ENZYMATIC SYNTHESIS OF MACROLACTONES

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CHAPTER 1

BIOCATALYSIS IN ORGANIC SYNTHESIS
1.1 Biocatalysis: a green tool in organic chemistry

Advances in both chemical catalysis and biocatalysis are determinants in reducing the environmental footprint of chemical processes. In the field of chemical catalysis (i.e. using catalysts of nonbiological origin) the principle of environmental catalysis is well established and has been accepted for decades, producing excellent research on how to decrease, catalytically, the amounts of contaminants.

In this context, biocatalysis, intended as the use of whole cells or enzymes for the catalysis of chemical reactions, fully participates in the “green chemistry” concept that was introduced in the 90s\(^1\), and its effect on sustainability is now established beyond question.

The term “Green chemistry” was first coined by the US Environmental Protection Agency. “Green chemistry” is defined as the design, development, and application of chemical processes and products to reduce or eliminate the use and generation of substances that can be hazardous to human health and environment.\(^2,3\) However, “Green chemistry” is not just about industrial production but essentially a way of thinking. It involves pulling together tools, techniques and technologies that can help chemists and chemical engineers in research, development and production to develop more eco-friendly and efficient products and processes, which may also have significant financial benefits.

The principles involved apply equally to the use of chemicals in laboratories and education. In practice “Green chemistry” embraces concepts such as:

- Atom efficiency - designing processes to maximise the amount of raw material that is converted into the product.

- Energy conservation – designing more energy efficient processes.

- Waste minimisation - recognising that the best form of waste disposal is not to create waste in the first place.

- Substitution - using safer, more environmentally benign raw materials and solvents or solvent free processes.

This involves concepts explained by Anastas et. al.\(^1\) in a set of 12 principles (Fig.1).
(1) Prevent waste: Design chemical syntheses to prevent waste, leaving no waste to treat or clean up.

(2) Design safer chemicals and products: Design chemical products to be fully effective, yet have little or no toxicity.

(3) Design less hazardous chemical syntheses: Design syntheses to use and generate substances with little or no toxicity to humans and the environment.

(4) Use renewable feedstock: Use raw materials and feedstocks that are renewable rather than depleting. Renewable feedstocks are often made from agricultural products or are the by-products of other processes; depleting feedstocks are made from fossil fuels (petroleum, natural gas, or coal) or are mined.

(5) Use catalysts, not stoichiometric reagents: Minimise waste by using catalytic reactions. Catalysts are used in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and work only once.

(6) Avoid chemical derivatives: Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.

(7) Maximise atom economy: Design syntheses so that the final product contains the maximum proportion of the starting materials. There should be few, if any, wasted atoms.

(8) Use safer solvents and reaction conditions: Avoid using solvents, separation agents, or other auxiliary chemicals. If these chemicals are necessary, use innocuous chemicals. If a solvent is necessary, water is a good medium as well as certain eco-friendly solvents that do not contribute to smog formation or destroy the ozone layer.

(9) Increase energy efficiency: Run chemical reactions at ambient temperature and pressure whenever possible.

(10) Design chemicals and products to degrade after use: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment.

(11) Analyse in real time to prevent pollution: Include in-process real-time monitoring and control during syntheses to minimise or eliminate the formation of by-products.

(12) Minimise the potential for accidents: Design chemicals and their forms (solid, liquid, or gas) to minimise the potential for chemical accidents including explosions, fires, and releases to the environment.

**Figure 1** - Twelve principles of green chemistry

Biocatalysts constitute a green alternative to traditional organic synthesis, offering appropriate tools for the industrial transformation of natural or synthetic materials under mild reaction conditions, low energy requirements and reduced problems of isomerisation and rearrangement. In addition, biocatalysts are biodegradable and may display chemo-, regio- and stereo-selectivity resulting in decreased by-product formation and avoiding the need for functional group activation, protection or deprotection. Large-scale industrial applications of biocatalysis include for example the thermolysin-catalyzed synthesis of the low calorie sweetener aspartame, the production of acrylamide and nicotinamide assisted by nitrile hydratases, the synthesis of isomaltulose – a non cariogenic sweetener - by sucrose mutases (Fig. 2).
Figure 2 - Examples of molecules manufactured using large-scale industrial applications of biocatalysts.

Good examples of the replacement of traditional organic processes by a “greener” biocatalytic alternative are the industrial synthesis of semisynthetic penicillins and cephalosporins, the transformation of natural and synthetic fibres, the pulp Kraft-bleaching and recycling of paper, and the development of pharmaceutical intermediates.

An example of the impact of biocatalysis on chemical manufacturing is a process recently launched by a Company for the manufacture of atorvastatin, a key ingredient in the cholesterol-lowering drug.

Figure 3 – Atorvastatin

They focused on the impact of the biocatalytic step to produce a key advanced intermediate in the manufacture of atorvastatin (Fig. 3).

Scheme 1 – Chemical route to the key diol precursor of atorvastatin
Table 1 shows the comparative data from both the chemical and biocatalytic processes. The enzymatic process operates at higher concentration with approximately 1% catalyst loading. In addition, the expense and energy of cryogenic conditions is avoided. Another important advantage of the biocatalytic process is the large reduction in solvent required – the biocatalytic process uses almost 90% less solvent than the chemical process, resulting in far lower waste generation. The process is not only greener and lower in cost but it produces a purer product. Top conversion is near 100% of theoretical, with perfect stereochemical purity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Enzymatic Process</th>
<th>Chemical Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude substrate load</td>
<td>300 g/L</td>
<td>100 g/L</td>
</tr>
<tr>
<td>Substrate / Catalyst (wt/wt)</td>
<td>100:1</td>
<td>1:1</td>
</tr>
<tr>
<td>Conversion</td>
<td>99.3%</td>
<td>Not provided</td>
</tr>
<tr>
<td>Diastereomeric excess</td>
<td>99.99%</td>
<td>~94%</td>
</tr>
<tr>
<td>Cryogenic reaction cond. required</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Solvent use</td>
<td>3.2 L/kg</td>
<td>27.5 L/kg</td>
</tr>
</tbody>
</table>

Table 1: Comparison of the biocatalytic and chemical processes for the production of atorvastatin precursor.

1.2 Enzymes in organic chemistry

1.2.1 Introduction

During the last two decades the general opinion about the use of enzymes in organic reactions has changed. In fact, some years ago, conventional biocatalysis had mainly been performed in aqueous solutions. Enzymes were considered to be most active in water and organic solvents were thought to destroy their catalytic activity. This view was radically changed in the late 70s and early 80s by reports of enzymes used in organic media\textsuperscript{15,16} and particularly by the publications by Klibanov who showed that lipases were highly active and stable in different organic solvents at low water concentrations. There are several advantages of conducting enzymatic conversions in organic solvents as opposed to water. Most organic compounds are more soluble in non aqueous media than in an aqueous one and the biocatalytic step is more easily integrated into a chemical process. Further, unwanted side reactions caused by water can be reduced and the equilibrium in hydrolytic...
reactions can be shifted towards synthesis. Since these pioneering work many publications have appeared on enzyme catalyzed kinetic resolutions in organic solvents, especially lipase catalyzed reactions and on related topics like the use of organic co-solvents in water, reactions in solvent-free medium or in compressed or supercritical gases, and the addition of salts or (thia)crown ethers to the lipase.

The use of enzymes in organic synthesis starts with the search for a selective enzyme for a certain reaction. Enzyme screening is a route to enantioselective enzymes. A change in enzyme stereoselectivity can in principle be achieved by protein engineering. However, by changing the reaction conditions (solvent, temperature, pressure) the enantioselectivity of enzymes can often be improved in a simpler manner. A no selective enzyme in one solvent may become a useful selective catalyst in another solvent. Sometimes, changing the solvent can even cause a reversal of enzyme enantioselectivity. Attempts to correlate the enantioselectivity with physical characteristics of the solvents like dipole moment, dielectric constant, and hydrophobicity can be found in several papers.

Water is absolutely necessary for the catalytic function of enzymes, because water participates, directly or indirectly, in all no covalent interactions that maintain the conformation of the catalytic site of enzymes. Hence, removal of all water from the enzyme should drastically distort its conformation and result in deactivation of the enzyme. However, only a minimal amount of water is necessary around the enzyme molecules, and as long as this layer of essential water is present, replacement of the rest of the water by an organic solvent should be possible without unfavorably affecting the enzyme. The amount of water available for the enzyme is best quantified by the thermodynamic water activity. The water activity can be controlled by addition of molecular sieves or mixtures of salt hydrates.

In this way the influence of the solvents can be studied separately from their ability to strip water from the enzyme. In synthetic organic chemistry commercially available freeze dried samples of lipases (containing a minimal amount of water) are probably the most accessible enzymes to use in organic solvents.

The nature of the solvent is crucial for maintaining the layer of essential water around the enzyme. The most hydrophobic solvents, such as hydrocarbons, are the most suitable for this purpose because in these solvents the essential water isn’t stripped from the enzyme molecules.

**1.2.2 Kinetic reasons that govern the selectivity**

In a catalyzed process an enzyme (E) accelerates a reaction lowering the energy barrier between substrate (S) and product (P): energy activation (Eₐ). This is attributed to the stabilization of the transition state by the enzyme (Fig. 4).
In theory all the stereoselectivity of enzymes originates from the difference in energy enzyme-transition state $[\text{ES}]^\neq$ (Fig. 5). For example, in an enantioselective reaction, the two enantiomers A and B or the two forms of a mirror oriented prochiral substrate (involving its faces or its enantiotopic groups) may compete for the active site: enzyme-substrate complexes formed diastereoisomeric $[\text{EA}]$ and $[\text{EB}]$ have different values of free energy ($\Delta G$) for the respective transition states $[\text{EA}]^\neq$ and $[\text{EB}]^\neq$. An enantiomer (or a particular orientation of a prochiral substrate) will be processed faster than another.

**Figure 4**

\[ \Delta \Delta G^\neq = \Delta \Delta H^\neq - T \Delta \Delta S^\neq \]
\[ \Delta \Delta G^\neq = -RT \ln \frac{V_a}{V_b} \]
The value of $\Delta \Delta G^\neq$ is of great importance because it determines the optical purity of product: the enthalpy of activation is usually dominated by the breaking and formation of ties when the substrate is transformed into the product. The entropic contribution includes energy balance resulting from the order of the system, as the orientation of the reagents, conformational changes during the approach to the active site and effects of solvation.

1.2.3 Advantages and drawbacks of biocatalysts

The approach to organic reactions catalyzed by enzymes are often accompanied by the assumption that enzymes are expensive. Some enzymes are indeed expensive, but many are produced cheaply on a large scale. Considering their high catalytic power and the fact that they are recyclable most reasonably price crude enzyme preparations are adequate when compared to chemical catalysts.

However, the following advantages can be listed:

– ‘Enzymes are very efficient catalysts’. Compared to their non-enzymatic reactions, enzyme-assisted processes are accelerated by a factor of $10^8-10^{10}$, far out seeding those of chemical catalysts. Consequently, whereas chemical catalysts are generally employed in concentrations of mole percentage 0.1-1%, enzymatic reactions can be performed at reasonable rates with mole percentage of 0.001-0.0001%.

– ‘Enzymes are environmentally acceptable.’ Biocatalysts are environmentally benign reagents since they are completely degradable, unlike heavy metals.

– ‘Enzymes act under mild conditions.’ Enzymes act in a pH range of about 5-8, typically around 7, and in a temperature range of 20-40°C, preferably around 30°C. This minimizes undesired side-reactions which often plague traditional methodology.

– ‘Enzymes are compatible with each other.’ Since enzymes normally function under similar conditions, biocatalytic reaction can be carried out in tandem making sequential reactions feasible in multienzyme synthetic methodology, particularly if isolation of unstable intermediates is not always possible.

– ‘Enzymes are not bound to their natural role.’ Enzymes can tolerate a large variety of man-made unnatural substrates. Often, aqueous medium can be replaced by an organic solvent.

– ‘Enzymes can catalyze a broad spectrum of reactions.’ There’s an enzyme-catalyzed process equivalent to almost every type of organic reaction. For example, as we have been previously described common examples range from:
(a) Hydrolysis-synthesis of esters, amides, lactones, lactams, ethers, acid anhydrides, epoxides and nitriles.
(b) Oxidation-reduction of alkanes, alkenes, aromatics, alcohols, aldehydes and ketones, sulfides and sulfoxides.
(c) Addition-elimination of water, ammonia, hydrogen cyanide.
(d) Halogenation and dehalogenation, alkylation and dealkylation, carboxylation and decarboxylation, isomerization, acyloin and aldol reactions. Even Michael-additions, Diels-Alder and Claisen-rearrangement reactions have been reported.

Enzymes display three major types of selectivities:
- **Chemoselectivity.** Since enzymes tend to act on a single type of functional group, other sensitive functionalities that are sensitive to chemical catalysis do survive. Thus, reactions tend to be cleaner and laborious purification can be largely omitted.
- **Regioselectivity and Diastereoselectivity.** The complexity of an enzyme’s three dimensional structure affords an enzyme to distinguish between functional groups which are situated in different regions of the same substrate molecule.\(^{32,33}\)
- **Enantioselectivity.** Since all enzymes are made from L-amino acids, they are thus chiral catalysts. Consequently, chirality present within a substrate molecule is ‘recognized’ upon enzyme-substrate complex formation. A prochiral substrate, as a consequence, may be transformed into an optically active product through asymmetrization and both enantiomers of a racemic substrate may react at different rates, affording a kinetic resolution. Collectively, these properties constitute the ‘specificity’ of an enzyme and represents its most important feature for selective and asymmetric exploitation.\(^{37-47}\)

On the contrary, there are certainly drawbacks to the use of biocatalysis:
- ‘**Enzymes are provided by Nature in only one enantiomeric form.**’ Since there’s no general way of creating mirror-image enzymes, it is impossible to invert the chiral induction of a given enzymatic reaction by choosing the ‘other enantiomer’ of the biocatalyst, a strategy possible if chiral chemical catalysts are involved.
- ‘**Enzymes require narrow operation parameters.**’ Working under mild reaction conditions can certainly have its drawback. For example, if a reaction has a narrow window of operation at a given pH and temperature, elevated temperature, extreme pH or even high salt concentration may deactivate the enzyme. Some enzymes, however, remain catalytically active even in ice.\(^{48}\)

Most enzymes sensitive to temperature but if certain precautions are met, enzymes are
remarkably stable. Some are known to tolerate hostile environments such as temperature
greater than 100°C and pressures beyond several hundred bar.\textsuperscript{40,41}

– ‘Enzymes display their highest catalytic activity in water.’ Enzymes display their highest
catalytic activity in water, but the majority of organic compounds are only poorly soluble.
Providing certain guidelines are followed, enzymes can function in organic solvents.
Although low activity may be expected, other advantages can be accrued.\textsuperscript{41-44}

– ‘Enzymes are bound to their natural cofactors.’ Although enzymes are extremely flexible in
accepting non-natural substrates, they are almost exclusively bound to their natural
cofactors. The majority of these ‘biological reagents’ are relatively unstable molecules and are
prohibitively expensive when used in stoichiometric amounts. Unfortunately, they cannot be
replaced by more economical man-made substitute.\textsuperscript{38}

– ‘Enzymes are prone to inhibition phenomena.’ The efficiency of a reaction may be limited if the
enzymatic reactions are prone to substrate or product inhibition. Whereas substrate
inhibition can be circumvented easily by keeping substrate concentration low, product
inhibition is a little more complicated.

– ‘Enzymes may cause allergic reactions.’ Enzymes may cause allergic reactions but careful
handling may minimize reaction.

In conclusion, many factors make enzymes especially attractive for organic synthesis,
although there are still certain restrictions in their applicability. We can expect that with the
progress of technology, some of the limitations will be overcome in the near future by using
novel (genetically engineered) enzymes and through the development of novel techniques.

\subsection{1.2.4 Biotransformations in organic chemistry}

The use of enzymes for the biotransformation of non-natural organic compounds is not at all
new as they have been used for about a century. What has changed, however, is the objective;
that is, ‘biocatalysis’ has evolved as a trend-setting segment of organic synthesis since the
mid-eighties thus providing a powerful tool to the arsenal of modern organic synthesis.
Whereas most of the early studies were directed towards the elucidation of biochemical
pathways and enzyme mechanisms, the enormous potential for employing enzymes to
transform non-natural organic compounds was only realized relatively recently, \textit{i.e.} during
the late 1980’s. As a result of this intense research, enzymes have been secured an important
place in contemporary organic synthesis.\textsuperscript{50-61}
Much of the early research was impeded by a tacitly accepted dogma which stated that enzymes are Nature’s own catalysts developed during evolution for the regulation of metabolic pathways. This narrow definition implied that man-made or non-natural organic compounds cannot be regarded as substrates. However, once this pedagogic problem was attacked by non-traditionalists, it was quickly shown that the substrate tolerance of enzymes is much wider than believed. An impressive number of biocatalysts have been shown to possess a wide substrate tolerance by keeping their exquisite catalytic properties with respect to chemo-, regio- and, most importantly, enantio-selectivity. For many of these enzymes, the natural substrates — if there are any — are not known. As a consequence, the frequency of use of a particular enzyme is not evenly distributed among the various types of biocatalysts but follows a pattern shown in figure 6.

The field of industrial enzymes is now experiencing major R&D initiatives, resulting in both the development of a number of new products and in improvement in the process and performance of several existing products. According to a report from Business
Communications Company, Inc. the global market for industrial enzymes was estimated at $2 billion in 2004.

With the increased awareness of environment and cost issues, biotechnology is gaining ground rapidly due to the various advantages that it offers over conventional technologies. Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential. They constitute the most important group of biocatalysts for biotechnological applications. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavour compounds.

1.2.5 Lipases

Enzymes are classified according to the type of the catalyzed reaction, and lipases (3.1.1.3) are a sub-class of enzymes within the esterase family whose natural function is to hydrolyze long chain triacylglycerols (i.e. oils or fats) as depicted in Figure 7.

![Figure 7](image)

Enzymatic hydrolysis of a triglyceride into fatty acids and glycerol.

Lipases are among the most commonly used enzymes in organic synthesis because of their stability, availability, and acceptance of a broad range of substrates. They are found widely in nature in microorganisms, animals, and plants where their function is to digest lipids in order to make these available as an energy source for the cells. Fungi and bacteria secrete lipases to their surroundings to facilitate nutrient absorption from the external medium. There are many potential applications of lipases as can be seen in Table 3.
leather processing, animal feed, pulp and paper processing, etc. \textsuperscript{72}, however the most significant industrial applications of lipases have mainly been found in the pharmaceutical sector, in food and in detergents.\textsuperscript{79} One very useful trait of lipases is their enantioselectivity; this is exploited in the pharmaceutical industry where lipases are used for the preparation of single-isomer chiral drugs, either by kinetic resolution of racemic alcohols, acids, esters or amines, or by the desymmetrisation of prochiral compounds.\textsuperscript{74}

<table>
<thead>
<tr>
<th>INDUSTRY</th>
<th>PRODUCTS</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food</td>
<td>Cocoa butter equivalent</td>
<td>Trans esterification</td>
</tr>
<tr>
<td></td>
<td>Human milk fat substitute</td>
<td>Trans esterification</td>
</tr>
<tr>
<td></td>
<td>Mono/diacyl glycerol</td>
<td>Emulsifier</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>Flavour development</td>
</tr>
<tr>
<td></td>
<td>Bread</td>
<td>Dough stability and conditioning</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Fatty acid ester</td>
<td>Emollient</td>
</tr>
<tr>
<td>Detergent</td>
<td>Stain removal</td>
<td>Lipid degradation</td>
</tr>
<tr>
<td>Leather</td>
<td>Degreasing</td>
<td>Lipid degradation</td>
</tr>
<tr>
<td>Paper</td>
<td>Pitch control,</td>
<td>Lipid degradation</td>
</tr>
<tr>
<td>Organic synthesis</td>
<td>Resolution of chiral alcohols and amide</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

![Figure 8](image.png)

The lipase can catalyse many synthetic reactions: perhydrolysis, amidation, glucose ester and esterification.

Lipases are prepared either by extraction from animal or plant tissue, or by cultivation of microorganism. Commercially available lipases are usually derived from microorganisms although there are some difficulties because many organisms produce mixtures of lipase isoforms which differ only marginally, for example by their glycosilation pattern. Limited proteolysis during maturation of a prolipase or during recovery may result in a heterogeneous mixture of enzymes with lipolytic activity, and ambiguities may exist in the taxonomic classification of the producing microorganism (table 4).
<table>
<thead>
<tr>
<th>Lipase source</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida rugosa</td>
<td>An organism that was formerly classified as <em>Candida cylindracea</em>. Protein purification and cloning of the enzyme revealed that the organism produces at least five related lipase isoforms.</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>Contains two isoforms differing in specificity towards Δ⁹-unsaturated fatty acids.</td>
</tr>
<tr>
<td>Penicillium camambertii</td>
<td>Classified as lipase from <em>P. cyclopium</em> until 1990. Contains four lipase isoforms which differ in their glycosilation pattern.</td>
</tr>
<tr>
<td>Pseudomonas cepacia</td>
<td>Reclassified in 1995 as <em>Burkholderia cepacia</em>. Cloning and sequencing revealed identity with lipase from <em>Pseudomonas sp. ATCC21808</em>.</td>
</tr>
</tbody>
</table>

**Table 4**
Some of ambiguities concerning commercially available lipases.

Fortunately, many pure lipases, often obtained by recombinant technology, can now be purchased from enzyme suppliers. In table 5 are presented commercially lipases.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Specificity</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammalian origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human pancreatic lipase</td>
<td>sn-1,3</td>
<td></td>
</tr>
<tr>
<td>Human gastric lipase</td>
<td>sn-1,3 (acid stable)</td>
<td></td>
</tr>
<tr>
<td>Porcine pancreatic lipase</td>
<td>sn-1,3</td>
<td>Organic synthesis, digestive aid</td>
</tr>
<tr>
<td>Guinea pig pancreatic lipase</td>
<td>sn-1,3</td>
<td>(phospholipase A1 activity)</td>
</tr>
<tr>
<td><strong>Fungal origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida rugosa</td>
<td>nonspecific</td>
<td>Organic synthesis</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>cis-Δ⁹ (unsaturated fatty acids)</td>
<td>Oleochemistry</td>
</tr>
<tr>
<td>Humicola lanuginosa</td>
<td>nonspecific</td>
<td>Detergents</td>
</tr>
<tr>
<td>Rhizomucor miehei</td>
<td>sn-1,3</td>
<td>Cheese manufacturing</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>sn-1,3</td>
<td>Cheese manufacturing</td>
</tr>
<tr>
<td>Penicillium camambertii</td>
<td>sn-1,3</td>
<td>Monoglycerides</td>
</tr>
<tr>
<td>Rhizopus oryzae</td>
<td>sn-1,3 (phospholipase A1 activity)</td>
<td>Oleochemistry</td>
</tr>
<tr>
<td><strong>Bacterial origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas glumae</td>
<td>nonspecific</td>
<td>Organic synthesis, detergents</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>nonspecific</td>
<td>Organic synthesis</td>
</tr>
<tr>
<td>Chromobacterium viscosum</td>
<td>sn-1,3</td>
<td>Organic synthesis</td>
</tr>
</tbody>
</table>

**Table 5**
Important commercially available lipases.

Common to all lipases is the α/β− hydrolase fold structure, which means that the enzyme is composed of a core of predominantly parallel β -strands surrounded by α− helices (Fig.9).
The folding pattern arranges the residues of the catalytic triad of His-Asp-Ser\(^{76}\) so that the nucleophilic serine rests at a hairpin turn between a \(\beta\)-strand and an \(\alpha\)-helix.\(^{77}\)

In figure 10, is represented the general mechanism of hydrolysis reactions by lipase enzyme, between an ester and an alcohol which can be a triglyceride.\(^{78}\)

![Figure 9](image)

![Figure 10](image)

In a) it’s shown the mechanism of action of a lipase to break the ester bond. After the substrate was anchored in the oxoanion hole (carbonyl oxygen site on the two remaining-NH), serine attacks the carbonyl nucleophilic to form the tetrahedral intermediate (b). The tetrahedral intermediate decomposes in acyl-enzyme (c). Then, the alcohol group formed by the enzyme is released and replaced with a water molecule that attacks the acyl-enzyme complex, very unstable to hydrolysis (d). The deacylation proceeds with a reverse mechanism.
(e) to that of the intermediate tetrahedral formation, resulting in the release of the carboxy group with the concomitant regeneration of free enzyme (f).

