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CHEMOENZYMATIC APPROACHES TO THE PREPARATION OF BIOLOGICALLY ACTIVE COMPOUNDS

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ABSTRACT

Biologically active compounds are usually polyfunctional molecules bearing one or more stereocenters. Enzyme catalyzed transformations are well suitable for their preparation since they act in a very mild and selective manner.

The study of three selected biocatalyzed transformations has been the aim of my PhD researches.

The first project, regards the development of an advantageous and mild method to selectively transform the 24-hydroxyl function of ***ascomycin***, a 23-membered macrolactam, bearing two secondary alcoholic moieties bounded at position 24 and 32 of its macrocycle. This selective protection is required to transform ascomycin into pimecrolimus, its 32-epi chlorine analogue, an immunomodulator useful for the treatment of inflammatory skin diseases. The advantage offered by enzymes, showed to be not only their ability to regioselective discriminate between the two secondary hydroxyl groups, but also, their chemoselectivity, *i.e.* the capacity to leave intact the other functional groups present in the structure of ascomycin. Among enzymes to screen for the desired transformation we selected lipases, taking into account their well know regioselectivity in the acylation of secondary alcohols and their efficiency to transform lipofilic compounds.

The second topic regards the ***clopidogrel***, a thienopyridine compound that inhibits platelet aggregation by selectively binding to adenylate cyclase-coupled ADP receptors. It is indicated for the reduction of atherosclerotic events. Its structure contains a stereocenter: since only the S*(+)* isomer is biologically active, the compound must be prepared in the enantiomerically pure form. Although it is known and used by several years, usually this target is achieved by means of time consuming and expensive fractional crystallization of the unnatural aminoacid easily recognizable in its structure, 2-chloro-phenylglycine. The aim of my work was to find an enzymatic approach to achieve the suitable resolution with a low cost and a good optical purity overcoming the disadvantages of fractional crystallization. Different kinds of hydrolases were screened on several amides and esters derivatives of 2-chloro-phenylglycine or of a more advanced intermediate of the preparation.

The third project, realized in Canada at Université Laval, deals with the resolution of the enantiomers of ***erhytro-p-nitrophenylserine***, a non proteogenic aminoacid bearing the opposite stereochemistry of the antibiotic chloramphenicol, the D(-)-*threo*-2-dichloroacetamido-p-nitrophenylserinol. The obtained optically pure aminoacids, by means of penicillin acylase, after their reduction to aminoalcohols were then N-acylated with the proper chiral L-methioninesulfone. The diastereoisomeric amides were tested as inhibitors for amidotransferase, an enzyme absent in humans and present in some archea, organella and bacterials, and then an ideal target for new antibacterial compounds.

The analytical methods employed to verify the outcomes of reactions are ^{1}H and ^{13}C, COSY and HSQC NMR and mass spectroscopy; the selectivity and the conversion of the enzymatic resolution were checked by means of reverse phase and chiral HPLC. The configuration of the final compounds was also proved by specific optical rotatory values.

1. Introduction

1.1 The discovery of a new science.

The enzymes (from greek, ἐν ζύμῳ [*en zýmō*], *inside the yeast*) are very specialized proteins that catalyze the reactions of the cellular metabolism.
The knowledge of enzymatic reactions is well before the XX century, despite the fact that the chemical processes catalyzed by agents of biological origin were understood only vaguely. Reactions such as fermentation of sugar solutions, the acidification and processing of milk into cheese, the putrefaction of urine were certainly observed by chance as accidental occurrences, but the use of grafts from previous fermentations as catalysts is probably very old . The concept of 'fermentation' is found in the Bible: “A little yeast leavens the whole lump” (*1st Letter at Corinthians*, **5**, 6). In addition to wine, whose production depends only by enzymes naturally present in the skin of grapes, even beer (or similar fermented beverage derived from barley) has been described by many classic authors, including Xenophon, Herodotus, Pliny and Tacitus. The Latin word fermentum, used by Virgil for example, refers to anything that causes a reboot and then also with beer or yeast itself.
Anyway, the basic concept of enzymology, *i.e.* catalysis of chemical reactions by agents of biological origin, began to consolidate in only seconds XIX century.

The idea of catalysis was formulated for the first time by the Swedish chemist J. J. Berzelius, such as "a force different from the forces that we know so far". In the third edition of his chemistry text published in 1837, he wrote: "This is a force likely to be more widespread than was previously thought and whose there is still hidden nature[...]. The catalytic ability seems to be that some bodies, only by virtue of their presence, not their similarities, can awaken dormant similarities at that temperature, and as a result, elements of a complex body settle down to a new order" (Berzelius, *Lehrbuch der Chemie*, vol. VI, 18373 Dresden-Leipzig, pp. 19-25). These chemical interpretations arrived at the right time, because several cases of biological catalysis were described in the previous 50 years. In 1783 L. Spallanzani, an Italian physiologist, showed that the flesh was melted by the gastric juice of crows, in 1830 R. Robiquet and A.-F. Boutron-Charard discovered the hydrolysis of amygdalin by bitter almonds, and in the same year A.-P. Dubrunfaut showed that an extract of malt starch turned into sugar. In an article, that for many it is the birth of enzymology, A. Payen and J. Persoz (in 1833) described the precipitation with alcohol,

from malt extract, of a substance that could be stored in the dry state while maintaining the ability to hydrolyze starch and which they called 'diastasis'. The sinigrinase and pepsin were described shortly thereafter. [1, 2]

The half century following was characterized by the dispute over the possibility that some of these biological catalysis depended, to some extent, on the presence of living organisms. The great work of L. Pasteur proved (on the basis of earlier contributions by T. Schwann and C. Cagniard de Latour) that yeast was a living thing and that many fermentations, both aerobic and anaerobic, were caused by living organisms. Despite their importance for overall development of biology, these results encouraged a trend 'vitalistic' in the study of fermentation. Pasteur made a distinction between 'soluble or unorganized ferments', such as diastase and pepsin, and 'organized ferments', such as yeast, as L. Traube suggested the hypothesis that the “organized ferment” properties belonged to the soluble enzymes present in them. On the other hand, Liebig and other chemists ridiculed the idea that all chemical reactions depend in some way by the presence of life: in a satirical article published anonymously by Liebig in 1839, they did mock the idea that the yeast cells had " a sucker facial for eat sugar, alcohol jet coming out anus, while carbon dioxide bubbling out enormous genitals " (Das Geheimnis der enträtselte geistigen Gärung in" Liebig's Annalen der Chemie, 1839, XXIX, pp. 100-104). The demonstration, given by E. Buchner (1897), that a juice-free extract from yeast cells could still ferment sugar, revealed that Pasteur and Liebig were both right, each from its own point of view. Yeast is made up of real organisms, but the chemical processes of fermentation are caused by a system of non-living catalysts produced in yeast cells. The term 'enzyme' was suggested by W. Kühne in 1878 to indicate the presence of this catalyst 'in yeast but he intended to apply also to more complex organisms which, he said, "are not so fundamentally different from single-celled organisms like many would have us believe." The word 'enzyme' replaced soon (except in German) the more ambiguous 'ferment'. [3]

After resolution of the dispute between Pasteur and Liebig, the XX century opened with the scene ready for the exceptional development of enzymology.

Following the demonstration of existence of enzymes independently of a living cell, the research focused on the chemical nature of enzymes themselves. Numerous evidences showed the close association between protein and enzyme activity, but an influential part of the scientific community of the early twentieth century (including the Nobel Laureate Richard Willstätter) argued that proteins were not only the transport of enzymes. The

scientist said that the enzymes were composed of one part colloidal protein called apoenzyme or apofermentum, and an active group called coenzyme or cofermentum. The cofermentum determines the specificity of the enzyme. In 1926, however, James Sumner showed how the enzyme urease was a protein itself crystallized and in 1937 he showed the same for catalase. However, were the work of Northrop and Stanley on the digestive enzymes pepsin, chymotrypsin and trypsin to definitively confirm the hypothesis of Sumner. The three researchers were awarded the Nobel Prize in 1946 . [4]

The discovery that the enzymes were crystallizable gave way to a tense race to define the three-dimensional structures of enzymes through techniques such as X-ray crystallography. The first macromolecule to be defined with this technique was lysozyme, the enzyme that digests the bacterial cell wall and contents in tears, saliva and in the album. The crystallization of lysozyme was carried out by the group coordinated by David Chilton Phillips in 1965 and this marked the beginning of structural biology. [5]

The XX century was characterized by the development of purified enzyme preparations to employ as catalysts in organic synthesis.

1.2 Enzymes as catalysts

Enzymes are proteins that acts as catalysts in the reactions of the living. Generally they are globular proteins but not all the catalysts are proteins: some RNA, called ribozymes, are also endowed with catalytic activity. Anyway the difference between the enzymes and the other catalysts is their specificity of substrate due to the presence, in their tridimensional structure of the *active site*, the portion of the macromolecule directly involved in the catalysis. In order to analyze the basic principles of the enzymes action, is essential to know the evolution of a reaction from a thermodynamic point of view. In 1935, Eyring proposed that for a reaction to proceed the reactants molecules must overcome a free energy barrier: once the reactants have reached this state of highest free energy, the so called *transition state*, they proceed into products at a fixed rate.
The free energy contains both enthalpic and entropic terms. In general, the lower is the activation energy, the faster the overall reaction will proceed.
The transition-state teory is summaryzed into the Eyring equation (eq 1)

$K = (\mathbf{k}T/h) \exp(-\Delta G/RT)$ (1)

In which K, **k**, h and R are the rate, Boltzmann, Planck and gas constants, respectively, ΔG and T represent the activation energy and the temperature for the reaction.
Since ΔG is correlated to ΔH and ΔS, the enthalpy and entropy of activation, by equation 2, equation 1 can be rearranged to equation 3:

$\Delta G = \Delta H - T\Delta S$ (2)

$K = (\mathbf{k}T/h) \exp(-\Delta H/RT) \exp(\Delta S/R)$ (3)

One role of enzymes can be considered to be the reduction in the free energy of activation by stabilizing the rate limiting transition state and this reduction in ΔG results in an acceleration in reaction rate. Enzymes accomplish this reduction by either reducing the entalpy of activation (ΔH), setting up more favorable interaction between substrates (an entropy effect, ΔS), or by modifying interactions with solvent, or all of these.

Many reactions of enzymes follow a pattern of kinetic behavior known as Michaelis-Menten kinetics.
They ideally subdivided the multistage reaction process of enzymatic catalysis in two phases: in the first one, the substrate (S) initially is bound noncovalently to the enzyme at a specific pocket called active site. This complex (ES), called Michaelis complex, provide the proper alignment of reactants and catalytic groups in the active site that is the place where the chemical step take place.
Because each molecule of enzyme has only a limited number of active sites, usually one, the number of substrate molecules that can be processed per unit of time is limited.
In the second step the reaction happens and the product (P) is released by the active site of the enzyme, who can be recovered unchanged at the end of the reaction (E).

$$\mathbf{E + S} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} \mathbf{ES^*} \xrightarrow{K_2} \mathbf{E + P}$$

After an enzyme (E) is mixed with a large excess of substrate (S) and before equilibrium is reached, the reactive intermediates have different concentrations than they do at equilibrium. This short time interval is called the pre-steady state. Once the concentrations of the intermediates have reached equilibrium, the system is considered to be in the steady state. This steady state is the period in which the concentration of the reactive intermediates change slowly and this conditions are called as steady state conditions. Steady state rates are a good approximation of conditions used in synthesis and steady state rates are measured because these data are easy to collect (as compared to the most pre-steady state rates) and generate the most reliable and relevant enzymatic constants.

