THESIS ON NATURAL AND EXACT SCIENCES B75

Stereoselective chemoenzymatic synthesis of deoxy sugar esters involving *Candida antarctica* lipase B

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Defence of the thesis: July 4, 2008

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Ly Villo

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Desoksüsuhkru estrite stereoselektiivne kemoensümaatiline süntees kasutades *Candida antarctica* lipaasi B

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CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numbers.

I Villo, L.; Metsala, A.; Parve, O.; Pehk, T. Chemical versus enzymatic acetylation of α-bromo-ω-hydroxyaldehydes: decyclization of hemiacetals by lipase. *Tetrahedron Letters*, **2002**, 43, 3203-3207.

II Ruiz, C.; Falcocchio, S.; Xoxi, E.; Villo, L.; Nicolosi, G.; Pastor, F. I. J.; Diaz, P.; Saso, L. Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Journal of Molecular Catalysis B: Enzymatic*, **2006**, 40, 138-143.

III Villo, L.; Danilas, K.; Metsala, A.; Kreen, M.; Vallikivi, I.; Vija, S.; Pehk, T.; Saso, L.; Parve, O. Synthesis of Deoxy Sugar Esters: A Chemoenzymatic Stereoselective Approach Affording Deoxy Sugar Derivatives Also in the Form of Aldehyde. *Journal of Organic Chemistry*, **2007**, 72, 5813-5816.

IV Villo, L.; Xoxi, E.; Ruiz, C.; Parve, O.; Saso, L. Influence of Prostaglandin Derivates on the Catalytic Activity of *Candida rugosa* Lipase. Manuscript.

INTRODUCTION

Sugars are the most abundant group of natural products. In living organisms they have several roles, such as the storage and transport of energy and as structural elements of many compounds. Deoxy sugars and their derivatives occur very widely in natural products. They can be used in therapy as antibiotics, antitumor agents, *etc*. but also in food and cosmetic industries. In addition, sugar derivatives like glycoproteins, glycolipids, glycosteroids, are involved in several key-processes taking place in living organisms, for instance in intercellular interaction and recognition processes. The investigation of glycoconjugates and their reactions could contribute to a better understanding of the processes taking place in Nature.

Besides deoxy sugars and their derivatives, an enzyme called lipase was subjected to study. Lipases hydrolyze triglycerides into fatty acids and glycerol. They can be found in animal tissues, plants, fungi and bacteria. Lipases are used in biotechnology, for example in food and cosmetic industries. They are also useful in organic synthesis because of their ability to retain the catalytic activity in organic media. In asymmetric synthesis lipases have still been mainly used for the resolution of stereoisomers.

Lipases can be also considered as potential targets for chemical compounds. These enzymes are involved in some serious diseases such as type 2 diabetes, obesity and arteriosclerosis. Pathogenic microorganisms like *Helicobacter pylori* (induces ulcer) and *Propionibacterium acnes* (causes acne) produce lipases that help them in colonization. Inhibition of lipases could help to prevent or treat lipase-related diseases.

The main goal of the present work was the development of a novel approach for the synthesis of deoxy sugar esters that could have an influence on the catalytic activity of lipases, especially as potential inhibitors.

In the initial stage of the work, synthesis of precursors of deoxy sugar derivatives with both the pyranose and furanose rings by chemoenzymatic method was carried out. The chemoenzymatic synthesis of deoxy sugar esters was performed to obtain stereochemically pure products. To this effect a lipase was used for the resolution of the stereoisomers formed.

As mentioned above, the goal of the research was to synthesize potential lipase inhibitors. The development of new inhibitors was based on the assumption that the molecule should consist of two components – a chemically reactive part, that reacts with an active site of a lipase, and a “carrier molecule”, that mimics the specificity determining parts of a substrate and provides correct position orientation for the reactive part in the active site of the enzyme. Deoxy sugar moiety was considered as putative reactive part. The efficiency of several compounds belonging to different types as candidates for the carrier molecule was tested.

ABBREVIATIONS

BEH	bacterial epoxide hydrolase
CA	cholic acid
CALB	*Candida antarctica* lipase B
CRL	*Candida rugosa* lipase
DBDMH	1,3-dibromo-5,5-dimethylhydantoin
DCA	deoxycholic acid
DHF	2,3-dihydrofuran
DHP	3,4-dihydro-2*H*-pyran
DIPEA	*N*,*N*-diisopropyl-ethylamine
DKR	dynamic kinetic resolution
DOS	deoxy sugar
HLL	*Humicola* (*Thermomyces*) *lanuginosa* lipase
HPL	human pancreatic lipase
HSL	hormone-sensitive lipase
HSV	Herpes simplex virus
MA	mandelic acid
MPA	methoxyphenylacetic acid
β-NL	*β*-naphthyl laurate
p-NP	*p*-nitrophenol
p-NPL	*p*-nitrophenyl laurate
OA	(*E*)-2-octenoic acid
PE	petrol ether
PG	prostaglandin
PLA_2	phospholipase A_2
QC	quantum chemical
THF	tetrahydrofuran
THP	tetrahydropyran
THPMA	THP-protected mandelic acid
p-TSOH	*p*-toluenesulfonic acid

1. REVIEW OF THE LITERATURE

In this chapter a short overview of the most intriguing investigations carried out in the fields of the current work and also in closely related fields will be presented.

The first part of the chapter will briefly characterize deoxy sugars (DOS) and their derivatives. The synthesis of DOS and their derivatives, as well as of α-bromoaldehydes and *O*-alkylation of carboxylic acids will be touched.

Next part will treat of lipase-catalyzed reactions. Important issues such as the mechanism of the reactions; use of lipases for the resolution of stereoisomers and interactions of the lipase with carboxylic acids, *viz*. bile acids, in particular deoxycholic acid, prostaglandins and sugars will be dealt with. Also some examples of cascade reactions triggered by lipase will be presented.

The importance of lipase inhibitors will be treated of in the final section. Also the structures of some antilipolytic compounds and a short description of the methods for measuring the change in enzyme activity will be presented.

1.1. Deoxy sugars and their derivatives

Sugars are the most abundant group of natural products. They are prime biological substances and major constituents of shells as well as the supporting tissue of plants. Sugars are present as parts of cell walls and are an important source of energy.[1,2]

Several DOS and their derivatives can be found in many natural products. Dideoxy and trideoxy sugars such as 3,6-dideoxyhexoses are components of the antigenic determinants contained in bacterial lipopolysaccharides. 2,6-Dideoxy hexoses are present in many steroidal glycosides, antibiotics and antitumor compounds.[3] Deoxy sugars are also frequently found in many bioactive compounds either as single structural elements or as part of oligosaccharide moieties in glycoconjugates. The glycoconjugates composed of a deoxy sugar moiety and an aglycon are of high clinical importance. The alterations of the saccharide structures may improve biological activity, in particular, against drug-resistant microorganisms.[4]

In organic chemistry carbohydrates and their derivatives have been used as chiral auxiliaries.[5,6] The functionalized tetrahydrofurans and tetrahydropyrans, which represent the simple parent ring systems for carbohydrate furanosides and pyranosides,[5] are embedded in many biologically important natural products[7] and are also successfully used as chiral auxiliaries.[8, 9]

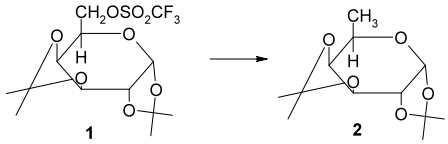
1.1.1. Synthesis of deoxy sugars

There are several review articles on the synthesis of deoxy sugars. The one by Lederkremer and Marino[3] was published in 2007 and that of Kirschning *et al.*[10] in 2001, to name just the most recent.

In his work Kirschning *et al.* have described five main strategies for the synthesis of DOS:

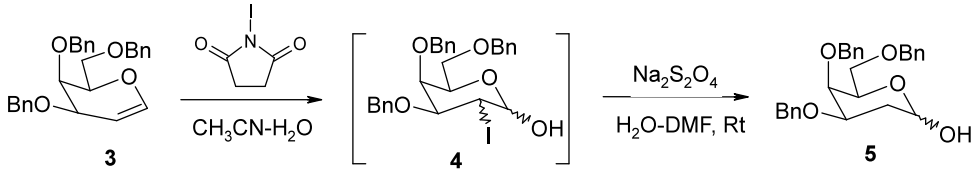
1. a "classical" ex-chiral-pool strategy (from amino and hydroxy acids)
2. an asymmetric oxidation (epoxidation, dihydroxylation)
3. cycloaddition
4. an organometallic-based ring-closing technique
5. biotransformation

Other methods such as a radical aldehyde cyclization[11]; and deoxygenation[12]; have been also published. The reduction of the triflate of galactopyranose **1** gave 6-deoxy sugar **2** (Scheme 1).[13]



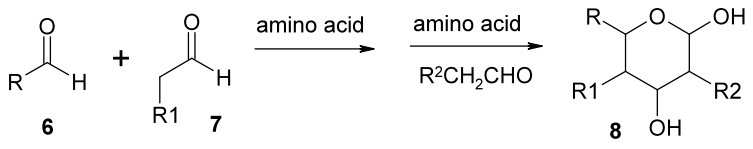
Scheme 1. Reduction of the triflate group of galactopyranose

Glycals[4] can be converted into the corresponding deoxy sugars. Glycal **3** has been converted into the corresponding derivative of 2-deoxysugar **5** by treatment with *N*-iodosuccinimide and removal of the iodide group (Scheme 2).[14]



Scheme 2. Synthesis of DOS from glycals

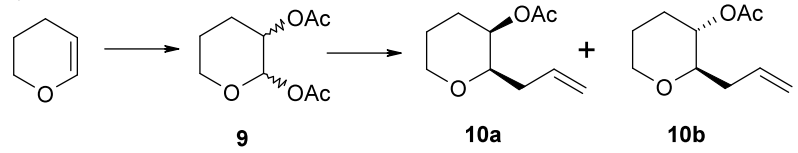
Cordova *et al.* have established the ability of amino acid to catalyze a sequential cross-aldol reaction to form L- and D-hexoses and their analogues (Scheme 3).[15]



Scheme 3. Organocatalytic synthesis of DOS

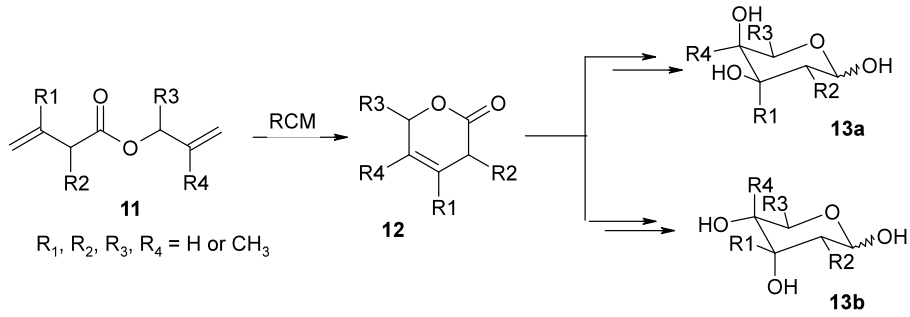
According to Mash,[5] tetrahydrofuran (THF) and tetrahydropyran (THP) are considered as base structures of furanosides and pyranosides. For this reason also strategies for the synthesis of functionalized pyrans (deoxy pyranoses) and furans (deoxy furanoses) are considered.[16, 17, 18]

The hydroxylation of dihydropyran followed by the formation of acetate and "anomeric allylation" gives a 2.4:l mixture of acetoxypyrans **10a** and **10b** (Scheme 4).[19]



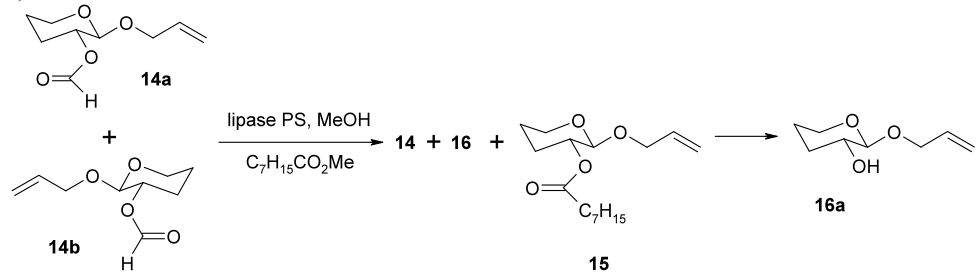
Scheme 4. Synthesis of acetoxypyrans

Ruthenium-catalyst complexes have been successfully utilized in the construction of *β,γ*-unsaturated *δ*-lactones (**12**). The asymmetric dihydroxylation followed by the reduction leads to 3,4-*cis*-dihydroxy-2,6-dideoxypyranoses (**13**) (Scheme 5).[20]



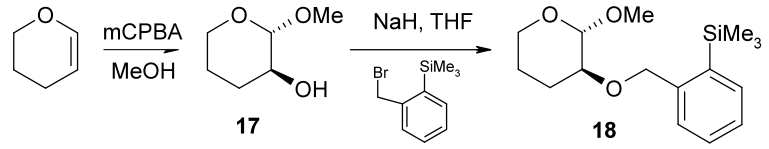
Scheme 5. Dideoxypyranoses from β,γ-unsaturated δ-lactones

Preparing both enantiomers of (2*R*,3*S*)-2-allyloxy-3,4,5,6-tetrahydro-*2H*-pyran-3-ol (**16a**), a precursor of Charette's chiral auxiliary,[8] *Pseudomonas* Amano PS lipase catalyzed the transesterification the best way in a preparative manner. (Scheme 6).[21]



Scheme 6. Synthesis of chiral auxiliaries

The epoxidation of dihydropyran in MeOH has been used for the synthesis of hydroxy-methoxy-THP (**17**),[22] followed by benzylation of alcohol **17** with the requisite bromide which yields methyl dideoxy pyranoside **18** (Scheme 7).[23]



Scheme 7. Synthesis of methyl dideoxy pyranoside

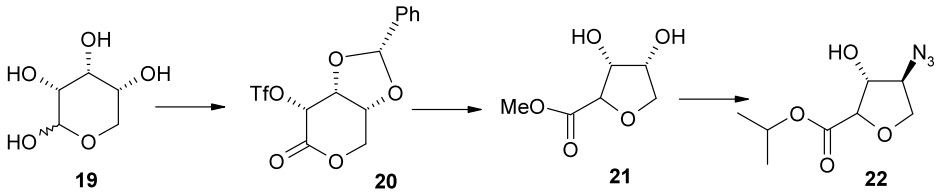
Dideoxy sugars such as 2,3-, 2,4- and 3,4-dideoxy sugars have not been found in natural products. Some of them have been synthesized to study of structure-activity relationships.[3] They exhibit antiviral activity and have been intermediates for the synthesis of other compounds. However, in the literature examples of the synthesis of these dideoxy sugars are quite few.[4]
As the majority of deoxy monosaccharides are not readily available from natural sources, new synthetic pathways are always welcome.

1.1.2. Synthesis of sugar derivatives – esters, glycosides, nucleosides

The amino acyl esters of carbohydrates are used as sweetening agents, surfactants, in the preparation of microcapsules of pharmaceuticals, active nucleoside amino acid esters, and as antibiotics as well as in the delivery of biologically active agents.[24]

The fatty acid esters of sugars and related compounds have been used as low calorific sweeteners and biosurfactants in the food, pharmaceutical and cosmetic industries. Recently, some of the fatty acid esters have shown antitumor activity, inhibiting activity towards some transferases, as well as plant growth inhibiting and antibiotic activities.[25,26]

The synthesis of sugar esters is predominantly carried out by the enzyme-catalyzed acylation.[27,28] In this work only some methods of chemical synthesis will be discussed. The lipase-catalyzed acylation of sugars will be reviewed in section 1.4.8.



Scheme 8. Synthesis of tetrahydrofuran-based γ-azido esters

The synthesis of sugar esters was carried out under the Mitsunobu conditions.[26] The tetrahydrofuran-based γ-azido esters (**22**) were obtained by the ring contraction

of the suitably protected 2-*O*-triflates of pentono-1,5-lactones (D-ribose (**19**) and L-arabinose) with the subsequent introduction of the azide via 4-*O*-triflate (Scheme 8).[29]

For the synthesis of glycoside several methods have been developed.[30] The oldest method is Koenigs-Knorr reaction in the course of which glycosides are obtained from glycosyl bromides with the help of silver salts.[1]

Glycoglycerolipids (the derivatives of 2-deoxy sugars having one or two fatty acid chains) have been synthesized by the catalyzed addition of the glycerol moiety to glycal.[31] The $SnCl_4$-catalyzed coupling of silyl ethers of the desired aglycon residue with the 1,6-lactone derivative of glucuronic acid provides different glucuronides.[32]

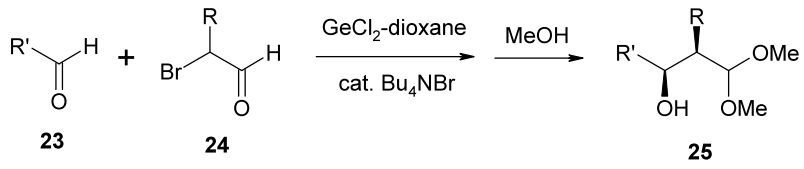
N-glycosides as nucleosides have an important part to play in the DNA and RNA. The analogues of nucleosides find application as anti-viral (HIV, HSV) compounds in medicine. The synthesis of different N-glycosides, in particular nucleosides has been described in several publications.[33, 34]

1.2. Synthesis and importance of α-bromoaldehydes

α-Bromoaldehydes, important organic intermediates used in the pharmaceutical and fine chemical industries, are usually synthesized by the direct bromination of aldehydes or bromination of the dimethylacetal of aldehyde followed by hydrolysis of the acetal.[35] For the α-bromination of aldehyde 5,5-dibromobarbituric acid has also been used.[36]

α-Bromoaldehydes are often used as intermediates for the synthesis of several important compounds like pyrrolo-[2,3-*d*]pyrimidine-based antitumor agents,[36,37] functionalized vinylepoxides,[38,39] adenosine 3′,5′-cyclic phosphate analogs[40] and cyclooxygenase inhibitors[41].

The reaction of α-bromoaldehyde **24** with aldehyde **23** in the presence of $GeCl_2$-dioxane gave the *syn*-selective cross-aldol equivalent **25** (Scheme 9).[42]



23 **24** **25**

Scheme 9. The reaction of α-bromoaldehyde with aldehyde

1.3. *O*-alkylation of carboxylic acids

O-alkylation is an important method in the preparation of esters.

The derivatization of carboxylic acids in the presence of tertiary amine (*N,N*-diisopropyl-ethylamine (DIPEA)) with *p*-bromophenacyl bromide as chromophore

has been used for the quantitative chromatographic determination of the composition of fatty acid mixture.[43]

Recently the microwave-assisted *O*-alkylation of carboxylic acids with (un)substituted ω-haloacetophenones has been described.[44] The polymer-supported reagents were used to transform carboxylic acids into the corresponding methyl, benzyl, allyl, and *p*-nitrobenzyl esters.[45] The cesium carbonate mediated *O*-alkylation of carboxylic acids was efficiently carried out under mild *in situ* conditions to give exclusively the corresponding esters.[46]

1.4. Lipase-catalyzed reactions

1.4.1. Lipases; *Candida antarctica* lipase B

Lipases are enzymes that hydrolyze triglycerides into fatty acids and glycerol. They belong to the family of serine hydrolases and they are produced by animal tissues, plants, fungi and bacteria.[47,48]

Lipases are used in detergents and as bleachers in paper and textile industries, as well as in the food and cosmetic industries and in organic synthesis. In biotechnology lipases play an important role – ca 35% of biotransformations is accounted for by lipases. Of natural lipases, those contained in human, pig and horse pancreas; as well as in several microorganisms (like bacteria and fungi) have been studied most thoroughly.

In human organism lipases can be found for example in gastro-intestinal system – lingual lipase, gastric lipase, pancreatic lipase and different phospholipases. Lipoprotein lipase, hormone sensitive lipase and endothelial lipase are present in vascular system. In human organism lipases perform several functions and are involved in some diseases such as obesity and type 2 diabetes. The lipases excreted by pathogenic microorganisms help their colonization and therefore cause severe inflammations. For example, *Helicobacter pylori* induces ulcer and *Propionibacterium acnes* lipase produce fatty acids thus causing inflammation on skin. This makes lipases a potential target in drug-discovery.

In organic synthesis lipases are especially important because they hydrolyze and synthesize also other esters (artificial substrates), besides glycerides. They retain catalytic activity in media of low water content (organic solvents, CO_2 under super-critical conditions, ionic liquids). Since lipases from bacteria and fungi do not require cofactors they can be used in either free or immobilized form. They are readily available, inexpensive, highly stable, and accept a broad range of substrate structures, retaining at the same time a high stereoselectivity.

One of the most versatile lipases is obtained from the *basidomycetous* yeast *Candida antarctica*. The yeast was found in the Antarctica. It produces two isoenzymes – A and B which differ in some extent. Lipase A is more termostable and Ca^{2+}-dependent; B is less termostable and metal-dependent. Also their substrate-specificity is different. For example, lipase B is much more active towards unnatural esters than lipase A.

In this work, *Candida antarctica* lipase B (CALB) was used. It is produced by using a recombinant DNA technology. The enzyme is attached (immobilized) onto the solid carrier, acryl polymer resin. It tolerates well even temperatures as high as 80°C but the optimum temperature is 40-60°C and it is possible to work with CALB in a solvent-free environment and also in inert solvents as hexane.

Candida antarctica lipases have neither the effect of interfacial activation nor the amphiphilic „lid" like most known lipases do. This could be the explanation why the selectivity of CALB is quite well predicted by computer modelling.

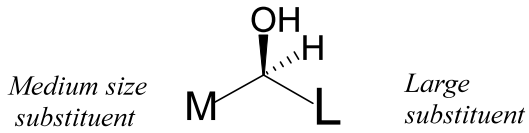


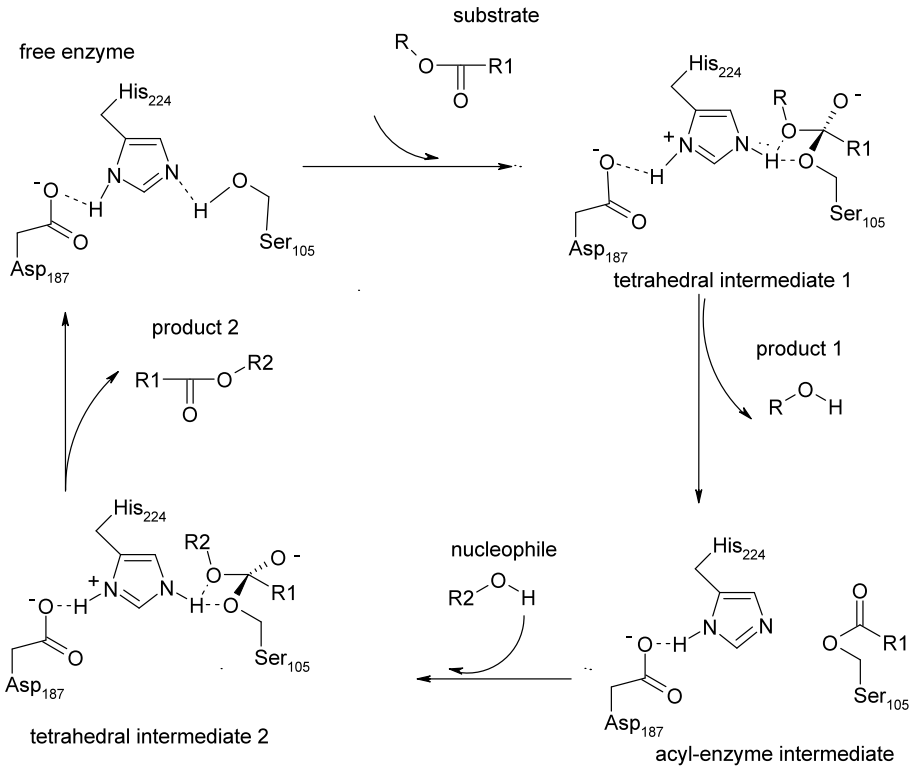
Figure 1. The Kazlauskas rule for the secondary alcohol nucleophiles – the configuration of the model enantiomer preferred by lipase

To the majority of nucleophiles (Figure 1).and substrates the Kazlauskas rule may be applied. This rule describes the enantiopreference of lipase towards the configuration of substrates and nucleophiles.[49]

1.4.2. Mechanism of the CALB-catalyzed acyl transfer

CALB is a serine hydrolase and in its active centre there is a catalytic triad – Ser-His-Asp. The mechanism of CALB-catalyzed reaction of acyl transfer can be described as belonging to a "bi-bi ping-pong" type (Scheme 10).[50] "Bi-bi ping-pong" means that two substrates are entering the reaction and two products are leaving it; the first product leaves before the second substrate binds on the enzyme.

Acylation begins when the substrate (acyl donor) binds on the enzyme by a nucleophilic attack of the serine γOH group on the carbonyl group of the substrate 1. His224 initiates the base-catalyzed withdrawal of a proton from serine and the first tetrahedral intermediate is formed. At the same time the charge is redistributed and the positively charged histidine residue is stabilized by Asp187. The oxyanion is stabilized by three hydrogen bonds (this so-called "oxyanion hole" is not shown in the Scheme) which are formed by Gln106 and Thr40. Then His224 initiates the breaking of the tetrahedral intermediate by acid catalysis (donates a proton to the leaving group of the substrate), product 1 is released and an acyl-enzyme intermediate is formed. After that the deacylation process begins which is reversed to the acylation of the enzyme. The nucleophile attacking is activated by the His224 base-catalysis while the carbonyl group of the acyl bound by the enzyme is polarized by the "oxy-anion hole" and tetrahedral intermediate 2 is formed. Due to the protonation of Ser105 γO this intermediate is broken down, product 2 is released from the enzyme and a new cycle may begin.



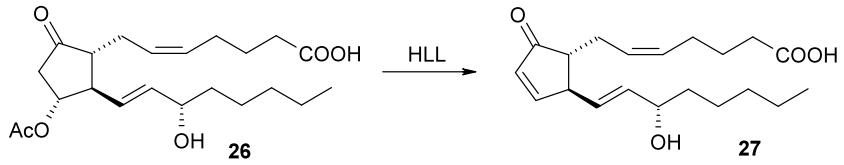
Scheme 10. Mechanism of the CALB-catalyzed reaction of acyl transfer[51]

1.4.3. Lipase-catalyzed formation of the C-C bond, racemization and elimination reactions

In addition to acyl transfer reactions characteristic to lipases (hydrolysis, ester synthesis, inter-esterification, amidation), which are common in organic synthesis, also some unusual reactions catalyzed by the lipases have been reported.

Lipases have been observed to catalyze the formation of the carbon-carbon bond via an aldol-type condensation.[52] A mutant of CALB catalyzes the Michael addition of 1,3-dicarbonyls to α,β-unsaturated carbonyl compounds.[53]

Elimination is an unusual reaction for lipases, but in our group it has been described in the case of prostaglandin (PG) derivatives.[54] The incubation of 11-acetyl-PGE_2 (**26**) with *Humicola* (*Thermomyces*) *lanuginosa* lipase (HLL) in methanol leads exclusively to PGA_2 (**27**) via the elimination of acetic acid from the acetyl-β-ketol moiety. The treatment of 11-acyetyl-PGE_1 under identical conditions afforded no elimination product. This confirmed the catalytic role of lipase in elimination. The reaction mechanism has also been proposed.



Scheme 11. Lipase-catalyzed elimination reaction with prostaglandins

A unique CALB-catalyzed racemization reaction along with proposed reaction mechanism has been described by Sheldon and co-workers. The acylation of a racemic aminonitrile with ethyl acetate produced almost a racemic mixture of the product but the unconverted substrate showed a considerable *e.e.* indicating that the product must be racemized.[55]

1.4.4. Selectivities of lipase-catalyzed reactions

In enzyme-catalyzed reactions different kind of selectivities can be achieved. Below some of them will be briefly characterized:

substrate selectivity – the ability of the enzyme to distinguish and act on a subset of compounds within a larger group of chemically related compounds;

functional group selectivity – the ability of the enzyme to act on one functional group selectively in the presence of other equally reactive or more reactive functional groups in the molecule;

stereoselectivity – the ability of the enzyme to act on a single enantiomer or diastereomer selectively – this is the most important feature of lipase-catalysis allowing kinetic resolution of enantiomers and diastereomers;

regioselectivity – the ability of the enzyme to act on one location in a molecule selectively allowing regioselective protection and/or deprotection of complex molecules – polyols, etc.;

chemoselectivity – the ability of the enzyme to produce a certain product selectively upon acting on a functional group; in some cases more than one chemically different alternative product can be formed upon acting on one certain functional group (for example, hemiacetal ester versus hydroxyaldehyde ester).[56]

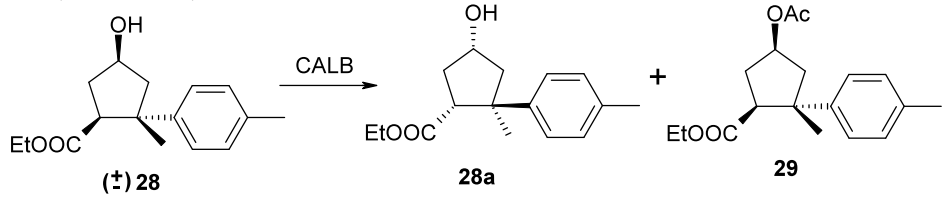
1.4.5. Lipase-catalyzed kinetic resolution of stereoisomers

In organic synthesis a single enantiomer is mostly obtained by the resolution of racemates. The approaches to the resolution of enantiomers can be divided into four types: direct preferential crystallization, crystallization of diastereomeric salts or other derivatives, chromatography of diastereomeric derivatives or enantiomers, and kinetic resolution. The lipase-catalyzed kinetic resolution is one of the most attractive techniques used to obtain enantiomerically pure compounds, transesterification in organic solvents being the most dominant.

