride is more effective as an acyl donor for synthesis of MAGs than some alkanoic and aromatic acid esters[6]. We therefore expected that the replacement of the acyl donors with their anhydrides would increase the yield and optical purities of MAGs produced by the enzyme reaction. In this study we examined various aliphatic and aromatic anhydrides $(RCO)_2O$ as substrates for the lipase-catalyzed esterification of glycerol (Fig. 1). We synthesized several anhydrides (R = cyclohexane, $Ph(CH)_2$, $PhCH_2$, *p*-Me-Ph, *p*-MeO-Ph, *p*-Cl-Ph, *p*-NO_2-Ph) and used another commercially available ones (R = Ph, Me, Et, *n*-Pr, *i*-Pr, *n*-Bu, *i*-Bu, *t*-Bu). Under optimal conditions (100 mM glycerol, 100 mM acid anhydride, dioxane, 15 °C) Chirazyme® L-2 was active towards all the anhydrides to give the corresponding MAGs. The enzyme preferred acting on the aliphatic substrates, especially acetic and propionic anhydrides which afforded the short-chain monoacylglycerols. The yields and enantiomeric excess were determined for aliphatic MAGs by GLC or HPLC without derivatization and for aromatic acylglycerols by HPLC of their acetonides. The authentic samples were synthesized by reaction of *(R,S)*-solketal and acid anhydrides followed by suitable deprotection of the resulted compounds.

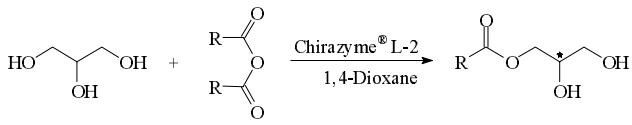

Fig 1. **Enzymatic esterification of glycerol by Chirazyme® L-2**

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P011 LIPASE-CATALYZED DIRECT CONDENSATION OF L-ASCORBIC ACID AND FATTY ACIDS IN IONIC LIQUIDS WITH ASSISTANCE OF HYDROPHOBIC ADDITIVES

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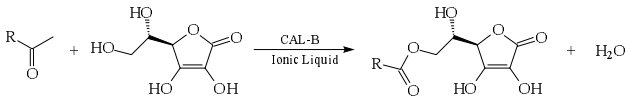
Keywords: ionic liquid, lipase, L-ascorbyl fatty acid ester

L-Ascorbic acid (Vitamin C) is a potentially useful compound for food and cosmetic chemistry because of its high reducing activity. However, it dissolves only in water and thus cannot be used in applications that require solubility in fats. Modification to its fatty acid esters would enable its use as an antioxidant. Researchers synthesized L-6-*O*-ascorbyl fatty acid esters under a mild reaction conditions such as lipase-catalyzed esterification, but the poor solubility of L-ascorbic acid in nonpolar organic solvents prevented an efficient synthesis. Polar ionic liquids permit a lipase-catalyzed esterification with high conversion because they readily dissolve L-ascorbic acid (e. g. ~ 130 mg.ml^{-1} in sBMIM × BF_4 at 60 °C) and to some extent fatty acids, but do not inactivate the lipase. The product, L-ascorbyl fatty acid esters, inhibited the reaction by precipitating on the lipase particles. To avoid a product inhibition, we added a hydrophobic additive such as hexane or polypropylene. With assistance of these additives, lipase B from *Candida antarctica* (immobilized on macroporous acrylic resin) catalyzed a direct esterification of ascorbic acid with 83 % conversion and 65 % yield to produce L-ascorbyl 6-oleate.



R = $(CH_2)_7CH=CH(CH_2)_7CH_3$

P012 CORK POLYESTERS AND THEIR BUILDING BLOCKS FROM PLANT OILS BY ENZYME CATALYSIS

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Keywords: cork, long chain dicarboxylic acid derivatives, synthesis, Novozym 435®

Long chain unsaturated, epoxidized and hydroxylated dicarboxylic acids as well as ω-hydroxy carboxylic acids are polymer building blocks of polyesters occurring in suberin, the main constituent of cork from cork oac[1]. We studied the synthesis of these and analogous structures and further unusual polyesters on the base of vegetable oils.

Natural unsaturated fatty acid methyl esters obtained from plant oils like rapeseed oil, crambe oil or castor oil were converted by metathesis with ethylene or pyrolysis to terminal unsaturated fatty acid esters (C_{10}, C_{14} and C_{11}). Cometatheses of these esters with equimolar amounts of their analogous alcohols yielded symmetrically unsaturated diols, hydroxy fatty acids and dicarboxylic acid derivatives (ratio: 1/2/1). The internal unsaturated derivatives were epoxidized using our new method of chemo-enzymatic epoxidation with methyl acetate/H_2O_2 and Novozym 435®, the immobilized lipase B from *Candida antarctica*, as biocatalyst[2].

Enzymatic hydrolysis of the epoxides under catalysis of epoxide-hydrolase which was prepared from soybeen as aceton-powder, demonstrate that this method can be used for selective epoxide-ring-opening with water. Alternatively the preparative synthesis of the diols could be achieved by dihydroxylation of the olefins using formic acid/H_2O_2 and following alkaline saponification. Similar to our enzymatic polycondensation of long chain dicarboxylic acids with diols[3], the mixtures mentioned above were polycondensated using Novozym 435® as catalyst. Due to the selectivity of the enzyme and equimolar amounts of acid- and primary hydroxyl-groups in the mixtures of diol-, hydroxy fatty acid- and dicarboxylic acid derivatives, polycondensation reactions led to linear polyesters (Mw up to 55,000 g.mol^{-1}).

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P013 LIPASE-MEDIATED PREPARATION OF CHIRAL BUILDING BLOCKS FOR TERPENES

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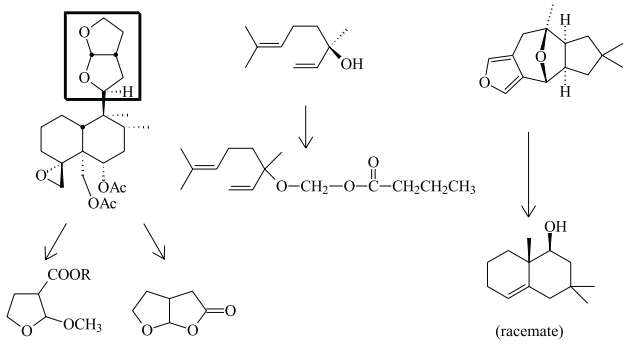
Keywords: terpenes, chiral building blocks, lipases

Terpenes are one of the major classes of secondary metabolites, widespread in the animal and plant kingdom as well as in microorganisms. Their natural functions range from chemical communication (*e. g.*, fragrances of flowering plants) to chemical defence (*e. g.*, insect antifeedants). Isolation from Nature is often not feasible for practical applications, so their total synthesis has been studied extensively. Almost all terpenes have one or more chiral carbon atoms and their absolute configuration is usually essential for biological activity, so the total syntheses have to be stereoselective.

This poster summarises our work on the lipase-mediated kinetic resolution of chiral building blocks for the synthesis of several terpenes.



P014 BIOGENERATION OF LIPOPHENOL COMPOUNDS IN HEXANE MEDIUM USING SELECTED LIPASES AND SUBSTRATE MODELS

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Keywords: phenolic compounds, triacylglycerols, lipophenols, lipases, esterification

Lipophenols are products of an esterification reaction between phenolic compounds and fatty alcohols, fatty acids or triacylglycerols. Since phenolic acids are hydrophilic, they exhibit little stability and solubility in different organic solvent systems and therefore have limited antioxidant efficiency in stabilizing oils and fats[1]. However, enzymatic esterification of phenolic compounds with free fatty acids and their acylglycerol esters could therefore be used to modify these physical properties by producing lipophenols with different solubility characteristics and making them more useful as food lipophilic antioxidants and emulsifiers[2]. Enzymes can act as biocatalysts in nearly anhydrous organic solvents, which is due to the fact that the water needed for enzymatic activity is bound tightly to the enzyme molecule even the bulk water is replaced with organic solvent[3].

These lipophenols have the potential to become excellent nutraceutical and functional products since they would posses the combined health benefits of polyunsaturated fatty acids and the phenolic antioxidant activity.

The biogeneration of lipophenols by enzymatic esterification of phenolic compounds, catechin and catechol, with the fatty acid caprylic and its triacylglycerol ester, tricaprylin, in organic solvent medium model, hexane, was investigated. Commercial lipase obtained from *Rhizopus niveus* (Lipase N) as well as immobilized ones obtained from *Mucor miehei* (Lipozyme IM) and *Candida antarctica* (Novozym 435) were used in throughout this study.

In order to determine the optimal conditions for enzymatic biocatalysis, the effect of reaction time, incubation temperatures and agitation speeds on enzyme activity were investigated. The optimal temperature for lipase activity was determined to be 37.5 °C for Lipase N and 55 °C for Lipozyme IM and Novozym 435. Maximum hydrolysis of tricaprylin, 1.7 μmol free fatty acid per ml, was obtained after 1.5 days of reaction time with Lipase N. However, Lipozyme showed higher hydrolytic activity after 1 and 4 days of reaction time with 8.1 and 8.8 μmol free fatty acid per ml, respectively, whereas Novozym 435 showed such activity after 2 and 9 days of reaction time, with 4.0 and 6.1 μmol free fatty acid per ml, respectively.

The high-performance liquid chromatography (HPLC) analysis indicated that there was no formation of lipophenol molecules from tricaprylin and catechin as substrates when Lipase N was used as biocatalyst. However, there were 47.8 and 37.3 % relative esterification yields using Lipozyme IM and Novozym 435 after 4 and 8 days of reaction time, respectively.

The use of caprylic acid and catechin as substrates resulted in relative esterification yields of 44.0 and 54.0 % after 3 and 1 days of reaction time, when Lipozyme IM and Novozym 435 were used, respectively, as biocatalysts. The relative esterification yields of tricaprylin and catechol, used as substrates, were 12.5 and 33.7 % after 8 days of reaction time using Lipozyme IM and Novozym 435, respectively, as biocatalysts. In addition, the use of caprylic acid and catechol, used as substrates, resulted in relative esterification yields of 26.3 and 70.4 % after 1 and 6 days of reaction time, using Lipozyme IM and Novozym 435, respectively, as biocatalysts.

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P015 A DIRECT ENZYMATIC ROUTE TO ENANTIOPURE ALICYCLIC β-AMINO ACIDS

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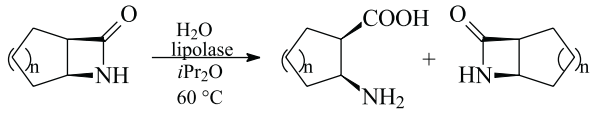
Keywords: cispentacin, β-amino acid, β-lactam, enantioselective ring-opening, Lipolase

The β-amino acids and β-lactams are of biological and chemical importance[1]. They can be used as building blocks for the synthesis of modified peptides with increased activity and stability, and in drug research. Consequently, in the past few years a large number of syntheses have been developed for enantiopure alicyclic β-amino acid derivatives. One good possibility for the preparation of enantiopure unactivated β-lactams is an enantioselective enzyme-catalyzed hydrolysis of β-lactams[2].

We now report a highly efficient and very simple method for the enantioselective ring opening of unactivated alicyclic β-lactams (±)–**1** – (±)–**4** (n = 1, 2, 3, 4), yielding the ring-opened valuable β-amino acids **1a** – **4a** (e. g. cispentacin) and unreacted β-lactam enantiomers **1b** – **4b** in an organic medium.



(±)-1 - (±)-4 **1a - 4a** **1b - 4b**

High enantioselectivity (*E* > 200) was observed when the Lipolase (lipase B from *Candida antarctica*)-catalyzed reactions were performed with H_2O (1 equiv.) in diisopropyl ether at 60 °C. The products, obtained in good chemical yield (36–47 %), could be easily separated. Transformations by ring opening of β-lactams **1b** – **4b** with 18 % aqueous HCl resulted in the corresponding β-amino acid hydrochloride enantiomers (ee ≥ 99 %).

