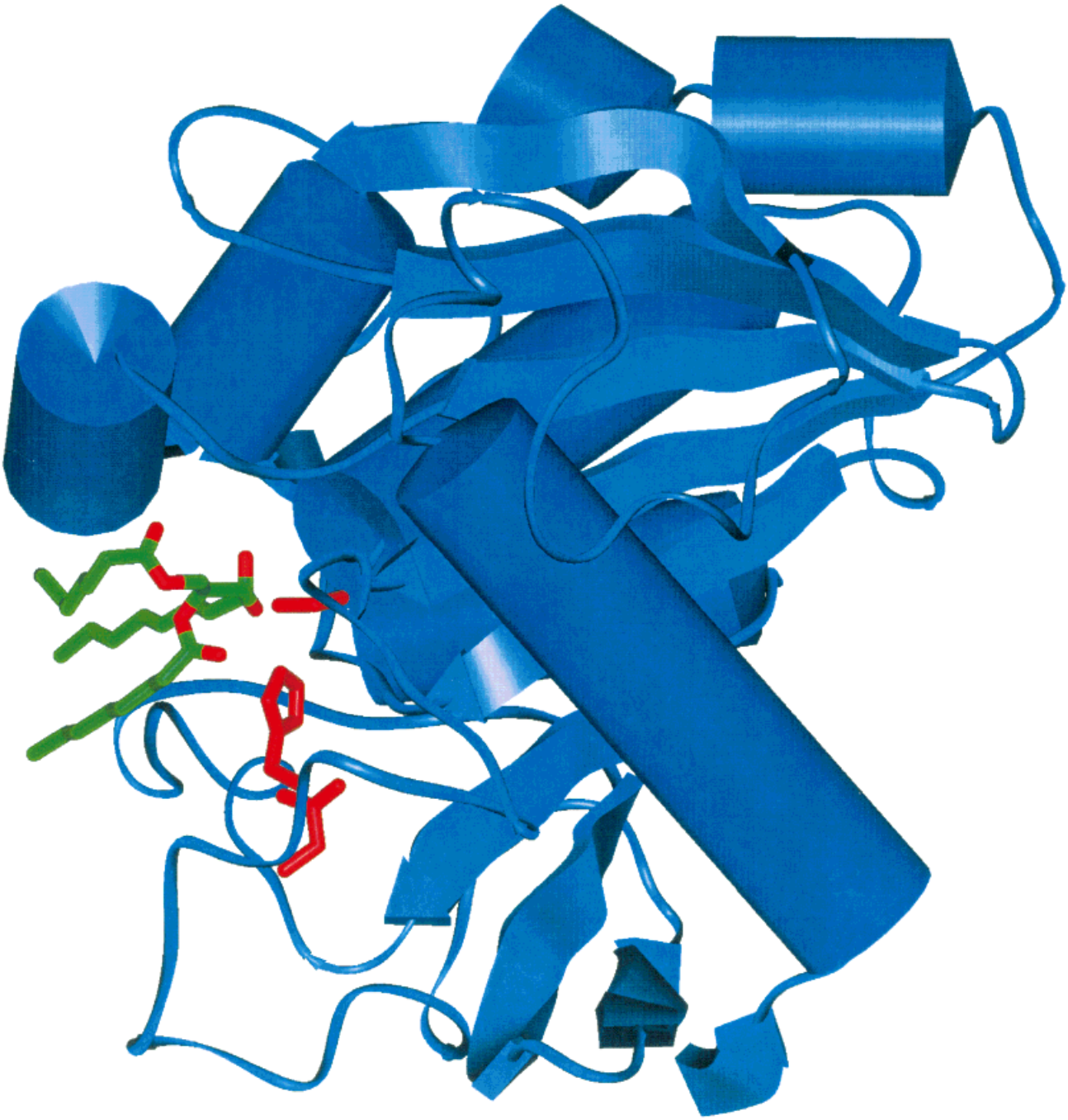


Structure of the *Mucor miehei* lipase in the open form. As with all lipases whose structures have been investigated, this lipase shows the "*α*/*β*-hydrolyse fold" motive known from serine proteases. When the "lid" is open, the catalytic triad at the active center (red) becomes accessible for a substrate (normally triglycerides).

Lipases: Interfacial Enzymes with Attractive Applications

Rolf D. Schmid* and Robert Verger

Lipases not only hydrolyze fat in the digestion tract or interesterify triglycerides on a technical scale, but are surprisingly flexible biocatalysts for the acylation or deacylation of a wide range of unnatural substrates. Lipase-catalyzed transformations of racemic and prostereogenic compounds usually proceed with high enantioselectivity. If several functional groups amenable to lipase catalysis are present, the reaction is mostly regioselective. Lipases are quite stable and can be obtained from animals and plants as well as from natural and recombinant microorganisms in good yields. Therefore, they are used industrially as detergent enzymes, in paper and food technology, in the preparation of specialty fats, and as biocatalysts for the synthesis of organic intermediates. From an enzymological point of view, lipases exhibit a unique tertiary structure that exposes the catalytically active site only in the presence of a lipid phase or, presumably, in an organic solvent. Recently, a large-scale European research project has led to an explosion in information about the structure and function of lipases. As a result, these interesting enzymes have become a preferred object for industrial optimization through the modern methods of genetic engineering. Finally, lipases play an important physiological role in the digestion of fat by mammals and humans, and lipase inhibitors may have a potential as antiobesity drugs.

Keywords: acylations · enzyme catalysis · industrial chemistry · lipases · metabolism

1. Introduction

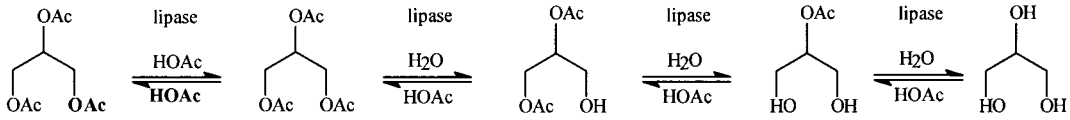
Lipids are key elements in the chemistry of life. Most organisms use the supramolecular chemistry inherent to phospholipids to form their exterior and compartimental membranes. Many plants and animals store chemical energy in the form of triglycerides, which are sparingly soluble in water. For the metabolic turnover of these and other biochemicals, they produce esterases, enzymes which can hydrolyze bonds of water-soluble esters. Esterases which can hydrolyze triglycerides at the water/oil boundary are termed lipases or, more systematically, triacylglycerol hydrolases [EC 3.1.1.3], and those which attack phospholipids are termed phospholipases (various entries under [EC 3.1.*x*]).[1]

Both types of enzymes have recently received considerable attention.[2–13] Whereas phospholipases are involved in key metabolic events such as membrane turnover and signal transduction (which will not be further discussed here), lipases have diverse functions in the degradation of food and fat; they have qualified as valuable drugs against digestive disorders and diseases of the pancreas. They also find applications in biotechnology (in particular as detergent additives) and as catalysts for the manufacture of specialty (oleo)chemicals and for organic synthesis. Their broad synthetic potential is largely due to the fact that lipases, in contrast to most other enzymes, accept a wide range of substrates, are quite stable in nonaqueous organic solvents, and thus, depending on the solvent system used, can be applied to hydrolysis reactions or ester synthesis (Scheme 1).

In addition, lipases can accomodate a wide range of substrates other than triglycerides such as aliphatic, alicyclic, bicyclic, and aromatic esters and even esters based on organometallic sandwich compounds. With respect to racemic esters or substrates with several hydroxyl groups, lipases react with high enantio- and regioselectivity.[14] Finally, the acyl enzyme intermediate in lipase-catalyzed reactions is not only formed from carboxylic esters but from a wide range of other substrates such as thioesters or activated amines, which expands the synthetic potential of lipases considerably.[15] As a result, chemists have long studied how to exploit these properties of lipases for the synthesis of useful intermediates. Today, they may choose their preferred lipase from a great variety of commercially available preparations or even explore a range of different lipases and esterases marketed as a kit.

[*] Prof. Dr. R. D. Schmid
Institut für Technische Biochemie der Universität
Allmandring 31, D-70569 Stuttgart (Germany)
Fax: (+49) 711-685-3196
E-mail: rolf.d.schmid@rus.uni-stuttgart.de
Dr. R. Verger
Laboratoire de Lipolyse Enzymatique
Centre de Biochimie et Biologie Moleculaire, CNRS
Chemin Joseph Aiguier, BP 71, F-13402 Marseille Cedex 9 (France)
Fax: (+33) 9-1220875
E-mail: verger@ir2cbm.cnrs-mrs.fr

  1433-7851/98/3712-1609 $ 17.50+.50/0



Scheme 1. Hydrolysis and ester synthesis with lipases.

2. Occurence, Preparation, and Analysis

2.1. Occurence and Preparation

Lipases are ubiquitous enzymes and have been found in most organisms from the microbial,[16–18] plant,[19, 20] and animal kingdom.[21, 22] They are prepared either by extraction from animal or plant tissue, or by cultivation of microorganisms.[2] Commercially available lipases are usually derived from microorganisms. Since the advent of genetic engineering techniques, an increasing number of lipases is being commercially manufactured from recombinant bacteria and yeasts. As an example, the "detergent lipase" from the fungus *Humicula lanuginosa* is commercially produced in large scale (several 100 tons per year) through fermentation of *Aspergillus oryzae* into which the gene coding for *Humicula lanuginosa* lipase was cloned.[23]

Usually, lipases are just one member of a "hydrolytic enzyme cocktail" elaborated by an organism with the objective to sustain its growth. Often, the lipase of interest must be separated from other esterases and proteases occurring in the crude enzyme preparation (Table 1).[24, 25]

Table 1. Purity of commercial lipases; many are impure.

Lipase source	Portion of protein [%]	Lipase activity[a] [U mg^{-1}]	Major SDS-PAGE bands [kDa]	Number of major SDS-PAGE bands	Number of esterase bands[b]	Protease activity[c] [%]
Rhizomucor miehei	3.5	16	25	5	2	1
Rhizopus spec.	4.8	32	43, 67	10	4	3
Humicola lanuginosa	2.9	10	20, 30	4	2	20
Candida rugosa I	4.2	11	20, 30, 43, 67, 90	13	2	<1
Candida rugosa II	6.4	14	43, 67	11	2	<1
Geotrichum candidum	4.3	8	67	3	0	<1
Pseudomonas fluorescens	1.2	30	14, 25, 40, 43	6	3	<1
Chromobacterium viscosum I	1.1	45	17, 30, 40	3	1	0
Chromobacterium viscosum II	9.5	154	17, 30, 40	2	1	12

[a] Towards triolein (pH 7.5, 37 °C). [b] Hydrolysis in the presence of α-naphtyl acetate and activity staining with FastRed. [c] As a percentage of proteinase K activity on azoalbumin.

R. Verger R. D. Schmid

Rolf D. Schmid was born 1942 in Salzburg, Austria, and studied chemistry from 1961 at the Universität München, Germany, and later in Freiburg, where he obtained his Ph.D. in 1970 for thesis work done in Hans Grisebach's laboratory. After postdoctoral studies in France and the USA, he joined the research laboratories of Henkel KGaA in Düsseldorf in 1972, where he became Director of the Biotechnology Laboratories. In 1987 he moved to the Gesellschaft für Biotechnologische Forschung (GBF) in Braunschweig, where he headed the Division of Enzyme Technology and Natural Products Research. In 1993 he accepted an invitation to build up the newly founded Institute for Technical Biochemistry at the Universität Stuttgart. He is also in charge of the Department of Molecular Biotechnology of the Fraunhofer Institute for Membrane Science and Biotechnology in Stuttgart, and he is chairman of the IUPAC Commission of Biotechnology.

Robert Verger was born 1944 in Maison Carrée, Algeria. He started to study chemistry at the University of Aix-Marseille, where in 1970 he obtained his Doctorat d'Etat in physics for a thesis under the guidance of Pierre Desnuelle. After a postdoctoral stay at the University of Utrecht, The Netherlands, he returned to Marseille and started his scientific career at the Centre National de la Recherche Scientifique (CNRS) in Marseille. Since 1990 he has held the title of "Directeur de Recherche 1ère classe" and has received the bronze and silver medals of the CNRS.

Purification protocols are often laborious as the affinity of lipases is high not only to the oil/water boundary, but to any interphase of lower polarity than water (e.g. water-immiscible organic solvents, glass and plastic surfaces, and air bubbles); lipases may irreversibly adsorb and denature at such interphases.[26, 27]

The critical discussion of experiments on lipase catalysis reach even beyond such difficulties, as many organisms produce mixtures of lipase isoforms which differ only marginally, for example by their glycosylation pattern.[28] Limited proteolysis during maturation of a prolipase or during recovery in the presence of protease impurities may result in a heterogenous mixture of enzymes with lipolytic activity, and ambiguities may exist in the taxonomic classification of the producing microorganism (Table 2).

Thus, quite a few results from the application of "lipases" to oleochemistry and organic synthesis may have originated from the use of a poorly defined enzyme cocktail which, among other activities, also included a lipase. Any result obtained with a commercial lipase should be scrutinized in view of potential masking effects from unrelated enzymes or other additives contained in the sample. Fortunately, many pure lipases, often obtained by recombinant technology, can now be purchased from enzyme suppliers. Table 3 summarizes commercially available lipases and their abbreviation codes used throughout this review.

Table 2. Some of the ambiguities concerning commercially available lipases.

Lipase source	Remarks
Candida rugosa (CRL)	An organism that was formerly classified as *Candida cylindracea*. Protein purification and cloning of the enzyme revealed that the organism produces at least five related lipase isoforms.
Geotrichum candidum (GCL)	Contains two isoforms differing in specificity towards Δ^9-unsaturated fatty acids.
Rhizopus (RAL, ROL, RDL, RNL)	Although literature data suggest different specificities of the lipases from *R. arrhizus*, *R. oryzae*, *R. delemar*, and *R. niveus*, cloning and sequencing revealed nearly complete identity of all four enzymes.
Penicillium camembertii (PeCL)	Classified as lipase from *P. cyclopium* until 1990. Contains four lipase isoforms which differ in their glycosylation pattern.
Pseudomonas glumae (PGL)	Cloning and sequencing revealed identity with lipase from *Chromobacterium viscosum* (CVL).
Pseudomonas cepacia (PCL)	Reclassified in 1995 as *Burkholderia cepacia*. Cloning and sequencing revealed identity with lipase from *P. sp.* ATCC 21808 (PSL).

2.2. Analytical Determination

Lipases are often analyzed by their hydrolytic action on triglycerides in a heterogeneous reaction medium of water and oil. The greatest caution must be exercised both when

Table 3. Important commercially available lipases.

Origin	Code[a]	*M* [kDa] (rounded)	Specificity (remarks)	Applications
of mammalian origin				
human pancreatic lipase	HPL	50	*sn*-1,3	
human gastric lipase	HGL	50	*sn*-3 (acid-stable)	
porcine pancreatic lipase	PPL	50	*sn*-1,3	organic synthesis, digestive aid
guinea pig pancreatic lipase	GPL-RP2	48	*sn*-1,3 (phospholipase A1 activity)	
of fungal origin				
Candida rugosa	CRL	60	nonspecific	organic synthesis
Candida antarctica B	CAL	60	*sn*-1,3	organic synthesis
Geotrichum candidum	GCL	60	cis-Δ^9 (unsaturated fatty acids)	oleochemistry
Humicola lanuginosa	HLL	30	nonspecific	detergents
Rhizomucor miehei	RML	30	*sn*-1,3	cheese manufacturing
Aspergillus oryzae	AOL			cheese manufacturing
Penicillium camembertii	PEL	30	*sn*-1,3	monoglycerides
Rhizopus delemar	RDL	41	*sn*-1,3 (phospholipase A1 activity)	oleochemistry
Rhizopus oryzae	ROL	41	*sn*-1,3 (phospholipase A1 activity)	oleochemistry
Rhizopus arrhizus	RAL	41	*sn*-1,3 (phospholipase A1 activity)	oleochemistry
of bacterial origin				
Pseudomonas glumae	PGL	33	nonspecific	detergent enzyme, organic synthesis
Burkholderia cepacia	PCL/BCL	33	nonspecific	organic synthesis
Pseudomonas pseudoalcaligenes	PPL	33	*sn*-1,3	detergents
Pseudomonas mendocina	PML	33	*sn*-1,3	detergents
Chromobacterium viscosum	CVL	33	*sn*-1,3	organic synthesis
Bacillus thermocatenulatus	BTL-2	43	*sn*-1,3 (thermoalkalophilic)	
Fusarium solani (hydrolyzes cutin)	FSL	22		detergents

[a] Other appreviations for lipases used in this article: PSL (*Pseudomonas species* lipase), PFL (*Pseudomonas flourescens* lipase), HLL (human lipoprotein lipase), LPL (lipoprotein lipase). Lipases can be obtained commercially from many suppliers. Important original producers are Novo-Nordisk (Baegsvard, Denmark), Genencor International B. V. (Delft, The Netherlands), Boehringer-Mannheim (Mannheim, Germany), and Amano Co. (Nagoya, Japan).

performing and interpreting kinetic measurements. Since the medium is heterogeneous, the addition of any amphiphilic compound to the system will have effects on the interface and often on the enzyme itself. Some lipases, such as gastric lipases, rapidly become denatured at an interface with pure tributyrin emulsion, and it is therefore impossible to assess the interfacial activation with substrates of this kind. Esters which are partly soluble in water may form monomolecular adsorption films on the surface of the air bubbles which are produced upon stirring the reaction mixture. This phenomenon is responsible for a great disparity between initial velocity measurements, depending on whether or not mechanical stirring methods are used. It has in fact been established that the "quality" of the lipid/water interface—in terms of the orientation and conformation of the film-forming molecules, the molecular and charge surface densities, the interfacial water structure, the surface viscosity, etc.—is one of the most decisive parameters when working with lipolytic enzymes.[29–31] This unfortunately means that valid comparisons can be made only between data obtained under strictly identical conditions, preferably within the same laboratory. Data on lipase activity obtained from different commercial manufacturers usually cannot be compared. For practical purposes, Table 4 gives an overview of the major procedures used in lipase analysis.