Lipases have been used to catalyze the resolution of racemic hydroxy-compounds such as hydroxy phenyl sulfones,[57] hydroxy tellurides,[58] aryl β-hydroxy ketones,[59] phenyllactic acid and hydroxy-phenylbutanoic acid,[60] aryl hydroxybutanenitriles,[61] different diols,[62,63] flavanols,[64] etc.

The lipase-catalyzed acylation of the secondary alcohol group is by far the most popular method to resolve stereoisomers because stereoselectivity of lipases towards secondary alcohols is much higher than that towards primary or tertiary alcohols.

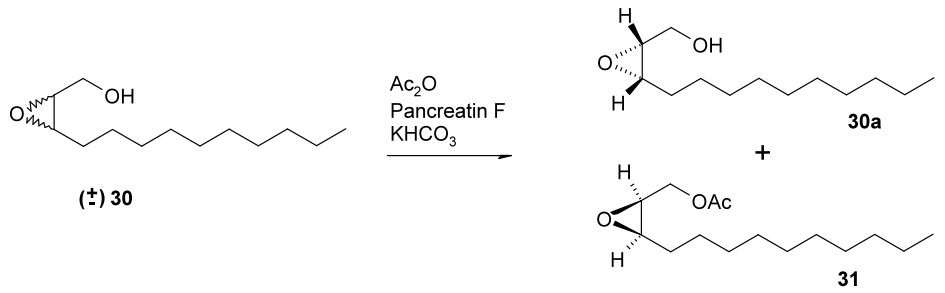
The enzymatic kinetic resolution of the hydroxy-cyclopentane carboxylate **28** was investigated by using the CALB-catalyzed transesterification of hydroxy carboxylate **28** with vinyl acetate affording both enantiomers in high enantiomeric excess (Scheme 12).[65]



Scheme 12. Lipase-catalyzed resolution of hydroxy-cyclopentane carboxylate

The effect of adjacent unsaturation on the enzyme-catalyzed kinetic resolution of secondary alcohols has been studied by Morgan *et al.*[66]

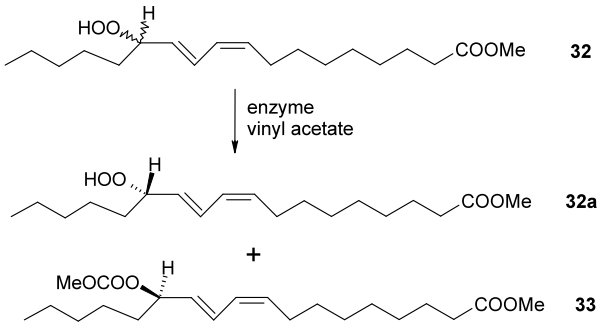
It is also possible to use the stereoselective acylation of the primary hydroxy group for kinetic resolution,[67,68,69] even though it is more difficult to achieve as lipase is less selective towards the primary hydroxyl group. For example, the acylation of epoxy-tridecanol **30** yielded (2R,3S)-2,3-epoxy-1-tridecanol (**30a**) as the remaining substrate with an optical purity of over 99% *e.e.* (Scheme 13).[70]



Scheme 13. Lipase-catalyzed resolution of epoxy-tridecanol

The lipase-catalyzed transesterification of glycerol derivatives enables the resolution of the corresponding stereoisomers.[71,72] Also carboxyl acid ester hydrolysis[73] and acetal hydrolysis[74] afford stereochemically pure compounds.

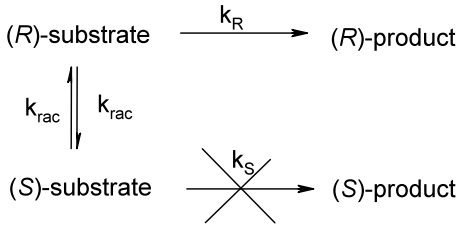
One interesting example is the lipase-catalyzed kinetic resolution of a racemic polyunsaturated hydroperoxy fatty acid (Scheme 14).[75]



Scheme 14. Lipase-catalyzed resolution of hydroperoxy fatty acid

Also nitrogen-containing functional groups – lactams,[76,77] secondary amines,[78] primary amines[79] and aminals[80] are resolved by lipase catalysis.

The conventional kinetic resolution often provides enantiomerically pure compounds. A drawback of this method is that the yield of the desired stereochemically pure product does not exceed 50%. This can be overcome by using a *dynamic kinetic resolution* (DKR) in the course of which the enantiomer (*R*)-substrate is transformed to the (*R*)-product faster than the enantiomer (*S*)-substrate (Scheme 15). The substrate is racemized *in situ* during the resolution process (or step-wise after separation). As a result, (*R*)- and (*S*)-substrates are in equilibrium, which will allow the conversion of almost all the starting material into the (*R*)-product that has to be stable under (*in situ*) racemizing conditions.[48,81]



Scheme 15. Dynamic kinetic resolution

For example, Bäckvall and co-workers have studied the chemoenzymatic DKR of *β*-hydroxy nitriles by using CALB and a ruthenium catalyst.[82]

1.4.6. Lipase catalyzed derivatization of prostaglandins

Natural PGs are short-lived local hormones (and metabolites) which are derived, by cyclooxygenase enzymes, from polyunsaturated fatty acids. PGs can be found in all

mammalian tissues. Several synthetic PGs are already used as medicines such as misoprostol, alprostadil, latanoprost, etc. In recent years much attention has been paid to the synthesis of new PG isomers and derivatives.[83] Just in a few publications lipase-catalyzed derivatization of prostanoids have been reported.

Taber *et al.* has used lipase for the resolution of the enantiomers of isoprostane derivatives regioselectively acetylating different hydroxyl groups in the molecule.[84,85]

Our research group has been interested in lipase–prostaglandin interactions for some time already. The lipase-catalyzed acetylation of cloprostenol (a veterinarian medicine), $PGF_{2\alpha}$,, PGE_1 and PGE_2 in organic media has been described for the first time.[54] The lipase-catalyzed (CALB) acetylation of the hydroxyl groups of five stereoisomeric PGs of type F and two of type E was thoroughly investigated using NMR and molecular dynamics simulations.[86,87] The calculation protocol allowing the distinction between the hydroxyl groups of PGs accessible and inaccessible to the lipase was presented.

1.4.7. Lipase catalyzed derivatization of steroids

Steroids are triterpenoids that are produced in plants and animal organisms. They are widespread in nature and perform several important biological functions. Steroids can be found in the same environment as lipases and, therefore, it is interesting to know what kind of reactions take place in organisms containing steroids and triglycerides.

Sterols (sitosterol, cholesterol, stigmasterol, ergosterol, 7-dehydrocholesterol) and sitostanol have been converted in high to near-quantitative yields to the corresponding long-chain acyl esters via ester synthesis or transesterification reactions using the lipase from *Candida rugosa* as biocatalyst.[88]

Using bile acid derivatives a 39-member library was prepared by Secundo *et al.*, starting from cholic acid methyl ester by employing a combinatorial biocatalytic approach. The investigators found that only the 3α-OH of the steroid can be enzymatically acylated.[89] Moreover, in many other articles it has been claimed that only the α-hydroxyl group at the 3rd position of the steroid skeleton of different steroids was acetylated by lipase.[90,91]

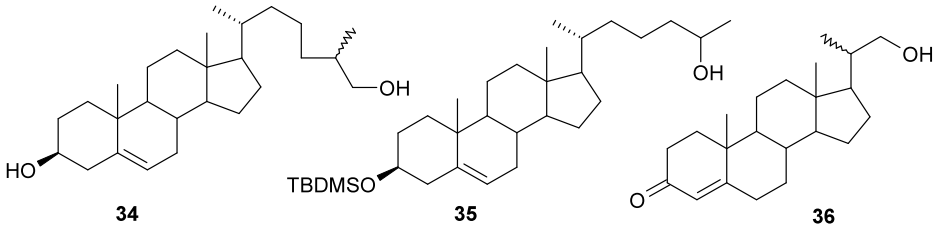


Figure 2. Lipase-catalyzed acetylation of hydroxyl groups in the side chain of steroid

In the side chains of steroid the lipase from *Pseudomonas cepacia* catalyzes the regio- and stereoselective acylation of primary and secondary hydroxy groups (Figure 2).[92]

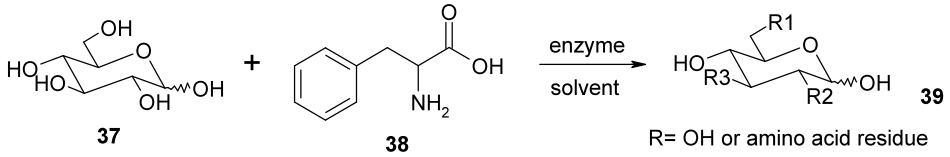
The lipase-catalyzed deacetylation has also been used for the synthesis of a series of acetoxy derivatives of androstane and pregnane in whose cases deacetylation is also possible in other positions than the 3rd one.[93]

1.4.8.Lipase catalyzed derivatization of sugars

The lipase-catalyzed derivatization of different sugars has been studied since the 80s when lipases became popular in organic synthesis enabling the selective modification of such polyhydroxy compounds.

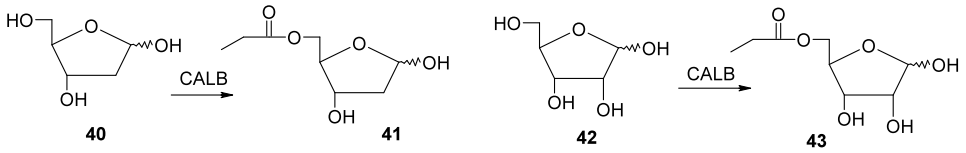
Most researchers engaged in the field have used mono- or disaccharides for the enzyme-catalyzed transformations[94,95] as tri- and polysaccharides produce too complex mixtures which are difficult to analyze. Enzymatic modifications have been performed using different lipases on the same sugar-compound as the enzymes display a notable selectivity towards different hydroxyl groups.[96] As acylation medium also ionic liquids have been used. In some cases this led to the increase of selectivity.[97,98] The lipase-catalyzed regioselective acylation has been used for the resolution of α- and β-anomers of glucopyranosides and galactopyranosides.[99]

The amino acid esters of sugars are used as antibiotics, detergents, sweetening agents, etc. Their regioselective synthesis with unprotected L-phenylalanine (**38**) and D-glucose (**37**) has been carried out in organic solvents by using lipases. Different mono- and diacylated sugars **39** were obtained (Scheme 16).[24,100]



Scheme 16. Synthesis of the amino acid esters of sugars

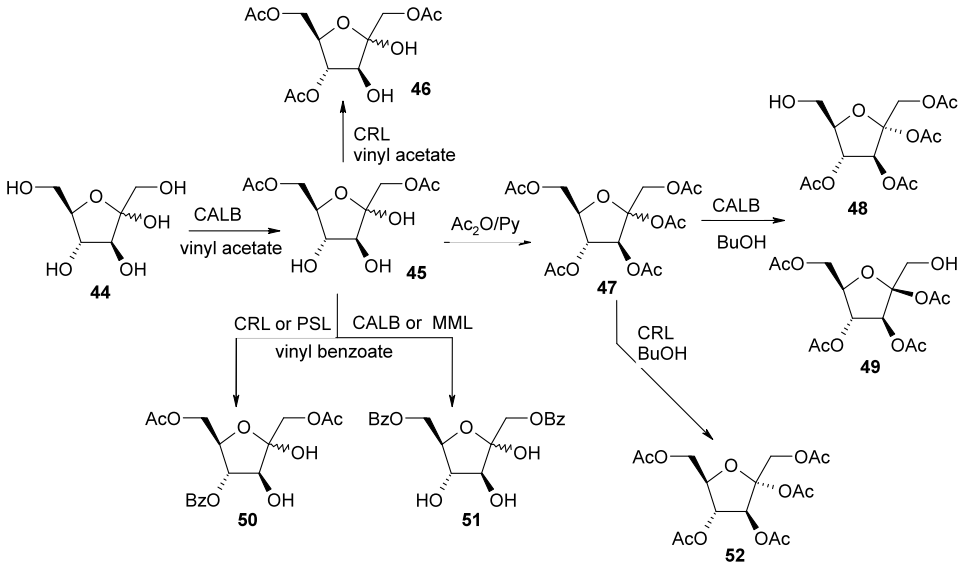
The primary hydroxyl group of sugars is enzymatically most easily acylated. [101] In several papers the acylation of monosaccharides (fructose, mannose, galactose, glucose) with porcine pancreatic lipase and *Candida rugosa* lipase (CRL), which yields mono-acylated sugars (deacetylated sugars in the case of fructose which has two primary OH-groups).[102,103] If the C-6 hydroxyl group is blocked, lipases exhibit a remarkable regioselectivity by discriminating among the four available secondary hydroxyl groups in the C-6 protected glucose, galactose and mannose. While some lipases exclusively acylate the C-3 hydroxyl group, others display an overwhelming preference toward the C-2 hydroxyl group.[104]



Scheme 17. Lipase-catalyzed acylation of 2-deoxy ribofuranose and ribofuranose

The regioselective CALB-catalyzed acylation of unprotected 2-deoxy-D-ribose (**40**) and D-ribose (**42**), by using propionic anhydride, afforded 5-*O*-monoacylated derivatives **41** and **43**, accordingly (Scheme 17).[105]

The lipase-catalyzed acylation of sugars with free hydroxyl groups and deacylation of peracylated sugars[106,107] are the main methods for their modification. Combining these techniques along with chemical modifications a vast amount of new sugar derivatives can be synthesized.[108] For example, D'Antona combined enzymatic reactions using different lipases and chemical acetylation which furnished seven new sugar derivatives (Scheme 18).[109]



Scheme 18. Combining of lipase-catalyzed reactions with chemical methods for the synthesis of fructose derivatives

The same approach has also been applied to glycosides. The advantage is that the number of products is reduced because of the fixation of the anomeric stereogenic center.

The regioselective deacylation of fully acylated methyl D-hexopyranosides (D-glucose, D-mannose, D-galactose) gave the 6-OH derivatives using the lipase from *Candida cylindracea*.[110]

The acetylation of corresponding D- and L-sugars shows that the regioselectivity of lipases towards the secondary hydroxyl groups of sugars is greatly influenced by the nature of aglycone and the stereochemistry of the glycosidic bond.[111] Ciuffreda *et al.* [112] used different lipases to regioselectively acylate methyl α-L- and α-D-rhamnopyranoside (Figure 3; **53** and **56**) and methyl α-L- and α-D-fucopyranoside (Figure 3, **55** and **54**). While D-sugars always gave 2-butyrate as the main product, their L-enantiomers showed a different regioselectivity. Selecting a proper enzyme, in the L-sugars the rather unreactive 4-OH group can be preferentially acylated. The best substrates were L-rhamnoside and D-fucoside, even though they were acylated at different positions. This may be explained by the arrangement of three central hydroxyl groups at the 2nd, 3rd and 4th positions.

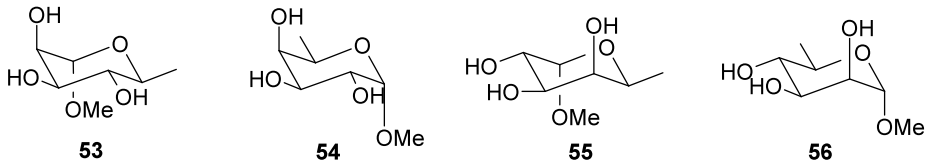


Figure 3. The structure of L-rhamnoside; D-fucoside; L-fucoside; D-rhamnoside

1.4.9. Lipase-catalyzed derivatization of glycoconjugates

Sugars that are covalently bound to non-carbohydrate natural products are called glycoconjugates. Many smaller glycoconjugates possess antibiotic activity and a lot of pharmaceuticals belong to this category. More complex carbohydrates are linked to proteins and lipids and such glycoproteins and glycolipids belong to the most important group of biomolecules in the cell.[1] Elucidating their biological functions and interactions with other biomolecules has become an important topic in carbohydrate research.

Below some examples of the lipase-catalyzed derivatizations of glycoconjugates will be presented.

Due to their rigid framework, potential for varying the levels of functionalization, broad biological activity and ability to penetrate the cell membrane[113] steroids are of interest in biochemistry and drug design. Steroidal glycoconjugates have been subjected to lipase-catalyzed acetylation.[114,115] For example, CALB has been shown to be an efficient catalyst for the regioselective acylation of the dammarane-type glucoside ginsenoside **57** (Figure 4), affording the corresponding 6'-*O*-acetyl derivative **58**.[116]

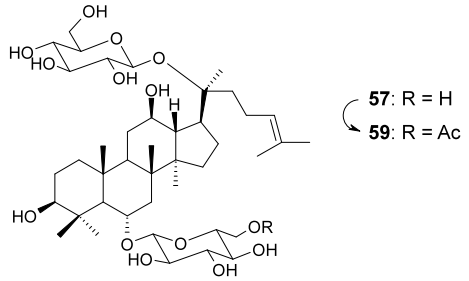
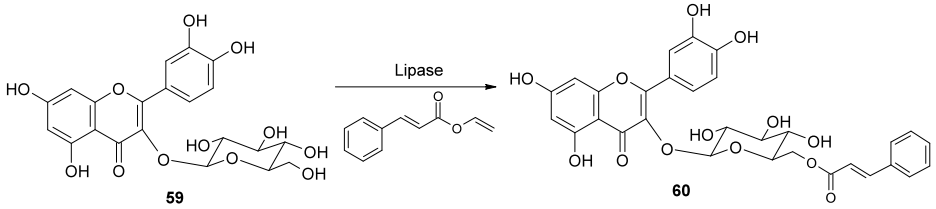


Figure 4. Lipase-catalyzed acylation of ginsenoside

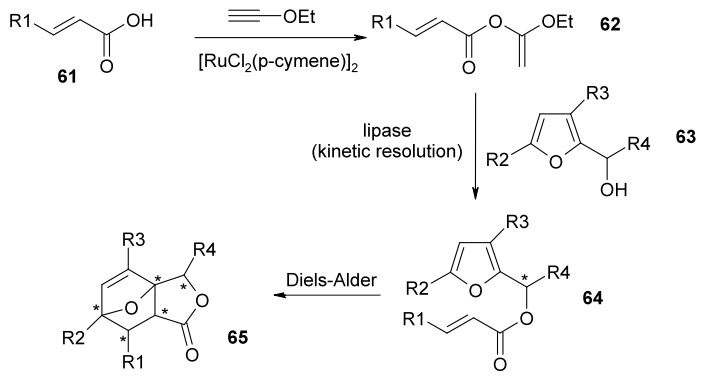
The lipase-catalyzed acylation of flavonoid glucosides which are used as food ingredients and in cosmetic preparations and possess antioxidant and antiviral activities has been described in several publications.[117,118] Using vinyl cinnamate as an acyl donor and lipase Nakajima *et al.* acylated isoquercitrin (**59**, quercetin 3-*O*-glucoside, Scheme 16) to the corresponding aromatic acid ester **60**.[119]



Scheme 19. Lipase-catalyzed acylation of isoquercitrin

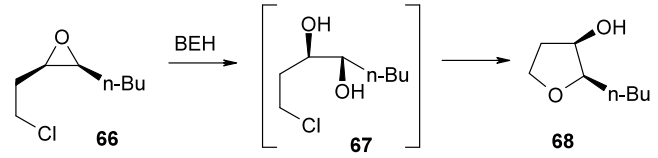
1.4.10. Cascade reactions triggered by lipase

In organic chemistry the synthesis is usually carried out stepwise. However, it would be more advantageous if different bonds could be formed in one sequence without addition of reagents. Reactions of this kind are called cascade or domino reactions, which are synonyms in a given context. This area has been reviewed by H. Pellissier [120] and L. Tietze.[121] The latter author has defined the reaction as follows: *a domino reaction is a process involving two or more bond-forming transformations (usually C-C bonds) which take place under the same reaction conditions without adding additional reagents and catalysts, and in which the subsequent reactions result as a consequence of the functionality formed in the previous step.* In the above reviews, also enzymatic domino reactions have been treated of. Below examples of the cascade reactions carried out using hydrolytic enzymes will be presented. [122]



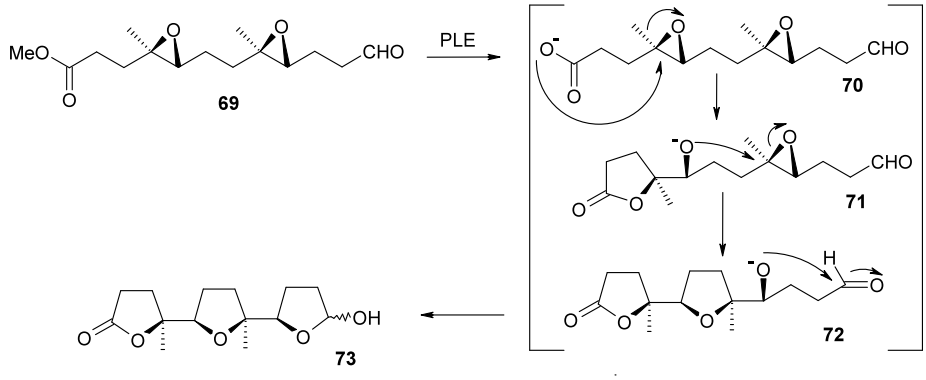
Scheme 20. Lipase-catalyzed cascade reaction

The cascade process, in which the acyl moiety formed during the enzymatic kinetic resolution of furfuryl alcohols **63** with 1-ethoxyvinyl ester **62,** was utilized as a part of the constituent structure for the subsequent Diels-Alder reaction. The preparation of ester **62** from carboxylic acid **61** and the subsequent domino process were carried out as a one-pot reaction (Scheme 20).[123]



Scheme 21. Cascade reaction triggered by an initial enzyme-catalyzed hydrolysis of epoxide: a spontaneous ring-closure

In the second example, both enantiomers of the racemic epoxide **66** were hydrolyzed at first by bacterial epoxide hydrolases (BEH) in an enantioconvergent fashion to furnish the corresponding diols **67**, which, underwent a spontaneous ring closure to yield the corresponding cyclic products **68** (Scheme 21).[124]



Scheme 22. Lipase-initiated cascade reaction

The third example describes a cascade starting with the enzymatic hydrolysis of ester **69**, which liberates a nucleophile (–COO^-) and opens an epoxide to furnish the corresponding lactone, together with a free alkoxy moiety in the δ-position. The latter alkoxide underwent another (intramolecular) nucleophilic attack on the second epoxide to afford a tetrahydrofuran derivative **72**. At the end of the cascade, the resulting alkoxide was trapped by forming a hemiacetal with an aldehyde, bringing the cascade to a halt (Scheme 22).[125]

1.5. Inhibition of lipases

1.5.1. The inhibition and activation of lipases

Lipase inhibitors play an important role in several medical conditions.

In human organism, pancreatic and gastric lipases are essential enzymes for an efficient fat digestion. The hydrolysis of dietary triacylglycerols by these enzymes is a necessary step for the absorption of fat by enterocytes. The inhibitors of these enzymes are of interest as anti-obesity agents.[126,127,128,129,130,131]

Hormone-sensitive lipase (HSL) is a vital enzyme in lipid metabolism in humans and in general energy homeostasis in mammals. HSL catalyzes the rate-limiting step in the hydrolysis of the triglycerides stored in the adipose tissue. The elevated level of free fatty acids has been shown to be associated with an increased insulin resistance which increases the risk to type 2 diabetes. The two products of HSL-mediated hydrolysis are glycerol and free fatty acids. Thus, the inhibition of HSL could potentially decrease the plasma level of free fatty acids implicated in a variety of pathological conditions. There is an interest in finding compounds that would modulate the activity of HSL, as this might help to develop medicines to treat diseases like type 2 diabetes.[132,133]

Several lipases produced by microbial pathogens play an important role in infectious diseases. For example, *Propionibacterium acnes* lipase and its inhibition by antiacne compounds have been studied because the fatty acids produced by *P. acnes* lipase activity on sebaceous triglycerides induce severe inflammation.[134] Also *Helicobacter pylori* lipase has been described to weaken the barrier properties of mucus by hydrolyzing endogenous lipids and inducing peptic ulcer.[135,136]

Also coronary heart diseases are connected with lipase activity. Arteriosclerosis is a chronic disease. A recent research suggests that inflammatory phenomena are the major determinants of its progression. The central role of phospholipase A_2 (PLA_2) in inflammation makes this enzyme a potential target for drug development. Theoretically, the inhibition of PLA_2 may block the formation of a wide variety of secondary inflammatory mediators. The cells contain different forms of PLA_2 that participate in the normal phospholipid metabolism. Therefore, when the inhibition of PLA_2 is considered for therapeutic use, the question of which PLA_2 plays the major role in the generation of pro-inflammatory lipid mediators is central.[137,138]

Lipase inhibitors are also used in investigating the mechanism of action of lipases,[139,140] their structural information[141] and for directed molecular evolution.[142]

There are many lipases that have been used to investigate the influence of lipase inhibitors. The most popular ones are digestive lipases[127,128,130,143] (pancreatic and gastric); hormone-sensitive lipase;[144,145] phospholipases[137,138]; microbial lipases;[146] which are the real target molecules in drug design. Model lipases have been used to test the influence of different compounds – potential inhibitors – on "lipolytic activity" of CRL being common for this purpose.[147,148]

Increasing the hydrolytic activity of lipases has been described as well. The activation of lipases by bile acids has been known for a long time already.[149] Also the presence of calcium ions usually increases the reaction rate, sodium and ferrous ions exhibit a similar effect on some lipases.

Stolzmann *et al.*[150] have measured the activity of glycerol ester hydrolase in the presence of simvastatin and found that the latter increases the hydrolytic activity of the enzyme. Therefore it may be suitable for the treatment of combined lipoprotein disorders characterized by the elevation of the level of triacylglycerols. The respective mechanism of action is not yet known.

1.5.2. Inhibitors of lipases

Lipases can be inhibited reversibly and irreversibly.[151] The first group includes compounds that are quasi-substrates which form stable acyl-enzyme. The other group includes substances (surfactants, bile salts, some proteins, metal-ions, boronic acid, etc.) that do not act at the active site of enzymes but change their conformation or interfacial surface properties. Several irreversible inhibitors are known to act at the active site of the enzyme.

Phosphorus-containing inhibitors. Some of the most common active-site-directed inhibitors of serine hydrolases are reactive phosphorus compounds. They react with the serine of a catalytic triad at the active site, thus forming irreversibly a stable transition-state-like product. Similarly, phosphorus-containing compounds such as diethyl p-nitrophenyl phosphate (Figure 5, **74**), and 3-guanidopropyl p-nitrophenyl methylphosphonate (**75**), have been used as inhibitors of lipase. Based on this information a very efficient inhibitor of lipase n-hexyl chlorophosphonate ethyl ester (**76**), was designed. A lot of today's knowledge of the mechanism and function of lipase has been acquired by using diethyl p-nitrophenyl phosphate, which has been shown to inhibit pancreatic and gastric lipases as well as some microbial lipases at high concentrations. The phosphonate compounds mimic both – the charge distribution of the substrate and geometry of the first transition state occurring during hydrolysis of carboxyl ester. Enantiomerically pure alkylphosphonate compounds have been synthesized[152] and investigated as potential inhibitors of human gastric and pancreatic lipases (HPL).