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P016 APPROACH FOR THE DYNAMIC KINETIC RESOLUTION OF CYCLIC α-AMINO ESTERS

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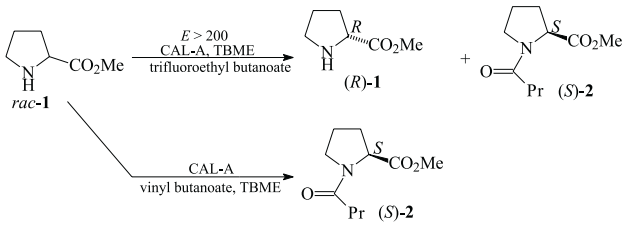
Keywords: DKR, CAL-A, amino acid, resolution

The present research introduces a dynamic kinetic resolution method based on the acylation of the amino group of amino acids with vinyl butanoate catalysed by *Candida antarctica* lipase A (CAL-A) in *tert*-butyl methylether. Racemization is induced by acetaldehyde through Schiff's base formation. Inexpensive and readily available methyl ester of proline (*rac*-**1**) was chosen as a model compound based on our previous work with methyl pipecolinate[1].



In order to develop a satisfactory method of dynamic kinetic resolution where product is formed with high yield and enantiomeric excess, the resolution reaction should be highly enantioselective and racemization of the starting material should take place fast enough. The normal kinetic resolution of *rac*-**1** with trifluoroethyl butanoate by CAL-A showed excellent enantioselectivity (*E* > 200). This prompted that also the resolution with vinyl butanoate could proceed with high enantioselectivity. In this case the racemizing agent, acetaldehyde, is elegantly released during the course of the reaction. Under optimised conditions the resolution led to highly enantiopure (ee = 97,5 %) product (*S*)-**2** with 90 % yield.

The developed method was also tested with other amino acids. The results and mechanisms are presented and discussed in the poster.

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P017 ACTIVATION AND PEPTIDE BOND FORMATION BY LIPASE-CATALYZED ACYL TRANSFERS

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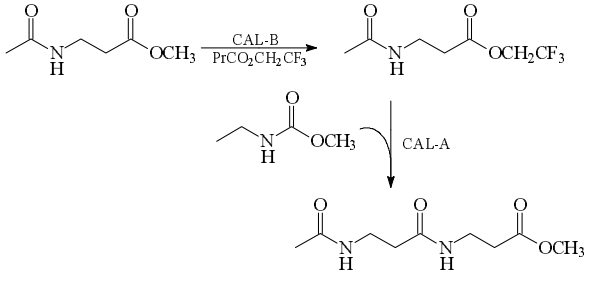
Keywords: lipase, enantioselective, dipeptide, kinetic resolution

Enzymes have gained acceptance as asymmetric catalysts in organic chemistry, especially with the aim of the preparation of enantiopure compounds. Our focus has long been on the lipase-catalyzed kinetic resolution of various types of β-amino esters employing lipases as catalysts[1–3]. In these reactions, CAL-A (lipase A from *Candida antarctica*) and CAL-B (lipase B from *Candida antarctica*), two lipases, which behave in highly different manners, have been especially useful. Thus, CAL-A is exceptionally effective and enantioselective for asymmetric acylation of β-amino esters in organic solvents whereas CAL-B shows various degrees of chemo- and enantioselectivity under the same conditions, substrate structure being a tool to control between the acylation of the amino group and interesterification of the ester group.

Enzymatic peptidomimetics synthesis is ever gaining greater significance because of the potential applications in pharmaceutical industry. In this work, we now report the application of CAL-A and CAL-B catalyses on the preparation of dipeptides through reactions where an amino ester is first activated by lipase-catalyzed interesterification with 2,2,2-trifluoroethyl ester and then a peptide bond is created by the lipase-catalyzed acylation of the amino group of another amino ester. The strategy of peptide synthesis is from *N*-terminus to *C*-terminus as is shown below by using achiral β-alanine methyl ester as a model substrate. Racemic and enantiopure β-amino esters as substrates will be described.



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P018 "EASY ON-EASY OFF TECHNOLOGY": A FULLY ENZYMATIC METHOD FOR KINETIC RESOLUTION OF CHIRAL AMINES

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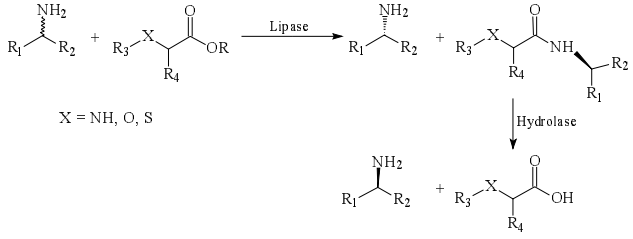
Keywords: enzymatic amine resolution, lipase, hydrolase

Enantiopure amines are used in the fine chemical industry as resolving agents, chiral auxiliaries and chiral synthetic building blocks. Some common methods to produce enantiopure amines are the crystallisation of diastereomeric salts and enantioselective reductive amination. Enzymatic resolution through enantioselective acylation, is another well-developed method. However, the chemical deacylation step to liberate the free chiral amine requires harsh reaction conditions that sometimes are incompatible with certain amines.

"Easy On-Easy Off Technology" is a newly elaborated and fully enzymatic strategy for chiral amine resolution, which involves a lipase-catalyzed acylation followed by a hydrolase catalyzed deacylation[1,2]. The enzymatic cleavage of the amide is quite facile due to the activating effect of the heteroatom in the acyl moiety. Thus, some results of amines resolution using this method will be presented.



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P019 CAL-B CATALYZED RESOLUTION OF 2-PHENYLCYCLOALKANAMINES

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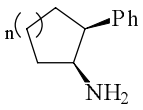
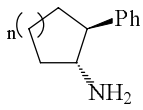
Keywords: resolution, cycloalkanamines, aminolysis, lipase

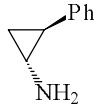
2-Phenylcycloalkanamines are interesting compounds because of their pharmacological properties and synthetic applications. For example, *cis* and *trans*-isomers of 2-phenylcyclopentanamine and 2-phenylcyclohexanamine are building blocks of potent hypoglycemic agents[1] and potentiators of AMPA receptors[2]. In addition, *trans*-2-phenylcyclopentanamine, "cypenamine", and 2-phenylcyclopropylamine, "tranylcypromine" are well-known antidepressives. In most cases, the pharmacological activities of these amines are related to the configuration of the stereogenic centers.



(±)-**1** : n=1
(±)-**3** : n=2



(±)-**2** : n=1
(±)-**4** : n=2



(±)-**5**

In the last years, we have investigated the utility of some lipases, specially, the lipase B from *Candida antarctica* (CAL-B) to catalyze the aminolysis of esters[3,4]. In these processes, optically active esters, amines and amides have been obtained. In the present work, we describe the resolution of some racemic 2-phenylcycloalkanamines following the methodology developed in our research group. Thus, the resolution of (±)–**1-5** was carried out by CAL-B catalyzed enantioselective acylation, using the most simple reaction conditions, that is, employing ethyl acetate as the acyl donor and solvent. Under these conditions the enzyme catalyzed acylation of the *(R)*-enantiomer of the amine preferentially, the resulting *(R)*-acetamides and the remaining *(S)*-amines being easily separated by selective extraction and isolated in very high yields (>85 %).

From the conversion values and enantiomeric excesses showed in Table I, it can be deduced that *trans*-isomers **2** and **4** are more suitable substrates for the enzyme in comparison with the *cis*-isomers **1** and **3**. In addition, the size of the cycle plays a key role in the enantioselectivity of these reactions, cyclopentanamines being transformed with higher *E* values than the corresponding cyclohexyl analogous. It is of note the high rate of conversion and the low *E* value achieved in the reaction with 2-phenylcyclopropanamine.

Table I
Conversion values and enantiomeric excesses of **1–5**

Amine	*t* (h)	*c* (%)	remaining susbstrate	E. e.[a]	acetamide	E. e[a].	*E*[b]
(±)–**1**	24	28	*(S,S)*-1	33	*(R,R)*-**6**	85	16
(±)–**2**	6.5	50	*(S,R)*-2	98	*(R,S)*-**7**	97	>200
(±)–**3**	47	5	*(S,S)*-3	1	*(R,R)*-**8**	13	1
(±)–**4**	20	41	*(S,R)*-4	67	*(R,S)*-**9**	98	200
(±)–**5**	2	90	*(S,R)*-5	96	*(R,S)*-**10**	11	3

[a] determined by chiral HPLC,
[b] enantiomeric ratio calculated according to Sih et al.[5]

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P020 TWO CHEMOENZYMATIC SYNTHESES OF BOTH ENANTIOMERS OF *trans*-CYCLOPENTANE-1,2-DIAMINE

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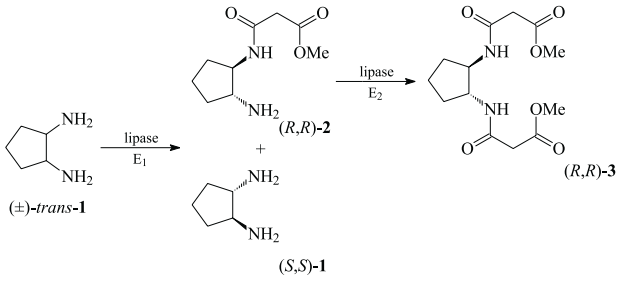
Keywords: diamines, enzymatic resolution, lipases

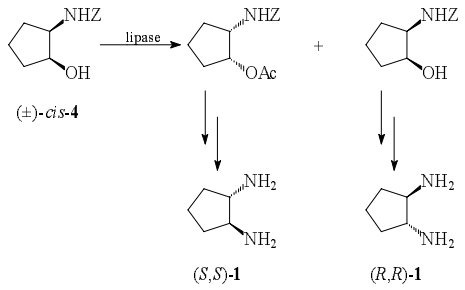
In recent years, chiral synthetic diamine derivatives have been employed as medicinal agents, in particular in chemotherapy[1]. Their use in organic synthesis has also increased considerably, specially in the field of catalytic asymmetric induction[2]. The easy availability of both enantiomers of the target compound in very high ee is one of the limitations for the use of some optically active diamines. Lipase-catalyzed kinetic resolution of racemates is one the most frequently used strategies for the preparation of optically active compounds[3]. Here we describe two different approaches to enantiomerically pure *trans*-cyclopentane-1,2-diamine **(1)**. The first one (Scheme 1) is through a one-pot sequential biocatalyitc resolution of racemic diamine. This strategy had been successfully used for the preparation of optically active *trans*-cyclohexane-1,2-diamine[4]. The enantioselectivities[5] of both steps have been studied and we conclude enantiopure products cannot be obtained with the first aminolysis reaction

(E_1 = 21), but the second biocatalytic process (E_2 > 200) is enantioselective enough for obtaining substrate and product both in enantiomerically pure forms[6].



Scheme 1

On the other hand, we had previously described the efficient resolution of (±)-*cis*-*N*-Cbz-2-aminocyclopentanol[7]. In this case product and substrate were recovered in 99 % ee. We carried out an alternative chemoenzymatic synthesis of **1** using these enantiomers as starting material (Scheme 2).



Scheme 2

Further synthetic applications of the enantiomerically pure obtained compounds are under investigation in our laboratory.