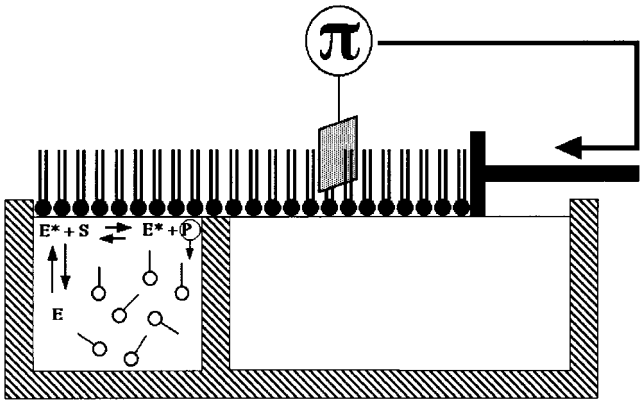


Figure 1. Drawing of a zero-order trough (a Langmuir–Blodgett film balance) composed of a substrate reservoir and a reaction compartment containing the enzyme solution. The two compartments, each covered with a monomolecular lipid film, are connected to each other by a narrow surface canal made in etched glass. The surface pressure of the film can be maintained constant automatically by the surface barostat method.[32] Fully automated monolayer systems of this kind are now commercially available (KSV, Helsinki, Finland) and have been found to have many advantages: a) With very little amount of lipid, it is possible to monitor one of several physicochemical parameters characteristic of the monolayer film (surface pressure, potential, area, etc). b) In contrast to bulk methods, it is possible to transfer the monomolecular film from one aqueous subphase to another. c) It is possible to obtain accurate pre-steady-state kinetic measurements with minimal pertubation caused by increasing amounts of reaction products. d) Inhibition of lipase by water-insoluble analogues can be precisely estimated using a zero order trough and mixed monomolecular films in the absence of any synthetic, non-physiological detergent.[33]

2.3. Interaction of Lipases with Lipids

As lipase catalytic action is strictly dependent upon the presence of a lipid interface, lipolytic enzymes provide a valuable model for studying protein–lipid interactions.[34–37] Most data dealing with the surface properties of lipases were obtained with the monolayer technique, by recording (either independently or simultaneously) the lipolytic activity, the amount of protein adsorbed to the lipid monolayer, and the variations in surface pressure following protein adsorption (Figure 1). Several non-enzymatic proteins were used as controls in order to determine how lipase behavior differs from that of other proteins. With zwitterionic monolayers, the amount of pancreatic lipase binding to the monolayer decreased with increasing initial surface pressure, as observed with control proteins such as bovine serum albumin and β-lactoglobulin A. However, pancreatic lipase as well as control proteins adsorbed to neutral lipid monolayers in a similar manner and independant from surface pressure. Lipase activity in the presence of various proteins was investigated with monomolecular films of glycerol didecanoate, either at constant surface area or at constant surface pressure. Depending upon the nature of the lipase and the protein, inhibition of lipase activity was either observed or not. Inhibition was correlated with a decrease in lipase surface concentration. The ability of the various proteins to inhibit lipolysis is a) a function of their concentration in comparison to the concen-

Table 4. Analytical methods for determining lipases.[16]

Method	Principle	Advantages	Disadvantages
pH stat	potentiometric determination of fatty acids liberated upon hydrolysis	• continuous kinetic methods for the determination of initial rates	• expensive equipment • added emulsifiers often modify reaction conditions • not applicable under acidic pH conditions
back titration	titration of liberated fatty acids after defined exposure to lipase action	• simple	• no continuous kinetic data
colorimetry	liberation of a reporter group from a synthetic ester (e.g. *p*-nitrophenyl palmitate or dilauryl glycerol resorufin ester)	• fast and automatic kinetic measurements	• unnatural substrates
determining the hydrolysis of lipid films spread at the air/water interface	hydrolysis of a lipid monolayer at constant surface pressure with continuous supply of substrate (zero-order trough)	• kinetic measurements in the absence of added emulsifier and under controlled "interfacial quality"	• expensive equipment • time-consuming • trained experimentalist necessary

tration of lipase in the bulk phase and b) correlated with their penetration capacity (i.e., the initial rate of increase in surface pressure of a glycerol didecanoate monolayer having an initial surface pressure of 20 dyn cm^{-1} after the injection of the protein). Since lipase inhibition was observed with low surface densities of inhibitory proteins, a long-range effect is probably involved in the mechanism of interfacial lipase inhibition.[34] It is hypothesized that a lack of lipase adsorption to, or desorption from, the lipid monolayer results from a change in the organization of the hydrocarbon moiety of the lipid.

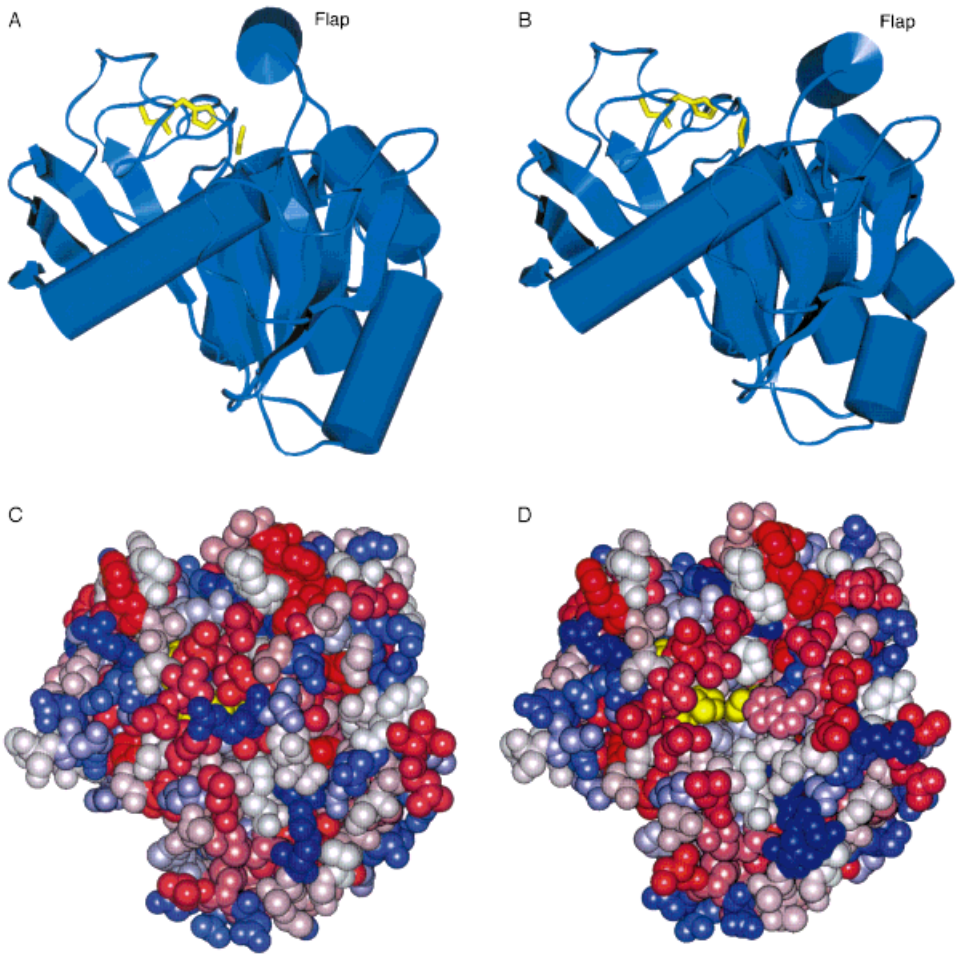


Figure 2. Structure of *Mucor miehei* lipase in closed (A, C) and open form (B, D). A and B (side view): the catalytic triad (yellow) and secondary structure elements showing the α/β-hydrolase fold common to all lipases. C and D (top view): space-filling model, colored by decreasing polarity (dark blue – light blue – white – light red – dark red). Upon opening of the lid, the catalytic triad (yellow) becomes accessible (D), and the region binding to the interphase becomes significantly more apolar.

3. Structure and Mechanism

What exactly is a lipase? In 1958 Sarda and Desnuelle defined lipases in kinetic terms, based on the phenomenon of interfacial activation.[38] It amounts to the fact that the activity of lipases is low on monomeric substrates but strongly enhanced once an aggregated "supersubstrate" (such as an emulsion or a micellar solution for instance) is formed above its saturation limit. This property is quite different from that of the usual esterases acting on water-soluble carboxylic ester molecules, and for a long time lipases were considered as a special category of esterases which are highly efficient at hydrolyzing molecules aggregated in water.

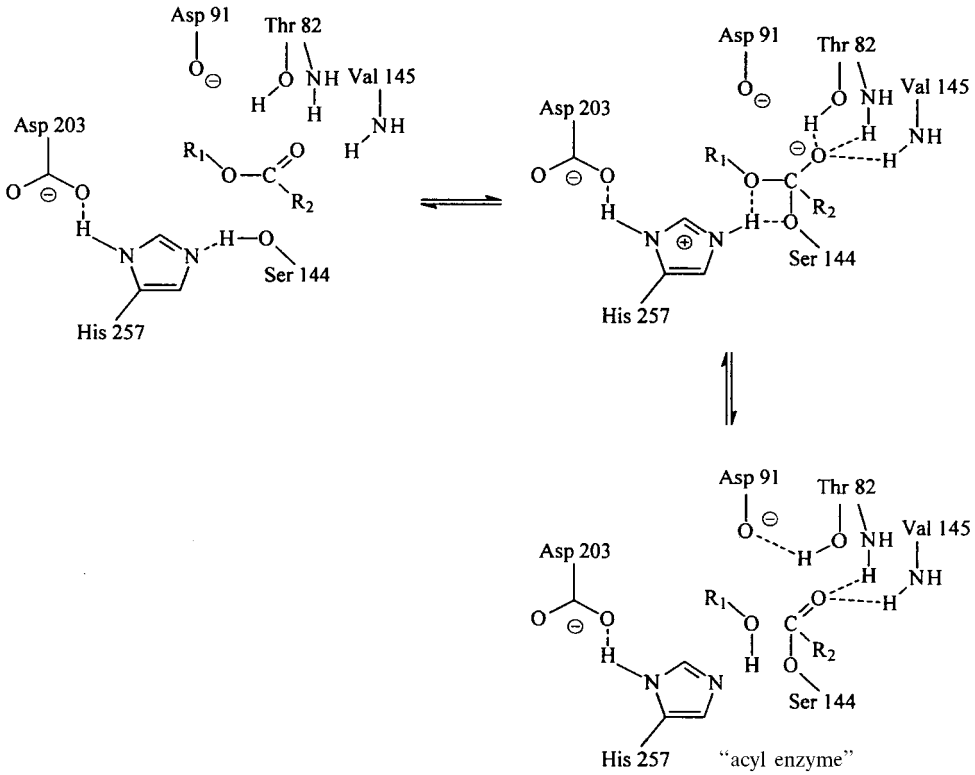
The protein structure underlying these observations remained a mystery until a few years ago. Only in 1990 were the first two lipase structures solved by X-ray crystallography. They revealed a unique mechanism, unlike that of any other enzyme: Their three-dimensional structures suggested that interfacial activation might be due to the presence of an amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid or flap.[39, 40] From the X-ray structure of cocrystals between lipases and substrate analogues, there is strong indirect evidence that, when contact occurs with a lipid/water interface, this lid undergoes a conformational rearrangement which renders the active site accessible to the substrate (Figure 2).[41]

However, within the framework of the European Lipase Project, carried out from 1990 to 1994 by 24 laboratories in 8 nations, structural and numerous biochemical data on highly purified lipases provided evidence that not all lipases subscribe to the phenomenon of interfacial activation.[42] Thus, the lipases from *Pseudomonas glumae*[43] and *Candida antarctica* (type B),[44] whose tertiary structure is known, both have an amphiphilic lid covering the active site but do not show interfacial activation. Among the pancreatic lipases whose tertiary structures have been solved, human pancreatic lipase contains a lid with 23 amino acid residues and shows interfacial activation,[45] whereas coypu lipase does not, although it has a lid of homologous size.[46] The lipase of the guinea pig, an enzyme which shows no interfacial activation, features a "mini-lid" composed of only five amino acid residues.[47] Thus, neither the phenomenon of interfacial activation nor the presence of a lid domain are appropriate criteria to classify an esterase into the lipase subfamily. For classifying an esterase as a lipase, the safest experimental evidence remains to be, as in the early days of research on this enzyme, whether or not it can hydrolyze long-chain acyl glycerols.[29]

All 12 lipases whose structure has hitherto been elucidated are members of the "α/β-hydrolase fold" family with a common architecture composed of a specific sequence of α helices and β strands.[39, 40, 43, 44, 48–53] They hydrolyze ester

bonds by means of a "catalytic triad", composed of a nucleophilic serine residue activated by a hydrogen bond in relay with histidine and aspartate or glutamate (Scheme 2). Serine proteases ("detergent proteases") follow essentially the same mechanism.

As pointed out already, a unique structural feature common to most lipases is a lid or flap composed of an amphiphilic α helix peptide sequence, which in its closed conformation (i.e., in the absence of an interphase or organic solvent) prevents access of the substrate to the catalytic triad. After the lid has opened, a large hydrophobic surface is created to which the hydrophobic supersubstrate (usually the oil drop) binds. This presumed mechanism is supported by the X-ray structures of lipases covalently complexed with hydrophobic inhibitors such as alkyl phosphonates, cycloalkyl phosphonates, or alkyl sulfonates. They reveal an open lid, suggesting that the phosphonates mimic the transition state for acylation and the sulfonates for deacylation of natural triacyl glycerol ester substrates.[54, 55]



Scheme 2. Catalytic mechanism of lipases based on a "catalytic triad" of serine (nucleophile), histidine, and aspartate or glutamate (connected through a hydrogen bond). The tetrahedral intermediate is stabilized by an "oxyanion hole". The numbering of amino acids in this example refers to lipase from *Rhizopus oryzae*.

To summarize these findings, all lipases investigated so far exhibit a surprising degree of structural and functional similarity, regardless of the organism from which they were isolated and even if the observed amino acid sequence homologies are low. The coherent features of lipases are summarized in Table 5.

In spite of these similarities, subtle variations in the architecture of the substrate binding site may have a strong effect on the catalytic properties, the temperature, and stability of a lipase in a solvent. As these differences are of paramount importance for the selection of an individual lipase for a desired application, recent research has emphasized this aspect, as further described in Section 6.

4. Function and Structure of Digestive Lipases

Human pancreatic lipase (HPL) is the major lipolytic enzyme involved in the digestion of dietary triglycerides,[56] and it amounts to around 3 % of the total proteins secreted by

Table 5. Important features of lipases.[54, 55]

Feature	Details	Remarks
Mechanism based on a "catalytic triad" made up of nucleophilic serine, histidine, and aspartate/glutamate	Found in over 30 lipases, including those from psychrophilic or thermophilic microorganisms; the only exception is replacement of aspartate for glutamate. Substitution of serine for cysteine by site-directed mutagenesis led to strongly reduced activity.	Related hydrolases show much greater variability in catalytic mechanism. In the case of proteases, the amide bond can be hydrolyzed through nucleophilic attack by hydroxyl (serine, threonine) or thiol groups (cysteine), or through electrophilic attack by a carboxyl group (aspartate/glutamate) or a metal ion (Zn^{2+}).
Consensus sequence at the active serine residue	The consensus sequence for over 30 cloned lipases is a "nucleophilic elbow" at the end of a σ sheet and is composed of -Gly/Ala-X-*Ser*-X-Gly-.	Other hydrolases show greater variability.
Most lipases feature a lid structure	A lid composed of an amphiphilic peptide loop covers the active site of the enzyme in the inactive state.	No lid was observed in esterases or proteases, but some lipases have no lid or just a small lid.
All lipases are members of the"α/β-hydrolase fold" family	The structure is composed of a core of predominantly parallel β strands surrounded by α helices. The active nucleophilic serine residue rests at a hairpin turn between a β strand and an α helix.	Many other hydrolases (esterases, acetylcholine esterases, serine proteases, carboxypeptidases, dehydrogenases) and even a haloperoxidase show a similar structural motive, which suggests evolutionary relationships.

the exocrine pancreas.[57] Pancreatic lipase hydrolyzes primary ester bonds of tri- and diglycerides to generate 2-monoglycerides and fatty acids, which are adsorbed through the intestinal barrier in the form of mixed micelles with bile salts. In contrast to most of the other pancreatic enzymes, which are secreted as proenzymes and further activated by proteolytic cleavage in the small intestine, pancreatic lipase is directly secreted as an active enzyme with 449 amino acid residues and a molecular weight of 50 kDa.