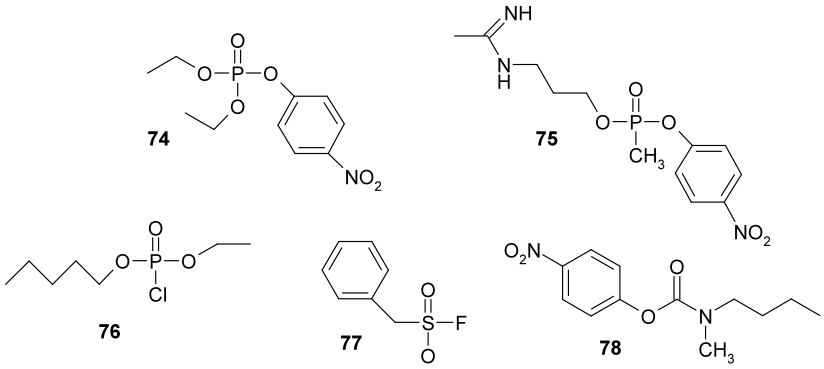


*Figure 5. The structure of phosphorus-containing inhibitors (**74**,**75**,**76**); phenyl methyl sulphonyl fluoride (**77**); carbamate (**78**)*

Non-phosphorus compounds. Phenyl methyl sulphonyl fluoride (Figure 5, **77**), has been used as an inhibitor of lipases but it is not as effective as phosphorus-containing inhibitors. A number of alkanesulfonyl halides (chlorides and fluorides) and esters have been synthesized and their effect on the activity of lipoprotein lipase has been studied.[153]

Carbamates. N-butyl-N-methyl-4-nitrophenyl carbamate (Figure 5, **78**) has been shown to be a specific inhibitor of bile-salt-dependent lipases from human pancreas, human milk and dog pancreas.

Substrate analogues. One strategy in inhibitor design is mimicking the Nature, i.e. to derive inhibitors from the natural substrates of lipases, triacylglycerols.

α-Keto amide moiety is of special interest since it is the most frequently encountered electrophilic ketone pharmacophore found in many potent inhibitors of proteolytic enzymes, e.g., serine protease and chymase. [154,155]

Simons *et al.*[156] have synthesized a series of α-keto triglyceride analogues as inhibitors of the lipase from *Staphylococcus hyicus*. Hydrolysis at positions 1 and 2 was prevented by replacement of the ester bonds by a nonhydrolyzable ether, carbamoyl or amide bonds. Also, an α-keto fatty acid has been introduced at position 3. Inhibition must be caused by the presence of the α-keto group, since the compounds containing an ester or a hydroxyl group at position 3 did not inhibit the enzyme. Other specific protein/inhibitor interactions, probably with the carbonyl oxygen at 1st and/or 2nd positions, will improve the binding of the inhibitor.

Kokotos *et al.*[157] have designed inhibitors having α-keto amide functionality for human cytosolic PLA_2, taking into consideration the chemical structure of phospholipids which are natural substrates of phospholipases. These inhibitors contain an α-keto amide and a free carboxyl group (Figure 6; **79**, **80**). The idea to insert α-keto amide group into the structure of natural substrate of lipases has been used to develop inhibitors of pancreatic and gastric lipases. This kind of α-keto amide triacylglycerol analogues have the α-keto amide group instead of the ester

group at the 1st or 3rd position and the ether bond at the other two positions (Figure 6, **81**).[158,159,160]

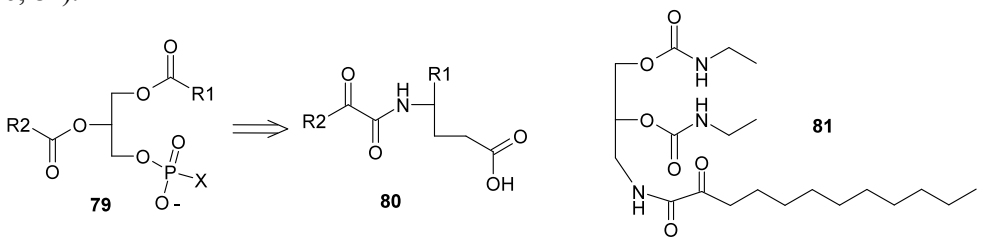


*Figure 6. Natural substrate of phospholipases (**79**), α-keto amide (**80**), α-keto triglyceride analogue (**81**)*

For human digestive lipases also aldehyde dialkyl and alkyl-acyl glycerol analogues have been developed using the aldehyde group as a reactive functional group at the 1st position (Figure 7).[127]

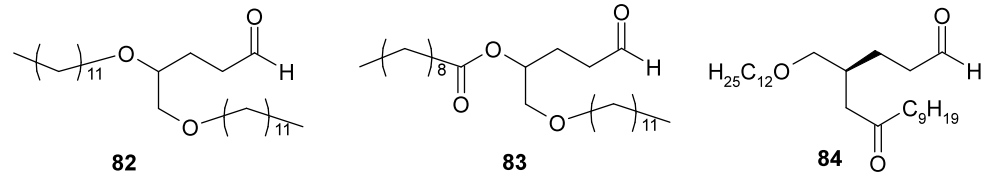


Figure 7. Aldehyde dialkyl and alkyl-acyl glycerol analogues

In addition, various sterically hindered triacylglycerols,[130] biotinylated substrate analogues[161] and triacylglycerol analogues containing 2-(*N-tert*-butoxycarbonyl-amino) fatty acids have been synthesized.[162]

β-Lactone-containing inhibitors (Figure 8). Several *β*-lactones of microbial origin have been isolated and tested as lipase inhibitors. Among them are lipstatin (**85**), esterastin (**87**), valilactone (**88**), vibralactone (**89**),[131] Panclicine D (**90**)[143] and ebelactone A (**91**). Although most of them are structurally similar, these *β*-lactones exhibit slightly different inhibition profiles towards lipases. Of these inhibitors, tetrahydrolipstatin (**86**), a saturated analogue of lipstatin, has been studied most thoroughly. It has been shown to be covalently bound to the active-site serine of HPL. By now this compound has passed clinical testing and can be purchased from pharmacies as an anti-obesity drug Xenical® (Orlistat).[163] Tetrahydrolipstatin has also been tested with microbial lipases and contrary to HPL, their inhibition is reversible.[146]

Also synthetic *β*-lactones have been developed for lipase inhibition.[164]

Natural substances. A lot of compounds exhibiting an inhibiting activity towards lipases have been extracted from natural sources.[165]

Different polyphenols from oolong tea leaves[166] and the crocin and crocetin isolated from the fructus of *Gardenia jasminoides* Ellis[167] were evaluated for their inhibitory activity towards pancreatic lipase.

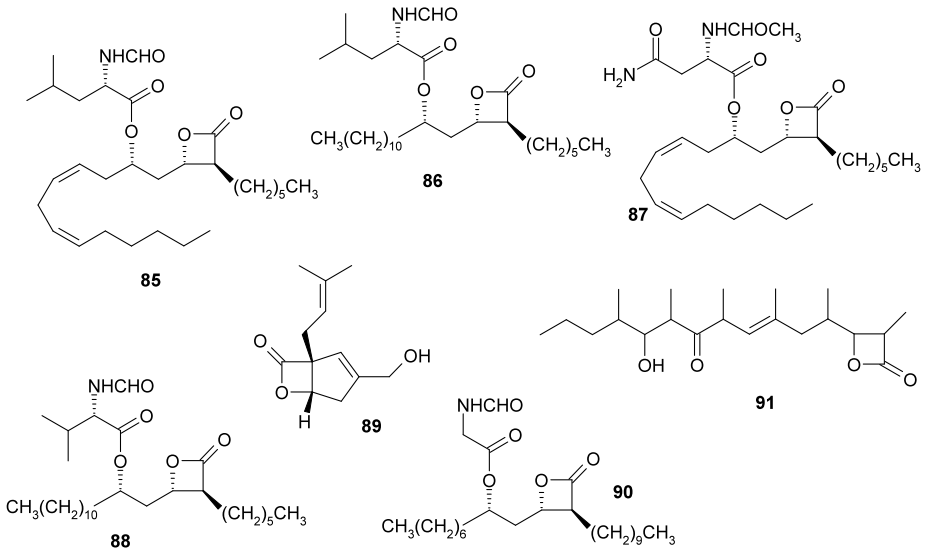


*Figure 8. β-Lactone-containing inhibitors: lipstatin (**85**), tetrahydrolipstatin (**86**), esterastin (**87**), valilactone (**88**), vibralactone (**89**), Panclicine D (**90**), ebelactone (**91**)*

The effect of several natural substances on *Propionibacterium acnes* lipase has been evaluated. The study revealed glycyrrhizic acid, (±)-catechin and kaempferol to be promising candidates for the treatment of acne due to their strong inhibitory activity towards lipase.[168]

Gatto *et al.* described the anti-lipase activity of quercetin (Figure 9, **92**) and its C2-C16 3-*O*-acyl-esters.[148]

Among other natural substances, Grippa *et al.* tested also berberin (Figure 9, **93**) and sanguinarin (**94**) which displayed no remarkable activity towards lipases, but nevertheless, these substances could be used for the inhibition of lipase-producing microorganisms.[147]

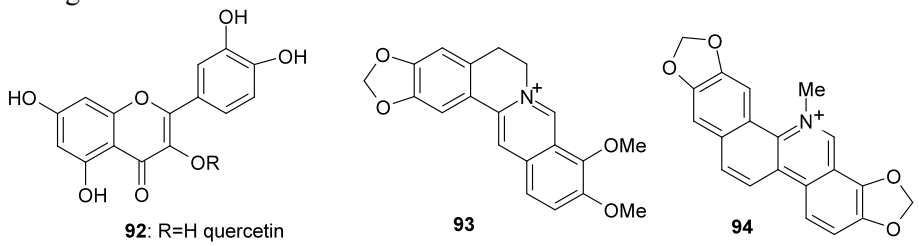
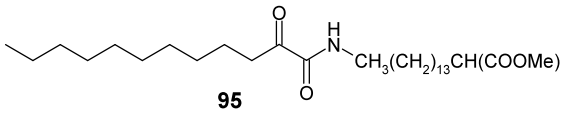


Figure 9. Quercentin; alkaloids – berberin and sanguinarin

Miscellaneous. Chiou *et al.* [169] synthesized an α-keto amine **95** that is not an analogue of its natural substrate, triacylglycerol, and tested it on porcine pancreatic lipase (Figure 10).

The effect of saturated fatty acids has been evaluated on *Bacillus*-related lipases.[136]



95

Figure 10. α-Keto amine

Methyl-phenyl-carbamoyl-triazoles were identified as potent and efficacious inhibitors of HSL. These compounds do not inhibit other hydrolases such as hepatic, lipoprotein and pancreatic lipases as well as butyrylcholine esterase.[144] For the inhibition of HSL different compounds, such as ε-polylysine,[170] pyrrolopyrazinediones,[132] (5-(2H)-isoxazolonyl) ureas,[171] carbazates[172] and some peptides[142] have been found to be suitable. Ebdrup *et al.*[133] tested the ability of aryl and heteroaryl boronic acids to act as inhibitors of the same lipase.

Tests have shown azetidinones to act as inhibitors of lipoprotein-associated PLA_2.[137]

1.5.3. Methods for monitoring lipase-catalyzed reactions

Lipase-catalyzed reactions have been monitored using several methods. These include[173]:

1. spectroscopy (spectrophotometry,[174] fluorimetry,[166,144,175] infra red spectroscopy; colorimetric assay,[136,176] NMR[86,177]);
2. chromatography (HPLC,[178] gas chromatography[179]);
3. titrimetry (pH-stat assay[180]);
4. radioactivity[181];
5. interfacial tensiometry (monolayer technique[155,162]);

Two methods, HPLC and colorimetric assay are relevant to this current work and therefore will be described in more detail.

In HPLC the reaction mixtures containing inhibitors of varying concentrations, β-naphthyl laurate (β-NL) as substrate, buffer and lipase, are incubated under a gentle mixing. Then, the hydrolysis product, β-naphtol, is extracted and aliquots are analyzed using a HPLC. The unspecific hydrolysis of β-NL is subtracted performing proper blanks.[147]

In a colorimetric assay the non-colored substrate, *p*-nitrophenyl laurate (*p*-NPL), is added to the preincubated mixture of the enzyme and inhibitor in different concentrations on a microtiter plate and incubated. The absorbance of each well is measured to detect the release of the yellow-colored product of the reaction, *p*-nitrophenol (*p*-NP). The proper blanks corresponding to the absorbance of the reaction mixture without the substrate are prepared and subtracted from the total absorbance.[136]

In both cases the inhibition of the lipase is calculated from the residual activity detected in the presence of a potential inhibitor. The residual activity of the lipase is compared with the activity of the lipase in the samples without the inhibitor. All the samples are prepared and analyzed under the same conditions.

2. AIMS OF THE PRESENT STUDY

The aims of the work were:

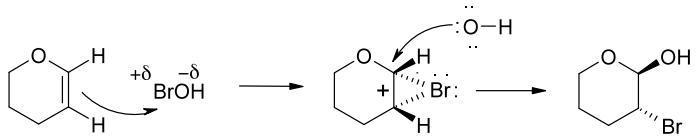
- Creation of a novel approach for the chemoenzymatic synthesis of stereochemically pure deoxy sugar esters.
- Investigation of the lipase-catalyzed derivatization of the polyhydroxy carboxylic acids used for the synthesis of deoxy sugar esters – potential inhibitors of lipases.
- Investigation of a selection of natural compounds including also polyhydroxy carboxylic acids and their chemoenzymatically prepared derivatives as putative lipase inhibitors.

3. RESULTS AND DISCUSSION

3.1. Synthesis and acetylation of α-bromo-ω-hydroxyaldehyde hemiacetals (Article I)

In the initial stage of the work, the synthesis of the alkyl glycosides of deoxy sugars having a carbon skeleton of four or five atoms was carried out using 2,3-dihydrofuran (DHF) and 3,4-dihydro-*2H*-pyran (DHP),* respectively. Starting with these compounds α-bromo-ω-hydroxyaldehyde hemiacetals, which are precursors of deoxy sugars having furanose and pyranose cycles, were synthesized. The experimental part has been described in Article I.

The first step of the synthesis was the bromohydroxylation of DHF or DHP by using 1,3-dibromo-5,5-dimethylhydantoin (DBDMH). An attempt was made to carry out the bromohydroxylation of DHP by using *N*-bromosuccinimide, which yielded some by-products, unlike DBDMH. The reactions resulted in racemic bromo-hemiacetals **96** having a pyranose cycle (Scheme 24) and bromo-hemiacetals **100** having a furanose cycle (Scheme 27). According to the established mechanism of the reaction the hemiacetal products should have been formed only in a *trans*-configuration (Scheme 23). Actually, due to its similarity to sugar, *i.e.* the unstable structure of the hemiacetal moiety under the conditions used, the hemiacetal product appears as an equilibrial mixture of anomers (*cis*- and *trans*-isomers in this case). In order to better understand isomeric ratios in different reactions quantum chemical (QC) calculations were performed. The ratio of isomers (*cis*- and *trans*-hemiacetals and an extended-chain aldehyde) was observed to correspond in good approximation to the thermodynamical distribution. This is the reason why the hemiacetal in a *cis*-configuration is present in the product. Considering the above, also the open-chain forms of hydroxyaldehydes corresponding to hemiacetals **97** and **101** should exist although detection of them failed. It is probable that the open-chain form of hydroxyaldehyde is converted to a cyclic hemiacetal very quickly.



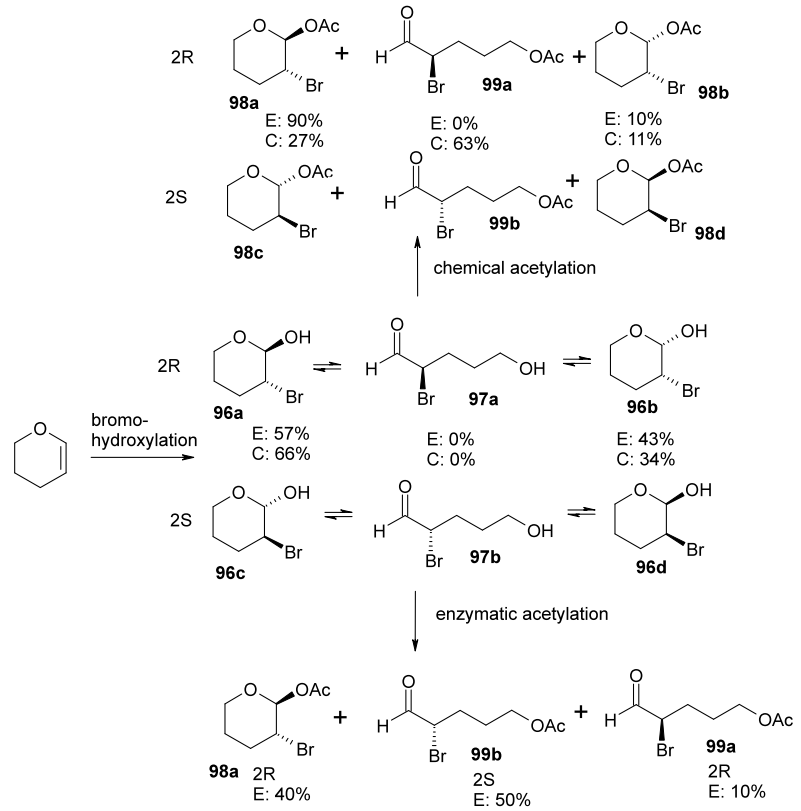
Scheme 23. The reaction mechanism of bromohydroxylation

In the case of the deoxy pyranose (Scheme 24), the equilibrial mixture of bromohemiacetals consists of 57% of *trans*- and 43% of *cis*-isomers (by an NMR experiment).

* Numeration of α-bromoaldehydes in hemiacetal as well as in open-chain form starts from carbonylic carbon (the same numeration has been used as for deoxy sugars) unlike for compounds named as pyran or furan rings.

For hemiacetals, their open-chain aldehyde forms and corresponding acetates, conformers with minimum energy were found. Based on these results the thermodynamical distribution of the isomers present in (hypothetical) equilibrial mixtures was calculated.

The second step of the synthesis was the CALB*-catalyzed acetylation of hemiacetals to elucidate the possibility of performing the lipase-catalytic kinetic resolution of enantiomers.[74,182]



Scheme 24. Acetylation of pyranose; E - the ratio of isomers according to experimental data; C - a thermodynamic distribution of isomers according to quantum chemical calculations

(2*R*)-*trans*-Hemiacetal **96a** (Scheme 24) gave acetylated cyclic bromohemiacetal **98a** (40%) in a short time as expected. This compound is acetylated as a cyclic secondary alcohol. According to the Kazlauskas rule, for the lipase this hemiacetal is the favored enantiomer. In the active site of the lipase the secondary hydroxyl group is deprotonated by histidine and a new bond is formed between the substrate oxygen and the acyl group of the acyl-enzyme (Figure 11).

* An immobilized enzyme preparation, Novozym 435, was used in all CALB-catalyzed experiments.

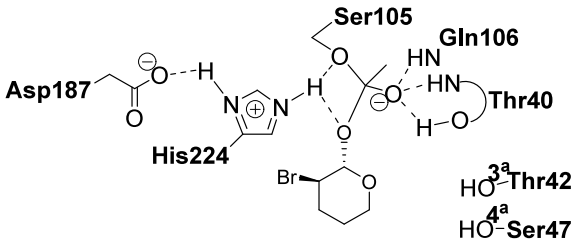


Figure 11. CALB-catalyzed acetylation of (2R)-trans-hemiacetal

Somewhat unexpectedly, the acetylation of the other enantiomer – (2*S*)-bromohemiacetals **96c** and **96d** underwent decyclization and gave an open-chain aldehyde with an acetylated ω-hydroxyl group – **99b** (50%) under the conditions used.

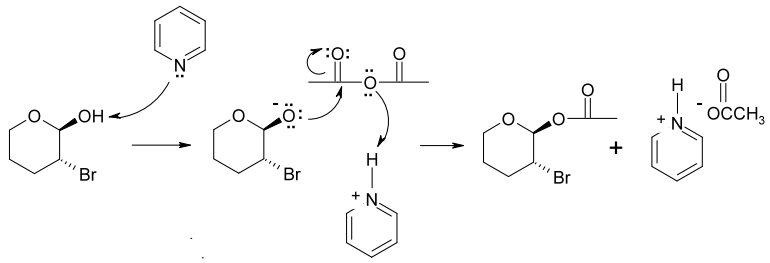
This kind of the lipase-catalyzed decyclization of the cyclic hemiacetal affording chemically more reactive aldehyde stabilized via acetylation of the terminal hydroxyl group has not been described earlier.

To enable acetylation of the ω-hydroxyl group of an α-bromo-ω-hydroxyaldehyde to take place the opening of the hemiacetal cycle is required. Considering that the lifetime of the extended-chain α-bromo-ω-hydroxyaldehyde in solution is probably too short for trapping preferentially just ω-hydroxyaldehyde by the enzyme, the decyclization must be performed by the lipase in its active site. The stabilization of the aldehyde by the hydrogen bonds only (formed in the oxy-anion hole) is not likely, and therefore transhemiacetalization could be expected to occur. Performing the latter, anomeric carbon (*i.e.* carbonyl) is probably attacked by the oxygen atom of the hydroxyl group of Thr40 under a simultaneous acid-catalytic destabilizing influence of an oxy-anion hole on the hemiacetal, leading to the formation of a covalent bond. This, in turn, leads to the decyclization of the pyranose cycle (Scheme 3, Article I). Thus, the extended-chain form of ω-hydroxyaldehyde is expectedly stabilized by the transhemiacetalization with the hydroxyl group of the residue of Thr40 (in this case simultaneous acetylation of the hydroxyl group of an aldehyde is geometrically possible), allowing the acetylation of the ω-hydroxyl group.

Also, a certain amount of (2*R*)-bromohemiacetal was observed to give an open-chain aldehyde **99a** (10%) probably because of the presence of the *cis*-isomer that could be recognized by the lipase (in the active site of the CALB) similarly to (2*S*)-bromohemiacetal for evident sterical reasons. During the lipase-catalyzed acetylation no formation of *cis*-isomeric hemiacetal esters was observed (according to the NMR analysis of the crude product). The enantiomeric excess of all acetylated bromoaldehydes was determined by a chiral HPLC of the bromohemiacetals obtained by a quantitative acid-catalyzed deacetylation of the samples. In some cases the results of analysis were confirmed by the NMR analysis

of mandelic acid diastereomeric esters of deoxy sugar methyl glycosides prepared starting from bromohemiacetals.

In order to further study the formation of the open-chain aldehydes starting from the hemiacetals, a chemical acetylation of the latter was carried out. This afforded cyclic hemiacetal esters only, acetylated open-chain products were not formed (no formation of an aldehyde was detected by the NMR of the crude acetylation product). The mechanism of the reaction is described in Scheme 25. Interestingly, the ratio of *trans*- to *cis*-isomers had significantly changed extent compared to the *cis*/*trans* ratio of the isomers of the starting material. In the mixture only 10% of an acetylated *cis*-isomer along with 90% of the *trans*-isomer was found to be present . This result is in good accordance with the thermodynamic distribution found by QC calculations for the acetate of the *cis*-isomer. However, a major constituent of the isomeric mixture predicted had to be an acetylated open-chain form which was not formed at all by chemical acetylation.



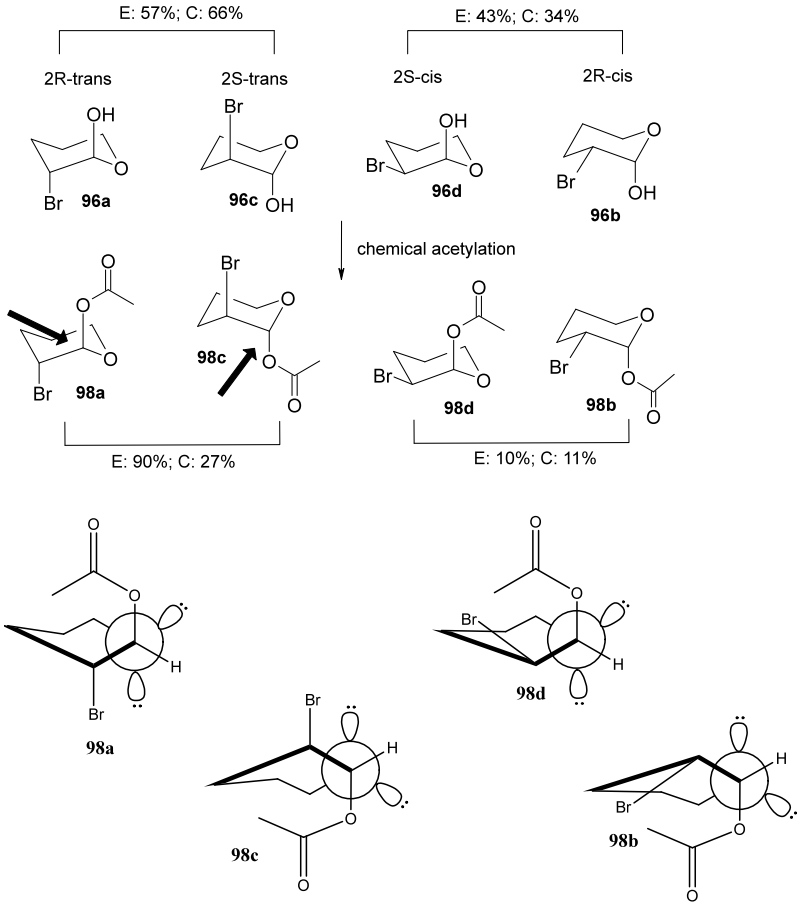
Scheme 25. The reaction mechanism of chemical acetylation

The changed ratio of *cis*- to *trans*-isomers may be accounted for the elements of kinetic control (allowing the realization of the thermodynamic preference of certain isomeric end-products) in the case of the *trans*-isomer. The latter evidently reacts faster than the *cis*-isomer, while the equilibrial ratio of *cis*/*trans*-hemiacetals is rapidly restored. This is an interesting case of the chemical kinetic dynamic resolution of *cis*- and *trans*-isomers affording a product of considerably higher concentration of the trans-isomer compared to starting material.

Thus, as can be concluded from the calculated thermodynamic distribution of isomeric acetates, the velocity of formation of the acetate of *cis*-hemiacetal is controlled by the relative stability of the latter. At the same time, the formation of the acetate of the extended-chain hydroxyaldehyde does not occur (upon chemical acetylation) according to the calculated thermodynamic distribution of isomeric products, evidently for kinetic reasons, *i.e.* due to too low a content of this isomeric form in the starting equilibrial mixture. Instead of an aldehyde only a *trans*-hemiacetal is formed.

The conformations of *cis*- and *trans*-isomers of acetylated bromohemiacetals using a Fisher-projection are presented in Scheme 26. Due to the anomeric effect only axial conformations of anomeric carbon substitutes are favoured. The higher acetylation rate of the *trans*-isomer along with the higher stability of the

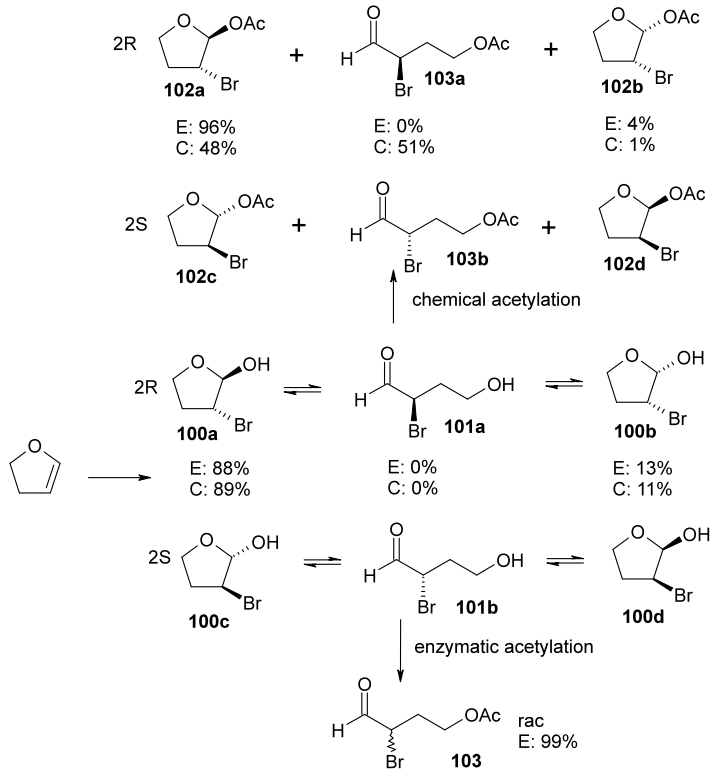
corresponding isomeric product may be explained by the sterically easier deprotonation and nucleophilic attack to anhydride by the hydroxyl group situated on the opposite side of quite large bromine atom.



Scheme 26. Conformers of trans- and cis-hemiacetals; Fisher-projections of trans- and cis-acetals viewed along the anomeric carbon-oxygen bond

The absolute configuration of deoxy pyranose (as well as furanose) bromohemiacetals has been determined by shielding effects observed in NMR spectra of diastereomeric Mosher`-esters.[182] In the current work, it was also determined via the absolute configuration of methyl glycoside **(2*S*)-5*trans*** by the results of the studies of differential shielding effects observed in the NMR spectra of the corresponding THP-mandelate diastereomers (Set A, Scheme 1, Article I).

For furanose bromohemiacetals **100a-d** the enzymatic acetylation yielded racemic open-chain aldehyde **103** only. The decyclization of the substrate was performed by the active site of the lipase and the open-chain aldehyde was trapped by the acetylation of the ω-hydroxyl group. The expected mechanism of the reaction has been discussed in the previous paragraph.