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P021 CALB-CATALYZED ASYMMETRIC AMINOLYSIS AND AMMONOLYSIS OF PROCHIRAL GLUTARATES

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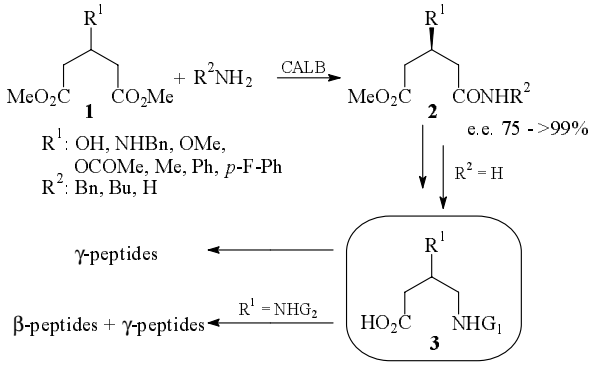
Keywords: desymmetrization, glutarates, ammonolysis, aminolysis, lipase

The enzymatic desymmetrization of *meso* and prochiral compounds constitutes an elegant approach to the synthesis of enantiomerically pure compounds[1]. This methodology has attracted considerable interest as it avoids the inherent 50 % yield limit and the difficult separations often encountered in the resolution of racemates. Enzymatic hydrolysis, transesterification and lactonization of prochiral diesters and diols have been largely applied to prepare chiral synthons in high enantiomeric excesses. However, the potential of enzymes to catalyze the aminolysis and ammonolysis of prochiral substrates has been recently reported by us. Lipase B from *Candida antarctica* (CALB) has shown to be an exceedingly effective catalyst either for the asymmetric aminolysis and ammonolysis of dimethyl 3-hydroxyglutarate (R^1 = OH, Scheme), thus affording enantiopure monoamidation products in very high yield[2].

Following on from these studies, we envisioned to generalize this methodology to different dimethyl 3-substituted glutarates, **1**, and to study the scope of this biocatalytic process as well as the effect of such substitution in the efficiency and enantioselectivity of the reaction.



CALB always showed a clear preference toward the *pro-R* ester group, leading to monoamides of *S* configuration, **2**. Diamides were never detected. However, both chemical yield and enantiomeric excesses strongly depends on the substrate structure (R^1) and, in a lesser extent, on the nucleophile (R^2). Glutarates with a heteroatom at C-3 position with possible hydrogen bonding acceptor behaviour, showed higher chemical yields and e.e.'s. In all the tested conditions, dimethyl 3-methylglutarate showed the poorer results. Aromatic derivatives were less reactive although high e.e.'s can be obtained when the reaction conditions are optimized.

Optically active monoamides **2** (R^2 = H) are interesting chiral compounds for the preparation of different *β*-substituted *γ*-amino acids **3**, which are useful starting materials in the synthesis of non-natural *β*- and *γ*-peptides.

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P022 KINETIC RESOLUTION OF NITRO-ALDOL ADDUCTS

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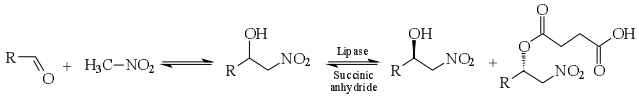
Keywords: β-nitro alcohols, chiral synthesis, succinic anhydride

The aldol reaction is one of the most important methods for C-C bond formation. An aldol-type condensation between an aldehyde and a nitro alkane (Henry reaction) will result in a β-nitro alcohol. The control of the formed stereochemistry is of importance for many synthetical purposes. Two routes that can be followed are direct chiral synthesis from prochiral substrates or resolution of the formed secondary alcohol.



Chiral nitro alcohols can be reduced to the hydroxy amines or undergo further C-C bond formation first on the α-carbon, giving acces to a wide variety of important chiral intermediates.

In this work the applicability of lipase catalysed resolution is studied for obtaining enantiopure β-nitro alcohols. The use of succinic anhydride as the acyl donor has some benefits for downstream product separation and showed much higher enantiomeric ratios for this type of components compared to other commonly used donors like vinyl acetate.

P023 ENANTIOSELECTIVE SYNTHESIS OF 1,4-DIHYDROPYRIDINE DERIVATIVES USING LIPASES

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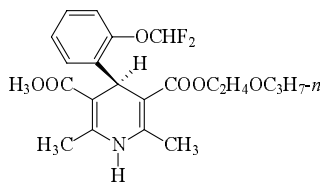
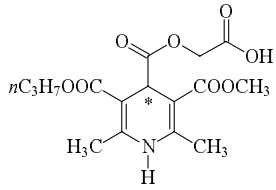
Keywords: 1,4-dihydropyridines, lipases, asymmetrisation, kinetic resolution

1,4-Dihydropyridines (1,4-DHPs) are an important class of drugs since they possess a wide range of biological activities. Since it is well known that enantiomers differ in their biological effects, we have been studying chemoenzymatic routes towards enantiopure building blocks for chiral 1,4-DHPs.

Since ester groups that are connected directly to the 1,4-DHP ring are not reactive towards enzymes, we have used spacers attached to a hydrolysable group in order to turn 1,4-DHPs into substrates[1]. We have found before that the $-CH_2O-$ spacer is advantageous in these reactions, since it splits off formaldehyde spontaneously after hydrolysis of the adjacent ester. In the same studies[1], an isobutyric acid ester was found to give the highest stereorecognition, because it has the optimum amount of steric hindrance. This spacer and ester are now used in a new versatile route for making 4-[2-(difluoromethoxy)phenyl]-1,4-DHP-3,5-diesters, as well as in some studies on 1,4-DHP-3,4,5-triesters (isonicotinic acid derivatives).



4-[2-(difluoromethoxy)phenyl] 1,4-DHP-3,5-diester



1,4-DHP-3,4,5-triester

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P024 *Candida rugosa* LIPASE-CATALYSED KINETIC RESOLUTION OF POLYCYCLIC ANALOGUES OF 1,4-DIHYDROPYRIDINES

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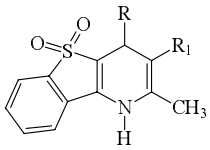
Keywords: 1,4-dihydrobenzthieno[3,2-b]pyridine-5,5-dioxide, 1,4-dihydropyridine, kinetic resolution, lipase

1,4-Dihydropyridines (1,4-DHPs) are an important class of calcium channel antagonists. Novel activities of 1,4-DHPs, such as neuroprotective, antineurodegenerative, cognition and memory enhancing, antidiabetic, anti-inflammatory and antiviral have been also described. Chirality plays an important role in the biological activity of 1,4-DHPs. The use of enantiopure and racemic drugs is regulated by specific rules in EC (ref.[1]). Classical Hantzsch synthesis of 1,4-DHPs is not enantioselective, so enantioselective synthetic methodologies have to be developed.

Polycyclic analogues of 1,4-DHPs in enantiopure form are desired for extended pharmacological studies, since racemic 1,4-dihydrobenzothieno[3,2-*b*]pyridine-5,5-dioxides **1** and 5-oxo-4,5-dihydro-1,4-indeno[1,2-*b*]pyridines **2** have exhibited coronary dilating and anticancer activities; they have been also found as active glutathione *S*-transferase inhibitors.

The standard resolution technique of monocyclic 1,4-DHPs, such as incorporation of an enzymatically labile acyloxymethyl group[2–4] has been successfully applied for the kinetic resolution of 3-(isobutyryloxy)methyl 4-[2-(difluoromethoxy)phenyl]-2-methyl-5,5-dioxo-1,4-dihydrobenzothieno[3,2-*b*]pyridine-3-carboxylate **3** (see Scheme).

Careful screening yielded *Candida rugosa* lipase (CRL) as the preferred biocatalyst. A remarkably good *E*-value was obtained for the CRL-catalysed resolution of this bulky 1,4-DHP derivative. The enantioselectivity of *C. rugosa* lipase can be improved by changing the reaction medium and the temperature. The transition from water-saturated IPE to a solution of *n*-butanol in toluene that was water-saturated at 45 °C resulted in an increase of enantiomeric ratio from $E = 12$ to $E = 28$. More derivatives of the polycyclic 1,4-DHP will be studied.



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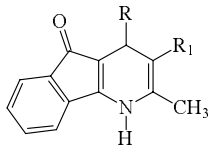

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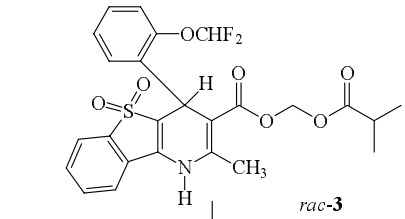
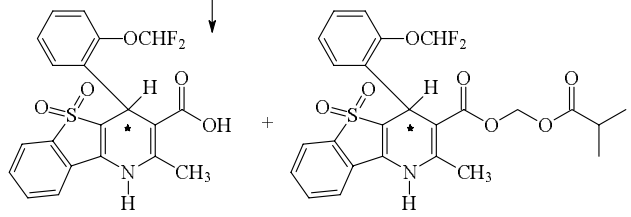


rac-**3**

Candida rugosa lipase E = 28



(-)-**4** (+)-**3**

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P025 ENZYME-CATALYZED KINETIC RESOLUTION OF PIPERIDINE HYDROXY-ESTER REGIOISOMERS

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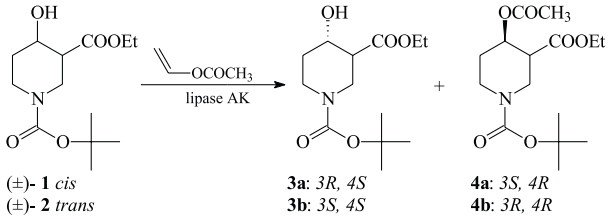
Keywords: piperidines, regioisomers, kinetic resolution, organic solvents, lipase AK

Esters and hydroxyesters possessing piperidine ring among alkaloids and their analogues can modify the neurotransmission resulting in wide-ranging effects on the central nervous system[1, 2].

Our aim was to obtain all four enantiomers of 1-(*tert*-butyl) 3-ethyl 4-hydroxypiperidine-1,3-dicarboxylate **(1, 2)** and of its regioisomer, 1-(*tert*-butyl) 4-ethyl 3-hydroxypiperidine-1,4-dicarboxylate through lipase-catalyzed kinetic resolution in organic media.



High enantioselectivity (E > 200) was observed in the acylation reaction of (±)-*cis* and *trans* 1-(*tert*-butyl) 3-ethyl 4-hydroxypiperidine-1,3-dicarboxylate **(1, 2)** using vinyl-acetate as acyl donor and *Pseudomonas fluorescens* lipase (lipase AK) as a catalyst in diisopropyl ether at 45 °C. In the lipase AK-catalyzed acylation of the sterically more hindered (±)-*cis* 1-(*tert*-butyl) 4-ethyl 3-hydroxypiperidine-1,4-dicarboxylate no product formation was observed even at higher temperature (60 °C). From the lipases screened only *Candida antarctica* A catalysed the reaction with low enantioselectivity.

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P026 SYNTHESIS OF ENANTIOMERS OF PROLINE-RELATED COMPOUNDS *via* HYDROLYTIC ENZYME-CATALYZED KINETIC RESOLUTION

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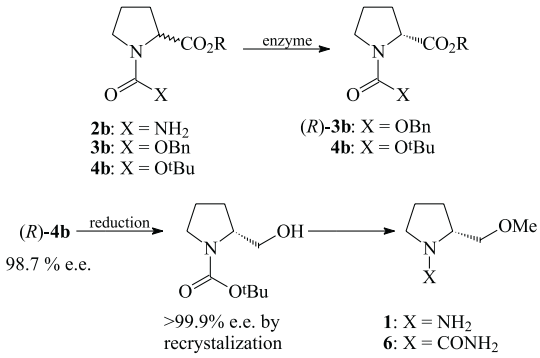
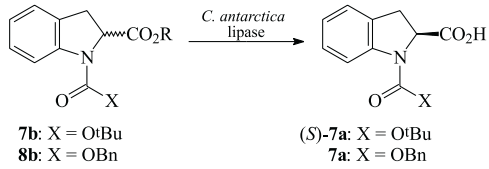
Keywords: *C. antarctica* lipase, protease, cyclic amino acid, hydrolysis, kinetic resolution

We examined approaches towards the preparations of SAMP and RAMP (1-amino-2-methoxymethylpyrrolidine **1**, important chiral auxiliaries) *via* enzyme-catalyzed enantiomeric resolution of racemic intermediates. *B. licheniformis* protease (subtilisin) preferentially hydrolyzed the (*R*)-carbamoylproline ester **(2b)** with an enantiomeric ratio *(E)* of 10. To a hydrophobic *N*-Cbz proline ester **(3b)**, subtilisin showed lower selectivity (E 2.8), and contrary to that, a purified earthworm protease isozyme *(Lumbricus rubellus)* showed the preference of (*S*)-enantiomer (E 13.6). Diverse enantiomeric preference was observed between purified isozymes. In turn, *C. antarctica* lipase B (Chirazyme L-2) was effective for the enantioselective hydrolysis of *N*-Cbz and *N*-Boc proline esters **(4b)** with E > 100 (*S* preferred). The methyl ester **(4b)** was obtained in 49 % yield (98.7 % ee) through a preparative-scale enzyme-catalyzed resolution.