To understand the molecular basis of substrate specificity, Schmid and co-workers analysed and compared six lipases with different substrate specificities for the acyl group.\textsuperscript{79}

It was found that the lipases have a large, hydrophobic fatty acid binding site but that differ in the geometry of their binding sites. The lipases were subdivided into three sub-groups:

1. lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from \textit{Rhizomucor} and \textit{Rhizopus});
2. lipases with a tunnel-like binding site (lipase from \textit{Candida rugosa});
3. lipases with a funnel-like binding site (lipases from \textit{Candida antarctica}, \textit{Pseudomonas} and mammalian pancreas and cutinase).

The shape and micro-environment of the binding site determine the substrate specificity of the lipase.

The reaction rate and enantioselectivity of enzymatic reactions are affected by the reaction temperature. In general, enhanced temperatures cause an increase in catalytic activity, but at a certain temperature the enzyme can become completely deactivated. Denaturation of proteins may be expected between 30 and 80°C and often leads to irreversible aggregation. Another problem with increasing temperature is the decrease in enantioselectivity. This is caused by the increasing mobility of the protein segments and the decreasing strength of hydrophobic interactions.\textsuperscript{80} However, there are certain enzymes that are still very active at very high (and very low) temperatures and enantioselectivities can still be very high. In particular several lipases are found to withstand rather high temperatures.\textsuperscript{81}

A way of increasing the (thermal) stability of lipases can be by immobilization.\textsuperscript{82} However, the use of immobilized lipases in organic solvents has many other advantages compared to their use in powder form\textsuperscript{83,84}

- Lipases are less sensitive to denaturation and therefore the observed reaction rate is increased.
- Immobilization by adsorbing the enzymes onto solid matrices leads to a higher surface area to volume ratio and the enzyme-substrate interaction may be improved.
- Their recovery is facilitated.
- Both activity and selectivity can be increased.
1.2.6 Candida antarctica lipase B

In the work covered in this thesis the lipase B from the yeast *Candida antarctica* has been mainly used because it resulted more efficient in lactonization than other lipases. *C. antarctica* was, as the name implies, originally found on Antarctica, isolated from a hypersaline lake called Vanda. The yeast expresses two lipases: lipase A (CALA) and lipase B (CALB). CALB consists of 317 amino acid residues which are folded into a globular $\alpha\beta$ type protein with the dimensions of 30Å x 40Å x 50Å.

CALB shares the common catalytic triad Ser-Asp-His of other lipases, however it differs from the typical lipase in that it is not activated by interfaces. The optimal pH of CALB is 7, however the enzyme is stable in the range of pH 3.5-9.5 and it shows an unusual pH profile with a broad isoelectric region ranging from pH 4 to 8. X-ray crystallography has revealed that the active site of CALB is composed of two channels, one hosting the acyl group, and the other hosting the nucleophile. The channels are described as deep and narrow. This has implications for the selectivity and specificity of the enzyme, which is known to be highly enantioselective and only accepting rather non-bulky substrates, especially for the nucleophilic substrate. Substrates are however not only restricted to the natural substrates fatty acids, water and glycerol; straight-chain primary alcohols are very good substrates for CALB and some secondary alcohols are also accepted as substrates while tertiary alcohols are not accepted. Along with alcohols, also hydrogen peroxide and amines works as nucleophiles in the synthesis of esters, peracids and amides, respectively.

![Figure 11](image)

To improve the industrial production efficiency, CALB is expressed in *Aspergillus oryzae* by submerged fermentation (http://www.novozymes.com). Usually CALB is used in its immobilised form on a macroporous acrylic resin (Lewatit VP OC 1600). In this shape it is marketed by Novozymes A/S as Novozym®435 (Fig. 13) and by Roche Molecular Biochemicals as Chirazyme L-2.
The temperature dependence of CALB/Novozyme®435 is very much determined by the conditions studied. There are reports stating that CALB denature already at 40°C in aqueous solutions, whereas when immobilised and kept dry it can withstand temperatures of beyond 100°C for an extended period of time. When used in an industrial application, the optimum temperature is a balance between productivity and stability of the enzyme. Novozym®435, has been suggested for the production of a number of different speciality chemicals as can be seen from table 6.  

<table>
<thead>
<tr>
<th>Product</th>
<th>Reaction</th>
<th>Temp. (°C)</th>
<th>Reaction rate (µmol/min/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emollient ester</td>
<td>C14 acid + C14 alcohol</td>
<td>60</td>
<td>7000</td>
<td>21, 22, 23</td>
</tr>
<tr>
<td>Wax ester</td>
<td>C16 acid + C16 alcohol</td>
<td>65</td>
<td>10,000</td>
<td>24</td>
</tr>
<tr>
<td>Ester</td>
<td>Decanoic acid + propane-2-ol</td>
<td>60</td>
<td>600</td>
<td>25</td>
</tr>
<tr>
<td>Biodiesel</td>
<td>TG + methanol</td>
<td>30</td>
<td>50</td>
<td>26</td>
</tr>
<tr>
<td>Sugar ester</td>
<td>Myristic acid + glucose</td>
<td>60</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>Polymer</td>
<td>e-Caprolactone ring opening polymerisation</td>
<td>70</td>
<td>130</td>
<td>28</td>
</tr>
<tr>
<td>Acrylation</td>
<td>Acrylic acid + octanol</td>
<td>50</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>t-acrylation</td>
<td>Ethyl acrylate + octanol</td>
<td>50</td>
<td>700</td>
<td>30</td>
</tr>
<tr>
<td>Acyl glycerols</td>
<td>Sunflower oil + glycerol</td>
<td>40</td>
<td>3000</td>
<td>31</td>
</tr>
<tr>
<td>Ceramides</td>
<td>2-hydroxy-propyl amine + octadeca-9,12-cis-cisdienoic acid</td>
<td>65</td>
<td>&gt;200</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 6  
Reaction rates and reaction conditions for some suggested applications of Novozym®435 for the synthesis of speciality chemicals

The ideal temperature will probably be as low as 30°C or lower for processes involving substrates that have a clear adverse effect on the enzyme, as methanol in biodiesel production. For processes that only involve hydrophobic reactants, as is the case in the synthesis of wax esters, the process can probably be run at 70–80°C without severe inactivation of the enzyme, especially if the system is kept relatively dry (particularly avoiding the formation of a separate water phase).  

Lipase B from *Candida Antarctica* (CALB) has been widely used as a catalyst in kinetic resolutions. The enantioselective performance of enzymes is expressed as the *enantiomeric ratio* E, which is a measure for the selectivity of a enzyme for one of the enantiomers of a substrate. Michaelis-Menten kinetics equations developed for enzyme catalyzed reactions relate the conversion of the (racemic) substrate, the enantiomeric excess, and the enantiomeric ratio (E-value).
Suppose that the substrate consists of enantiomers A and B which are, respectively, the fast and slow reacting enantiomers that compete for the same site the enzyme. Assuming the enzyme catalyzed reaction follows a simple three-step kinetic mechanism (Fig. 1), the reaction is virtually irreversible, and there is no product inhibition, the enantiomeric ratio is given in equation reported in figure 12.

\[ \begin{align*}
A + \text{enzyme} & \xrightarrow{k_1} \text{EA} \xrightarrow{k_3} \text{EP} \xrightarrow{k_4} P + \text{enzyme} \\
B + \text{enzyme} & \xrightarrow{k_1'} \text{EB} \xrightarrow{k_3'} \text{EQ} \xrightarrow{k_4'} Q + \text{enzyme}
\end{align*} \]

\[ E = \frac{\ln(A/A_0)}{\ln(B/B_0)} = \frac{V_A/K_A}{V_B/K_B} \]

Figure 12

\( V_A, K_A \) and \( V_B, K_B \) denote maximal velocities and Michaelis constants of the fast- and slow-reacting enantiomers, respectively. Equation in scheme 12 shows that the discrimination between two competing enantiomers (A and B) by enzymes is equal to the \( E \)-value. In particular, high enantiomeric ratios, \( E \), have been obtained with secondary alcohols. On this basis, it is important to understand what structural features in the substrate controls the enantiomeric discrimination of this enzyme.

Crystal structure of CALB shows that its active site is buried in the core of the enzyme, and the enzyme contains a catalytic triad (Ser105–His224–Asp187), an oxyanion hole (Thr40 and Gln106) that stabilizes the transition state as well as a cavity called the stereospecificity pocket. The bottom of the pocket is defined by Trp104. Binding site of CALB has a funnel-like shape and during a typical reaction, an ester is bound in the acyl- and alcohol-binding sites (Fig. 13). CALB does not seem to have a structural element that covers the active site, the so-called lid.
The alcohol-binding part, which contains the stereospecificity pocket, gives CALB high substrate selectivity towards secondary alcohols.\textsuperscript{93-97} It is important to realize that the enantiomeric ratio reflects the ratio of reactivity between the fast and slow reacting enantiomer. Therefore, it is not straightforward to explain the reason for a steric effect on $E$ on the basis of molecular modelling unless both enantiomers are taken into account.

This structural feature implies that CALB does not show interfacial activation which has been taken as the most characteristic property of lipases.\textsuperscript{98,99} Most researchers attributed lipase enantioselectivity to the differences of productive binding modes (PBM) between the fast-reacting enantiomer and the slow-reacting enantiomer (Fig.14).

Studies conducted by Roticci et al.\textsuperscript{100} were oriented about the enantioselectivity of \textit{Candida antarctica} lipase B (CALB) towards acyclic \textit{sec}-alcohols in order to delineate the substrate structure requirements and understand how CALB discriminates between enantiomers.
High enantiomeric ratios \((E)\) were found for long chain alcohols with an \(M\) substituent smaller than \(n\)-propyl and \(L\) substituent bigger than ethyl. Further extension of the \(M\) group entailed a drop in activity and enantioselectivity.

Thus, they concluded that the enzyme provides space enough to accommodate an \(M\) substituent smaller than an \(n\)-propyl group. In that work, all substrates tested with CALB followed the empirical rule proposed by Kazlauskas et al.\(^4\) for the prediction of the fast reacting enantiomer. When the \(M\) group was kept fixed, \(E\) increased while increasing the size of the large (\(L\)) substituent (Table 7).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enantiomeric ratio</th>
<th>Fast reacting enantiomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1</td>
<td>n.d.</td>
</tr>
<tr>
<td>b</td>
<td>7</td>
<td>(R^-(-))</td>
</tr>
<tr>
<td>c</td>
<td>9</td>
<td>(R^-(-))</td>
</tr>
<tr>
<td>d</td>
<td>390</td>
<td>(R^-(+))</td>
</tr>
<tr>
<td>e</td>
<td>705</td>
<td>(R^-(-))</td>
</tr>
<tr>
<td>f</td>
<td>350</td>
<td>(R^-(-))</td>
</tr>
<tr>
<td>g</td>
<td>97</td>
<td>(R^-(-))</td>
</tr>
</tbody>
</table>

Table 7
Enantioselectivity of CALB towards short chain alcohols.

A drastic difference in selectivity between 2-butanol (c) and 2-pentanol (d) or 3-methyl-2-butanol (e) was also noted even though their large groups differ only by one methyl. The dramatic increase in enantioselectivity for 2-pentanol \((E=390)\) compared to 2-butanol \((E=9)\) could be rationalised with the help of molecular modelling. \(R\) enantiomers bind with the \(M\) substituent in the stereospecificity pocket. The pocket provides enough space to accommodate each \(M\) substituent in docking mode I. In order to be catalysed, \(S\) enantiomers have to place the large substituent in the stereospecificity pocket. In the latter docking mode II, the pocket provides room enough to fit the \(L\) group of \((S)\)-2-butanol. Modes I and II can thus operate simultaneously on the \(R\) and \(S\) enantiomers respectively, resulting in a low \(E\).

On the other hand, the \(n\)-propyl substituent of \((S)\)-2-pentanol can hardly fit in the pocket thereby preventing it from being hydrolysed and thus inducing a high \(E\).
CHAPTER 2

MACROLACTONES: BIOACTIVITY AND SYNTHESIS
2.1 Introduction to macrolactones

Macrolactones are macrocyclic lactones with more than 12 atoms in the ring. Some macrolactones known also as macrolides, are generally acknowledged to be one of the safest class of antimicrobial agents used extensively in the treatment of upper respiratory tract and skin structure infections particularly for children and penicillin-allergic patients.

The chemistry of macrocyclic compounds originated in 1926 when Ruzicka\textsuperscript{101} elucidated the structures of civetone (1) and muscone (2) as large-ring ketones (Fig. 15).

Before this time it was believed on the basis of Baeyer’s strain theory\textsuperscript{102} that large-ring compounds would be too unstable to exist because the internal bond angles in large planar rings do not have tetrahedral geometry. In fact, large rings are able to adopt non-planar conformations and they are flexible and almost strain free.

In 1927 Kerschbaum isolated the first macrocyclic lactones, exaltolide (3) and ambrettolide (4), from angelica root and abelmoscus seed oil, respectively (Fig. 16).\textsuperscript{103}
The discovery of these vegetable musk oils aroused interest in finding synthesis routes to these and related macrolides owing to their commercial importance in the fragrance industry. The great breakthrough in macrolide chemistry came in 1950 when Brockmann and Henkel isolated the first macrolide antibiotic picromycin \( (5) \) from an *Actinomyces* culture.

![Picromycin (5)](image)

The growing interest in macrolides chemistry can be understood taking a look at the diversity of the structures and physiological effects of macrolides. Natural products containing a macrolactone framework are found in plants, insects, and bacteria and they may be of terrestrial or marine origin. The useful properties of macrolides range from perfumery to biological and medicinal activity. The new findings in the field of antitumour active and antibiotic macrolides, together with pheromones and plant growth regulators with macrolactone framework, are an inspiration to chemists to study macrolides.

### 2.1.1 Macrolactones of pharmaceutical interest

Macrolide antibiotics play a therapeutically important role. They are regarded as among the safest of antibiotics and they have successfully been used to treat infections caused by gram-positive organisms and certain gram-negative and anaerobic bacteria. The wide variety of macrolide structures in nature can be appreciated just by looking at some examples of different types of macrolide antibiotics (Fig.17).

The most important macrolide used as antibiotics include erythromycin \( (11) \), azithromycin \( (12) \) and clarithromycin \( (13) \). The prototypical macrolide, erythromycin, became available in the 1950s. Natural macrolide antibiotics can be classified according to the size of the aglycone ring, and are classed according to the number of lactone ring components; the 12-membered, 14-membered and 16-membered groups.
Erythromycin, oleandomycin and troleandomycin belong to the 14-membered group. Spiramycin, josamycin and tylosin are the only 16-member macrolides in clinical use. None of the 12-member macrolides are used clinically. Azithromycin and clarithromycin are semisynthetic derivatives of erythromycin that are characterized by increased tissue penetration and improved gastrointestinal tolerance.

Recent findings in the field of macrolides include the first isolated mycobacterial toxins, mycolactone A and its isomer B (14), containing a 12-membered lactone ring. Mycolactones are shown to be directly responsible for the necrosis and immunosuppression associated with the skin disease Buruli ulcer.
Most of the pharmacologically active macrolides have highly substituted structures as can be seen from the few examples above. However, complexity of the structure is not essential for antibiotic activity. Relatively simple macrolides such as macrolide A26771B \((\text{15})\) and patulolides A \((\text{16})\), B \((\text{17})\), and C \((\text{18})\) are antimicrobial compounds. Interestingly then, even very simple macrolides possess properties that make them worth studying.

### 2.1.2 Macrolactones as chemical signals

The chemical communication among several organisms is assured by the emission of chemical substances acting as signals (semiochemicals). The signals are chemical compounds capable of transmitting information to individuals of the same species or different species. This type of communication is very favorable in terms of energy because even small amounts of substance can cause a behavioral response that can last hours or even days. The duration depends on the molecular composition of the substance emitted, in turn closely linked to the type of message to be transmitted. Some messages, because they have to reach the maximum number of individuals and spread rapidly, such as warning signs, have very low molecular weight and therefore high volatility. Instead, signals that must persist for a longer time in the environment, such as signal trace, have a higher molecular weight and lower volatility. Depending on the type of interaction, the chemical signals are divided into:
PHEROMONES: used in intraspecific communication, are secreted outside causing a behavioral response in individuals of the same species. They are the basis of structure and function of insect societies. They mediate the recognition of kin and of belonging to their colony, reproduction, caste determination, behavior of alarm and recruitment.

ALLOMONS: generally mediate interspecific interactions and bring benefit to the emitter and the receiver. Belong to this type of signals, any substances secreted by predators (many species of vertebrates but also invertebrates and plants) to attract prey.

KAIROMONES: unlike of allomons bring benefit to the recipient and not the emitter.

These chemical stimuli play an important role in the search for food and no predator-prey relationship, encouraging such behavior in the prey to escape. Some pheromones, e.g. those of the saw-toothed grain beetle, *Oryzaephilus surinamensis*, and related cucujid species *O. mercator*, *Cryptolestes ferrugineus*, *C. turcicus*, and *C. pusillus*, have rather simple macrolactone structures (Figure 15). These beetles are major pests of cereal and grain on a world wide scale and some of these pheromones have been patented as attractants for insect traps in order to be used as natural pesticides.

Pheromones released by the stink bug *Piezodorus hybneri* are characterized by a mixture of three active compounds including \((R)-15\)-methyl hexadecanolid (24).
Compounds with allomones role were recently isolated from marine organisms more of other animal’s kingdom. These creatures are a particularly rich source of bioactive compounds because natural selection has forced them to develop an elaborate chemical arsenal in order to ensure their survival. Marine organisms generally more studied are sponges, algae, jellyfish, corals, echinoderms, tunicates, bryozoans and molluscs. In fact, these invertebrates, often brightly colored, constitute easy preys. However, during evolution, many of them have developed viable defense strategies, producing, or assimilated from the diet, unusual and interesting chemicals studied by the scientific community increasingly concerned with the search for new active ingredients. The origin of these compounds has been the subject of many studies. In many cases it was shown that they are byosynetized de novo; in others, these substances are taken from the diet, stored in the digestive gland and, when appropriate, used for defense. The defense mechanism used to deter predators involves the secretions or mucous concentrated in peripheral tissues. For example, molluscs such as Aplysia are devoid of physical protection: during evolution, in fact, have reduced or gradually lost their shell because this will restrict the movement and have developed more sophisticated alternative defensive strategies based on the use of toxic chemicals or repellents (allomones defense). Aplyolides (Fig. 19) are macrolactones isolated from the mantle of Aplysia depilans. Aplyolide A (25) derives from cyclization 15-(S)-hydroxyhexadeca-4(Z), 7(Z), 10(Z), 13(Z)-tetraenoic acid. The lactones B (26) and D (27), result from cyclization at the C-15 or C-16 of 15(S), 16(S)-dihydroxyoctadeca-9(Z), 12(Z)-dienoic acid and differ from lactones C (28) and E (29) only for the absence of double bond at C-6.
Other examples of macrolactones acting allomones are the class of azamacrolides\textsuperscript{(30, 31, 32)}, minor components from mexican bean beetle pupae secretions.

A novel class of antiviral and cytotoxic 24-membered lactones have been isolated from the culture broth of an apparently taxonomically unclassifiable marine bacterium.\textsuperscript{(124)} Macrolactine A (33) is active against several pathogenic viruses, including the human immunosuppressive virus (HIV).
A series of cytotoxic macrocyclics, named amphidinolides (34, 35), were isolated from symbiotic marine dinoflagellates of the genus *Amphidinium* which were separated from inside cells of marine flatworms. \(^{125}\)

2.1.3 Macrolactones (and other macrocycles) in fragrance industry

The musks are of central importance for the fragrance industry. They form the *bottom note* of a perfume composition, i.e. the musky undertone stays the longest on the skin or on the fabric. The *musk* odour is difficult to describe, it is often called *warm*, *sweet*, *powdery*, *animal*, *etc.*, it is longlasting, tenacious, and substantive. The use of musks goes back to antiquity, and is itself a chapter of human cultural history. \(^{126}\)

The chemistry of the natural musk odorants started with the discovery of the macrocyclic musks, ketones and lactones, during the first half of last century, when their isolation from animal and plant sources and subsequent structure elucidation took place.

Muscone, civetone, and Exaltone ® are the main odorous principles from the animal kingdom, whereas the lactones Exaltolide ®, and (Z)-Δ^7^-ambrettolide have been isolated from plants (Fig.21). \(^{127}\)
The discovery of these vegetable musk oils aroused interest in finding synthesis routes to these and related macrolides owing to their commercial importance in the fragrance industry. However, in past, musky molecules were characterized by a class of nitro-compounds discovered by serendipity during the search for new explosives a hundred years ago,\textsuperscript{128,129} when the structure of the above natural musks was not yet known, and their price was exorbitant. Musk xylol, Musk ketone, Musk Tibetene, Musk Ambrette, and Moskene® are the most prominent representatives of this class (Fig. 22).

It is a fascinating coincidence, that these pentagon and hexasubstituted polynitro benzene derivatives display a musky odour, with an \textit{ambery}, \textit{sweet vanilla}, \textit{powdery} character, and a touch of a highly esteemed \textit{animal} tonality. Because of these odour characteristics, and their very favourable price, especially compared to the natural and later synthetic macrocyclic musks, the nitro musks became high-volume industrial chemicals. During the 1980s their use began to decline. The reason is partly toxicological,\textsuperscript{130,131} partly practical, but mainly far the introduction of another class of high-performing musks to the market.\textsuperscript{132} This new class of synthetic musks consists of polycyclic, aromatic compounds. Phantolide® (42),\textsuperscript{133} Celestolide® (43),\textsuperscript{134} Traseolide® (44),\textsuperscript{135} Fixolide® (45) or Tonalide®,\textsuperscript{136} and
Galaxolide® (46)\textsuperscript{137} are the main representatives, the last two being the most important today.

Their chemistry is textbook aromatic chemistry, their odours are musky with fruity and woody effects, and an animal undertone. The polycyclic musks are today in the same price class as the nitro musks or even cheaper, \textit{i.e.} US $120-150 per kg, and several thousands of tons are manufactured per year. However, because of their low biodegradability, and their tendency to bioaccumulate, they are causing some concern today.\textsuperscript{158-160}

The chemistry of the nitro musks can be considered today as a closed chapter, and that of the nitro-free aromatic, polycyclic musks is also approaching an end.

On the other hand, in recent years, advances in the synthesis of macrocyclic compounds have driven cost down to competitive levels and popularity of macrocyclic musks amongst perfumers is growing steadily as their price/performance ratio increases favourably. Any synthesis of both macrocyclic ketones or lactones has its key step in the formation of the large ring (14–17 membered in the case of macrocyclic musks). The first approaches as well as the first industrial syntheses all used the technique of macrocyclization. In connection with the synthesis of antibiotic macrolides new macrocyclization strategies have been developed during the last 20 years.
2.2 Chemical synthesis of macrolactones

The key step in macrolactones synthesis is the formation of the macrocyclic ring. Macrolactones are prepared using several strategies and intramolecular cyclization of bifunctional acyclic molecules is the most frequently employed.

Intramolecular ring closure of open, long chain precursors is disfavoured entropically, owing to the loss of entropy associated with the formation of a usually more rigid ring structure.\(^{141}\) Illuminati and Mandolini, who have studied carefully factors controlling cyclization of omega-bromo acid, Br-(CH\(_2\))\(_n\)-CO\(_2\)H, have reported that macrolactonization is dependent on activation energy, related to energy developed by the tension ring in training and in terms of entropy on the probability of meeting the ends of the molecule as required for the reaction.\(^{142}\)

Generally, the enthalpic factor prevails entropy in the formation of lactones consisting of medium sized rings because of strain energy in the ring being formed; on the contrary, in reactions of intramolecular lactone ring of large scale, the entropy factor increases, while the enthalpy decreases until the strain of cycle is minimal. These results are shown in the profile of reactivity (Fig.24) whereby the cyclization to macrolactones molecules consisting of 8-11 carbon atoms are more difficult because of the combination of both factors enthalpic and entropic.

The main problem arising in the macrolactonization is the competition between intramolecular reactions leading to the formation of diolide and oligomers.

[Chemical structure diagrams]

The principal method for favoring intramolecular reactions in this competition is to use a "high dilution technique" first introduced by Ruggli and Ziegler.\(^{143, 144}\)
To assess the dominance between intramolecular and intermolecular reactions, the effective molarity (EM) is used as a fundamental quantity in cyclization reactions. $^{145}$ EM is expressed by the equation $EM = \frac{k_{\text{intra}}}{k_{\text{inter}}}$ where EM represents the reactant concentration at which cyclization ($k_{\text{intra}}$) and polymerization ($k_{\text{inter}}$) occur at the same rate. In practice this means that when the cyclization is done batch-wise, ring closure predominates over polymerization if the initial concentration of the substrate is lower than EM. The effective molarity depends on both the enthalpy of activation $\Delta H^\ddagger$ and the entropy of activation $\Delta S^\ddagger$. The enthalpy term decreases when the chain length of the bifunctional substrate increases, but the relationship between enthalpy of activation and the strain energy of the ring being formed is far from simple. It has been observed that the strain energies of transition states are lower than the strain energies of the formed lactones, which means that only a fraction of the product ring strain shows up in the transition state. The entropy effects of the cyclization are more straightforward. As long ago as 1935, Ruzicka proposed that the probability of end-to-end reactions in a bifunctional linear precursor decreases as the chain length increases. This postulate was later fully confirmed with entropy of activation data for cyclization reactions.