Surprisingly, pancreatic lipase is inactive with respect to an emulsified triglyceride substrate in the presence of micellar concentrations of bile salts, such as those found in the small intestine during digestion. Bile salts are amphiphilic molecules which are mainly found absorbed to the lipid/water interface or dispersed as micelles in solution. Experimental evidence supports the notion that the negatively charged surface of triglyceride globules coated by bile salts inhibits the adsorption and activation of pancreatic lipase. To counteract this effect, a specific lipase-anchoring protein, colipase, is present in the exocrine pancreatic juice. It forms a 1:1 complex with the lipase, which facilitates its adsorption to lipid/water interfaces covered with bile salts.[45] Thus, through the concerted action of colipase, HPL displays a high specificity towards insoluble triglycerides. Lipases have a key function in the catabolism of fat. Low levels of pancreatic lipase have been described in diseases such as pancreatitis and cystic fibrosis. As a consequence, preparations of lipases from porcine pancreas and other origins are offered, and have been for a long time, as substitutes in enzyme therapy. On the other hand, the excessive intake of dietary fat in the industrialized countries may lead to overweight, which constitutes a risk factor for health. Intact, nonhydrolyzed long-chain triglycerides cannot be absorbed through the gastrointestinal tract and therefore do not contribute to the energy balance of an organism. Lipase inhibitors might thus constitute a principle to prevent obesity in spite of normal dietary fat intake. We will discuss the application of lipase and lipase inhibitors to pharmacology further in Section 7.

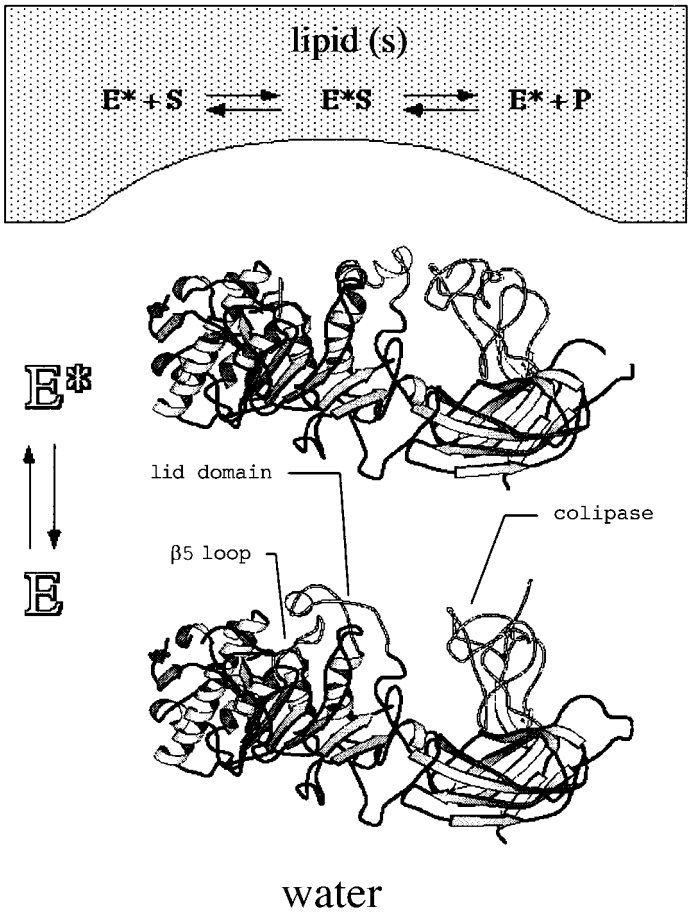


Figure 3. Structure of the HPL – procolipase complex in the closed (E) and open conformatioin (E*S). These two figures show the conformational changes in the lid—that is, in the β5 loop—and the colipase during interfacial activation.

4.1. Mechanism of Action of Human Pancreatic Lipase

The general features of the interfacial catalysis by the pancreatic lipase – colipase complex have been particularily well investigated and refined at a molecular level. X-ray studies of the three-dimensional structure of HPL by Winkler et al. confirmed the existence of two distinct domains in pancreatic lipase: a larger N-terminal domain comprising residues 1 – 335, and a smaller C-terminal domain made up of residues 336 – 449 (Figure 3).[39]

The large N-terminal domain is a typical α/β hydrolase fold dominated by a central parallel β sheet.[39] It contains the active site with a catalytic triad formed by Ser 152, Asp 176, and His 263, all of which are conserved in other members of the mammalian lipase family (e.g. in lipoprotein lipase and hepatic lipase). The active site is covered by a surface loop between the disulfide bridge Cys 237 and 261. This surface loop includes a short one-turn α helix with a tryptophan residue in position 252, which is completely buried inside the protein and sitting directly on top of the active site Ser 152. Under this closed conformation, the lid prevents the substrate from having access to the active site. Spectroscopic studies of tryptophan fluorescence with HPL have shown that large spectral changes are induced by acylation of pancreatic lipase with the inhibitor tetrahydrolipstatin (**16**, see Scheme 18) in the presence of bile salt micelles.[58] The X-ray crystal structure analyses of cocrystals of the pancreatic lipase – procolipase complex in the presence of mixed bile salt/phospholid micelles[45] as well as in the presence of a monoalkyl phosphonate inhibitor[59] showed that the lid was shifted to one side, exposing both the active site and a larger hydrophobic surface. This motion is induced when binding of the enzyme to the lipidic interface occurs and is probably the structural basis for interfacial activation of pancreatic lipase.

The open structure of the lipase – procolipase complex illustrates how colipase might anchor the lipase at the interface in the presence of bile salts: Colipase binds to the noncatalytic β sheet of the C-terminal domain of HPL and exposes the hydrophobic tips of its fingers at the opposite side of its lipase-binding domain. This hydrophobic surface, along with the hydrophobic back side of the lid as well as the β9 loop, helps to bring the catalytic N-terminal domain of HPL into close contact with the lipid/water interface.

Apart from the apolipoprotein CII activating lipoprotein lipase, no colipase has been found in other organs or organisms. Thus, the mechanism discussed above seems to be special to the specific case of the pancreatic lipase/colipase system.

5. Applications of Lipases in Oleochemistry, in Detergents, and in the Paper and Food Industry

5.1. Lipases in Oleochemistry

5.1.1. Soaps and Fatty Acids

While the bulk of the 60 million tons of fats and oils produced every year are directly used in food, over five million tons are a renewable chemical feedstock for non-food-related applications ("oleochemistry"). One major chemical application of triglycerides is in the preparation of soap: Todays preferred chemical processes (Monsavom, Sharples, or De Laval – Centriput processes) operate continuously and provide quantitative yields within minutes at 100 °C, independent from the origin of the fat or oil. Energy consumption is further minimized by energy recycling.[60]

At present, about two million tons of soap are produced every year by these processes. As early as 1902, however, Connstein described the use of lipase extracted from *Ricinus* seed for the preparation of pure fatty acids from tallow and plant oils.[61] The process took 34 hours at 30 °C, and yields were between 60 and 95 %, depending on the triglyceride used. While this is hardly competitive to todays chemical processes, points in favor of lipase-catalyzed soap production are the lower depreciation costs incurred (as the equipment is limited to a stirred reactor), the better color of the soap, and the generation of 20 % glycerol water as a by-product (instead of more dilute glycerol in the steam splitting process). Miyoshi Yushi, a company in Nagoya, Japan, seems to be the only company in the world which produces sizable amounts of soap through lipase-based hydrolysis of oils and fats with *Candida rugosa* lipase. They use stirred reactors at around 40 °C, in a batch mode lasting less than 48 hours.

Another key chemical intermediate produced from triglycerides are fatty alcohols, which are prepared from the fatty acid methyl esters (obtained by interesterification of the parent triglycerides), by hydrogenation in fixed-bed reactors at 200 – 300 bar and 200 – 300 °C.[60, 62] Yields of the process are nearly quantitative, regardless of the fat or oil used, and energy recycling has been optimized. However, raffination steps may be necessary to remove products of thermal degradation, and heat-sensitive oils such as fish oils have to be processed separately. Companies spezialized in oleochemistry—such as Unilever, Henkel, Procter & Gamble, and others—have therefore explored lipase-based fat splitting as an alternative to generate the raw material for production of fatty alcohols. These studies revealed major drawbacks; for example a) the reaction time depends strongly on the type of fat or oil used (the rate of hydrolysis at 40 °C decreases in the following order for oils from olive > soya = sunflower > palm > coconut > tallow), b) the space-time yields compare poorly with those for chemical process and necessitate the construction of expensive multistage reactors, c) expensive techniques are required for the separation of the glycerol water from the oil/lipase phase, and d) continuous use of the expensive lipase catalyst is limited by its inherent instability. At present, Nippon Oils & Fats, a medium-sized company in Tokyo, seems to be the only firm which operates with an enzyme-based process for the preparation of highly pure unsaturated fatty acids (oleic, linoleic, linolenic, etc.); they use lipase from *Candida rugosa*.[63] However, even these speciality products, which are sold only on the kilogram scale, compete with the highly pure fatty acids available today from the oils obtained from genetically modified plants.

5.1.2. Transesterified Triglycerides

Many lipases exhibit *sn*-1,3 specificity, and can therefore be used to regioselectively interesterify positions 1 and 3 of a natural triglyceride; however, the tendency towards acyl migration from the *sn*-2 to the *sn*-1 or *sn*-3 postions must be supressed. So far, three types of modified triglycerides have gained in commercial importance: a) fats with improved spreadability, b) cocoa-butter equivalents, and c) highly digestive triglycerides.

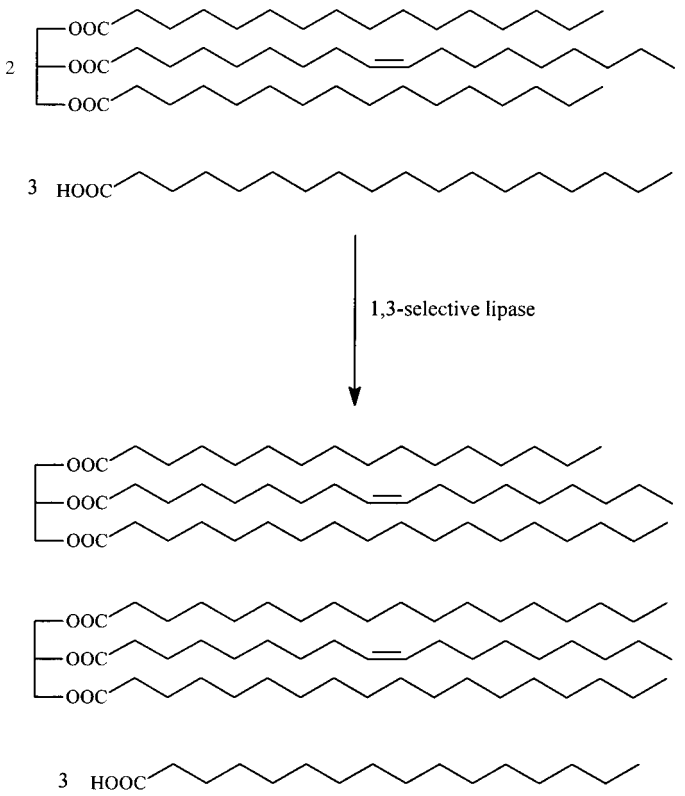
5.1.2.1. Fats with Improved Spreadability

The melting point of an oil can be modulated by the degree of catalytic hydrogenation of double bonds in unsaturated fatty acids. This is done on a large scale for the preparation of margarines and shortenings from plant oils. Alternatively, the desired melting behavior can be achieved through interesterification of suitable triglyceride mixtures with the use of *sn*-1,3-specific lipases or a combination of both procedures.[64, 65]

5.1.2.2. Cocoa-Butter Equivalents

In 1995 several 100 000 tons of cocoa butter were imported from tropical countries, mainly Kenya and Malaysia. Since its melting point is around human body temperature (37 °C), cocoa butter is well suited as a matrix for suppositories. The main application, however, is in the production of chocolates, where the rapid melting conveys a desirable "mouth feel". The predominant triglycerides of cocoa butter are glycerols with oleic acid in the *sn*-2-position and stearic and palmitic acids in the *sn*-1- and *sn*-3-positions (SOS and SOP). Cocoa-butter equivalents can be prepared either chemically or by lipase catalysis through the interesterification of suitable natural triglycerides, for example, the middle fraction of palm oil (POP) or sunflower oil with a high content of oleic acid (high-oleic sunflower oil; OOO) with stearic acid or tristearin (SSS; Scheme 3).[66, 67]

Since the primary hydroxyl groups of glycerol in positions *sn*-1 and *sn*-3 are more reactive than the secondary hydroxyl group in *sn*-2, triglycerides of the type SOP or SOS are predominantly formed. From the standpoint of food regulation, enzyme-catalyzed transesterifications of natural oils are "natural processes" and therefore do not require labeling of the triglyceride. The lipase-catalyzed preparation of cocoa-butter equivalents has been investigated by many companies.

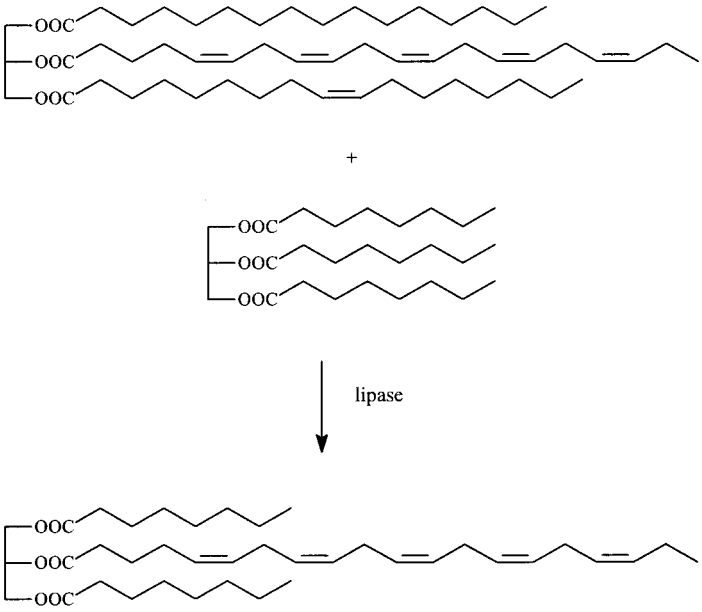


Scheme 3. Interesterfication of a natural triglyceride substrate with a *sn*-1,3-regioselective lipase.

At present, Unichema, which is a former subsidiary of the Unilever group and was recently acquired by ICI, produces several hundred tons of "chocolate fat" (SOS) by transesterification of high-oleic sunflower oil with stearic acid in a solvent-free packed-bed reactor; the immobilized lipase from *Rhizomucor miehei* is used as a catalyst.[68] In Japan, Fuji Oil has established a production capacity of several thousand tons per year for SOS.[69] The process is similar to the Unichema procedure, but *Rhizopus* lipase is used as a catalyst.

5.1.2.3. Highly Digestive Triglycerides

The absorption of triglycerides from the small intestine strongly depends on the structure of the triglyceride. Thus, triglycerides containing palmitic acid are well adsorbed only when this fatty acid is located in the *sn*-2-position, like in human milk. A commercial product of this type is Unichema's Betapol (OPO), a diet additive for premature infants. It is prepared by interesterification of tripalmitin with oleic acid with use of immobilized *Rhizomucor miehei* lipase. Alternatively, triglycerides of the type MLM, with long-chain saturated or unsaturated fatty acids (L) in the *sn*-2-position and medium-chain fatty acids (M) in positions *sn*-1 and *sn*-3, provide a rapid delivery of energy through enhanced hydrolysis and resorption; pancreatic lipase hydrolyzes medium-chain triglycerides preferentially, and the resulting monoglycerides are efficiently absorbed from the large intestine. Several products of this type are commercially available (e.g. Caprenin (C_6-$C_{22:0}$-C_8) from Procter & Gamble), but are synthesized by chemical interesterification. Triglycerides of the MLM type containing essential fatty acids, for example eicosapentaenoic or docosahexaenoic acid, have shown beneficial effects against cardiovascular and inflammatory diseases. Functional triglycerides of this composition are preferentially prepared by means of *sn*-1,3-specific lipases (Scheme 4), as chemical interesterification of highly unsaturated triglycerides (e.g. fish oil) promotes side reactions such as oxidation, *cis*–*trans* isomerization, or migration of the double bond.[70, 71]



Scheme 4. Preparation of a functional triglyceride with a *sn*-1,3-specific lipase.

5.1.3. Monoglycerides

In 1993 about 120000 tons of monoglycerides and mixtures of mono- and diglycerides were manufactured through glycerolysis of triglycerides. After short-path distillation, monoglycerides of greater than 90% purity are obtained. Monoglycerides are mild emulsifiers (HLB value 3.4, HLB = hydrophilic lipophilic balance) permitted for use as food additives. Industrial applications include emulsification in food, cosmetics, and drug preparations.[62, 72, 73] Many reports deal with the preparation of monoglycerides through lipase catalysis, but the key problem remains that mixtures of mono- and diglycerides are formed.[74, 75] Table 6 offers a survey of enzymatic preperation procedures.

Under commercial aspects, none of these procedures has been able to compete with the chemical manufacture of monoglycerides outlined above. Even when a lipase obtained from *Penicillium roquefortii* providing greater than 90% monoglycerides in a one-step glycerolysis reaction was used, the economic data were unsatisfactory. The use of lipases has proven advantageous, however, for the synthesis of partial glycerides containing labile substituents such as 8′-apo-β-carotinoic acid, which would not withstand chemical procedures.[76]

Table 6. Lipase-catalyzed synthesis of monoglycerides.