C. antarctica lipase-catalyzed hydrolysis could also be applied on the bicyclic substrates. Enantioselectivities (E >100) were shown in the case of the hydrolysis of *N*-Boc **(7b)** and *N*-Cbz indoline-2-carboxylic esters **(8b)**. An elevated reaction temperature over melting point of the substrate was required, so that the reaction proceeds promptly up to nearly 50 % conversion, to give enantiomerically pure acid **7a** (49 % yield, >99.9 % ee) and ester **7b** (50 % yield, 97.6 % ee).



P027 LIPASE-CATALYSED RESOLUTION OF CYCLIC β-HYDROXYESTERS

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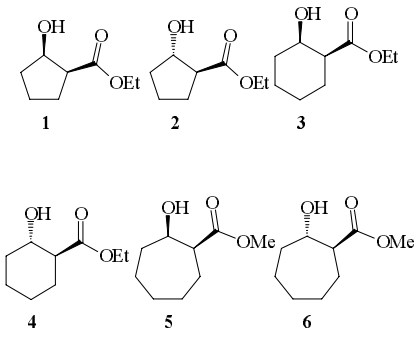
Keywords: lipase, resolution, *Candida antarctica*, cyclic β-hydroxy esters

The importance of optically active β-hydroxy acid derivatives as versatile building blocks for the synthesis of biologically active compounds is well established[1]. Amongst them,

those bearing a substituent in α-position are especially interesting, since they are bifunctionalised molecules with two vicinal stereogenic centres. Biocatalytic approaches to these compounds have been mainly *via* enantio- and diastereoselective reduction of the corresponding β-keto esters[2], amides or nitriles using whole cells, or by kinetic resolution of the racemic mixtures with hydrolases (*i. e.* lipases or esterases)[3]. Although in recent years, a number of elegant strategies have been developed to address the problems associated with the above mentioned methods, β-hydroxy acids are still not readily available. Hence, an efficient methodology for their preparation is still required.



Encouraged by the excellent results obtained in our research group in the enzymatic kinetic resolution of cyclic amino alcohols[4] and diamines[5], over the last few years, we decided to examine the lipase-catalysed enantioselective acylation of the cyclic β-hydroxy esters **1-6** . The resolution of the racemic alcohols (±)–**1-6** was carried out by CALB-catalysed enantioselective acylation using vinyl acetate (VA) as the acyl donor and *tert*-butyl methyl ether as the solvent. In all cases the acyl donor was used in moderate excess (molar ratio VA: alcohol 3 : 1), and the temperature was kept at 30 °C. This acylation procedure afforded excellent enantioselectivities (E = 200) reaching in all cases (apart from substrate (±)–**3**) the maximal conversion of 50 %, at which both substrate and product were isolated enantiomerically pure in high yields. Quite surprisingly, the activity of CALB towards substrate (±)–**3** was very low (and thus unsatisfactory for preparative purposes). After two days, a conversion of only 5 % was reached, although it should be pointed out that this enzyme showed again complete enantiodiscrimination, (product (+)–**3** was obtained in >99 % ee. After testing a number of different solvents and higher temperatures without success, we considered the possibility of using a biocatalyst whose binding pocket can accept more sterically hindered substrates. Another isozyme from *Candida antarctica* (CALA) belongs to the group of lipases which are highly active towards tertiary alcohols. Very satisfyingly, under the reaction conditions described above for CALB-catalysed resolution, a 34 % conversion was reached after just 30 minutes affording product (+)–**3** in >99 % ee and the remaining substrate in 51 % ee.

Thus, we have shown that lipases from *Candida antartica* are excellent biocatalysts for the enantioselective transesterification of five to seven-membered ring β-hydroxy esters. This constitutes for the first time, a direct access to all four stereoisomers of these interesting building blocks in enantiopure form and high yield.

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P028 CHEMOENZYMATIC SYNTHESIS OF OPTICALLY PURE α-HYDROXYALDEHYDES AND KETONES

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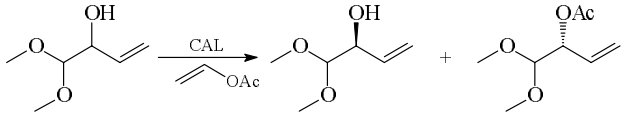
Keywords: enzymatic kinetic resolution, lipase, enol ester epoxide

We have been interested, for years, in the possibility to use transketolase and fructose-1,6-diphosphate aldolase for the production of ketoses with the (3*S*,4*R*) D-*threo* configuration[1, 2]. These enzymes accept a wide range of aldehydes as acceptor substrates. Transketolase appears very enantioselective towards α-hydroxyaldehydes. However when a racemic aldehyde is used as substrate, fructose-1,6-diphosphate aldolase gives both diastereoisomers. We have therefore decided to study the enantioselective synthesis of α-hydroxyaldehydes. Two methodologies have been developped, both based on lipase-catalysed kinetic resolution.

Alcohol **1**, easily prepared from glyoxal dimethyl acetal is a versatile synthon for applications with aldolase and trans-

ketolase catalysed reactions. This alcohol can be converted into α-hydroxyaldehydes by ozonolysis of the double bond or hydrolysis of the acetal moiety. The kinetic resolution of this product was accomplished by acylation with vinyl acetate in the presence of *Candida antarctica* lipase.



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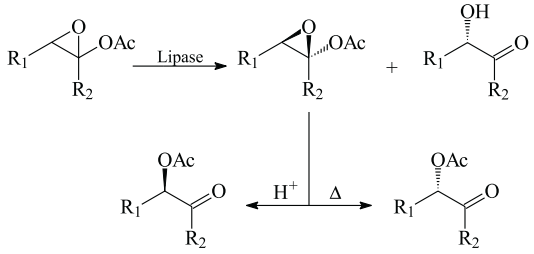
Enol ester epoxides are usefull intermediates in organic synthesis. They can rearrange to α-acyloxyaldehydes or ketones under thermal or acidic conditions[3]. The chemical enantioselective synthesis of enol ester epoxides has been reported. We studied the enzymatic kinetic resolution of these compounds. The epoxides are prepared by epoxidation of enol esters or oxidation of α,β-unsaturated ketones. The lipase-catalysed hydrolysis of enol esters epoxides gives the desired α-hydroxy carbonyl compounds. The remaining chiral epoxides can undergo stereospecific rearrangements to the enantiomerically enriched α-acyloxy carbonyl compounds.



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P029 THE ENANTIOSELECTIVITY CHANGES WITH SUBSTRATE CONVERSION DURING KINETIC RESOLUTION OF SECONDARY ALCOHOLS AND THEIR BUTANOATES CATALYSED BY CALB

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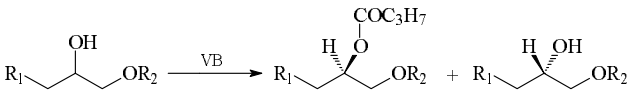
Keywords: kinetic resolution, *Candida antarctica* lipase, hydrolysis, transesterification

Enzyme catalyzed kinetic resolution is characterized by the enantiomeric ratio (*E*-value), which, according to the theory, should be constant during the reaction and independent of the degree of conversion[1]. However, *E*-values increasing with conversion, both in transesterifications[2] and hydrolysis[3, 4] have been reported.



R_1 = Me, Et, Br, Cl
R_2 = Ph, CH_2Ph

The substrate structure has been varied as follows: R_1 = = Me, R_2 = Ph **(1a, 1b)**, R_1 = Et, R_2 = Ph **(2a, 2b)** R_1 = Br, R_2 = Ph **(3a, 3b)**, R_1 = Me, R_1 = Cl, R_2 = Ph **(4a, 4b)** R_1 = = Cl, R_2 = CH_2Ph **(5a, 5b)** where **1a**-**5a** are the alcohols and **1b**-**5b** are the respective butanoates. During kinetic resolution of **1**-**5** catalyzed by *Candida antarctica* lipase B the *E*-value was found to decrease with increasing degree of conversion when the reaction was transesterification. However, when the resolution was performed as hydrolysis of the corresponding butanoates, the *E*-values increased by increasing conversion. Since the composition of the reaction mixture changes during the resolution process, the observed effect may be due to enantioselective inhibition or activation either by substrate or product.

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P030 THIOL FUNCTIONALIZATION OF ALCOHOLS BY CHEMOSPECIFIC LIPASE CATALYSIS

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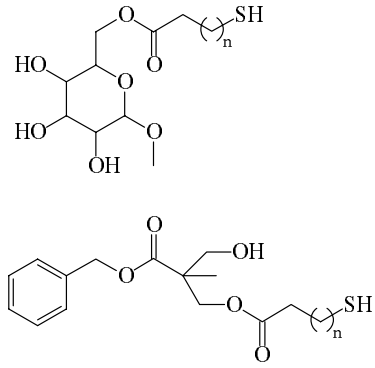
Keywords: thiol functionalization, lipase, chemospecificity

Thiols are highly interesting compounds due to their ability to form self-assembled monolayers (SAMs) on gold surfaces, with applications in areas such as biosensors and nano-electronics. They also have widespread interest in material science in form of polymers with thiol functionalities. Thiol functionalization of alcohols can be performed via ester bond formation using *Candida antarctica* lipase B. This study is focused on the acylation of methyl-β-D-glucopyranoside and the benzyl ester of bis(hydroxy)-propionic acid using 3-mercaptopropionic acid and γ-thiobutyrolactone as acyl donors. This resulted in the thiol functionalized compounds shown below. In contrast to acyl donors such as lactones and hydroxy acids no polymerisation were observed with the corresponding thiolactone and mercapto acid.



n = 1 for 3-mercaptopropionic acid, n = 2 for γ-thiobutyrolactone

P031 *Candida antarctica* B LIPASE CATALYSED ALCOHOLYSIS OF 3',5'-DIACETYL-2'--DEOXYNUCLEOSIDES

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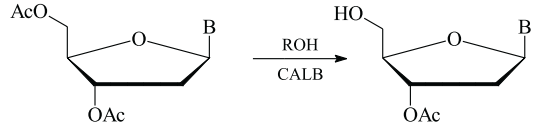
Keywords: deacylation, enzymatic alcoholysis, lipases, nucleosides

Biotransformations catalysed by hydrolytic enzymes provide convenient methods to achieve regioselective transformations in the sugar moiety of nucleosides. Most of the examples described and reviewed deal with the enzymatic regioselective acylation and alkoxycarbonylation of nucleosides[1,2]; in a lesser extent, the hydrolase-catalysed deacylation of nucleosides has also been studied, mainly through enzymatic hydrolysis[1–3].

Over the last years we have been studying the enzymatic deacylation of peracylated ribonucleosides through enzymatic alcoholysis and we have found that *Candida antarctica* B lipase catalyses efficiently the formation of the corresponding 2',3'-di-*O*-acylribonucleosides[4–6]. Taking into account these results, we considered of interest to study the *Candida antarctica* B lipase (CAL B)-catalysed alcoholysis of 2',3'-di--*O*-acetyl-2'deoxy-nucleosides **(1a-e)** :



1a-e **2a-e**

a B: Uracil
b B: Thymine
c B: Cytosine
d B: Guanine
e B: Adenine

R: C_2H_5-, *n*-C_3H_7-, *n*-C_4H_9-

According to the regioselectivity displayed by CAL B in the enzymatic acylation and deacylation of nucleosides[1–6], the selective cleavage of the 5'-*O*-acetate of the substrates was observed, giving the corresponding 3'-*O*-acetyl-2'-deoxynucleosides **2a-e** . The enzymatic butanolysis of 3',5'-di-*O*--acetyl-2'deoxyguanosine **(1d)** afforded the best result: after 48 hours at 30 °C, **2d** was formed in 96 %.