Because very dilute solutions are impractical in synthetic work, the high dilution is generally obtained by the influxion procedure, where the reactant or reactants are slowly introduced into the reaction medium over a long time. The optimal situation is that the starting material flows into the reaction flask at the same rate as the cyclized product is formed; that is to say, high dilution does not simply mean the use of a large volume of solvent but rather the establishment of a stationary concentration of substrates. Flow equilibrium of substrate influx and product outcome may also be achieved with relatively small solvent volumes. In the influxion procedure the rate of feed is a critical parameter and should be smaller than $EM \cdot k_{\text{intra}}$. The rate of feed controls the duration of the process and so describes its efficiency. Even though physicochemical studies have been made on the effective molarity of cyclization reactions, the high dilution principle is in general used empirically.

Several extensive reviews on methods of macrolactonization in the total synthesis of natural products.$^{146}$

Generally, direct cyclization of a long chain hydroxyl acid cannot be easily obtained and activation of one or both functional groups is needed.

### 2.2.1 Macrolides by ring closure through acid activation

The most famous reaction involve the “double activation” method described in 1974 by Corey and Nicolaou.$^{147}$ The mechanism involves the initial formation of a 2-pyridine thioester of the
ω-hydroxy acid via a Mukaiyama oxidation-reduction condensation with PySSPy and triphenylphosphine.\textsuperscript{148} Internal proton transfer then affords an intermediate in which both the carbonyl and the hydroxyl group have been activated, leading to the “electrostatically driven” macro lactonization (scheme 3).

![Scheme 3](image)

This “double activation” has been confirmed, and a mechanism involving ketene formation was ruled out by deuterium labeling and kinetic studies.\textsuperscript{149,150} The “classical” Corey-Nicolaou method has been used in a large number of total syntheses and synthetic applications\textsuperscript{151} such as aplyolide A\textsuperscript{151r} (25) (scheme 4).

![Scheme 4](image)

A substantial increase in the rate of cyclization in the presence of metal ions (Ag, Hg, Cu) has been reported.\textsuperscript{152} The activating effect of the thiol ester is also utilized in the Masamune\textsuperscript{153} macro lactonization reaction. A rapid lactonization occurs when S-\(\text{t}-\)butyl thiolester of hydroxy acid reacts with an electrophilic Hg(II)-compound such as mercuric trifluoroacetate. The mechanism of the Masamune method (Scheme 5) has not been fully clarified and two intermediates, a) and b), have been suggested.
In 1976 Mukaiyama\textsuperscript{154} showed a method involving the hydroxy acid activation with 1-methyl-2-chloropyridinium iodide. The mechanism of this reaction is fairly similar to that of the Corey double activation method. The mechanism involves (Scheme 6) chloride substitution by the carboxylate ion to give a highly activated acyloxy pyridinium species which then undergoes macrolactonization.

Syntheses using this methodology of various macrolactones have also been described. Among them there is the synthesis of gloeosporone (48)\textsuperscript{155}, in which the 14-membered macrolactone is formed (Scheme 7), various aggregation pheromones\textsuperscript{156}, and nine membered asci diatrienolide\textsuperscript{157} and jatrophone analogues.\textsuperscript{158}

Another very practical method for macrolactonization was described by E. P. Boden and G. E. Keck\textsuperscript{159}, who used DCC, DMAP, and DMAP•HCl to inhibit the formation of N-acylurea in the Steglich esterification.\textsuperscript{160} Recently, was developed an efficient macrolactonization protocol using benzotriazole esters and DMAP.\textsuperscript{161}
2.2.2 Ring closure through the formation of anhydride intermediate

A very common way to activate the carboxyl group of a long chain hydroxy acid is to convert it to a mixed anhydride. The anhydride reacts with the hydroxy group under base-catalysed reaction conditions, leading to a lactonized product. One of the most commonly used mixed anhydride methods is the Yamaguchi lactonization.\textsuperscript{162} It has been applied for the synthesis of more than 200 naturally occurring macrolides. This method has been utilized with success, for example, in the total syntheses of patulolides (Fig.25).\textsuperscript{163}

The Yamaguchi reaction consists of two steps: the formation of the mixed anhydride and the alcoholyis of this anhydride (Scheme 9).
The mixed anhydride is prepared to accelerate the reaction and overcome the unfavourable entropy factors leading to the formation of polymeric products. 2,4,6-Trichlorobenzoyl chloride is used to prepare the mixed anhydride, for two reasons: it forms a good leaving group and it is sterically hindered towards nucleophilic attack. The alcoholysis reaction is catalysed by 4-dimethylaminopyridine (DMAP), which is highly active in acyl transfer reactions. In the preparation of large-ring lactones the alcoholysis step of the reaction is done under high dilution conditions, usually in refluxing benzene or toluene, and the amount of DMAP is from three to six equivalents. In a modification of the Yamaguchi lactonization reaction, 2,4-dichlorobenzoyl chloride has been used instead of 2,4,6-trichlorobenzoyl chloride to prepare the mixed anhydride.\textsuperscript{166}

The main drawback of the Yamaguchi procedure is the use of the highly basic DMAP and high temperature. These factors sometimes lead to undesirable side reactions such as $\alpha/\beta$ to $\beta/\gamma$ isomerization of conjugated double bonds, epimerization of sensitive chiral centers\textsuperscript{165}, and $Z/E$ isomerization of conjugated double bonds.\textsuperscript{166,167} The latter problem is usually solved by performing the macrolactonization on the ynoic seco-acid $50$ and then reducing the triple bond, as illustrated in the synthesis of laulimalide ($51$) (scheme 10).\textsuperscript{168}

\textbf{2.2.3 Macrolides by ring closure trough alcohol activation}

Macrolactonization can further be achieved by activating the hydroxy group of the hydroxy acid. This methodology, presented in 1976 by Mitsunobu, is based on the activation of the seco-acid alcohol using diethyl azodicarboxylate (DEAD) and triphenylphosphine.\textsuperscript{169,170} Initially, diolides were usually obtained as the major products for medium ring lactones,\textsuperscript{171,172} and the Mitsunobu reaction has long been considered as a selective method to obtain diolides. A modification was introduced by Steglich in 1991 during the synthesis of combrestatin analogues.\textsuperscript{173} Using the classical Mitsunobu protocol, the diolide was obtained as the major
product (diolide 40%, macrolactone 2%), but with slow addition of the seco-acid to DEAD-triphenylphosphine, the macrolactone was the major product (macrolactone 59% yield, diolide trace yield).

Scheme 11

In the reaction mechanism, the key intermediate is an alkoxyphosphonium salt produced in situ, and the macrolactonization proceeds via an intramolecular SN$_2$ reaction and with inversion of the alcohol configuration. This reaction has been used in a number of 11- to 16-membered macrolactones, for example in the total syntheses of natural products such as (+)-amphidinolide K (53) (Scheme 12),

Scheme 12

2.2.4 Ring closure by C–C and C=C bond formation

In addition to macrolactonization reactions, also several other methods have been used for the preparation of macrocyclic lactones through ring closure. Many reactions involving C–C or C=C bond formation can be utilized intramolecularly to afford macrocles. Metal-catalysed coupling reactions, such as oxidative coupling of o,o'-diacetylenic esters with Cu(OAc)$_2$, coupling of allylic dibromides with nickel carbonyl, intramolecular palladium catalysed coupling of stabilized anions with allylic acetates, and intramolecular palladium
catalysed coupling of acid chlorides and ω-stannylalkenoates in the presence of CO, have been utilized to prepare macrolides of different size.\textsuperscript{177,178}

Also other intramolecular C–C bond formation reactions such as Dieckmann condensation and Diels–Alder reaction have been applied in the preparation of macrolides.\textsuperscript{175} Ring closure by Diels–Alder reaction is particularly useful in the construction of cytochalasans and their skeleton structures.\textsuperscript{176}

For macrolide ring closure by C=C bond formation, intramolecular Wittig like reactions are useful and generally give good yields.\textsuperscript{179}

A relatively new but very efficient route to macrolides through C=C bond formation is the ruthenium carbene (55) catalysed reaction of 1,ω-dienes by ring-closing metathesis (RCM) (Scheme 13).\textsuperscript{180}

The RCM method for macrolide ring closure has been utilized in several synthesis of natural products.\textsuperscript{181} An application of RCM is the syntheses of natural odoriferous macrolides such us Exaltolide,\textsuperscript{182} a valuable musk-odored olfactory ingredient of \textit{Archangelica officinalis} (Scheme 14). Acylation of 1-hex-5-enol with 10-undecenoyl chloride with 5-hexenoic acid gave dienes (56). Slow addition of a CH\textsubscript{2}Cl\textsubscript{2} solution of these compounds to a solution of the Grubbs carbene (4 mol %) in the same solvent at ambient temperature led to the corresponding 16-membered lactones (57) (\textit{Z,E}) in excellent yields. Hydrogenation of these compounds afforded Exaltolide (3) almost quantitatively.
Olefin metathesis is now an established technique for ring-closure of macrocycles, and recently new catalytic systems have emerged to give new impetus to this field. By a ring-closing metathesis employing the Grubbs catalyst, diene was transformed in 68% yield to (+)-(12R)-12-methyl-13-tridecanolide; the stereogenic centre was created via Oppolzer's bornane 10,2-sultame (Scheme 15). While the enantiomers of muscone smell very similar, the enantiomers of 12-methyl-13-tridecanolide, which is a natural constituent of angelica root oil (*Archangelica officinalis*), differ significantly in their odour characteristics: (+)-(12R)-(60) possesses a musk note with a sandalwood tonality, while (-)-(12S)-(60) has an animal musk odour with camphoraceous aspects.

2.2.5 Other methodologies

Other methodology used in macrolactones preparation are below described. Examples of ring expansion procedures include the Baeyer-Villiger oxidation of macrocyclic ketones and the Fe²⁺ ion promoted fragmentation of alkoxy hydroperoxides. Except for symmetrical ketones, the Baeyer-Villiger oxidation is not generally applicable for macrolactones synthesis because of the formation of isomers and the relatively harsh conditions that are required (peracids). Although a highly region- and stereoselective synthesis of recifeiolide was achieved by the fragmentation of an alkoxy hydroperoxide precursor, this method has not been tested on molecules with sensitive functional groups.
For the musk specialities this route became important when cyclododecanone (43) came to the market, at a reasonable price, as one of the downstream products of the butadiene oligomerization in the early sixties. A few examples shall demonstrate here the utility of cyclododecanone (61) as a starting material. 61 can be transformed in two steps into the bicyclic enolether 62, which upon treatment with hydrogen peroxide (70%) yields the hydroperoxide 63. The labile 63 then undergoes a fragmentation reaction (64) to a mixture of exaltolide® (3), and the dehydro-exaltolides (Scheme 16). This fragmentation has been studied in more detail under iron-copper catalysis. The mixture of the dehydro-exaltolides has recently been introduced to the market under the name habanolide® (64).

The discovery of vegetable musk oils aroused interest in finding synthesis routes to these and related macrolides owing to their commercial importance in the fragrance industry. Among methodologies used to obtain odour compounds, the Story’s synthesis, is one of most important and it’s the current industrial process to prepare exaltolide (3) by cyclohexanone (66) and involving the thermal decomposition of trisperoxide (68).
Despite the obvious hazardous nature of the intermediates, this approach presents a very economical approach to macrocyclic lactones.\textsuperscript{197}

Another industrial route for the large-scale synthesis of C15 and C16-lactones involves a polymerisation-depolymerization process.\textsuperscript{198,199} Hydroxyacid is initially dehydrated to give a polyester. Then, in the presence of a catalyst, heating under vacuum, depolymerization gives macrocyclic lactone (Scheme 18). This method was patented by Carothers at DuPont in 1930s.\textsuperscript{200}
CHAPTER 3

Enzymatic approach to the synthesis of macrolactones
3.1 Introduction
The ester forming ability of lipases has been used for regiospecific interesterification of tryglicerides and preparative resolution of chiral acids and alcohols via enantiospecific esterifications. Of course, the ability of lipases in enzyme catalyzed esterification and transesterification has prompted some investigations on their use for intramolecular reactions.

First applications of lipase for the catalysis of intraesterification of hydroxyacids or hydroxyesters dates back in the 80s. In 1984, Gatfield was the first to observe that a lipase (Mucor miehei) is able to catalyze the intramolecular esterification of 15-hydroxypentadecanoic acid to pentadecanolide. This observation was confirmed by Yamada et al. in 1987. They found that only Pseudomonas sp. and Porcine pancreatic lipases catalyze efficiently the lactonization of methyl 16-hydroxyhexadecanoate (71) producing, with high reaction yield, the monolactone 72 (Table 7).

\[
\text{Scheme 19}
\]

<table>
<thead>
<tr>
<th>Lipase used</th>
<th>Yield of lactone (%)</th>
<th>Lipase used</th>
<th>Yield of lactone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas sp.</td>
<td>78</td>
<td>Rhizopus delmer</td>
<td>0</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>73</td>
<td>Candida cylindracea</td>
<td>0</td>
</tr>
<tr>
<td>Rhizopus japonicus</td>
<td>2</td>
<td>Wheat germ</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7
Efficiency of several lipases in lactone synthesis from methyl 16-hydroxyhexadecanoate (1mM in dry benzene, 500mg of each lipase, 40°C, 72h).

They reported that lactonization was also dependent on the length of the $\omega$-hydroxyester. In fact, the cyclic dimer formation decreases and the monolactone yield increases with increasing chain length of $\omega$-hydroxyesters, going from C13-C16 (Table 8).

\[
\text{Table 8}
\]

<table>
<thead>
<tr>
<th>$\text{HO-(CH}_2\text{)}_n\text{-COOCH}_3$</th>
<th>Yield of lactone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=12</td>
<td>38 (25)*</td>
</tr>
<tr>
<td>n=13</td>
<td>64</td>
</tr>
<tr>
<td>n=14</td>
<td>78</td>
</tr>
<tr>
<td>n=15</td>
<td>80 (3)*</td>
</tr>
</tbody>
</table>

* cyclic dimer yield.

Substrate specificity of lipase P in lactonization reaction (1mM in dry benzene, 3g of lipase, 40°C, 72h).
Yamada also has investigated the effect of solvents on the lactonization, observing that best results are obtained using non-polar solvents. It was observed that initial reaction rate was different for various solvents although the lactone yield is often the same. In fact, the initial rate for lactonization of methyl hydroxyhexanoate in n-heptane was almost 7-fold higher than rate in benzene, but the same amount of lactone was obtained in both cases (table 9).

<table>
<thead>
<tr>
<th>Solvent used</th>
<th>Reaction rate (nmoles mg⁻¹ min⁻¹)</th>
<th>Yield of lactone (%)</th>
<th>Solvent used</th>
<th>Reaction rate (nmoles mg⁻¹ min⁻¹)</th>
<th>Yield of lactone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-heptane</td>
<td>0.67</td>
<td>78</td>
<td>Benzene</td>
<td>0.09</td>
<td>78</td>
</tr>
<tr>
<td>n-hexane</td>
<td>0.65</td>
<td>80</td>
<td>Dimethylsulfoxide</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>0.59</td>
<td>79</td>
<td>Ethyl acetate</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 9
Effect of solvent on the reaction rate and the yield in lipase P catalyzed lactonization (1 mM methyl 16-hydroxyhexadecanoate).

Similarly, Sih et al., exploring the relationship of substrate structure of different ω-hydroxyacids, found that product profile varies with the length of carbon chain. Furthermore, a complex of di-, tri, tetra and penta-lactones were formed in different ratios, depending on lipase used. For examples, the lipases of Pseudomonas sp. and Porcine pancreas produce di- and trilactones while the lipase of Candida cylindracea and Mucor mehei gave mostly tri- and tetralactones, starting from 10-hydroxydecanoic acid (table 10).

\[
\text{OH} \quad \text{C} \quad \text{OH} \quad \text{Lipase} \quad (\text{CH}_2)_n \quad \text{O} \\
\text{O} \quad \text{C} \quad \text{O} \quad + \text{di} + \text{tri} + \text{tetra} + \text{penta} \\
\text{CH}_2 \\
\text{Scheme 20}
\]

<table>
<thead>
<tr>
<th>n</th>
<th>Lipase</th>
<th>Mono</th>
<th>Di</th>
<th>Tri</th>
<th>Tetra</th>
<th>Penta</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Pseudomonas sp. AK</td>
<td>0</td>
<td>55</td>
<td>16</td>
<td>4</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp. K-10</td>
<td>0</td>
<td>33</td>
<td>14</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Porcine pancreas</td>
<td>0</td>
<td>57</td>
<td>20</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Candida cylindracea</td>
<td>0</td>
<td>4</td>
<td>26</td>
<td>11</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Mucor mehei</td>
<td>0</td>
<td>2</td>
<td>20</td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td>12</td>
<td>Pseudomonas sp. AK</td>
<td>66</td>
<td>26</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp. K-10</td>
<td>62</td>
<td>30</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porcine pancreas</td>
<td>46</td>
<td>21</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Candida cylindracea</td>
<td>19</td>
<td>15</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mucor mehei</td>
<td>3</td>
<td>43</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10
Lipase catalyzed lactonization of ω-hydroxyacids

O’Hagan et al. reported that 16-hydroxyhexadecanoic acid incubated (65°C, 48 h) with porcine pancreatic lipase in hexane furnished 34-membered diolide in 25% yield.²⁰⁷
A enzymatic methodology for the construction of macrocyclic lactones via direct condensation of diacids with diols has been also reported (scheme 21).  

\[
\begin{align*}
\text{HO}_2\text{C(CH}_2\text{)}_m\text{CO}_2\text{H} + \\
\text{HO}_2\text{(CH}_2\text{)}_n\text{OH} & \rightarrow \\
\text{O} & \text{(CH}_2\text{)}_m\text{O} + \\
\text{O} & \text{(CH}_2\text{)}_n\text{O}
\end{align*}
\]

\textbf{Scheme 21}

Experiments on specificity of lipase were subsequently conducted by Robinson \textit{et al.} in fact in a screening of 33 esterases and lipases. They showed that only microbial lipases from \textit{Pseudomonas fluorescens} and \textit{Candida antarctica} were able to catalyze efficiently the intramolecular esterification, especially in methylene chloride, chloroform and benzene. Antczak \textit{et al.} found that from among seven tested lipase preparations, \textit{Lipozyme} IM20 and \textit{Mucor javanicus} L46 and \textit{Mucor racemosus} L45 show ability to catalyze lactonization of \( \omega \)-hydroxyacids (C15 and C16) to macrocyclic lactones (pentadecanolide and hexadecanolide), with yields of monolactones range from a few percent to over 30%, depending on the substrate concentration, enzyme concentration, solvent type, time, temperature of system reaction, pH of the essential water layer and the addition of aprotic solvents.

Hydroxyacids or hydroxyesters containing a secondary alcohol moiety are very interesting substrate because these chiral compounds allow an investigation on the asymmetric nature of the lipase catalyzed lactonization, however they have been scarcely used as substrate. Two investigations on lipase catalyzed lactonization of short chain (C8-C13) (\( \omega \)-1)-hydroxyacids showed that mainly di- and trilactones were obtained. The reaction conducted using racemic methyl 10-hydroxyundecanoate yielded an optically pure diolide. The analysis of its optical purity allowed the conclusion that lipase P reacted preferentially with (\( R \)) isomer of the starting hydroxyacid methyl ester. This result is consistent with the empirical rule of Kazlaukas on \( R \)-preference of lipase towards secondary alcohols.

Studies on substrate with unsaturations along carbon chain, were conducted by research’s group of Ohta examining the effect of structure of the substrate with fixed ring size. They found that in lipase-catalyzed macrolactonization the presence of a double bond in the chain increases the formation of the monomeric product (Table 11).
An important factor in enzymatic macrolactonization is the product concentration. Robinson et al. have demonstrated that the presence of hexadecanolid product is inhibitory to the lactonization of C16 ω-hydroxy acids. They also demonstrated that the addition of water decreased the rate and amount of lactone formation from C16 ω-hydroxy acids. So the water content can be considered an important parameter for these catalyzed reactions. Efforts were also directed to improve catalytic efficiency by changing properly reaction environment. With this aim, Rees et al. screened some microbial lipases from Chromobacterium viscosum, Candida cylindracea, Pseudomonas and lipoprotein lipase ex Microbial, for lactonization activity towards 16-hydroxyhexadecanoic acid in a variety of different water / oil microemulsion systems. With the exception of Candida cylindracea, all the lipases exhibited lactonization activity although they were inherently more active in microemulsion systems based on the anionic surfactant sodium bis (2-ethylhexyl) sulphosuccinate (AOT) than in those based on the cationic surfactant cetyltrimethylammonium bromide (CTAB). Lactone yields were typically 50-60% and were markedly better than those reported using microemulsions in combination with chemical catalysts (Table 12).

<table>
<thead>
<tr>
<th>Lipase</th>
<th>% Yield of hexadecanolid in AOT incubations</th>
<th>% Yield of hexadecanolid in CTAB incubations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R=10</td>
<td>R=40</td>
</tr>
<tr>
<td>Chromobacterium viscosum</td>
<td>55</td>
<td>58</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>57</td>
<td>50</td>
</tr>
<tr>
<td>Microbial</td>
<td>54</td>
<td>49</td>
</tr>
<tr>
<td>Candida cylindracea</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 12
Hexadecanolid synthesis by lipase in w/o microemulsions based on AOT/n-heptane and CTAB/1:1 chloroform/n-heptane. R= measure of water content in microemulsion. Incubation temperature: 40°C.

Although direct lipase macrolactonization appeared an appealing methodology in organic synthesis only rare applications can be found in literature.

One of the first application of enzymatic lactonization to a synthetic process was carried out by Mandapur et al.; they developed in 1993 a novel synthetic strategy to obtain (3Z, 6Z)-
dodecanolide (74), a pheromone produced by Cucujid beetles. At final step, there was an ester with an hydroxy group at a suitable position to lactonize (scheme 22). Although in enzymatic lactonization of hydroxyesters with short length chain diolide predominates, reducing the yield of desired macrolide, in this case Porcine pancreatic lipase was used in benzene to provide the target compound in yield of 78%.

\[
\text{Scheme 22}
\]

Other synthetic applications were directed to the synthesis of Ferrulactone II (76). Chattopadhyay et al. found that among a series of lipase, only Porcine pancreatic lipase could effect the transformation in benzene (scheme 23), albeit in very poor yield (15%).

\[
\text{Scheme 23}
\]

Lipase catalyzed lactonization was used in the final step of the synthesis of the phytotoxic lactone herbarumin III (77). Immobilized lipase from Pseudomonas sp. provided the lactone in 31% yield (Scheme 24).

\[
\text{Scheme 24}
\]

Another study of enzymatic lactonization reported in literature concerns the synthesis of lactone 79 from 13(S)-hydroxy-(9Z,11E)-linoleic acid (coriolic acid-78) carried out by Gargouri et al. They obtained, in the presence of lipase N-435, a new lactone containing a 13 carbon ring with 2 unsaturations and 5 carbon lateral chain (Scheme 25). Testing different solvents lactonization yield ranged between 11.9% (acetone) and 42.2% (diisopropyl-ether), when a 2 mM concentration was used. Significative enhancement (73.8% yield) were obtained conducting the reaction in very diluted conditions (0.25 mM).
In this work, the immobilized lipase N-435 from *Candida Antarctica* was the most adequate catalyst offering a high yield of lactonization. The stability and especially the thermostability is one of the advantage of this enzyme that catalyze highly enantioselective transformations of many chiral alcohols in esterification, transesterification and hydrolysis reactions. In previous work, the immobilized lipase from *Candida Antarctica* (N-435) showed also important production of intramolecular monolactone (hexadecanolide). Lipase catalyzed macrolactonization has been recently used in the synthesis of some glycolipids (82, 83) containing macrolide subunit (scheme 26).
The observation that short chain hydroxyacids give mainly di- and trilactones has been applied to the preparation of (R,R)-pyrenophorin (84), an antifungal metabolite from *Pyrenophora avenae*. Lipase catalyzed dimeric lactonization allowed the preparation of a compound which was than transformed to obtain 44% of (R,R)-pyrenophorin (scheme 27).

![Scheme 27]

The main advantages of using enzymes in organic chemistry are related to their favourable unique properties, basically their biodegradability and high chemo-, regio- and stereoselectivity resulting in low byproduct formation. In particular, lipases hold considerable promise in synthetic organic chemistry and have found several practical applications in this field.