Procedure	Details	Results and comments
Hydrolysis or alcoholysis	1,3-Specific lipases	Acyl migration from *sn*-2 to *sn*-1/*sn*-3 may lead to total hydrolysis.
Esterification	a) With glycerol and fatty acid and use of any type of lipase b) With isopropylidene-protected glycerol	a) Since monoglycerides are better nucleophiles than glycerol, mixtures of mono- and diglycerides are formed. b) Leads to pure monoglycerides, but necessitates additional protection and deprotection.
Highly selective lipase	The use of lipase from *Penicillium roquefortii* allows the production of up to 90% monoglycerides from triglycerides.	
Glycerolysis and improved process engineering	The use of an enzyme membrane reactor with counter-current extraction allows the removal of monoglycerides from the reaction mixture. Alternatively, monoglycerides are precipitated or cryoprecipitated.	Provides the best results, but process engineering is more complex than a chemical procedure.

5.2. Lipases in Detergents

The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains[77–80] and in situ generation of peracid bleach by perhydrolysis.[81]

5.2.1. Removal of Fat Stains

After the great commercial success of proteases as detergent additives, the enzyme industry undertook major efforts to introduce lipases as a second group of detergent enzymes. It was hoped that lipases could compete with chemical surfactants in the detergent formulation, and thus respond to changing detergent formula in view of lower wash temperatures and ecologically benign components. Standard wash liquids contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 50 °C, which is a rather hostile environment for enzymes. As a result, major screening programs for lipases stable under such conditions were initiated. Early systematic studies with the then available lipases showed that the marginal effect of lipases could be substituted, at lower price, by the addition of suitable chemical surfactants to the formulation.[82] It later turned out, however, that suitable lipases can prevent redeposition of fat material on washed textiles, thus exerting a secondary effect on laundry cleaning. In various experiments based on the removal of dyed soils from textiles, detergent formulations including lipase showed an improved performance in the elimination of stains such as lipstick. Novo-Nordisk was the first company to commercialize these results. The product Lipolase, a recombinant fungal lipase from *Humicola lanuginosa* expressed in a host strain of *Aspergillus oryzae*, was produced on an industrial scale in Hokkaido, Japan, and later exported to North America and Europe.[23] This was probably the first genetically engineered protein to obtain permission by regulatory bodies in Europe to be used in consumer products and be discharged, after use, into the environment. Today, lipases are used in most detergent formulations. The major commercial products are summarized in Table 7.

All the lipases mentioned above show a good secondary washing behavior. However, cutinase, a long-chain cleaving esterase, has an outstanding performance in the removal of wax esters, which are found in lipstick.

Table 7. Commercial detergent lipases.

Brand name	Company	Origin of lipase	pH optimum	*T* optimum
Lipolase	Novo-Nordisk	*Humicola lanuginosa*, cloned and expressed in *Aspergillus oryzae*	10.0	40 °C
Lipomax	Gist-Brocades[a]	*Pseudomonas pseudoalcaligenes*, recloned and expressed in the same organism	11.0	45 °C
Lumafast	Genencor[a]	*Pseudomonas mendocina*, cloned and expressed in *Bacillus spec.*	10.5	40 °C

[a] The enzyme business of Gist-Brocades was acquired in 1995 by Genencor International B. V. (Delft, The Netherlands).

5.2.2. Production of Peracid Bleach by Perhydrolysis

Peracids of a chain length C_8-C_{12} are excellent oxidants under the slightly alkaline conditions of washing and thus have potential as a low-temperature bleach. Owing to their limited stability, they cannot easily be mixed into a detergent formulation. The in situ lipase-catalyzed generation of peracids has been investigated as an alternative. However, native lipases show only a marginal tendency to perhydrolyze esters in the presence of water/hydrogen peroxide, probably because hydrolysis of the initial "peracid-acyl" enzyme is much faster at alkaline pH than its formation.[81]

5.3. Lipases in Paper Manufacture

Nihon Seishi Co., a Japanese paper manufacturer, has developed a process by which the triglycerides contained in raw lumber are hydrolyzed by the addition of lipase. This results in better "pitch control", that is, an easier processing of the lumber to low-grade paper. The process is carried out at a scale of several hundred tons of lumber per day, and two other Japanese paper manufacturers are using the same procedure.[83]

5.4. Lipases in the Dairy Industry

Rennet paste, isolated from the stomach of ruminant animals such as cows or goats, is an enzyme mixture traditionally used for the preparation of cheese.[84] The active

component of rennet is chymosin, an aspartate protease involved in the clotting of milk through the hydrolysis of κ-casein; however, lipases and esterases contained in rennet also contribute to cheese ripening. In 1955 the US Food and Drug Administration issued a ban on further import of calf rennet pastes from Europe because of their poor quality. Since then, proteases and lipases from other sources have increasingly been used in cheese making. Depending on the chain-length specificity of a given lipase, its addition to a milk product may enhance the flavor of the cheese, accelerate the cheese ripening, or assist in the preparation of "enzyme-modified cheeses" (EMC), an important commercial flavor used in the USA for the manufacture of dips, sauces, dressings, crackers, etc. EMC is produced from cheese curd by the addition of lipases at elevated temperatures, increasing the content of free fatty acids about tenfold. Table 8 contains some examples of lipases presently used in cheese making and in accelerated cheese ripening.

Table 8. Examples of lipases used in cheese making and ripening.

Cheese type	Lipase
romano, domiati, feta	pregastric lipase from lamb or goat, *Mucor miehei*
mozarella, parmesan, provolone	pregastric lipase from lamb or goat
fontina, ras, romi	*Mucor miehei*
cheddar, manchego, blue	*Aspergillus oryzae*, *Aspergillus niger*

Depending on the chain-length specificity of the lipases used, there is either predominant liberation of short-chain fatty acids (C_4-C_6)—which results in a sharp, tangy flavor—or of medium- and long-chain fatty acids ($>C_{12}$)—which convey a soapy taste and are readily metabolized by the microbial consortia found in cheese to other flavor ingredients such as acetoacetate, β-keto acids, methyl ketones, and flavor esters and lactones. Addition of lipases to pasteurized cow's milk can generate flavors similar to goat, sheep, or raw milk if used with the appropriate microbial consortia.

6. Lipases as Biocatalysts in Organic Synthesis

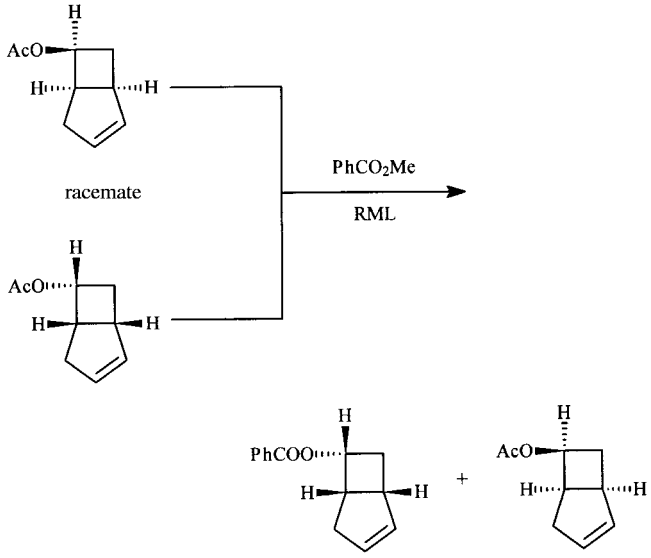
In view of their specific and limited function in metabolism, one should expect lipases to be of limited interest for the organic chemist. However, the opposite is true: Chemists have discovered lipases to be one of the most versatile classes of biocatalysts in organic synthesis[15, 85–90] for a few simple reasons:

1. Owing to the large enzyme domains required for acyl group binding and the unpronounced structural features of acyl chains, lipases can accomodate a wide variety of synthetic substrates, while still showing regioselectivity or chiral recognition.
2. Lipases act at the water/lipid boundary, which exhibits high interfacial energy. To withstand the denaturing effect of the interface, lipases have evolved unusually stable structures that may survive even the effect of organic solvents.
3. The free energy of fat hydrolysis is close to 0 kJ mol^{-1}.[91, 92] As a result, thermodynamic equilibria are largely governed by reactant concentrations, and lipase-catalyzed ester hydrolysis in water can easily be reversed, in nonaqueous media, into ester synthesis or interesterification.
4. The acyl lipase formed in the first step of the enzymatic reaction can formally be considered as an acylating agent. The wide substrate specificity of this enzyme class allows acylation of nucleophiles other than those with hydroxyl groups, for example hydroperoxides, amines, and thiols.

As a result of this unique combination of properties, chemists have used lipases for a plethora of synthetic reactions.[93–103] In a recent comprehensive monograph on biotransformations in organic synthesis, almost one-third of all reactions were carried out with lipases.[85, 89] When polyfunctional compounds, isomers, or racemic or prostereogenic substrates are used, lipases usually exhibit regio- and/or stereoselectivity. Thus, PPL (for abbreviations, see Table 3)—designed by nature to adhere to an oil drop in the presence of bile salts and hydrolyze triglycerides—was found to carry out the regioselective reaction shown in Scheme 5,[104] and RML catalyzes enantioselective transesterifications in high yields (Scheme 6).[105]

83 % yield
99 % monoacetate

Scheme 5. Regioselective esterfication by PPL.



Scheme 6. Example of an enantioselective transesterification by RML; $E=206$ (acylation), $E=8$ (hydrolysis).

The only major drawback of lipase selectivity is, in fact, their somewhat restricted specificity towards the acyl group of esters: Most lipases accept linear aliphatic moieties much better than bulky aromatic groups.

6.1. Selectivity of Lipases Towards Non-Glyceride Substrates

6.1.1. Kinetic Resolution

As shown in Schemes 5 and 6, lipases often are capable of recognizing enantiomers or enantiotopic groups of prostereogenic molecules in a highly enantioselective manner. In the case of a racemate, this property can be used to achieve kinetic resolution with a theoretical yield of 50%. However, as lipase-catalyzed reactions are usually reversible, it must be kept in mind that the reverse reaction may lead to racemization. Thus, if acylation of a racemic alcohol provides predominantly the (*S*)-alcohol and the (*R*)-ester in the forward reaction, the reverse reaction of the achiral product (R^3OH) with the (*R*)-ester will result in the formation of the (*R*)-alcohol (Scheme 7). When the reverse reaction starts to

$$\underset{(R,S)}{R^1OH} + R^2CO_2R^3 \rightleftharpoons \underset{(S)}{R^1OH} + \underset{(R)}{R^2CO_2R^1} + R^3OH$$

Scheme 7. Kinetic resolution of an alcohol by lipase.

take place to a significant extent (which depends on the position of the equilibrium), the enantiomeric purity of both the substrate and the product is decreased.[106, 107] Kinetically, this type of reaction can be described by Equation (1). For the case of an irreversible reaction without substrate inhibition up to 40% conversion, the conversion *U* can now be calculated according to Equation (2). The "E value" (a measure of the enantioselectivity) is independent of both substrate concentration and conversion.[106, 107] It is calculated according to Equation (3) and indicates the discrimination of an enzyme

$$K = \frac{1 - c_e}{c_e} \quad (1)$$

(c_e = conversion at equilibrium)

$$U = \frac{ee(S)}{ee(S) + ee(P)} \quad (2)$$

(*ee* = enantiomeric excess for the substrate S or product P)

$$E = \frac{\ln[(1-U)(1-ee(S))]}{\ln[(1-U)(1+ee(S))]} = \frac{\ln[(1-U)(1-ee(P))]}{\ln[(1-U)(1+ee(P))]} \quad (3)$$

between the two enantiomers. The enantiomeric excess that can be reached is shown in Figure 4.

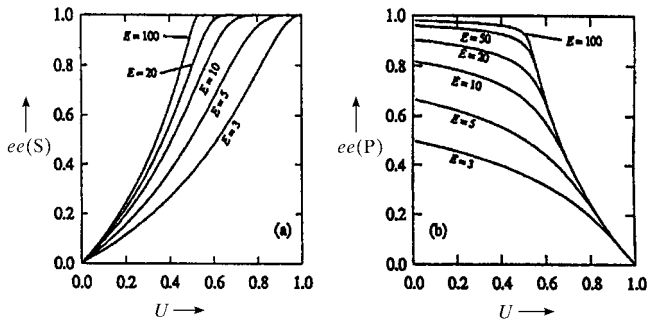
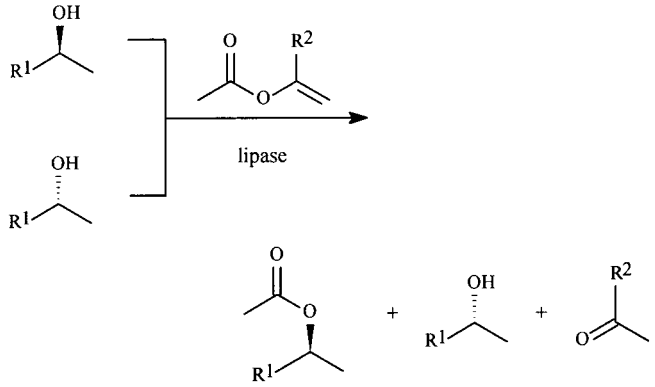


Figure 4. Enantiomeric excess *ee* as a function of conversion *U* and the "E value" for a substrate (a) and a product (b) for an irreversible reaction.[14]

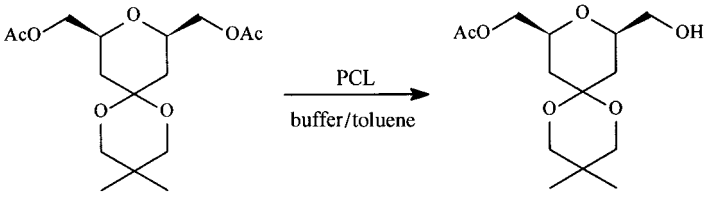
As lipase catalysis leads to reaction equilibria, measures must be taken to drive the reaction in the desired direction. Thus, water liberated during esterification can be removed in vacuo or absorbed in a molecular sieve. Alternatively, a large excess of the acyl donor can be introduced, for example, use of ethyl acetate as both the acyl donor and the solvent. Good leaving groups such as trifluoro- or trichloroethyl acetate and cyclic anhydrides have been successfully employed. Irreversible transesterification reagents such as oxime esters may also push product formation exclusively in the desired direction. The method of choice, nearly simultaneously introduced by de Jeso et al.[108] and Wong et al.,[109] is the use of enol esters (usually vinyl or isopropenyl ester). The primary alcohol formed during hydrolysis tautomerizes to give a carbonyl compound, and thus cannot participate in the reverse reaction (Scheme 8).



Scheme 8. Irreversible acylation with enol esters (R^1, R^2 are general residues).

6.1.2. The "Meso Trick"

In kinetic resolutions, theoretical yields are limited to 50%. Yields of up to 100% are possible with *meso*-diesters or diol reagents, which undergo enantioselective hydrolysis or transesterification through enantiotopic group differentiation (Scheme 9 and Table 9).[110]

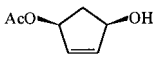
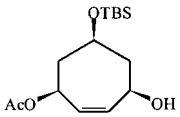
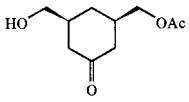
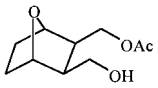
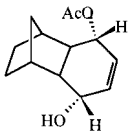


Scheme 9. Application of the "meso trick".

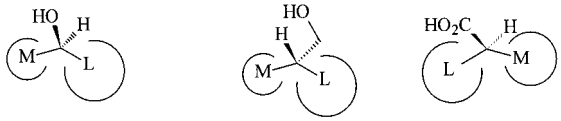
6.1.3. Semiquantitative Predictions of Lipase Stereoselectivity

Following a thorough survey of the literature on chiral resolutions with lipases from *Candida rugosa* (CRL) and *Pseudomonas cepacia* (PCL), Kazlauskas et al. put forward tentative rules for the enantiopreference of these two enzymes based on the spatial requirements of the substituents

Table 9. Lipase-catalyzed asymmetrization of *meso*-diesters.

Product	Lipase/solvent	Yield [%]	*ee* [%]	Ref.
	pancreatin/ vinyl acetate, THF/Et_3N	65	> 99	[132]
	PSL/ isopropenyl acetate	81	> 99	[133]
	PCL	80	70	[134]
	PPL/ vinyl acetate, MeCN	92	> 99	[135]
	PPL/ vinyl acetate	92	> 99	[136]

on the reagent. These rules are often dubbed "Kazlauskas rules";[111] the basics are presented in Scheme 10. Experience has shown that the rule is highly predictive for lipase action on secondary alcohols, but less accurate for lipase-catalyzed transformations of primary alcohols and acids.



Scheme 10. The "Kazlauskas rules" (M = medium-sized substituent, L = large substituent).

Recently, the Kazlauskas model was supported by crystallographic evidence. Cygler et al. cocrystallized two complexes of CRL inhibited by the two enantiomers of a menthyl phosphonate inhibitor (CRL is able to discriminate between enantiomeric menthyl esters and prefers the *R* enantiomer).[112] The preferred binding of the R inhibitor was attributed to the higher number of hydrogen bonds (Figure 5).