Scheme 27. Acetylation of furanose bromohemiacetals

The chemical acetylation of furanose bromohemiacetals **100a-d** resulted in isomeric products of a changed ratio of *cis*- to *trans*-isomers again. The major compound detected was the *trans*-acetate while only 4% of the *cis*-isomer was detected in the crude product. This was in good correlation with QC calculations. However, the calculated thermodynamical distribution of isomeric products indicated that the favored isomer should have been the open-chain aldehyde as earlier calculated for pyranose bromohemiacetal esters and corresponding aldehyde, but experimentally no formation of the product in the reaction mixture was observed. Instead, the acetylation of the *trans*-isomer took place.

3.2. Investigation of lipase-catalyzed reactions of bile acids and their derivatives as potential specific lipophilic substituents for inclusion into lipase inhibitors

A strategy has been proposed for designing lipase inhibitors. *A lipase inhibitor should consist of two components: a chemically reactive moiety, which reacts with the active site serine of the enzyme, and a part that contains chemical motifs for specific interactions and orientation in the enzyme binding pocket.*[127] Proceeding from this suggestion the search for the proper substituents was the next task. The

reactive moiety could be a deoxy sugar in hemiacetal or in extended-chain form attached to carboxyl group of the carrier. The compounds under investigation in the current work as potential specific substituents were bile acids and other natural compounds such as saponins, alkaloids, flavonoids as well as prostaglandins.

The main objectives of the investigation of especially the lipase-catalyzed reactions (but also others) of the potential carrier molecules are:

1. determination of the accessibility of functional groups (suitable for the attaching of potential "serine traps") to lipases (they should be accessible, otherwise the traps introduced may be inaccessible to the active site of the enzyme; these results are also important for the use of the lipase-catalyzed reactions in synthesis, *e.g.* for the selective protection of functional groups);
2. determination of the influence of the potential carrier molecules and their model derivatives on the catalytic activity of lipases in order to evaluate the inhibitory effects caused just by the traps introduced.

3.2.1. Lipase catalyzed derivatization of cholic and deoxycholic acid

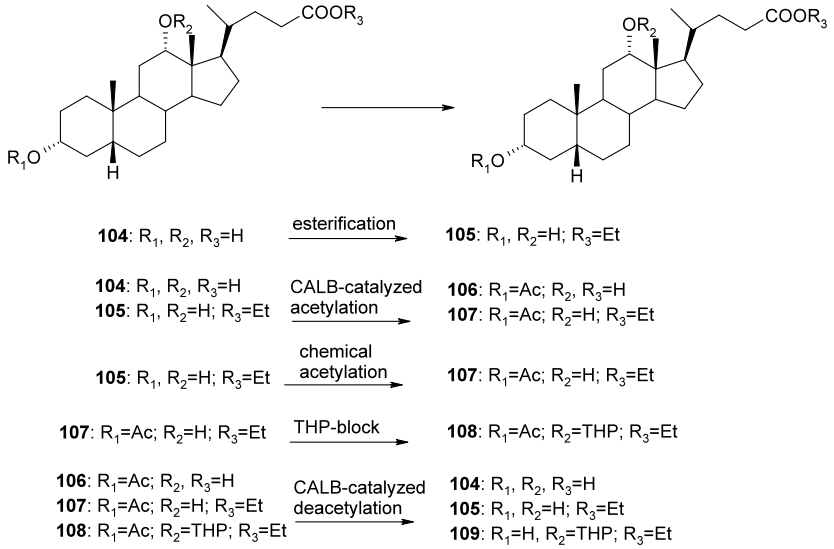
Bile acids are a group of chemical compounds with a multitude of pharmacological properties. Among their applications as drugs, they are used to dissolve cholesterol gallstones, improve the absorption of hydrophilic drugs and as anti-HIV agents. They can be administered to patients having altered cholesterol metabolism or diseases related to irregular cholesterol or bile acid biosynthesis.[89,183]

First the investigation of the availability of the functional groups of cholic (CA, Scheme 28) and deoxycholic acid (DCA, Scheme 29) as well as their derivatives to the lipase under different conditions was undertaken. It was planned to use lipase-catalyzed acetylation/deacetylation reactions in the synthesis of deoxy sugar esters of bile acids.

The synthesis of bile acid derivatives (see section 4.1. for experimental data) started with the CALB-catalyzed esterification of the carboxylic acid group, which, however, under the conditions used during 6 days of incubation failed. Instead, a chemical esterification was performed. DCA and CA ethyl esters **105** and **111** were obtained in good yield. (Later it was observed that under specific conditions CALB was effective in ester hydrolysis.)

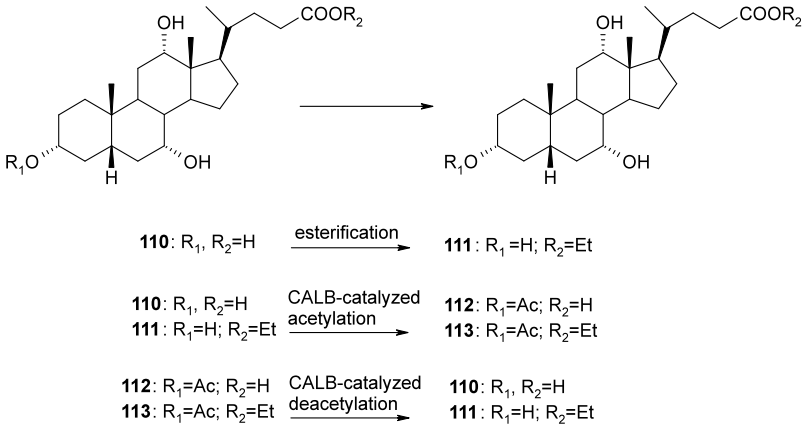
The lipase-catalyzed acetylation of DCA (**104**), CA (**110**) and their esters **105** and **111** yielded 3-acetyl products **106**, **112**, **107** and **113**, leaving the hydroxyl group at the 12th carbon atom and the 7th carbon atom for CA free. Comparing the acetylation of esters and free acids, the difference in reaction time was notable. The esters were converted to acetylated products in 24 hours *vs.* 72 hours in case of free acids (in all cases, the conversion of the starting material was ~95%).

The idea was to block the hydroxyl at the 12th carbon atom to prevent the possible formation of an intramolecular cycle during the synthesis of an ester with α-bromoaldehydes **99** or **103**.



Scheme 28. Derivatization of DCA

To further investigate the availability of the hydroxyl groups, also the chemical acetylation with DCA ester **105** was carried out. 3-Acteyl product **107** was obtained in good yield. After 140 hours also traces of another product, possibly diacetyl-DCA, were detected on TLC but the amount remained between 3-4% and the product was not purified. The chemical acetylation of **107** gave only traces of the same product, thus, the attempt to block the hydroxyl group at the 12th carbon atom by acetylation failed. Protecting the hydroxyl group at the 12th carbon atom by a bulkier THP-block gave the desired product **108** in good yield.



Scheme 29. Derivatization of CA

The CALB-catalyzed deacetylation of DCA and CA derivatives were carried out in different environments, changing the solvent ($CHCl_3$, Et_2O, CH_3CN), the

nucleophile (MeOH, EtOH, H_2O) and the concentration of the nucleophile. The results are summarized in Table 1.

The results show that at the 3rd carbon atom deacetylation took place (at least 50% of the conversion after 96 h of incubation was reached) in case of all derivatives, except for the compound **108**. The enzymatic deacetylation was successful at low concentrations (2%) of the nucleophile in reaction media (Run 1, 3, 7) and also when neat ethanol was used as nucleophile (Run 6). It is important to point out that there was no or almost no deacetylation when neat methanol was used (Run 5). Also no deacetylated products or formation of only traces (< 2%) of those products were detected at a 50% concentration of the nucleophile after 240 h of incubation with the lipase (Runs 2 and 4). Deacetylation of compound **107** using 2% of ethanol in acetonitrile (Run 7) or the diethyl ether saturated with water (Run 8) produced a deacetylated product **105** as well as a product, in which the hydroxyl group at the 3rd carbon atom was deacetylated and the ester group was hydrolyzed (DCA **104**). On the other hand, the THP-blocked derivative **108** did not give the desired deacetylated product **109** under any conditions tested.

Table 1. Lipase-catalyzed deacetylation of CA and DCA derivatives at different concentrations of the nucleophile

Run	1	2	3	4	5	6	7	8
Substrate	Reaction medium							
	$CHCl_3$				MeOH 100%	EtOH 100%	CH_3CN	H_2O/ Et_2O
	MeOH		EtOH				EtOH	
	2%	50%	2%	50%			2%	
106	**104**	**tr**[a]	**104**	**tr**	**tr**	**104**	**104**	**104**
107	**105**[b]	**-**[c]	**105**	**tr**	**tr**	**105**	**104, 105**	**104, 105**
108	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
112	**110**	**tr**	**110**	**tr**	**tr**	**110**[b]	**110**	**110**
113	**-**	**-**	**112**[b]	**-**	**-**	**112**[b]	**112**	**tr**

[a] – *traces (< 2%) of the deacetylated product after incubation for 240 h;*
[b] – *~30% of the deacetylated product after incubation for 240 h;*
[c] – *no products detected after incubation for 240 h;*

In conclusion:

1. The content of the alcoholic nucleophile (2, 50 or 100%) in the reaction medium is crucial for the catalytic activity of the lipase.
2. The catalytic performance of CALB in neat MeOH and neat EtOH greatly varied. The almost non-existent catalytic activity of CALB observed in methanol towards steroidal substrates could be explained, for instance, by the higher ability of MeOH molecules compared to EtOH to exchange (or extract)

the H_2O molecules in (from) the protein structure, which are essential for maintaining the catalytic activity of the enzyme.

3. The deacetylation of CA derivatives is more complicated to perform than that of DCA ones. Therefore DCA is selected for further testing.

3.2.2. Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids (Article II)

Saponins, flavonoids and alkaloids are plant secondary metabolites which are present in herbal drugs and food. The compounds have shown to be very useful in the prevention and treatment of several diseases. The plant extracts rich in the above substances have been used for the treatment of diseases like ulcer and acne, in whose development lipases play an important role.

In this work *Candida rugosa* lipase was used to test the inhibitory effect of saponins, flavonoids and alkaloids (the tested compounds are listed and their structures presented in Figure 1, Article II). Saponins have been demonstrated to have antimicrobial, hypocholesterolemic and anti-inflammatory properties. Flavonoids are capable of modulating the activity of several enzymes. They are components of antiacne preparations and some of them have been shown to possess antiulcer, antitumoral and cardioprotective activity. Different alkaloids have also displayed antimicrobial and anti-HIV effects.

CRL is widely used in biotechnology and it was chosen to serve as a model for fungal pathogenic lipases.

The inhibition of the lipase in the presence of the compounds under evaluation was measured by using HPLC. To this effect, the reaction mixtures containing inhibitors of varying concentrations, β-naphthyl laurate as a substrate, buffer and lipase, were incubated for a determined period. Then, the hydrolysis product, β-naphtol, was extracted and aliquots were analyzed by using HPLC. The inhibition of the lipase was calculated from the residual activity detected in the presence of the potential inhibitor. The residual activity of the lipase was compared with the activity of the lipase in the samples containing no inhibitor. The concentrations of potential inhibitors leading to the lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the curves of the inhibition rate versus the concentration of the inhibitor (Table 1, Article II).

The effect of saponins on CRL. The effect of β-aescin, digitonin, glycyrrhizic acid and *Quillaja* saponin was investigated. All the saponins tested strongly inhibited CRL, digitonin and β-aescin (IC_{50}=0.8-2.4×10^{-5}M) being the most active. The inhibiting activity of glycyrrhizic acid was the lowest.

The effect of flavonoids on CRL. The inhibition of the lipase by (±)-catechin, kaempferol, 5-hydroxyflavone and 3-hyroxyflavone was evaluated. The inhibiting capability of flavonoids was lower than that of saponins (except for glycyrrhizic acid). All the flavonoids tested had a similar IC_{16}, while IC_{50} was displayed only by (±)-catechin and kaempferol.

The effect of alkaloids on CRL. Eight alkaloids – aspidospermine, papaverine, physostigmine, pilocarpine, raubasine, rescinnamine, reserpine and trigonelline, were tested for their inhibitory effect on the lipase. Measured by HPLC assay the rate of inhibition of CRL by alkaloids highly varied. This is probably because alkaloids greatly differ in structure. The most active compounds were rescinnamine and reserpine, while papaverine, physostigmine, pilocarpine and raubasine showed no inhibitory activity towards CRL.

The mechanism of inhibition of lipases by the compounds tested should be studied further in the future using proper techniques.

In conclusion, among the compounds tested, some saponins were the most potent inhibitors of the lipase. Digitonin, β-aescin, rescinnamine, reserpine, (±)-catechin and kaempferol revealed a significant rate of inhibition. The potential use of rescinnamine and reserpine for the treatment of lipase-related diseases is limited because these compounds are active ingredients in hypertension medicines. Some flavonoids are considered toxic at high concentrations, however, the daily human diet contains up to 1g of such compounds. β-Aescin is currently used in the treatment of the peripheral vascular disease. Therefore, as the latter substances exhibit low toxicity they can be promising candidates for the prevention and treatment of lipase-related diseases.

Moreover, the most active compounds could be derivatized further to improve their inhibitory effect. One of the possible approaches for the derivatization of these compounds is an addition of lipase-catalytically activated deoxy saccharides (*e.g.* deoxy saccharides in aldehyde form).

3.2.3. Influence of prostaglandins and their derivatives on *Candida rugosa* lipase (Appendix I, manuscript)

The HPLC method is precise but the procedure of analysis is tedious and time-consuming. The colorimetric assay was introduced in the laboratory of the University of Rome "La Sapienza" to measure lipase inhibition and it proved to be a fast, simple and sensitive method.[136] Therefore, the colorimetric assay was chosen for the preliminary testing of the influence of prostaglandins and their derivatives on the catalytic activity of CRL in hydrolysis reaction.

Several prostaglandin derivatives were prepared and tested along with some native ones. The most active prostaglandin derivatives were found to be among the $F_{2\alpha}$ series, which displayed inhibition as well as activation effects on CRL. The results of testing the influence of different PGs and their derivatives on CRL are described in detail in manuscript (Appendix I).

3.3. Synthesis of DOS esters

The investigation of the synthesis of DOS esters was started from the approach based on the acylation of the methyl glycoside of 3,4-dideoxy ribose (the synthesis

is described in Article I). In this approach the hydroxyl groups of a polyhydroxy carboxylic acid have to be protected prior to acylation by using routine techniques (a carbodiimide method and related ones). That is a significant inconvenience in several cases, *e.g.* for sterols, prostanoids, etc. Furthermore, an acidic cleavage of methyl glycoside appeared to be difficult to perform selectively, avoiding a simultaneous hydrolysis of ester group.

Therefore, for the synthesis of DOS esters (as potential lipase inhibitors) the *O*-alkylation of carboxylic acids with an α-bromo-ω-hydroxyaldehyde followed by lipase-catalytic steps in order to produce stereochemically pure compounds was chosen. The carboxylic acids used were – deoxycholic acid (a sterically demanding polyhydroxycarboxylic acid that is part of several bioactive molecules) and prostaglandin $F_{2\alpha}$ (a large flexible polyhydroxycarboxylic acid that is a physiologically highly active compound). In addition, two more carboxylic acids were involved: a THP-mandelic acid for the assignment of an absolute configuration of the deoxy sugar moiety (in the mandelate diastereomers) using NMR spectroscopy and 2-octenoic acid as a representative of nonchiral carboxylic acids.

DOS esters were synthesized by the *O*-alkylation of the above carboxylic acids with α-bromopentanal **99** and α-bromobutanal **103**. It should be noted that in order to obtain both diastereomers of DOS esters the time of incubation in the synthesis of α-bromopentanal **99** was prolonged which resulted in bromoaldehyde of low enantiomeric excess. α-Bromobutanal **103** was obtained as racemic product under the conditions used.

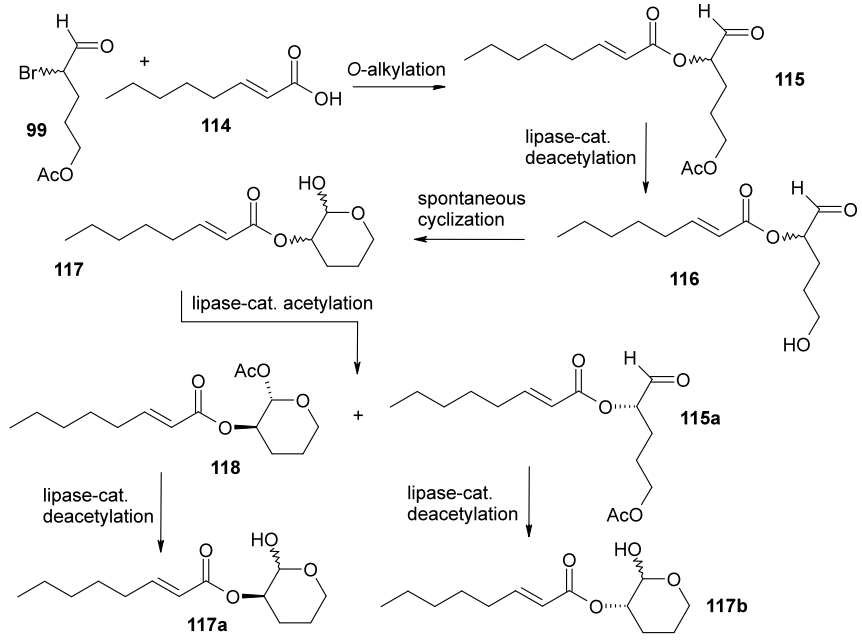
3.3.1. Synthesis of DOS esters with α-bromo-ω-acetoxypentanal (Article III)

The synthesis of DOS esters started with the *O*-alkylation of carboxylic acid with α-bromo-ω-acetoxypentanal (**99**) followed by the lipase-catalyzed deacetylation that was immediately followed by a spontaneous formation of cyclic hemiacetals. The subsequent reaction, the CALB-catalyzed acetylation, was a key-step in the resolution of stereoisomers.

The reactions will be outlined step-wise for every carboxylic acid to point out significant novel effects. Experimental data are presented in Article III and the Supporting Information file available online.[184]

(E)-2-Octenoic acid (OA, **114**) was chosen as a non-chiral compound to investigate a lipase-catalyzed kinetic resolution of 3,4-dideoxy ribose ester enantiomers via acetylation. All reactions with OA proceeded smoothly affording the desired products with good yield (Scheme 30). The *O*-alkylation of OA afforded ester **115**, adding water and CALB to the same reaction mixture resulted upon incubation in four stereoisomeric hemiacetals **117** (because of anomers). The latter step of this "one-pot-synthesis" is the cleavage of the terminal acetyl group followed by the spontaneous cyclization into hemiacetal.

A kinetic (dynamic) resolution of hemiacetal stereoisomers **117** upon the stereoselective lipase-catalyzed acetylation afforded two chemically different compounds of high enantiomeric excess: aldehyde **115a** and acetylated *trans*-hemiacetal **118** which were separated by chromatography over silica. The stereochemistry of deoxy sugar moieties corresponding to these chemically different compounds was determined by the NMR investigation of the products of analogous synthesis: diastereomeric mandelic acid 3,4-dideoxy ribose esters (Scheme 34). The desired deoxy sugar esters of OA, **117a** and **117b**, were obtained with high *e.e.* after the lipase-catalyzed deacetylation.



Scheme 30. Synthesis of DOS esters of (E)-2-octenoic acid

Deoxycholic acid (**104**) was a good example of a rigid bulky molecule to test the capabilities of the lipase in kinetic resolution. Besides, bile acids are possible drug-candidates for several diseases.[183]

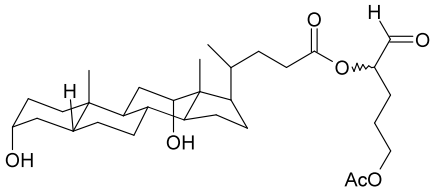
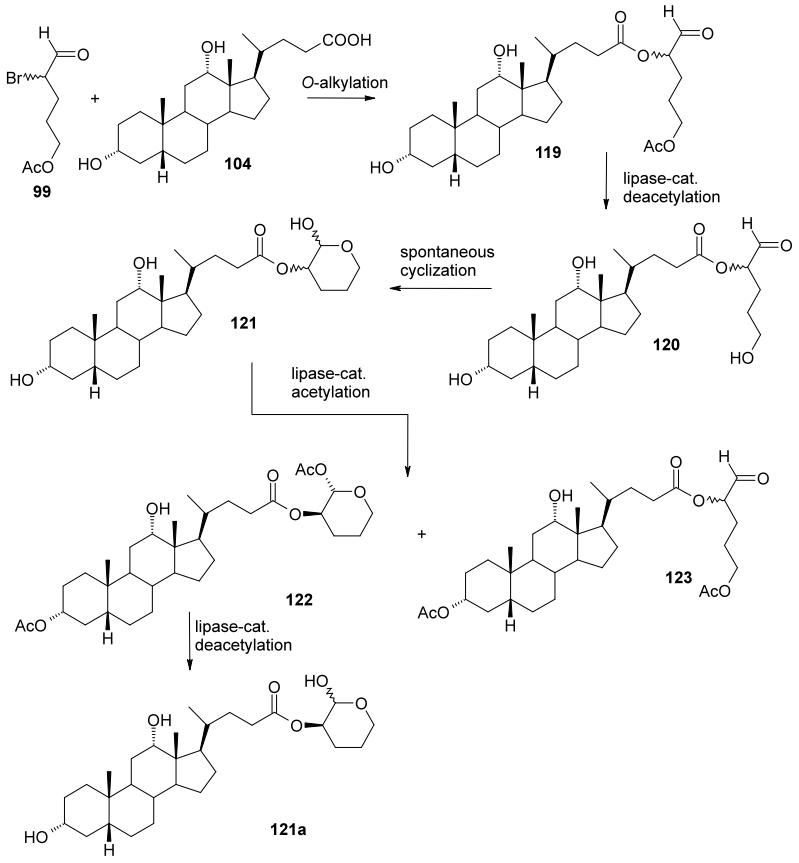


Figure 12. Conformational structure of DCA moiety in DOS ester

O-alkylation was conducted with unprotected hydroxyl groups of the latter (Scheme 31) as it was found that the aldehyde group of the target ester is not

capable of forming an (expectedly) undesired intramolecular hemiacetal cycle with any of the hydroxyl groups of DCA because these functional groups are too far away from each-other (Figure 12). The possible prevention of the lipase-catalyzed deacetylation of the *O*-alkylation product was expected by the formation of macrocyclic hemiacetals. Actually, the enzymatic deacetylation of ester **119** gave an ester **120** of the extended-chain deoxy sugar which underwent spontaneous cyclization and resulted in four diastereomeric hemiacetals **121** inseparable on silica.

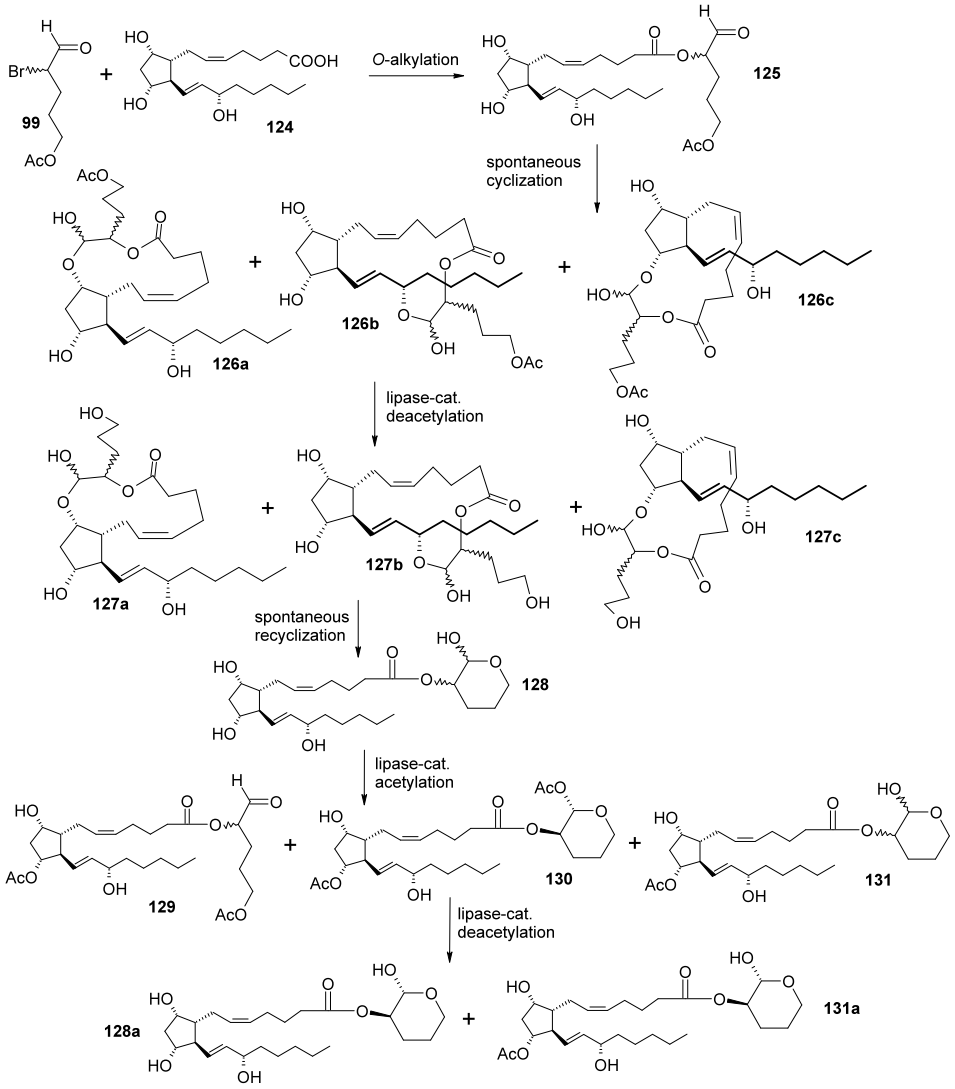


Scheme 31. Synthesis of DOS esters of DCA

The lipase-catalyzed acetylation afforded stereochemically pure acetylated hemiacetal **122** and aldehyde **123.** The latter was found (by NMR) to be a mixture of two diastereomers. During this reaction the usual amount of the enzyme preparation was used but interestingly the reaction rate slowed down remarkably rapidly. As a result, a product was produced only in poor yield and therefore several additional portions of the enzyme preparation were added periodically in order to reach about 95% conversion of the starting material. The inhibition of some lipases by bile acids (just themselves) has been described earlier.[185] In our case the deactivation of the lipase by any of the DCA esters involved needs further

investigation to find out which compound could be the real cause of the inhibition. As shown before, CALB catalyzed also the acetylation of the hydroxyl group at the 3rd carbon atom of the DCA skeleton. This acetyl group was cleaved in the next step, the enzymatic deacetylation that afforded an homogeneous DOS ester of DCA, **121a**.

PGs are polyhydroxy carboxylic acid with a large flexible skeleton. They are used as medicines for the treatment of several diseases. Therefore, they were found to be of especial interest for being included in the target molecules of potential lipase inhibitors. Moreover, the acetyl-derivatives of $PGF_{2\alpha}$ themselves proved, in our hands, to be potent candidates for lipase inhibitors.

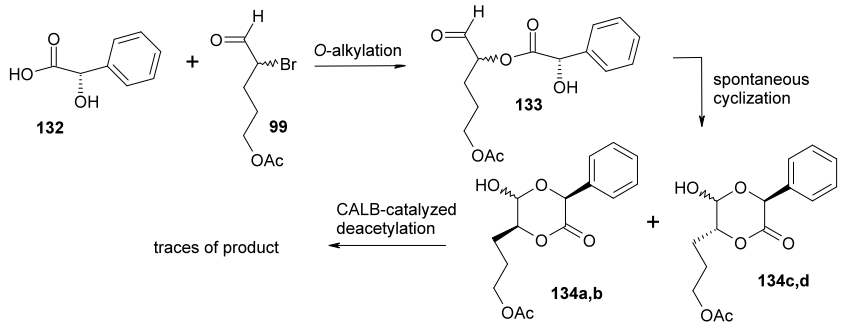


Scheme 32. Synthesis of DOS esters of $PGF_{2\alpha}$

$PGF_{2\alpha}$ bears three hydroxyl groups and the idea was to try *O*-alkylation without protecting these hydroxyls (Scheme 32). The reaction resulted in the formation of a complex mixture of products as established by TLC, consisting of a trace amount of the initial aldehyde **125** and most probably a mixture of several stereo- and regioisomeric macrocyclic hemiacetals **126a**, **126b** and **126c**. The following CALB-catalyzed deacetylation triggered a cascade of reactions where the cleavage of the terminal acetyl group initiates decyclization of macrocyclic hemiacetals **127** followed by the formation of thermodynamically more stable six-membered deoxy sugar cycle. This product was purified and characterized.