However, most applications involve intermolecular esterification while only few attempts on intramolecular esterification leading to macrolactones have been described. The reason for the lack of data on the use of this method in macrolactone synthesis is probably due to the difficulty of finding suitable conditions for obtaining products with yields competitive with conventional synthesis methods.

Scope of this PhD work is the development of protocol for the improvement of the enzymatic approach to synthetize macrolactones, a class of organic compounds with a wide range of natural features that make them attractive for their biological fragrance, phytotoxicity, pesticide or pheromone activity.

### 3.2 Improvement of the methodology for enzymatic lactonization

Although first approaches were reported, applications of enzyme catalysis in lactonization reactions are rare and, in most cases with low amounts of lactones to the point of preferring the chemical synthesis.\(^{216,217}\)

The difficulty of finding suitable conditions for obtaining yields competitive with conventional synthesis methods led us to develop a protocol for the improvement of this type of biocatalyzed synthesis.

In the course of this investigation we improved the enzymatic methodology by optimizing the following parameters:
3.2.1 Choice of substrate

16-Hydroxyhexadecanoic methyl ester was used as substrate for the study. We have chosen this compound, in order to better compare the results with the experiences reported in the literature. Furthermore, the obtained lacton, 16-hexadecanolide (85), is an important macrocyclic musk lactone with good stability, good odour characteristics and fixative properties to volatile fragrance oils.

The reaction scheme is shown in the scheme 28.

The hydroxy ester 71 was easily prepared by Fisher esterification of commercial 16-hydroxy hexadecanoic acid (85). Then the ester lactonize in presence of lipase in organic medium.

3.2.2 Enzyme choice

Lactonization activity is not a common feature of lipases, but only a specific characteristic of some lipases. Thus we started a screening to evaluate which enzyme is most efficient to catalyze lactonization reaction. Initially we applied a general method used by Yamada 205 who synthesized hexadecanolide starting from methyl 16-hydroxyhexadecanoate at 1mM and 40°C.

In the first instance, we chose lipase from Pseudomonas Cepacea because, as reported in literature, it appears to be one of the most efficient in terms of yields of macrolactones, although long time (72 hours) are required. Results were not encouraging; in fact yields were low and long time was required to obtain significative quantity of lactone.

Subsequently we extended the investigation to other commercial enzymes, choosing those that showed significant enzymatic activity (table 13).
Among them, we used some immobilized enzymes because in literature is reported that immobilization often improves efficiency of catalytic activity. The efficiency of immobilized catalyst was already observed in the utilization of *Candida Antarctica* (CALB) immobilized on acrylic resin (commercially known as Novozym 435®).

High yields of lactone were obtained after few minutes, increasing up a maximum value (79%) after 1h. But this good result was the only one in the class of immobilized enzyme tested. In fact, with immobilized lipase from *Mucor miehei* and lipase immobilized on Immobead 150 from *Pseudomonas Cepacea* we had small amounts of hexadecanolide similar to that obtained with Amano Lipase PS from *Burkholderia cepacia* (*Pseudomonas Cep*); this wasn´t caused by less activity of both immobilized enzyme compared to CALB, because long time didn´t afford any increase. Probably these enzymes were not specific towards lactonization reaction, in accordance to initial definition that lactonization activity is not a common feature of lipases.

This aspect was confirmed by using other enzymes, not immobilized, but with higher value of activity compared to CALB; in some case no lactonization activity was demonstrated, even for long time. Results of test are reported in Table 13 and show that CALB was best enzyme for our experiments.

To better confront the specific activity of each enzyme, we decided to set the ratio to 44 U (where U= units of enzyme defined as µmol of acid formed in a minute by the enzyme during its hydrolytic action for mg of dihydroxy ester).

![Table 13](image)

According with these evidences, we chose Lipase from *Candida Antarctica* (CALB) immobilized on acrylic resin (commercially known as Novozym 435®).

Regarding the relationship between the amount of enzyme and the amount of hydroxy ester substrate, we performed tests that allowed to consider equally effective even low
concentrations on efficient lactonization by lipase (Table 14). In fact, reducing amounts of CALB, catalytic activity was still efficient in hexanolide production without significative variation. This evidence is very important because allows to reduce the amount of catalyst decreasing costs of a lactonizing reaction.

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>g enzyme / mmol hydroxy ester</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Yield (%) of 16-hexadecanolide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>0,3</td>
<td>40</td>
<td>6</td>
<td>66</td>
</tr>
<tr>
<td>1 mM</td>
<td>0,7</td>
<td>40</td>
<td>6</td>
<td>65</td>
</tr>
<tr>
<td>1 mM</td>
<td>1,3</td>
<td>40</td>
<td>6</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 14

3.2.3 Solvent choice

Different solvents were used in the lipase catalyzed reaction on methyl 16-hydroxy hexadecanoate. It is well known that the best choice for enzymes in organic solvents is to use "hydrophobic" solvents.

The criteria for determining hydrophobicity of a solvent are subject to dispute. Most important indicators of hydrophobicity are: the parameter of Hidebrand (δ), the dielectric constant (Σ), the dipole moment (µ) and the coefficient partition (P). The best classification proposal was based in logP, where P is the partition coefficient of solvent in a mixture octanol / water. The partition coefficient (P) of a compound is generally described as the ratio of its concentration in aqueous and organic phases. To obtain high levels of product is essential to use an organic solvent in which organic partition coefficient is high. This implies an efficient extraction of the product for the organic phase, which produces a higher conversion. The most suitable solvents are those with logP greater than 2. Biocatalysis for synthesis reactions, such as esterification are generally considered possible in solvents which are immiscible in water (logP greater than 4). Examples of logP of some organic solvents are shown in table 15.

<table>
<thead>
<tr>
<th>Log P for common organic solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>Propyl acetate</td>
</tr>
</tbody>
</table>

Table 15
However, this classification cannot be applied to all enzymes, because it doesn’t consider specific interactions between the enzyme and solvent, which are due to the dielectric constant. The decrease in dielectric constant of the solvent, allows increased electrostatic interactions between ionizable residues of the enzyme molecule, which may cause a reduction in internal flexibility of the protein. Whereas the molecular mobility is essential for catalytic activity of the enzyme, reducing its flexibility is usually accompanied by a decrease in activity of enzyme. Changing the value of the dielectric constant also alters the value of pKa of ionizable residues on the surface of the protein. If this change occurs in the active site or close to it, a change in the bond and / or conversion of substrates is possible, and when the change in dielectric constant is dramatic, three-dimensional structure of the enzyme can be transformed.

The addition of substrates and the formation of products during the reaction may also change the hydrophobicity of the environment and, consequently, the water content around the enzyme. In our work we chose to use the cyclohexane, although similar results were obtained with isoctane.

### 3.2.4 Water content of organic medium

As known, water plays a key role in biocatalysis in organic environments, since it is involved in noncovalent interactions essential for maintaining the active conformation of the enzyme molecule.

The addition of water to solid enzyme preparations in organic solvents can increase the activity of enzyme by increasing the polarity and flexibility of the active site. However, the excess water facilitates the aggregation of the enzyme and may cause a decrease in its activity. The amount required for maintenance of enzyme structure varies with the nature of the enzyme. The hydration of polar and charged groups of molecules of enzyme seems to be a prerequisite for enzymatic catalysis. It’s possible that without water, these groups interact to produce a structural conformation inactive. The function of water in the maintenance of enzyme activity in a non aqueous environment, appears to be related with its ability to form hydrogen bonds with these functional groups, thus electrostatic interactions between groups ionize dipole-dipole interactions between unit peptide and near polar groups of protein. Since water is distributed between different phases, it is useful to describe the water in the system in terms of water activity, “aw”, where the aw of a system "sealed" is often given by the ratio of its vapor pressure (Pw) and vapor pressure of pure water (P° w): aw = Pw / P° w. The “aw” should be adjusted to obtain optimal activity of an enzyme. This can be achieved through the use of salt hydrates that facilitate the exchange of water with the
system. Some pairs of hydrates salts may be effective in achieving the desired value of aw226 (Table 16).

<table>
<thead>
<tr>
<th>Salt</th>
<th>aw</th>
<th>Salt-hydrate pair</th>
<th>aw</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>0.113</td>
<td>NaI anh./2H2O</td>
<td>0.12</td>
</tr>
<tr>
<td>MgCl2</td>
<td>0.328</td>
<td>Na₂HPO₄ anh./2H₂O</td>
<td>0.16</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>0.452</td>
<td>NaAc anh./3H₂O</td>
<td>0.28</td>
</tr>
<tr>
<td>Mg(NO₃)₂</td>
<td>0.529</td>
<td>NaBr anh./2H₂O</td>
<td>0.35</td>
</tr>
<tr>
<td>NaBr</td>
<td>0.576</td>
<td>Na₂P₂O₇ anh./10H₂O</td>
<td>0.49</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.753</td>
<td>Na₂HPO₄·2H₂O/7H₂O</td>
<td>0.61</td>
</tr>
<tr>
<td>KCl</td>
<td>0.843</td>
<td>Na₂SO₄ anh./10H₂O</td>
<td>0.80</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.973</td>
<td>Na₂HPO₄·7H₂O/12H₂O</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 16

As the catalytic efficiency of an enzyme depends on the value of aw,227 we decided to use a mixture of salt Na₂HPO₄·2H₂O/7H₂O to obtain an intermediate value of aw (0.61). This condition was achieved by placing the solvent and the enzyme, for 24 hours, under stirring in a closed chamber in indirect contact with a saturated aqueous solution of salts (3g/20ml H₂O), as in Fig.26.

This time is considered sufficient to achieve equilibrium in water solution at room temperature.

3.2.5 Concentration of substrate

As expected, high concentrations of hydroxyester appear to favour inter-molecular reactions and are not favourable to the formation of monolactone.

In order to check this aspect we evaluated the reactions conducted on 1 mM and 5 mM of substrate. Results (Fig. 27) demonstrated that optimum concentration to lactonize methyl 16-hydroxy hexadecanoate is 1 mM while 5 mM concentration is unfavourable in obtaining the desired monolactone and leaded preferentially to polymeric products.
We tested the effect of temperature in enzymatic reaction of synthesis of hexadecanolide. As in literature there are evidences that enzymes in organic solvents appear to be thermostable, we performed reactions at 40°C and 65°C. Several trials were conducted at different reaction time (Fig. 28). For short times (15'-30') we didn’t note marked differences between results obtained at both temperature, while after 1h the reaction at 40°C was by far the most efficient. In fact, the yield in hexadecanolide at temperature of 40°C in 1h, reached 79% while at 65°C, in the same time and concentration of substrate, monolactone yield was only 41%.

3.2.7 Reaction time

The tests were conducted in a range of time from minutes up to 24 hours. The evolution of the lactonization of the substrate showed that the enzyme acts in a relatively short time; In fact, as shown in Table 17, a decreasing trend over time was observed, during which oligomeric products are formed. Large quantity of hexadecanolide was already obtained after
1h. These results weren’t consistent with what reported in the literature \( ^{205} \), were high yields are described for reactions carried out for long time (up to 72h).

<table>
<thead>
<tr>
<th>Time</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>15'</td>
<td>66</td>
</tr>
<tr>
<td>30'</td>
<td>69</td>
</tr>
<tr>
<td>1 h</td>
<td>79</td>
</tr>
<tr>
<td>2 h</td>
<td>76</td>
</tr>
<tr>
<td>3 h</td>
<td>74</td>
</tr>
<tr>
<td>6 h</td>
<td>69</td>
</tr>
<tr>
<td>8 h</td>
<td>50</td>
</tr>
<tr>
<td>24 h</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table 17**
Variation in time of yield at 40°C with Enzyme CALB at 1mM concentration of methyl 16-hydroxy hexadecanoate.

**3.2.8 Ciclization of 16 hydroxy decanoic acid**

In literature are reported some enzymatic lactonizations using hydroxy acid, instead esters. So we performed reaction with 16-hexadecanoic hydroxy acid (85) as substrate (scheme 29), in the same condition of developed methodology. Results showed a clear reduction in efficiency of cyclization compared to the corresponding hydroxy ester (Fig.29). In fact, after 1h, the ratio of product obtained with hydroxy ester and that obtained by hydroxy acid was 2.6.

![Scheme 29](image)

**Figure 29**
Comparison of variation in time of yield at 40°C with CALB at 1mM concentration of methyl 16-hydroxy hexadecanoate and 16-hydroxy hexadecanoic acid.
3.2.9 Cyclization without biocatalyst

In order to prove the essential role of enzyme, we performed reactions using the usual conditions but without enzyme. There were no phenomena of cyclization of methyl 16-hydroxy hexadecanoate in absence of lipase (Scheme 30). The reaction was monitored for several days, without any results.

[Scheme 30]

3.2.10 Recovery of enzyme

One of the advantages in using immobilized enzymes is their recovery from reaction environment. We evaluated this aspect by filtering the enzyme at the end of each reaction. Recovered enzyme was then introduced in the reaction flask in order to react with a new solution of substrate, for the same time and same temperature.

Recovery operations were performed for 4 times; after fourth filtration it was noted that the enzyme had lost the initial particle size, indicating that acrylic resin was ruined. However, after 4 recovery, CALB showed sufficient activity for synthetize hexadecanolide giving a yield of 58% (Table 18). Additional recovery with satisfactory yields would have been probably possible if we had used more mild stirrer instead that magnetic stirrer.

<table>
<thead>
<tr>
<th>Yield of Hexadecanolide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial reaction</td>
</tr>
<tr>
<td>79%</td>
</tr>
</tbody>
</table>

Table 18

3.2.11 Summary of results

The evaluation of all these parameter allows the following conclusion for the optimum conditions for lactonization to hexadecanolide from methyl 16-hydroxyhexadecanoate:

- Establishment aw for enzyme activation (solvent and enzyme with saturated solution of salt hydrate pair for 24 hours).
- Enzyme: Candida antarctica lipase immobilized on acrylic resin
- Concentration: 1 mM
- Solvent: Cyclohexane
- Time: 1h
Temperature: 40 °C

The optimized methodology can be now applied to lactonization of other ω-hydroxy esters. Furthermore, in our work, the enzymatic intraesterification based on developed condition has been used to evaluate the effect of CALB toward secondary hydroxy esters and poly-hydroxy esters.

3.3 Macrolactones from ω-hydroxy esters. Synthesis of musky compounds

The synthetic protocol developed for obtaining hexadecanolide from 16-hydroxy hexadecanoic ester was applied to the lactonization of some long chain ω-hydroxy esters. The odour of musk has been highly valued since immemorial time. Odorous compounds play an important role in life processes. The odorous compounds, first used in religious ceremonies, have been used for centuries as pharmaceutical ingredients and odorants. Three musky macrolactones of vegetable origin, hexadecanolide, exaltolide and ambrettolide, are very well known. However, these compounds are found as intricate mixture in nature and in minute quantities. The constant demand for these musks odorants in perfumery industry, has led to develope artificial musks. However, the artificial musks, as nitroaromatic compounds and polycyclic musks are either suspected to be carcinogenic or nonbiodegradable. Hence, in recent years, the demand for artificial musks is fading whereas synthetic natural musks are gaining importance. Actually, industrial process to synthesize these compounds are based on methodologies that are characterized by their objective hazard. For example, in the Story’s synthesis, used for preparation of exaltolide and hexadecanolide, hazard formation and thermal decomposition of trisperoxide are key steps. We decided to apply enzymatic lactonization to the synthesis of hexadecanolide (85), exaltolide (3) and ambrettolide (4) as a green approach to their preparation.

3.3.1 Hexadecanolide

Dihydro-ambrettolide, hexadecanolide (72), although significantly less musky and less tenacious than ambrettolide, has also found its way into the perfumers armoury of raw materials.
Application of enzymatic lactonization on methyl 16-hydroxy hexadecanoate has been already described being object of development of our methodology. Results have demonstrated that lipase CALB catalyzes hexadecanolid formation by a very simple step in high yield (79%) after only 1h at 40°C.

### 3.3.2 Exaltolide

Exaltolide (Cyclopentadecanolide), was isolated for the first time from the root of *Angelica archangelica* (Fig. 30); exaltolide, having musk-like odour and fixative properties is highly valued in the perfumery industry.

![Angelica archangelica](image)

Figure 30

Several syntheses of 3 have been reported in the literature, including a Japanese patent which reports the acquisition of 77.8% of exaltolide obtained using lipase from *Pseudomonas P* in benzene at 30°C for over 24 hours, starting from a solution of 15-hydroxy pentadecanoic acid.

The synthetic approach we have chosen is very simple and is shown in Scheme 31.

![Scheme 31](image)

Compound 86 was obtained by Fisher esterification of 15-hydroxy pentadecanoic acid (70). Lactonization of methyl 15 hydroxy pentadecanoate (86), using our conditions, furnished exaltolide (3) in yield of 88% after 2h (Fig. 31).
Several macrolactonizations were conducted at different times in order to look for the best yield. A maximum was reached after 2h, then a progressive decrease was observed up to a stable value which suggests the establishment of a balance between monolacton and starting hydroxy ester. Various tests were also carried out to determine whether higher concentrations of substrate could have a significant effect in terms of quantity of monolacton obtained. Result at 5 mM concentration, however, have confirmed a substantial reduction in yield of exaltolide, according to previous result observed for hexadecanolide.

### 3.3.3 Ambrettolide

(Z)-16-Hexadec-7-enolide (ambrettolide®) (4) is the principal odorous constituent of *Hibiscus abelmoschus*, isolated from ambrette seed oil by Kerschbaum in 1927.\(^{229}\) Owing to their importance in the perfume industry, several methods have been developed for its synthesis.\(^ {230}\)

The compound used in perfumery today is of synthetic origin and is an isomer of the natural product: (E)-16-hexadec-9-enolide (isoambrettolide®).
The retro-synthetic scheme we have developed for the synthesis of (Z)-16-Hexadec-7-enolide (Ambrettolide ®) (4) is depicted in scheme 32. The unsaturated hydroxy ester needed for enzymatic lactonization (93) was envisioned as obtained by Wittig reaction. Z-selective olefination by Wittig reaction of aldehyde 89 with the phosphorane derived from triphenylphosphonium salt 91 appeared to offer the most convergent assembly of 94. In turn, 81 comes from reduction with Dibal-H of hydroxy ester 88, that is originated by protection of alcohol group of commercially available 87.

The synthesis started with protection of alcohol group of 87 with a solution of tert-butyl dimethyl silyl chloride with imidazole in dry dichloromethane, at room temperature; the next step was the carefully reduction of 88 to aldehyde 89. In order to reach an intermediate
degree of oxidation, it is necessary to pay attention to the addition of the reducing agent to avoid transformation of the ester 88 in alcohol. With most reducing agents, carboxylic esters give the primary alcohols and not the corresponding aldehydes. In case of complete reduction to alcohol, reoxidation to aldehyde, is often carried out. As alternative to this lengthy two steps procedure, the partial reduction of carboxylic esters with Dibal-H, has been reported. In our experiment, a careful addition of a diluted solution of Dibal-H to a diluted solution of ester in dry dichloromethane, in two hours at -78°C, allowed to obtain a mixture whose main product was the desired aldehyde (scheme 33).

![Scheme 33](image)

Ethyl 7-bromo heptanoate (90) was transformed in the phosphonium salt 91 under reflux, at 90°C for 48h in presence of triphenylphosphine in 90% yield (scheme 34).

![Scheme 34](image)

The subsequent step was olefination by Wittig reaction, followed by deprotection of alcohol group (scheme 35). In the Wittig reaction the phosphonium ion is deprotonated by potassium of bis(trimethylsilyl)amide and dry THF was used because such strong base requires moisture-free conditions. Once the strongly nucleophilic carbanion/ylide is formed, attacks aldheyde's carbonyl just like other strong nucleophiles, producing an alkoxide. This rapidly closes onto the phosphorus to form a 4-membered ring, which rapidly fragments to give the desired alkene and triphenylphosphine oxide as a side product.

One interesting aspect of Wittig reactions is that normally the carbanion/ylides are colored, often intensely (many are a deep, blood red or sometimes grape-juice purple) while the product alkene and phosphine oxides are normally not colored. Thus it could be possible often monitor Wittig reactions by observing the color of the solution. In our experience, only a sluggish fade of the orangegred color of ylide was observed and TLC was used for monitoring when the ylide has reacted and gone on to final products.
At the end, the olefin $Z-92$ was formed exclusively and isolated in yields of 60%. The geometry of double bound was evaluated by observing the coupling constants between vinylic protons in H-NMR spectra ($J=9.2$ Hz). Subsequent removal of t-BDMSi protecting group was directly executed treating $92$ with fluoridric acid 48%. After 3h the reaction gives 90% of $93$.

\[
\begin{align*}
89 + 91 & \xrightarrow{TMSA-K} 1-\text{BDMSiO} \\
 & \xrightarrow{\text{THF dry}} \text{92} \\
 & \xrightarrow{\text{HF48\%}} \text{93}
\end{align*}
\]

Scheme 35

With hydroxyl ester $93$ in our hand, we were ready for the cyclization step. Enzymatic lactonization of $93$, was conducted in presence of CALB immobilized on acrylic resin and under the established conditions of developed methodology (scheme 36). The best yield, 79% of (Z)-16-Hexadec-7-enolide (ambrettolide ®) $4$, was obtained after 3h.

\[
\begin{align*}
93 & \xrightarrow{\text{CALB}} \text{4} \\
 & \xrightarrow{1 \text{ mM} / 40^\circ \text{C}/2h}
\end{align*}
\]

Scheme 36

Again we found that the enzyme acts immediately on hydroxy ester which begins to lactonize already in the first few minutes until it reaches the maximum in terms of percentage yield of ambrettolide after 3h (Fig. 33).

Figure 33
Variation in time of yield of $4$ at $40^\circ$C with Enzyme CALB at 1mM concentration of $93$.
3.4 Macrolactones from hydroxyester owning secondary alcohol

Lipase B from *Candida Antarctica* (CALB) has found several applications as a catalyst in kinetic resolutions. However, all these investigations were directed to study intermolecular reactions between a secondary alcohol and a carboxylic acid (ester), while in a macrolactonization process both reacting moieties are part of the same molecule. Starting from these considerations, we wanted to test the reactivity and the enantioselectivity of CALB in lactonization reactions of hydroxy esters owning a secondary alcohol. For our investigation we choose as synthetic targets 15-methyl hexadecanolide and 15-propyl hexadecanolide, the former being a semiochemical isolated from the stink bug *Piezodorus hybneri*.

3.4.1 Chemo-enzymatic synthesis 15-hexadecanolide

Male-released pheromones of the stink bug *Piezodorus hybneri* (Heteroptera: Pentatomidae) (Fig. 34) were isolated from the airborne volatiles of males by flash chromatography, with the activity monitored by GC-EAD and behavioral bioassay. The pheromone system was characterized as a mixture of three active compounds including \((R)-15\)-methyl hexadecanolide. Enantiomerically pure samples of the \(R\) and \(S\) macrolactone were obtained by Yamaguchi's and Mitsunobu's macrolactonization of a key intermediate, \((R)-15\)-hydroxyhexadecanoic acid. The unnatural \(S\) stereoisomer was neither a beneficial nor a behavioural antagonist. Individual constituents or binary mixtures were active, but the optimal male response was elicited only by the full mixture. Behavioural observation and the fact that the onset of pheromone production is coincident with ovarian development strongly suggest that these semiochemicals are, in fact, sex pheromones.

The retro-synthetic scheme follows a similar approach previously described for the synthesis of ambrettolide (Scheme 37). The desired lactone 103 derives from hydrogenation of lactone 102 which can be obtained by lipase catalyzed lactonization on 101, an hydroxy ester unsaturated with double bond in \(Z\) stereochemistry. This hydroxy ester results by olefination with Wittig reaction of aldehyde 99 with the phosphorane derived from...
triphenylphosphonium salt 96. In turn, 99 comes from reduction with DIBAL-H of hydroxy ester 98, that is originated by protection of alcohol group of 97 with a tert-butyl dimethyl silyl chloride solution. Instead, fragment 99 is a phosphonium salt obtained starting from 95, obtained by Fisher esterification on 12-bromo dodecanoic acid 94.

In order to ascertain the enantioselectivity of enzymatic lactonization we used racemic hydroxyester 97 as starting material.

Scheme 37
In the first part of the synthesis the two reagents needed for the Wittig reaction were prepared. At the beginning of the synthesis, the secondary alcohol group of rac-3-hydroxy butyrate 97 was protected using a solution of tert-butyl dimethyl silyl chloride with imidazole in dichloromethane dry, at room temperature, obtaining 95% of 98; this ester was then reduced to aldehyde 99, by slow addition of a diluted solution of Dibal-H at -78°C (CO₂/acetone bath). In about two hours we obtained a mixture whose main product is the desired aldehyde (scheme 37) with 60% of yield.

Scheme 37

The preparation of phosphonium salt 96 starts with Fisher esterification of bromoacid 94 to give 12-bromo dodecanoate methyl ester (95). Then, bromoester 95, in the presence of triphenylphosphine, at 90°C for 72h, was transformed in the phosphonium salt 96 (Scheme 39).