As showed in the eq. 4 and represented in the figure 1.1, the inizial rate v_0, for low concentration of substrate S, is directly proportional to the concentration of enzyme and substrate (S). As the concentration of the substrate increases, a point will be reached where further increase in the substrate concentration does not further increase v.

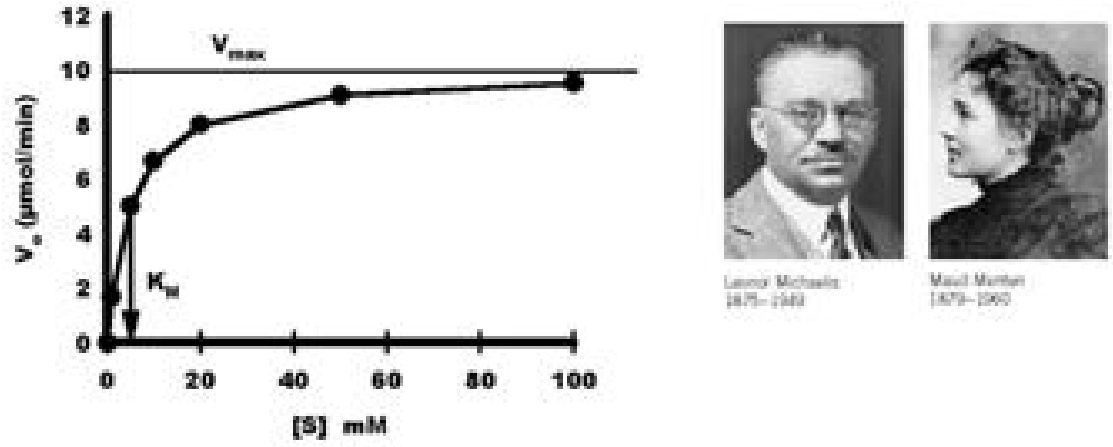


Figure 1.1. Relationship between the initial rate and substrate concentration in the Michaelis-Menthen kinetics.

$$vo = \frac{\text{kcat [E]0 [S]}}{\text{Km+[S]}} \qquad \text{(eq. 4)}$$

$$K_{cat}\,[E]_0 = v_{max} \qquad \text{(eq. 5)}$$

$$v = (k_{cat}/K_m)[E]_0[S] \qquad \text{(eq. 6)}$$

In this equation k_m represents the concentration of substrate at which $v = v_{max}/2$ and k_{cat} is the apparent first-order enzyme rate constant for the conversion of the enzyme-substrate complex to product, also called the turnover number.
At high concentration of substrate, equation 4 simplifies to eq. 5. Correspondingly, at low concentration of substrate, equation 4 simplifies to equation 6.

By applying the Michaelis-Menten kinetics, the measured reaction rates or velocities (v) can be transformed into rate constants that describe the enzymatic mode of action.
Useful costants, such as k_{cat}, k_m, k_{cat}/k_m, can be easily determined. Very important to compare the efficiency and selectivity of two enzymes is the value of k_{cat}/k_m, that, for the above system is equal to $k_1k_2/(k_{-1}+k_2)$.[6]

1.3 Nomenclature of enzymes

From zymase characterized by Buchner, all enzymes were named using the suffix-ase. Commonly, the name of the enzyme is composed by the fusion of the substrate with the suffix. For example, lactase is able to split the lactose molecule, DNA polymerase is involved in the formation of polymers of DNA.

The need of clear rules for defining the name of enzymes, led the International Union of Biochemistry and Molecular Biology to meet in the Enzyme Commission, established in 1955. They proposed a classification of enzymes based on EC numbers: to each enzyme is assigned a code composed of four numbers and a systematic name according to the reaction catalyzed.
On the basis of the forst EC number, all enzymes are classified into six different classes.

-EC 1 oxidoreductase: catalyze redox reactions;
-EC 2 - Transferase: catalyze the transfer of a functional group;
-EC3 hydrolase: catalyze the hydrolysis of various types of chemical bonds;
- EC 4 - lyase: catalyze the breakage of covalent bonds through hydrolysis ;
-EC 5 - Isomerases: catalyze isomerization within a molecule, catalyze the intramolecular reorganization;
- EC 6 - Ligase: catalyze the bond between two molecules through a covalent bond.[7]

1.4 Chirality and selectivity

The main reason of the wide application of enzymatic catalysis in organic synthesis is the emphasis upon their selectivity. The synthesis of optically active materials is an argument of great actuality. The well understanding of biological process and the general recognition that chirality plays a crucial role in nature and in biological properties fostered a tremendous efforts in enantioselective synthesis. Opposite enantiomers interact differently within an organisms and can display various activities, such as different flavor and smell or different pharmacological actions. Probably the most famous evidence of this latter behaviour was the theratogenic effect showed by the racemic drug thalidomide. Around 1960, its racemic administration to pregnant women caused severe birth defects in approximately 10.00 children. After this sadly famous event, the Food and Drug Administration requires that adequate information be provided to establish the effectiveness and safety of new drug: the choice of a racemic synthesis over the development of an enantiopure drug must be justified and, anyway, investigation on the bioavailability and pharmacological effects for both the single enantiomer and the racemic mixture are required (Figure 1.2).

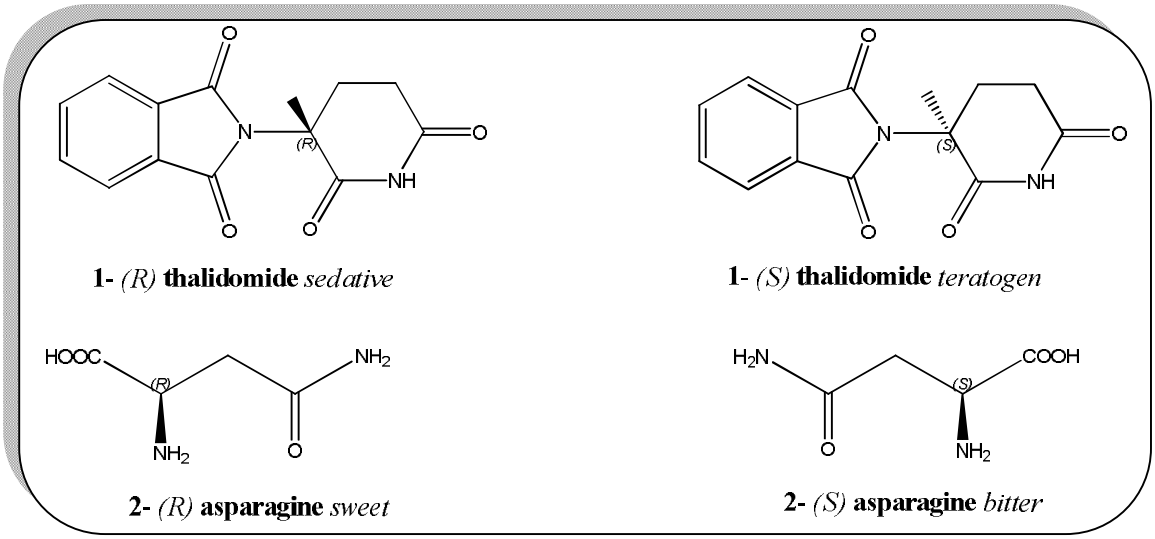


Figure 1.2. Examples of the effects of chirality in nature.

In chemical reactions selectivity take many forms: at the simplest level, the chemist is concerned with the ensuring that, as far as possible, only the desiderate functional group is transformed, leaving unreact the unwanted (*chemoselectivity*) (Figure 1.3). At the next level of control, the chemist wishes to determine at which of two or more positions of

similar reactivity, a reaction will occur (*regioselectivity*) (Figure 1.3). Perhaps the most subtle control required by the organic chemist is the stereoselectivity and, in particular, the formation of only one optical isomer (*enantioselectivity)* (Figure 1.4). Enzymes, do to the presence of the active site in quaternary structure of a protein, are able to show all these kinds of selectivity.

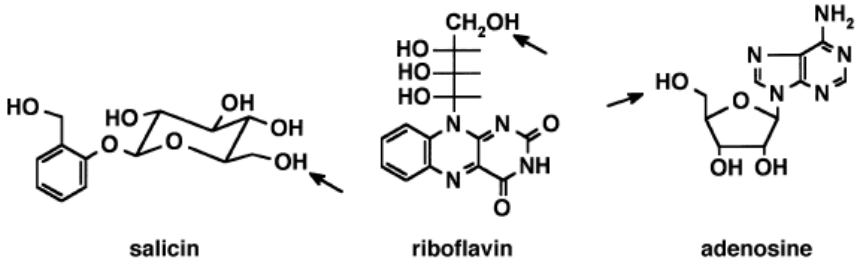


Figure 1.3 . Acylation of sugar and natural glycosides by protease subtilisin occurs at the indicate sites: example of regio- and chemoselectivity of subtilisin.

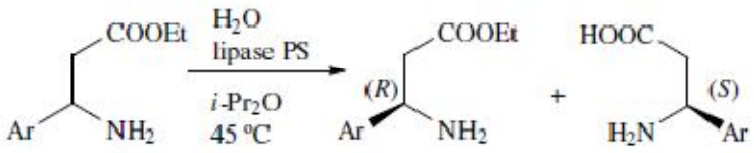
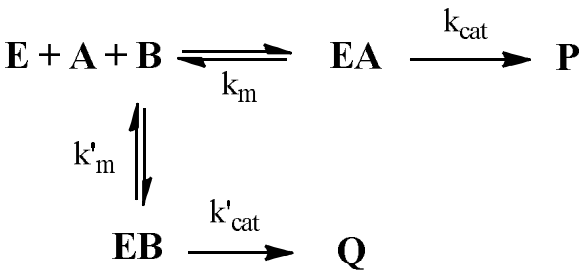


Figure 1. 4. Lipase PS-enantioselective hydrolysis of β-amino ester.

The reason of enzymes selectivity must be correlate to their substrate specificity. Obedience to Michaelis-Menten kinetics yields interesting conclusions about the specificity of enzyme-catalysed reactions. In vitro "non-specific" substrates are sometimes described as "poor" because they show a low value of k_{cat} or a high value of k_m. However, in vivo specificity results from a competition by the substrates for the active site of the enzyme. If two substrates, A and B, compete for the same enzyme different conclusion could be reached about their relative specificity if rate of reaction or the k_ms of the individual substrates are compared instead of their relative values of k_{cat}/k_m. If the enzyme catalyses the reaction of both A and B, the relevant equations may be obtained by the usual procedures (eq.11)



$$(EA) = \frac{(Eo)(A)K'm}{km\ k'm+km\ (A)+ K'm(A)} \quad \text{(eq.8)}$$

$$(EB) = \frac{(Eo)(B)K'm}{km\ k'm+km\ (B)+ K'm(B)} \quad \text{(eq.9)}$$

$$\frac{d(P)}{d(P')} = \frac{Kcat(A)\left[K'm+(B)+ K'm(A)/Km\right]}{k'cat(B)[Km+(A)+ Km(B)/K'm]} \quad \text{(eq.10)}$$

$$\frac{v}{v'} = \frac{\frac{Kcat}{Km}(A)}{\frac{k'cat}{K'm}(B)} \quad \text{(eq.11)}$$

Whether the enzyme is working below or above saturation for both substrates, the relative rate of the two reactions is given by the last equation (eq.11). Specificity between competing substrates is therefore given simply by relative values of K_{cat}/K_m and not by the individual values of K_{cat} or K_m. Specificity can apparently be reflected in poor binding (high K_s) and/or slow catalytic steps (low K_{cat}) but specificity between competing substrates is controlled only by their relative value of K_{cat}/K_m.