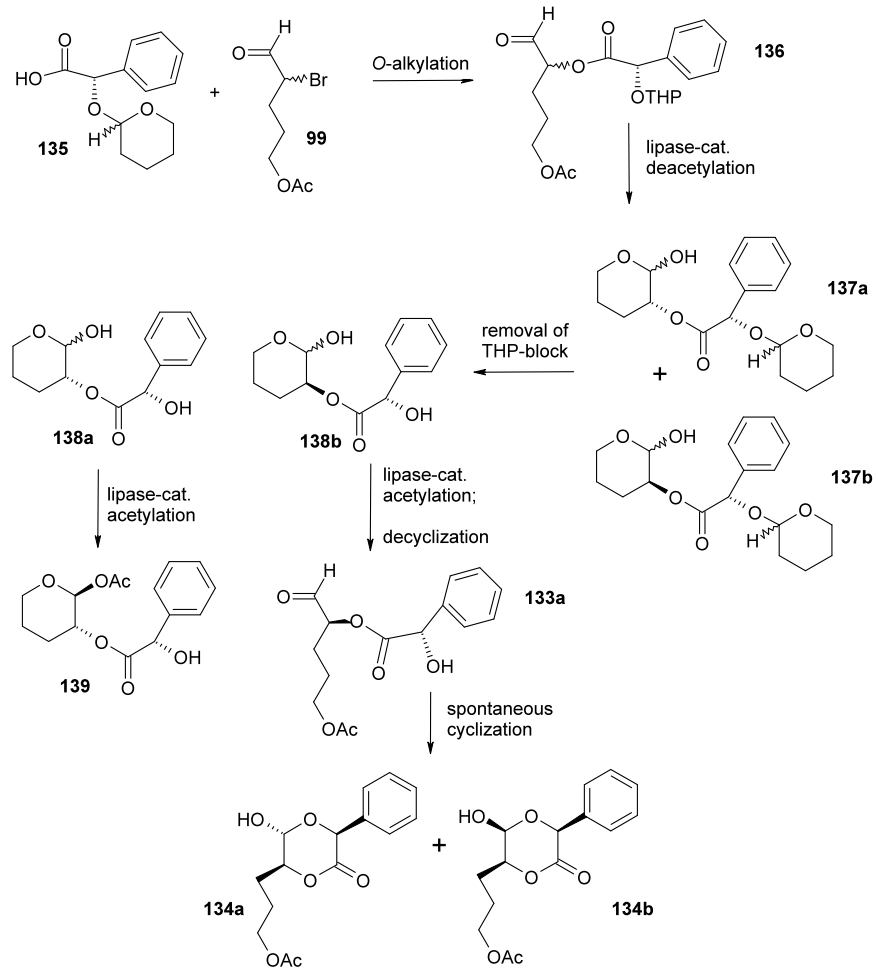
The lipase-catalyzed acetylation of the mixture of diastereomers **128** afforded acetylated hemiacetal **130** as a major product. A trace amount of aldehyde **129** with undetermined stereochemistry and stereoisomers of compound **131** were detected by NMR of the crude product. For some reason acetylation had not proceeded with the deoxy sugar moiety of compound **131**, only the hydroxyl group at the 11th carbon atom was acetylated. Also the previously mentioned acetylated hemiacetal **130** and aldehyde **129** were acetylated at the PG skeleton at the 11th carbon atom when the other hydroxyls at the 9th and 15th positions were intact. The final step, the deacetylation of **130** yielded stereochemically pure DOS esters **128a** and additionally **131a** where the hydroxyl group at the 11th carbon atom was still acetylated. The reaction conditions were not optimized and therefore deacetylation was incomplete, but the reaction had to be terminated as the lipase started to slowly hydrolyze the ester bond of the carboxylic acid of PG.

(*S*)-Mandelic acid (**132**, MA) was included because it enables the determination the absolute configuration of the deoxy sugar moiety of diastereomeric esters by using NMR. Preliminary testing showed that the protection of the alcoholic hydroxyl group of MA is necessary. Otherwise the aldehyde group in compound **133** (the product of *O*-alkylation of unprotected MA with α-bromopentanal **99**) immediately gave a stable 1,4-dioxane ring upon hemiacetalization with an alcoholic hydroxyl group of MA (Scheme 33). The lipase-catalyzed selective deacetylation of such compounds (**134**) is almost impossible even during a long incubation time (only traces of the product were detected before the deactivation of the enzyme) making this derivative one candidate compound in search for potential lipase inhibitors.



Scheme 33. O-alkylation of unprotected MA followed by the lipase-catalyzed deacetylation

The alcoholic hydroxyl group of MA was protected with the THP group.[186] The THP-protected mandelic acid (THPMA) was *O*-alkylated with α-bromopentanal **99** and the resulting ester **136** was deacetylated by lipase in an "one-pot synthesis" (Scheme 34). DOS esters **137a** and **137b** (both mixtures of four stereoisomers) can be separated on silica gel according to the absolute configuration at the C_2 of the deoxy sugar moiety. A separate removal of the THP protecting group from the products and the flash chromatography afforded the desired pure mandelates **138a** and **138b**.



Scheme 34. Synthesis of DOS esters of MA

The absolute configurational assignment of mandelates **138a** and **138b** was based on the differential shielding effects caused by the phenyl group in NMR spectra. The determined absolute configuration is in agreement with the prognosticated stereochemistry of the lipase-catalyzed acetylation of mandelates **138a** and **138b**,

occurring in accordance with the Kazlauskas rule. (2'*R*)-Isomer **138a** afforded a pure *trans*-acetylated hemiacetal **139** when (2'*S*)-isomer **138b**, with an unfavoured configuration for the lipase, underwent a novel three-step cascade reaction. The cascade started with the lipase-catalyzed decyclization of the deoxy sugar moiety (1) followed by an immediate acetylation of the terminal hydroxyl group (2). After that the aldehyde formed gave a new cycle with the MA hydroxyl group (3) affording hemiacetal **134a** with a certain amount of isomeric **134b**. As the lipase acetylated 2'*R*-isomer much faster (about 10 times) than 2'S-isomer it suggests a good possibility for a kinetic resolution of stereoisomers.

In summary, a desired novel stereoselective chemoenzymatic approach for the synthesis of stereochemically pure deoxy sugar esters was elaborated and the pure enantiomeric (2'*R*)-3,4-dideoxy ribose esters were obtained with all carboxylic acids used. The lipase-catalyzed acetylation also afforded a stereochemically pure (2'*S*)-3,4-dideoxy ribose ester of OA, a mixture of diastereomers of the aldehyde in the case of DCA and 1,4-dioxane hemiacetals for MA.

The target DOS esters described in Article III are the first representatives of a novel type of organic compounds.

In interpreting the results of this work the following observations should be taken into consideration:

1. The protection of hydroxyl groups is not necessary for some carboxylic acids (PG, DCA), but for mandelic acid this is obligatory because an intramolecular cyclization (hemiacetalization) in the *O*-alkylation product leads to the formation of products that deactivate lipase (that, in turn, makes impossible selective deacetylation of these products);
2. The lipase-catalyzed selective deacetylation that follows *O*-alkylation should be monitored carefully because the prolonged incubation of the DOS ester under basic conditions (in a "one-pot" procedure) could lead to the partial hydrolysis of the ester;
3. The optimization of reaction conditions could improve yields in some cases.

3.3.2. Synthesis of DOS esters with α-bromobutanal

Sugars as well as deoxy sugars form two types of thermodynamically favorable cyclic hemiacetals, *i.e.* pyranoses and furanoses. If both of them can be formed, then usually the pyranose form prevails because of its higher stability. The five- and six-membered rings significantly differ in molecular geometry and, therefore, also in accessibility of the corresponding hydroxyl groups to enzymes.

The ratio of sugar or DOS derivatives in the furanose or pyranose form has been observed to change along the lipase-catalyzed acetylation. The assistance of lipase in this process has been suggested.

In the current work, the lipase-catalyzed acetylation of the hemiacetal moiety of several pyranose DOS esters has been successfully used for the kinetic resolution of diastereomers. An important question is whether this technique could also be

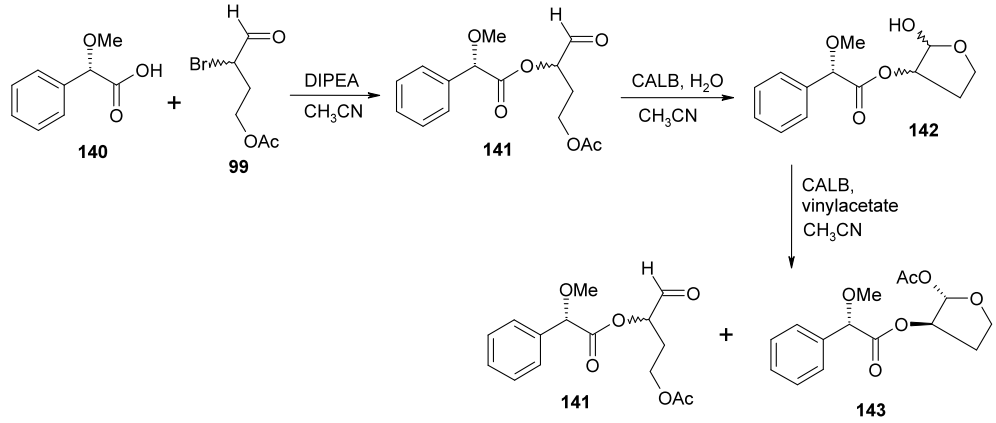
employed for the resolution of the stereoisomers of furanose DOS esters. Preliminary investigations gave promising results. Unlike the lipase-catalyzed acetylation of α-bromo-ω-hydroxybutanal in hemiacetal form, which results solely in the formation of an extended-chain acetoxyaldehyde, the acetylation of the corresponding DOS esters gave a cyclic hemiacetal ester in high diastereomer excess (>90%).

The synthesis of furanose DOS esters using α-bromo-ω-acetoxybutanal (**103**) was carried out using the same synthetic approach as when using α-bromo-ω-acetoxypentanal (**99**) in Article III.

The carboxylic acids used for the synthesis of deoxy furanose esters were methoxyphenylacetic acid (MPA, **140**) and deoxycholic acid.

MPA was chosen because it allows the determination of the stereochemistry of the products of lipase catalyzed reactions by using NMR[187] and it has a protected hydroxyl group. The *O*-alkylation of MPA and the following lipase catalyzed deacetylation of the aldehyde derivative **141** formed was carried out according to the protocol A given in Article III. The formation of ester **141** was fast and after adding the enzyme and the nucleophile (water) in 25 hours the lipase-catalyzed deacetylation afforded hemiacetals **142** inseparable on silica gel. If *O*-alkylation proceeded without the formation of a significant amount of by-products, then the enzymatic deacetylation gave also about 30% of non-desired compound(s) which need to be studied in more detail in the future.

The subsequent lipase-catalyzed acetylation gave the desired (2'*R*)-deoxy furanose ester in approximately 30% yield as an acetylated *trans*-hemiacetal **143** in good diastereomeric excess along with a diastereomeric mixture of aldehyde **141** in 72 hours.



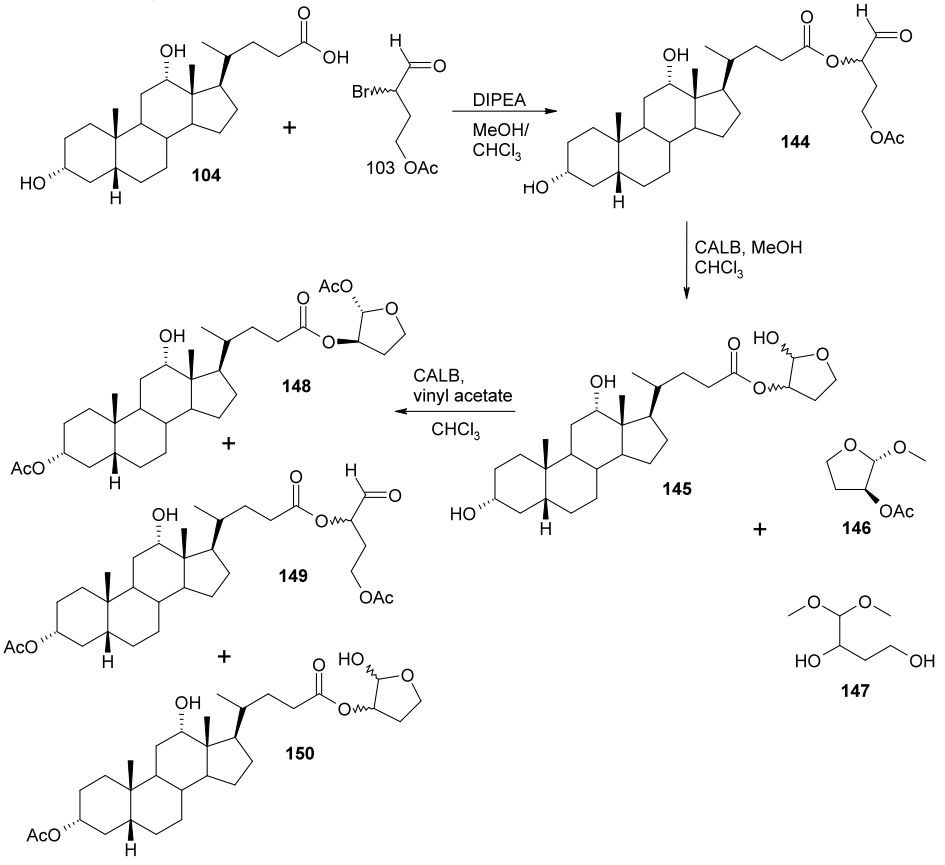
Scheme 35. Synthesis and the kinetic resolution of diastereomers of furanose DOS esters of MPA

The *O*-alkylation of DCA was performed (Scheme 36) in a mixture of chloroform and methanol as reaction medium. The reaction gave aldehyde **144** along with a

certain amount of unidentified by-products in 24 hours. In this case the removal of the solvent before adding the enzyme and a nucleophile was necessary because the concentration of methanol was too high for the lipase-catalyzed deacetylation as proven in preliminary tests. In the lipase-catalyzed deacetylation reaction chloroform was used as a solvent and 2% of methanol as a nucleophile. A 120-hour incubation afforded the desired hemiacetals **145**. Also several by-products were formed, of them, the major fraction consisted of compounds **146** and **147** and a certain amount of DCA methyl ester.

The resolution of diastereomers by the lipase-catalyzed acetylation of hemiacetals **145** resulted in an acetylated cyclic *trans*-hemiacetal **148**, a stereoisomeric mixture of aldehyde **149** and a cyclic hemiacetal ester **150** in which only the hydroxyl group at the 3rd carbon atom of the DCA skeleton was acetylated.

As already mentioned above, the lipase-catalyzed acetylation of bromohydroxy THF (**100**) afforded only an acetylated open-chain aldehyde **103.** While after the transformation of the compound **100** into the furanose DOS ester **145** the lipase-catalyzed acetylation of the latter affords, besides the aldehyde **149**, also the stereochemically pure acetylated cyclic hemiacetal **148** in a significant amount (more than 30%).



Scheme 36. Synthesis of DOS esters of DCA

In conclusion, the synthesis of DOS esters starting from α-bromobutanal with the carboxylic acids (MPA and DCA) tested was successful since the desired pure stereoisomeric esters with a (2'*R*)-configuration were obtained. However, the reactions involving furanose DOS were more than those with pyranose ones due to the formation of by-products, and lower yields. Also a specific effect stands out in comparison with pyranose and furanose esters. In the lipase-catalyzed acetylation of pyranose hemiacetals the first product formed is always a cyclic acetylated hemiacetal. Thereafter, the aldehyde starts slowly to form. In the case of furanose hemiacetals, the lipase-catalyzed acetylation produces the aldehyde first and then the cyclic product is formed.

4. EXPERIMENTAL

4.1. Synthesis of CA and DCA derivatives

Chemical esterification of CA and DCA. 0.2 eq of pTSOH was added to the mixture of 1 eq of CA or DCA, 10 eq of EtOH and chloroform. The reaction mixture was stirred at rt for 6 days and monitored by TLC. The reaction was stopped by adding Et_3N, the mixture was evaporated and the product purified by column chromatography over silica.
105: TLC: R_f=0.49 (20% isopropanol/hexane); yield 92%
111: TLC: R_f=0.29 (20% isopropanol/hexane); yield 93%

CALB-catalyzed acetylation of CA, DCA and their esters. Acid or ester (1 eq) was dissolved in the mixture of vinyl acetate (20 eq) and chloroform (forming 0.15-0.2 M solution), then Novozym 435 was added (400 mg for 1 mmol of substrate). The reaction mixture was kept at rt without stirring for 72 h for acids and 24 h for esters and monitored by TLC. Then the enzyme preparation was filtered off, the mixture was evaporated and purified by column chromatography over silica. The yield for all acetylated products was between 50-60%.
106: TLC: R_f=0.25 (20% isopropanol/5% water/hexane)
107: TLC: R_f=0.63 (20% isopropanol/5% water/hexane)
112: TLC: R_f=0.38 (20% isopropanol/5% water/hexane)
113: TLC: R_f=0.50 (20% isopropanol/5% water/hexane)

Chemical acetylation of DCA ester. DCA ester (1 eq) was dissolved in dichloromethane resulting in 0.1 M solution; 3 eq of acetic anhydride and 5 eq of pyridine were added to the solution. The reaction was monitored by TLC for 6 days (144 h).

Protecting hydroxyl group at 12th carbon atom of 3-acetyl-DCA ethyl ester with THP-block. 1 eq of ester **107** was dissolved in dichloromethane, 5 eq of DHP and 0.1 eq of pTSOH were added. The reaction was monitored by TLC, after 15 minutes reaction was stopped by adding Et_3N. The mixture was evaporated and purified by column chromatography over silica. The yield of the desired product was 85%. The product was determined by NMR but full assignment was not done due to the complicated spectra caused by THP-group.

Lipase-catalyzed deacetylation of the derivates of CA and DCA. Substrate (20-50 mg) was added to a mixture of a solvent and a nucleophile at different concentrations (2% or 6 eq; 50% or 230 eq; or just a nucleophile) resulting in a 0.05 M solution, then 100 mg of Novozym 435 was added per 0,108 mmol of the

substrate without stirring at rt. The reaction mixture was incubated for 240 h at rt. The results are summarized in Table 1.

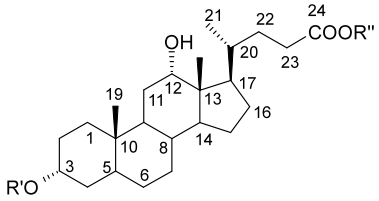


Table 2. Chemical shifts of DCA and CA derivatives

	105	**106**	**107**	**112**
C_1	35.17	34.83	34.82	34.83
C_2	30.43	26.42	26.44	28.14
C_3	71.75	74.31	74.24	74.38
C_4	36.38	32.11	32.10	35.12
C_5	42.02	41.81	41.80	41.12
C_6	27.08	26.91	26.90	34.26
C_7	26.08	25.97	25.97	68.36
C_8	35.07	35.04	35.02	39.46
C_9	33.60	33.57	33.58	26.56
C_{10}	34.07	34.09	34.08	34.68
C_{11}	28.60	28.60	28.62	26.52
C_{12}	73.10	73.20	73.09	73.10
C_{13}	46.45	46.45	46.45	46.48
C_{14}	48.22	48.20	48.23	41.86
C_{15}	23.61	23.57	23.56	23.09
C_{16}	27.42	27.41	27.39	27.40
C_{17}	47.29	47.26	47.34	47.05
C_{18}	12.70	12.69	12.69	12.48
C_{19}	23.12	23.06	23.07	22.43
C_{20}	35.98	35.94	35.93	35.15
C_{21}	17.27	17.23	17.29	17.22
C_{22}	30.84	30.96	30.84	30.63
C_{23}	31.31	30.64	31.29	31.03
C_{24}	174.27	174.44	174.23	179.90
$C_{1'}$	-	170.78	170.69	177.00
$C_{2'}$	-	21.40	21.42	21.40
$C_{1''}$	60.18	-	60.18	-
$C_{2''}$	14.23	-	14.22	-

4.2. The synthesis of DOS esters with bromobutanal

The experimental data for α-bromo-ω-acetoxybutanal (**103**) are given in the Article I.

The synthesis of DOS esters of MPA. *O*-alkylation of MPA and the following lipase-catalyzed reactions were carried out accordingly to the General procedures A and B given in the Article III.
The reaction time of *O*-alkylation was 3 hours.
141: TLC R_f= 0.14 (30% EtOAc/PE)
The incubation time for the lipase-catalyzed deacetylation was 25 hours. The yield of the product **142** was 25% (for two reactions).
142: TLC: R_f= 0.36 (50%EtOAc/PE)
The incubation time for the lipase-catalyzed acetylation was 72 hours. The yield of hemiacetal ester **143** was 30%.
143: TLC: R_f= 0.38 (30%EtOAc/PE)

The synthesis of DOS esters of DCA. Using DCA as a carboxylic acid General procedure A was modified. The solvent was exchanged for chloroform/MeOH mixture (1:1) in *O*-alkylation reaction. Afterwards the reaction mixture was washed with 1M $NaHSO_4$ and brine, dried on Na_2SO_4 and evaporated. The crude product was used for CALB-catalyzed deacetylation in chloroform containing 2% of MeOH as nucleophile. Otherwise the General procedures A and B given in the Article III were followed.
The reaction time of *O*-alkylation was 24 hours.
144: TLC R_f= 0.40 (100% EtOAc)
Side-product of *O*-alkylation: TLC R_f= 0.57 (100% EtOAc)

The incubation time for the lipase-catalyzed deacetylation was 120 hours. Side-products formed: methyl ester of DCA (TLC: Rf=0.23, 100% EtOAc), 4,4-dimethoxybutane-1,3-diol (**147**) and methyl deoxy furanoside **146**. The yield of the product **145** was 29% (for two reactions).
145: TLC: R_f= 0.19 (100% EtOAc)

The incubation time for the lipase-catalyzed acetylation was 96 hours (full acetylation was not achieved).
148: TLC: R_f= 0.79 (20% iPrOH/PE)
149: TLC: R_f= 0.71 (20% iPrOH/PE)
150: TLC: R_f= 0.61 (20% iPrOH/PE)

Identification of the products of the above-described preliminary syntheses of furanose DOS esters was performed by dr. T. Pehk by using NMR spectroscopic investigation of the crude products (as well as those after chromatographic fractionation) in order to discover also minor side products formed.

CONCLUSIONS

1. A chemoenzymatic stereoselective approach for the synthesis of pure stereoisomers of deoxy sugar esters was developed. The lipase-catalyzed acetylation of hemiacetals was used for the resolution of stereoisomers.
2. A novel lipase-catalyzed decyclization reaction of cyclic hemiacetals was described. The different behavior of pyranose and furanose hemiacetals in this reaction was established. A mechanism for this lipase-catalyzed reaction was suggested.
3. The chemical acetylation of bromohydroxy-THP and bromohydroxy-THF was investigated, the change in the ratio of *cis*- to *trans*-isomers was observed to take place, an explanation to this phenomenon was proposed.
4. It was shown that deoxycholic acid is a proper candidate among bile acids for the synthesis of deoxy sugar derivatives as potential inhibitors of lipases.
5. In order to find lipase inhibitors and suitable structures for further modification preliminary tests with saponins, alkaloids, flavonoids, some prostaglandins and their derivatives were carried out. The best inhibitors belonged to the group of saponins.
6. Some prostaglandin derivatives displayed controversial effects activating the lipase-catalyzed hydrolysis at lower concentrations and inhibiting the same reaction at higher concentrations. The prostaglandin $F_{2\alpha}$ was chosen for further derivatization with the deoxy sugar moiety.
7. Four different carboxylic acids were tested to synthesize DOS esters with α-bromopentanal. At least one of the stereoisomers of the target compound was obtained as a stereochemically pure material in all cases.
8. Two carboxylic acids were also tested to synthesize DOS esters with α-bromobutanal. Preliminary results indicate the possibility to use the same method as with α-bromopentanal in order to obtain stereochemically pure compounds.

There are several possibilities of carrying on this investigation in the future.

1. The by-products of the synthesis of DOS esters with α-bromobutanal should be studied thoroughly.
2. The development of potential lipase inhibitors is promising due to the intriguing findings during the synthesis of DOS esters.
3. The lipase-catalyzed derivatization of sugars has already been started in order to find out possible isomerization reactions that may be indicative to corresponding decyclization reactions.

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ARTICLE I

Villo, L.; Metsala, A.; Parve, O.; Pehk, T. Chemical versus enzymatic acetylation of α-bromo-ω-hydroxyaldehydes: decyclization of hemiacetals by lipase. *Tetrahedron Letters*, **2002**, 43, 3203-3207.

ARTICLE II

Ruiz, C.; Falcocchio, S.; Xoxi, E.; Villo, L.; Nicolosi, G.; Pastor, F. I. J.; Diaz, P.; Saso, L. Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Journal of Molecular Catalysis B: Enzymatic*, **2006**, 40, 138-143.

ARTICLE III

Villo, L.; Danilas, K.; Metsala, A.; Kreen, M.; Vallikivi, I.; Vija, S.; Pehk, T.; Saso, L.; Parve, O. Synthesis of Deoxy Sugar Esters: A Chemoenzymatic Stereoselective Approach Affording Deoxy Sugar Derivatives Also in the Form of Aldehyde. *Journal of Organic Chemistry*, **2007**, 72, 5813-5816.

SUMMARY

In this work the main fields under investigation were stereoselective chemoenzymatic synthesis of deoxy sugars and their derivatives as well as synthetic applications of novel catalytic properties of lipases. Lipase-catalyzed reactions were investigated along with the goal to find novel potential lipase inhibitors.

The main goal of the work was the synthesis of stereochemically pure deoxy sugar esters. Firstly, the chemoenzymatic synthesis of the deoxy sugar moieties of tetrahydropyran and tetrahydrofuran rings was carried out. The enzymatic acetylation of the cyclic hemiacetals of THP and THF type revealed a novel lipase-catalyzed decyclization affording open-chain α-bromo-ω-acetoxy aldehydes.

Unexpectedly, also chemical acetylation of the cyclic hemiacetals of α-bromoaldehydes resulted in the formation of the products with changed ratio of isomeric acetates compared to the starting equilibrial mixture of hemiacetals. A possible explanation of such an effect has been presented.

α-Bromopentanal and α-bromobutanal were used for the *O*-alkylation of carboxylic acids (deoxycholic acid, prostaglandin $F_{2\alpha}$, 2-octenoic acid and mandelic acid) followed by the lipase-catalyzed deacetylation of the terminal acetyl group which yielded the esters of cyclic hemiacetal compounds. The key-step was the resolution of stereoisomers by the lipase-catalyzed acetylation affording at least one of the desired pure stereoisomers in case of all the carboxylic acids tested.

In the synthetic process some novel intriguing cascades of reactions triggered by initial lipase-catalyzed reaction were observed with prostaglandin and mandelic acid derivatives.

The second part of the work focused on the investigation of the lipase-catalyzed derivatization of the carboxylic acids used in the ester synthesis. Preliminary tests to study the influence of some compounds on the catalytic activity of lipases were performed. Several natural products were studied, among them also prostaglandins and bile acids which were used as “carrier molecules” for the synthesis of deoxy sugar esters as potential lipase inhibitors. It should be emphasized that the synthesis of such compounds provided some derivatives that remarkably decreased the rate of the lipase-catalyzed transesterification or hydrolysis reactions. These derivatives will be investigated further for their antilipolytic properties.

KOKKUVÕTE

Doktoritöö peamisteks uurimisaladeks olid desoksüsuhkrute ja nende derivaatide stereoselektiivne kemoensümaatiline süntees ning lipaasi uudsete katalüütiliste omaduste rakendus sünteesis. Lipaaskatalüütilisi reaktsioone uuriti ka eesmärgiga leida uusi lipaasi inhibiitoreid.

Esmane ülesanne oli stereokeemiliselt puhaste deoksüsuhkru estrite süntees. Esimese etapina sünteesiti kemoensümaatiliselt tetrahüdropüraani (THP) ja tetrahüdrofuraani (THF) tsüklitest lähtudes desoksüsuhkru „ehitusblokid" kasutamiseks estrite sünteesil. THP ja THF tsükliliste poolatsetaalide ensümaatilisel atsetüleerimisel kirjeldati uudne lipaaskatalüütiline detsükliseerimisreaktsioon, mille produktidena saadi avatud ahelaga α-bromo-ω-atsetoksüaldehüüdid.

Üllatuslikult andis ka α-bromoaldehüüdide tsükliliste poolatsetaalide keemiline atsetüleerimine produktid, mille isomeersete atsetaatide suhe võrreldes lähteaine poolatsetaalide tasakaalulise seguga oli muutunud. Töös on antud sellele nähtusele ka tõenäoline seletus.