6.2. Lipases as Acylating Agents

The enzymatic mechanism of lipases (see Scheme 2) leads to the acylation of a serine residue in the catalytic triad of the enzyme. The subsequent reactions can formally be viewed as acylation of water (hydrolysis) or an alcohol (esterification). In the following, we will discuss acylation of oxygen-containing and other nucleophiles as well as unusual acyl donors. We will show that lipases are not only acylated when they hydrolyze carboxyl esters, but also upon reaction with

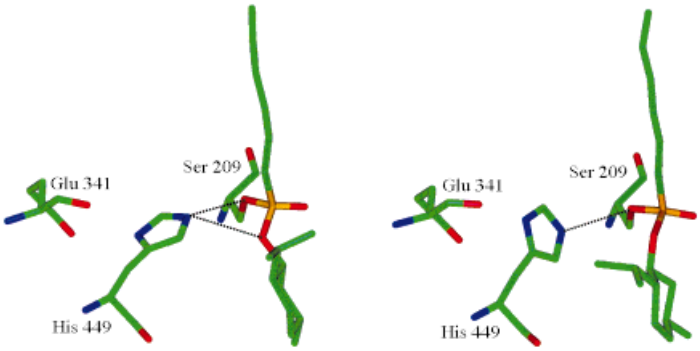


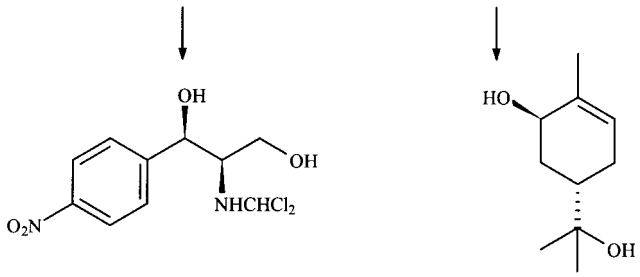
Figure 5. Binding of (*R*)- (left) and (*S*)-menthyl phosphonate esters (right) to the active site of *Candida rugosa* lipase. In the complex with the *R* enantiomer, catalytic His449 forms two hydrogen bonds (dashed lines), one to catalytic Ser209 and another to the alcohol group of menthol, while in the complex with the *S* enantiomer, His449 rotates out of the plane and thus loses its capacity to bind to menthol.

carbonates and other substrates. Finally, some examples for lipase-catalyzed reactions of industrial relevance will be summarized.

6.2.1. Acylation of Hydroxyl or Alcohol Groups

6.2.1.1. Selective Esterification of Hydroxyl Compounds

Hydroxyl compounds such as chloramphenicol (**1**), sobrerol (**2**), or oligopeptide esters were selectively acylated with PPL (Scheme 11).[85, 113–115] As sugars are the most important



1 **2**

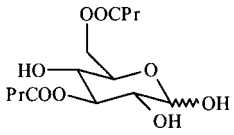
Scheme 11. Position of the regioselective acylation of chloramphenicol (**1**) and sobrerol (**2**).

examples of polyhydroxyl compounds, many studies have been devoted to their regioselective acylation or deacylation by means of lipases.[115–117] The major focus was on the elaboration of protection and deprotection in carbohydrate chemistry and, in the industrial domain, the preparation of sugar esters, a class of detergents based on renewable resources.

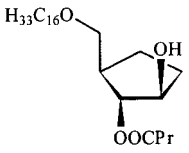
6.2.1.2. Protection and Deprotection of Sugars

Unprotected mono- or disaccharides in polar organic solvents (e.g. DMF, 2-methyl-2-butanol) and in the presence of a lipase such as PPL,[118] CAL,[119] or RML[120] react readily

with long- or short-chain fatty acids or their esters to form the 6-O-acylated sugars; the regioselectivities are higher than 70%, and the conversions between 40 and 100%. The yields could be increased by protecting the secondary hydroxyl groups (as isopropylidene derivatives or phenyl borates) or by working in the solid or molten phase, thus circumventing the large difference in solubility of the reaction partners. Protection of the primary hydroxyl group (at C6) by acylation allowed for the esterification of the secondary hydroxyl functionalities, and various lipases differed in their regiopreference. Thus, butyroylation of 6-*O*-butyroylglucose with trichloroethyl butyrate in THF by CVL led to 3,6-di-*O*-butyroylglucose (**3**, 98% regioselectivity),[121] whereas a lipase from *Rhizopus japonicus* (similar to CRL) catalyzed butyroylation of 1,4-anhydro-5-*O*-hexadecyl-D-arabinitol (**4**) with trichloroethyl butyrate in benzene at C3 in 79% yield (Scheme 12).[122]



3



4

Scheme 12. Esters **3** and **4** obtained by lipase-catalyzed butyroylation of sugars and sugar esters.

6.2.1.3. Sugar Ester Detergents

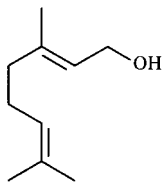
Sugar esters are chemically prepared on a scale of several thousand tons per year by the acid-catalyzed esterification of sorbitans and fatty acids (Span, if ethoxylated Tween). They are widely used as chemical emulsifiers, and the extent of acylation and ethoxylation determines the detergency of the products. Some of them have been registered as food additives, but lipases seem better suited to assist in the preparation of food-grade sugar esters. As a consequence, lipase-catalyzed synthesis of sugar esters has been explored by many researchers; a few examples are given in Table 10. In view of the different solution properties of the two reaction partners, however, it proved quite difficult to achieve high reaction rates that were competitive with chemical procedures using nonderivatized sugar reagents in food-grade solvents. Further competition arose from chemically prepared alkyl glucosides, which are now being produced on a large industrial scale for nonfood applications and have properties similar to the sugar esters. Lipase-catalyzed production of monoesters of ethylglycoside has been commercialized on the 100-kg scale by Unichema, a company of the ICI group.

Table 10. Lipase-catalyzed synthesis of sugar esters (examples); v = reaction rate.

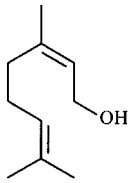
Sugar	Acyl donor	Solvent	Lipase	v [mmol g^{-1} h^{-1}]	Ref.
glucose	fatty acid trichloro ethyl ester	pyridine	PPL	0.015	[118]
mannose	fatty acid oxime ester	pyridine	PCL	0.028	[123]
sorbitol	fatty acid	2-pyrrolidone	CVL	1.4	[124]
fructose	fatty acid	2-methyl-2-butanol	RML CAL-B	0.048	[120]
glucose ethyl glycoside	fatty acid lauryl and octanoic acid	acetone *tert*-butanol	CAL-B CAL-B	0.2–0.4 undisclosed	[125]

6.2.1.4. Stereoisomers

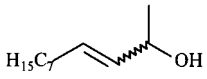
The *E*/*Z* stereoisomers of allylic alcohols were resolved by acylation in the presence of lipases (PPL, PSL), and the *E* isomer was always preferred. In most cases, however, selectivities were rather modest. Thus, a mixture of (*E*)-geraniol (**5**) and (*Z*)-nerol (**6**) was separated by acylation with trifluoroethyl butyrate or hexanoic anhydride in diethyl ether in the presence of PPL; **5** was acylated faster at a ratio of 4:1 (Scheme 13).



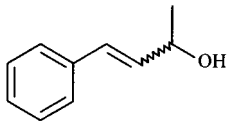
5



6



7



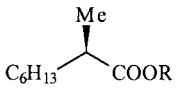
8

Scheme 13. Alcohols **5**–**8** used for enzymatic transesterfications.

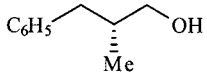
The *E* isomers of secondary allylic alcohols such as 3-undecen-2-ol (**7**) or 4-phenyl-3-buten-2-ol (**8**), however, reacted 20–40 times faster than the *Z* isomers under the same reaction conditions. The *S* enantiomer of the *E* isomer did not participate in the reaction, due to the high enantioselectivity of this enzymatic transesterification ($E > 100$ for the *E* isomers, see Scheme 13).[126]

6.2.1.5. Resolution of Racemic Alcohols and Acids

Most publications on lipase catalysis deal with this type of reaction. Two examples in which the enantiomers **9** and **10** were obtained in high kinetic resolution and good yields are given in Scheme 14. Further examples will be discussed in Section 6.3.4, which deals with industrial applications.

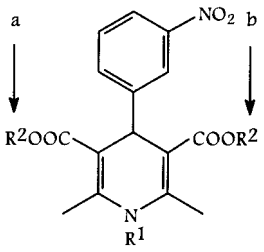


9



10

Scheme 14. Enantiopure substances **9** (ROH = 1-eicosanol, 1-dodecanol; CRL, 42% conversion, *ee* = 97%[127]) and **10** (PFL, 45% conversion, *ee* = 97%[128]) obtained by resolution of racemic compounds.



11

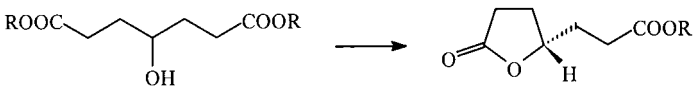
Scheme 15. Asymmetrization of prostereogenic diester 1,4-dihydro-4-(3-nitrophenyl)-2,6-dimethyl-pyridinedicarboxylate (**11**, $R^2 = CH_2OCOCH_3$): a) $R^1 = CH_2OCH_3$, lipase AK, 63% (*S*)-monoester, 95% *ee*; b) $R^1 = CH_2Ph$, Candida OF-360, 24% (*R*)-monoester, 73% *ee*.[129]

6.2.1.6. Asymmetrization of Prostereogenic Alcohols and Esters

Lipases have been shown to catalyze both types of reactions. An example for asymmetrization (the formation of enantiometrically pure compounds) is shown in Scheme 15. Asymmetrization of diesters (**12**) with lipase has also been used for the synthesis of enantiomeric lactones (**13**, Scheme 16).[130, 131]

6.2.1.7. Asymmetrization of meso-Diols

As this procedure may lead to quantitative conversions of the substrate, preventing racemization of the "wrong" enantiomer, much work has been done in this area. As an example, the transesterification of various cyclic *meso-cis*-diols is indicated in Table 9.



12 **13**

Scheme 16. Asymmetrization of diester **12** with lipase for the formation of the γ-lactone **13** (R = Et, PPL, hexane: 100% conversion, >98% *ee*; R = Et, PFL, hexane: 100% conversion, 32% *ee*; R = Bn, PPL, hexane: 100% conversion, >95% *ee*).

6.3.2. Acylation of Other Nucleophiles

Lipases not only form ester bonds, but acylate a wide range of nucleophiles such as ROOH or RNH_2. Table 11 gives a survey.

6.3.3. Acylations with Unusual Acyl Groups

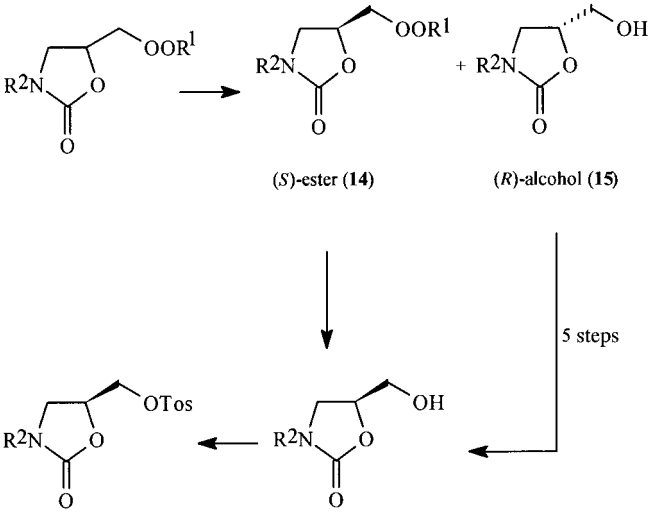
Apart from unusual acyl acceptors, unusual acyl donors generating the catalytically active acyl serine residue in a lipase have been observed. Bulky acylating reagents usually react more slowly, as the natural acylating agent of a lipases is a slim fatty acid. Depending on the configuration of their substrate binding sites, however, different lipases may show quite different enantiopreferences towards bulky acyl groups (Table 12).

These examples illustrate that lipases are unusally versatile biocatalysts. Indeed, some of the lipase-based transformations shown in Table 12 have been optimized in view of application in industry.

6.3.4. Examples of the Industrial Relevance of Lipases

In view of the ever-increasing need of the pharmaceutical, agrochemical, and fine-chemical industry for enantiomerically pure products and intermediates, lipases have been extensively studied for their catalytic potential in the preparation of chiral alcohols and acids. Some of the target products, the reagents, and the chiral intermediates are listed in Table 13. However, few of these processes have found industrial applications. This is due to several important reasons:

1. In many cases (e.g. pyrethroids, β-blockers) often no undesirable side effect has been found for the wrong enantiomer, and the racemic product can therefore be marketed as it is.
2. In other cases chemical methods such as preferential crystallization (naproxen, L-menthol), catalytic asymmetric synthesis (Diltiazem), or procedures based on fermentation or synthons from the chirality pool (α-phenoxypropionic acid herbicides) are more economical.
3. A major drawback for the industrial use of lipases is the need to racemize the wrong enantiomer. While this is feasible in most cases, it puts an economic burden on the process. For example, (*S*)-ester **14**, a useful enantiomer intermediate for the synthesis of β-blockers, can be industrially prepared by the action of LPL with greater than 99% *ee*. However, racemization of the unwanted (*R*)-alcohol **15** requires five additional chemical steps (Scheme 17), which renders the economics of the method questionable.[168]



Scheme 17. Preparation of (*S*)-ester **14** with LPL and racemization of (*R*)-alcohol **15** (R^1 = hexyl, R^2 = *iso*-propyl). Tos = $H_3CC_6H_4SO_2$.

While the use of prostereogenic or *meso* precursors within a retrosynthetic symmetrization–asymmetrization concept is a principal solution to this problem, it is often not possible to define such precursors for a given synthetic problem. In spite of these limitations, there are at present at least two processes in which lipase catalysis has been industrialized: the preparation of Diltiazem by Tanabe Seiyaku Co. and the preparation of several chiral amines by BASF AG.

Table 11. Acyl acceptors accesible by lipase catalysis.

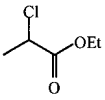
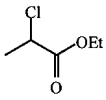
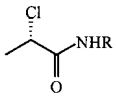
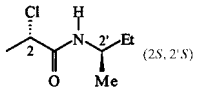
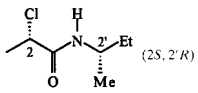
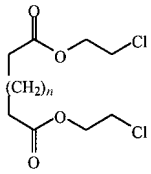
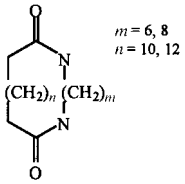
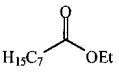
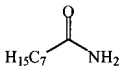
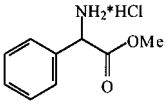
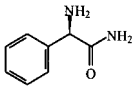
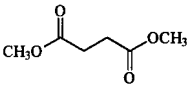
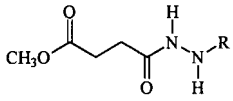
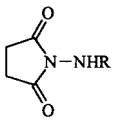
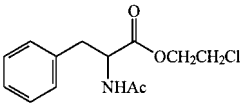
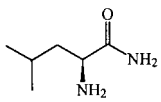
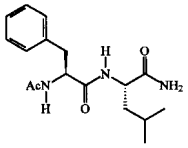
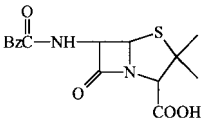
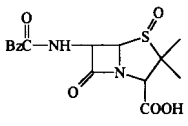
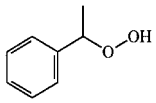
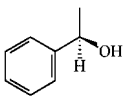
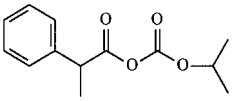
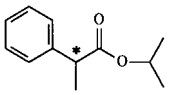
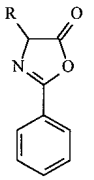
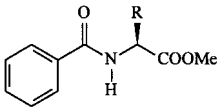
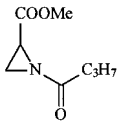
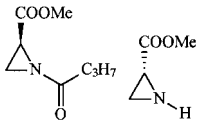
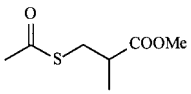
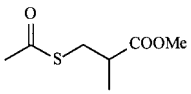
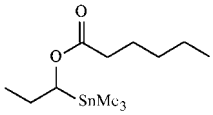
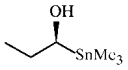
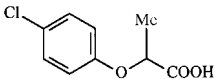
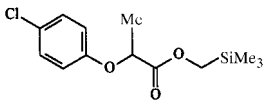
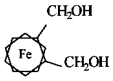
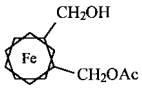
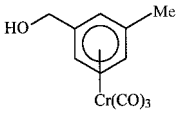
Acyl donor	Acyl acceptor	Product	Conditions	Yield [%]	*ee* [%]	Ref.
amine as acyl acceptor						
	*n*-butylamine phenylamine allylamine		CRL hexane or CCl_4	52 – 62	80 – 95	[137]
	*rac*-2-aminobutane	 	CAL	45	90	[138]
	1,10-diaminohexane 1,12-diaminododecane		CRL CH_2Cl_2	35 – 40	–	[139]
ammonia as acyl acceptor						
	NH_3		CAL, HSL 0.5 – 2.5 M NH_3 *t*BuOH, 40 °C	85 – 95	–	[140 – 142]
	NH_3		CAL *t*BuOH	39	91 (amide) $E = 38$	[142]
hydrazine as acyl acceptor						
	substituted hydrazides	 	PCL, R = CHO a) THF or iPr_2O, RT, 3 d b) 60 °C, 4 d	69 – 80 20 – 60	–	[140]
amino acid derivatives as acyl acceptors						
			PPL toluene 45 °C, 3 d	83	–	[137]
hydrogen peroxide and peracids as acyl acceptors						
	H_2O_2		CAL heptanoic acid, 4 °C	74	–	[143]
vinyl acetate			LPL cyclohexane	62	100	[144]

Table 12. Lipase catalysis with unusual acyl donors or acceptors.