According to transition-state theory the second-order rate constant k_{cat}/k_m is directly related to the free-energy difference (ΔG^*) between the enzyme-substrate transition state (ES^*) and the free unbound substrate and enzyme.

$$\mathbf{E + S} \overset{\Delta G^*}{\rightleftharpoons} \mathbf{ES^*}$$

$$\frac{Kcat}{Km} = \frac{kT}{h}\exp(-\Delta G*/RT) \quad \text{(eq.12)}$$

It follows that the maximum relative rate of the enzyme-catalyzed reaction of two substrate S_1 and S_2, of the same chemical reactivity, is given by the difference in the free of binding the non- reacting parts of the substrates to the enzyme in the transition state minus the difference between their free energies in the ground state. The latter difference may be insignificant in some cases but in others, e.g. replacing a hydrophobic by an hydrophilic group, it may make the major contribution. Ground state difference could be reflected in the solubility of the two substrates.[8]

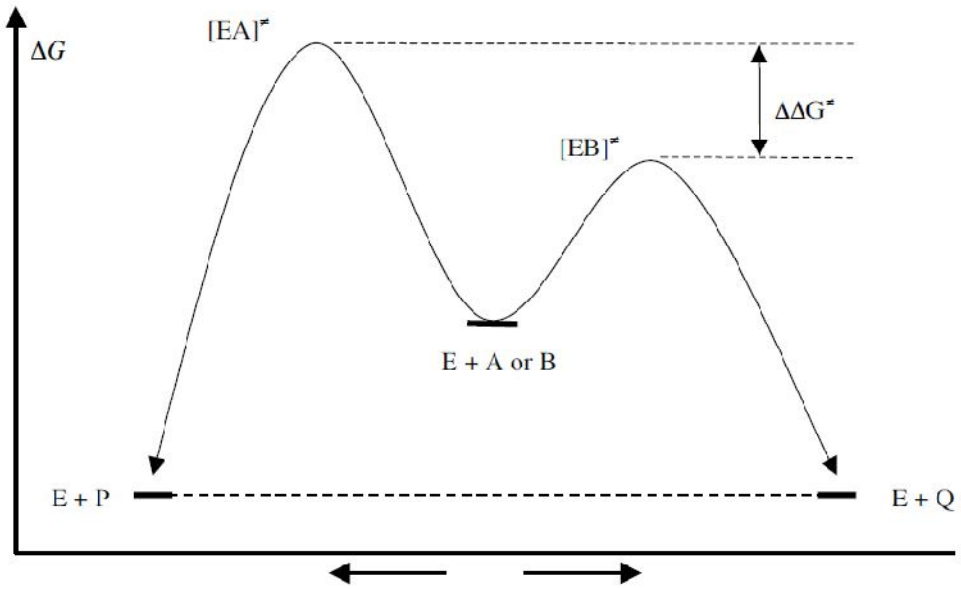


Figure 1. 5. Energy diagram for an enzyme –catalyzed reaction. E = enzyme; A and B = substrates; P and Q = products; [EA] and [EB][9] = enzyme-substrates complexes:$\Delta\Delta G^{\neq}$ = difference in free energy; $^{\neq}$ denotes a transition state.

In the specific case in which A and B are two enantiomers of a racemic mixture, whose relative configurations are *(R)* and *(S)*, the selectivity of the enzyme is always expressed as E, enantioselectivity, also called enantiomeric ratio, because is the ratio of specificity constant for the two enantiomers.(eq.12)

For an irreversible enzymatic reaction, E value can be related to the extent of conversion c, (eq.15), and to the enantiomeric excess, ee, (eq.13), a value that describes the enantiomeric purity of any compound.

$$E = \frac{(\frac{K_{cat}}{K_m})_R}{(\frac{K_{cat}}{K_m})_S} \qquad \text{(eq.12)}$$

$$\%\, ee_R = \frac{R-S}{R+S} \text{ X } 100 \qquad \text{For R > S} \qquad \text{(eq.13)}$$

$$E = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1+ee_p)]} = \frac{\ln[1-c(1+ee_s)]}{\ln[1-c(1+ee_s)]} \qquad \text{(eq.14)}$$

$$c = \frac{ee_s}{ee_s + ee_p} \qquad \text{(eq.15)}$$

1.5 Water versus organic solvents

From the first asymmetric synthesis, realized by Emil Fisher when he applied the cyanohydrin reaction to L-arabinose, the tremendous potential of enzyme has been widely discovered and improved to be more functional for they employ in organic chemistry. The most important discovery in the field of the technological utility of enzyme was probably the concept of the employment of *enzyme* in an *organic medium*. As long as the use of enzyme is restricted to their natural, aqueous reaction media, their application in the field of organic chemistry is necessarily limited by a series of consideration. Most such compounds are insoluble in water and water, often give rise to unwanted side reaction; moreover, the thermodynamic equilibria of many processes are unfavorable in water and product recovery is sometimes difficult from this medium. All these considerations suggested to add varying portion of water-miscible co-solvents to achieve and enhanced concentration of the substrate in the reaction medium, work that was pioneered by Jones and coworker in the late 1970s 1980s.[10] Soon it became clear that the presence of the organic medium was not detrimental, but, on the contrary, it can improve the enzymatic activity and, surprisingly, influence the stereoselectivity of the enzyme. The rational of these experimental results, was that, for the enzymatic activity, it is not important that the reaction happens in aqueous medium but the water bounded to the protein. Approximately, for some enzyme the maximal enzyme activity is verified when 1000 molecules of water, a monolayer, is bounded to its surface. A more rigorous way to correlate enzyme activity with the water present in the reaction medium was proposed by Halling and Goderis, who expressed the water in the medium no longer in terms of content but in terms of thermodynamic water activity (a_w):

$a_w = \chi_w \gamma_w$

χ_w = molar fraction of water.

γ_w = water activity coefficient; it increases as a fraction of solvent hydrophobicity. It follows that a given value of a_w will be obtained at a lower water concentration in a hydrophobic than in a hydrophilic medium. This essential water acts as a lubrificant providing the enzyme molecule with the flexibility necessary for the catalysis. That's for water ability to form hydrogen bonds with the functional group of the enzyme which before were bound one to each other "unlocking" the structure.[11]

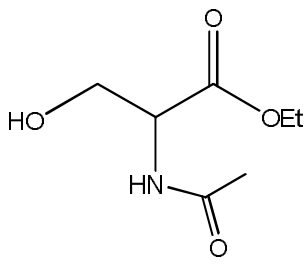
Further studies in this field, conducted by Klibanov, revealed that the stability of enzymes was superior in pure organic solvents where they lack the pliability and the conformational flexibility to denature and maintain their native conformations.

1.6 The choice of solvent and medium engeneering

Substrate selectivity is manifested in the ability of an enzyme to discriminate between two distinct, albeit structurally similar functional groups.

Besides the advantages on the stability of the enzyme, the organic medium was also advantageous in terms of selectivity. The term "medium engineering", that is the possibility to affect enzyme selectivity simply by changing the solvent in which the reaction is carried out, was coined by Klibanov, who indicated it as an alternative or an integration to protein engeneering.[12]

Concerning the most critical characteristic of the enzyme, their selectivity, there are many documented cases of switching of enzyme selectivity in dependence of the solvent. Kinetic studies carried out mostly with hydrolases have shown that enzymes in organic solvent follow conventional models, for example, Chatterije and Russel, demonstred the identity of mechanism of subtilisin catalyzed ester-hydrolysis and transesterification in organic solvents. Considering the kinetic parameters in organic solvents, the values may be very different from solvent to solvent and for water; similarly, *substrate specificity* may change dramatically. A clear example of this is offered by subtilisin, that in dichloromethane prefers, as substrate, N-acetyl-L-serine ethyl-ester, while in *tert*-butylamine it prefers N-acetyl-L-phenylalanine ethyl ester (Table 1.1).



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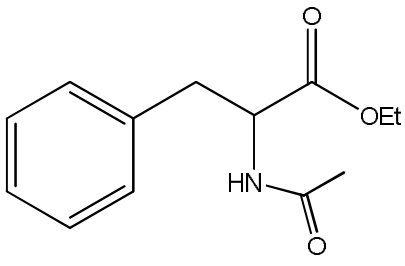


6

solvent	S	solvent	S
dichloromethane	8.2	*tert*-butyl methyl ether	2.5
chloroform	5.5	octane	2.5
toluene	4.8	isopropyl acetate	2.2
benzene	4.4	acetonitrile	1.7
N,N-dimethylformamide	4.3	dioxane	1.2
tert-butyl acetate	3.7	acetone	1.1
N-methylacetamide	3.4	pyridine	0.53
diethyl ether	3.2	*tert*-amyl alcohol	0.27
carbontetrachloride	3.2	*tert*-butyl alcohol	0.19
ethyl acetate	2.6	*tert*-butylamine	0.12

Table 1.1. Substrate specificity (S, $(K_{cat}/K_m)_{Ser}/(K_{cat}/K_m)_{Phe}$) in the transesterification of N-Ac-L-Ser-OEt (Ser) **5** and N-Ac-L-Phe-OEt (Phe) **6** with 1-propanol, catalyzed by subtilisin Carlsberg in various anhydrous organic solvents.

All these effects can be at least partly explained by the different solvation of the substrates as the reaction medium change, often based on differences in their hydrophobicity. For instance, the main driving force of enzyme-substrate binding for many proteases, such as α-chymotripsin and subtilisin, is the hydrophobic interaction between the side chain of the amino acids substrate and the active site of the enzyme. Consequently, in water, an hydrophobic substrates is more reactive than an hydrophilic one simply because this driving force is greater. This situation should change markedly, however, when an organic solvent (in which by definition there are no hydrophobic interaction) is used instead of water.

In 1986, Zaks and Klibanov reported that the substrate specificity of α-chymotripsin in octane was reversed compared to that in water.[13] Since hydrophobic interaction contributes significantly to substrate binding to chymotripsin in water, it was suggested that the reversal of substrate specificity in organic solvent was due to the lack of hydrophobic interactions in non-aqueous media.

By a practical application, the possibility to influence the selectivity of a particular enzyme by the medium of reaction regards especially the *enantiomeric* and *prochiral* stereoselectivity. One of the most famous example of this, is the asymmetric hydrolysis of dihydropyridine diesters reported by Hirose *et al.* and represented in the Figure 1.6.[14] He reported that in the *Pseudomonoas* lipase- catalyzed desymmetrization of prochiral dihydropyridine dicarboxylates the *(S)*-monoesters as high as 99% ee were obtained in water-satured *di*-isopropyl ether, whereas the *(R)*-isomers were formed preferentially (88-91% ee) in water saturated cyclohexane. Since the log P values of the solvent were similar,

it seemed unlikely that the inversion of enantioselectivity was brought about by a difference in the portioning of the substrate between the enzyme and the media. The authors attributed the change in enantioselectivity to direct interaction between the solvent and the enzyme.

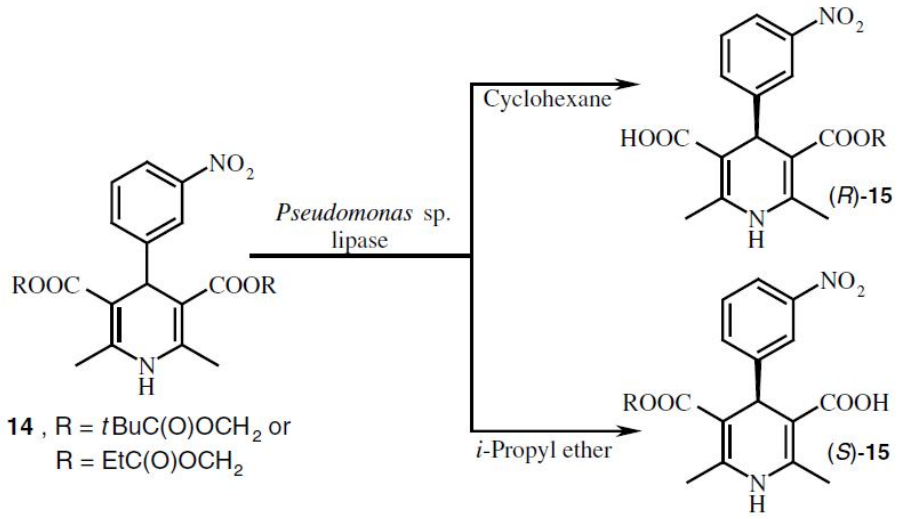


Figure 1.6. Asymmetric hydrolysis of dihydropyridine diesters depending on the solvents.