α-Bromopentanaali ja α-bromobutanaali kasutati karboksüülhapete (desoksükoolhappe, prostaglandiin $F_{2\alpha}$, 2-okteenhappe ja mandelhappe) *O*-alküleerimisel, millele järgnes aldehüüdi terminaalse hüdroksüülrühma selektiivne lipaaskatalüütiline deatsetüleerimine, mis omakorda andis tsüklilise poolatsetaali estrid. Selle sünteesiskeemi võtme-etapiks oli stereoisomeeride lahutamine lipaaskatalüütilise atsetüleerimise teel, mille kaudu saadi kõikide kasutatud karboksüülhapete korral vähemalt üks stereoisomeerselt puhas desoksüsuhkru ester.

Prostaglandiini ja mandelhappe derivaatide sünteesi käigus kirjeldati huvitavad ensüümi poolt vallandatud kaskaadreaktsioonid.

Töö teises osas uuriti estrite sünteesil kasutatud karboksüülhapete lipaaskatalüütilist derivatiseerimist ning viidi läbi eelkatsed, kus mõõdeti mõningate ühendite mõju lipaasi katalüütilisele aktiivsusele. Uuriti mitmeid looduslikke ühendeid, nende hulgas ka prostaglandiine ja sapihappeid, mida kasutati kui „kandja-molekule" desoksüsuhkrute estrite kui lipaasi inhibiitorite sünteesil. Tähelepanu väärib asjaolu, et selliste ühendite sünteesil leitigi mõned derivaadid, mis vähendasid oluliselt lipaaskatalüütilise transesterdamise või hüdrolüüsi reaktsiooni kiirust. Leitud ühendeid uuritakse edasi, et määrata nende antilipolüütilisi omadusi.

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1. Villo, L.; Danilas, K.; Metsala, A.; Kreen, M.; Vallikivi, I.; Vija, S.; Pehk, T.; Saso, L.; Parve, *O*. Synthesis of Deoxy Sugar Esters: A Chemoenzymatic Stereoselective Approach Affording Deoxy Sugar Derivatives Also in the Form of Aldehyde. *Journal of Organic Chemistry* **2007**, 72, 5813-5816.
2. Ruiz, C.; Falcocchio, S.; Xoxi, E.; Villo, L.; Nicolosi, G.; Pastor, F. I. J.; Diaz, P.; Saso, L. Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Journal of Molecular Catalysis B: Enzymatic* **2006**, 40, 138-143.
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Publications

1. Villo, L.; Danilas, K.; Metsala, A.; Kreen, M.; Vallikivi, I.; Vija, S.; Pehk, T.; Saso, L.; Parve, O. Synthesis of Deoxy Sugar Esters: A Chemoenzymatic Stereoselective Approach Affording Deoxy Sugar Derivatives Also in the Form of Aldehyde. *Journal of Organic Chemistry* **2007**, 72, 5813-5816.
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APPENDIX I

Manuscript

Influence of Prostaglandin Derivates on the Catalytic Activity of *Candida rugosa* Lipase

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Abstract
Influence of different prostaglandins and their derivates on the catalytic activity of *Candida rugosa* lipase in hydrolysis reaction is evaluated by using a simple colorimetric assay. Of the substances tested, the derivatives of prostaglandins $F_{2\alpha}$ were the most potent inhibitors and activators. 9,15-Diacetyl-$PGF_{2\alpha}$ and 9,11,15-triacetyl-$PGF_{2\alpha}$ methyl ester revealed the highest inhibitory effect on lipase-catalyzed hydrolysis.

Keywords
lipase-catalyzed hydrolysis; colorimetric assay; prostaglandin; *Candida rugosa* lipase; inhibition; activation

Introduction
Lipases are glycerol ester hydrolases (E.C. 3.1.1.–) which hydrolyze acylglycerols to liberate fatty acids and glycerol. Several lipases produced by microbial pathogens play an important role in the development of infectious diseases. It has been described that a variety of extracellular enzymes such as proteases, lipases and phospholipases, elaborated by *Helicobacter pylori* (HP) cause the weakening of the integrity of gastric mucus coat by hydrolyzing endogenous lipids.[†] HP plays a central role in the development of gastritis, peptic ulcer disease and possibly

[†] Slomiany, B. L.; Slomiany, A. Mechanism of *Helicobacter pylori* pathogenesis: focus on mucus. *Journal of clinical gastroenterology* **1992**, 14, 114-121.

gastric carcinoma.[‡] Therefore, agents capable of counteracting the detrimental effects of HP, *i.e.* inhibiting lipolytic enzymes, are gaining in importance in the treatment of a gastric disease. Also *Propionibacterium acnes* lipase and its inhibition by antiacne compounds have been studied. The fatty acids produced by *P. acnes* lipase from sebaceous triglycerides induce severe inflammation.[§]

In addition, the inhibitors of lipolytic enzymes could contribute to a better understanding of the mechanism of the action of the latter and, as a result, to design new enzymes. This information is valuable because of an outstanding biotecnologycal potential of lipolytic enzymes.

Prostaglandins (PGs) belong to the class of lipids. They are synthesized from polyunsaturated fatty acids (arachidonic acid, etc) by cyclooxygenase. They serve as signaling molecules and stimulate a variety of responses in their target cells, including blood platelet (de)aggregation, inflammation and smooth-muscle contraction. Several prostaglandins and their derivatives are used as medicines.[**] For example, misoprostol, a PGE_1 analogue, is an agent for the prevention and treatment of the peptic ulcer in patients who take non-steroidal anti-inflammatory drugs.

The direct effect of PGs on lipases is not known very well yet. Some investigations *in vivo* and *in vitro* were carried out in the 60s and 70s. The study of the influence of PGEs, PGAs and $PGF_{2\alpha}$ on the pancreatic lipase demonstrated the inhibitory effect of PGE_1 and PGA_1; PGE_2 and $PGF_{2\alpha}$ to be low. The direct action of PGE_1 on pancreatic lipase and an interference of PGE_1 with the binding capacity or turnover rate of the enzyme have been suggested.[††] PGE_1 has been reported to inhibit a lipase in human serum[‡‡] and hormone-sensitive lipase in adipose tissue.[§§] In contrast, the activity of myocardial lipoprotein lipase (LPL) was increased by PGE_1 *in vivo*[***] and the latter had no effect *in vitro* on the LPL in human plasma.[†††]

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[§§] Stock, K.; Westermann, E. Inhibition of lipase by α- and β-sympatholytic agents, nicotinic acid, and prostaglandin E_1. *Archiv for Experimentelle Pathologie und Pharmakologie* **1966**, 254, 334-354.

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In the current work, changes in the catalytic activity of *Candida rugosa* lipase (CRL) caused by different prostaglandins and their derivates evaluated by a simple colorimetric assay[‡‡‡] will be reported.

The investigation had the following objectives:

1. When designing novel inhibitors of lipases by means of introducing a serine trap into the prostanoid molecule background information about the influence of the prostanoid residue on the catalytic activity of a lipase is needed. CRL, a well-known enzyme widely used in biotechnological applications,[§§§] was used in the work as a model lipase.
2. In the synthetic schemes of potential inhibitors the lipase-catalyzed selective protection of functional groups of PGs was used. Another aim of the investigation was to provide information about the feasibility of the above steps, *e.g.* influence of the starting prostanoids as well as products on the catalytic activity of the lipase to be used in biocatalytic steps.

Experimental

Reagents. Methanol was purchased from Lab-Scan. *p*-Nitrophenol (*p*-NP), *p*-nitrophenyl laurate (*p*-NPL), vinyl acetate, pyridine, dMAP, acetic anhydride, acetonitrile and 2-propanol were purchased from Fluka, *Candida rugosa* lipase and misoprostol from Sigma. PGE_1, PGE_2, PGD_2, $PGF_{2\alpha}$ were purchased from Kevelt. Novozym 435 was a kind gift from Novozymes AS. All reagents were used without further purification.

Synthesis of prostaglandin derivates

General procedure A (enzymatic esterification): 2.62 mmol of prostaglandin (or its derivative) was dissolved in 25 ml of benzene, then 1 ml of methanol and Novozym 435 (900 mg) were added to the mixture. The reaction was kept at rt without stirring for 72 hours. The immobilized enzyme was filtered off, the solution was evaporated and the product purified by column chromatography over silica. The yield of **2**, **6** and **8** was 92, 93 and 83% respectively.

General procedure B (enzymatic acetylation): 0.35 mmol of the prostaglandin derivative was dissolved in 6 ml of benzene, then and 2 ml of vinyl acetate and 260 mg of Novozym 435 were added to the mixture. The reaction was kept at rt without stirring for 60 hours. The immobilized enzyme was filtered off, the solution was

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[†††] Boehle, E.; Doebert, R.; Merkl, I. M. Metabolic activities of prostaglandins. III. Effect of prostaglandin E_1 and insulin on heparin-induced triglyceride hydrolysis. *Zeitschrift fuer die Gesamte Experimentelle Medizin Einschliesslich Experimenteller Chirurgie* **1967**, 144, 285-299.

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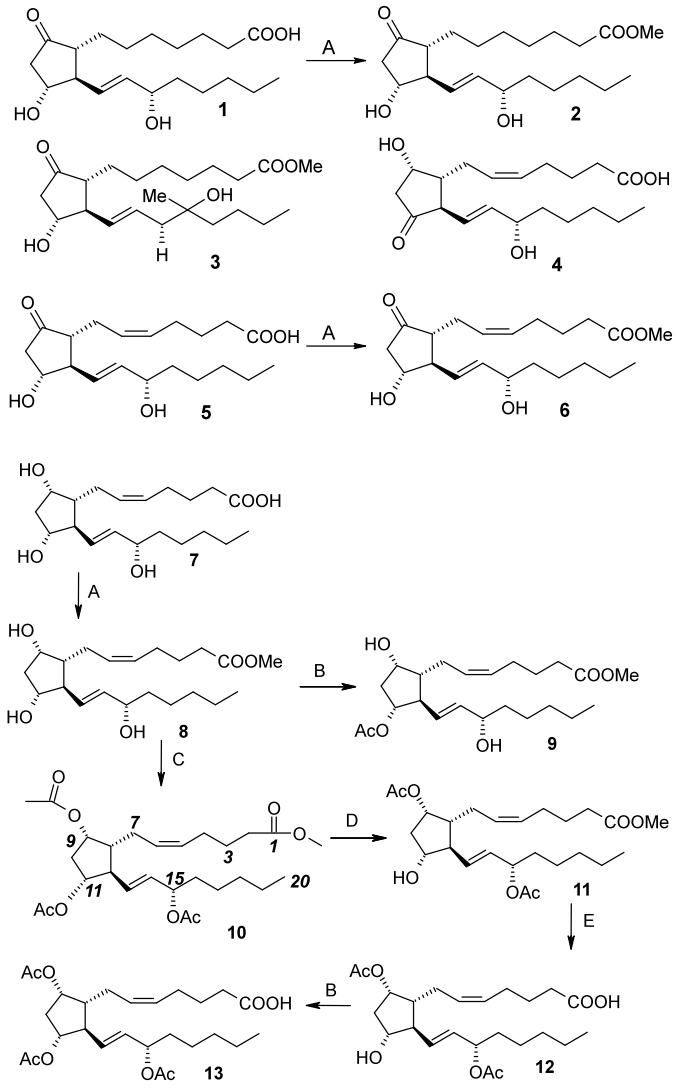
evaporated and the product purified by column chromatography over silica. The yield of **9** and **13** was 81 and 86% respectively



Scheme 1. Synthesis of prostaglandin derivatives. The structure of the prostanoids tested

Procedure C (chemical acetylation): 0.95 mmol of $PGF_{2\alpha}$ methyl ester (**8**) was dissolved in 19 ml of benzene; 2,3 ml of pyridine, 1,3 ml of acetic anhydride and 1 mg of dMAP were added on magnetic stirrer with a simultaneous cooling on a water bath at rt. The reaction was stirred at rt for 24 hours. To terminate the reaction methanol was added. The mixture was evaporated and purified by column chromatography over silica; 0.83 mmol of 9,11,15-triacetyl $PGF_{2\alpha}$ methyl ester (**10**) was gained (yield 87%).

Procedure D (enzymatic deacetylation): 0.85 mmol of 9,11,15-triacetyl-$PGF_{2\alpha}$ methyl ester (**10**) was dissolved in 38 ml of CH_3CN; 1.9 ml of methanol and 0.8 g of Novozym 435 were added. The reaction was kept at rt for 24 hours. The immobilized enzyme was filtered off, the solution was evaporated and the product purified by column chromatography over silica. The yield of PG derivative **11** was 95%.

*Table 1. ^{13}C NMR chemical shifts of PG derivatives **10**, **11** and **12***

C no	10		11		12	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
C1	173.8	-	173.9	–	178.2	–
C2	33.3	2.26	33.4	2.27	33.2	2.33
C3	24.6	1.65	24.7	1.65	24.4	1.67
C4	26.5	2.01	26.5	1.96/2.08	26.4	1.99/2.10
C5	129.9	5.31	129.9	5.29	129.6	5.33
C6	127.9	5.29	127.9	5.31	128.2	5.34
C7	24.8	2.00	24.8	2.06	25.1	2.08
C8	47.4	1.65	47.4	1.64	47.7	1.65
C9	74.2	5.04	74.3	5.07	74.4	5.11
C10	38.9	1.66/2.52	38.9	1.64/2.45	40.8	1.67/2.47
C11	77.7	4.48	77.7	3.91	76.4	3.95
C12	51.8	2.50	51.8	2.31	55.7	2.33
C13	132.1	5.50	132.1	5.49	133.6	5.52
C14	131.4	5.48	131.5	5.50	131.7	5.55
C15	73.9	5.18	74.0	5.17	74.9	5.21
C16	34.2	1.56/1.61	34.3	1.52/1.62	34.5	1.55/1.66
C17	24.7	1.25	24.7	1.28	24.8	1.31
C18	31.4	1.25	31.5	1.26	31.5	1.28
C19	22.4	1.20	22.5	1.27	22.5	1.30
C20	13.9	0.85	13.9	0.86	13.9	0.88
9AcC'	170.3	-	170.4	-	170.9	-
11AcC'	170.5	-	170.5	-	-	-
15AcC'	170.2	-	170.2	-	170.6	-
9AcC''	21.2	2.02	21.1	2.02	21.4	2.06
11AcC''	21.1	2.01	21.2	-	-	-
15AcC''	20.9	1.98	21.0	2.02	21.2	2.06
OCH_3	51.4	3.62	51.4	3.64	-	-

Procedure E (enzymatic ester hydrolysis): 0.82 mmol of 9,15-diacetyl-$PGF_{2\alpha}$ methyl ester (**11**) was dissolved in 30 ml of CH_3CN; 0.6 ml of water and 0.8 g of Novozym 435 were added. The reaction was kept at rt for 7 days. The immobilized enzyme was filtered off, solution was evaporated and purified by column chromatography over silica. The yield of PG derivative **12** was 63%.

Evaluation of the effect of prostaglandins on lipase activity

PGs were dissolved at several concentrations in methanol by a gentle agitation. The non-colored substrate *p*-NPL was dissolved in 20 mM 2-propanol by sonication for 3 minutes. A 1:10 (v/v) dilution in the phosphate–Triton X-100 buffer was prepared with a gentle agitation until an optically clear and stable emulsion was obtained. 50 µl of the mixture were dispensed into a 96-well microtiter plate and preincubated for 15 minutes at 37°C. The solution of enzyme (50 µl; 10^{-2} mg/ml) and PG at different concentrations, prepared in 50 mM phosphate buffer (pH 7 at 22°C) and preincubated for 15 minutes at 37°C, was added to the previously prepared substrate mixture to obtain 100µl of a final reaction mixture. The latter was incubated for a further 15 minutes at 37°C. The absorbance of each well at λ = 405 nm was measured using a microtiter plate reader to detect the release of *p*-NP, a yellow reaction product. Proper blanks were performed to subtract the absorbance that was not produced by *p*-NP.

Lipase inhibition or activation was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples, subtracting the absorbance of the reaction mixtures with proper blanks. The concentrations of PG yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) and a maximum lipase activation (A_{max}%) were calculated. Calculation was performed from the inhibition or activation percentage *vs*. concentration curves of prostaglandins by using regression analysis using the Sigma-Plot 8.0 software. All experiments were done at least in triplicate. Capric acid was used as an internal method control.

Results and discussion

In the present study, the effect of several prostaglandins and their derivates on CRL was investigated. Their potential role as a constituent of the molecule of an antilipolytic drug was evaluated by using a colorimetric assay. In this work, the direct effect of PGs on the fungal lipase of biotechnological interest has been described for the first time. In Table 2 the maximum solubility of the PG derivative (S_{max}), the concentrations of PGs leading to the lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}), and a maximum activation of lipase (A_{max}%) along with the corresponding concentration of PG are presented.

The compounds of PGE_1 group, including PGE_1 (**1**), its methyl ester **2** and a PGE_1 derivative – misoprostol (**3**), showed no inhibition of CRL. On the contrary, – at lower concentrations PGs **2** and **3** activated the hydrolysis of *p*-NPL up to 37%.

PGD_2 (**4**) had almost no influence on the rate of hydrolysis. PGE_2 (**5**) was also inactive, its methyl ester **6** showed no inhibitory effect. However, at lower concentrations of the latter hydrolysis was increased up to 29%.

In the $PGF_{2\alpha}$ group compounds **7**, **9** and **13** had almost no influence on the rate of hydrolysis, **8** and **11** showed a weak activation of the lipase. The most active PG derivatives were **10** and **12**. Triacetyl-$PGF_{2\alpha}$ methyl ester (**10**) had IC_{50} at the

concentration of 4.08 x 10^{-3} M which was almost the highest solubility of the compound achieved. This derivative had also the highest impact on the lipase activation, increasing hydrolysis up to 40%. A similar effect was observed in case of 9,15-diacetyl-$PGF_{2\alpha}$, IC_{50} was achieved roughly at the same concentration and the compound activated hydrolysis at low concentrations of about 30%.

Table 2. Inhibition and activation of the CRL-catalyzed hydrolysis by different prostaglandin derivates

PG	S_{max}(M)[a]	IC_{16}(M)[b]	IC_{50}(M)[c]	[PG] M[d]	A_{max} (%)[e]
1	2.0 x 10^{-3}	inactive		1.94 x 10^{-3}	13
2	6.8 x 10^{-4}			6.80 x 10^{-4}	37
3	6.6 x 10^{-4}			6.54 x 10^{-4}	35
4	1.2 x 10^{-3}	inactive			
5	9.9 x 10^{-2}	inactive			
6	1.4 x 10^{-3}	inactive		1.40 x 10^{-3}	29
7	6.0 x 10^{-3}	2.61 x 10^{-3}	inactive		
8	3.6 x 10^{-3}	inactive		3.60 x 10^{-3}	29
9	1.1 x 10^{-3}	inactive			
10	6.5 x 10^{-3}	1.52 x 10^{-3}	4.08 x 10^{-3}	1.63 x 10^{-4}	40
11	3.7 x 10^{-3}	inactive		1.00 x 10^{-3}	23
12	5.7 x 10^{-3}	7.70 x 10^{-4}	4.0 x 10^{-3}	1.60 x 10^{-5}	30
13	1.2 x 10^{-2}	7.25 x 10^{-4}	inactive		

[a] *– the highest final concentration of prostaglandins achieved in reaction mixtures*
[b] *– the concentrations leading to the lipase inhibition of 16%*
c – the concentrations leading to the lipase inhibition of 50%
d – the concentration of PG yielding a maximum activation of CRL
e – the maximum activation of CRL at the corresponding concentration of prostaglandin

In Figure 1 the activity of lipase depending on the concentration of four PG derivatives is shown in comparison with that of esters and free acids. $PGF_{2\alpha}$ (**7**) and $PGF_{2\alpha}$ methyl ester (**8**) have the opposite effect on the lipase activity. Triacetyl-$PGF_{2\alpha}$ methyl ester **10** activates hydrolysis at low concentrations, inhibiting it at higher concentrations, while triacetyl-$PGF_{2\alpha}$ (**13**) shows no activating effect on hydrolysis at lower concentrations and decelerates it also at higher concentrations.

In summary, five inactive PG derivatives out of six were free acids. Six out of seven PG derivatives, which activated hydrolysis (from 13 to 40%) were esters (they have an enhanced lipid solubility). Acetylated hydroxyl groups versus unprotected ones do not seem to show a very high influence on activity of the lipase as in both groups the derivatives with free hydroxyls and also peracetylated compounds can be found. One of the two most active PG derivatives with inhibitory properties was an ester and the other, carboxylic acid, but both of them had acetylated hydroxyl groups.

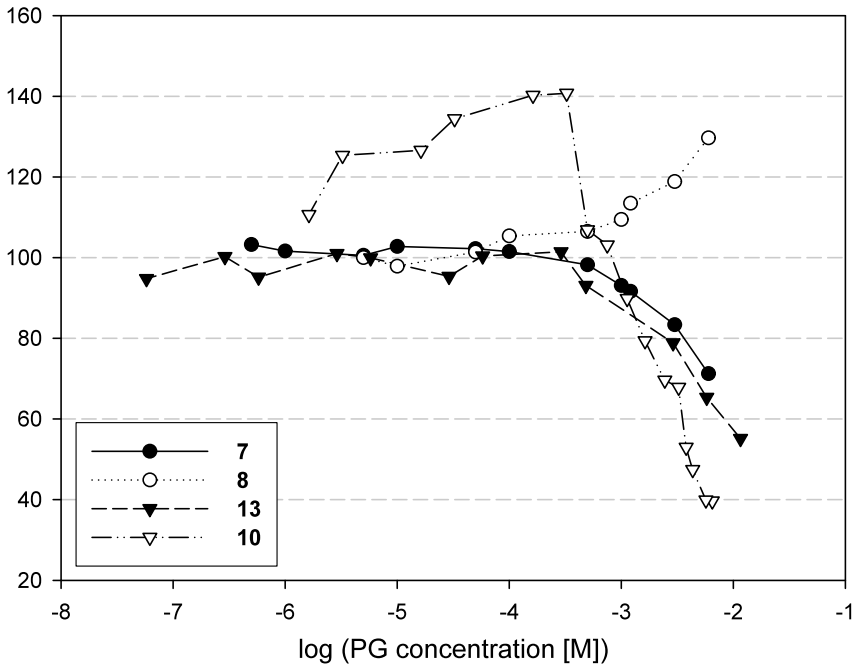


*Figure 1. The influence of $PGF_{2\alpha}$ (**7**), $PGF_{2\alpha}$ methyl ester (**8**), 9,11,15-triacetyl-$PGF_{2\alpha}$ (**13**) and 9,11,15-triacetyl-$PGF_{2\alpha}$ methyl ester (**10**) on the lipase activity*

The methyl ester group evidently increases the hydrophobicity of the molecule, therefore the activation of hydrolysis can probably be accounted for PGs altering the interface of the water/lipid phase. But to make conclusive statements about the importance of a certain type of functional groups or mechanisms of activation and inhibition, further tests with different PG derivatives will be required.

In conclusion, judging by the results of this work prostanoid structural units included in the molecules of potential inhibitors influence dramatically neither the velocity of the lipase-catalyzed synthetic steps nor the inhibitory activity of the target compounds in the lipase-catalyzed hydrolytic reactions.

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TETRAHEDRON LETTERS

Chemical versus enzymatic acetylation of α-bromo-ω-hydroxyaldehydes: decyclization of hemiacetals by lipase

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Abstract—Lipase-catalyzed decyclization of hemiacetals of α-bromo-ω-hydroxyaldehydes followed by trapping upon acetylation was observed. Quantum chemical investigations were performed to explain the energetic background of the reactions. The stereocontrolled synthesis of enantiopure *trans*-(2*S*,3*S*)-2-methoxy-tetrahydropyran-3-ol was elaborated.

Deoxysugars play a significant role in living organisms where they are mainly found as subunits of oligosaccharide moieties of glycoconjugates. A number of different deoxymonosaccharides are present in antibiotics and other physiologically active compounds. Because of their pharmaceutical importance, the synthesis of deoxysugars has become an important field of natural product research.[1] Moreover, deoxysugars act as efficient chiral auxiliaries,[2] probably because of the high structural rigidity which is characteristic of carbohydrates and their deoxyanalogs.

On the other hand, studies of activation of (deoxy)-saccharides by lipase probably allowing isomerization, transfer, etc. of these moieties could be useful for understanding the nature of these processes occurring in some systems in vivo.

For the synthesis of alkyl glycosides of some deoxysugars we chose a chemoenzymatic approach (Scheme 1) consisting of three steps:

1. Bromohydroxylation[3] of 3,4-dihydro-2*H*-pyran (DHP) (Scheme 1) and 2,3-dihydrofuran (DHF) (Scheme 2).
2. *Candida antarctica* lipase B (CALB)-catalyzed kinetic resolution[4,5] of enantiomers of α-bromo-ω-hydroxyaldehydes[6,7] upon acetylation or deacetylation followed by chromatography.
3. Treatment of optically resolved compounds (bromohemiacetals or their acetates) with base in alcohol.[8]

The bromination[9] of DHP resulted in *rac*-1*trans* in high yield (≥90%), while DHF afforded *rac*-3*trans* in moderate yield (~50%). The products[10] are formed as *trans* isomers according to the mechanism of the reaction. Actually, we observed (by NMR, HPLC) the equilibria between anomers characteristic of a certain structure. It is probable that all the isomeric forms could exist (including open-chain forms, despite our failure to detect those experimentally) pictured as Set C (Scheme 1) and Set F (Scheme 2) in solution.

The single anomers obtained by HPLC separation were observed to undergo a rapid re-establishment of the initial equilibrium under the conditions used (rt; then evaporation at 35°C under reduced pressure). The dynamic equilibration observed explains the high discrimination of the *cis* anomer along with a high total yield of the products in both the chemical and enzymatic reactions performed. No *cis* isomer was detected in the product of the lipase-catalyzed acetylation (conv.: ≥98%) of bromohemiacetals: Set C→Set D (Scheme 1). The optically pure material (Set A; (2*R*)-2*trans* of Set D) was treated with LiOH in methanol affording *trans*-glycoside (2*S*)-5*trans* upon the double S_N2 process (Scheme 1) in >90% yield and >98% iso-

Keywords: lipase; deoxysugar alkyl glycoside; decyclization of hemiacetal by lipase; α-bromo-ω-hydroxyaldehyde.

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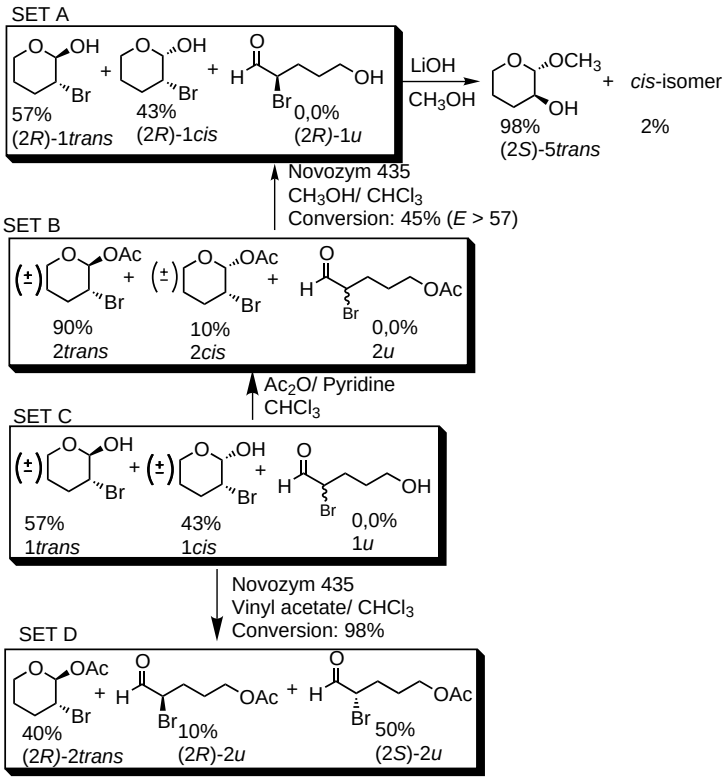
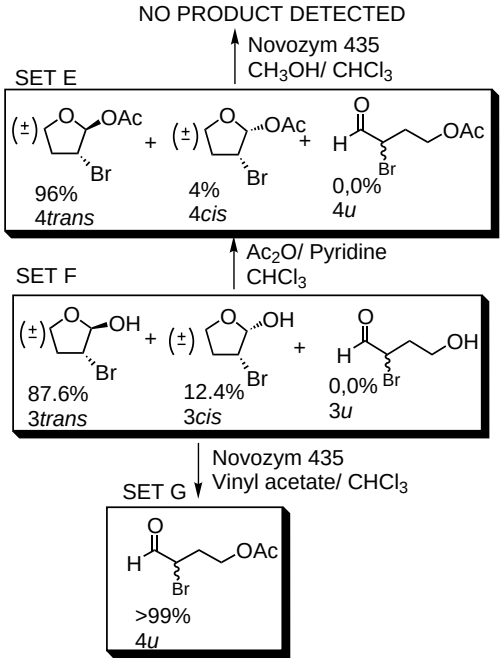


Scheme 1. Synthesis of deoxysugar methyl glycoside (2*S*)-5*trans*. Chemical and lipase-catalyzed acetylation (deacetylation) of *rac*-2-bromo-5-hydroxypentanal.