Acyl donor	Acyl acceptor	Product	Conditions	Yield [%]	*ee* [%]	Ref.
carbonates						
	*n*-propanol		HLL MTBE	55	90	[145]
oxazolidines						
	methanol	 a: R = 4-HOC$_6$H$_4$ b: PhCH$_2$SCH$_2$	a: PSL b: PCL	52 61	75 90	[146]
amides						
	water		CRL	60	90	[147]
acyl donors or acceptors in structures with unusual heteroatoms						
			PSL PrOH, 25 °C	31	93	[148]
			PPL buffer	40	83	[149]
	Me_3SiCH_2OH		CRL 19 h	52	96 (acid)	[150]
vinyl acetate			PCL	80	100	[151]
vinyl acetate				47	100	[152]

7. Applications of Lipases in Medicine

We will now briefly discuss the function of lipases in the digestion of normal fat and mention some applications of these enzymes and their inhibitors in human therapy.

7.1. Physiological Function of Lipases in the Digestion of Dietary Fat

Dietary fats are composed of about 95% triacylglycerols (TG).[169] Until recently, the hydrolysis of fats was thought to begin in the intestinal lumen and to be catalyzed entirely by pancreatic lipase. In this view, the stomach was a transient storage organ, whose function was limited to mixing and dispersing lipids with the other nutrients. Although many authors have observed the occurrence of preduodenal lipolysis in humans and other species, gastric lipolysis was assumed to be negligible and even attributed to pancreatic contamination after a gastric reflux in the duodenum.

Today, the picture has changed, and strong experimental evidence supports the view that the gastric and pancreatic lipases act in synergy. Preduodenal lipases have been purified and biochemically characterized, for example rat lingual,[170] human gastric lipase (HGL),[171, 172] calf and lamb

Table 13. Examples of industrial relevance.

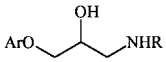
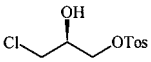
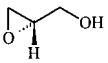
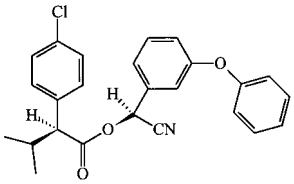
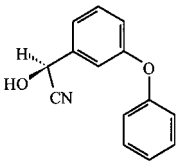
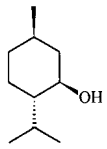
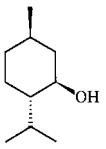
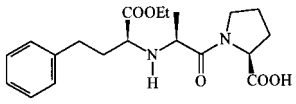
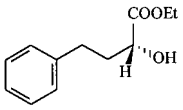
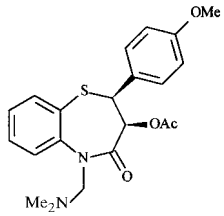
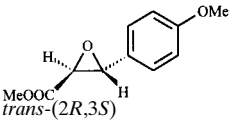
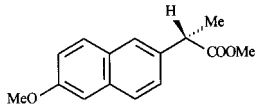
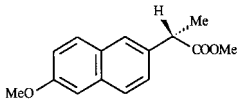
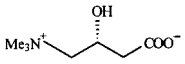
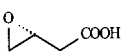
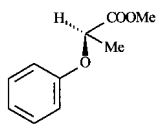
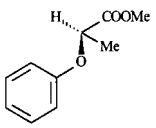
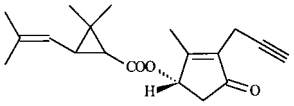
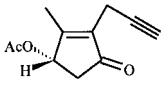
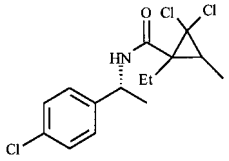
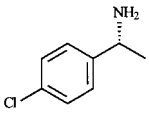
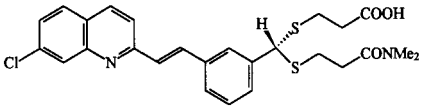
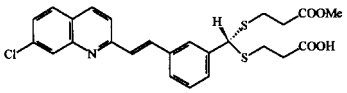
Desired end product	Chiral intermediate or end product	Optimized yields	Ref.
chiral alcohols			
 (*S*)-beta blocker	 "C_3 chiron"	LPL/H_2O: > 99 % *ee*; PPL/BuOH: 97 % ee, 49 % conv.; PCL/vinyl acetate: > 99 % *ee*, 50 % conv.	[153] [154]
		PPL/H_2O: very high substrate concentration, solvent-free	[155]
 (*S,S*)-fenvalerate, a pyrethroid insecticide		L/H_2O: 99.8 % *ee*, 49 % conv.	[156]
 L-menthol		CRL: > 98 % ee, 42 % conv.	[157]
chiral acids			
 enalapril, an ACE inhibitor			[158, 159]
 diltiazem, a calcium antagonist	 *trans*-(2*R*,3*S*)	*Serratia marcescens*: 100 % *de*	[153, 160, 161]
 (*S*)-naproxen, a nonsteroid inflammatory agent		esterase NP: 98 % *ee*, 39 % conv.	[162]
 (*R*)-carnitine, a vitamin-like nutrient		PPL: 95 % *ee*	[163]
 (*R*)-α-phenoxypropionic acid, a herbicide		CCL: 95 % *ee*	[164]

Table 13. Examples of industrial relevance (continued).

Desired end product	Chiral intermediate or end product	Optimized yields	Ref.
		PFL: 99.8% *ee*	[165]
prallethrin, a pyrethroid insecticide			
chiral amines			
		PCL: 99% *ee*, 43% conv.	[166]
substituted phenethylaminees, herbicides			
		PCL: 98% *ee*, 90% yield	[167]
LTD_4 antagonist			

pharyngeal,[173] rabbit gastric (RGL),[174] and dog gastric (DGL).[175]

Recently, investigations on healthy volunteers without any pancreatic or gastric deficiency allowed the definition of the respective physiological contributions of preduodenal and pancreatic lipases in the global hydrolysis of dietary TG. During the digestion of a liquid test meal, gastric and pancreatic lipase hydrolyze 18 and 48% of the meal TG acyl chains, respectively (66% hydrolysis of TG is sufficient for complete absorption). In other words, of the four acyl chains of two TG molecules which must be hydrolyzed for complete intestinal absorption, pancreatic lipase hydrolyzes three, and preduodenal lipase(s) one acyl chain.

It is now well established that the gastric lipolysis of long-chain triacylglycerols is of paramount importance in the physiological absorption of dietary fat, especially in patients suffering from exocrine pancreatic insufficiency, where it could partially compensate for the absence of the pancreatic lipase.[176] Furthermore, it was demonstrated that human and rabbit gastric lipases can potentiate in vitro the hydrolysis of triglycerides by human pancreatic lipase.[177]

7.2. Lipases in Substitution Therapy

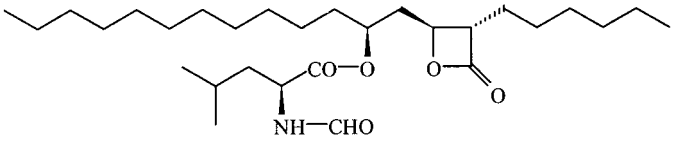
Exocrine pancreatic insufficiency, often found in cystic fibrosis patients, results in two major problems: malnutrition and steatorrhea. These problems can be partly solved by the administration of porcine pancreatic lipase extracts as a replacement therapy for these patients. In the past, such preparations were far from satisfactory, since a large proportion of the enzymes administrated were denaturated in the stomach due to the extreme acidity of the gastric juice. The coadministration of a lipase which could hydrolyze dietary lipids under acidic conditions would probably help in improving some of the problems of malabsorption and steatorrhea. Various clinical studies have been conducted both in animals and humans to assess the efficacy of acid-resistant lipases of fungal origin as enzymatic replacement therapy in exocrine pancreatic insufficiency.[178] Although some significant effects were observed in weight gain and reduction of steatorrhea, the use of such enzymes is limited as a result of their sensitivity to the proteolytic action of gastric pepsin.

With the advent of genetic engineering techniques, human gastric lipase (HGL) cDNA was synthesized, amplified, and cloned into a baculovirus transfer vector. This vector was cotransfected with a baculovirus which had been deleted from essential parts of the virus genome. High Five cells were used to express recombinant HGL; the level of HGL secretion was about 32 mg l^{-1} of culture medium. The protein migrated with an apparent molecular mass of 45 kDa as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE) analysis (50 KDa in the case of native HGL), which indicates that the level of the glycosylation was lower for the insect cells than for human gastric chief cells. The maximum specific activities of the recombinant lipase were 434 U mg^{-1} on long-chain triacylglycerols (Intralipid) and slightly higher on medium-chain and short-chain triglycerides.[179]

In contrast to the human enzyme, which preferentially hydrolyzes short-chain triacylglycerols, dog gastric lipase has an exceptionally high specific activity on long-chain triacylglycerols, which represent the majority of dietary fat in humans.[180] Dog gastric lipase would probably facilitate the absorption of lipids when given as an enzyme supplement to cystic fibrosis patients together with classical pancreatic extracts.[181].

7.3. Lipase Inhibitors as Antiobesity Agents

Conventional treatment for obesity has focused largely on strategies to control energy intake. Under clinical circumstances, the use of an inhibitor of digestive lipases which reduces dietary fat adsorption holds great promise as an antiobesity agent. Tetrahydrolipstatin (THL, **16**; Scheme 18), derived from lipstatin produced by *Streptomyces toxytricini*, acts in vitro and in vitro as a potent inhibitor of pancreatic and gastric lipases as well as of cholesterol ester hydrolase.[182, 183]



16

Scheme 18. Tetrahydrolipstatin (THL, **16**) as a potent inhibitor of pancreatic and gastric lipases as well as of cholesterol ester hydrolase.

It has been suggested that a stoichiometric enzyme–inhibitor complex in the form of an acyl enzyme is produced, which is slowly hydrolyzed (with water as the final acceptor) to leave an intact enzyme and the inactive form of THL.[184] A reactivation of inactivated HPL was observed, as evidenced by kinetics showing a lag phase; this is probably due to a slow deacylation in the pH-stat assay system of the inhibited enzyme.

Therefore, THL is an amphiphilic inhibitor that reacts with the essential serine residue of the lipase active site. While micellar partitioning of lipases in the presence of bile salts renders many amphiphilic inhibitors inefficient, THL is mostly associated with the triacylglycerol phase, even in the presence of sodium taurodeoxycholate. This property is probably prerequisite for a lipase inhibitor to be effective under physiological conditions, that is, in the presence of bile and lipids.[185]

As THL inhibits hydrolysis and thus adsorption of triacylglycerols in the duodenum, it is being developed by Hoffmann–La Roche as an antiobesity agent. In one set of experiments, the effect of THL on the secretion and activity of pancreatic lipase and on the hydrolysis of fat in duodenal aspirates of human volunteers (with polyethyleneglycol 4000 as a nonabsorbable recovery marker) was measured. THL was infused in an oil/egg emulsion in parallel with ingestion of a meal, and duodenal juice was aspirated at the ligament of Treitz for determination of exocrine pancreatic enzymes as well as triglycerides that remained intact and their hydrolysis products. It could be demonstrated that an intraduodenal infusion of THL in humans strongly reduced the activity of pancreatic lipase, both by reducing its catalytic activity and pancreatic secretion.[186]

8. The Future: Highly Pure and Genetically Engineered Lipases

8.1. Highly Pure Lipases

Lipases hold considerable promise in synthetic organic chemistry and have found practical applications already in detergents, oleochemistry, cheese production, medical therapy, and industrial synthesis of specialty chemicals. By now, lipases from over 30 biological sources have been cloned, sequenced, and expressed in host organisms. Pure recombinant lipases from *Humicola lanuginosa*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas aeruginosa*, *Mucor miehei*, *Candida antartica* (type B), *Bacillus thermocatenulatus*, and other sources are now commercially available in free or immobilized form, or as part of a screening set (e.g. Chirazyme from Boehringer Mannheim). Even cross-linked crystals of *Candida rugosa* and of *Pseudomonas cepacia* lipase (ChiroCLEC) are commercially available from Altus (Cambridge, MA). The tertiary structures of twelve lipases have been resolved, and due to their application potential, this number will grow rapidly. As a result, a more rational approach for how to modify lipases for detergents, oleochemical applications, and organic synthesis is emerging.

8.2. Protein Engineering of Lipases

With our increasing knowledge on lipase structure and function, it has become clear that substrate binding domains vary greatly from one lipase to another, providing a more rational explanation for their varying substrate specificities. In Figure 6, the substrate binding sites of four different lipases are shown in their activated (open-lid) conformation. While these drawings are based on homology modeling of lipase–inhibitor complexes and molecular dynamics calculations and not strictly proven by X-ray crystallograpy, they clearly suggest the different steric and electronic environment prevailing in lipases of different origin. As an example, the distance of the active serine residue from the surface varies between 5 Å (ROL) and 16 Å (PCL) in the three examples shown. Information on genetically engineered lipases (mostly taken from the patent literature) is summarized in Table 14.

As detergents are still the commercially most important field of lipase applications, most pertinent patents deal with enhancing lipase stability and activity in a household detergent matrix.[78] The scientific literature on modified lipases for oleochemistry and organic synthesis is still scarce. One case deals with the chain-length specificity observed in *Rhizopus delemar* lipase, which may be related to steric effects involved in the binding of acyl groups. Indeed, site-directed mutagenesis of F95, F112, V206, and V209, sterically demanding points of acyl chain interaction in this lipase, led to a significant shift in the preference of the mutant lipase for the hydrolysis of medium-chain triglycerides.[196]

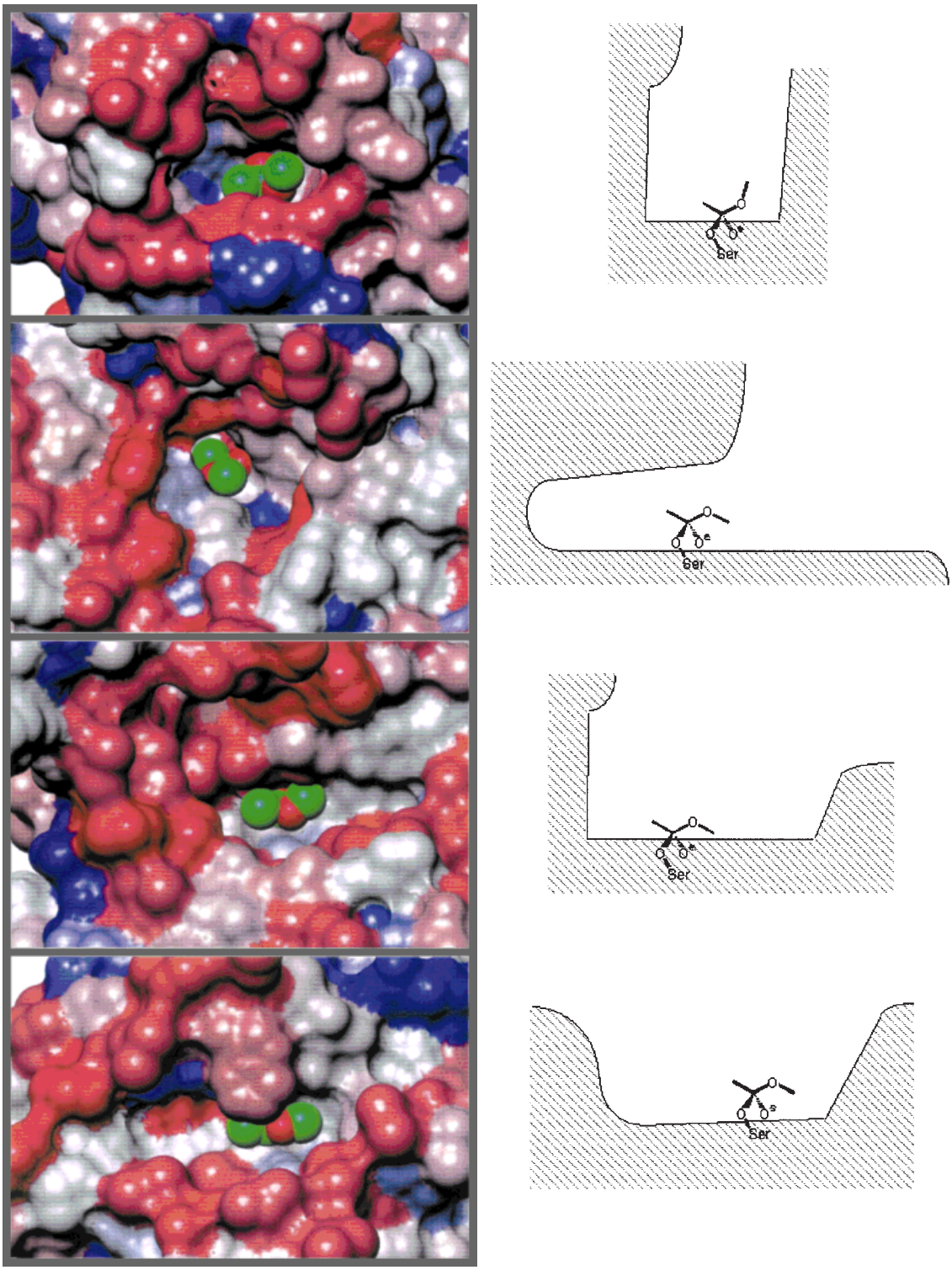


Figure 6. Polarity and geometry of the binding sites of four lipases differ significantly. From the top: *Candida antarctica* lipase (type B), *Candida rugosa* lipase, *Pseudomonas cepacia* (renamed *Burkholderia cepacia* lipase), *Rhizomucor miehei* lipase. Left: space-filling models (plan view), colored as for Figure 2. For comparison, the tetrahedral intermediate of ethyl acetate was docked into the active sites, guided by the experimental structures with substrate analoguous covalent inhibitors. Right: sketch of the two dimensional cross-sections (side view) close to the active serine residues ("Conolly surface").