Scheme 39

The subsequent step was olefination by Wittig reaction, using potassium of bis(trimethylsilyl)amide in THF dry. Anhydrous conditions are essential for effective formation of ylide. Thus, the phosphonium salt, anhydried with dry toluene, was dissolved, under nitrogen, in dry THF. Then the solution of potassium bis(trimethylsilyl)amide in THF was added dropwise and the solution was stirred for half an hour until ylide (orange dark color) was formed. At the end, the olefin Z-100 was exclusively formed and isolated in yields of 60%. Subsequent removal of t-BDMSi protecting group was done using 48% aqueous hydrofluoric acid. After 3h the reaction gives 90% of 101 (scheme 40).
Enzymatic lactonization of 101 was conducted according to the conditions of the developed methodology and gives 41% of 15-methyl hexadecanolide 102 after 3h. The reaction time was optimized conducting several reactions (Figure 35).

The subsequent hydrogenation with Pd/C under pressure of hydrogen for 1 hour leads to 90% of 103 (scheme 41).

When we measured optical rotation of the obtained lactone we found a negative value ([α]D = −13.7). Therefore, starting from a racemic substrate, optical active lactone was obtained. In particular the negative sign of the measured optical rotation indicates that (R)-15-methyl exadecanolide is the main component obtained. In fact the measured optical reported for (R)-15-methyl exadecanolide (103), obtained by other synthetic routes, is
\[ \alpha^\circ = -18.2 \cdot \]

This result confirms the enantioselectivity of the enzyme in agreement with the reported empirical rule of Kaslaukas which for a resolution of racemic secondary alcohols by lipase catalyzed esterification indicates the R enantiomer as the faster one. However, the issue about the presence of the lactone derived from the slow reacting substrate remains to be solved. In fact, it is well known that the success in enzymatic kinetic resolution depends on the careful control of the reaction times. We firstly tried to calculate the e.e. of the obtained product by HPLC using chiral column. In this analysis we were able to detect only one peak but this doesn’t exclude the presence of both enantiomers in the same peak eventually due to scarce separation in our condition. At this point we considered the analysis of the mixture of alcohols recovered at the end of reaction. However, derivatization by Mosher's method followed by spectroscopy NMR analysis did not allow us to determine e.e. In fact, two set of signals, with marked different intensities, were observed but with great superimposition of many of them.

Chromatographic analysis in HPLC using chiral column on the products obtained by Mosher esterification showed that the unreacted alcohol mixture was rich in S-enantiomer (see experimental). This indicates that R-isomer did not reacted completely but doesn’t exclude that a small part of S-enantiomer could have reacted. These results were not conclusive so we decided to verify the enantioselectivity of the reaction synthesizing the \((R)\)-101 and \((S)\)-101 starting from the two pure enantiomers ethyl \((R)\)-3 hydroxy butyrate \((R)\)-97 and ethyl \((S)\)-3 hydroxy butyrate \((S)\)-97, using the same synthetic route (Schemes 43 and 44).
With (R) and (S) 15-hydroxyhexadecenoic ester in our hands, we could submit them separately to enzymatic lactonization. It was found that only R-enantiomeric is capable of lactonization after 1h (scheme 44) while S-enantiomer was recovered unreacted in the same conditions. Furthermore, even after 24h no lactone formation was detected using (S)-101. From these data we can conclude that the lactone obtained, by enzymatic lactonization of racemic hydroxy ester, is enantiomerically pure.
3.4.2 Synthesis of 15-octadecenolide

Studies on lipase catalyzed esterifications have shown that enzymatic activity is strongly influenced by substituents of alcohol. In fact, it has been proposed that for long chain secondary alcohols high activity is found when the medium substituent (M) is smaller than n-propyl (see section 1.2.6). In order to verify this influence we considered the enzymatic cyclization of propyl 15-hydroxy pentadecenoate (111).

The retro-synthetic scheme follows the same way developed for the synthesis of 15-hexadecanolide (Scheme 46). The desired lactone 112 derives from lactonization catalyzed by CALB on 111, an hydroxy ester unsatured with double bond in Z stereochemistry. Protected compound 110 can be obtained by Wittig olefination using aldehyde 109 and the phosphorane derived from triphenylphosphonium salt 106. In turn, 109 comes from reduction with DIBAL-H of hydroxy ester 108, originated by protection of alcohol group of ethyl (R,S)-3-hydroxyhexanoate (107). Instead, fragment 106 comes from 105, obtained by Fisher esterification on 12-bromo dodecanoic acid (104).
108 was prepared by simple protection of the alcohol group of ethyl (R,S) 3-hydroxy exanoate (107) using a solution of tert-butyl dimethyl silyl chloride with imidazole in dry dichloromethane, at room temperature. In the following step, the reduction to aldehyde 109, the reducing agent was added very slowly in a CO₂/acetone bath, to avoid transformation of the starting ester in alcohol. Thus, a diluted solution of Dibal-H was added to a diluted solution of ester 108 in dry dichloromethane, dropwise in two hours at -78°C. In this way it was possible to obtain a mixture whose main product is the desired aldehyde (scheme 46).
Preparation of 106 is depicted in the following scheme 47.

Finally, olefin (Z)-110 was prepared, in 60% yields, by Wittig reaction between 106 and 109, followed by deprotection of alcohol group (scheme 48).

Enzymatic lactonization of 111, conducted in presence of CALB immobilized on acrylic resin and under the established conditions of developed methodology, proved to be very slow and gave, after 96h, 15-octadecenolide (112) with a yield of 26% (scheme 49). Thus, cyclization is slower when the size of substituent linked to chiral carbon is equal or larger than propyl. This result is consistent with the model described by Roticci in which the enzyme provides space enough to accommodate a substituent smaller than an n-propyl group.

In conclusion, we can observe that the model for the active site of CALB described studying intermolecular transesterifications, can be confidently used for intramolecular reactions.
3.5 Macrolactones from di-hydroxy esters

One of the problems encountered in the synthesis of a macrolacton starting from a di-hydroxy ester (or acid) is related to the regiocontrol of the reaction. In some cases, the size of the cyclic compound to be formed can control its formation. Sometimes, it appears that macrolactonization is favored if a properly conformation is easily adopted during cyclization. For example, selective macrolactonization has been described for the synthesis of polycavernoside, a macrolide isolated by Yasumoto from the red alga *Polycavernosa tsudai* and showning to possess lethal toxic properties. Macrolactonization, using Yamaguchi methodology, afforded exclusively 143 resulting from closure at the C15 hydroxyl group, with no trace of lactones derived from the hydroxyl substituents at either C10 or C13 (Scheme 50).

![Scheme 50](image.png)

Scheme 50

Yamaguchi lactonization in the total synthesis of polycavernoside.

However, in a part few cases, generally when more than one macrolide can be formed, the solution consists in the protection of the alcoholic function whose reaction is unwanted in order to allow macrolactonization only on the free alcohol position. Of course, a deprotection step is then needed in order to obtain the desired hydroxy macrolacton. However, this procedure requires the incorporation in the synthetic strategy of two additional steps (protection, deprotection).

In this context, an investigation was undertaken on the ability of lipases to control the regiochemistry of macrolactonization reaction. This expected behavior was based on the specific constrains required by the catalytic site of the enzyme.

Really, in literature the only reported results on the regioselectivity of lipase are related to regioselective acylations of 1,6-diols. In these cases, a regioselective acylation of a primary...
hydroxyl group is observed, allowing discrimination between primary and secondary alcohols.

For our study lipase catalyzed macrolactonization for the synthesis of aleuritic lactone and the synthesis of alyolides were chosen.

3.5.1 Lactone from poly-hydroxylated substrates: synthesis of aleuritic lactone
In order to apply regioselectivity of CALB towards poly hydroxylated substrates, we conducted a lipase catalyzed reaction starting from erythro-aleuritic ester, prepared by Fisher esterification from corresponding acid. The ester showed low solubility and thus the lactone was obtained in poor amount. In order to overcome this problem, we tried to improve the solubility by dissolving the ester adding few microliters of acetonitrile and using high temperature (65°C). In these conditions, lactonization was possible, leading to formation of 48% of lactone after 1h. As expected, only primary hydroxyl group reacted.

![Scheme 51]

3.5.2 Synthesis of alyolides B and D
With the aim to study the competition between two secondary alcohols we decided to apply our methodology to the synthesis of alyolides.

Aplyolides B and D are two ichthyotoxic macrolactones, isolated from the marine mollusk Aplysia depilans, containing 18 carbon atoms, characterized by two homo-conjugated double bonds and two stereogenic centers of absolute configuration S at C-15 and the C-16. They derive from the cyclization, respectively at C-15 and C-16, of the same precursor, the 15(S), 16(S)-dihydroxy octadeca-9 (Z), 12(Z)-dienoic acid. They look like very thick light yellow oil with the following optical rotatory power: \( [\alpha]_D^{\infty} = -42.8 \), for the alyolide B, and \( [\alpha]_D^{\infty} = +28 \), for the alyolide D.
Retrosynthetic analysis follows a convergent approach (scheme 52). The first step is the breaking of the ester bond to form compound 120. Dihydroxyacid is derived from the compound 119, where two double bonds in Z stereochemistry have been converted to triple bonds. The next disconnection of dynoate splits in fragments 115 and 118, comparable in size, which, when properly protected, will be paired in the synthetic route by coupling reaction via nucleophilic substitution. The first of the two fragments, 115, contains two stereogenic centers of S configuration introduced by asymmetric dihydroxylation on alchene stereochemically E, 114. This fragment can be generated by the corresponding alcohol (113), which can be further disconnected to propargyl alcohol and 1-bromo-pent-2(E)-ene. Compound 118 will be derived from esterification of 117 which is easily obtained by oxidation of alcohol 116; this last one is directly obtained from decyn-1-3-ol, by “migration” of the triple bond.

The synthesis begins with a first coupling, involving 1-bromo-pent-2-(E)-ene and propargyl alcohol, followed by the bromination of the mixture of the two alcohols, 113 and 113* and the dihydroxylation on the obtained mixture of bromides, 114 and 114*. The double bond stereochemistry of the compound 114 is suitable for the reaction of Sharpless asymmetric dihydroxylation (A.D.), to obtain the compound 115 (scheme 53). The use of ligands, such as the phtalazine (Phal), and the presence of sulfonamides accelerating the hydrolysis of organic esters of osmium, have recently improved the application of this reaction, allowing the development a procedure for a wide range of olefinic substrates. For our enantioselective synthesis, we used the AD-mix-α™, a preparation of standard reagents that achieves two centers in carbinol stereochemistry S. For this diol, the absolute configuration was assigned in accordance with the rule of "AD face-selection”. The mixture is commercially available, however, affordable only for small-scale reactions (up to 5mmol). The conversion of 1 mmol of alkene requires 1.4g of AD-mix-α™, containing
Scheme 52: Retrosynthetic scheme of aplyolides B and D.

Scheme 53: Synthesis of fragment 115.
0.980g of K$_2$Fe(CN)$_6$, 0.410g of K$_2$CO$_3$, 7.8 mg of (DHQ)$_2$-Phal (or (DHQD)$_2$-Phal for AD-mix-β™), 0.74 mg K$_2$OsO$_4$(OH)$_4$ as a non-volatile source of osmium. The process actually took only catalytic amount of OsO$_4$ and one of the two diastereoisomers of a chiral amine, such as dihydroquindine (DHDQ) or dihydroquinine (DHQ). DHDQ and DHQ are diastereoisomers and not enantiomers: the two alkaloids "pseudoenantiomeric" give diols of opposite configuration, but with e.e. usually not identical. To limit the catalytic amount of expensive and toxic OsO$_4$, is used a secondary oxidant able to form osmium in situ. This is the potassium ferrocyanide which is located in stoichiometric amounts in a biphasic mixture, together with potassium carbonate. Coordination to osmium of the chiral ligand amine, (DHQ)$_2$PHAL, leads to the formation of a stable chiral adduct that can distinguish between the two faces of the prochiral olefin substrate, leading to the formation of the cis-diol on one side than the other. The degree of e.e. reflects the skill with which this complex can distinguish between the two prochiral faces of alkene thanks to the difference in energy of the transition states of resulting diastereoisomers. However, use of methansulphonamide cause dihydroxylation of only the desired compound, as in his presence, terminal olefins react very slowly. In this way, even if 113 cannot be separated from its isomer, does not react within 20 hours and is separated at this time.

The yield of 115 is 60%, with an enantiomeric excess of 95%, as estimated by HPLC.

The fragment 38, containing a terminal triple bond and an ester group, is obtained starting from 3-decyn-1-ol. 9-decyn-1-ol, 116, was prepared by the reaction of triple bond migration on 3-decyn-1-ol, using NaH in 1,3-diaminopropane (DAP). In these circumstances, bifunctional "Superbase", 3-aminopropilamide, is formed. The 3-aminopropilamide is completely soluble in the excess of amine from which it derives: this high solubility may result from solvation power of the diamine and / or "internal solvation" amide itself. The reaction involves a multi-position rapid isomerization of the triple bond which, at the end of a process with a series of alkyne-allene interconversion, remains locked at the end of the chain. This migration to the end position is "anti-thermodynamics", since, at equilibrium, the internal alkynes are thermodynamically more stable. In fact, the driving force that moves the balance involved in the base mediated isomerization, is the precipitation, once formed, of alkaline acetylide. Better efficiency is attributed to KH: more reactive (isomerization instantaneous) but more difficult to handle.
Scheme 54: Isomerization of triple bond in presence of NaH and 1,3-diaminopropane (DAP). Down is shown the mechanism “zipper” of superbase, the 3-aminopropylamide, that migrates along carbonious chain.

Therefore, 3-decyn-1-ol, in the presence of strong base, transposes at 55°C, gave the sodium salt of terminal alkyne, and the subsequent acidification produces 9-decyn-1-ol with a yield of 95%. Subsequently, 9-decynoic acid 117 is prepared by oxidation of alcohol function with Jones reagent. After esterification, we obtain 118 in 99% yield (scheme 55).

Scheme 55

The next step provides 119 by direct coupling between 115 and 118 using the procedure already described for the other couplings. Finally, hydrogenation using Pd/BaSO₄ as catalyst affords 15(S),16(S)-dihydroxy-octadeca-9(Z),12(Z)-dienoic acid (120) (scheme 56).

Scheme 56: Synthesis of 120.

15(S),16(S)-dihydroxy-octadeca-9(Z),12(Z)-dienoic acid (120) was transformed into lactone via the enzymatic method developed and described in previous chapter. The reaction, monitored chromatographically, leads to the synthesis of smaller size aplyolid (28) (with traces of aplyolide B (26)) in 48 hours at 40°C in yield of 37%.
Lactonization reaction was very slow and needed of long time for obtain 28 in appreciable amount. This result confirms that \((S,S)\)-120 is the slow reactant enantiomer, according with previous observed enantioselectivity of CALB. Moreover, even in long time, there was noted no evidence of dimer formation.

Compounds obtained have spectroscopic characteristics consistent with those described in the literature.

**3.5.3 Synthesis of enantiomers of aplyolides B and D**

With the aim to verify the enantiomeric preference of CALB we undertook the enantioselective synthesis of enantiomers of aplyolides B and D. We followed a retrosynthetic similar to scheme 52. The only step that differs from that reported for the synthesis of aplyolides B and D was the use of AD-\textit{mix-\textbeta}™, for the Sharpless asymmetric dihydroxylation, in order to obtain the \(R,R\)-isomer 123.
Starting from \((R,R)-123\) enantiomer, lipase catalyzed lactonization afforded in one hour larger aplyolide \((124)\), in contrast to that observed for \((S,S)\)-enantiomer which produced smaller lactone in long reaction time.

![Figure 37](image)

\textbf{Figure 37}

Results of lactonization to enantiomers of aplyolides B and D.

### 3.5.4 Synthesis of Aplyolides C and E

The aplyolides C \((28)\) and E \((29)\) are two macrolactones with 18 carbon atoms characterized by three double bonds homo-conjugated and two stereogenic centers of absolute configuration \(S\) at C-15 and the C-16. They derive by cyclization, respectively, at the C-15 and C-16 of the same precursor, the \(15(S),16(S)\)-dihydroxyoctadeca-6(Z),9(Z),12(Z)-tryenoic acid, \(135\). They appear as very dense yellow oil with the following optical rotatory power: 

\[
\left[\alpha\right]_b^{25} = -26.7 \quad \text{for the aplyolide C} \quad \text{and} \quad \left[\alpha\right]_b^{25} = +46.3 \quad \text{for the aplyolide E}.
\]

The two lactones aplyolides C and E differ from B \((26)\) and D \((27)\) only for the presence of further double bond at C-6. Compared with the synthetic route adopted for B and D, the synthesis of aplyolides C \((28)\) and E \((29)\) requires an extra step for the introduction of the third triple bond and isomerization reaction of the triple bond was no longer necessary. In the synthetic strategy used, in addition, the portion with the two stereogenic centers was used as a terminal acetylene in the coupling reaction and not as a halide. The analogy with the previous retrosynthetic schemes is evident. Once again, the precursor of the polyene is a poliynic system, the triyne \(133\). While fragment \(128\) derives from coupling of 1-bromo-pent-2\((E)\)-ene \((126)\) with trimethylsilyl-acetylene \((127)\), compound \(133\) is obtained from coupling between 4-chloro-2-butin-1-ol \((131)\) and \(132\) (Scheme 59).259
The stereogenic centers of 130 are once again introduced by Sharpless asymmetric dihydroxylation.

As provided in Scheme 60, we proceed to the synthesis of fragments 130 and 133. The first reaction of the synthesis is based on a nucleophilic substitution made by the trimethylsilyl-acetylide on 1-bromo-pent-2-(E)-ene: under normal conditions, the compound 128 is obtained with its isomer 128*. The mixture of the two, in a 2:1 ratio, then is subjected to the subsequent Sharpless dihydroxylation (scheme 60).

Scheme 60: In scheme isomers 128 and 128* are obtained and made to react in mixture of 2:1. The e.e. value was valuated by NMR.
Differently from the synthesis of analogous 114, here we saw that after only five hours also 128 began to react. The total yield of 129 is 60% in two steps. The diol is then deprotected in the presence of a solution of tetrabutylammonium fluoride. 133 was subsequently prepared using a coupling reaction between 4-chloro-2-butyn-1-ol and heptinoate 132 followed by bromination (scheme 61).

![Scheme 61](image)

**Scheme 61**: Reagents and conditions: a) Cs₂CO₃, CuI, NaI, DMF, 90%; b) CBr₄, PPh₃, CH₂Cl₂, 85%.

Synthetic sequence for the synthesis of dihydroxyester 135 and aplyolides C and E, followed the usual scheme: coupling reaction, reduction obtained triyne, 134 and enzymatic lactonization of dihydroxyester, 135 (scheme 62).

![Scheme 62](image)

The reaction, monitored chromatographically, led to the synthesis of smaller aplyolide E (29), with presence of traces of aplyolide C, in 48 hours at 40°C in yield of 39% (Scheme 64). There was no evidence of dimer formation.

The compounds obtained showed spectroscopic characteristics perfectly in agreement with those described in literature. 123

![Scheme 63](image)

39% aplyolide E (29)
As previously observed in the synthesis of aplyolides B/D from di-hydroxy ester at stereochemistry S,S smaller lactone (E) was obtained.

### 3.5.5 Synthesis of enantiomers of aplyolides C and E

Similarly, we proceeded to the synthesis of enantiomers of aplyolides C and E following a retrosynthetic scheme in which the only step that differs from that reported for the synthesis of aplyolide C and E was the use of AD-\textit{mix}-\textbeta\textsuperscript{TM}, for the Sharpless asymmetric dihydroxylation.

\begin{center}
\includegraphics[width=\textwidth]{Scheme_64.png}
\end{center}

\textbf{Scheme 64} : Chemoenzymatic synthesis of enantiomers of aplyolides C and E.

Lactonization of \textbf{138} shows, also in this occasion, that \textit{R,R}-\textbf{138} is the fast reactant enantiomer which led to obtain 81% of enantiomer aplyolide C after 3h, at 1mM and 40°C (Figure 38). As previously observed in the synthesis of enantiomers of aplyolides B/D, using di-hydroxy ester with \textit{R,R} stereochemistry larger size lactone is formed.

\begin{center}
\includegraphics[width=\textwidth]{Figure_38.png}
\end{center}

\textbf{Figure 38}  
Results of lactonization to enantiomers of aplyolides C and E.
3.5.6 Macrolactonization of 15(\(R\)),16(\(R\))-dihydroxy-octadecanoic acid methyl ester.

It is well known that the presence of double bond in secoesters has an influence in macrolactonization.\(^{240}\) In order to study this effect on our substrates we decided to prepare 15(\(R\)),16(\(R\))-dihydroxy-octadecanoic acid methyl ester (141) for its lactonization via the enzymatic method developed and described in previous chapter. Compound 141 was easily obtained by hydrogenation of 138.

The formation of larger lactone 142, as usually resulted from (\(R/R\))-dihydroxy esters, was obtained from 15(\(R\)),16(\(R\))-dihydroxy-octadecanoic acid methyl ester (141) (Scheme 66). However, the reaction was very slow, giving after 3h only 36% yield of lactone. Longer time were required to obtain higher amount of lactone. Variation of yield at different reaction time is depicted in Figure 39. Only after 12h, lactone 142 was produced in over 60% of yield. These data suggest that the reaction is more difficult due to the absence of double bounds which could direct cyclization.
3.5.7 Final considerations on lipase catalyzed lactonization of dihydroxyesters

The results obtained can be resumed as follows:

1) Substrates containing unsaturations are more reactive than saturate ones.
2) The enzyme CALB lactonizes $R,R$-dihydroxyesters faster than corresponding $S,S$-substrate.
3) Stereochemistry of stereocentres controls the regiochemistry of lactonization: $R,R$-enantiomes give large lactone while $S,S$-enantiomers furnish the small cycle.

This last result deserves a comment. It is really impressive how in these reactions the stereochemistry of secondary alcohol can direct the regiochemistry of the ring closure. However, considering the reported description of the active site of CALB we can propose an explanation of this result. We know that high activity of CALB is observed when one substituent (M) is an ethyl group or smaller and the other substituent (L) bigger than ethyl (Figure 40). When $R,R$ enantiomer reacts we can imagine two productive binding modes (PBM): one for lactonization at C-16 (PMB I) and another for cyclization at C-15 (PMB II). Only PMB for the formation of the largest ring contains the right sized substituents at the right pockets. When we consider the $S,S$ enantiomer the needed orientation in the active site causes the presence of a group larger than ethyl in the stereospecificity pocket in both cases PMB III and PMB IV. However, PMB IV shows at list a L substituent larger than a propyl while PMB III in both pockets are filled with wrong substituents, being ethyl in the pocket generally requiring a substituent larger than propyl (Figure 41).
Figure 41
3.6 Conclusions

In this work an efficient methodology has been optimized for the enzymatic macrolactonization of hydroxyesters. The synthesis of several compounds has been accomplished using lipase CALB as biocatalyst. CALB catalyzed intramolecular lactonizations seems to fit very well with the proposed model of active site developed for intermolecular transesterification. The lipase catalyzed reactions on dihydroxyesters have shown an interesting regioselectivity directed by the stereochemistry of the alcoholic stereocenters.

All these data show how lipase mediate macrolatonization, although with some limitations, can be a useful green tool in organic synthesis.
All reactions were conducted, where the procedure demanded, in an inert atmosphere, using solvents anhydrified with proper drying, the glassware used was flamed under vacuum. The reactions were monitored by thin layer chromatography (TLC) and the products were visualized by UV light or by spraying of indicators, such as phosphomolybdic acid and cerium sulfate, or by exposure to iodine vapor.

The products were purified using silica gel (Merck 70-230 mesh and Merck 230-400 mesh). The NMR spectra were performed on Brucker AM 250 spectrometers (250.13 MHz for \(^1\text{H}\) and 62.89 for \(^{13}\text{C}\)), Bruker DRX 300 (300 MHz for \(^1\text{H}\) and 75 MHz for \(^{13}\text{C}\)) and Brucker DRX 400 (400.135 MHz for \(^1\text{H}\) and 100.03 for \(^{13}\text{C}\)) of the Department of Chemistry, University of Salerno. The chemical shifts were reported in ppm: CHCl\(_3\) signal (7.26 ppm for \(^1\text{H}\)) and CDCl\(_3\) (77.0 ppm for \(^{13}\text{C}\)) were used as internal standard.

Enantiomeric excess were measured by HPLC using a Modular HPLC System JASCO LC-NET II/ADC equipped with a JASCO Model PU-2089 Plus Pump, a JASCO MD-2010 Plus UV-vis multiple wavelength detector and a Waters refractive index detector.