Scheme 2. Synthesis and chemical and lipase-catalyzed acetylation of *rac*-2-bromo-4-hydroxybutanal.

meric purity. In this reaction only the *trans* hemiacetal anomer is reactive. The absolute configuration of (2*S*)-5*trans* was assigned by the results of the studies of differential shielding effects observed in the NMR spectra of THP–mandelate diastereomers[11] confirming also the absolute configuration of (2*R*)-1*trans*.

The chemical acetylation of bromohemiacetals (Set C→Set B; Set F→Set E) resulted in isomeric stable acetates of a nearly 3–4-fold diminished content of the *cis* anomer.

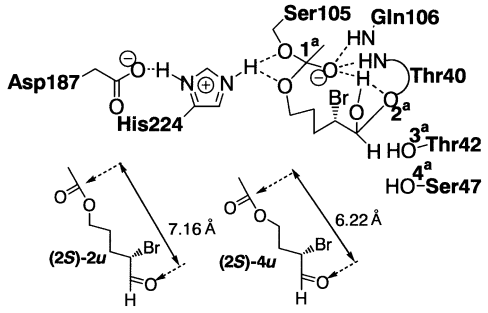
The open-chain α-bromo-ω-hydroxyaldehyde (2*S*)-1*u* together with some amount of (2*R*)-1*u*; (Scheme 1) both of minute thermodynamic probability (Table 1) were stabilized by CALB (Scheme 3) followed by trapping of these isomeric forms by acetylation[12] occurring during the prolonged incubation (60 h; conversion rate $\geq$98%), while the enantioselective acetylation (E$\geq$67) of cyclic *trans*-hemiacetal (2*R*)-1*trans* was found to be rapid (3 h; conversion rate=45%) under the same conditions. In the case of C_4–aldehyde hemiacetals none of the isomeric cyclic acetates (of Set E) was formed nor cleaved upon CALB-catalytic reactions[13] in our hands. The product obtained from the lipase-catalyzed acetylation in ~98% yield was an almost racemic mixture of open-chain acetates (Set G).

QC calculations were performed in an attempt to explain the anomer ratio dynamics as well as the logic of acetylation of open-chain hydroxyaldehydes.

For all hemiacetals, open-chain aldehydes and the corresponding acetates (Table 1) an attempt to find conformers with minimum energy was made with the help of Tinker's[14] SCAN program.

Table 1. Thermodynamic characteristics calculated for the most probable conformers of isomeric forms of α-bromo-ω-hydroxyaldehydes and the corresponding acetates. Thermodynamic and experimentally (NMR) determined distribution of isomeric forms for products of chemical synthesis

Set	Compound	Heat of formation (kcal/mol)	Difference of heat of formation between conformers of all isomers	Percentage of an isomeric form	
				Calculated	Experimental
C	1*trans*	−96.19	0.00	65.9	57
	1*cis*	−95.66	0.53	34.1	43
	1*u*	−86.73	9.46	0.0	0.0
F	3*trans*	−89.82	0.00	89.3	87.5
	3*cis*	−88.38	1.44	10.7	12.5
	3*u*	−81.23	8.59	0.0	0.0
B	2*trans*	−137.34	0.00	26.6	90
	2*cis*	−136.80	0.54	10.6	10
	2*u*	−136.37	0.97	62.8	0.0
E	4*trans*	−131.38	0.00	47.7	≥96
	4*cis*	−128.99	2.39	1.4	≤4
	4*u*	−130.26	1.12	50.9	0.0



Scheme 3. Probable tetrahedral intermediate of CALB-catalytic ω-acetylation of (2*S*)-2-bromo-5-hydroxypentanal. [a]: (1) C_1 of acetyl docked to CALB; (2) oxygen atom (OA) of the hydroxyl group (HG) of Thr40; (3) OA of the HG of Thr42; (4) OA of the HG of Ser47. The distances between labeled atoms: (1 and 2) 3.9 Å; (1 and 3) 7.65 Å; (1 and 4) 8.00 Å. The distances measured are based on the crystal structure of CALB structure 1 TIB[19] acquired from Protein Data Bank.[20]

This program performs a general conformational search for the entire potential energy surface via a basin hopping algorithm,[15] and it also minimizes each conformer after its generation. The force field chosen, MM3,[16] is known to be quite successful in reproducing molecular IR spectra.

All the conformers generated were additionally minimized using the Alchemy 2000[17] program and the same MM3 method therein. This was performed because the MM3 force field within the latter program has improvements upon Tinker's version, viz. the anomeric and Bohlmann correction terms[18] have been implemented.

The percentage of isomeric forms in the equilibrium of α-bromo-ω-hydroxyaldehydes calculated for Set C and Set F is in approximate agreement with experimental results.

The chemical acetylation of α-bromo-ω-hydroxyaldehydes as an isomeric equilibrium mixture of cyclic hemiacetals afforded isomeric stable acetates of a changed ratio. The content of *cis* acetates, 2*cis* and 4*cis*, in the products (Set B and Set E, respectively) was found to correspond to the thermodynamical distribution of isomeric acetates whereas instead of open-chain acetates expected by calculation only *trans* acetates were formed.

The cyclic *trans* hemiacetal (2*R*)-1*trans* favored by lipase is acetylated by CALB rapidly, but even this aldehyde enantiomer was partially acetylated as an open-chain form, probably because of the presence of the *cis* anomer not favored by CALB, thus allowing coordination of the aldehyde molecule for transhemiacetalization/decyclization. Another aldehyde enantiomer not favored by CALB, in both cyclic forms (hemiacetals), was almost totally acetylated as an open-chain isomer.

Conclusions

1. The observed thermodynamic control of *cis* acetate formation during the chemical acetylation suggests that the activated intermediate complex of the hemiacetal acetylation has to proceed via an open- or quasi-open-chain state allowing realization of intramolecular interactions characteristic of acetate molecules. By using chemical methods no acetylation of the ω-OH group of the open-chain isomer of any hydroxyaldehyde was observed.
2. During the enzymatic acetylation of the C_5–aldehyde (Set C) the 2*S*-enantiomer was stabilized by the lipase as the open-chain isomer followed by trapping by acetylation, while the 2*R*-enantiomer afforded preferentially the cyclic *trans* acetate (2*R*)-2*trans*. CALB-catalyzed acetylation of C_4–aldehyde (Set F) resulted in an almost racemic mixture of open-chain acetates (Set G).

3. The asymmetric chemoenzymatic synthesis of *trans*-(2*S*,3*S*)-2-methoxy-tetrahydropyran-3-ol was elaborated.
4. Differences in the heat of formation between conformers of all isomers of 2-bromo-4-hydroxybutanal and 2-bromo-5-hydroxypentanal allow the estimation of the hemiacetal decyclization energies: 7.15–8.59 kcal/mol for the C_4–aldehyde and 8.93–9.46 kcal/mol for the C_5–aldehyde. These values correspond to the total energies of 4–5 hydrogen bonds seeming improbable even with the help of the 'oxy-anion hole' of CALB[21] which, besides, has to be engaged in a simultaneous stabilization of the tetrahedral intermediate of acetylation of the ω-hydroxyl group (involving Ser105).
5. Based on the results of measurements of molecular geometry in the active site of CALB, we expect the stabilization of the open-chain form of the hydroxyaldehyde molecule to occur covalently upon transhemiacetalization with the hydroxyl group of Thr40. (However, this process could be evoked by initial influence of the 'oxy-anion hole' of lipase on the hydroxyaldehyde.)

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9. **Experimental**. **Chemical acetylation**. Chloroform was added to a racemic mixture (1 equiv.) of bromohemiacetals resulting in a 0.4 M solution. Acetic anhydride (1.5 equiv.) and pyridine (5.4 equiv.) were added at rt with stirring. After stirring overnight the reaction mixture was washed with $NaHCO_3$ and brine and dried over Na_2SO_4, filtered and evaporated. Crude products were investigated by using NMR spectroscopy (conversion rate stated: >98%; the isomer ratios are presented in Table 1). Samples were purified over silica (15% EtOAc in hexane). Total yield of products was about 50%. Reaction time: 18 h (Set C→Set B; Set F→Set E). **Lipase-catalyzed acetylation**. Substrate (100 mg) was added to a mixture of chloroform and vinyl acetate (3:1) resulting in a 0.138 M solution. Novozym 435 (50 mg) was added per 0.552 mmol of substrate at rt. The solution was stored without stirring at rt. The reaction was terminated at the conversion rate according to the goal of the experiment by filtering off the enzyme, the solution was evaporated and the residue was studied using NMR spectroscopy, further purified over silica and characterized. **Lipase-catalyzed deacetylation**. Substrate (100 mg, 1 equiv.) and methanol (44 equiv.) were added to chloroform resulting in a 0.138 M solution. Novozym 435 (76 mg) was added per 1 mmol of substrate without stirring at rt. After reaching the conversion rate of 45% the solution was filtered, evaporated (studied by NMR) and purified over silica. Reaction time: 6 h (Set B→Set A; Set E→no product was detected after 60 h of incubation). **Synthesis of deoxy-sugar methyl glycoside**. 3-Bromo-tetrahydro-pyran-2-ol (1 equiv., 4.42 mmol) and 2.5 equiv. LiOH were added to methanol (16 ml) at rt. After stirring for 1 h at rt the solution was evaporated and purified over silica (1/1 EtOAc/hexane).
10. **Characterization of compounds**. **(2*R*)-1*trans*+(2*R*)-1*cis* (57/43)**: TLC R_f=0.4 (1/9 acetone/C_6H_6); $[\alpha]_{546}^{20}$ −4.76 (*c* 0.01; C_6H_6); **(2*R*)-1*trans***: ^{13}C and ^1H NMR C_1 97.00 (4.84), C_2 50.93 (3.87), C_3 31.86 (1.93/2.40), C_4 25.11 (1.59/1.80), C_5 64.46 (3.58/4.04); $^3J_{H1,H2}$=6.4 Hz; **(2*R*)-1*cis***: ^{13}C and ^1H NMR C_1 92.55 (4.76), C_2 53.76 (4.24), C_3 29.67 (2.09/2.32), C_4 23.04 (1.58/1.88), C_5 63.01 (3.56/4.04); $^3J_{H1,H2}$=1.8 Hz; **(2*S*)-5*trans***: TLC R_f=0.35 (5/5 EtOAc/hexane); $[\alpha]_{546}^{20}$ +46 (*c* 0.005; C_6H_6); ^{13}C and ^1H NMR C_1 102.80 (4.23), C_2 67.85 (3.43), C_3 27.45 (1.50/1.90), C_4 22.08 (1.39/1.69), C_5 62.86 (3.40/3.75), C_{CH3} 55.48 (3.35); $^3J_{H1,H2}$=5.1 Hz; **3*trans***: TLC R_f=0.4 (4/6 EtOAc/hexane); ^{13}C and ^1H NMR C_1 103.29 (5.58), C_2 50.82 (4.22), C_3 33.27 (2.22/2.68), C_4 66.95 (4.15/4.16); $^3J_{H1,H2}$ <1 Hz; **3*cis***: TLC R_f=0.4 (4/6 EtOAc/hexane); ^{13}C and ^1H NMR C_1 96.62 (5.26), C_2 50.33 (4.26), C_3 33.20 (2.38/2.52), C_4 66.45 (3.89/4.16); $^3J_{H1,H2}$=3.6 Hz. **(2*R*)-2*trans***: TLC R_f=0.5 (3/7 EtOAc/hexane); $[\alpha]_{546}^{20}$ −74.8 (*c* 0.01; C_6H_6); ^{13}C and ^1H NMR C_1 94.22 (5.85), C_2 47.09 (3.99), C_3 30.18 (2.01/2.40), C_4 23.21 (1.60/1.95), C_5 64.30 (3.72/3.96), C_1' 169.03, C_2' 20.80 (2.11); $^3J_{H1,H2}$=5.2 Hz; **2*cis***: TLC R_f=0.5 (3/7 EtOAc/hexane); ^{13}C and ^1H NMR C_1 90.78 (6.06), C_2 46.89 (4.16), C_3 29.01 (2.15/2.25), C_4 26.10 (1.80), C_5 61.02 (3.68/3.85), C_1' 169.03, C_2' 20.72 (2.16); $^3J_{H1,H2}$=2.8 Hz; **(2*S*)-2*u***: TLC R_f=0.54 (3/7 EtOAc/hexane); $[\alpha]_{546}^{20}$ −67.4 (*c* 0.02; C_6H_6); ^{13}C and ^1H NMR C_1 192.26 (9.45), C_2 54.60 (4.27), C_3 28.05 (1.75/2.10), C_4 26.10 (1.75/2.10), C_5 63.21 (4.10), C_1' 170.64, C_2' 20.04 (2.05); **4*trans***: TLC R_f=0.5 (3/7 EtOAc/hexane); ^{13}C and ^1H NMR C_1 102.48 (6.28), C_2 48.82 (4.27), C_3 33.01 (2.24/2.57), C_4 68.06 (4.17/4.18), C_1' 169.37, C_2' 20.89 (1.99); $^3J_{H1,H2}$ <1 Hz; **4*cis***: TLC R_f=0.5 (3/7 EtOAc/hexane); ^{13}C NMR C_1 95.30, C_2 43.76, C_3 32.20, C_4 67.36; $^3J_{H1,H2}$=4.1 Hz. **4*u***: TLC R_f=0.37 (3/7

EtOAc/hexane); ^{13}C and ^{1}H NMR C_1 191.75 (9.48), C_2 51.46 (4.40), C_3 30.73 (2.18/2.45), C_4 61.07 (4.20/4.25), C'_1 170.60, C'_2 20.72 (2.05).

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Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids

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Abstract

Lipase inhibitors have generated a great interest because they could help in the prevention or the therapy of lipase-related diseases. Therefore, the aim of the work was to evaluate by HPLC, and using *Candida rugosa* lipase as model, the inhibitory effect of several saponins: β-aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (QS); flavonoids: 3-hydroxyflavone, 5-hydroxyflavone, (±)-catechin and kaempferol; and alkaloids: aspidospermine, papaverine, physostigmine, pilocarpine, raubasine, rescinnamine, reserpine and trigonelline.

The inhibition produced by most of these compounds is described here for the first time. Saponins appeared very active, being β-aescin and digitonin the most active compounds (IC_{50} = 0.8–2.4 × 10^{-5} M). The inhibitory activity of flavonoids was lower than that of saponins (except GA), and (±)-catechin and kaempferol were the most active. Alkaloids was the most heterogeneous group assayed, varying from rescinnamine, with an IC_{16} similar to that of digitonin, to papaverine and others which showed almost no inhibition.

In conclusion, β-aescin, digitonin, kaempferol or (±)-catechin, strong lipase inhibitors with a low toxicity and present herbal drugs used for lipase-related diseases such as acne or ulcer, are promising candidates for the prevention or the treatment of these diseases.

Keywords: *Candida rugosa* lipase; Inhibition; Saponins; Flavonoids; Alkaloids

1. Introduction

Lipases and esterases are glycerol ester hydrolases (E.C. 3.1.1.–) acting on acylglycerols to liberate fatty acids and glycerol. Several lipases produced by microbial pathogens play an important role in infective diseases. Indeed, *Propionibacterium acnes* lipase and its inhibition by antiacne compounds have been studied because the fatty acids produced by *P. acnes* lipase activity on sebaceous triglycerides induce severe inflammation [1]. It has also been described that *Helicobacter pylori* lipase activity can weaken the barrier properties of mucus by hydrolyzing endogenous lipids [2,3], and it is inhibited by sucralfate and other antiulcer drugs [4,5]. Furthermore, lipase-producing fungal dermatophytes can efficiently colonize the keratinized layers of the skin producing cutaneous diseases [6]. Therefore, research on new lipase inhibitors for the therapy of these diseases and also for other pathologies like obesity, has generated a great interest.

On the other hand, lipolytic enzymes are currently attracting an increasing attention because of their biotechnological potential [7,8]. Inhibition studies on lipases could contribute to better understand their mechanism of action in order to design novel substrate specificities for improving the biotechnological applications of these enzymes [9].

Plant secondary metabolites present in herbal drugs and food have shown to be very useful in the prevention and treatment of many diseases [10]. Among these metabolites, saponins, flavonoids and alkaloids are a promising source of lipase inhibitors since they are present in high concentrations in plant extracts capable of inhibiting porcine pancreatic lipase activity [11]. Furthermore, these compounds are also present in several plant extracts that have been used for the treatment of diseases in which lipases could play an important role like ulcer [12] and acne [1]. Recent studies have also demonstrated that saponins like platycodin D [13], flavonoids like quercetin [14] and alkaloids like berberine and sanguinarine [15] are

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good lipase inhibitors. However, further studies are necessary to elucidate the effect of other purified plant metabolites on lipolytic enzymes in order to select the most suitable ones for therapeutic or preventive pharmacological treatments.

Here, we report the inhibitory effect of several saponins, flavonoids and alkaloids (Fig. 1) on *Candida rugosa* lipase (Crl). This enzyme is well known and widely used in biotechnology [7] and the analysis of its activity and inhibition could help in controlling and increasing its effectiveness in a wide range of biotechnological processes. Moreover, Crl could serve as a model for fungal pathogenic lipases, and its inhibition could be of interest in the treatment against *C. rugosa* strains refractory to antifungal therapy involved in veterinary mycology and in emerging pathogenesis on immunocompromised patients [16]. For these reasons, Crl has been recently used as model for lipase inhibition assays [14,15,17].

2. Experimental

2.1. Reagents

Methanol, acetonitrile and water for HPLC were purchased from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid and ethyl acetate (EA) were obtained from Aldrich (Milwaukee, WI, USA). β-Naphthyl laurate (β-NL), β-naphthol and kaempferol were purchased from Fluka (Buchs, SG, Switzerland). Raubasine, rescinnamine and aspidospermine were obtained from Simes Spa (Milan, Italy). Digitonin was purchased from ICN Biomedicals Inc. (Irvine, CA, USA). *C. rugosa* lipase (cat. No. L-1754), β-aescin, glycyrrhizic acid (GA), *Quillaja* saponin (QS), (±)-catechin, 3-hydroxyflavone and 5-hydroxyflavone, reserpine, pilocarpine, physostigmine and the other reagents were from Sigma (St. Louis, MO, USA). All reagents were used without further purification.

2.2. Evaluation of lipase inhibition by HPLC

Lipase inhibition assays by HPLC were performed as previously described [15]. Essentially, the substances under evaluation were dissolved at their maximum solubility in a proper solvent such as water (trigonelline, papaverine and pilocarpine), acetone (3-hydroxyflavone and 5-hydroxyflavone), dimethyl sulfoxide (DMSO; GA and kaempferol), or methanol (the rest of substances evaluated). Then, 2020 μl reaction mixtures containing varying inhibitor concentrations (from 0 to their maximum solubility), 3.75% inhibitor solvent, 0.46 mM β-naphthyl laurate (β-NL), 1.25% acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.4 at 22 °C) and 10 μg ml^{-1} of Crl, were incubated for 30 min at 37 °C under gentle mixing. Then, β-naphthol was extracted with 2 ml of ethyl acetate, and 500 μl of the organic phase were withdrawn, evaporated at room temperature under a nitrogen stream and redissolved in 1 ml methanol. Aliquots of 50 μl were analyzed at room temperature using a C-18 reversed-phase column (4.6 mm × 250 mm; 5 μm particle size, 90 Å pore size; Beckman) equilibrated at a flow rate of 1 ml min^{-1}, with a mobile phase consisting of 40% (v/v) acetonitrile in water, containing 0.1% trifluoroacetic acid. The eluate was monitored at a wavelength of 230 nm with a sensitivity of 0.8 A.U.F.S. The chromatographic system consisted of a precision pump (Waters, model 515) and a variable wavelength monitor (Waters, model 2487). The area under the chromatographic peak was measured using the Millennium 32 chromatography manager 4.0 software package for Windows®. β-NL unspecific hydrolysis was subtracted performing proper blanks.

2.3. Statistical analysis

Lipase inhibition was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples (without inhibitor but prepared and analyzed under the same conditions than the inhibitor-treated samples, and including the inhibitor solvent to take into consideration the effect of each solvent in Crl activity). The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the inhibition rate versus inhibitor concentration curves by regression analysis performed using the software Sigma-Plot 8.0 (SPSS, Chicago, IL, USA). Three or more replicates of regression curves with *R*-square coefficients higher than 0.99 were used for IC calculations, being each replicate the result of an independent HPLC assay performed in duplicate.

3. Results and discussion

The effect of several saponins, flavonoids and alkaloids on *C. rugosa* lipase was analyzed to evaluate their potential as antilipase drugs. Among the compounds tested, saponins were very active, flavonoids displayed a lower inhibition, and alkaloids, the most heterogeneous group, showed a wide inhibition range (Table 1; Fig. 2). The different inhibition produced by these substances is probably related to their different structure (Fig. 1) and physicochemical properties, as explained below.

3.1. Effect of saponins on C. rugosa lipase

Saponins are glycosidic compounds containing a steroid or triterpenoid sapogenin nucleus. They are characterized by antimicrobial, hypocholesterolemic, anti-inflammatory and other activities and by a low toxicity [10,18,19]. They also have a certain activity against ulcer [12] and human pathogenic *Candida* spp. [20]. In vitro inhibition by saponin-containing formulations on *P. acnes* lipase [21] and by purified saponins (platycodin D and dioscin) on pancreatic lipases [13,22] has also been reported recently. However, saponins from *Medicago sativa* activated pancreatic lipase [23].

Here, we have analyzed the effect of β-aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (QS) on Crl using an HPLC assay (Table 1; Fig. 2). All saponins showed a high inhibition on Crl, being the most active group of inhibitors in general terms. Both digitonin and β-aescin were the most active compounds (IC_{50} = 0.8–2.4 × 10^{-5} M). β-Aescin was the most active inhibitor at concentrations below 2–5 × 10^{-5} M, and digitonin displayed the highest inhibition above this concentration.

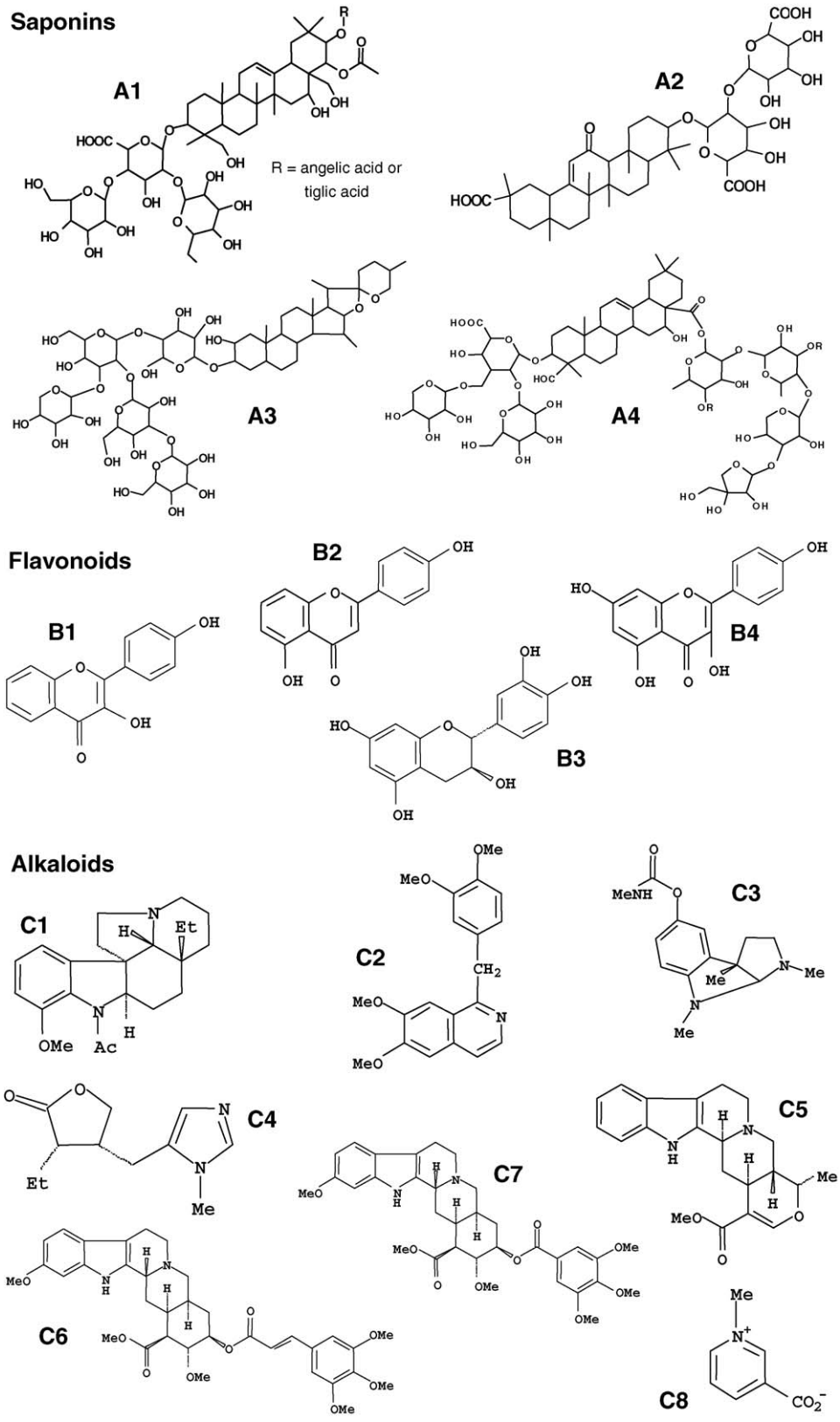

Fig. 1. Natural substances assayed on *Candida rugosa* lipase. (A) Saponins: (1) β-aescin, (2) glycyrrhizic acid, (3) digitonin, (4) *Quillaja* saponin; (B) flavonoids: (1) 3-hydroxyflavone, (2) 5-hydroxyflavone, (3) (±)-catechin, (4) kaempferol; (C) alkaloids: (1) aspidospermine, (2) papaverine, (3) physostigmine, (4) pilocarpine, (5) raubasine, (6) rescinnamine, (7) reserpine, (8) trigonelline.

Table 1
Effect of natural substances on *Candida rugosa* lipase evaluated by HPLC assay

Substance	S_{max}[a] (M)	IC_{16} (M)[b]	IC_{50} (M)[b]
(I) Saponins			
β-Aescin	4.0×10^{-4}	7.5×10^{-7}	8.0×10^{-6}
Digitonin	1.5×10^{-4}	5.9×10^{-6}	2.4×10^{-5}
Glycyrrhizic acid	2.0×10^{-3}	8.5×10^{-4}	$>S_{max}$
Quillaja saponin	1.4×10^{-3}	1.0×10^{-4}	5.5×10^{-4}
(II) Flavonoids			
3-Hydroxyflavone	1.5×10^{-3}	1.1×10^{-3}	$>S_{max}$
5-Hydroxyflavone	2.5×10^{-3}	4.5×10^{-4}	$>S_{max}$
(±)-Catechin	3.0×10^{-2}	8.3×10^{-4}	6.3×10^{-3}
Kaempferol	1.4×10^{-2}	2.8×10^{-4}	7.5×10^{-3}
(III) Alkaloids			
Aspidospermine	2.5×10^{-3}	6.3×10^{-4}	1.0×10^{-3}
Papaverine	2.0×10^{-3}	Inactive	
Physostigmine	3.8×10^{-2}	Inactive	
Pilocarpine	1.0×10^{-2}	Inactive	
Raubasine	1.2×10^{-3}	Inactive	
Rescinnamine	8.0×10^{-4}	2.3×10^{-6}	1.6×10^{-4}
Reserpine	4.5×10^{-4}	1.1×10^{-4}	4.0×10^{-4}
Trigonelline	5.0×10^{-2}	6.8×10^{-3}	2.0×10^{-2}

[a] Highest concentration at which each substance was tested expressed as mol/l (M).
[b] Inhibitory concentrations (IC) 16% and 50% calculated from the inhibition vs. inhibitor concentration curves.

On the other hand, GA produced the lower inhibition among the saponins evaluated ($IC_{16} = 8.5 \times 10^{-4}$ M). QS showed an increasing inhibition at concentrations up to $1.5–2.2 \times 10^{-4}$ M (about the IC_{50}). However, above these concentrations the inhibition by QS remained stable between 50 and 60%, in contrast to the other saponins which always produced a higher inhibition when increasing concentrations were assayed (Table 1; Fig. 2).

Direct inhibition of a lipase by these saponins is described here for the first time. Only inhibition of the hydrolysis of acetyl fluorescein (substrate of lipases and esterases) by β-aescin on several bacterial strains [24], inhibition of a cholesterol esterase by digitonin [25], inhibition of pancreatic lipase by GA-containing plant extracts [11], and the presence of GA and QS in plant formulations that inhibited *P. acnes* lipase [21] have been reported before.