8.2.1. Combinatorial Design

In view of their broad applications and the rapidly advancing knowledge about their structure and function, lipases are interesting candidates for combinatorial approaches to modify their stability and substrate specificity. The esterase activity of *Rhizomucor miehei* lipase could already be enhanced with this technique.[198] Recently, proteases have been subjected to an evolutionary screening,

Table 14. Engineered lipases.

Strain	Application	Effect of mutations	Ref.
Humicola lanuginosa	detergent additive	improvement of washing performance	[187]
		increase in thermostability	[188]
		improvement of resistance to proteolytic degradation increase in specific activity	[189]
Pseudomonas glumae	detergent additive	improvement of resistance to oxidative degradation	[190]
		improvement of proteolytic resistance	[191]
Pseudomonas mendocina/ putida ATCC 53552	detergent additive in peracid bleaching systems	alteration of substrate specifity	[192]
		improvement of perhydrolysis to hydrolysis ratio	[193]
Pseudomonas pseudoalcaligenes	detergent additive	improvement of washing performance	[194]
		enhanced stability towards anionic surfactants	[195]
Rhizopus delemar	biocatalysis	alteration of chain length selectivity	[196]
Pseudomonas mendocina SD702	detergents, food, and paper	increase of thermostability	[197]

resulting in enhanced solvent stability[199] and modified substrate specificity.[198, 201] It is safe to predict that before long lipases will become an eminent example of those enzymes which have been modified by rational and combinatorial design for use in industrial processes.

R.D.S. wishes to acknowledge critical discussions of the manuscript with Kurt Faber (University of Graz, Austria) and Wilhelm Tischer (Boehringer-Mannheim, Penzberg). Valuable contributions by members of the group in Stuttgart, in particular by Uwe Bornscheuer and Jürgen Pleiss, are gratefully acknowledged, as is the untiring help of Markus Enzelberger and Frank Zocher in editing the manuscript. Mr. M. Kawashima, Itopia, provided important information from Japan. Financial support by the Fonds der Chemischen Industrie has been of great help. R.V. wishes to thank Frédéric Carrière and Claude Rivière for designing the illustrations of the pancreatic lipase/colipase complex and the zero-order through. Financial support from the BIOTECH G lipase programs (contracts BIO2-CT94-3041 and BIO2-CT94-3013) of the European Union as well as the CNRS-Imabio project are particularly acknowledged.

Received: April 14, 1997 [A225IE]
German version: *Angew. Chem.* **1998**, *110*, 1694 – 1720

[1] *Enzyme Nomenclature* (Ed.: International Union of Biochemistry and Molecular Biology), Academic Press, New York, **1992**.
[2] P. Wooley, S. B. Petersen, *Lipases: Their Structure, Biochemistry and Application*, Cambridge University Press, Cambridge, **1994**.
[3] F. D. Gunstone, J. L. Harwood, F. B. Padley, *The Lipid Handbook*, Chapman & Hall, London, **1995**.
[4] *Lipases: Structure, Mechanism and Genetic Engineering*, *GBF Monographs, Vol. 16* (Eds.: L. Alberghina, R. D. Schmid, R. Verger), VCH, Weinheim, **1991**.
[5] H. Brockerhoff, R. G. Jensen, *Lipolytic Enzymes*, Academic Press, New York, **1974**.
[6] B. Borgström, H. L. Brockman, *Lipases*, Elsevier, Amsterdam, **1984**.
[7] M. Waite in *The Phospholipases*, *Vol. 5* (Ed.: D. J. Hanahn), Plenum, New York, **1987**.
[8] *Phospholipases*, *Methods Enzymology*, *Vol. 197* (Ed.: E. A. Dennis), Academic Press, New York, **1991**.
[9] *Engineering of/with Lipases* (Ed.: F. X. Malcata), Kluwer, London, **1996**.
[10] *J. Mol. Catal. B* **1997**, *3* (special issue devoted to "Microbial Lipases in the Biocatalysis").
[11] B. Rubin, *Structural Biology* **1994**, *1*, 568 – 572.
[12] P. E. Sonnet, *J. Am. Oil Chem. Soc.* **1988**, *65*, 900 – 904.
[13] *Methods in Enzymology*, *Vol. 284* (Eds.: B. Rubin, E. A. Dennis), Academic Press, San Diego, **1997**.
[14] C.-S. Chen, C. J. Sih, *Angew. Chem.* **1989**, *101*, 711 – 724; *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 695 – 708.
[15] A. L. Gutman, M. Shapira in *Synthetic Applications of Enzymatic Reactions in Organic Solvents*, *Adv. Biochem. Eng. Biotechnol., Vol. 52* (Ed.: A. Fiechter), Springer, Heidelberg, **1995**, pp. 87 – 128.
[16] K. E. Jaeger, S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, O. Misset, *FEMS Microbiol. Rev.* **1994**, *15*, 29 – 63.
[17] E. J. Gilbert, *Enzyme Microb. Technol.* **1993**, *15*, 634 – 645.
[18] S. Wohlfahrt, K. E. Jaeger, *BioEngineering* **1993**, *9*, 39 – 46.
[19] A. H. C. Huang in ref. [6], pp. 419 – 442.
[20] K. D. Mukherjee, M. J. Hills in ref. [2], pp. 49 – 76.
[21] F. Carrière, Y. Gargouri, H. Moreau, S. Ransac, E. Rogalska, R. Verger in ref. [2], pp. 181 – 205.
[22] F. Carrière, S. Bezzine, R. Verger in ref. [10], pp. 55 – 64.
[23] E. Boel, T. Christensen, H. Woldike (Novo Nordisk AS), US-A 5536661, **1996** [*Chem. Abstr.* **1996**, *125*, 160364].
[24] M. M. Soumanou, U. T. Bornscheuer, U. Menge, R. D. Schmid, *J. Am. Oil Chem. Soc.* **1997**, *74*, 427 – 433.
[25] H. K. Weber, H. Stecher, K. Faber in *Preparative Biotransformations: Some Properties Of Commercially Available Crude Lipase Preparations* (Ed.: S. M. Rakels), Wiley, New York, **1995**.
[26] M. R. Aires-Barros, M. A. Taipa, J. M. S. Cabral in *Isolation And Purification Of Lipases* (Eds.: P. Wooley, S. B. Petersen), Cambridge University Press, Cambridge, **1994**, pp. 243 – 270.
[27] S. Ransac, F. Carrière, E. Rogalska, R. Verger, F. Marguet, G. Buono, E. P. Melo, J. M. S. Cabral, M.-P. E. Egloff, H. van Tilbeurgh, C. Cambillau in *The Kinetics, Specificities And Structural Features Of Lipases*, *Nato ASI Series*, *Vol. H 96* (Ed.: J. A. F. Op den Kamp), Springer, Berlin, **1996**, pp. 265 – 304.
[28] K. Isobe, K. Nokihara, S. Yamaguchi, T. Mase, R. D. Schmid, *Eur. J. Biochem.* **1992**, *203*, 233 – 237.
[29] R. Verger, *Trends Biotechnol.* **1997**, *15*, 32 – 38.
[30] R. Verger, *TIBTECH* **1997**, *15*, 32 – 38.
[31] J. G. T. Kierkels, L. F. W. Vlengels, E. T. F. Gelade, D. P. Vermeulen, J. Kamphuis, C. Wandrey, W. J. J. van den Tweel, *Enzyme Microbiol. Technol.* **1994**, *16*, 513 – 521.
[32] M. M. Momsen, M. Dahim, H. L. Brockman, *Biochemistry* **1997**, *36*, 10073 – 10081.
[33] S. Ransac, M. G. Ivanova, R. Verger, I. Panaiotov in *Methods in Enzymology*, *Vol. 286* (Eds.: B. Rubin, E. A. Dennis), Academic Press, San Diego, **1997**.
[34] G. Piéroni, Y. Gargouri, L. Sarda, R. Verger, *Adv. Colloid Interface Sci.* **1990**, *32*, 341 – 378
[35] L. de la Fournière, M. G. Ivanova, J. P. Blond, F. Carrière, R. Verger, *Colloids Surf. B* **1994**, *2*, 585 – 593.

[36] W. E. Momsen, M. M. Momsen, H. L. Brockman, *Biochemistry* **1995**, *34*, 7271 – 7281.
[37] W. Nitsch, R. Maksymiw, H. Erdmann, *J. Colloid Interface Sci.* **1991**, *141*, 322 – 328.
[38] L. Sarda, P. Desnuelle, *Biochim. Biophys. Acta* **1958**, *30*, 513 – 521.
[39] F. K. Winkler, A. D'Arcy, W. Hunziker, *Nature* **1990**, *343*, 771 – 774.
[40] L. Brady, A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, U. Menge, *Nature* **1990**, *343*, 767 – 770.
[41] A. M. Brzozowski, U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar, L. Thim, *Nature* **1991**, *351*, 491 – 494.
[42] B. Nieuwenhuis in *The CEC Bridge Lipase Project* (Eds.: L. Alberghina, R. D. Schmid, R. Verger), VCH, Weinheim, **1990**, pp. 3 – 6.
[43] M. E. M. Noble, A. Cleasby, L. N. Johnson, L. G. J. Frenken, M. R. Egmond, *FEBS Lett.* **1993**, *331*, 123 – 128.
[44] J. Uppenberg, M. T. Patkar, S. Hansen, A. Jones, *Structure* **1994**, *2*, 293.
[45] H. van Tilbeurgh, M.-P. Egloff, C. Martinez, N. Rugani, R. Verger, C. Cambillau, *Nature* **1993**, *362*, 814 – 819.
[46] K. Thirstrup, R. Verger, F. Carriére, *Biochemistry* **1994**, *33*, 2748 – 2756.
[47] A. Hjorth, F. Carriére, C. Cudrey, H. Wöldike, E. Boel, D. M. Lawson, F. Ferrato, C. Cambillau, G. G. Dodson, L. Thim, R. Verger, *Biochemistry* **1993**, *32*, 4702 – 4707.
[48] P. Grochulski, Y. Li, J. D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, M. Cygler, *J. Biol. Chem.* **1993**, *268*, 12843 – 12847.
[49] J. D. Schrag, M. Cygler, *J. Mol. Biol.* **1993**, *230*, 575.
[50] U. Derewenda, L. Swenson, R. Green, Y. Wei, G. G. Dodson, S. Yamaguchi, M. J. Haas, Z. S. Derewenda, *Nat. Struct. Biol.* **1994**, *1*, 36 – 47.
[51] C. Martinez, P. de Geuss, M. Lauwereys, G. Matthyssens, C. Cambilleau, *Nature* **1992**, *356*, 615 – 618.
[52] J. D. Schrag, Y. Li, M. Cygler, D. Lang, T. Burgdorf, H. J. Hecht, R. D. Schmid, L. C. Strickland, S. B. Larson, J. Day, A. Mc Pherson, *Structure* **1997**, *5*, 187 – 202.
[53] D. L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. Franken, M. Harel, S. J. Remington, I. Silman, *Protein Eng.* **1992**, *5*, 197 – 211.
[54] U. Derewenda, Z. S. Derewenda, *Biochem. Cell. Biol.* **1991**, *69*, 842 – 851.
[55] Z. S. Derewenda, A. M. Sharp, *TIBS* **1993**, *18*, 20 – 25.
[56] F. Carriére, J. A. Barrowan, R. Verger, R. Laugier, *Gastroenterology* **1993**, *105*, 876 – 888.
[57] G. Scheele, H. Kern in *The Exorcine Pancreas, Molecular and Cellular Basis of Digestion* (Ed.: P. Desnuelle), Elsevier, Amsterdam, **1986**, pp. 173 – 192.
[58] Q. Lüthi-Peng, F. K. Winkler, *Eur. J. Biochem.* **1992**, *205*, 383 – 390.
[59] M. P. Egloff, F. Buono, R. Marguet, R. Verger, C. Cambillau, H. van Tilbeurgh, *Biochemistry* **1995**, *34*, 2751 – 2762.
[60] *Encylopaedia of Chemical Technology* (Eds.: R. E. Kirk, D. F. Othmer), Wiley, New York, **1978**.
[61] W. Connstein, E. Hoyer, H. Wartenberg, *Chem. Ber.* **1902**, *34*, 3988 – 4006.
[62] N. O. V. Sonntag in *Baileys Industrial Oil and Fat Products* (Ed.: D. Swern), Wiley, New York, **1982**, pp. 97 – 111.
[63] R. Hashizume, Y. Tanaka, T. Funada (Nippon Oil and Fats Co.), JP-A 03019693 A2, **1991** [*Chem. Abstr.* **1991,** *115*, 90690].
[64] H. Moore (Unilever PLC), GB-B 2185990 A1, **1987** [*Chem. Abstr.* **1987**, *107*, 174780].
[65] P. M. J. Holemans, R. Schijf, K. P. A. M. van Putte, T. de Mans (Unilever N.V.; Unilever PLC), EP-B 209176 A1, **1987** [*Chem. Abstr.* **1987**, *106*, 118502].
[66] S. Bloomer, P. Adlercreutz, B. Mattiasson, *J. Am. Oil Chem. Soc.* **1990**, *67*, 519 – 524.
[67] A. R. Macrae, *J. Am. Oil Chem. Soc.* **1983**, *60*, 291 – 294.
[68] M. H. Coleman, A. R. Macrae (Unilever N.V.), DE-B 2705608, **1977** [*Chem. Abstr.* **1977**, *87*, 166366].
[69] T. Matsuo, N. Sawamura, Y. Hashimoto, W. Hashida (Fuji Oil Co.), EP-B 0035883 A2, **1982** [Chem. Abstr. **1982**, *96*, 4958y].
[70] C. C. Akoh, *INFORM* **1995**, *6*, 1055 – 1061.
[71] B. Ö. Gudmundsson, Ö. Almarsson, G. G. Haraldsson, *Tetrahedron Lett.* **1993**, *34*, 5791 – 5794.
[72] H. Baumann, M. Bühler, H. Fochem, F. Hirsinger, H. Zoebelein, J. Falbe, *Angew. Chem.* **1988**, *100*, 41 – 62; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 41 – 62.
[73] N. O. V. Sonntag, *J. Am. Oil Chem. Soc.* **1982**, *59*, 795A – 802A.
[74] U. T. Bornscheuer, *Enzyme Microb. Technol.* **1995**, *17*, 578 – 586.
[75] S. Moore, P. Quinlan, *INFORM* **1993**, *4*, 579 – 583.
[76] V. Partali, L. Kvittingen, H.-R. Sliwka, T. Anthonson, *Angew. Chem.* **1996**, *108*, 342 – 344; *Angew. Chem. Int. Ed. Engl.* **1996**, *35, 329 – 330.*
[77] A. M. Wolff, M. S. Showell in *Enzymes in Detergency* (Eds.: J. H. Van Ee, O. Misset, E. J. Baas), Marcel Dekker, New York, **1997**, pp. 93 – 106
[78] O. Misset in ref. [77], pp. 107 – 132.
[79] E. Gormsen, M. Lykke, C. F. Jensen, *Rev. Quim. Text.* **1994**, *120*, 78 – 81.
[80] B. Kottwitz, H. Upadek, G. Carrer, *Chim. Oggi* **1994**, *12*, 21 – 24.
[81] O. Kirk, M. W. Christensen, T. Damhus, S. E. Godtfredsen, *Biocatalysis* **1994**, *11*, 65 – 77.
[82] H. Andree, R. W. Müller, R. D. Schmid, *J. Appl. Biochem.* **1980**, *2*, 218 – 229.
[83] Y. Fujita, H. Awaji, M. Matsukura, K. Hata, H. Shimoto, M. Sharyo, H. Skaguchi, K. Gibson, *Tappi J.* **1992**, *75*, 117 – 122.
[84] E. N. Vulfson in ref. [2], pp. 271 – 286.
[85] K. Faber, *Biotransformations in Organic Chemistry*, Springer, Heidelberg, **1995**.
[86] K. Drauz, H. Waldmann, *Enzyme Catalysis in Organic Synthesis*, VCH, Weinheim, **1995**.
[87] R. A. Sheldon, *Chirotechnology: Industrial Synthesis of Optically Active Compounds*, Marcel Dekker, New York, **1993**.
[88] R. Kazlauskas, U. T. Bornscheuer in *Biotechnology*, *Vol. 8a* (Eds.: H. J. Rehm, G. Reed, A. Pühler, P. J. W. Stadler, D. R. Kelly), VCH, Weinheim, **1998**, pp. 37 – 191.
[89] K. Faber, *Pure Appl. Chem.* **1997**, *69*, 1613 – 1632.
[90] A. M. P. Koskinen, A. M. Klibanov, *Enzymatic Reactions in Organic Media*, Blackie, London, **1996**.
[91] H. P. Kaufmann, M. C. Keller, *Fette Seifen* **1937**, *44*, 105 – 107.
[92] H. P. Kaufmann, M. C. Keller, *Fette Seifen* **1937**, *44*, 42 – 47.
[93] J. S. Dordick, *Biotechnol. Prog.* **1992**, *8*, 259 – 267.
[94] M. N. Gupta, *Eur. J. Biochem.* **1992**, *203*, 25 – 32.
[95] E. Santaniello, P. Ferraboschi, P. Grisenti, *Enzyme Microb. Technol.* **1993**, *15*, 367 – 381.
[96] F. Effenberger, B. Gutterer, T. Ziegler, E. Eckhardt, R. Aichholz, *Liebigs Ann. Chem.* **1991**, 47 – 54.
[97] M. Schneider, E. H. Reimerdes, *Forum Mikrobiol.* **1987**, 65 – 71.
[98] F. Theil, *Chem. Rev.* **1995**, *95*, 2203 – 2227.
[99] K. Mori, *Synlett* **1995**, 1097 – 1109.
[100] C. H. Wong, J. M. Fang, *Synlett* **1994**, 393 – 402.
[101] A. L. Margolin, *Enzyme Microb. Technol.* **1993**, *15*, 266 – 279.
[102] C. Froessl, M. Lorenz, W. Boland, *Synthesis* **1991**, 1049 – 1072.
[103] G. M. Whitesides, C.-H. Wong, *Angew. Chem.* **1985**, *97*, 617 – 620; *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 617 – 620.
[104] H. P. Schär, X. F. Busquets, O. Ghisalba, G. M. R. Tombo, *Tetrahedron Lett.* **1986**, *27*, 5707 – 5710.
[105] E. L. A. Mac Farlane, S. N. Roberts, N. J. Turner, *J. Chem. Soc. Chem. Comm.* **1990**, 569 – 571.
[106] C. S. Chen, Y. Fujimoto, C. J. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294 – 7299.
[107] C. S. Chen, S. H. Wu, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1987**, *109*, 2812 – 2817.
[108] M. Castaing-Degueil, B. de Jeso, S. Drouillard, B. Maillard, *Tetrahedron Lett.* **1987**, *28*, 953 – 954.
[109] Y. F. Wang, J. J. Lalonde, M. Momongan, D. E. Bergbreiter, C. H. Wong, *J. Am. Chem. Soc.* **1988**, *110*, 7200 – 7205.
[110] H. M. R. Hoffmann, U. T. Bornscheuer, T. F. J. Lampe, *Tetrahedron: Asymmetry* **1996**, *7*, 2889 – 2900.
[111] R. Kazlauskas, A. N. E. Weissfloch, A. T. Rappaport, L. A. Cuccia, *J. Org. Chem.* **1991**, *56*, 2656 – 2665.
[112] P. Grochulski, F. Bouthillier, R. Kazlauskas, A. N. Serreqi, J. D. Schrag, E. Ziomek, M. Cygler, *Biochemistry* **1994**, *33*, 3494 – 3500.