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P032 INFLUENCE OF RING D SUBSTITUTION ON LIPASE-CATALYSED DEACETYLATION OF STEROID DERIVATIVES

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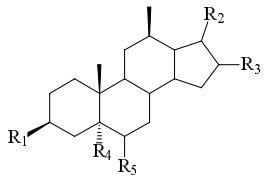
Keywords: androstane, pregnane, deacetylation, lipase-catalysed

The pharmaceutical properties and high cost of steroids made the research on these natural compounds a noteworthy achievement. In the last years, highly selective synthetic procedures for the transformation of complex polyfunctional steroids were developed. Among them, the enzyme-catalysed approach was applied succesfully in regioselective acylation and deacylation reactions producing new compounds with added value to their physiological properties[1–4]. In previous works we have reported the enzyme-catalysed alcoholysis of steroids 3β-acetates containing labile funcional groups in the molecule[5] and the regioselective deacetylation of some androstane derivatives[6]. We have observed that lipases from two yeasts catalysed the alcoholysis of acetyl groups located at different positions of the steroid skeleton. *Candida rugosa* lipase (CRL) and *Candida antarctica* lipase (CAL) have affinity for different regions of the rigid steroid molecule. While CRL removed acetyl groups situated in ring A, CAL was preferentially active on substituents located in ring D. Considering the good performance of CAL in alcoholysis of 16β-*O*-Ac with a carbonyl group at C(17) **(1a)** and the poor activity when the keto at C(17) is replaced by an acetoxy group, the activity of CAL seems to be conditioned by the occurrence of an sp^2-hybridized C-atom in ring D (ref.[6]).

To learn more about the regio and stereoleselective behavior of lipases in the deacetylation of steroids we have performed this reaction using different androstanes and pregnanes **2a**-**10a** as substrates. These substrates were prepared in our laboratory, some of them being novel steroidal compounds.



1a R_1 = AcO; R_2 = =O; R_3 = β-AcO; R_4 = R_5 = H
2a R_1 = AcO; R_2 = =O; R_3 = α-AcO; R_4 = R_5 = H
3a R_1 = AcO; R_2 = $=CH_2$; R_3 = β-AcO; R_4, R_5 = Δ^5
4a R_1 = AcO; R_2 = $=CH_2$; R_3 = α-AcO; R_4, R_5 = Δ^5
5a R_1 = TBDMSO; R_2 = $=CH_2$; R_3 = α-AcO; R_4, R_5 = Δ^5
6a R_1 = AcO; R_2 = (*E*) $=CHCH_3$; R_3 = β-AcO; R_4, R_5 = Δ^5
7a R_1 = AcO; R_2 = (*E*) $=CHCH_3$; R_3 = α-AcO; R_4, R_5 = Δ^5
8a R_1 = AcO; R_2 = (*E*) $=CHCH_2OAc$; R_3 = H; R_4, R_5 = Δ^5
9a R_1 = TBDMSO; R_2 = (*Z*) $=CHCH_2OAc$; R_3 = α-AcO; R_4, R_5 = Δ^5
10a R_1 = AcO; R_2 = (*Z*) $=CHCH_2OAc$; R_3 = α-AcO; R_4, R_5 = Δ^5

CAL was not very stereoselective in the deacetylation of position 16 of androstanes. On treatment of 16α-*O*-Ac **2a** with this lipase, the 3β-acetate of **2a**, unknown till now, was obtained in such a good yield as we had previously obtained with 3β-acetate from **1a** (ref.[6]).

The replacement of the carbonyl group by a carbon-carbon double bond at position 17 showed a remarkable decrease or no activity at all of CAL on the α and β 16-*O*-Ac of substrates **3a**-**7a** . The corresponding 3β-acetate-16-hydroxy compounds were obtained in poor yield or not obtained at all. The primary acetyl group in **8a** was easily removed by both enzymes (CAL and CRL). CAL kept its regioselectivity in the formation of the 3β-monoacetyl derivative.

In **9a** and **10a** the primary acetyl group in carbon 21 was more reactive than the 16α-*O*-Ac. This result shows that the *tert*-butyldimethylsilyloxy in 3β position **(9a)** does not produce inactivation of the enzyme.

The behavior of CRL was the expected one in all substrates being removed the acetyl group in position 3β in the acetylated derivatives. By enzymatic catalysis with CRL the *tert*-butyldimethylsilyl derivatives **5a** and **9a** remained unaltered.

In conclusion, we show that lipases from *C. rugosa* and *C. antarctica* removed acetyl groups in a regioselective fashion from di- and triacetylated androstane and pregnane derivatives. These results could lead us to say that the substitution in ring D with a polar carbon double bond such as the carbonyl group could be necessary for CAL activity. Some of the reported compounds were proved to be useful intermediates in the synthesis of biologically active steroids.

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P033 DYNAMIC RESOLUTION OF *(R,S)*-NAPROXEN 2,2,2-TRIFLUORO ESTER *via* LIPASE-CATALYZED HYDROLYSIS IN MICRO-AQUEOUS ISOOCTANE

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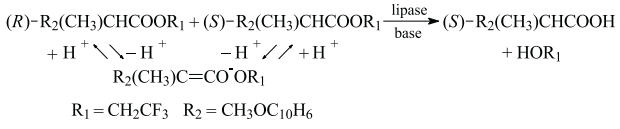
Keywords: dynamic resolution, lipases, naproxen, hydrolysis

Candida rugosa lipases immobilized on polypropylene powders were employed as the biocatalyst for the enantioselective hydrolysis of *(R,S)*-naproxen 2,2,2-trifluoroethyl ester in micro-aqueous isooctane at 45 °C, in which TBD immobilized on polystyrene crosslinked with 2 % DVB was added as an in situ racemization catalyst for the remaining *(R)*-ester (Scheme). The kinetic behaviors of the lipase and the base were first investigated, respectively, by considering of the enzyme stability and product inhibition as well as the base deactivation. The results for the dynamic resolution process were then compared with the kinetic resolution process without adding the base. The results were also compared with the dynamic resolution process by employing *(R,S)*-naproxen 2,2,2-trifluorothioethyl ester and trioctylamine as the substrate and the base, respectively, for producing the desired *(S)*-naproxen.



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P034 ENZYMATIC RESOLUTION OF TERPENOID LACTAMS TOWARDS NEW CHIRAL COMPOUNDS WITH POTENTIAL NEUROACTIVITY

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Keywords: (+)-3-carene, chiral compounds, terpenoid lactams, enzymatic resolution, pharmacological activity

Optical active lactams are valuable building blocks in organic synthesis and find wide application as synthons for biologically active compounds. It is known that some of them are resoluted into stereoisomers by lipase-catalysed transesterification[1, 2].

We have obtained the isomeric mixtures of bicyclic terpenoid lactams, starting from (+)-3-carene, naturally occurring monoterpene, component of many turpentines[3], followed by conversion into new amino acids[4]. Pharmacological studies showed that these amino acids possess neuromodulatory activity[5].

As a continuation of our investigation we have developed lipase-catalyzed resolution process for diastereoisomeric mixture of terpenoid lactams which are intermediates in the synthesis of novel chiral piracetam analogues with nootropic activity.

Screening of lipases and the effects of solvent, influence of donor group and temperature will be widely discussed with special emphasis on their biosynthetic aspects.

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P035 A NEW CHEMOENZYMATIC METHOD FOR THE SYNTHESIS OF *(S)*-(+)-ZOPICLONE

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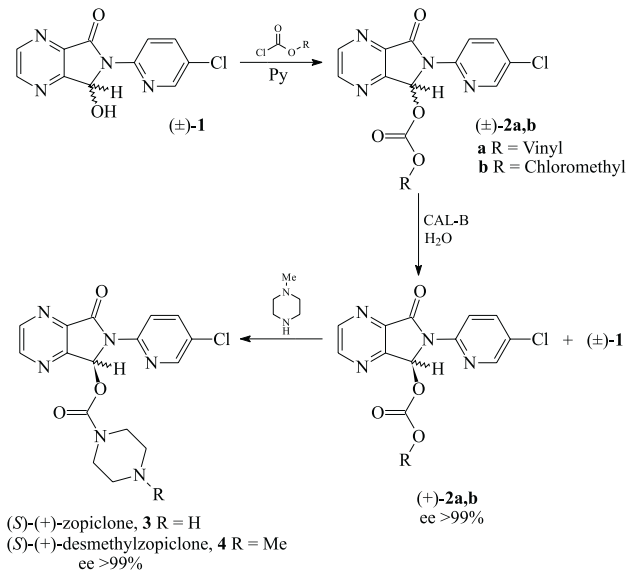
Keywords: hydrolysis, lipase, zopiclone, hypnotic, pharmaceutical industry

Racemic zopiclone is a hypnotic agent of the cyclopyrrolone class, which has been commercialized for the treatment of insomnia[1]. However, the *(S)*-configured enantiomer is more active and less toxic than the *(R)*-isomer. In a previous report we described a chemoenzymatic method for the preparation of enantiomerically pure *(S)*-(+)-zopiclone, **3** (Scheme)[2]. The key step in this procedure is the resolution of the vinyl carbonate intermediate (±)–**2a** by lipase-catalyzed hydrolysis. Although the enzymatic step is efficient, two facts make the method expensive for the kilogram preparation of enantiopure *(S)*-(+)-zopiclone. Firstly, the high cost of vinyl chloroformate as a reagent and, secondly, the low 30 % yield of the last step in the synthesis, i. e. the treatment of the vinyl carbonate (+)–**2a** with *N*-methylpiperazine.



Herein, we describe an improvement of this method based on the preparation and enzymatic resolution of a new carbonate intermediate (±)–**2b**, via *Candida antarctica* lipase B-catalyzed hydrolysis. The *(R)*-configured product **1** of the enzymatic reaction undergoes spontaneous racemization in the reaction medium. Therefore, it can be directly recycled after work-up of the enzymatic reaction. Thus, the overall formal yield of the enzymatic process is 100 %, even though this enzymatic step is a kinetic resolution process.

It is noteworthy the low cost of the reagents employed in the synthesis of (±)–**2b** as well as the high yield of the last step in the synthesis of *(S)*-(+)-zopiclone by the reaction of the enantiomerically pure carbonate (+)–**2b** with *N*-methylpiperazine.

This methodology has been also successfully used in the synthesis of *(S)*-(+)-desmethylzopiclone, **4**, a metabolite of zopiclone, which has been recently reported as an interesting compound for its biological activities in preclinical anxiolytic evaluation[3].

Taking into account the simplicity and easy scale-up of lipase-catalyzed reactions, it is noteworthy the applicability of this method to the industrial preparation of the hypnotic *(S)*-(+)-zopiclone.

Furthermore, in order to prove the economic efficiency of this biocatalytic method, we are investigating the feasibility of reusing the immobilized *Candida antarctica* lipase B.

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P036 ALCOHOL AND ACYL DONOR STRUCTURE EFFECT ON THE ENZYMATIC ACYLATION OF FLAVONOIDS

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Keywords: flavonoids, fatty acids, enzymatic, acylation, *Candida antarctica*

Flavonoids are natural compounds with interesting properties due to their antioxidant, antimicrobial, anticarcinogenic activities[1]. However, they are characterised by a low solubility and stability, which could limit their use. In view to improve these properties chemical and enzymatic acylations with lipophilic groups have been suggested[2,3]. The aim of this work is to investigate the behaviour of enzymatic acylation of different flavonoids and to study the carbon chain length effect of the acyl donor (fatty acids).

The enzymatic acylations were led in *tert*-amyl alcohol as solvent at 60 °C and with immobilized lipase of *Candida antarctica* as catalyst. The substrate used in this study are rutin, hesperidin and esculin as flavonoids and caproic, pelargonic, oenanthic, lauric, myristic, palmitic, stearic and oleic acid as acyl donor. The reactions were conducted in Chemspeed automated parallel synthesis workstation at low level of water content in the reaction medium (< 300 ppm). The substrates and products were quantified by HPLC system equipped by UV, RI and LS detectors. The structures of some obtained products were characterised by ^{1}H NMR analysis.