Concerning the effect of solvents on *enantioselectivity*, that represented an important aspect of my work, as an old example of kinetic resolution of racemic mixtures, mention must be made on the report of Kise an Tomiuchi on the significant effect of acetonitrile on the enantioselectivity of different proteases toward the kinetic resolution of (L)-DOPA that was obtained with 99% of enantiomeric excess in the presence of 90% of acetonitrile (Figure 1.7)[15].

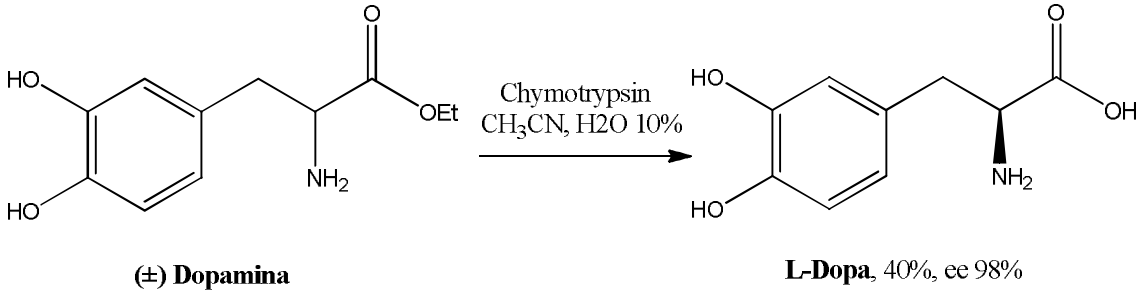


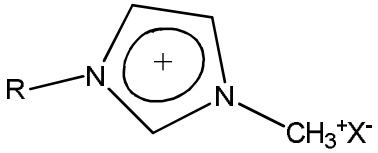
Figure 1.7. Kinetic resolution of L-Dopa by chymotripsin.

Three different hypotheses were formulated to try to rationalize the effects of the solvent on enzymatic enantioselectivity. The first suggests that the solvent, depending on its polarity, could modify the biocatalyst conformation and, thus, influence the selectivity by

altering the molecular recognition process between substrates and enzyme.[16] The second theory tries to explain the solvent effects on enantioselectivity by means of the contribution of substrate solvation to the energetic development of the reaction.[17] An example of the contribute of this substrate solvation was also claimed in the case of subtilisin-catalyzed resolution of secondary alcohols.[18] In a third model it has been proposed that solvent molecules could bind within the active site and, depending on their structure, interfere with the association or transformation of one enantiomer more than the other one and the van der Waals volume was suggested as one of the parameters that govern solvents effects on enzyme selectivity. In these works was demonstrated that the tertiary structure of enzymes was the same once the proteins had been dissolved in water or suspended in organic solvent. It was found that, both in organic solvents and in mixtures of organic solvents and water, molecules of different solvents were found to be located in different region of the active site. An example of this is offered again by subtilisin, whose active site was found to be penetrate by organic solvents with the displacement of some molecules of water.[19]

1.7 Environmentally friendly solvents: ionic liquids.

Ionic liquids can be defined as salts that do not crystallize at room temperature and their recently intensive investigation is correlated to their property to be environmental respect that include them in the green solvents. They are composed of a 1,3 dialkylimidazolium or N-alkylpyridinium cation; and a noncoordinating anion; they have no vapor pressure and, in principle, can be reused more efficiently than conventional solvents (Figure 1.8).



$R = C_nH_{2n+1}$; $X = BF_4$, PF_6, Br, NO_3

Figure 1.8. Chemical structure of the 1,3 dialkilimidazolium ionic liquids.

Their polarity is in the range of lower alcohol or formamide and their miscibility with water varies widely and unpredictably and is not strictly correlated with their polarity.[20] Even though the miscibility of ionic liquids and organic solvents is not yet well documented, generally they mix with lower alcohols and ketones, dichloromethane, and THF, whereas they do not mix with alkanes and ethers. [21] Many application of this solvents

in biocatalysis were reported in the last year, principally because their high polarity increase the solubility of polar substrates and extend the range of application of biocatalysis. Moreover, both increased enzyme stability and activity have been reported as compared to conventional organic solvents media for the lipase from *C. antarctica* and α-chymotripsyn. It has been proved that ionic liquids are able to positive influence enzymes enantioselectivity when employed both in mixture with water or in plain organic solvents. As described in chapter 3, ionic liquids-*tert*-butanol cosolvent systems markledy boosted the activity, stability and enantioselectivity of *C.antarctica* lipase B[22] and, similarly, cosolvents system consisting of ionic liquids and chloroform or *tert*-butanol increased the selectivity of the same enzyme in the resolution of racemic mandelate.[23]

1.8 Modes of using enzyme in organic media: immobilization on supports.

The introduction of organic medium for enzymatic reactions, has raised the question about the solubility of biocatalysts. In fact, in most solvents, enzymes and other proteases are insoluble and form a solid phase in the bulk organic phase: therefore heterogeneous catalytic system are commonly used for enzymes catalysis in organic media. The most common way to use solid enzyme in organic media is to suspend the solid enzyme directly in the solvent. The solid enzyme could derived by a process of *lyophilization* of the solution of the enzyme. This process is very critical and might inactivate the enzyme that is probably caused, at least partly, by a reversible conformational change in the enzyme. To avoid this and thereby increase the activity of lyophilized enzymes in dry organic solvents, the liophylization can be carried out in the presence of lyoprotectants such as sorbitol.[24] The second important aspect is the pH of the solution to lyophilize. Protonation and deprotonation of enzymes influence their catalytic activities and, the same enzyme, depending on the kind of reaction that need to catalyze, requires different condition of "pH control", as we faced for the enzyme penicillin acylase, (see chapter 3). For this reason, is important to adjust the pH value of the enzyme solution prior to lyophilization because of the enzyme keeps its ionization state from the aqueous solution in which it was present before the removal of water. This property has been called "pH memory" of enzymes in organic media and was theorized by Zaks and Klibanov in 1985. The wanted pH control of the system could be obtain by adding buffers in the solution of the enzyme before the lyophilization or buffering substances such as trisoctylamine and triphenyl acetic acid that, being soluble in organic solvent, can be added directly to the medium of reaction.[25] The majority of the enzymes used for the experimental work correlate to this thesis belongs to

this class of powder-lyophilized. They are often inexpensive, if compared with other preparation, easy commercially available and suitable for many applications; however, the enzyme particles sometimes tend to aggregate and attach to the walls of the reactor, decreasing the catalytic surface.

Instead of lyophilized enzyme podwer, *enzyme crystals* can be used as catalysts in organic media. In order to increase the stability, crosslinking with glutaraldehyde has been carried out, for example, with the enzyme thermolisine: this enzyme preparation is more stable towards thermic inactivation and exogeneous proteolysis.

Enzymes immobilized on solid supports are one of the more recent innovation in the mode of use of enzymes, provides great advantageous in their employment in organic medium and are used to a large extend also for preparative purposes. They allow to overcome the problem of diffusion cited above and, furthermore, often they show considerably higher catalytic activity than enzyme powder. Many could be the reasons of this: the enzyme, spread to a wide area, which makes a larger proportion of the active sites available for catalytic function and facilitates mass transfer of substrate and products. Another possible reason is that the support can protect the enzyme from possible inactivation during drying or lyophilization and that the support provides a favorable micro-environment for the enzyme during catalysis increasing the catalytic activity.[26]

Several materials of quite different types and with different properties have been successfully used for different applications of enzymes in organic media and the choice of the kind of material represents a critical aspect. In the choosing of a support, both their morphological and chemical characteristic are of importance. In particular, for practical application, the most important morphological characteristics are particle size, pore size and specific surface area and compressibility. By a chemical point of view is important to value the amount of water, products and substrate partition and the direct effects of the support on the enzyme. The methods of immobilization are also very important in the final performance of the enzyme and range from the simple mix of a solution of the enzyme and the support and the subsequent dryness at reduced pressure or the linkage of the enzyme at the support.

Talking about the kind of supports, there is Celite, a kind of diatomaceous earth very common that can be use to support the enzyme immediately before use. It results to be a good support for chymotripsin and lipases. Among the inorganic supports there are also

allumina and silica gel. Synthetic polymers have been showed to be suitable material for the enzyme: among these the most common are hydrophobic supports, such as polystyrene, very useful for *Candida* lipase that show an increase in the esterification activity with the hydrophobicity of the modifying reagent. For the use in aqueous media are frequently used agarose gel and other polysaccharide.

By a practical point of view, the use of supported enzyme simplify the work up of the reaction: the enzyme can be easily and completely removed by a simple filtration avoiding tedious formation of emulsion.

1.9 Applications of biocatalysts

The described peculiar properties of enzymes, expecially their high selectivity and their ability to work under mild conditions, appear to be particularly suitable for supporting the synthesis and the modification of natural products. The raising importance that enzymatic transformation is assuming in synthesis is mainly due to the economical vantage of biocatalytic method when compared with the classical organic procedures, due to the low cost of biocatalysts and mostly, the absence of collateral side reaction that avoid tedious and time consuming purification and, by a preparative point of view, simplify the protocol of impurity profile. In the course of the second half of the XX secol, the literature provides us a wide and detaild range of examples that prove the predominant role that biocatalisys covers in the field of organic chemistry.

In 1995, Nicolaou *et al.* reported the chemoenzymatic synthesis of (-) Taxol, an approved drug for the treatment of cancer, originally isolated from *Taxus brevifolia*. By means of the recombinant lipase showed in the Figure 1.9, throw double step of subsequently acylation of the primary alcohol by the acyldonor isoprophenyl-acetate, the key intermediate was obtained with an excell enantiopurity.[27]

This example is very interesting because underlines that the selected enzyme, is able to discriminate both the functional groups that underwent acylation, leaving inalterated the secondary alcohol (chemoselectivity) and the stereoisomer (enantioselectivity).