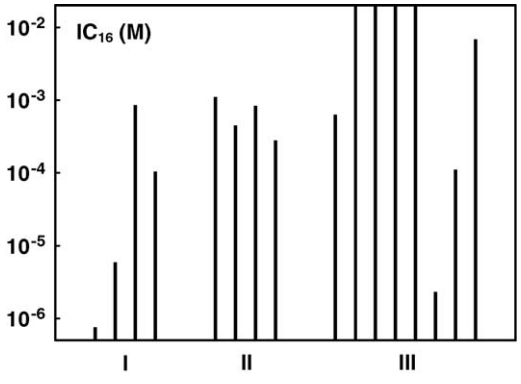

Fig. 2. Comparison of the effect of natural substances on *Candida rugosa* lipase. The effect of several saponins, flavonoids and alkaloids on Crl was evaluated by HPLC assay. The concentrations of these substances that produced a 16% inhibition (IC_{16}) on Crl activity are compared, since the IC_{50} was not reached in some of them. The compounds assayed are plotted in the same order as reported in Table 1. (I) Saponins; (II) flavonoids; (III) alkaloids. The compounds being inactive are plotted as vertical bars reaching the top of the graphic.

Crl displays complex substrate-binding site, since the polypeptide chain folds over this site forming a deep tunnel penetrating towards the centre of the molecule. The tunnel has an unusual "L" shape, it is 25 Å long and it has a diameter of 4 Å, thus it can only accommodate a single acyl chain not longer than 18 carbon atoms, whereas the positions of the other acyl chains of the substrate would be located in hydrophobic patches under the tunnel entrance. A phenylalanine-rich area near the tunnel entrance contains the catalytic serine, whereas the tunnel walls are lined with hydrophobic residues [33–35]. The fact that the aglicone nucleus of saponins is too large to enter into the tunnel, together with the higher inhibition produced by the saponins with larger and more branched carbohydrate side chains (β-aescin and digitonin) seems to indicate that the carbohydrate chains of saponins could enter, at least partially, into the tunnel of Crl, being thus the responsible for Crl inhibition, although further experiments are necessary to confirm this hypothesis.

3.2. Effect of flavonoids on Candida rugosa lipase

Flavonoids are benzo-γ-pyrone derivatives that can be grouped according to the presence of different substituents and the degree of benzo-γ-pyrone ring saturation. They are capable of modulating the activity of many enzymes and cell systems producing, among others, antitumoral, cardioprotective or anti-inflammatory effects [27]. Flavonoids are also components of antiacne formulations [1], and some of them, like kaempferol, have shown antiulcer activity [28]. Furthermore, some flavonoids such as quercetin [14] and hesperidin or neohesperidin [29] are known lipase inhibitors.

Here, we have evaluated the inhibition produced by (±)-catechin, kaempferol, 5-hydroxyflavone and 3-hydroxyflavone on Crl (Table 1; Fig. 2). All of them produced a lower inhibition than saponins (except GA). All flavonoids displayed a similar IC_{16} (from 2.8×10^{-4} to 1.1×10^{-3} M), whereas the IC_{50} was only achieved for (±)-catechin and kaempferol (IC_{50} of 6.3×10^{-3} and 7.5×10^{-3} M, respectively). At low concentrations kaempferol was the most active flavonoid, although (±)-catechin was the most efficient at high concentrations. 5-Hydroxyflavone showed an intermediate inhibition at low concentrations, halfway between (±)-catechin and kaempferol. However, 3-hydroxyflavone caused an inhibition more than two-fold lower than its isomer 5-hydroxyflavone (Table 1; Fig. 2).

It has previously been reported that catechin-rich extracts or catechin-related compounds like (−)-epicatechin inhibit pancreatic or gastric lipases and rat adipose tissue-derived lipoprotein lipase, although they do not inhibit hormone-sensitive lipase [11,30,31]. Inhibition of pancreatic lipase by kaempferol, a compound similar to quercetin, or by extracts of *Thea sinensis* (rich in kaempferol) has also been reported [11,32]. However, microbial lipase inhibition by (±)-catechin and kaempferol, and lipase inhibition by 5-HF and 3-HF, are described here for the first time.

The mechanism of enzyme inhibition by these compounds is unclear. The flavonoids analyzed have a similar structure,

and are small enough to enter in the tunnel of Crl [33–35]. However, (±)-catechin and kaempferol produced a higher inhibition than 5-hydroxyflavone, which in turn is more active than 3-hydroxyflavone. These results suggest that a higher number hydroxyl groups, as well as their disposition (mainly the presence of the hydroxyl group at position 5 of ring A, which is missing in the less active flavonoid 3-hydroxyflavone, as well as the presence of the hydroxyl group at position 7 of ring A, only present in the most active flavonoids (±)-catechin and kaempferol), would be the responsible for the different inhibition produced by these compounds on Crl. The mechanism of action of these compounds and the importance of their hydroxyl groups will be analyzed in the future, although the requirement of polyhydroxylated flavonoids for the inhibition of Crl is in agreement with the inhibition of the H^+, K^+-ATPase by polyhydroxylated flavonoids [36].

3.3. Effect of alkaloids on Candida rugosa lipase

Alkaloids are very diverse natural substances (and their related synthetic compounds) that contain nitrogen, usually as a part of a cyclic system. They are active on many enzymes and biological systems, like the central nervous system, producing a wide range of effects [37]. Moreover, berberine and other alkaloids are known lipase inhibitors [15].

The inhibition obtained by HPLC assays on Crl by trigonelline and several heterocyclic alkaloids was very different among the compounds assayed, even for those belonging to the same structural group. Among the indole-benzopyrrole alkaloids, rescinnamine was the most active, displaying an IC_{16} of 2.3×10^{-6} M, similar to those of digitonin and β-aescin. However, this alkaloid was less active than the mentioned saponins at high concentrations ($IC_{50} = 1.6 \times 10^{-4}$ M). Reserpine was the second most active alkaloid, showing an inhibition higher than flavonoids. Aspidospermine displayed an inhibition rate similar to most flavonoids at low concentrations (IC_{16}), but it was more active than flavonoids at high concentrations ($IC_{50} = 1.0 \times 10^{-3}$ M), and physostigmine and raubasine caused almost no inhibition of CRL (Table 1; Fig. 2). Among the non-indole-benzopyrrole alkaloids, only trigonelline (pyridine and piperidine group) was active, but it caused a low inhibition, whereas papaverine (isoquinoline group) and pilocarpine (imidazole or glyoxaline group) did not inhibit Crl (Table 1; Fig. 2).

Lipase inhibition by the mentioned alkaloids is reported here for the first time, except for reserpine and physostigmine. Among other effects, reserpine increased lipoprotein lipase activity of heart tissue [38] and inhibited epididymal hormone-sensitive lipase but not that of other tissues [39]. Physostigmine, a known esterase inhibitor, inhibited also lipases like bile-salt stimulated lipase [40] or pancreatic lipase [41], but not lipolytic liver extracts [42]. Moreover, some isoquinoline-group alkaloids similar to papaverine like palmatine were inactive on Crl, although berberine and others inhibited Crl [15].

Inhibition by alkaloids is difficult to explain as they have very different structures. Except trigonelline, most of them have a large structure with several rings that makes difficult or not possible their entering into the narrow tunnel of Crl [33–35], which could explain the lack of inhibition of some of them. However, further considerations are possible with respect to the indole-benzopyrrole alkaloids. Raubasine (inactive) and rescinnamine or reserpine (the most active) share the same indole nucleus (Fig. 1: C5–C7). Thus, it is clear that the inhibition produced by rescinnamine and reserpine is caused by their lateral groups. As we suggested for saponins, the lateral groups probably enter into the active site of Crl interfering with the activity of the enzyme, whereas the indole nucleus remains out of the tunnel, since it is too large to enter. However, further assays are in progress to establish more accurately the mechanism of action of these compounds.

3.4. Conclusions and clinical implications

Saponins, (±)-catechin and kaempferol, produced a high Crl inhibition, and some of them have been described to be active against acne, ulcer or pancreatic lipase, as explained before. Moreover, almost all of them have a low toxicity. For example, β-aescin is currently used in the treatment of peripheral vascular diseases as it displays more effectiveness and tolerability than the conventional therapy [19]. At high concentrations, (±)-catechin produces toxic effects like haemolysis, and kaempferol is genotoxic. Nevertheless, they are some of the most occurring compounds among the approximately 1 g of flavonoids that contains the daily human diet [27]. Therefore, these inhibitors are promising candidates for the prevention (creams, shampoos, etc.) and the therapy of diseases in which lipases play an important role like acne, *H. pylori*-associated ulcers, obesity, or fungal diseases. In fact, further assays in our research group have demonstrated that some of these inhibitors are also effective on *P. acne*s lipase and other lipases involved in virulence (unpublished data). However, further studies are necessary to confirm their pharmacological potential. On the contrary, the potential use of rescinnamine and reserpine to treat lipase-related diseases is more limited by their other marked pharmacological activities [43].

In conclusion, knowledge about the effect on Crl of these compounds will be helpful with respect to their application in pharmacology, in the designing of new lipase inhibitors, and in the evolution of the active site of the enzyme in order to carry out new biotechnological processes.

Acknowledgements

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JOC*Note*

Synthesis of Deoxy Sugar Esters: A Chemoenzymatic Stereoselective Approach Affording Deoxy Sugar Derivatives Also in the Form of Aldehyde

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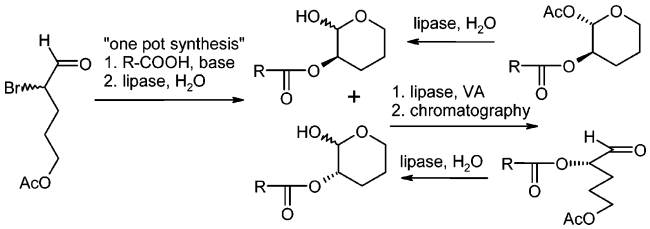
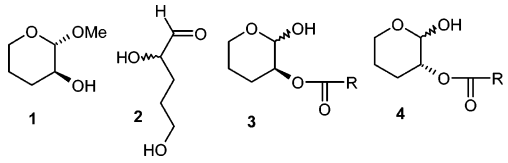


A chemoenzymatic synthesis of deoxy sugar esters is described. The synthesis is based on the *O*-alkylation of carboxylic acid with 2-bromo-5-acetoxypentanal. The method allows treatment of hydroxy carboxylic acids without protection of alcoholic hydroxyl groups. Several stereoisomeric deoxy sugar esters were resolved (up to ee or de > 98%) using a lipase-catalyzed acetylation of hemiacetals that in certain cases afforded deoxy sugar derivatives in the form of aldehydes. The stereochemistry of the reactions was determined by the NMR spectra of mandelic acid derivatives.

Deoxy sugars (DOS) play a significant role in many active compounds of medicines such as antibiotics, antiviral drugs,[1] glycosylation inhibitors,[2] etc. Some of the DOS derivatives have been used as chiral auxiliaries in organic synthesis.[3] Considering the above, the development of diverse strategies for the preparation of DOS derivatives[4] has become an important field of synthetic research.

Several researchers have used methyl glycoside **1** as a source for 3,4-dideoxy ribose (**2**) for inclusion in conjugates. Racemic *trans*-glycoside **1** has been synthesized by the epoxidation of



2,3-dihydropyran in methanol[5] and used in the synthesis of polycyclic ethers: (a) upon a Friedel−Crafts cyclization of 2-*O*-benzyl ethers,[6] (b) by means of a cation-mediated cyclization of the thioglycoside derived from glycoside **1** to afford a ketooxetane,[7] (c) for the preparation of 2,7-dioxabicyclo[4.4.0]-decane and 2,8-dioxabicyclo[5.4.0]undecane.[8]

Derivatives of **2** have been synthesized with high enantiomeric purity starting from L-glutamic acid or D- or L-arabinose.[1,12] The synthesis of (2*S*,3*S*)-2-methoxytetrahydropyran-3-ol[9] (**1**) by bromohydroxylation of 2,3-dihydropyran followed by treatment with LiOH in methanol has been described.[10] In this synthesis, the enantiomers of bromohemiacetal **5** were resolved by lipase-catalyzed acetylation.[11] 3,4-Dideoxy ribose in the form of a glycoside has been included in several conjugates to be used as a chiral auxiliary in an asymmetric modification of the parent structure.[12−14]

For the synthesis of sugar esters, several enzymatic processes have been developed.[15] For the synthesis of hydroxy carboxylic acid esters using routine acylation techniques, the alcoholic hydroxyl groups of the acid have to be protected prior to acylation.[16] For a chemoselective esterification of unprotected hydroxy carboxylic (phenolic) acids the Mitsunobu reaction has been used.[17]

The aim of the present work was to develop a synthetic approach for the preparation of stereochemically pure 3,4-

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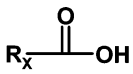
TABLE 1. Structures of the Carboxylic Acids Used



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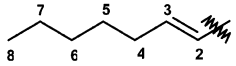
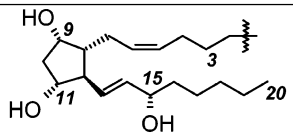
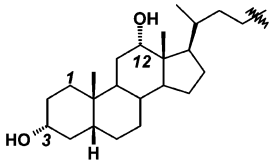
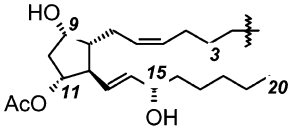
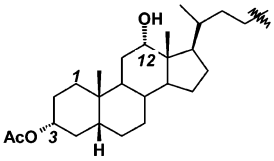
1	
2	
3	
4	
2a	
3a	

dideoxy ribose esters of general formulas **3** and **4** to be used as building blocks in the synthesis of putative medicines. The hemiacetal (and especially the aldehyde group) of the deoxy sugar enables the linking of the derivative to a proper functional group of another synthetic building block.

A chemoenzymatic synthesis of the 3,4-dideoxy ribose esters of selected carboxylic acids (Table 1) was performed to test the chemoselectivity and stereoselectivity of the novel procedure.

We started from (*E*)-2-octenoic acid (OA) as a nonchiral compound to investigate a kinetic resolution of 3,4-dideoxy ribose ester enantiomers upon the lipase-catalyzed acetylation of the hemiacetal group. The inclusion of (*S*)-mandelic acid (MA) in the test set was necessary for NMR-based stereochemical studies.[16,18] Deoxycholic acid (DCA) was chosen to test the limitations of lipase-catalyzed kinetic resolution of the diastereomers of sterically demanding hemiacetal products. We also bore in mind that the glycoconjugates of bile acids are of interest in several cases.[19] Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) is a carboxylic acid with a large flexible hydrocarbon skeleton. Despite their complex structure, several prostanoids have been smoothly esterified or acylated by lipases.[20] In general, prostaglandins enjoy diverse clinical applications, reflecting their wide-ranging physiological and pharmacological properties.

Bromoaldehyde **6** (Scheme 1) was synthesized by the incubation of racemic bromohemiacetal **5** with Novozym 435 (*Candida antarctica* lipase B (CALB) immobilized on an acrylic resin) in a chloroform/vinyl acetate (VA) mixture at rt.[10] Under these conditions, the conversion of the starting material was >98%, and two chemically different products were formed. After chromatography the yield of the *trans*-acetyl bromohemiacetal (acetic acid (2*S*,3*R*)-3-bromo-tetrahydropyran-2-yl ester) was 50%, and that of aldehyde **6**, 40%. Both products were, somewhat unexpectedly, gained with a low (<50%) enantiomeric excess upon the prolonged process, while the faster process reported earlier afforded the above *trans*-acetyl bromohemiacetal with a higher ee.[10] However, we found that starting from bromoaldehyde **6** of low ee and resolving the stereoisomers of target compounds are advantageous in some cases, allowing the preparation of both the individual enantiomers or diastereomers of the DOS esters.

The stereoisomeric mixtures of DOS esters were synthesized using *O*-alkylation of carboxylic acids with bromoaldehyde **6** followed by lipase-catalyzed deacetylation (Scheme 1).

The *O*-alkylation of OA afforded ester **7**, to the same reaction mixture were added water and the CALB, resulting in hemiacetals **8**. In this "one-pot synthesis" the latter step includes a selective lipase-catalyzed deacetylation of the terminal hydroxyl group, affording hydroxyaldehyde, which spontaneously transformed to a hemiacetal as a thermodynamically more stable form. The procedure afforded **8** as a mixture of four stereoisomers (because of the anomers) in a near quantitative yield.

The *O*-alkylation of the carboxyl group of $PGF_{2\alpha}$ resulted in the formation of diastereomeric esters **9**, actually existing as a complex mixture of products, very probably intramolecular macrocyclic hemiacetals (visible as four spots on TLC). The following lipase-catalyzed deacetylation selectively cleaved the acetyl group and afforded a mixture of four hemiacetal diastereomers **10** (inseparable on silica gel) in less than 18 h. The process triggered by the lipase-catalyzed deacetylation evidently involves also the decyclization of macrocyclic hemiacetals and the formation of more stable six-membered hemiacetals as cascade reactions. This five-step reaction sequence, including also the former reactions—the *O*-alkylation followed by spontaneous acetalizations, all taking place as a "one-pot synthesis"—is a reliable way to hemiacetals **10** (overall yield 60%).

The *O*-alkylation of DCA afforded ester **11**, and the acetoxy group was selectively hydrolyzed by adding the CALB and H_2O to the same reaction mixture. This resulted in the formation of ester **12** consisting of four stereoisomers inseparable on silica.

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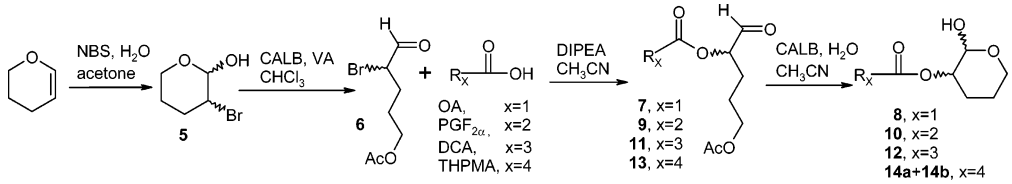
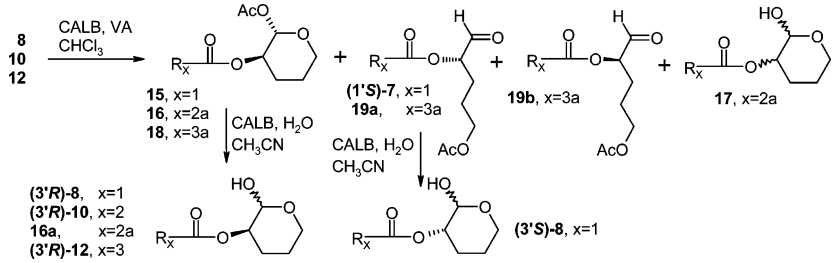
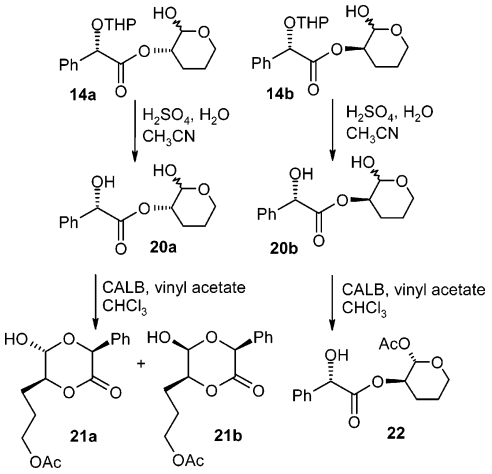
SCHEME 1. Synthesis of Esters 8, 10, 12, 14a and 14b



SCHEME 2. Synthesis of Deoxy Sugar Esters (3′S)-8, (3′R)-8, (3′R)-10, and (3′R)-12



SCHEME 3. Synthesis of Mandelic Acid 3,4-Dideoxy Ribose Esters



The THP-protected MA was *O*-alkylated to afford ester **13** followed by lipase-catalyzed deacetylation to afford hemiacetals **14a** and **14b** in a near quantitative yield.

The synthesis of stereochemically pure DOS esters of OA, $PGF_{2\alpha}$, and DCA using lipase-catalyzed resolution of the stereoisomeric hemiacetals is described in the Scheme 2.

A kinetic resolution of **8** by the lipase-catalyzed acetylation resulted in the simultaneous formation of two chemically different compounds—hemiacetal **8** with 3′*S* configuration afforded aldehyde **(1′*S*)-7**, whereas hemiacetal **8** with 3′*R* configuration afforded acetylated hemiacetal **15** that were separated by chromatography over silica. The stereochemistry of the CALB-catalyzed acetylation of hemiacetals was determined by the NMR assignment of diastereomeric MA 3,4-dideoxy ribose esters (Scheme 3). We found it to be in accordance with the Kazlauskas rule.[21] Also, the exclusive formation of only the *trans*-isomer of acetylated hemiacetal was observed. The subsequent deacetylation of individual products **(1′*S*)-7** and **15** catalyzed by CALB gave the desired, deoxy sugar esters of OA **(3′*S*)-8** and **(3′*R*)-8** in an almost quantitative yield. The enantiomeric excess of the target ester stereoisomers (both of them existing as an equilibrium mixture of anomers) was high.

For the resolution of $PGF_{2\alpha}$ deoxy sugar ester diastereomers **10** the lipase-catalyzed acetylation afforded acetylated hemiacetal **16** as a major product separated by column chromatography on silica. It was gained as an individual compound, whereas **17** (15% of the material gained) was a mixture of several isomers. In esters **16** and **17** the hydroxyl group at the C_{11} of the $PGF_{2\alpha}$ skeleton was acetylated as well. The lipase-catalyzed deacetylation of **16** gave (under the nonoptimized reaction conditions) stereochemically pure deoxy sugar esters **(3′*R*)-10** and **16a** with the ratio of 1:1.4.

The enzymatic acetylation of DCA ester **12** afforded acetylated hemiacetal **18** (38%) as a pure stereoisomer after flash chromatography. The formation of aldehyde **19** in a significant quantity (46% of the separated material) as a mixture of diastereomers was observed. The low stereoselectivity of the lipase-catalyzed decyclization and/or racemization of the α-substituted aldehyde could be assumed. Nevertheless, the result revealed an interesting ability of lipase to produce 3,4-dideoxy ribose bile acid conjugates in the form of a chemically rather active aldehyde starting from less active hemiacetals. In esters **18** and **19**, the hydroxyl group at the C_3 of the DCA skeleton was acetylated as well. The enzymatic deacetylation of acetylated hemiacetal **18** afforded the desired homogeneous deoxy sugar ester **(3′*R*)-12**.

The separation of MA deoxy sugar esters **14a** and **14b** (Scheme 3), both being mixtures of four stereoisomers, proceeded in accordance with an absolute configuration at the C_2 of the deoxy sugar moiety; the diastereomers related to the stereogenic center of the THP protecting group were inseparable on silica gel. Afterward, the THP protecting group was cleaved with dilute H_2SO_4 to afford, after flash chromatography of the products, pure mandelates **20a** and **20b**, respectively. The absolute configurational assignment of mandelates **20a** and **20b** was based on the differential shielding effects caused by the phenyl group in NMR spectra. The assignment was confirmed by the results of their acetylation catalyzed by CALB, which occurred in accordance with the Kazlauskas rule. A separate acetylation of individual hemiacetals **20a** and **20b** exclusively afforded chemically different products. Diastereomer **20b** afforded a pure (>99%) *trans* acetylated hemiacetal **22** when diastereomer **20a,** unfavored in the CALB configuration,

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TABLE 2. Yields and Stereoisomeric Purities of the Products

cmpd	yield (%)	ee or de	cmpd	yield (%)	ee or de	cmpd	yield (%)	ee or de
5	90	rac	**12**	66	low	**18**	38	>98
6	40	<50	**(3′*R*)-12**	71	>98	**19a**+**19b**	46	low
(1′*S*)-7	32	>98	**14a**	43	mix[a]	**20a**	85	>98
8	94	nd[b]	**14b**	37	mix[a]	**20b**	86	>98
(3′*R*)-8	92	>92	**15**	48	>98	**21a**	92	nd
(3′*S*)-8	94	>86	**16**	52	>98	**22**	85	>98
10	60	low	**16a**	49	>98			
(3′*R*)-10	36	>98	**17**	15	low			

[a] A complex mixture of diastereomers. [b] Not determined.

underwent a novel three-step cascade of the reactions. The cascade started with the lipase-catalyzed decyclization of the hemiacetal followed immediately by the acetylation of the terminal hydroxyl group. Thereafter, the MA ester of the extended-chain deoxy sugar acetate underwent a spontaneous cyclization, affording thermodynamically preferable cyclic hemiacetal **21a** together with a low amount of isomeric **21b**. The cascade process consuming **20a** was at least 10 times slower than that consuming mandelate **20b**, thus offering a possibility for a kinetic resolution as well (Table 2).

In summary, a novel approach for the synthesis of stereochemically pure deoxy sugar esters has been developed. The hydroxy carboxylic acids of complex structure ($PGF_{2\alpha}$, DCA) were treated without protection of alcoholic hydroxyl groups. The synthesis began with *O*-alkylation of the carboxylic acid with bromoaldehyde affording deoxy sugar esters in the form of an aldehyde. The latter was deacetylated selectively by CALB, and DOS esters, in the form of hemiacetals, were gained. Integration of the *O*-alkylation and CALB-catalyzed deacetylation into a "one-pot synthesis" is justified because of the lability of aldehydes. However, the lipase-catalyzed hydrolytic deacetylation should be carefully performed because an uncontrolled incubation of the DOS esters under basic conditions may lead to partial hydrolysis of the product. The resolution of stereoisomeric DOS esters has been performed upon lipase-catalyzed acetylation of the hemiacetals. In all cases, the derivatives corresponding to (2′*R*)-3,4-dideoxy ribose afforded individual acetylated hemiacetals. Depending on structure, the CALB-catalyzed acetylation of (2′*S*)-3,4-dideoxy ribose esters led to a stereochemically pure aldehyde (OA ester), a diastereomeric mixture of the aldehyde (DCA ester), or novel 1,4-dioxane hemiacetal products (MA ester).

Experimental Section

General Procedure A: The *O*-Alkylation of a Carboxylic Acid Followed by a Lipase-Catalyzed Deacetylation. Carboxylic acid (1 mmol) was dissolved in CH_3CN (8 mL), 4 equiv of DIPEA was added, followed by 0.7 mmol of bromoaldehyde **6** dissolved in 2 mL of CH_3CN. After stirring the mixture for 24 h Novozym 435 (600 mg) and H_2O (5 mmol) were added, the mixture was shaken at rt for 18−48 h, and the reaction was monitored by TLC. The solution was diluted with Et_2O, the enzyme was filtered off, and the solution was washed with water, 1 M $NaHSO_4$, water, and brine and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the product was purified by column chromatography over silica.

General Procedure B: The Lipase-Catalyzed Acetylation of Hemiacetal Compounds (8, 10, 12, 20a, 20b). To the solution of 0.2 mmol of hemiacetal in 4 mL of $CHCl_3$ were added 1 mL of vinyl acetate and 200 mg of Novozym 435. The reaction mixture was shaken at rt for 24−96 h, and the reaction was monitored by TLC. After the process had been completed, the enzyme was filtered off and the solution evaporated. The products were separated by column chromatography over silica.

General Procedure C: The Lipase Catalyzed Deacetylation of (1′*S*)-7, 15, 16 and 18. To the solution of 0.5 mmol of an acetylated compound in 8 mL of CH_3CN (containing 2% of H_2O) was added 350 mg of Novozym 435. The reaction mixture was shaken at rt, and the process was monitored by TLC. The enzyme was filtered off, the reaction mixture was evaporated, and the products were purified by column chromatography over silica.

(*E*)-Oct-2-enoic Acid (2′*S*,3′*R*)-2′-acetoxy-tetrahydropyran-3′-yl Ester (15). The synthesis was carried out following General Procedure B to yield 126 mg (48%) of **15**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.0, 5.87, 5.82, 4.79, 3.88, 3.70, 2.20, 2.10, 2.04, 1.93, 1.82, 1.53, 1.46, 1.31, 1.29, 0.89. ^{13}C NMR (500 MHz, $CDCl_3$) δ 169.1, 165.5, 150.7, 120.7, 91.5, 67.3, 62.8, 32.2, 31.3, 27.5, 24.3, 22.4, 20.9, 13.9. MS (*m*/*z*): 225.05, 168.25, 142.30, 125.15, 100.10. IR (neat, cm^{-1}): 1198, 1264, 1362, 1440, 1468, 1654, 1723, 1757. $[\alpha]^{20}_{546} = -63$ (*c* 1.8, EtOAc). Anal. Calcd for $C_{15}H_{24}O_5$ (284.39): C, 63.35; H, 8.52. Found: C, 63.21; H, 8.54. TLC: R_f = 0.46 (eluent: 20% EtOAc/hexane). Flash chromatography eluent: 15% EtOAc/hexane.

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Supporting Information Available: General experimental methods and compound characterization data including copies of ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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