The enzymatic acylation of the three flavonoids tested indicated that the performance of this reaction depends of the presence or not of a primary hydroxyl group on the glycosyl moiety. The highest conversion yields was obtained with esculin and it is about of 82 %. When rutin was acylated with different fatty acids, the results showed that the conversion yield increases with carbon chain length up to C12. For higher carbon number, no effect was detected except for C14. The ^{1}H NMR analysis showed that for glycosylated flavonoids the acylation took place on the glycoside moiety.

Flavonoids acylation performance was affected by alcohol and acyl donors structure. The highest conversions (> 80 %) were obtained for glycosylated flavonoids with a primary hydroxyl group. Only fatty acids with short carbon chain affect the kinetic of this reaction. These enzymatically acylated flavonoids will be evaluated for their properties and biological activities.

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P037 ENZYMATIC PREPARATION OF FLAVONOID DERIVATIVES

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Keywords: flavonoid, antioxidant, esterification

The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to ring structures, conferring the antioxidant activity. The practical exploitation of their antioxidant properties, for instance as protective agents for fats and oils against oxidation, is limited by the hydrophilic nature of these compounds that may hinder solubility in lipidic matrices. This problem can be solved by esterification of the polyhydroxylated molecules catalysed by enzymes, in organic solvent[1, 2, 3].

In this work acylated derivatives of plant derived flavonoids as catechin, hesperetin, were synthesised in a reaction catalysed by lipase B from *Candida antarctica*, using *tert*-butanol as solvent whereas vinyl acetate and vinyl laurate were used as the acyl donors. The various parameters affecting the reactions were extensively studied.

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P038 DIRECTED EVOLUTION OF A *Pseudomonas fluorescens* LIPASE FOR RESOLUTION IN ORGANIC SOLVENTS

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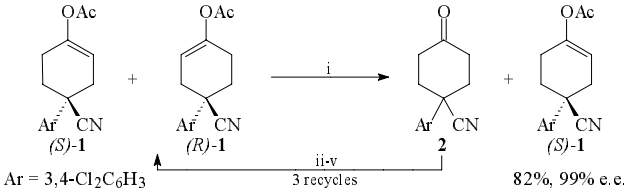
Keywords: lipase, directed evolution, organic solvents

A novel biotransformation has been developed in our laboratory for the preparation of homochiral enol acetates derived from prochiral ketones. 4-Cyano-4-aryl substituted cyclohex-1-enyl acetates such as 1 have been resolved with *Pseudomonas fluorescens* lipase (PFL) by transesterification with *n*-butanol in THF giving good to excellent enantiomeric purities (e.e.'s) for the chiral enol acetates. With an efficient recycling method (Scheme), even with a relatively low *E* value of 13, it is possible to isolate enantiomerically pure enol acetate *(S)*-1 in 82 % yield after 4 cycles[1, 2]. This is a successful approach and has been scaled up to provide synthetically useful quantities (20 g) of the *(S)*-enol ester 1 which was used for the synthesis of a tachykinin NK-2 antagonist used in the treatment of neuro-inflammatory conditions[3, 4].

The aim of this project is to use this lipase resolution as a model system for developing a high throughput directed-evolution strategy for obtaining lipases with improved enantioselectivity for use in organic solvents. The *Pseudomonas fluorescens* lipase gene, *lipA*, has been cloned with a histidine tag, expressed in E. coli, purified under denaturing conditions and refolded directly on the Ni-NTA resin. This gene will be subjected to a radical mutagenesis protocol (ep-PCR) to

generate a library of mutant enzymes. Due to the tag, the enzymes can be absorbed from the crude cell lysate using Ni-NTA coated microtitre plates, facilitating a high-throughput enzyme screening strategy in organic solvents. The e. e. screen is based on the use of pseudo-racemic d^3-labelled enol ester 1 with electrospray ionisation mass spectrometry (ESI-MS) to detect M and M + 3 (ref.[5]). A protocol is being developed to maximise the spectrum of mutations in the *lipA* gene, thereby increasing the probability of creating and isolating mutant enzymes with improved enantioselectivity. Interesting mutants will then be sequenced to identify key mutations and may be subjected to further rounds of random mutagenesis or gene shuffling.



i: Pseudomonas fluorescens lipase (PFL), nBuOH, THF; ii: Remove PFL; iii: add isopropenyl acetate and ^{t}BuOK; iv: Add Dowex H^+ resin; v: Remove resin and add PFL and nBuOH

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P039 PRESERVING THE ACTIVITY OF LIPASE FROM *Pseudomonas fluorescence* BY HYDROPHYLISATION IN THE PRESENCE OF SODIUM DODECYL SULFATE

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Keywords: lipase, thermoinactivation, chemical modification

The industrial interest in lipase activity is very high, especially as component of household detergents. Especially the temperature stability of enzymes used in laundry detergents is an important factor. Enzyme engineering is a variable tool to enhance the thermostability of enzymes.

The hydrophile-lipophile balance (HLB) of an enzyme seems to be essential for most of its properties (recognition of substrate, binding, stability, etc). In our work, the HLB of lipase (Lip) from bacteria *Pseudomonas fluorescence* has been changed by chemical modification. Lipase was modified by glucose with primary amino group of lysine and the Schiff base formed in the reaction was reduced by the addition of sodium borohydride.

The comparative study of the behavior of the native Lip and Lip-Glu modified by hydrophylisation showed that glucosylation of lipase does not affect on the stability of these enzymes in buffer solution. The rate of thermoinactivation of lipases at 58 °C is the same.

In the presence, however, of sodium dodecyl sulfate (SDS) both lipases are less stable, but hydrophylisation of lipase provides a better protection for thermoinactivation. This stabilizing effect (ratio of the rate constant of thermoinactivation of the modified and native enzymes) is about 2-fold.

P040 REAL-TIME MONITORING OF LIPASE-CATALYSED CARBOHYDRATE MODIFICATION BY ^{1}H-NMR SPECTROSCOPY

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Keywords: enzymatic, NMR spectroscopy, lipase, carbohydrate

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive analytical method that can be used to study biocatalytic reactions *in situ*. In this study, ^{1}H-NMR was used to monitor on-line lipase-catalysed transesterification reactions in organic solvents without the need to sample the reaction medium. The synthesis of acryl esters of a range of monosaccharides and their α-methoxy derivatives catalysed by lipase (Novozym 435) in *tert*-butan(ol-d) has been monitored. The time-course of sugar acrylate and acetaldehyde formation as well as the disappearance of vinyl acrylate was monitored, allowing for simultaneous quantification of each reactant and product species in the reaction mixture. The kinetic parameters obtained from real-time ^{1}H-NMR data were comparable with those obtained by classical methods consisting of sampling the reaction mixture and quantitative determination by GC.

This study shows that ^{1}H-NMR can be used for real-time monitoring of heterogeneous biocatalytic reactions.

P041 CLONING AND CHARACTERIZATION OF A NOVEL LIPASE FROM *Archaeoglobus fulgidus* DSM 4304

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Keywords: *A. fulgidus*, hyperthermophilic, lipase, alcalophilic

A novel lipase was identified in the genome of the hyperthermophilic archaeon *Archeaoglobus fulgidus*[1]. *Archaeoglobus fulgidus* is a sulphur-metabolizing organism with an optimal growth temperature of 83 °C. We have cloned and overexpressed a novel lipase from this organism in *E. coli* and purified it using immobilized metal affinity chromatography combined with anion exchange chromatography.

The activity measurements performed in a pH-Stat and spectrophotometrically demonstrated that this enzyme preferentially uses *p*-nitrophenylesters and esters of short chain fatty acids as substrates. Tributyrin was only weakly hydrolysed while triolein and other typical substrates for lipases were not used. The location of the catalytic triad Ser-Asp-His was identified by site-directed mutagenesis and the properties of the enzyme concerning the temperature and pH stability have been evaluated. The enzyme showed optimal activity and stability at pH values of 10–11. The temperature dependent activity measurements revealed a temperature optimum of 70 °C and a rapid loss of activity at higher temperatures. This leads to the assumption that compatible solutes of *Archaeoglobus fulgidus* like di-glycerolphosphate are a crucial factor for the temperature stabilization of the enzyme *in vivo* as it is in other thermophilic organisms[2].

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P042 PRODUCTION OF ETHYL BUTYRATE BY LIPASE-CATALYSED ESTERIFICATION IN BATCH AND IN CONTINUOUS REACTORS

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Keywords: batch reactor, continuous reactor, esterification, lipase, polyurethane

Ethyl butyrate is an important flavouring ester widely used in food and pharmaceutical products. The aim of this study was the production of ethyl butyrate in *n*-hexane catalysed by *Candida rugosa* lipase immobilized in biocompatible hydrophilic polyurethane foams prepared with the pre-polymers "Hypol FHP 2002™" (toluene diisocyanate) and "Hypol FHP 5000™" (diphenylmethane-4,4'-diisocyanate). The immobilised preparations were used in batch and in continuous reactors.

Batch experiments were conducted in 12 cm^3 of *n*-hexane solutions, at 30 °C under magnetic stirring, following a central composite rotatable design, as a function of the initial butyric acid concentration, *A* (0.078–0.57 M) and of the molar ratio ethanol/acid, *R* (0.43–2.27).

For both immobilised preparations, at 18 hours reaction time, production of ethyl butyrate and conversion could be fitted to flat or convex surfaces, described by first or second order polynomial models.

Theoretically, a maximum ester production of 0.23M is expected after 18 hours reaction time, at 0.35 M of *A* and *R* equal to 1.5, for the lipase in "FHP 2002™". When the immobilized lipase in "FHP 5000™" foam is used, a maximum of 0.27M ethyl butyrate is expected at 0.53 M of *A* and 0.78 of *R*. In reality, with these initial substrates concentrations, 0.28 M and 0.32 M ethyl butyrate concentrations were achieved, after 48 hours reaction time, when the lipase was immobilised in "FHP 2002™" and "FHP 5000™" foams, respectively. These concentrations correspond to conversions of 80.7 % and 78.2 %, respectively.

For both immobilized preparations, the highest conversion values were observed in acid-limited reactions, namely for *A* equal to 0.078 M and *R* equal to 1.5. Under these conditions, after 15 hours reaction time, 74.4 % and 97.1 % conversions were attained with the lipase in "FHP 2002™" and "FHP 5000™, respectively. However, these high conversions correspond to very low concentrations of ester (0.058 M and 0.076 M, respectively) and therefore to low productivities.

For the continuous process, an up-flow (0.1 $ml.min^{-1}$) fixed-bed reactor consisting of a thermostated (30 °C) glass

column (16 mm internal diameter; 20 cm height) filled with the immobilized biocatalyst (about 60 mg of lipase per ml of reaction medium) was used. The reaction mixture residence time was, in average, 260 min. As in batch reactions, when the reaction medium composed by 0.078 M *A* and 1.35 *R* was used, high conversions and low ester concentrations were also obtained with both immobilised preparations.

The bioreactor was continuously operated for 1 month. During this period, no activity decay was observed with either immobilized preparation.

Using the reaction medium formulation predicted to maximize ester production, the ethyl butyrate concentration obtained with the biocatalyst in "FHP 2002™" was only 0.097 M, corresponding to a conversion of 27.6 %. These unexpected results might be ascribed to an insufficient residence time, rather than to an inactivation of the biocatalyst.

The authors are grateful to Amano Enzyme Europe, for the gift of lipase AY and to Dow Chemical Company, U. K., for the gift of the polyurethane pre-polymers.

P043 PRODUCTION OF MARGARINE BASESTOCKS BY TRANSESTERIFICATION OF BLENDS OF THREE VEGETABLE FATS CATALYZED BY A COMMERCIAL IMMOBILIZED LIPASE

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Keywords: lipase, margarine, response surface modelling, transesterification

The functional properties of fats are determined by the distribution pattern of fatty acid radicals in their molecules. The modification of that pattern, by transesterification, is a route to improve certain properties of natural fats and to implement their nutritional value. This is of much interest for applications in margarine, confectionary and bakery industries, as well as for pharmaceutical purposes.