[113] R. Bovara, G. Carrea, L. Ferrara, S. Riva, *Tetrahedron: Asymmetry* **1991**, *2*, 931 – 938.
[114] R. Chenevert, R. Pouliot, P. Bureau, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2941 – 2944.
[115] S. J. Phythian, A. J. Reason, S. M. Roberts, N. B. Bashir, *J. Chem. Soc. Perkin Trans. 1* **1995**, 2203 – 2222.
[116] C. H. Wong, *Pure Appl. Chem.* **1995**, *67*, 1609 – 1616.
[117] J. Chopineau, A. P. G. Kieboom, A. M. Klibanov, S. Riva, *J. Am. Chem. Soc.* **1988**, *110*, 584 – 589.
[118] M. Therisod, A. M. Klibanov, *J. Am. Chem. Soc* **1986**, *108*, 5638 – 5640.
[119] R. Pulido, V. Gotor, *J. Chem. Soc. Perkin Trans. 1* **1991**, 491 – 492.
[120] C. Scheckermann, A. Schlotterbeck, M. Schmid, *Enzyme Microb. Technol.* **1995**, *17*, 157 – 162.
[121] A. M. Klibanov, M. Therisod, *J. Am. Chem. Soc.* **1987**, *109*, 3977 – 3981.
[122] S. Riva, F. Secundo, L. Zucchelli, F. Nicotra, *Tetrahedron Lett.* **1989**, *30*, 1703 – 1704.
[123] R. Pulido, F. O. Lòpez, V. Gotor, *J. Chem. Soc. Perkin Trans. 1* **1992**, *1*, 2981 – 2988.
[124] A. E. M. Janssen, C. Klabbers, M. C. R. Franssen, K. van't Reit, *Enzym Microb. Technol.* **1991**, *13*, 565 – 571.
[125] L. Cao, A. Fischer, U. T. Bornscheuer, R. D. Schmid, *Biocatal. Biotrans.* **1997**, *14*, 269 – 283.
[126] B. Morgan, A. C. Oehlschlager, T. M. Stokes, *J. Org. Chem.* **1992**, *57*, 3231 – 3236.
[127] H. Edlund, P. Berglund, M. Jensen, E. Hedenstroem, H.-E. Hoegberg, *Acta Chim. Scand.* **1996**, *50*, 666 – 671.
[128] A. L. Margolin, *Tetrahedron Lett.* **1990**, *31*, 6797.
[129] L. Salazar, C. J. Sih, *Tetrahedron: Asymmetry* **1995**, *6*, 2917 – 2920.
[130] M. M. Enzelberger, U. T. Bornscheuer, I. Gattfield, R. D. Schmid, *J. Biotechnol.* **1997**, *56*, 129 – 134.
[131] A. L. Gutman, T. Bravdo, *J. Org. Chem.* **1989**, *54*, 4263 – 4265.
[132] F. Theil, H. Schick, G. Winter, G. Reck, *Tetrahedron* **1991**, *47*, 7569 – 7582.
[133] C. R. Johnson, A. Golebiowski, D. H. Steensma, *J. Am. Chem. Soc.* **1992**, *114*, 9414 – 9418.
[134] V. Sunjic, *Croat. Chem. Acta* **1994**, *67*, 365 – 392.
[135] C. Andreu, J. A. Macro, G. Asensio, *J. Chem. Soc. Perkin Trans. 1* **1990**, 3209 – 3210.
[136] S. Takano, M. Moriaya, Y. Higashi, K. Ogasawara, *J. Chem. Soc. Chem. Comm.* **1993**, 177 – 178.
[137] S. Puertas, R. Brieva, F. Rebolledo, V. Gotor, *Tetrahedron* **1993**, *49*, 4007 – 4014.
[138] R. Brieva, F. Rebolledo, V. Gotor, *J. Chem. Soc. Chem. Commun.* **1990**, 1386 – 1387.
[139] A. L. Gutman, E. Meyer, X. Yue, C. Abell, *Tetrahedron Lett.* **1992**, *33*, 3943 – 3946.
[140] M. C. de Zoete, F. van Rantwijk, R. A. Sheldon (Delft University of Technology), WO-A 9507359 A1, **1995** [*Chem. Abstr.* **1995**, *122*, 313055].
[141] M. C. de Zoete, A. C. Kock-van Dalen, F. van Rantwijk, R. A. Sheldon, *J. Chem. Soc. Chem. Commun.* **1993**, 1831 – 1832.
[142] M. C. de Zoete, A. C. Kock-van Dalen, F. van Rantwijk, R. A. Sheldon, *Biocatalysis* **1994**, *10*, 307 – 316.
[143] M. C. de Zoete, F. van Rantwijk, L. Maat, R. A. Sheldon, *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 462 – 463.
[144] N. Baba, M. Mimura, J. Hiratake, K. Uchida, J. Oda, *Agric. Biol. Chem.* **1988**, *52*, 2685 – 2687.
[145] E. Guibe-Jampel, M. Bassir, *Tetrahedron Lett.* **1994**, *35* , 421 – 422.
[146] J. Z. Crich, R. Brieva, P. Marquart, R. L. Gu, S. Flemming, C. J. Sih, *J. Org. Chem.* **1993**, *58*, 3252 – 3258.
[147] M. Bucciarelli, A. Forni, I. Moretti, F. Prati, G. Torre, *Tetrahedron: Asymmetry* **1993**, *4*, 903 – 906.
[148] P. Cesti, D. Bianchi, *J. Org. Chem.* **1990**, *55*, 5657 – 5659.
[149] J. M. Chong, K. M. Mar, *Tetrahedron Lett.* **1991**, *32*, 5683 – 5686.
[150] S. Kurita, S. Ueji, T. Yamada, S. Kuwata, T. Miyazawa, *Biotechnol. Lett.* **1992**, *14*, 941 – 946.
[151] G. Nicolosi, R. Morrone, A. Patti, M. Piatelli, *Tetrahedron: Asymmetry* **1992**, *3*, 753 – 758.
[152] K. Nakamura, K. Ishihara, A. Ohno, M. Uemura, H. Nishimura, Y. Hayashi, *Tetrahedron Lett.* **1990**, *31*, 3603 – 3604.
[153] L. A. Hulshof, J. H. Roskam (Stamicarbon B.V.), EP-B 343714 A1, **1989** [*Chem. Abstr.* **1990**, *113*, 76603].
[154] K. Wünsche, U. Schwaneberg, U. T. Bornscheuer, H. H. Meyer, *Tetrahedron: Asymmetry* **1996**, *7*, 2017 – 2022.
[155] M. Kloosterman, V. H. M. Elferink, J. van Iersel, J. H. Roskam, E. M. Meijer, L. A. Hulshof, R. A. Sheldon, *TIBTECH* **1988**, *6*, 251 – 256.
[156] S. Mitsuda, S. Nabeshima, H. Hirohara, *Appl. Microbiol. Biotechnol.* **1989**, *31*, 334 – 337.
[157] C. S. Chen, S. Hsiung, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1987**, *109*, 2812 – 2817.
[158] D. Bianchi, T. Sugai, H. Ohta, *Agric. Biol. Chem.* **1991**, *55*, 293 – 294.
[159] P. Cesti, E. Battistel, D. Bianchi, *J. Org. Chem.* **1988**, *53*, 5531 – 5534.
[160] S. Brandt, R. F. Rossi, D. R. Dodds, J. L. Lopez (Sepracor Inc.), WO-A 9004643 A1, **1990** [*Chem. Abstr.* **1990**, *113*, 76620].
[161] T. Shibatani, Yukagaku **1995**, *44*, 862 – 868 [*Chem. Abstr.* **1995**, *123*, 312256].
[162] H. R. Sonawane, N. S. Bellur, J. R. Ahuya, D. G. Kulkarni, *Tetrahedron: Asymmetry* **1992**, *3*, 163 – 192.
[163] D. Bianchi, W. Cabri, P. Cesti, F. Francalanci, M. Ricci, *J. Org. Chem.* **1988**, *53*, 104 – 107.
[164] J. M. Moreno, J. V. Sinisterra, *J. Mol. Catal. A* **1995**, *98*, 171 – 184.
[165] S. Mitsuda, S. Nabeshima, H. Hirhora, *Appl. Microbiol. Biotechnol.* **1989**, *31*, 334 – 337.
[166] F. Balkenhohl, K. Ditrich, B. Hauer, W. Ladner, *J. Prakt. Chem.* **1997**, *339*, 381 – 384.
[167] D. L. Hughes, J. J. Bergan, J. S. Amato, P. J. Reider, E. J. J. Grabowski, *J. Org. Chem.* **1989**, *54*, 1787 – 1788.
[168] S. Hamaguchi, M. Asada, J. Hasegawa, K. Watanabe, *Agric. Biol. Chem.* **1985**, *49*, 1661 – 1667.
[169] R. L. Rizek, B. Friend, L. Page, *J. Am. Oil Chem. Soc.* **1974**, *51*, 244 – 250.
[170] M. Hamosh, D. Ganot, P. Hamosh, *J. Biol. Chem.* **1979**, *254*, 12121 – 12125.
[171] M. Aoubala, I. Douchet, R. Laugier, M. Hirn, R. Verger, A. de Caro, *Biochim. Biophys. Acta* **1993**, *1169*, 183 – 189.
[172] C. Tiruppathi, K. A. Balasubramanian, *Biochim. Biophys. Acta* **1982**, *712*, 692 – 697.
[173] J. de Caro, F. Ferrato, R. Verger, A. de Caro, *Biochim. Biophys. Acta* **1995**, *1252*, 321 – 329.
[174] H. Moreau, Y. Gargouri, D. Lecat, R. Verger, J. L. Junien, *Biochim. Biophys. Acta* **1988**, *960*, 286 – 293.
[175] F. Carriére, H. Moreau, V. Raphel, R. Laugier, C. Benicourt, J. L. Junien, R. Verger, *Eur. J. Biochem.* **1991**, *202*, 75 – 83.
[176] C. K. Abrams, M. Hamosh, V. S. Hubbard, S. K. Datta, P. Hamos, *J. Clin. Invest.* **1984**, *73*, 374 – 382.
[177] Y. Gargouri, H. Moreau, R. Verger, *Biochim. Biophys. Acta* **1989**, *1006*, 255 – 271.
[178] A. Suzuki, A. Mizumoto, M. J. Sarr, E. P. Dimagno, *Gastroenterology* **1997**, *112*, 2048 – 2055.
[179] C. Wicker-Planquart, S. Canaan, M. Riviére, L. Dupuis, R. Verger, *Protein Eng.* **1996**, *9*, 1225 – 1232.
[180] F. Carriére, H. Moreau, V. Raphel, R. Laugier, C. Bénicourt, J. L. Junien, R. Verger, *Eur. J. Biochem.* **1991**, *202*, 75 – 83.
[181] C. Bénicourt, C. Blanchard, F. Carriére, R. Verger, J. L. Junien in *Clinical Ecology of Cystic Fibrosis* (Eds.: H. Escobar, C. F. S. Baquero), Elsevier, Amsterdam, **1993**, pp. 291 – 295.
[182] P. Hadváry, H. Lengsfeld, H. Wolfer, *Biochem. J.* **1988**, *256*, 357 – 361.
[183] B. Borgstrom, *Biochim. Biophys. Acta* **1988**, *962*, 308.
[184] P. Hadváry, W. Sidler, W. Meister, W. Vetter, H. Wolfer, *FEBS Lett.* **1992**, *299*, 111 – 115.
[185] C. Cudrey, H. van Tilbeurgh, Y. Gargouri, R. Verger, *Biochemistry* **1993**, *32*, 13800 – 13808.
[186] S. Ransac, Y. Gargouri, F. Marguet, G. Buono, C. Beglinger, P. Hildebrand, H. Lengsfeld, P. Hadváry, R. Verger in ref. [33], pp. 190 – 231.
[187] A. Svendsen, I. G. Clausen, S. A. Patkar, E. Gormsen (Novo Nordisk), WO-A 9205249 A1, **1992** [*Chem. Abstr.* **1993**, *118*, 35131].

[188] A. Svendsen, I. G. Clausen, S. A. Patkar, E. Gormsen (Novo Nordisk), WO-A 9311254 A1, **1993** [*Chem. Abstr.* **1993**, *119*, 176669].
[189] A. Svendsen, I. G. Clausen, S. A. Patkar, E. Gormsen (Novo Nordisk), WO-A 9425577 A1, **1994** [*Chem. Abstr.* **1995**, *122*, 155213].
[190] A. M. Batenburg, M. R. Egmond, L. G. J. Frenken, C. T. Verrips (Unilever PLC), EP-B 407225 A1, **1991** [*Chem. Abstr.* **1991**, *115*, 25076].
[191] M. L. F. Giuseppin, M. T. S. Lopes, R. J. Planta, J. M. A. Verbakel, C. T. Verrips (Unilever PLC), WO-A 9100920 A2, **1991** [*Chem. Abstr.* **1991**, *115*, 134185].
[192] A. J. Poulose, S. A. Anderson (Clorox Co.), EP-B 375102 A2, **1990** [*Chem. Abstr.* **1990**, *113*, 174516].
[193] G. L. Gray, S. D. Scott, A. J. Ayrookaran (Genencor Inc.) US-A 5389536 A, **1995** [*Chem. Abstr.* **1995**, *122*, 234312].
[194] J. M. Van der Laan, H. B. M. Lenting, L. J. S. M. Mulleners, M. M. J. Cox (Gist-Brocades N.V.), WO-A 9425578 A1, **1994** [*Chem. Abstr.* **1995**, *122*, 127550x].
[195] W. Aehle, G. Gerritse, H. B. M. Lenting (Gist-Brocades B.V.), WO-A 9530744 A2, **1995** [*Chem. Abstr.* **1996**, *124*, 110997s].
[196] R. D. Joerger, M. J. Haas, *Lipids* **1994**, *29*, 377 – 384.
[197] T. Yoneda, Y. Miyota, K. Ohno, J. Sasuga (Showa Denko), WO-A 9514783 A1, **1995** [*Chem. Abstr.* **1995**, *123*, 221785].
[198] D. J. H. Gaskin, A. H. Bovagnet, N. A. Turner, E. N. Vulfson, *Biochem. Soc. Trans.* **1997**, *25*, 158.
[199] J. C. Moore, F. H. Arnold, *Nature Biotechnol.* **1996**, *14*, 458 – 467.
[200] D. L. Mc Beth, B. Hauer, *Appl. Environ. Microbiol.* **1996**, *62*, 3538 – 3540.
[201] Note added in proof (May 13, 1998): Since the completion of the manuscript one year ago, the amount of literature on lipases increased in all areas ranging from basic research to applications. A first example for the directed evolution of a lipase from *Pseudomonas aeruginosa* PAO1 was described: M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, K.-E. Jaeger, *Angew. Chem.* **1997**, *109*, 2961 – 2963; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2830 – 2832.