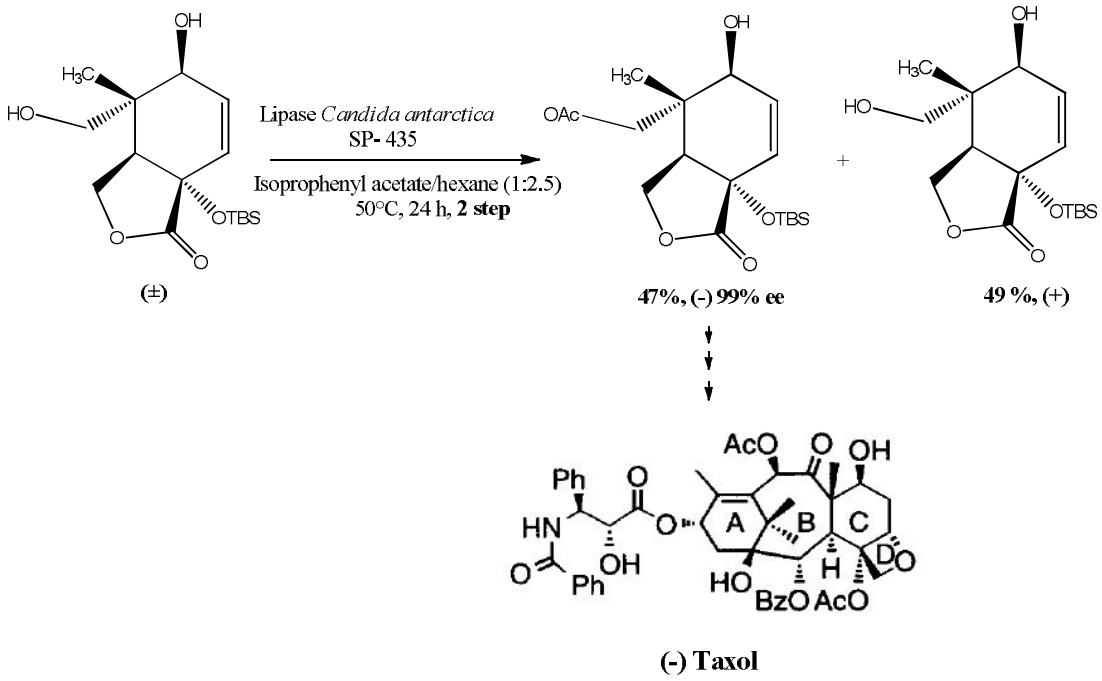


Figure 1.9. Asymmetric preparation of the (-) Taxol.

In the same period Chênevert *et al.* reported the preparation of baclofen, an analogue of GABA (γ-aminobutirryc acid), active as selective agonist for the $GABA_B$ receptor. Racemic baclofen is somministered for its antispastic activity but its two enantiomers show different pharmacological and toxicological properties: the (-) enantiomer is more active but also more toxic than the (+). For this reason the preparation of the enantiopure (-) R-baclofen was object of study. As underlined in the Figure 1.10, the desiderate stereochemistry was introduce in the prochiral di-ester throw the enantiotopic hydrolysis of the ester moiety achieved by α-chymotrypsin.[28]

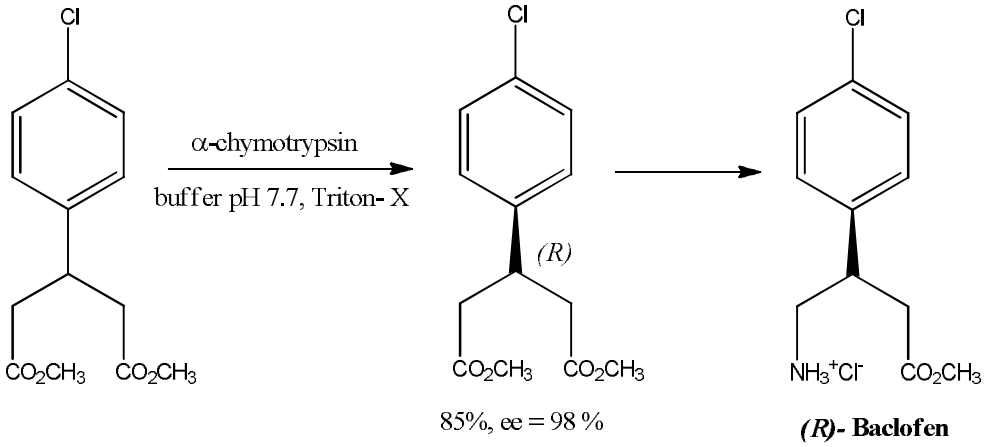


Figure 1.10. α-chymotrypsin enantiotopic ester hydrolysis in the route to *(R)* baclofen .

Always with the auxilium of a lipase, from *Candida antarctica* was accomplished, in our laboratory, the preparation of oxandrolone, a synthetic anabolic hormon, administered in severe burns and to improve the quality of life of patients with HIV infection. (Figure 1.11). The vantage of the biotransformation is the regioselective removal of the ester group bound at the less hindered position three, very difficoult to perceive by a classical ester hydrolysis in basic condition. [29]

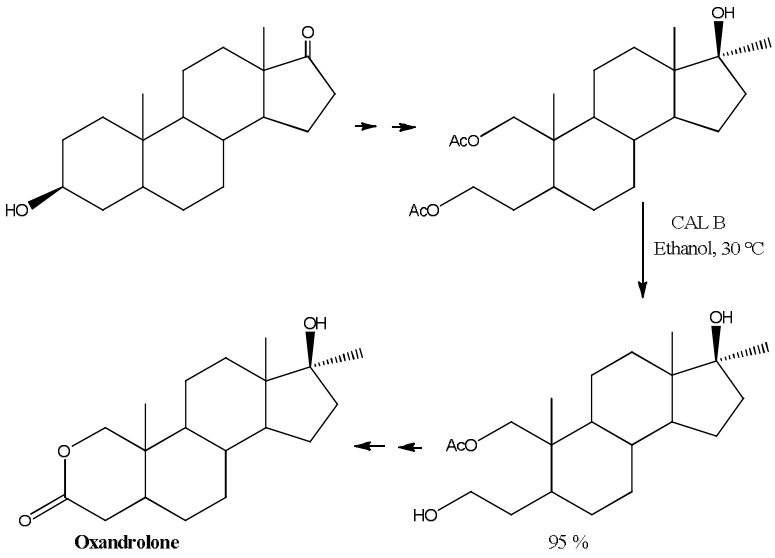


Figure 1.11. Regioselective *Candida antarctica* lipase B ester-hydrolysis in the preparation of oxandrolone .

In our laboratory the potentialities of biocatalysis were also employed to accomplish the preparation of several natural compounds, *i.e.* frontaline, a pheromone, mevalonolactone and a new potential antiandrogen (Figure 1.12 a-b-c).[30-32]

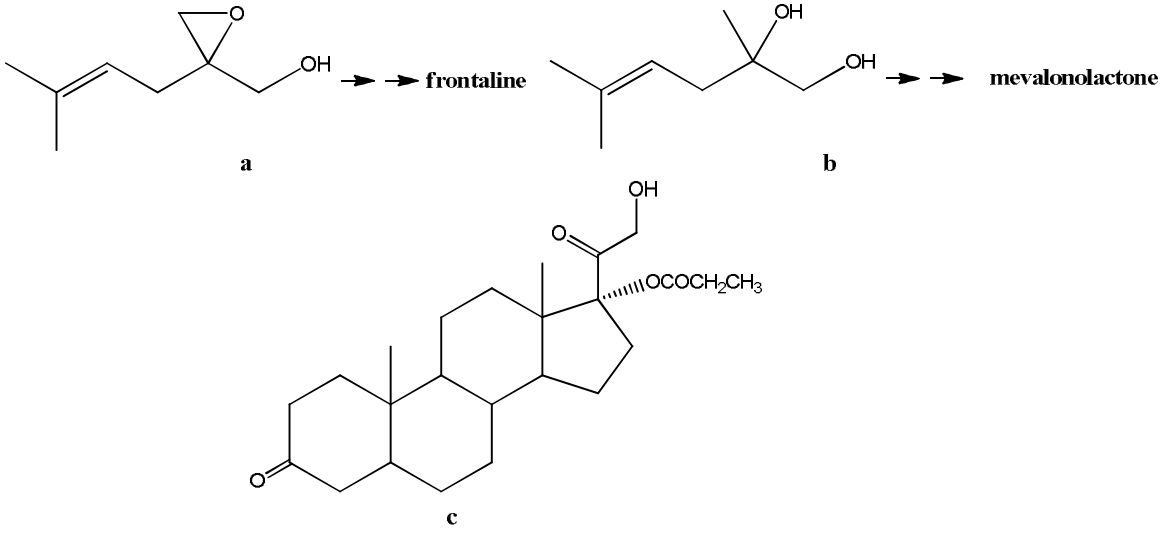


Figure 1.12. Regioselective *Candida antarctica* lipase B ester-hydrolysis in the preparation of oxandrolone .

1.10 Enzymes useful for biocatalysis

Reviewing the books and publications dealing with biotransformation, we can see that the hydrolases are the most prominent enzymes in both academic and industrial applications.[33]

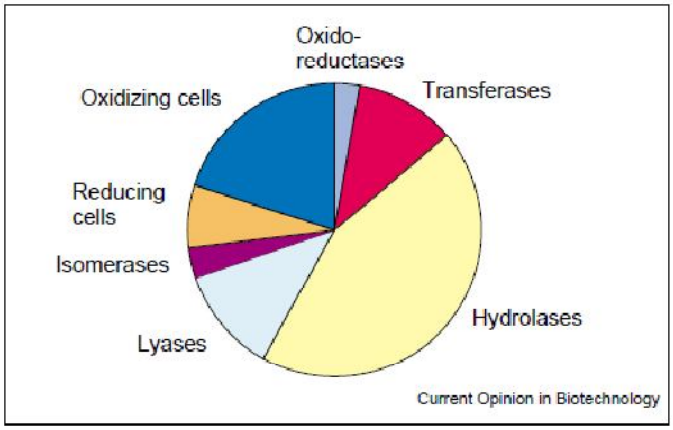


Figure 1.13. Enzymes type used in biotransformations.

This wide class comprehends amidases, proteases, esterases, lipases, nitrilases, phosphatases and epoxide hydrolases. Of particular interest are amidases, proteases, esterases and lipases because they catalyze the hydrolysis and the formation of esters and amides, functional groups ubiquitous in intermediate and building blocks of biological interest. Their widespread utilize is mainly do, as underline above, to their wide substrate specificity, their stability and their commercially availability, together with their low cost and their indipendence from cofactors. Many application of this hydrolytic enzymes can be found in the peptide synthesis, modification of sugar as well as desymmetryzation of *meso* and prochiral compounds. To this class belong many enzymes that I used for my experiments, the great part of which are serine hydrolases, term related to their active site and to their consequent mechanism of action.

- LIPASES

Many different kind of lipases will be cited in the course of the thesis, mainly with a microbial origin. In the Figure 1.14, is shown the structure of the active site of one of the most famous and effective lipases, lipase from *Candida antarctica*, CAL B, not only because offers a good example to exemplify the mechanism of action of serine proteases, but because it is the enzyme that allowed us to obtain the best results for the realization of the first project of this thesis.

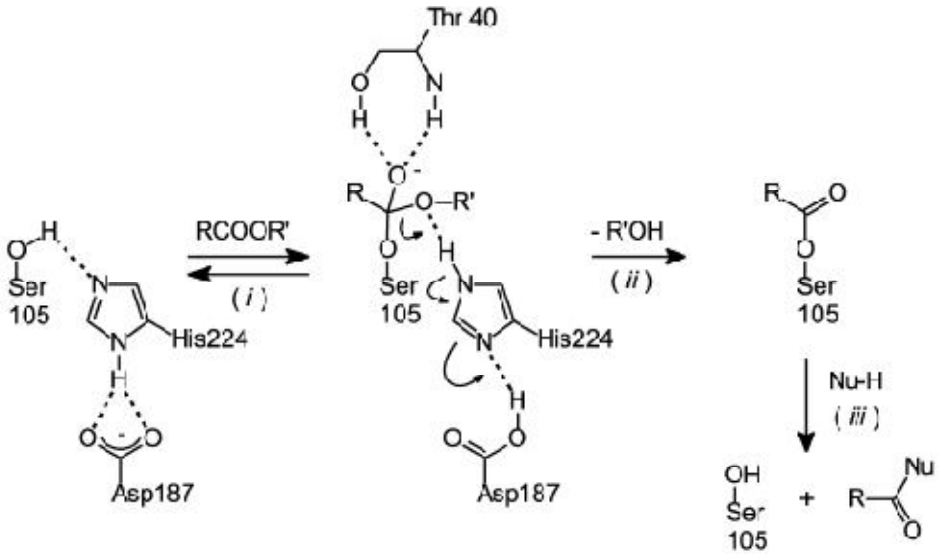


Figure 1.14. Active site of CAL B[34] and reaction mechanisms of lipase-catalysis.