ESIMS spectra were performed on a Micromass Quattro micro API™ mass spectrometer equipped with an electrospray ionization source operating in positive mode. IR spectra were obtained at a resolution of 2.0 cm\(^{-1}\) with a Vector 22 Bruker Spectrometer. Optical rotations were measured with a JASCO DIP-1000 polarimeter. Elemental analyses were performed on Flash EA 1112 (Thermo Electron Corporation) analyzer.
4.1 Synthesis of 16-hexadecanolide

4.1.1 Synthesis of 16-hydroxy hexadecanoic methyl ester (71)

16-Hydroxy hexadecanoic methyl ester (71) was prepared by Fisher esterification reaction. A solution of 16-hydroxy hexadecanoic acid (2mmol) in 10mL of methanol was acidified with 5 drops of sulfuric acid 96% and refluxed at 70° C for 20 hours. After extraction with diethyl ether and water, ethereal phases were combined, anhydridified on Na₂SO₄ and concentrated under vacuum. The reaction is almost quantitative. The ester 71 was purified on silica, eluting with petroleum ether/diethyl ether 7/3.

16-Hydroxy hexadecanoic methyl ester (71)

\[ \text{1H-NMR (CDCl}_3, 400 MHz): \delta 3.67 (3H, s, \text{–OCH}_3), 3.53 (2H, m), 2.30 (2H, t, J= 7.5 Hz), 1.94 (1H, t, J= 2.7 Hz), 1.62 (2H, br t, J= 7.5 Hz), 1.48 (2H, m), 1.29 (22H, m). \]

\[ \text{13C-NMR (100MHz CDCl}_3): \delta 174.4 (s), 68.0 (t), 52.3 (q), 34.0 (t), 32.3 (t), 29.0 (t), 28.8 (t), 28.4 (t), 28.1 (t), 24.8(t), 18(t). \]

4.1.2 Synthesis of 16-Hexadecanolide (72)

The reaction flask containing a solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40mL of cyclohexane was placed in a chamber containing a saturated solution of salt hydrates (Na₂HPO₄ · 2H₂O/7H₂O). The flask was left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester 71 were added to the reaction flask and it was immersed in a bath at 40° C, under vigorous agitation, for 1h.
At the end, the enzyme was removed from the solution by filtration and the filtrate was concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4, affording 79% of 16-hexadecanolide (72).

16-Hexadecanolide (72)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 4.13 (2H, dd, $J = 5.6$ Hz, $J = 5.2$ Hz), 2.33 (2H, t, $J = 6.6$ Hz), 1.66 (4H, m), 1.32 (22H, br s).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 173.4 (s), 67.0 (q), 34.4 (t), 32.3 (t), 29.9 (t), 27.4 (t).

ES-MS (m/z) = 254 (M+).

IR (cm$^{-1}$): 2923, 2859, 1732, 1457, 1164

4.2 Synthesis of exaltolide

4.2.1 Synthesis of 15-hydroxy pentadecanoic methyl ester (86)

86 was prepared by Fisher esterification reaction. A solution of 15-hydroxy hexadecanoic acid (2mmol) in 10mL of methanol was acidified with 5 drops of sulfuric acid 96% and refluxed at 70° C for 20 hours. After extraction with diethyl ether and water, ethereal phases were combined, anhydrified on Na$_2$SO$_4$ and concentrated under vacuum. The reaction is almost quantitative. The ester 86 was purified on silica, eluting with petroleum ether/diethyl ether 7/3.

15-Hydroxy pentadecanoic methyl ester (86)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.67 (3H, s), 3.53 (2H, m), 2.30 (2H, t, $J = 7.5$ Hz), 1.94 (1H, t, $J = 2.7$ Hz), 1.62 (2H, br t, $J = 7.5$ Hz), 1.48 (2H, m), 1.29 (18H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.4 (s), 68.0 (t), 52.3 (q), 34.0 (t), 32.3 (t), 29.0 (t), 28.8 (t), 28.4 (t), 28.1 (t), 24.8(t), 18(t).
4.2.2 Synthesis of exaltolide (3)

The reaction flask containing a solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40mL of cyclohexane was placed in a chamber containing a saturated solution of salt hydrates (Na$_2$HPO$_4$ · 2H$_2$O/7H$_2$O). The flask was left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester 86 were added to the reaction flask and it was immersed in a bath at 40° C, under vigorous agitation, for 1h. At the end, the enzyme was removed from the solution by filtration and the filtrate was concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4, affording 88% of exaltolide (3).

**Exaltolide (3)**

$^1$H-NMR (CDCl$_3$, 400 MHz): δ 3.93 (2H, t, $J$= 5.2 Hz), 2.17 (2H, t, $J$= 7.0 Hz), 1.66 (4H, m), 1.32 (20H, br s).

$^{13}$C-NMR (100MHz CDCl$_3$): δ 173.4 (s), 67.0 (t), 34.4 (t), 32.3 (t), 29.9 (t), 27.4 (t).

ES-MS (m/z) = 240 (M+).

IR (cm$^{-1}$): 2923, 2859, 1732, 1437, 1164
4.3 Synthesis of ambrettolide

4.3.1 Synthesis of compound 88

Alcohol group was protected by reaction with t-BDMS-Cl.
A solution was prepared by mixing 5.31 mmol of 9-hydroxy nonanoate methyl ester, 7 mmol of t-BDMS-Cl, 7 mmol of imidazole and 5.3 ml of dichloromethane dry. The reaction goes at room temperature, stirring vigorously, in 20h.
50ml of H₂O were added and the mixture was extracted with ethylic ether/H₂O. Ethereal phases were anhydrified on Na₂SO₄, concentrated in vacuo and purified by silica gel chromatography, eluting with petroleum ether/diethyl ether 97/3.
96% of pure 88 is obtained.

Ester 88

¹H-NMR (CDCl₃, 400 MHz): δ 3.65 (3H, s, –OCH₃), 3.58 (2H, m), 2.29 (2H, t, J= 7.7 Hz), 1.62 (2H, m), 1.49 (2H, m), 1.29 (8H, m), 0.88 (9H, s), -0.03 (6H, s).

4.3.2 Synthesis of compound 89

5.1 mmol of 88 were diluted with 10ml of CH₂Cl₂ dry. Then, under N₂ atmosphere, during 2h, at -78°C (CO₂/acetone bath), 5mmol of DIBAL-H (1.0M in CH₂Cl₂) diluted 1/1 with CH₂Cl₂ dry, were added to solution.
Subsequently, 10ml of a sature solution of Na/K tartrate were added to neutralize DIBAL-H; the mixture was extracted with diethyl ether/H₂O. Ethereal phases were anhydrified on Na₂SO₄, concentrated in vacuo and purified by silica gel chromatography, eluiting with pentane/diethyl ether 99/1.
Aldehyde 89 was obtained in a yield of 80%.

Aldehyde 89

$^1$H-NMR (CDCl$_3$, 250 MHz): $\delta$ 9.75 (1H, s), 3.58 (2H, t, J= 6.5 Hz), 2.30 (2H, dt, J= 7.4 1.8 Hz), 1.62 (2H, m), 1.49(2H, m), 1.29 (8H, m), 0.88 (9H, s), -0.03 (6H, s).

$^{13}$C-NMR (62.89 MHz CDCl$_3$): $\delta$ 202.7 (d), 63.0 (t), 43.7 (t), 32.6 (t), 29.1 (t), 28.9 (t), 25.8 (q, 3C), 25.5(t), 21.9 (t), 18.2 (t), -5.4 (q, 2C).

4.3.3 Synthesis of phosphonium salt of ethyl 7-bromo heptanoate (91)

\[
\begin{align*}
\text{BrPh}_3\text{P} & \quad \text{O} \\
& \quad \text{O} \\
& \quad 91
\end{align*}
\]

A mixture containing 6.3mmol of 90 bromo ethyl heptanoate, 6.3mmol of triphenylphosphine and 15ml of CH$_3$CN was placed under reflux, at 90°C for 48h. Then, at room temperature, the solution was concentrated in vacuo and purified by silica gel chromatography and eluted with petroleum ether/ diethyl ether 97/3 (to retrieve the starting bromo ester) and then with chloroform/methanol 95/5.

Phosphonium salt 91 was obtained in a yield of 90%.

Compound 91

$^1$H-NMR (CDCl$_3$, 250 MHz): $\delta$ 7.77-7.64 (15H, m), 3.82 (2H, q, J= 7.1 Hz), 3.43 (2H, m), 1.99 (2H, m), 1.42 (4H, m), 1.29 (2H, m), 1.18 (2H, m), 0.96 (3H, t, J= 7.1 Hz).
4.3.4 Synthesis of compound 92

5.1 mmol of 91 were placed in an anhydrided flask and washed 3–4 times with toluene dry. Toluene was then removed in vacuo and flask was maintained under N₂. 10 ml of THF dry were added to phosphonium salt at 0°C stirring for some minutes. At this temperature 5 mmol of a solution of TMSA-K (0.5M in toluene) were diluted with 10 ml of THF dry, are added to reaction flask; then the system was maintained at room temperature for 30 minutes.

The system was freeze at 0°C and a solution of 4.0 mmol of aldehyde 89, diluted with 7 ml of THF dry, was added dropwise. After 2h, reaction was stopped by acidification with 10 ml of HCl 1.0M; then solution was extracted with diethyl ether/H₂O. Ether phase was anidryfied on Na₂SO₄, concentrated in vacuo and purified by silica gel chromatography, eluting with petroleum ether/diethyl ether 99/1.

Wittig product 92 was obtained in a yield of 60%.

**Compound 92**

¹H-NMR (CDCl₃, 300 MHz): 6 5.33 (2H, m), 4.12 (2H, q, J = 7.1 Hz), 3.58 (2H, t, J = 6.3 Hz), 2.27 (2H, t, J = 7.6 Hz), 2.00 (4H, m), 1.62 (2H, m), 1.49 (2H, m), 1.36-1.24 (12H, m), 0.88 (9H, s), 0.86 (3H, t, J = 7.1 Hz), 0.04 (6H, s).
4.3.1 Synthesis of (Z)-ethyl 16-hydroxyhexadec-7-enoate (93)

The removal of protecting group t-BDMSi was directly executed by making, in a plastic container, a mixture of 3.0 mmol of 92 with 30 ml of HF 48%, and 100 ml of CH₃CN. The reaction was monitored and after 3h it was completed. HF was neutralized by adding NaHCO₃ until the end of effervescence.

The obtained solution was extracted with ethyl acetate/H₂O. Organic phase was anidryfied on Na₂SO₄, concentrated in vacuo and purified by silica gel chromatography, eluiting with petroleum ether/ diethyl ether from 85/15 to 7/3.

Yield of 93 was of 90%.

(Z)-Ethyl 16-hydroxyhexadec-7-enoate (93)

¹H-NMR (CDCl₃, 400 MHz): δ 5.33 (2H, m), 4.11 (2H, q, J= 7.1 Hz), 3.63 (2H, t, J= 6.6 Hz), 2.28 (2H, t, J= 7.6 Hz), 2.00 (4H, m), 1.57 (4H, m), 1.34-1.28 (14H, m), 1.24 (3H, t, J= 7.1 Hz).

¹³C-NMR (100.03 MHz CDCl₃): δ 174.1 (s), 130.3 (d), 129.5 (d), 63.7 (t), 60.2 (t), 29.7 (t), 29.5 (t), 29.4 (t), 29.3 (t), 29.2 (t), 28.8 (t), 27.2 (t), 27.0 (t), 25.7 (t), 24.9 (t), 14.2 (q).

ES-MS (m/z)= 321 (M+Na⁺).
4.3.5 Synthesis of Ambrettolide (4)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 • 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. Then, 0.04mmol of hydroxy-ester 93 were added to the reaction flask and it was immersed in a bath at 40° C for 3h.

When the reaction finished, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with petroleum ether/chloroform 8/2.

79% of ambrettolide 4 was obtained.

Ambrettolide (4)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 5.33 (2H, m), 4.13 (2H, m), 2.32 (2H, t, J= 6.64 Hz), 2.03 (4H, m), 1.61 (4H, m), 1.38 - 1.25 (14H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.2 (s), 130.4 (d), 130.2 (d), 63.9(t), 34.8 (t), 29.9 (t), 29.6 (t), 28.9 (t), 28.7 (t), 28.6 (t), 28.5 (t), 27.9 (t), 27.2 (t), 27.0 (t), 25.6 (t), 25.5 (t).

MS ($m/z$): 252, 104, 96.

IR ($cm^{-1}$): 2915, 2849, 1748, 1468, 1388, 1360.
4.4 Synthesis of 15-hexadecanolide

4.4.1 Synthesis of tert-butyl dimethyl silil ethyl butirrate (98)

Alcohol group was protected by reaction with t-BDMS-Cl.
A solution was prepared by mixing 75 mmol of ethyl 3-hydroxy butirrate, 99 mmol of t-BDMS-Cl, 99 mmol of imidazole and 74 ml of dichloromethane dry. The reaction goes at room temperature, stirring vigorously, in 20h.
After adding 50 ml of H₂O, mixture was extracted with diethyl ether/H₂O. Ethereal phases were anhydridied on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography, and eluted with petroleum ether/ diethyl ether 97/3.
98% of pure 98 was obtained.

tert-butyl dimethyl silil ethyl butirrate (98)

¹H-NMR (CDCl₃, 400 MHz): δ 4.26 (1H, m), 4.08 (2H, m), 2.45 (1H, dd, J = 14.5 and 7.6 Hz), 2.34 (1H, dd, J = 14.5 and 5.3 Hz), 1.24 (3H, t, J = 7.2), 1.17 (3H, d, J = 6.1), 0.85 (9H, s), 0.06 (6H, s).

¹³C-NMR (62.89 MHz CDCl₃): δ 171.8 (s), 66.1 (d), 60.4 (t), 45.2 (t), 25.9 (q, 3C), 24.1 (t), 18.1 (q), 14.4 (q), -5.4 (q, 2C).
4.4.2 Synthesis of *tert*-butyl dimethyl silil butirraldehyde (99)

65 mmol of 98 were diluted with 60 ml of CH₂Cl₂ dry. Then, under N₂ atmosphere, during 2h, at -78°C (CO₂/acetone bath), 65 mmol of DIBAL-H (1.0 M in CH₂Cl₂) diluted 1/1 with CH₂Cl₂ dry, were added to solution. At the end, 30 ml of a saturated solution of Na/K tartrate were added to neutralize DIBAL-H; the mixture was extracted with diethyl ether/H₂O. Ethereal phases were anhydrided on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography and eluted with pentane/diethyl ether 99/1. Aldehyde 99 was obtained in a yield of 80%.

*tert*-butyl dimethyl silil butirraldehyde (99)

1H-NMR (CDCl₃, 400 MHz): δ 9.79 (1H, br s), 4.34 (1H, m), 2.54 (2H, ddd, J = 15.7 Hz, J = 6.9 Hz, J = 2.7 Hz), 1.23 (3H, d, J = 6.1 Hz), 0.86 (9H, s), 0.07 (3H, s), 0.05 (3H, s).

13C-NMR (62.89 MHz CDCl₃): δ 202.1 (d), 64.4 (d), 52.9 (t), 25.7 (q, 3C), 24.1 (t), 17.8 (q), -5.4 (q, 2C).

4.4.3 Synthesis of methyl 12-bromo dodecanoate (95)

Bromo-ester 95 is prepared by Fisher esterification reaction, performed preparing a solution of 4 mmol 12-hydroxy dodecanoic acid in 20 ml of methanol and acidified with 10 drops of sulfuric acid 96%.
It leads to reflux at 70° C for 20 hours. After we performed an extraction with diethyl ether and water. Ethereal phases were combined, anhydridied on Na2SO4 and concentrated under vacuum. The reaction is almost quantitative, but the ester 17 was purified on silica and eluted with petroleum ether/diethyl ether 95/5.

methyl 12-bromo dodecanoate (95)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.47 (23H, s), 3.23 (1H, t, $J = 6.9$ Hz), 2.11 (2H, t, $J = 7.5$ Hz), 1.67 (2H, m), 1.43 (2H, m), 1.24 (2H, m), 1.11 (12H, m).

4.4.4 Synthesis of phosphonium salt of methyl 12 bromo dodecanoate (96)

A mixture containing 3mmol of 95, 3mmol of triphenylphosphine and 10ml of CH3CN was placed under reflux, at 90°C for 72h. After the system went at room temperature, solution was concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/ diethyl ether 97/3.

Phosphonium salt 96 was obtained in a yield of 90%.

Phosphonium salt of methyl 12 bromo dodecanoate (96)

$^1$H-NMR (CDCl$_3$, 250 MHz): $\delta$ 7.68-7.58 (15H, m), 4.57 (3H, s), 2.17 (2H, t, $J = 7.4$ Hz), 1.49 (6H, m), 1.09 (12H, m).
4.4.5 Synthesis of compound 100

1.5 mmol of 96 were placed in an anhydrided flask and washed 3-4 times with toluene dry. Toluene was then removed in vacuo and flask was maintained under N₂.

5 ml of THF dry were added to phosphonium salt at 0°C stirring for some minutes. At this temperature 1.5 mmol of a solution of TMSA-K (0.5 M in toluene) were diluted with 5 ml of THF dry, are added to reaction flask; then the system was maintained at room temperature for 30 minutes. Then, a solution of 1.2 mmol of aldehyde 99 diluted with 3 ml of THF dry was added at 0°C, drowsy. After 2 h, reaction was stopped by acidification with 2 ml of HCl 1.0 M; than solution was extracted with ethyl ether/H₂O. Ether phase was anidryfed on Na₂SO₄, concentrated in vacuo and purified by silica gel chromatography, eluting with petroleum ether/ethyl ether 99/1.

Wittig product 100 was obtained in a yield of 60%.

**Compound 100**

¹H-NMR (CDCl₃, 250 MHz): δ 5.38 (2H, m), 3.78 (1H, m), 3.65 (3H, s), 2.29 (1H, t, J= 7.4 Hz), 2.18 (2H, m), 2.06 (2H, m), 1.60 (2H, m), 1.26 (14H, m), 1.11 (1H, d, J= 6.1 Hz), 0.87 (9H, s), 0.03 (6H, s).
4.4.6 Synthesis of (Z)-methyl 15-hydroxyhexadec-12-enoate (101)

The removal of protecting group t-BDMSi was directly executed by making, in a plastic container, a mixture of 0.6 mmol of 100 with 6 ml of HF 48%, and 20 ml of CH$_3$CN. The reaction was monitored and after 3 h it was completed. HF was neutralized by adding NaHCO$_3$ until the end of effervescence. The obtained solution was extracted with ethyl acetate/H$_2$O. Organic phase was anhydrified on Na$_2$SO$_4$, concentrated in vacuo, purified by silica gel chromatography and elute with petroleum ether/ethylc ether from 85/15 to 7/3.

Yield of 101 was of 90%.

(Z)-methyl 15-hydroxyhexadec-12-enoate (101)

$^1$H-NMR (CDCl$_3$, 250 MHz): $\delta$ 5.49 (1H, m), 5.34 (1H, m), 3.77 (1H, m), 3.62 (3H, s), 2.26 (1H, t, $J$ = 7.5 Hz), 2.18-2.10 (2H, m), 2.00 (2H, m), 1.60 (2H, m), 1.22 (14H, m), 1.15 (1H, d, $J$ = 6.2 Hz).
4.4.7 Synthesis of 15-hexadec-12-enolide (102)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 · 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester 20 was added to the reaction flask and it was immersed in a bath at 40° C for 1h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with Petroleum ether/diethyl ether from 95/5 to 8/2.

41% of 102 was obtained together with unreacted alcohol.

**Compound 102**

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 5.50 (1H, m), 5.34 (1H, m), 4.94 (1H, m), 2.46 (1H, m), 2.22-1.96 (4H, m), 1.63 (2H, m), 1.32 (14H, m), 1.26 (3H, d).

ES-MS ($m/z$= 256 (M+).

In order to investigate on the enantiomeric purity of the recovered unreacted alcohol, Mosher esters were prepared following a described procedure starting from (R)-MTPA-Cl.$^{241}$ Chromatographic analysis by HPLC (ODH chiral column, hexane-isopropanol 97/3 at 1.0 ml/min and refractive index as detector) of Mosher ester derivatives of racemic and recovered unreacted alcohols are showed respectively in Fig. 42 and 43.
Figure 42
Chromatogram of ester Mosher on raceme 101.

Figure 43
Chromatogram of ester Mosher on no-lactonized residue.
4.4.7 Synthesis of 15 methyl-pentadecanolide (103)

![Diagram of 15 methyl-pentadecanolide (103)]

The double bond of 102 was saturated by catalytic hydrogenation to give 103. 0.016 mmol of 102 was anhydrified (vacuum/N2) in a flask and diluted with 1ml of ethanol. Then a little quantity of Pd/C (spatula tip) was added and maintained under pressure of hydrogen for 1 hour and stirred vigorously. At the end of time, the crude of reaction was filtered on stopper of celite, and washed with chloroform. The solution was concentrated in vacuo, purified by silica gel chromatography and eluted with Petroleum ether/diethyl ether 95/5.

Was recovered 90 % of 103

**15-methyl pentadecanolide (103)**

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 4.93-4.99 (1H, m), 2.32 (1H, ddd, $J = 6.3$ Hz, $J = 8.3$ Hz, $J = 14.5$ Hz), 2.28 (1H, ddd, $J = 6.1$ Hz, $J = 6.8$ Hz, $J = 14.5$ Hz), 1.67-1.76 (1H, m), 1.47-1.63 (1H, m), 1.25-1.42 (20H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 171.6 (s), 71.5 (d), 34.9 (t), 34.4 (t), 24.8 (t), 21.0-27.3 (t), 21.7 (t).

ES-MS ($m/z$) = 254 (M+).

IR (cm$^{-1}$): 2930, 2860, 1735, 1460, 1375,

$[\alpha]_D^{25} = -13.7$ (c = 1.0, CHCl$_3$).
4.4.8 Synthesis of \( \text{-butyl dimethyl silil ethyl butirrate (R-98)} \)

![Chemical Structure](image)

A solution was prepared by mixing 7.5 mmol of ethyl 3-(\(R\))-hydroxy butirrate, 9.9 mmol of \( \text{-BDMS-Cl} \), 9.9 mmol of imidazole and 8 ml of dichloromethane dry. The reaction goes at room temperature, stirring vigorously, in 20h.

After adding 50ml of H\(_2\)O, mixture was extracted with diethyl ether/H\(_2\)O. Ethereal phase was anhydrified on Na\(_2\)SO\(_4\), concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/ethylc ether 97/3.

98% of pure (\(R\))-98 is obtained.

4.4.9 Synthesis of \( \text{tert-butyl dimethyl silil butirraldehyde (R-99)} \)

![Chemical Structure](image)

6.5 mmol of (\(R\))-98 were diluted with 6.0 ml of CH\(_2\)Cl\(_2\) dry. Then, under N\(_2\) atmosphere, during 2h, at -78°C (CO\(_2\)/acetone bath), 6.5 mmol of DIBAL-H (1.0 M in CH\(_2\)Cl\(_2\)) diluted 1/1 with CH\(_2\)Cl\(_2\) dry, were added to solution.

After the time passed, 3.0 ml of a sature solution of Na/K tartrate were added to neutralize DIBAL-H; the mixture was extracted with ethylic ether/H\(_2\)O. Ethereal phase was anhydrified
on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography, and eluted with pentane/ethyl ether 99/1. Aldehyde \((R)-99\) was obtained in a yield of 80%.

### 4.4.10 Synthesis of compound \((R)-101\)

1.5 mmol of 96 were introduced in an anhydridied flask and washed 3–4 times with toluene dry. Toluene was then removed in vacuo and flask was maintained under N₂. 5 ml of THF dry were added to phosphonium salt at 0°C stirring for some minutes. At this temperature 1.5 mmol of a solution of TMSA-K (0.5 M in toluene) were diluted with 5 ml of THF dry, are added to reaction flask; then the system was maintained at room temperature for 30 minutes. After this time was passed, we returned system at 0°C and added, drop to drop, a solution of 1.0 mmol of aldehyde \((R)-99\) diluted with 3 ml of THF dry. After 2h, reaction was stopped by acidification with 2 ml of HCl 1.0 M; than solution was extracted with diethyl ether/H₂O. After purification, Wittig product was obtained.

t-BDMSi was removed by making, in a plastic container, a mixture of 0.6 mmol of Wittig product with 6 ml of HF 48%, and 20 ml of CH₃CN. The reaction was monitored and after 3h it was completed. HF was neutralized by adding NaHCO₃ until the end of effervescence. The obtained solution was extracted with ethyl acetate/H₂O. Organic phase was anhydridied on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/diethyl ether from 85/15 to 7/3. Product \((R)-101\) was obtained in a yield of 50%.
4.4.11 Synthesis of 15 (R) hexadec-12-enolide (102)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 · 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester (R) 101 was added to the reaction flask and it was immersed in a bath at 40° C for 1h.