In this study, a commercial immobilized thermostable 1,3-specific lipase from *Rizomucor miehei*, "Lipozyme™ IM", kindly donated by Novozymes, Denmark, was used as a catalyst for the transesterification of fats in a solvent free medium. The reaction medium was composed of palm oil stearin (POS), palm kernel oil (PK) and rapeseed oil (RP). Transesterification reactions were carried out in capped 100 ml cylindrical thermostated reactors, under magnetic stirring with a biocatalyst load of 5 % *(w/w)*. The biocatalyst was used at its original water activity (a_w) of 0.248, at 25 °C.

A set of 26 experiments was carried out following a Central Composite Rotatable Design, as a function of 4 variables: POS and RP content (reaction medium formulation), temperature *(T)* and reaction time *(t)*. Temperature varied from 55 °C to 75 °C; *t* from 15 to 105 minutes; POS content from 45 % to 85 % *(w/w)* and RP from 5 % to 25 % *(w/w)*. The proportion of PK used was calculated from the amounts of the other two fats. Five levels were considered for each variable inside the tested range.

The reaction time course was indirectly followed by the "Solid Fat Content" of the blend assayed by NMR at different temperatures ($SFC_{10\,°C}$; $SFC_{20\,°C}$ and $SFC_{35\,°C}$), which are related with the rheological behaviour of fats at storage ($SFC_{10\,°C}$), packing ($SFC_{20\,°C}$) and consumption temperatures ($SFC_{35\,°C}$), respectively. The $SFC_{35\,°C}$ of transesterified fats should be lower than that of their original counterparts, to prevent a sandy and coarse texture. Also, the extent of the competing reactions of hydrolysis and lipid oxidation was evaluated along the transesterification reaction.

Reaction time showed to have significant linear and/or quadratic effects on all SFC values and on the formation of oxidation products (initial and final oxidation products). A linear effect of reaction temperature on $SFC_{35\,°C}$, on FFA and oxidation products was also observed. SFC values showed to depend on the initial POS and on RP content. Also, a linear effect of the initial POS concentration on the formation of oxidation products was observed. Only the linear interactions ($t \times T$) and ($t \times POS$) were significant on $SFC_{20\,°C}$ and $SFC_{35\,°C}$.

Response surfaces, described by first or second order polynomials, can be well fitted to the final SFC values, $SFC_{35\,°C}$ reduction and oxidation products. The lowest $SFC_{35\,°C}$ values (lower than 8) and highest reduction levels of $SFC_{35\,°C}$ (higher than 50 %) were achieved under temperatures and reaction times higher than 65 °C and 70 minutes, respectively, and for POS between 45 and 60 %.

Starting from blends with 0.2 % FFA, transesterified fats with 2 % to 4 % FFA were obtained. A lack of fit was observed between FFA experimental data and the model. This suggests that the production of FFA is mainly a result of the first-step of lipase-catalysed transesterification and not very much dependent on reaction conditions[1].

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P044 RESPONSE SURFACE MODELLING OF THE TRANSESTERIFICATION OF FAT BLENDS, RICH IN ω-3 POLYUNSATURATED FATTY ACIDS, BY A COMMERCIAL IMMOBILIZED LIPASE

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Keywords: hardness, immobilized lipase, modelling, solid fat content, transesterification

In this study, a commercial immobilized thermostable lipase from *Thermomyces lanuginosa*, Lipozyme™ TL IM, kindly donated by Novozymes, Denmark, was used for the transesterification of fats in a solvent free medium, starting from different blends. The reaction medium was composed of palm oil stearin (POS), palm kernel oil (PK) and a commercial concentrate of triglycerides enriched in omega-3 fatty acids (ω-3 PUFA). The reaction medium formulation, temperature *(T)* and reaction time *(t)* varied according to a central composite rotatable design (CCRD). In CCRD 1, the commercial concentrate ("EPAX 2050TG") contained 20 % eicosapentaenoic (EPA) and 50 % docosahexaenoic acids (DHA). In CCRD 2, the concentrate "EPAX 4510TG", with c. a. 45 % EPA and 10 % DHA was used. Transesterification reactions were carried out in small cylindrical thermostated reactors, under magnetic stirring with a biocatalyst load of 5 % (w/w). The reaction time course was indirectly followed by the "Solid Fat Content" of the blend assayed by NMR at different temperatures ($SFC_{10\,°C}$; $SFC_{20\,°C}$; $SFC_{30\,°C}$ and $SFC_{35\,°C}$). A decrease in $SFC_{35\,°C}$ is desirable since this parameter is related to the decrease in the extent of fat crystallisation at the same temperature. Hardness of the fats was assayed at 20 °C by a TA-XT2 Texture Analyzer, using a 8 mm stainless steel cylinder probe. The SFC values of the transesterified fats depended on the POS and EPAX content, on the reaction temperature and time, and also on the linear interactions between these variables, for both experimental designs. The transesterification resulted in higher hardness values for high POS and low EPAX content, high *t* and *T* values. Response surfaces, described by first or second order polynomials, can be well fitted to the final SFC values, SFC reduction and final hardness values, either when "EPAX 2050TG" or "EPAX 4510", were used. Hardness at 20 °C can be described by second order polynomials as a function of $SFC_{10\,°C}$ or $SFC_{20\,°C}$.

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P045 THE INTERACTION BETWEEN *Candida antarctica* LIPASE AND BRANCHED CHAIN FATTY ACIDS: A KINETIC AND MOLECULAR MODELLING STUDY

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Keywords: lipase, branched chain fatty acids, molecular modeling, kinetic resolution, esterification

Branched chain fatty acids are widespread in Nature, occurring in several plant and animal tissues. They play an important role in the flavour and fragrance of mutton and sheep's cheese[1]. Most of them are chiral, depending on the substituent and its position at the chain. Earlier research in our laboratory has revealed that immobilised *Candida antarctica* lipase B (Novozym 435®) is the best catalyst for the kinetic resolution of 4-methyloctanoic acid. An enantiomeric ratio *(E)* of 57 was obtained in a direct esterification reaction with ethanol in a solventless system[2] which is quite high regarding the distance between the chiral centre and the position where the enzymatic attack takes place (the carbonyl group).

In order to gain more information about enzymatic stereorecognition at more or less remote chiral centres, we have synthesised all positional isomers of methyloctanoic acid and subjected them to Novozym 435®-mediated esterification with ethanol under the same conditions as previously used for 4-methyloctanoic acid. Remarkably, there appeared to be no clear relation between the distance to the chiral centre and the enantiomeric ratio. Using molecular dynamics we were able to explain our results and give a good estimate of *E*-values.

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P046 *Georichum candidum* LIPASE: ACTIVATION AND ITS ENANTIOSELECTIVITY TOWARDS XENOBIOTIC SUBSTRATES

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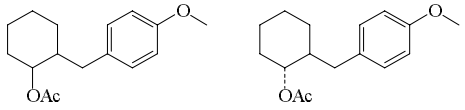
Keywords: *Geotrichum candidum*, lipase, enantioselectivity

Production of lipase by four strains of *Geotrichum candidum* (48, 0302, 4012, 4013) in liquid medium was investigated under addition of olive oil, which has been used as activator of the activity.

Calculated differences in lipase activity values were used as a basis for optimizing of fermentation conditions. Olive oil and peptone were the best sources of carbon and nitrogen. Majority of activated lipases represented extracellular lipases, which penetrated through cellular membrane into the medium. The activity of intracellular lipases was determined as well. The most remarkable growth of the cell biomass was also observed using a medium with peptone as a source of nitrogen. The highest specific activity of all activated lipases was found in the second half of the period of exponential (log) phase cultures. Strain 48 displayed the highest extracellular and intracellular lipase activity after 80 h of growth on a shaker at 30 °C. All activated lipases were used as biocatalysts of the hydrolytic resolution of racemic *cis*- and *trans*-isomers of 2-(4-methoxybenzyl)cyclohex-1-yl acetates (**1** and **2**)[1]. Enantiomeric purity and absolute configuration of the products, chiral isomers of 2-(4-methoxybenzyl)cyclohexanol, were studied.



1 **2**

The substrate **2** was more favoured by most of the activated lipases employed due to the higher conversion rates found. The optimal enantioselectivity of the enzymes were found when the compound **2** was used as the substrate of hydrolytic reaction.

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P047 NEW EFFICIENT LIPASE FROM *Yarrowia lipolytica* FOR THE RESOLUTION OF 2-SUBSTITUTED CARBOXYLIC ACID ESTERS

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Keywords: lipase, *Yarrowia lipolytica* culture, enantioselectivity

A new extracellular lipase from the yeast *Yarrowia lipolytica* was recently characterized[1]. This acid resistant and thermostable lipase was demonstrated to be of great interest in lipid waste process and in human pancreatic deficiency treatments[2]. Moreover, *Y. lipolytica* appears to be one of the most attractive organisms for heterologous enzyme production and new expression plasmids have been developed[3]. It combines the facility of single cell use, of high secretion abilities and efficient tools for post-translational modifications[4]. New *Y. lipolytica* strains were constructed by introducing multiple copies of the lipase gene in the genome (~15 copies/genome)[5]. The lipase gene is under the control of a promoter POX2 inducible by oleic acid.

A mineral medium was developed for lipase production which fulfilled *Y. lipolytica* nutritional requirements and enabled in fed-batch mode 100 $g.l^{-1}$ of biomass to be obtained with the tremendous production of 60 000 lipase $U.ml^{-1}$ (1U = 1 micromol triolein hydrolyzed per minute). The lipase represented the main enzyme in the broth.

New potentialities of this lipase were discovered in the field of resolution of racemic compounds. *Y. lipolytica* lipase is active and selective for resolution of 2-bromo phenyl and tolyl acetic acid esters. These types of compounds are important intermediates in the synthetic pathways of drugs such as prostaglandin, prostacyclin, semi-synthetic penicillin, thiazolium salts, etc. Methyl and ethyl ester derivatives of 2-bromo-*o*-tolylacetic acid are used as precursors for the synthesis of analgesics, and non-peptide angiotensin II-receptor antagonists[6]. Activity and enantioselectivity performances of *Y. lipolytica* lipase were compared with those of *Burkholderia cepacia* lipase, which is one of the preferred enzymes for

these reactions[6]. *Y. lipolytica* lipase has shown higher activities, an inverse enantioselectivity preference (*S* preference) and similar enantioselectivity.

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P048 ENANTIOSELECTIVITY IMPROVEMENT OF *Yarrowia lypolitica* LIPASE FOR RESOLUTION OF 2-SUBSTITUTED CARBOXYLIC ACID ESTERS

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Keywords: site-directed mutagenesis, lipase, *Yarrowia lipolytica*, enantioselectivity, 2-substituted carboxylic acid esters

A new extracellular lipase from the yeast *Yarrowia lipolytica* was recently demonstrated as efficient for resolution of 2-bromo phenyl and tolyl acetic acid esters (intermediates in the synthetic pathways of drugs such as prostaglandin, prostacyclin, semi-synthetic penicillin, thiazolium salts, etc.) and resolution of nonsteroidal anti-inflammatory drugs (ibuprofen, ketoprofen, naproxen) (see **P047**).

The objective of this work was to improve the lipase enantioselectivity by site-directed-mutagenesis. An original approach was used because of the non availability of the 3D-structure. By homology modelling (primary and secondary structure) with *Rhizomucor miehei* lipase (identity 32 %) the 3D-structure of the lipase from *Y. lipolytica* was determined *in-silico*. Four potential targets for mutagenesis were identified using this structural model. Twenty five variants of the protein were constructed containing one, two or three amino acid changes. An original reaction medium was developed in order to easily and rapidly quantify enantioselectivity from an Erlenmeyer culture of a *Y. lipolytica* strain containing only one copy of the variant lipase gene in its genome.