The hydroxy group of serine, the imidazole ring of histidine and an acid amino acid residue, aspartate or glutamate, (the so called catalytic triade) at the active site of serine hydrolases are mainly responsible for the catalytic process itself. The reactions catalysed by serine hydrolases proceed through a tetrahedral acyl-enzyme intermediate where the acyl group of the substrate (RCOOR') is covalent bound to the serine hydroxy-group present in the active site. In the natural process, water act as nucleophile, (Nu-H); the nucleophilic compound (Nu-H) can be water and, in this case, the ester hydrolysis is observed, but many other reaction can be carried out by a simply change of nucleophile (Figure 1.15)[35].

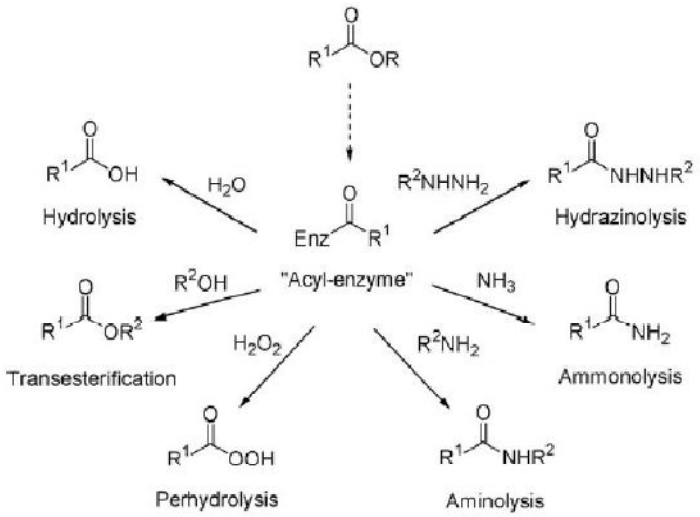


Figure 1.15. Different catalyzed reactions depending on the nature of the nucleophile.

The biological function of lipases, as expressed in their denomination (triacylglycerol acylhydrolases), is the hydrolysis and the transesterification of triglycerids. Therefore, their first application is the formation or hydrolysis of an ester moiety. For esterification (acylation) of alcoholic groups, a reaction of equilibrium, active compounds were employed as acyl donor. Trichloroethyl or trifluoroethyl esters, anhydrides, oxime esters are some of these but the best methods employ vinyl acylates. In this case, in fact, the formed enol tautomerizes to acetaldehyde making the reaction irreversible (Figure 1.16).[36-37]

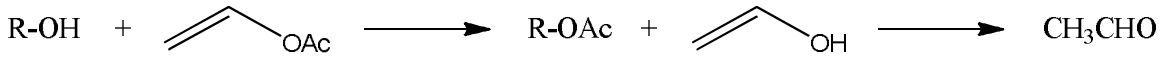


Figure 1.16. Irreversible esterification.

This process have found wide applications, at the beginning, in the resolution of compounds bearing a secondary alcohol, for which, according with the Kaslauskas' rule, the L-enantiomer is the favorite to be accepted in the active site of enzyme (Figure 1.17).

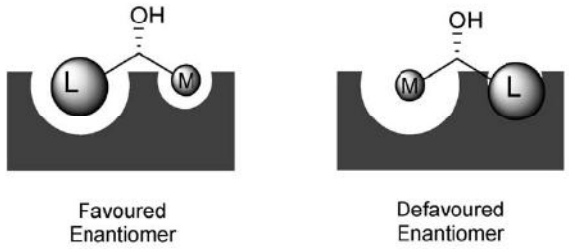


Figure 1.17. Kazlauskas' rule for the resolution of secondary alcohols by lipases.

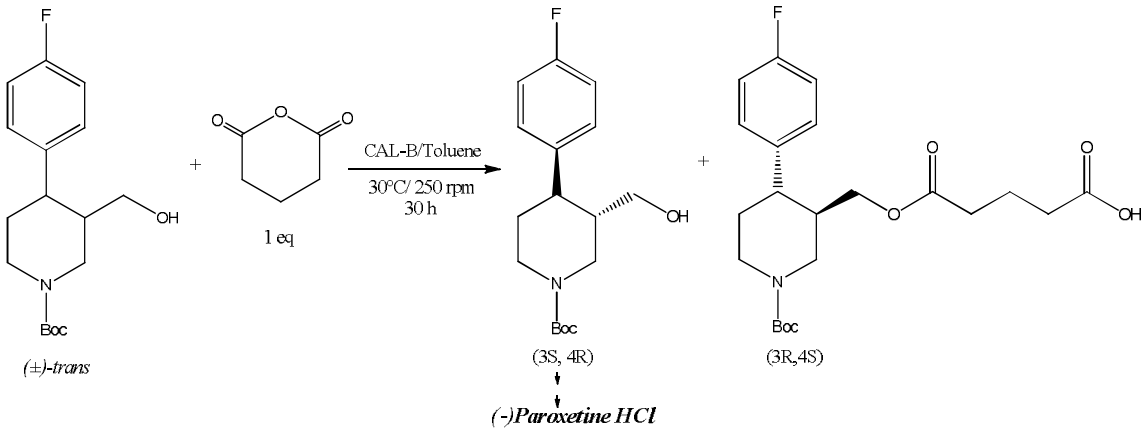


Figure 1.18. Cyclic anhydride as acyl donor in the chemoenzymatic synthesis of (-) Paroxetine

Later, also primary alcohols bearing a stereocenter at position 2 were successfully resolved (Figure 1.18). Also in this case, Kazlauskas, analyzing the great number of examples reported in literature, can suggest a rule about the lipases enantiopreference.

Beside lipase B, the yeast *Candida antarctica* produces also lipase A. The use of CAL A was neglected by the second isoenzyme CAL B, and a possible explanation is that a relative high CAL A loading seems to be needed in comparison with the B isoform.[38] Anyway this is an enzyme that shows unique peculiarities, high thermo-stability, (major than 90°C), specificity in the hydrolysis of triglycerides, its particular recognition of the *trans-trans* fatty acids and, moreover, its preference in the recognition of hindered secondary alcohols. Not only alcohol can be resolved by means of lipases but also aliphatic and aromatic amines.[39] Amino alcohols and amino acids can be discriminated through enantioselective N-acylation.[40]

- ESTERASE

As example of this family I propose Pig liver esterase, a serine type esterase that catalyzes the stereoselective hydrolysis of a wide variety of esters. The commercially available enzyme contains several isoenzymes; they behave, however, similarly in term of stereoselectivity. Of several models developed for interpreting and predicting the specificity of pig liver esterase, the cubic-space active site model reported by Jones seems mostly broadly useful, and has been applied to a number of prochiral, cyclic and acyclic meso esters (Figure 1.19).[41]

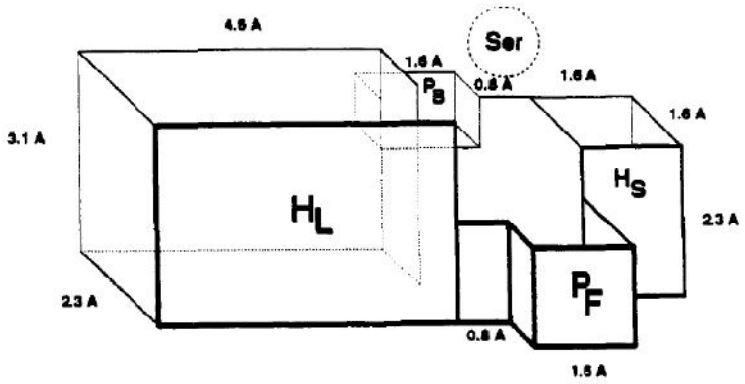


Figure 1. 19. Active-site model for PLE. H_L = large hydrophobic pocket; H_s = small hydrophobic pocket; P_F = polar front; P_B = polar back.

- PROTEASES

Proteases are essential constituents of all form of life on earth, including prokaryotes, fungi, plants and animals. These enzymes have become widely used in the detergent industry, since their introduction in 1914, as detergent additives. They are classified into various groups, dependent on whether they are active into acid, basic or neutral condition and on the characteristic of the active site group of the enzyme, i.e. metallo-, aspartic-, cysteine-, sulphidryl- or serine-type. Microorganisms elaborate a large array of proteases, which are intracellular/and or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell free environments and enable the cell to adsorb and utilize hydrolytic products. At the same time, these extracellular proteases have been commercially exploited to assist protein degradation in various industrial processes and as biocatalyzators for asymmetric synthesis.[42]

We reported here a short description of some alkaline serine-type proteases, the most commercially exploited, deeply studied in experiments explained later.

α-Chymotrypsin, consisting of three linear peptide chains, was firstly recognized by its ability to cause the clotting of milk.[43] Its natural role is the hydrolysis of amide bonds of proteins of aromatic amino acids such as Phe, Tyr and Trp. It also catalyzes the hydrolysis of various esters. An active-site model of the enzyme was proposed by Cohen to explain the enantioselectivity. The model contains four pockets: each correspond to one of the four group with different size attached to the α-carbon of a substrate. More recently, was reported the molecular modeling of the enzyme-substrate interactions in the calculation of enantioselectivity.[44] The enzyme was employed for the hydrolysis of several N-protected amino acid methyl ester to give the corrisponding L-enantiomer of the acid and the enzyme mantains this activity also for α-substituted amino acids (α-methyl and alkenyl).

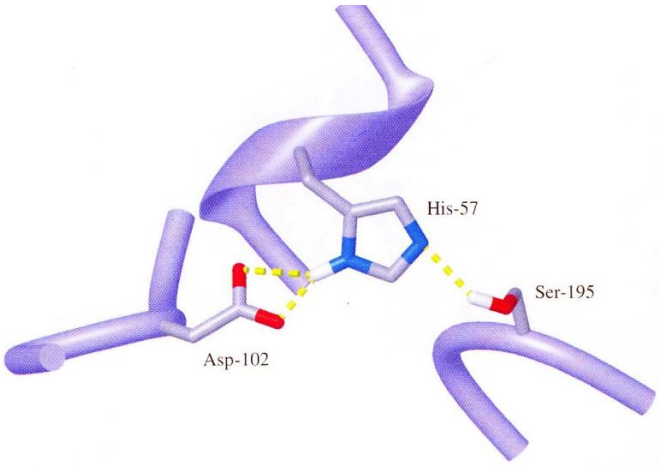


Figure 1.20. Active site of chymotripsin.

Other important proteases belong to the class of subtilisin. These are generally extracellular enzyme in order to assolve their function of scavenging nutrients. They all origin from *Bacillus* species and the most utilyzed in the field of biocatalysis are the subtilisin NOVO or BPN', produced by the *Bacillus amyloliquefaciens*, subtilisin Carlsberg, produced by *Bacillus licheniformis* and so called by the name of the laboratory where was discovered in 1947. There is also the subtilisin produced by *Bacillus subtilis* whose activity is close to that of subtilisin Carlsberg. The protease activity of subtilisin is similar to that of chymotrypsin but with a broader substrate specificity.[45] Their active site is a triad made up of serine, histidine and aspartate and consequently, their mechanism of catalysis is similar to that described for lipases. Beside its natural peptidase activity, other important applications of this enzyme are the resolution of chiral amine by enantioselective acylation. The selectivity of this enzyme is not limited to amides but is also high effective in the resolution of N-protected aminoacid esters by their L-enantioselective hydrolysis: these features make this enzyme a catalyst of first choice when racemic amino acids have to be resolved.