After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with Petroleum ether/diethyl ether from 95/5 to 8/2. 80 % of 102 was obtained.

15 (R) hexadecenolide (102)

1H-NMR (CDCl₃, 400 MHz): δ 5.50 (1H, m), 5.34 (1H, m), 4.94 (1H, m), 2.46 (1H, m), 2.22g 1.96 (4H, m), 1.63 (2H, m), 1.32 (14H, m), 1.26 (3H, d).

MS (m/z)= 256 (M+).
4.4.12 Synthesis of 15 (R) hexadecanolid (103)

The double bond of 102 was saturated by catalytic hydrogenation to give 103.

0.016 mmol of 102 was anhydrified (vacuum/N2) in a flask and diluted with 1ml of ethanol. Then a little quantity of Pd/C (spatula tip) was added and maintained under pressure of hydrogen for 1 hour and stirred vigorously. At the end of time, the crude of reaction was filtered on stopper of celite, and washed with chloroform. The solution was concentrated in vacuo, purified by silica gel chromatography and eluted with Petroleum ether/diethyl ether 95/5.

Was recovered 90 % of 103.

(R) hexadecanolid (103)

1H-NMR (CDCl3, 400 MHz): \( \delta \ 4.93-4.99 \) (1H, m), 2.32 (1H, ddd, \( J = 6.3 \ Hz, J = 8.3 \ Hz, J = 14.5 \ Hz \)), 2.28 (1H, ddd, \( J = 6.1 \ Hz, J = 6.8 \ Hz, J = 14.5 \ Hz \)), 1.67-1.76 (1H, m), 1.47-1.63 (1H, m), 1.25-1.42 (20H, m), 1.22 (30H, d, \( J = 6.3 \ Hz \)).

13C-NMR (100MHz CDCl3): \( \delta \ 171.6 \) (s), 71.5 (d), 34.9 (t), 34.4 (t), 24.8 (t), 21.0-27.3 (t), 21.7 (t).

MS (m/z)= 254 (M+).

IR (cm\(^{-1}\)): 2930, 2860, 1735, 1460, 1375, 1340, 1260, 1205, 1180, 1130, 1110, 790

\( [\alpha]_D^{25} = -14.8 \ (c = 1.0, CHCl_3) \).
4.4.13 Synthesis of $t$-butyl dimethyl silil ethyl butirrate ($S$-98)

Alcohol group was protected by reaction with $t$-BDMS-Cl. A solution was prepared by mixing 7.5 mmol of ethyl 3-$(R)$-hydroxy butirrate, 9.9 mmol of $t$-BDMS-Cl, 9.9 mmol of imidazole and 8 ml of dichloromethane dry. The reaction goes at room temperature, stirring vigorously, in 20h. After adding 50 ml of H$_2$O, mixture was extracted with diethyl ether/H$_2$O. Ethereal phase was anhydrified on Na$_2$SO$_4$, concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/ethylc ether 97/3. 98% of pure ($S$)-98 is obtained.

4.4.14 Synthesis of $tert$-butyl dimethyl silil butirraldehyde ($S$-99)

6.5 mmol of ($S$)-98 were diluted with 6.0 ml of CH$_2$Cl$_2$ dry. Then, under N$_2$ atmosphere, during 2h, at -78°C (CO$_2$/acetone bath), 6.5 mmol of DIBAL-H (1.0 M in CH$_2$Cl$_2$) diluted 1/1 with CH$_2$Cl$_2$ dry, were added to solution. After the time passed, 3.0 ml of a sature solution of Na/K tartrate were added to neutralize DIBAL-H; the mixture was extracted with ethylic ether/H$_2$O. Ethereal phase was anhydrified.
on Na$_2$SO$_4$, concentrated in vacuo, purified by silica gel chromatography, and eluted with pentane/ethyl ether 99/1.
Aldehyde (S)-99 was obtained in a yield of 80%.

4.4.15 Synthesis of compound (S)-101

\[ \text{(S)-101} \]

1.5 mmol of 96 were introduced in an anhydridified flask and washed 3–4 times with toluene dry. Toluene was then removed in vacuo and flask was maintained under N$_2$. 5 ml of THF dry were added to phosphonium salt at 0°C stirring for some minutes. At this temperature 1.5 mmol of a solution of TMSA-K (0.5 M in toluene) were diluted with 5 ml of THF dry, are added to reaction flask; then the system was maintained at room temperature for 30 minutes. After this time was passed, we returned system at 0°C and added, drop to drop, a solution of 1.0 mmol of aldehyde (S)-99 diluted with 3 ml of THF dry. After 2 h, reaction was stopped by acidification with 2 ml of HCl 1.0 M; than solution was extracted with diethyl ether/H$_2$O. After purification, Wittig product was obtained.
t-BDMSi was removed by making, in a plastic container, a mixture of 0.6 mmol of Wittig product with 6 ml of HF 48%, and 20 ml of CH$_3$CN. The reaction was monitored and after 3 h it was completed. HF was neutralized by adding NaHCO$_3$ until the end of effervescence. The obtained solution was extracted with ethyl acetate/H$_2$O. Organic phase was anhydridified on Na$_2$SO$_4$, concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/diethyl ether from 85/15 to 7/3.
Wittig product (S)-101 was obtained in a yield of 50%.
4.4.15 Synthesis of 15 (S) hexadecenolide (102)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na₂HPO₄ · 2H₂O/7H₂O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester (S)-101 was added to the reaction flask and it was immersed in a bath at 40° C monitoring up to 24h. Product 102 wasn’t formed.
4.5 Synthesis of 15-octadec-12-enolide

4.5.1 Synthesis of \( t \)-butyl dimethyl silil methyl hexanoate (108)

![Chemical Structure](image)

Alcohol group was protected by reaction with t-BDMS-Cl.

A solution was prepared by mixing 13 mmol of methyl 3 hydroxy hexanoate, 16 mmol of \( t \)-BDMS-Cl, 16 mmol of imidazole and 14 ml of dichloromethane dry. The reaction goes at room temperature, stirring vigorously, in 20h.

After adding 50 ml of \( \text{H}_2\text{O} \), mixture was extracted with diethyl ether/\( \text{H}_2\text{O} \). Ether phase was anhydrified on \( \text{Na}_2\text{SO}_4 \), concentrated in vacuo, purified by silica gel chromatography and eluted with hexane/diethyl ether 95/5.

90% of pure 108 is obtained.

\( t \)-butyl dimethyl silil ethyl hexanoate (108)

\( ^1 \text{H-NMR (CDCl}_3, \text{ 400 MHz): } \delta \begin{align*} 
4.12 (1\text{H, m}), 
3.65 (3\text{H, m}), 
2.43 (2\text{H, dd, } J= 1.9 \text{ and } 6.8 \text{ Hz}), 
1.48-1.36 (2\text{H, m}), 
1.34-1.26 (2\text{H, m}), 
0.92 (3\text{H, t}), 
0.87 (9\text{H, s}), 
0.03 (6\text{H, s}).
\end{align*} \)

\( ^13 \text{C-NMR (62.89 MHz CDCl}_3): \delta \begin{align*} 
173.2 (s), 
69.2 (d), 
51.3 (t), 
42.4 (t), 
39.7 (t), 
25.5 (q, 3\text{C}), 
18.1 (q), 
14.0 (q), 
-5.4 (q, 2\text{C}).
\end{align*} \)
4.5.2 Synthesis of \( \tau \)-butyl dimethyl silil butirraldehyde (109)

\[
\begin{align*}
\text{Si} & \quad \text{O} \\
\text{O} & \quad \text{H}
\end{align*}
\]

6.1 \text{mmol} of 108 were diluted with 6 \text{ml} of CH\(_2\)Cl\(_2\) dry. Then, under N\(_2\) atmosphere, during 2h, at \(-78^\circ\text{C} (\text{CO}_2/\text{acetone bath})\), 6.1 \text{mmol} of DIBAL-H (1.0 \text{M} in CH\(_2\)Cl\(_2\)) diluted 1/1 with CH\(_2\)Cl\(_2\) dry, were added to solution. Subsequently, 3 \text{ml} of a saturated solution of Na/K tartrate were added to neutralize DIBAL-H; the mixture was extracted with diethyl ether/H\(_2\)O. Ether phase was anhydridied on Na\(_2\)SO\(_4\), concentrated in vacuo, purified by silica gel chromatography and elute with petroleum ether/diethyl ether 99/1. Aldehyde 109 was obtained in a yield of 80%.

\( \tau \)-butyl dimethyl silil butirraldehyde (109)

\(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta\) 9.79 (1H, br s), 4.16 (1H, m), 3.45 (2H, m), 2.48 (2H, m), 1.25 (2H, m), 1.18 (3H, d, \(J=6.1\ Hz\)), 0.86 (9H, s), 0.07 (3H, s).

\(^13\)C-NMR (62.89 MHz CDCl\(_3\)): \(\delta\) 203.6 (d), 69.3 (d), 52.0 (t), 41.3 (t), 26.79 (q, 3C), 19.6 (t), 15.3 (q), -5.4 (q, 2C).
4.5.3 Synthesis of compound 110

**110**

4.4 mmol of 106 were placed in an anhydrided flask and washed 3–4 times with toluene dry. Toluene was then removed in vacuo and flask was maintained under N₂.

15 ml of THF dry were added to phosphonium salt at 0°C stirring for some minutes. At this temperature 4.4 mmol of a solution of TMSA-K (0.5 M in toluene) were diluted with 10 ml of THF dry, are added to reaction flask; then the system was maintained at room temperature for 30 minutes.

Then, a solution of 4.1 mmol of aldehyde 109 diluted with 10 ml of THF dry was added, dropwise at 0°C. After 2 h, reaction was stopped by acidification with 10 ml of HCl 1.0 M; then solution was extracted with diethyl ether/H₂O. Ether phase was anhydrided on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography and eluted with petroleum ether/diethyl ether 99/1.

Wittig product 110 was obtained in a yield of 60%.

**Compound 110**

¹H-NMR (CDCl₃, 400 MHz): δ 5.47 (1H, m), δ 5.35 (1H, m), 3.61 (3H, s), 2.25 (2H, t, J= 7.6 Hz), 2.17 (2H, t, J= 6.6 Hz), 1.57 (2H, m), 1.39 (2H, m), 1.22 (16H, m), 0.87 (9H, s), 0.03 (6H, s).
4.5.4 Synthesis of compound (Z)-methyl 15-hydroxyocta dec-12-enoate (111)

\[ \text{OH} \]
\[ \text{RS} \]
\[ \text{O} \]
\[ \text{O} \]

111

The removal of protecting group t-BDMSi was directly executed by making, in a plastic container, a mixture of 1.2 mmol of 110 with 12 ml of HF 48%, and 40 ml of CH₃CN. The reaction was monitored and after 3h it was completed. HF was neutralized by adding NaHCO₃ until the end of effervescence.

The obtained solution was extracted with ethyl acetate/H₂O. Organic phase was anhydrified on Na₂SO₄, concentrated in vacuo, purified by silica gel chromatography and elute with petroleum ether/diethyl ether from 95/5 to 8/2.

Yield of 111 was of 90%.

(Z)-methyl 15-hydroxyocta dec-12-enoate (111).

\[^1\text{H-NMR} \text{ (CDCl}_3, 400 MHz): \delta \text{ 5.47 (1H, m), } \delta \text{ 5.35 (1H, m), } 3.62 \text{ (3H, s), } 2.25 \text{ (2H, t, } J= 7.6 \text{ Hz), } 2.17 \text{ (2H, t, } J= 6.6 \text{ Hz), } 2.0 \text{ (1H, bs), } 1.57 \text{ (2H, m), } 1.39 \text{ (2H, m), } 1.22 \text{ (16H, m).}\]
4.5.4 Synthesis of 15-octadec-12-enolide (112)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na₂HPO₄ · 2H₂O/7H₂O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxy-ester 111 was added to the reaction flask and it was immersed in a bath at 65°C for 24h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane/diethyl ether from 95/5 to 1/1.

28% of 112 was obtained.

15-octadec-12-enolide (112)

$^1$H-NMR (CDCl₃, 400 MHz): $\delta$ 5.51 (1H, m), 5.36 (1H, m), 4.95 (1H, m), 2.88 (2H, m), 2.30-2.22 (4H, m), 2.02 (2H, m), 1.59 (2H, m), 1.55 (2H, m), 1.37 (14H, t), 0.91 (3H, t, $J$= 7.3 Hz).

$^{13}$C-NMR (100MHz CDCl₃): $\delta$ 132.7 (s), 124.52 (d), 73.4 (d), 35.7 (t), 32.3 (t), 34.3 (t), 32.5 (t), 27.7 (t), 26.6 (t), 26.5 (t), 26.3 (t), 26.2 (t), 26.1 (t), 26.0 (t), 25.8 (t), 24.4 (t), 18.4 (t), 13.7 (q).

MS (m/z) = 280 (M+).
4.6 Synthesis of aplyiolides B and D

4.6.1 Synthesis of 8-Bromo-6-octyn-3,4-diol (115)

![Chemical Structure of 115](image)

A mixture is prepared with 33.5mmol cesium carbonate, 33.5mmol of sodium iodide, 33.5mol of cuprous iodide, 60ml of N,N-dimethylformamide, 33.5mmol of propargyl alcohol and 33.5mmol of (E)-1-bromo-pent-2-ene.

After 20 hours, we added a saturated aqueous solution of ammonium chloride and we extracted with diethyl ether. The combined organic phases were anhydried on Na2SO4 and concentrated under vacuum. The crude was purified rapidly by DMF on silica gel with petroleum ether. Alcohol mixture of the two isomers, 113 and 113*, was diluted with anhydrous CH2Cl2; after 55mmol of carbon tetrabromide were added to it. To this solution at a temperature of 0° C was added dropwise a solution consisting of 55mmol of triphenylphosphine in dichloromethane. We kept stirring and monitoring by TLC; then we pulled 90% of dry CH2Cl2, obtaining a dense mixture that was loaded on silica. Then we eluted with petroleum ether rapidly achieving the blend of only the bromides 114 and 114*.

20ml of t-butanol, 20ml of water and 5.0g of AD-mix-α™ were placed in a flask. The agitation at room temperature forms a biphasic system of light colored; so we added the 3.6mmol of metansulfonammide. The mixture was cooled to 0° C to dissolve precipitated salts. Then the mixture of olefins were slowly added and left stirring for 20 hours at 0° C, monitoring the course of the reaction by TLC. While mixture was still at 0° C, 2.5g of Na2SO3 were added, the reactor was brought to room temperature, stirring for 1 hour. Ethyl acetate was added and the aqueous phase was extracted, anhydrified and purified on flash silica eluting with acetate / hexane. Product 115 was obtained with a total yield of 60% in three steps. The bromodiol was a white solid.
8-Bromo-6-octyn-3,4-diol (115)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.92 (2H, t), 3.65 (1H, dddd, $J= 6.4$ Hz, $J= 5.9$ Hz, $J= 4.7$ Hz, $J= 5.7$ Hz), 3.52 (1H, ddd, $J= 4.8$ Hz, $J= 4.7$ Hz, $J= 5.6$ Hz), 2.50-2.56 (2H, ddt, $J= 5.9$ Hz, $J= 2.2$ Hz, $J= 17.0$ Hz), 2.28 (1H, d, $J= 5.7$ Hz), 1.99 (1H, d, $J= 5.6$ Hz), 1.60 (1H, ddq, $J= 4.8$ Hz, $J= 7.4$ Hz, $J= 14.2$ Hz), 1.51 (1H, dq, $J= 7.4$ Hz, $J= 14.2$ Hz), 1.00 (1H, t, $J= 7.4$ Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 84.0 (s), 77.6 (s), 74.5 (d), 71.8 (d), 26.3 (t), 24.5 (t), 15.2 (t), 10.0 (q).

4.6.2 Synthesis of 9 decyn-1-ol (116)

46ml of DAP were added to 34mmol of NaH. The mixture was stirred at 80° C. There was evolution of hydrogen and one hour after the solution became clear dark for the formation of aminopropilammid. At room temperature we added a solution of 4.2mmol 3-Decin-1-ol in 16ml of DAP and left overnight at 70° C. After cooling, water was added and extracted several times with diethyl ether. The combined ethereal phases were washed with water, diluted HCl and a solution of NaCl, then anhydrided on Na$_2$SO$_4$. Filtration and subsequent concentration leaded to 4mmol of 116 already pure. The yield is 95%.

9 decyn-1-ol (116)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.64 (2H, t, $J= 6.3$ Hz), 2.18 (2H, dt, $J= 7.2$ Hz, $J= 2.7$ Hz), 2.94 (1H, t, $J= 2.7$ Hz), 1.54 (4H, m), 1.42-1.30 (8H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 84.3 (s), 68.0(d), 62.1 (t), 32.3 (t), 28.1-29.0 (t), 25.4 (t), 18.0 (t).
**4.6.3 Synthesis of 9 decynoic acid (117)**

9-decynoic acid was obtained by oxidation of alcohol function of 116 with Jones reagent. This was prepared dissolving from 2.14g of CrO₃ in 6.2ml of water which were added, at 0° C, 1.84ml of concentrated H₂SO₄. It was left to stir for 1 h, then diluted with 60ml of acetone. 8mmol of alcohol were diluted in acetone (60ml) and, at 0°C, Jones reagent was added dropwise over 4 hours. Green color of solution showed Cr (III); color turned red in the first excess of Cr (VI). Addition of the oxidizer was suspended to the first red finish. Isopropanol was added slowly to reduce the excess of Cr (VI) until the solution turns green; the resulting mixture was filtered on Celite and washed with acetone. Solution was concentrated and diluted with dichloromethane, extracted the carboxylate with a solution in 1M NaOH. Then solution was acidified with 8-9ml of concentrated sulfuric acid to pH 1-2 and extracted with dichloromethane. At the end, solution was anhydrified on Na₂SO₄ and concentrated. It is not necessary purification: yield 95%.

**9 decynoic acid (117)**

¹H-NMR (CDCl₃, 400 MHz): δ 2.34 (2H, t, J = 7.4 Hz), 2.18 (2H, dt, J = 7.2 Hz, J = 2.7 Hz), 1.93 (1H, t, J = 2.7 Hz), 1.62 (2H, tt, J = 7.4 Hz, J = 7.2 Hz), 1.50 (2H, tt, J = 7.5 Hz, J = 7.2 Hz) 1.44-1.29 (6H, m).

¹³C-NMR (100MHz CDCl₃): δ 180.0 (s), 84.6 (s), 68.1(t), 34.0 (t), 28.4-28.9 (t), 24.5 (t), 18.3 (t).
4.6.4 Synthesis of Methyl 9-decynoate (118)

To a solution consisting of 3.8mmol of 9-decinoic acid 117 and 20ml methanol were added a few drops of sulfuric acid 96%. After 15 hours reflux at 80° C, the reaction mixture is extracted with pentane and the combined organic phases were anhydrified on sodium sulfate and concentrated under vacuum. The yield is quantitative.

Methyl 9-decynoate (118)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.66 (3H, s), 2.28 (2H, t, $J= 7.5$ Hz), 2.17 (2H, dt, $J= 7.1$ Hz, $J= 2.7$ Hz), 1.60 (2H, tt, $J= 7.5$ Hz, $J= 7.2$ Hz), 1.48 (2H, tt, $J= 7.4$ Hz, $J= 7.1$ Hz) 1.44-1.29 (6H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.0 (s), 84.4 (s), 68.0(t), 51.2 (q), 33.8 (t), 28.2-28.8 (t), 24.7 (t), 18.2 (t).

MS (m/z): 182 (M+)
4.6.5 Synthesis of methyl 15(S),16(S)-dihydroxy-octadeca-9,12-dynoate (119)

To a suspension consisting of 3.6mmol of cesium carbonate, 3.6mmol sodium iodide, cuprous iodide 3.6mmol in 10ml of DMF, were added 3.6mmol of 118, then 3.6mmol of 115, leaving under stirring for 20 hours. After adding a saturated solution of ammonium chloride, it was extracted with diethyl ether, anhydrified and concentrated under vacuum. The crude was purified on silica gel, eluenting with petroleum ether / diethyl ether (from 9 / 1 to 1 / 1). 3mmol of 119 are obtained (85%).

Methyl 15(S),16(S)-dihydroxy-octadeca-9,12-dynoate (119)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.66 (3H, s), 3.60 (1H, ddd, $J = 4.3$ Hz, $J = 5.3$ Hz, $J = 6.4$ Hz), 3.50 (1H, ddd, $J = 4.3$ Hz, $J = 8.3$ Hz, $J = 4.6$ Hz), 3.12 (2H, tt, $J = 2.3$ Hz, $J = 2.3$ Hz), 2.41-2.46 (2H, ddt, $J = 2.3$ Hz, $J = 5.3$ Hz, $J = 6.4$ Hz, $J = 16.7$ Hz), 2.30 (2H, tt, $J = 7.5$ Hz), 2.13 (2H, tt, $J = 7.0$ Hz, $J = 2.3$ Hz), 1.62 (2H, bt, $J = 7.5$ Hz), 1.52-1.58 (2H, ddq, $J = 7.4$ Hz, $J = 8.3$ Hz, $J = 4.6$ Hz, $J = 14.1$ Hz), 1.47 (2H, bt, $J = 7.0$ Hz), 1.40-1.24 (6H, m), 0.98 (3H, bt, $J = 7.4$ Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.4 (s), 74.5 (d), 74.0-80.8 (s), 71.9 (d), 51.4 (s), 34.0 (t), 28.5-29.0 (t), 26.4 (t), 24.8 (t), 24.4 (t), 18.6 (t), 10.0 (t), 9.7 (t).

MS (m/z): 323 (M+)

$\alpha^E_{D} = + 0.5$ (c = 1.7 in CHCl$_3$).
4.6.6 Synthesis of methyl 15(S),16(S)-dihydroxyoctadeca-9(Z),12(Z)-dyenoate (120)

To a solution of 2.6 mmol of 119 in 10 ml of methanol was added a catalytic amount of Lindlar and a few drops of quinoline. We left react under mild hydrogen pressure for a night; the reaction monitored by TLC, was practically quantitative. It was purified on silica only to remove the quinoline and the catalyst, eluting with hexane / ethyl acetate 8:2.

**Methyl 15(S),16(S)-dihydroxyoctadeca-9(Z),12(Z)-dyenoate (120)**

\[\text{H-NMR (CDCl}_3, 400 \text{ MHz): } \delta \ 5.54 (1\text{H, bdt, } J = 10.7 \text{ Hz, } J = 6.6 \text{ Hz}), \delta \ 5.40 (1\text{H, dt, } J = 10.7 \text{ Hz, } J = 6.0 \text{ Hz}), \delta \ 5.35 (1\text{H, bdt, } J = 10.7 \text{ Hz, } J = 6.5 \text{ Hz}), \delta \ 3.66 (3\text{H, s}), \delta \ 3.51 (1\text{H, dt, } J = 4.9 \text{ Hz, } J = 6.8 \text{ Hz}), \delta \ 3.40 (1\text{H, ddd, } J = 8.8 \text{ Hz, } J = 8.3 \text{ Hz, } J = 4.9 \text{ Hz}), \delta \ 2.81 (2\text{H, dd, } J = 6.6 \text{ Hz, } J = 6.5 \text{ Hz}), \delta \ 2.33 (2\text{H, bdd, } J = 7.5 \text{ Hz, } J = 6.8 \text{ Hz}), \delta \ 2.30 (2\text{H, t, } J = 7.5 \text{ Hz}), \delta \ 2.06 (2\text{H, bdt, } J = 6.0 \text{ Hz, } J = 6.7 \text{ Hz}), \delta \ 1.62 (2\text{H, bt, } J = 7.5 \text{ Hz}), \delta \ 1.61 (1\text{H, m}), \delta \ 1.51 (1\text{H, ddq, } J = 8.3 \text{ Hz, } J = 7.5 \text{ Hz, } J = 15.0 \text{ Hz}), \delta \ 1.36-1.28 (8\text{H, m}), \delta \ 0.99 (3\text{H, t, } J = 7.5 \text{ Hz}).

\[\text{C-NMR (100MHz CDCl}_3): \delta \ 174.4 (s), 131.6 (t), 130.6 (d), 127.3 (d), 125.1 (d), 75.2 (d), 73.4 (d), 71.9 (t), 51.5 (q), 34.1 (t), 31.8 (t), 29.1-29.5 (t), 26.5 (t), 25.8 (d), 24.9 (t), 10.0 (q).

**MS (m/z):** 326 (M+)

**IR (cm\(^{-1}\)):** 3400; 1737
4.6.7 Synthesis of aplyolide B/D

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na₂HPO₄ · 2H₂O/7H₂O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of dihydroxy-ester 120 was added to the reaction flask and it was immersed in a bath at 40° C until 48h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. The total yield is of 37% of 27 (with traces of 26).