Enantioselectivity was increased 12 times (E = 48) with only one amino acid change for resolution of the 2-bromophenylacetic acid ethyl ester, and 5 times (E = 40) with two amino acid changes for resolution of 2-bromo-*o*-tolylacetic acid ethyl ester. Surprisingly, this improvement in enantioselectivity was accompanied by an tremendous increase in activity (fourteen and one hundred times respectively for the two substrates). One mono mutant enabled enantioselectivity to be reversed for both substrates.

A molecular model, based on accurate kinetic studies, is proposed to explain the observed enantioselectivity and activity gains.

P049 MOLECULAR MODELING STUDIES ON THE ENANTIOSELECTIVE ESTERIFICATION OF (+/-)-PERILLYL ALCOHOL CATALYSED BY LIPASES

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Keywords: lipase, enantioselectivity, structure-function relationship, molecular dynamics simulation

The use of biocatalysis by employing hydrolytic enzymes such as lipases, esterases and proteases in non-aqueous media for the synthesis of compounds of biological interest has gained particular interest during the last years mainly because of the advantages they present over the use in aqueous solutions. One of the most important properties of enzymes is the enantioselectivity they present towards a variety of substrates. In this work several lipases have been tested as far as their catalytic efficiency is concerned in the esterification of the two enantiomeric forms of perillyl alcohol, *(S)*-(–) and *(R)*-(+) and the findings showed a different preference of the enzymes towards the two enantiomers.

In an attempt to explain the molecular basis of the enantioselectivity, we have initiated molecular dynamics simulation by using the program CNS based on the known X-ray crystal structures of the lipases used.

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P050 ENZYME STORAGE STABILITY IN ORGANIC SOLVENTS

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Keywords: stability, lipase, stability, organic solvents, transesterification

This laboratory has recently reported the low storage stability of different preparations of subtilisin Carlsberg suspended in several organic solvents[1]. It was also reported that the highly efficient co-lyophilized preparation with methyl-beta cyclodextrin[2,3] losses its activity most rapidly (as compared to the CLEC and lyophilized powder). Curiously, the enantioselectivity of all preparations is conserved during the long incubation period (7 days).

To explain the reasons for these observations, we decided to study the stability of a very different enzyme: a lipase. The data to be presented compares the above mentioned results with the storage stability of different preparations (colyophilized with MßCD, CLEC and lyophilized) of a lipase, measured in different organic solvents. The substrates used for these transesterification reactions were *sec*-phenetyl and benzyl alcohols, and vinyl butyrate. Our results show that under certain conditions, and for some preparations, the lipase is very stable, and actually in some cases its activity increases after the first day of incubation in one organic solvent. An explanation for these observations will be presented.

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P051 CHARACTERIZATION OF ENRICHED LIPOXYGENASE EXTRACT FROM *Aspergillus niger* IN TERMS OF ACTIVITY AND SPECIFICITY

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Keywords: lipoxygenase, *Aspergillus niger*, activity, specificity, flavor precursors

Lipoxygenases (EC 1.13.11.12) are distributed widely in nature and are responsible for the enzymatic conversion of 1*(Z)*,4*(Z)*-pentadiene containing PUFAs into stereospecific hydroperoxide isomer(s), by antarafacial insertion of molecular oxygen at the methylene carbon[1]. However, there has been a paucity of studies investigating the role of this dioxygenase in microorganisms[2]. LOX has been implicated in the biogeneration of volatile desirable and undesirable flavor compounds in foods, stemming from an initial oxidation of polyunsaturated fatty acid[3]. There is an ongoing research work in our laboratory aimed at the biotechnological applications of microbial enzymes for the production of natural flavor precursors and flavor compounds from lipids[4].

The mold *Aspergillus niger* was grown and cultivated on day 6 which corresponded to its maximal dry biomass and lipoxygenase activity. Mycelia were washed of the growing media, and pH adjusted with sodium phosphate buffer. Lipoxygenase (LOX) enzymatic extract was recovered and partially purified by ammonium sulfate precipitation. Enzymatic fraction precipitated by ammonium sulfate at 30–70 % of saturation showed the highest LOX activity, with a purification fold of 2.2. The enriched enzymatic fraction was assayed for its LOX activity using selected polyunsaturated fatty acids (PUFAs) as substrates, including linoleic, linolenic and, arachidonic acids. Two pH optima were determined, in the acidic range (pH 5.0) and in the basic range (pH 10.5). The K_m and V_{max} values indicated that LOX from *A. niger* displayed preferential activities toward linolenic and linoleic acids at low and high pH, respectively. Using high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analyses, the amounts of hydroperoxides produced were separated and quantified. Substrate specificity of the microbial LOX demonstrated a preferential affinity for the free linoleic acid over its mono-, di- and triacylglycerol esters. It was also shown that the LOX activity of *A. niger* produced all positional isomers (monohydroperoxy regioisomers) of the assayed PUFAs. In addition, there was a predominance of conjugated diene

hydroperoxides, with approximately 60 % of end products. Significant production of the unconjugated 10-hydroperoxide of linoleic and linolenic acids was demonstrated for this enriched LOX extract, ranging from 15 to 21 % of total hydroperoxide produced, with a greater proportion attributed to the more saturated fatty acid among the assayed PUFAs.

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P052 SELECTIVE ENZYMATIC ACYLATION OF *N*-ACETYLHEXOSAMINES AND THEIR DERIVATIVES

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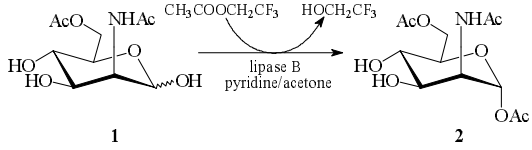
Keywords: enzymatic acylation, lipase, 1-*O*-acetates

Selective chemical modifications of carbohydrates always involve multistep reaction sequences, and despite high yield in each reaction step (e. g. 90 %), the overall yield is usually quite low (e. g. after 5 steps the total yield is only 59 %), regardless labour and material costs. Therefore, one-step selective modification, such as enzymatic acylation, is always advantageous.

The goal of our project was to acylate selectively *N*-acetylhexosamines using enzymes. For this aim we tested lipases (lipase PS from *Burkholderia cepacia*, lipase B from *Candida antarctica*, porcine pancreatic lipase) and protease subtilisin from *Bacillus licheniformis*. Acetylation and butyrylation of GalNAc, ManNAc and *p*-NP-*β*-GalNAc were carried out. Acylation of GalNAc with subtilisin yielded 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose and interestingly also its furanose form, i. e. 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose, which were obtained in a pure and stable form. Acylation of *p*-NP-*β*-GalNAc with lipase B yielded selectively *p*-nitrophenyl 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranoside. Acylation of ManNAc was considerably less feasible than that of GalNAc. ManNAc could be acylated exclusively by subtilisin to afford 2-acetamido-6-*O*-acyl-2-deoxy-D-mannopyranose. However, butyrylation with the same enzyme (trichloroethyl butyrate as donor) proceeded further to 2-acetamido-3,6-di-*O*-butyryl-2-deoxy-D-mannopyranose. 2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose **(1)** could be further acylated also by other enzymes, e. g. lipase B, yielding a quite unusual anomeric diacylderivative 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-a-D-mannopyranoside **(2)**. 2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose treated with lipase B gave 2-acetamido-4,6-di-*O*-butyryl-2-deoxy-D-mannopyranose.



1 **2**

Support by the COST D25/0001/02, Ministry of Education of the Czech Republic OC D 25.002, by the grant 203/01/1018 from the Grant Agency of the Czech Republic and the bilateral project between CNR and Academy of Sciences of the Czech Republic is acknowledged.

P053 NEW TRANSGLYCOSYLATION REACTIONS WITH MODIFIED SUBSTRATES CATALYSED BY β-*N*-ACETYLHEXOSAMINIDASES

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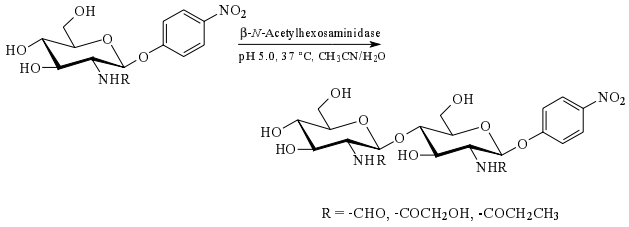
Keywords: β-*N*-acetylhexosaminidase, modified substrates, transglycosylation

There have been several hints in the literature suggesting that glycosidases exhibit a broad substrate specificity, which enables them to cleave and even to transfer modified glycosidic moieties yielding oligosaccharides[1–3]. In our project we have studied five *N*-acyl modified glycosides considering their ability to serve as glycosyl donors for fungal β-*N*-acetylhexosaminidases from our library.

The *p*-nitrophenyl glycosides bearing different substituents at the 2-C amino group instead of the common acetyl (formyl, glycoloyl, propionyl, trifluoroacetyl and hydrogen)

were tested as substrates for 32 β-*N*-acetylhexosaminidases belonging to the genera of *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces*. The enzymes were considered regarding the following criteria: hydrolysis and transglycosylation potential, yield of transglycosylation products and selectivity and stability in acetonitrile, used as a cosolvent due to the limited solubility of substrates. On the basis of the screening, β-*N*-acetylhexosaminidases from *A. oryzae*, *P. oxalicum* and *Talaromyces flavus* were selected for the catalysis of semi-preparative transglycosylations, which gave yield to *p*-nitrophenyl oligosaccharides. This rather novel concept widely extends the synthetic capability of glycosidases.



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P054 *N*-ACETYLMANNOSAMINE CONTAINING SACCHARIDES: PRODUCTION USING β-*N*-ACETYLHEXOSAMINIDASES AND THEIR SEPARATION BY ION-EXCHANGE/EXCLUSION CHROMATOGRAPHY

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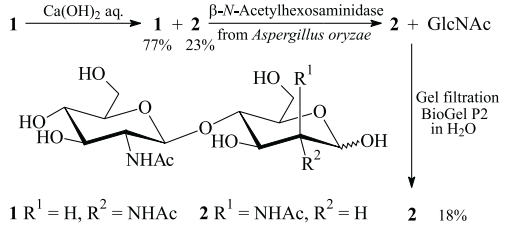
Keywords: acetamidosugars, alkali catalysed epimerization, *Aspergillus oryzae*, separation

Derivatives of 2-acetamido-2-deoxy-D-mannopyranose (ManNAc) are quite rare in nature, however they are rather important. β-ManNAc occurs in some surface structures of bacteria, e. g., those attaching teichoic acid. Occurrence of ManNAc is often linked to virulence and evading from the immunity surveillance. ManNAc and its derivatives were identified to be strong activators of the natural killer cells (NK cells)[1]. Preparation of the ManNAc derivatives, especially of those with β-configuration is one of the greatest challenge of the carbohydrate synthetic chemistry.

GlcNAcβ(1→4)ManNAc **(2)** was prepared from chitobiose **(1)** by 2-epimerization under catalysis of $Ca(OH)_2$. Resulting mixture is hardly separable only by analytical HPLC. Chitobiose in reaction mixture was selectively removed by hydrolysis β-*N*-acetylhexosaminidase from *Aspergillus oryzae*; GlcNAcb(1→4)ManNAc **(2)** is resistant to the enzyme hydrolysis. Resulting disaccharide can be then easily isolated by gel filtration. The enzyme was cloned and the molecular modelling explains the mode of discrimination of these two saccharides.



New, original and more effective methodology based on separation of the GlcNAc and ManNAc derivatives in borate buffer on the various gel materials (Sephadex, Toypearl, Cellufine) was developed. This method enables effective large-scale production of rather rare oligosaccharides containing ManNAc with important immunoactivity. This method has been extended to all common aminosugars (GlcNAc, GalNAc, ManNAc, TalNAc and their di- and trisaccharides). Analogous disaccharide as GalNAcβ(1→4)ManNAc and trisaccharides GlcNAcβ(1→4)GlcNAcβ(1→4)ManNAc and GalNAcβ(1→4)GlcNAcβ(1→4)ManNAc were prepared by the above methodologies as well.

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