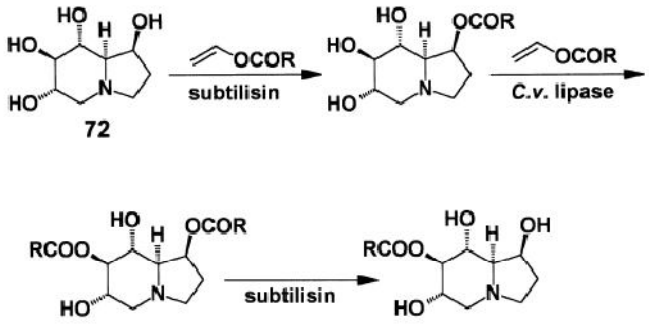


Figure 1.21. Different regioselectivity of lipases and proteases in then acylation of the alkaloid castanospermine .

Notheworthy is the employ of subtilisin to prepare specific esters of natural glycosides (Figure 1.3). Concerning its ability to acylate secondary alcohols, as clearly reported in the figure , the protease shows different selectivity than the lipase (Figure 1.21). The explanation can be found in the models proposed for their rispective active site, that accommodate the two substituent (L and S), bounded to the secondary alcohol, in a specular manner (Figure 1.22). [46]

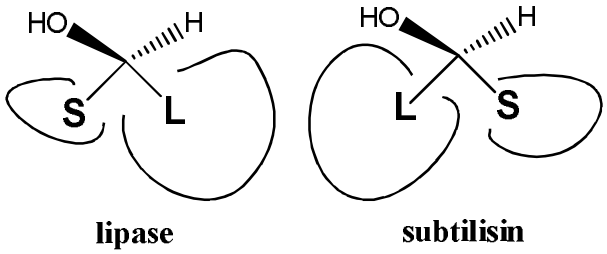


Figure 1.22. Differences of the active site of lipase and subtilisin.

Other important features that we could test directly are its high stability and its versatility to adapt to different kinds of medium, that, anyway, could be improved by immobilization, or cross linked crystallization, as reported in the third chapter.

Other proteases tested in our screening, *i.e.* papain and chymopapain, and protease from *Aspergyllus oryzae*, and from *Streptomyces griseus* were chosen for their well documented use in the stereoselective hydrolysis of esters of N-protected amino acids.

- AMIDASES

Finally penicillin acylase, the enzyme that allow us to achieve the best results for the researches carried out in Canada, belongs to the class of amidases. It is a serine hydrolase and consequently its mechanism of action is very similar of that described for the proteases. By a structural point of view is, on the contrary, completely different since it belongs to the class of the N-terminal nucleophile hydrolases, which have not the catalytic triad but an N-terminal serine that is activated by bridging water molecule. (Figure 1.23)

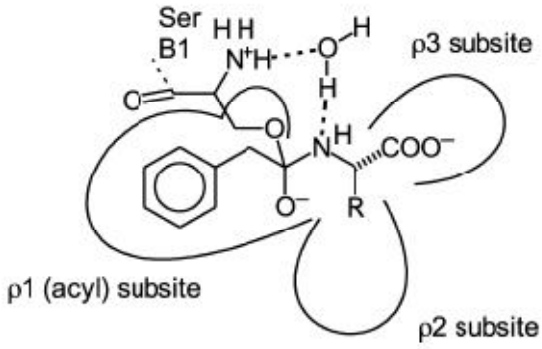
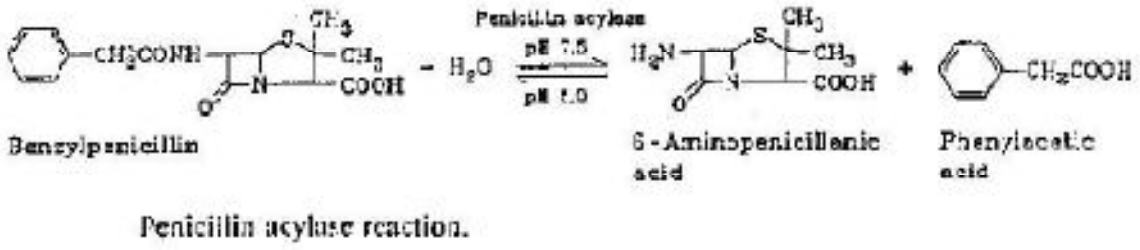


Figure 1.23. Schematic representation of the active site of penicillin acylase.

Penicillin acylase has a somewhat complex substrate specificity. The acyl binding (ρ1) sub-site is highly specific for phenylacetic acid and for this reason penicillin acylase is mainly used in the manufacturing of the β-lactam building block 6-aminopenicillanic acid from penicillin G (Figure 1. 24.)



Penicillin acylase reaction.

Figure 1.24. Penicillin acylase in the preparation of semisynthetic penicillines.

The ρ2 sub-site mainly interacts with the reactant through hydrophobic and steric forces. The ρ3 sub-site, in contrast, specifically binds negatively charged groups, which explains the high specificity of penicillin acylase for L-amino acid residues, but it also recognize ester groups. The commonly used penicillin acylase from *E. Coli* is commercially available in various carried-bound formulations. Crossed link enzyme maintains its activity even in pure organic solvents but, compared with lipases or subtilisin, is less stable and lose its catalytic activity upon dehydratation. [9]

1.11 References

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2. IMMUNOMODULATOR PIMECROLIMUS

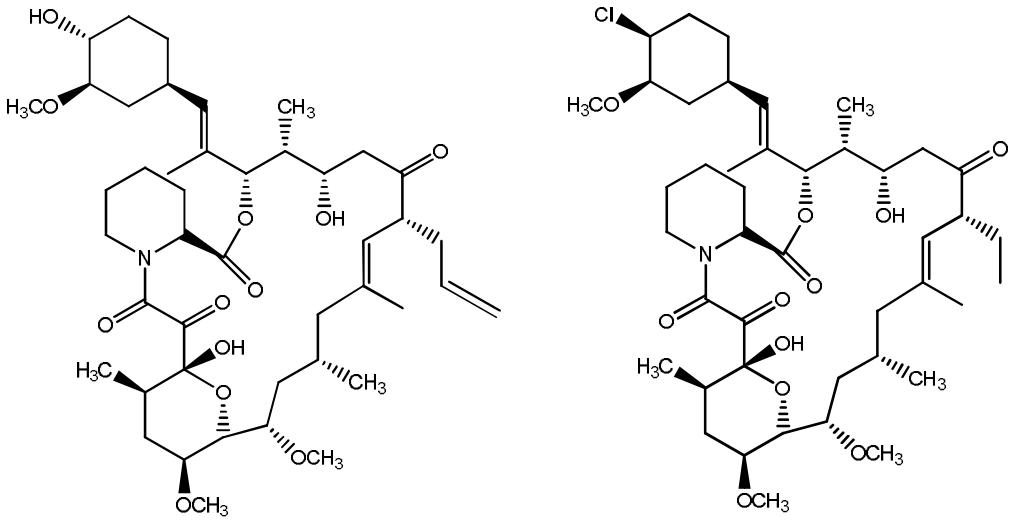
2.1 Introduction

Patients with chronic inflammatory skin diseases suffer from a markedly impaired quality of life.

In dermatological practice is increasing the demand for an alternative to topical corticosteroids, that for fifty years represented the gold standard topical therapy against inflammation. In fact, the use of topical corticosteroids is notoriously limited by side effects local and systemic (ie, skin atrophy, striae and telangiectases relaxing, diabetes mellitus, osteoporosis and inhibition of the hypothalamic-pituitary-adrenal axis).

Therefore, in recent decades, the efforts in the search for new dermatological drugs have been directed to discover topical therapeutic alternatives with immunomodulating properties, but devoid of known side effects of corticosteroids.

Particular emphasis was therefore given to a new class of macrolide lactones molecules with effect immunomodulatory which act selectively inhibiting the expression of genes for interleukin 2 (IL2) in T lymphocytes: tacrolimus and pimecrolimus (Figure 2.1) are the two macrolides prototypes of this new class of topical immunomodulators.[1]



Tacrolimus FK 506 *Pimecrolimus ASM 981*

Figure 2.1. The two immunosuppressive macrolactam tacrolimus and pimecrolimus.

2.2 Previous compounds

Because topical applications of drugs frequently reduces the unwanted effects of systemic administration, these are generally preferred for the treatment of dermatological inflammatory skin diseases. In the course of the years, many topical formulations of classical systemic antiinflammatory drugs were developed. To date, topical corticosteroids have been the mainstay of drug therapy for atopic dermatitis, because of their broad immunosuppressant and anti-inflammatory effects. The first report of the topical use of hydrocortisone (Figure 2.2, compound *a*) were made in 1952. Since then, there have been numerous modifications of the corticosteroid molecule, such as halogenation, hydroxylation and modification of side chains, allowing the increase of its pharmacological effect.(Figure 2.2)[2]

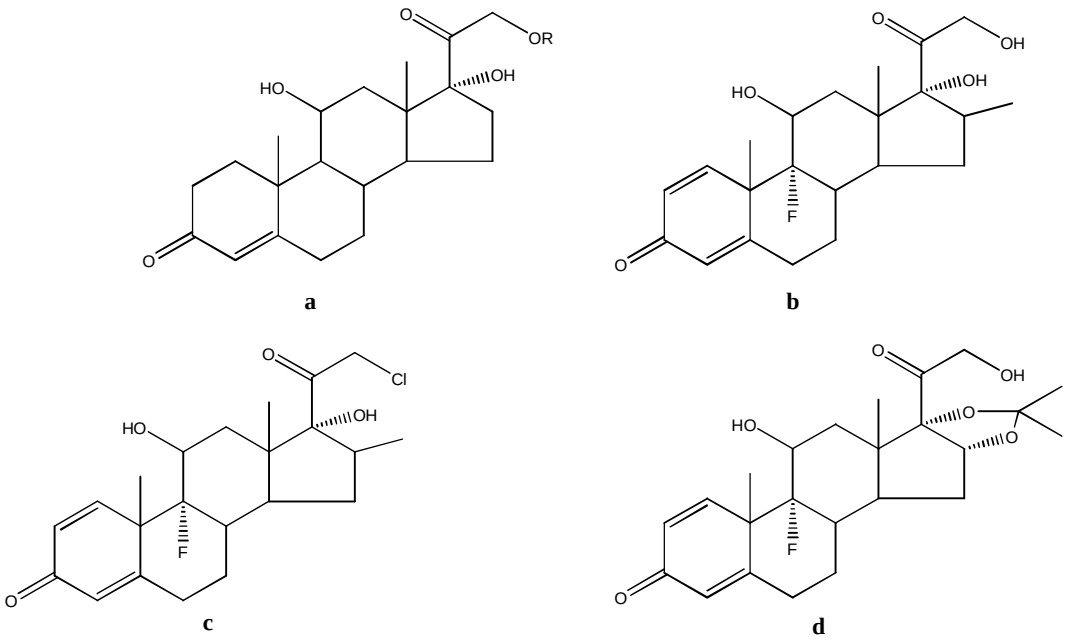


Figure 2.2. *a.* Hydrocortisone, *b.* Betametasone, *c.* Clobetasole, *d.* Triamcinolone acetonide.

At present, topical corticosteroids are classified into groups according to their potency. The European classification system has four levels numbered I to IV (from very potent, as clobetasol, to mild, as hydrocortisone), in descending order of potency.[3] The increase in steroids potency was accompanied by the occurrence of more frequent and severe side effects, both locally, as skin atrophy, glaucoma and some cases of allergic contact dermatitis and also systematically, due by the suppression of the hypothalamic-pituitary-adrenal axis. These steroids-induced side effects can become a major concern in patients receiving therapies for long periods of time, as in the case of atopic dermatitis. These

concerns are even more important in children, both because growth suppression can be a sequela of systemic corticosteroids excess and because a greater body surface area-to-weight ratio makes the potential for systemic absorption a greater concern. In addition, application to certain anatomic areas, such as the face, especially the eyelids and groin, has a particular potential for adverse events. For all these reasons, alternative treatments for atopic dermatitis (AD) are greatly needed.