Aplyolide D (27)

\[ \delta \] 5.51 (1H, m), 5.38 (1H, m), 4.96 (1H, m), 3.57 (1H, m), 2.90-2.76 (2H, m), 2.51-2.43 (2H, m), 2.38-2.31 (2H, ddd, J= 15.9 Hz, J= 9.0 Hz, J= 4.1 Hz), 2.15-1.95 (2H, m), 1.73-1.62 (2H, m), 1.50 (2H, m), 1.34 (8H, m), 0.99 (3H, t, J= 7.5 Hz).

\[ ^{13}\text{C-NMR (100MHz CDCl}_3\text{):} \delta \]
173.4 (s), 131.8 (d), 129.9-127.6 (d), 124.6 (d), 75.4 (d), 73.6 (d), 34.1 (t), 33.8 (t), 28.0-27.1 (t), 26.9 (t), 25.9 (t), 25.7 (t), 24.5 (t), 10.0 (q).

MS (m/z): 294 (M+)

IR (cm⁻¹): 1730

\[ \alpha_{D}^{24} = +22.0 \pm 0.1 \text{ (c=0.7 in CHCl}_3\text{)} \]
4.7 Synthesis of enantiomers of aplyolides B and D

Enantiomers of aplyolides B and D were prepared following a similar synthetic scheme but using AD-\textit{mix-β}™ for the Sharpless asymmetric dihydroxylation. At the end, obtained methyl 15(R),16(R)-dihydroxyctadeca-9(Z),12(Z)-dyenoate (123) was submitted to enzymatic macrolactonization.

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na$_2$HPO$_4$ · 2H$_2$O/7H$_2$O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of dihydroxy-ester 123 was added to the reaction flask and it was immersed in a bath at 40°C for 3h.

After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. Enantiomer of aplyolide B (124) was obtained in yield of 81%.
Enantiomer of aplyolide B (124)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 5.62 (1H, m), 5.41 (1H, m), 5.37 (2H, m), 4.83 (1H, m), 3.71 (1H, m), 2.85-2.78 (2H, ddd, $J$= 16.0 Hz, $J$= 7.0 Hz, $J$= 7.0 Hz), 2.47-2.38 (2H, ddd, $J$= 15.3 Hz, $J$= 4.9 Hz, $J$= 7.0 Hz), 2.31-2.18 (2H, ddd, $J$= 14.7 Hz, $J$= 9.0 Hz, $J$= 9.0 Hz), 2.04-1.97 (2H, m), 1.77-1.65 (2H, m), 1.76 (2H, m), 1.37 (8H, m), 0.92 (3H, t, $J$= 7.5 Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 173.4 (s), 131.8 (d), 130.4 (d), 127.7 (d), 124.9 (d), 76.7 (t), 71.3 (d), 34.1 (t), 32.5 (t), 28.0-27.3 (t), 26.2 (t), 26.0 (t), 24.9 (t), 20.4 (t), 10.0 (q).

MS ($m/z$): 294 (M+)

IR ($cm^{-1}$): 1720

$[\alpha]_D^{24} = + 16.0 \pm 0.1$ (c=0.8 in CHCl$_3$)
4.8 Synthesis of aplyiolides C and E

4.8.1 Synthesis of 4(E)-epten-1-ynil-trimethylsilane (128)

![Diagram of 4(E)-epten-1-ynil-trimethylsilane](image)

It was prepared a mixture of 33.6mmol of cesium carbonate, 33.6mmol of sodium iodide, 33.6mmol cuprous iodide and 70ml of N, N-dimethylformamide; to this were added 33.6mmol of trimethylsilyl-acetylene and 33.6mmol of (E)-1-bromo-pent-2-ene. A saturated aqueous solution of ammonium chloride was added after 20 hours. At the end, it was extracted with diethyl ether. The combined organic phases were anhydried on sodium sulphate, concentrated and purified on silica gel eluting with petroleum ether / diethyl ether (95 / 5). It was obtained 17mmol of 128 (yield ~ 60%) mixed with 128* (~ 30%), in turn 1:1 enantiomeric mixture. Isomers are not separable: their relationship were derived by integration of NMR signals.

**4(E)-epten-1-ynil-trimethylsilane (128)**

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 5.71 (1H, ddt, $J= 15.2$ Hz, $J= 6.4$ Hz, $J= 1.7$ Hz), 5.38 (1H, ddt, $J= 15.2$ Hz, $J= 5.7$ Hz, $J= 1.7$ Hz), 2.95 (2H, ddt, $J= 1.2$ Hz, $J= 5.7$ Hz, $J= 1.7$ Hz), 2.04 (2H, bdt, $J= 1.2$ Hz, $J= 6.4$ Hz, $J= 1.7$ Hz, $J= 7.5$ Hz), 0.99 (3H, t, $J= 7.5$ Hz), 0.16 (9H, s).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 133.7 (s), 122.6 (d), 104.5 (q), 85.8 (t), 25.2 (d), 21.5 (d), 13.4 (s), 0.04 (q).

128*
Compound 128*

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 5.75 (1H, ddd, $J= 17.1$ Hz, $J= 10.0$ Hz, $J= 6.0$ Hz), 5.29 (1H, ddd, $J= 17.1$ Hz, $J= 1.6$ Hz, $J= 1.3$ Hz), 5.08 (1H, ddd, $J= 1.3$ Hz, $J= 10.0$ Hz, $J= 1.3$ Hz), 3.02 (1H, bddd, $J= 7.5$ Hz, $J= 1.6$ Hz, $J= 6.0$ Hz, $J= 1.3$ Hz), 1.61 (1H, ddq, $J= 7.5$ Hz, $J= 7.4$ Hz, $J= 13.0$ Hz), 1.55 (1H, ddq, $J= 7.4$ Hz, $J= 13.3$ Hz, $J= 6.0$ Hz), 0.98 (3H, t, $J= 7.4$ Hz), 0.13 (9H, s).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 137.4 (d), 115.1 (d), 107.1 (s), 87.0 (s), 37.9 (d), 28.3 (d), 11.1 (q), 0.03 (q).
4.8.2 Synthesis of 7-trimethylsilanil-3(\(S\))-4(\(S\))dihydroxy-6-eptyn (129)

18ml of t-butanol, 18ml of water and 5.0g of AD-mix-\(\alpha^{\text{TM}}\) were placed into a flask. The agitation at room temperature forms a biphasic system of light colored; so we added the 5mmol of metansulfonammide. The mixture was cooled to 0\(^{\circ}\) C to dissolve precipitated salts. Then the mixture of olefins were slowly added and left stirring for 20 hours at 0\(^{\circ}\) C, monitoring the course of the reaction by TLC. While mixture was still at 0\(^{\circ}\) C, 7.5g of Na2SO\(_3\) were added, the reactor was brought to room temperature, stirring for 1 hour. Ethyl acetate was added and the aqueous phase was extracted, anhydrified and purified on flash silica eluting with acetate / hexane 4/6. Product 129 was obtained with a yield of 95% (~60% from bromopentene). The diol is a solid stable, white and low melting point.

7-trimethylsilanil-3(\(S\))-4(\(S\))dihydroxy-6-eptyn (129)

\(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta\) 3.60 (1H, ddd, \(J = 5.7\) Hz, \(J = 6.4\) Hz, \(J = 4.5\) Hz), 3.48 (1H, ddd, \(J = 4.8\) Hz, \(J = 8.3\) Hz, \(J = 4.5\) Hz), 2.49 (1H, dd, \(J = 5.7\) Hz, \(J = 17.5\) Hz), 2.46 (1H, dd, \(J = 6.4\) Hz, \(J = 17.5\) Hz), 1.55 (1H, ddq, \(J = 4.8\) Hz, \(J = 7.4\) Hz, \(J = 17.5\) Hz), 1.50 (1H, ddq, \(J = 8.3\) Hz, \(J = 7.4\) Hz, \(J = 21.5\) Hz), 0.96 (3H, t, \(J = 7.5\) Hz), 0.13 (9H, s).

\(^{13}\)C-NMR (100MHz CDCl\(_3\)): \(\delta\) 103.1 (s), 87.0 (s), 74.3 (d), 71.6 (d), 26.2 (t), 25.3 (t), 9.9 (q), 0.13 (q).

\([\alpha]_D^{20} = + (1.30 \pm 0.01), c = 5.2\) in CHCl\(_3\).
4.8.3 Synthesis of $3(S),4(S)$-dihydroxy-6-epyn (130)

To a solution prepared with 6.4mmol of 129 in 10ml of THF dry were slowly added 8mmol of TBAF. Deprotection was monitored by TLC: it was over in an hour. After we added ethyl acetate, we extracted several times with water until elimination of fluoride salts. Chromatographic purification is not necessary. The yield is almost quantitative.

$3(S),4(S)$-dihydroxy-6-epyn (130)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.65 (1H, ddd, $J = 5.7$ Hz, $J = 5.6$ Hz, $J = 4.2$ Hz), 3.58 (1H, ddd, $J = 4.7$ Hz, $J = 8.2$ Hz, $J = 4.2$ Hz), 2.50 (1H, ddd, $J = 5.5$ Hz, $J = 2.7$ Hz, $J = 17.0$ Hz), 2.46 (1H, ddd, $J = 6.5$ Hz, $J = 2.7$ Hz, $J = 17.0$ Hz), 1.59 (1H, ddq, $J = 7.5$ Hz, $J = 4.7$ Hz, $J = 14.8$ Hz), 1.52 (1H, ddq, $J = 8.2$ Hz, $J = 7.5$ Hz, $J = 14.8$ Hz), 1.00 (3H, t, $J = 7.5$ Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 80.7 (s), 74.3 (d), 71.6 (d), 70.6 (d), 26.2 (t), 23.9 (t), 9.9 (q).
4.8.4 Synthesis of methyl 6-epynoate (132)

To a solution consisting of 5g of 6-epynoic acid and 25ml of methanol was added dropwise 1 ml of sulfuric acid 96%.
After 15 hours reflux at 80°C, methanol was removed under vacuum and an extraction with pentane was performed; the combined organic phases were anhydritied on sodium sulphate and concentrated under vacuum. **132** was obtained in yield of 100%. The ester was presented as a yellow liquid, stable over time.

**methyl 6-epynoate (132)**

\[^1\text{H-NMR (CDCl}_3, 400 MHz): \delta \ 3.67 (3H, s), 2.34 (2H, t, J= 7.5 Hz), 2.20 (2H, dt, J= 7.0 Hz, J= 2.4 Hz), 1.95 (1H, t), 1.75 (2H, m), 1.56 (2H, m).\]

\[^{13}\text{C-NMR (100MHz CDCl}_3): \delta \ 171.3 (s), 83.5 (s), 68.4 (d), 51.1 (q), 33.1 (t), 27.6 (t), 23.6 (t), 17.8 (t), 9.9 (q).\]
4.8.5 Synthesis of methyl 11-bromo-undeca-6,9-dynoate (133)

To a suspension consisting of 10mmol of cesium carbonate, 10mmol of sodium iodide, 10mmol of cuprous iodide and 20ml of N, N-dimethylformamide (DMF) in a nitrogen atmosphere at room temperature, were added 10mmol of 132 and 10mmol of 4-chloro-2-butin-1-ol. After 20 hours a saturated aqueous solution of ammonium chloride was added and it was extracted with diethyl ether. The combined organic phases were anhydried on sodium sulfate and concentrated under vacuum. The crude reaction was rapidly purified on silica gel with an eluent consisting of a mixture of ethyl ether / petroleum ether 1/1.

Then, 9.0mmol of obtained compound and 15.3mmol of carbon tetrabromide were diluted with 35ml of anhydrous dichloromethane; a solution composed of 15.2mmol of triphenylphosphine in 30ml of dichloromethane was added dropwise at 0° C. After a few minutes, at the first excess of PPh3, the reaction mixture turns from yellow to brown. It was held under agitation for 30-60min, then it was concentrated, obtaining a dense mixture that was purified on silica, eluting 8/2 mixture of pentane and diethyl ether. Usually you can separate the CHBr3 early fractions (and UV-visible) phosphine salts, insoluble in the eluent mixture, remaining on the column. Compound 133 was synthesized in a yield of 85%.

Bromide obtained was a rather thick orange oil; it tended to decompose more slowly than starting alcohol if stored under nitrogen in ether solution.

Methyl 11-bromo-undeca-6,9-dynoate (133)

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.93 (2H, t, $J = 2.3$ Hz), 3.67 (3H, s), 3.21 (2H, tt, $J = 2.3$ Hz), 2.34 (2H, t, $J = 7.3$ Hz), 2.19 (2H, tt, $J = 7.1$ Hz, $J = 2.3$ Hz), 1.75 (2H, m), 1.56 (2H, m).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.0 (s), 80.4-73.1 (s), 81.7-75.1 (s), 51.3 (q), 33.3 (t), 27.7 (t), 23.9 (t), 18.2 (t), 14.6 (t), 9.8 (t).
4.8.6 Synthesis of methyl 15(S),16(S)dihydroxy-octadeca-6,9,12-trynoate (134)

To a suspension consisting of 4mmol of cesium carbonate, 4mmol of sodium iodide, 4mmol of cuprous iodide in 10ml of DMF, we added 4mmol of 133. It was left under stirring for 20 minutes then, 4mmol of 54 were added, leaving under stirring for 20 hours. After adding a saturated solution of ammonium chloride, it was extracted with diethyl ether, and anhydrified and concentrated under vacuum. The crude reaction was purified on silica gel with an eluent consisting of a mixture of petroleum ether and diethyl ether (from 9/1 to 1/1). We obtained 3.6mmol of 134 (90%).

**Methyl 15(S),16(S)dihydroxy-octadeca-6,9,12-trynoate (134)**

$^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.67 (3H, s), 3.60 (1H, bdd, $J_1=5.7$ Hz, $J_2=6.5$ Hz), 3.50 (1H, m), 3.15 (2H, bdd, $J_1=2.1$ Hz, $J_2=2.7$ Hz, $J_3=2.7$ Hz), 3.12 (2H, tt, $J_1=2.1$ Hz, $J_2=2.1$ Hz), 2.46 (2H, ddt, $J_1=5.7$ Hz, $J_2=2.7$ Hz, $J_3=16.9$ Hz), 2.42 (2H, ddt, $J_1=2.7$ Hz, $J_2=6.5$ Hz, $J_3=16.9$ Hz), 2.31 (2H, t, $J=7.5$ Hz), 2.18 (2H, tt, $J_1=7.1$ Hz, $J_2=2.1$ Hz), 1.72 (2H, m), 1.54 (4H, m), 1.00 (3H, t, $J=7.4$ Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): $\delta$ 174.2 (s), 79.5-73.8 (s), 73.4 (d), 71.6 (d), 50.8 (q), 32.9 (t), 27.5 (t), 25.9 (t), 23.6 (t), 23.2 (d), 17.8 (t), 9.7 (q), 9.1 (t).

MS (m/z): 318 (M+)
4.8.7 Synthesis of methyl 15(S),16(S)-dihydroxyoctadeca-6(Z),9(Z),12(Z)-tryenoate (135)

To a solution of 3.6mmol of 134 in 10ml of methanol was added a catalytic amount of Pd/BaSO₄ and a few drops of quinoline. We left react under mild hydrogen pressure for a night: the reaction monitored by TLC, was practically quantitative. It was purified on silica only to remove the quinoline and the catalyst, eluting with hexane / ethyl acetate 8:2.

Methyl 15(S),16(S)-dihydroxyoctadeca-6(Z),9(Z),12(Z)-tryenoate (135)

1H-NMR (CDCl₃, 400 MHz): δ 5.56 (1H, bdt, J= 10.7 Hz, J= 6.4 Hz), δ 5.46 (1H, bdt, J= 10.7 Hz, J= 7.3 Hz), 5.42-5.32 (4H, m), 3.66 (3H, s), 3.50 (1H, bdt, J= 5.3 Hz, J= 4.6 Hz), 3.39 (1H, bdd, J= 6.6 Hz, J= 4.6 Hz), 2.85 (2H, dd, J= 6.4 Hz, J= 6.0 Hz), 2.80 (2H, dd, J= 5.3 Hz, J= 5.2 Hz), 2.33 (2H, bdd, J= 7.5 Hz, J= 6.8 Hz), 2.31 (2H, t, J= 7.4 Hz), 2.06 (2H, bdt, J= 5.6 Hz, J= 7.5 Hz), 1.64 (2H, m), 1.61 (1H, m), 1.59-1.51 (2H, m), 1.39 (2H, bt, J= 7.5 Hz), 0.99 (3H, t, J= 7.4 Hz).

13C-NMR (100MHz CDCl₃): δ 174.2 (s), 131.6 (d), 129.5-127.6 (d), 125.4 (d), 75.0 (d), 73.3 (d), 33.8 (q), 31.6 (t), 288 (t), 26.3 (t), 25.6 (t), 25.5 (t), 24.4 (t), 9.9 (q), 5.2 (q).

MS (m/z): 324 (M+)

\[ [\alpha]_D^{20} = -(1.1 \pm 0.2), \text{ c = 12 in CHCl}_3 \]
4.8.8 Enzymatic lactonization of methyl 15(S),16(S)-dihydroxyoctadeca-6(Z),9(Z),12(Z)-tryenoate (135)

![Aplyolide C (28)](image)

![Aplyolide E (29)](image)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 · 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of dihydroxy-ester 135 was added to the reaction flask and it was immersed in a bath at 40° C for 48h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. 39% of aphyolide E (29) was obtained.

**Aplyolide E (29)**

1H-NMR (CDCl3, 400 MHz): δ 5.56 (1H, m), 5.47 (1H, m), 5.39 (2H, m), 5.33 (2H, m), 4.96 (1H, m), 3.57 (1H, m), 2.89-2.77 (2H, m), 2.58-2.29 (2H, m), 2.41-2.32 (2H, ddd, J= 15.4 Hz, J= 7.3 Hz, J= 6.6 Hz), 2.20-1.98 (2H, m), 1.73-1.63 (2H, m), 1.45 (8H, m), 1.42 (2H, m), 0.99 (3H, t, J= 7.5 Hz).

13C-NMR (100MHz CDCl3): δ 173.2 (s), 131.7-125.0 (d), 128.5 (d), 128.4 (d), 128.1 (d), 75.4 (d), 74.2 (d), 33.8 (t), 28.0 (t), 26.7 (t), 26.5 (t), 25.7 (t), 25.5 (t), 24.2 (t), 10.0 (q).

MS (m/z): 292 (M+)

IR (cm⁻¹): 1730

\[\alpha\]D = +40.4 ± 0.3 (c=0.4 in CHCl₃)
4.9 Synthesis of enantiomers of aplyiolides C and E

Again, we performed Sharpless asymmetric dihydroxylation reaction using the AD-\textit{mix-\textbeta TM} to obtain the enantiomers aplyiolides C and E. The obtained Methyl 15(\textit{R}),16(\textit{R})-dihydroxyoctadeca-6(\textit{Z}),9(\textit{Z}),12(\textit{Z})-tryenoate (138) was then submitted to enzymatic lactonization.

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 · 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of dihydroxy-ester 138 was added to the reaction flask and it was immersed in a bath at 40° C for 3h.
After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. 81% of enantiomer of aplyolide C (139) was obtained.

**Enantiomer of aplyolide C (139)**

$^1$H-NMR (CDCl$_3$, 400 MHz): \( \delta \) 5.64 (1H, m), 5.50 (1H, m), 5.39 (1H, m), 5.37 (1H, m), 5.34 (2H, m), 4.80 (1H, m), 3.72 (1H, m), 2.86-2.75 (2H, m), 2.48-2.34 (2H, ddd, \( J = 15.0 \) Hz, \( J = 7.5 \) Hz, \( J = 7.3 \) Hz), 2.32-2.16 (2H, m), 2.06-2.01 (2H, m), 1.80-1.67 (2H, m), 1.74 (2H, m), 1.46 (2H, m), 0.92 (3H, t, \( J = 7.5 \) Hz).

$^{13}$C-NMR (100MHz CDCl$_3$): \( \delta \) 173.4 (s), 131.8 (d), 130.4 (d), 127.7 (d), 124.9 (d), 76.7 (t), 71.3 (d), 34.1 (t), 32.5 (t), 28.0-27.3 (t), 26.2 (t), 26.0 (t), 24.9 (t), 20.4 (t), 10.0 (q).

MS (m/z): 292 (M+)

IR (cm$^{-1}$): 1720

\([\alpha]_D^{20} = + (20.7 \pm 0.2) \) (c=0.4 in CHCl$_3$)
4.10 Lactonization of methyl 15 \( (R) \),16\( (R) \) –dihydroxy-octadecanoate

4.10.1 Synthesis of methyl 15\( (R) \),16\( (R) \)-dihydroxyoctadecanoate (141)

To a solution of 3.6mmol of 138 in 10ml of methanol was added a catalytic amount of Pd/BaSO\(_4\) and a few drops of quinoline. We left react under mild hydrogen pressure for a night: the reaction monitored by TLC, was practically quantitative. It was purified on silica only to remove the quinoline and the catalyst, eluting with hexane / ethyl acetate 8:2.

Methyl 15\( (R) \),16\( (R) \)-dihydroxyoctadecanoate (141)

\(^1\)H-NMR (CDCl\(_3\), 400 MHz): \( \delta \) 3.66 (3H, s), 3.41 (1H, m), 3.33 (1H, m), 2.29 (2H, t, \( J = 7.6 \) Hz), 1.97 (1H, t, \( J = 6.8 \) Hz), 1.60 (4H, m), 1.45 (4H, m), 1.27 (20H, m), 0.99 (3H, t, \( J = 7.4 \) Hz).

\(^{13}\)C-NMR (100MHz CDCl\(_3\)): \( \delta \) 173.1 (s), 78.8 (d), 76.3 (d), 51.9 (q), 33.6 (t), 31.7 (t), 30.0 (t), 29.6 (t, 6C), 29.4 (t), 29.1 (t), 25.1 (t), 24.5 (t), 8.2 (q).
4.10.2 Enzymatic lactonization of methyl 15(R),16(R)-dihydroxyoctadecanoate (141)

A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na₂HPO₄ · 2H₂O/7H₂O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of dihydroxy-ester 141 was added to the reaction flask and it was immersed in a bath at 40°C for 24h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. 65% of 142 was obtained.

**Compound 142**

¹H-NMR (CDCl₃, 400 MHz): δ 4.03 (1H, m), 3.80 (1H, m), 2.26 (2H, m), 1.68 (2H, m), 1.57 (2H, m), 1.32-1.29 (22H, m), 0.99 (3H, t, J = 7.4 Hz).

¹³C-NMR (100MHz CDCl₃): δ 173.4 (s), 76.7 (d), 73.6 (d), 34.7 (t), 31.2 (t), 27.4 (t), 25.2 (t), 21.2 (t), 9.1 (q).
4.11 Synthesis of aleuritic lactone

\[
\text{HO} \quad \text{OH} \quad \text{OCH}_3
\]

\[
\text{O} \quad \text{145}
\]

was prepared by Fisher esterification reaction, performed preparing a solution of 2mmol erythro aleirtic acid in 10ml of methanol and acidified with 5 drops of sulfuric acid 96%.

It leads to reflux at 70° C for 20 hours. After you perform an extraction with diethyl ether and water. Ethereal phases were combined, anhydrided on Na2SO4 and concentrated under vacuum. The reaction is almost quantitative, but the ester 1 is purified on silica, eluting with petroleum ether/diethyl ether 7/3.

\[
\text{A solution of 50mg of enzyme (CALB Immobilized on acrylic resin) in 40ml of cyclohexane was prepared. Reaction flask was placed in a chamber containing a saturated solution of salt hydrates (Na2HPO4 · 2H2O/7H2O) and it is left in indirectly contact, under vigorous agitation, for 24h. After this time, 0.04mmol of hydroxyester 1 was added to the reaction flask and it was immersed in a bath at 40° C for 1h. After the reaction, the enzyme was removed from the solution by filtration and concentrated under vacuum. The crude was purified on silica and eluted with hexane / chloroform 6 / 4. We obtained 48% of lactone (145).}
\]

**Aleuritic lactone (145)**

\(^1\text{H-NMR (CDCl}_3, 400 \text{ MHz)}: 3.47 (2\text{H, m}), 4.13 (2\text{H, t, J= 4.5 Hz}), 2.32 (2\text{H, t, J= 6.1 Hz}), 1.81 (2\text{H, -OH}), 1.63 (4\text{H, m}), 1.39 (20\text{H, m}).
\]

\(^13\text{C-NMR (100MHz CDCl}_3): \delta 173.4 (s), 75.7 (t), 77.2 (2), 67.0 (t), 71.6 (d), 51.3 (q), 34.4 (t), 31.0 (t), 29.9 (t), 27.4 (t), 26.8 (t), 25.5 (7), 23.6 (t), 21.5 (t).
REFERENCES