Another compound that has been an important role in antiinflammatory therapy certainly is cyclosporine (Figure 2.3) that belongs to the family of cyclic polypeptides.

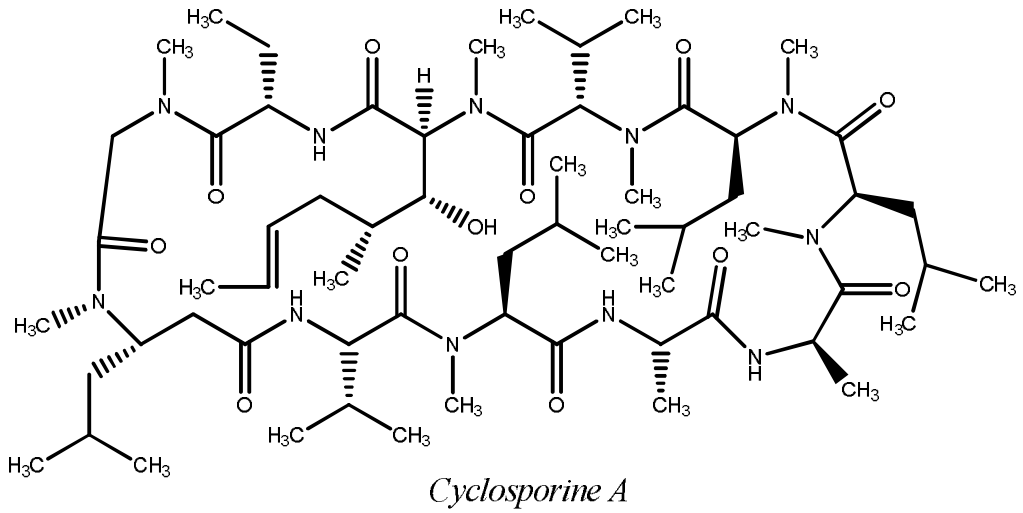


Figure 2.3. The cyclic polypeptide cyclosporine A.

CyA was isolated from the fungus *Tolyplocadium inflatum* and since its introduction in early 1980s has revolutionized immunosoppressive therapy in transplantation medicine. It inhibits T-cells activation and was employed for the treatment of atopic dermatitis, psoriasis, pyoderma gangrenosum only by sistemic somministration; in fact the drug is not active topically probably for its large molecular size, 1202 Da, and low liphofily which impede its ability to penetrate skin. Its oral administration associated with the risks of serious systemic effects, particularly renal toxicity, doesn't encourage its systemic use.[1]

Tacrolimus (FK506) (Figure 2.1) is a neologism composed of letters found in the following words: Tsukuba-macrolide-immunosoppressive. It is consider the first of a new class of topical calcineurine inhibitors. In fact t. was isolated in 1984 from the bacterium, *Streptomyces tsukubaensis,* a species discovered in Tsukuba, Japan. Although t. shows similar biological properties to CyA, there is no structural relationship: tacrolimus is a macrolide, the chemical term that refers to presence of a lactone in the cyclic carbone

backbone of the structure, with an atomic weight of 822 Da. Despite this high molecular weight, t., contrary to cyclosporine, is very hydrophobe, insoluble in water and may be easily dissolved in methanol, chloroform, acetone or ethanol. Since t. is unstable in aqueous solution, it has been marketed in dermatology for patient with moderate to severe AD, since early 2001, by Fujisawa Healthcare Inc as an ointment under the trade name Protopic. Tacrolimus has been used also intravenously and orally for the prevention of organ rejection after allogeneic kidney or liver and in bone marrow transplantation.[4]

Unlike tacrolimus and CyA, pimecrolimus (Figure 2.1) was developed specifically to treat inflammatory skin conditions. P. is an ascomycine derivative with a molecular weight of 810 Da. Ascomycin (Figure 2.7), the parent compound, was originally isolated in the early 1960s from the fermentation product of *Streptomyces hygroscopicus var ascomyceticus* and showed only antifungal activity. Preclinical studies in animal models have found that although p. exhibits high anti-inflammatory activity in skin, it has only a low potential for systemic immunosuppression. In fact, in animal studies, p. demonstrated cutaneous local anti-inflammatory properties equivalent or slightly superior to those of cyclosporine A and tacrolimus in model of allergic contact dermatitis while, in the transplanted kidney model, was three times less potent than CyA and fifteen times less potent that tacrolimus at preventing organ rejection.

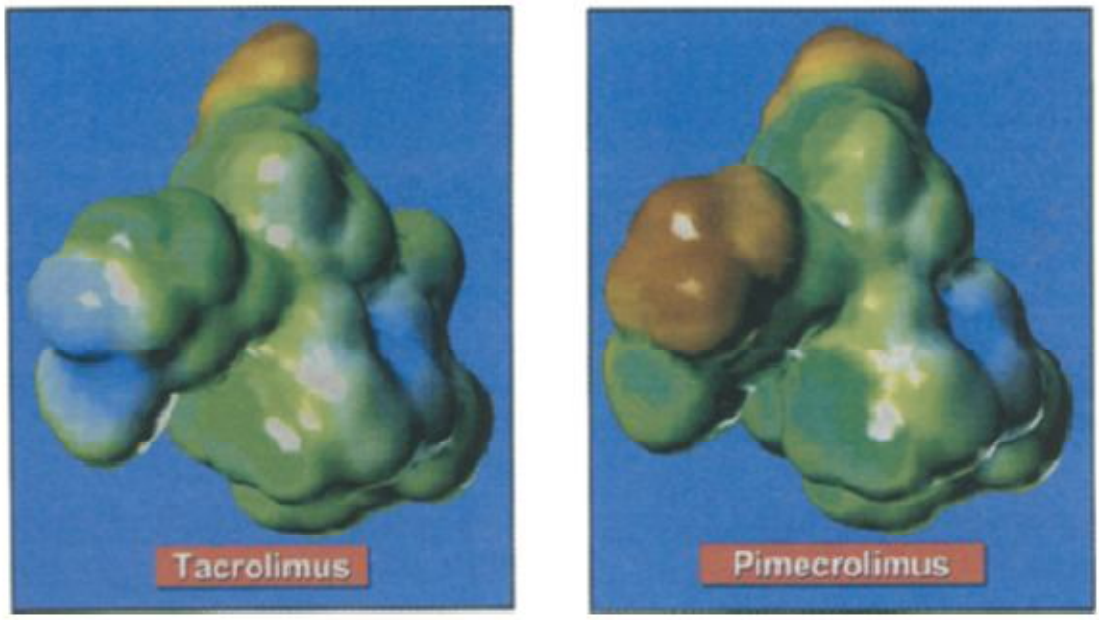


Figure 2.4. Lipophilicity distribution of tacrolimus and pimecrolimus. *Blue* indicates hydrophilic regions; *Brown* lipophilic regions.

Since is proved that the two macrolactams pimecrolimus and tacrolimus share a similar mechanism of action (described later), a possible explanation for the above-describes differences in their pharmacologic properties lies in their structures. Actually, although the two compounds are both macrolactams, pimecrolimus exhibits a higher overall lipophilicity, compared with tacrolimus and this property makes pimecrolimus more selective for skin and endowed with a large therapeutic index for safely and effectively treating cutaneous inflammatory diseases. [5]

2.3 Mechanism of action and pharmacokinetic of pimecrolimus

The mechanism of action of pimecrolimus (ASM 981), that it shares with tacrolimus (FK 506) is the blockage of T-cell activation. Ascomycin macrolactams are immunophilin ligands that bind to a specific cytosolic receptor. Pimecrolimus, like tacrolimus and rapamycin binds to immunophilin macrophilin-12 also known as FK506 binding protein and FKBP-12. X-ray crystal structure and NMR analysis showed that the left hand part of the macrolactam, containing the tricarbonylic portion, is responsible for the binding with this macrophilin and for this reason is called “binding domain”. Then, the right part of the macrocicle, together with elements of the immunophilin, interacts with the protein phosphatase calcineurin, which plays a key role in the Ca^{2+} dependent activation of lymphocytes, and thus represents the “effector domain”(Figure2.5). [6]

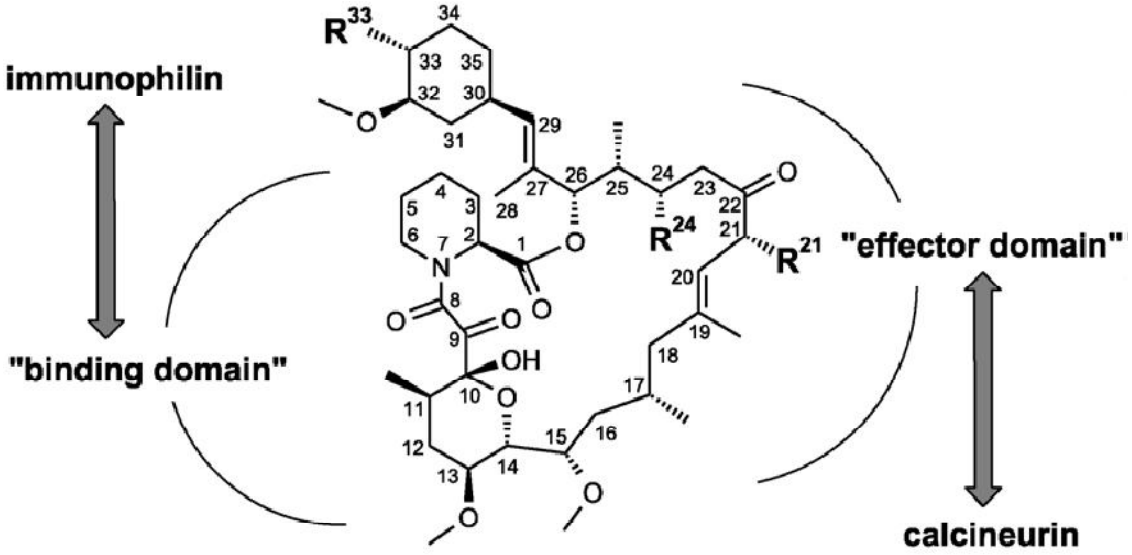


Figure 2.5. Functional domains in the structure of macrolactams’ family.

Calcineurine is a ubiquitous Ca^{2+}/calmodulin-dependent protein phosphatase necessary for survival by regulating the responses to stresses such as high salt and by controlling cell division in many eukaryotic microorganisms. In mammals, calcineurine is required for many functions in a variety of tissues: learning and memory, renal function and, of course, the immune response. The selective sensitivity of immune function to this drug is thought to reflect the low level of expression of calcineurin in lymphocytes relatives to cells in other tissues (eg. neurons) in which calcineurin is more abundant and was originally characterized. By an evolutionistic point of view, for bacterial organisms that secrete an agent which would inhibit a neighbor's calcineurine would gain an important competitive growth advantage. When a T-lymphocyte is activated by binding peptide antigen in the presence of a major histocompatibility protein, intracellular calcium is released and calcineurine is activated to dephosphorylate certain target protein. One critical target of calcineurine is a transcription factor called NF-AT (nuclear factor of activated T-cells). Upon this dephosphorylation, the cytoplasmic subunit of NF-AT traslocates to the nucleus and there it can binds its nuclear counterpart to form an active transcription factor, required for the production of a whole family of cytokines central to initiating an immunoresponse.

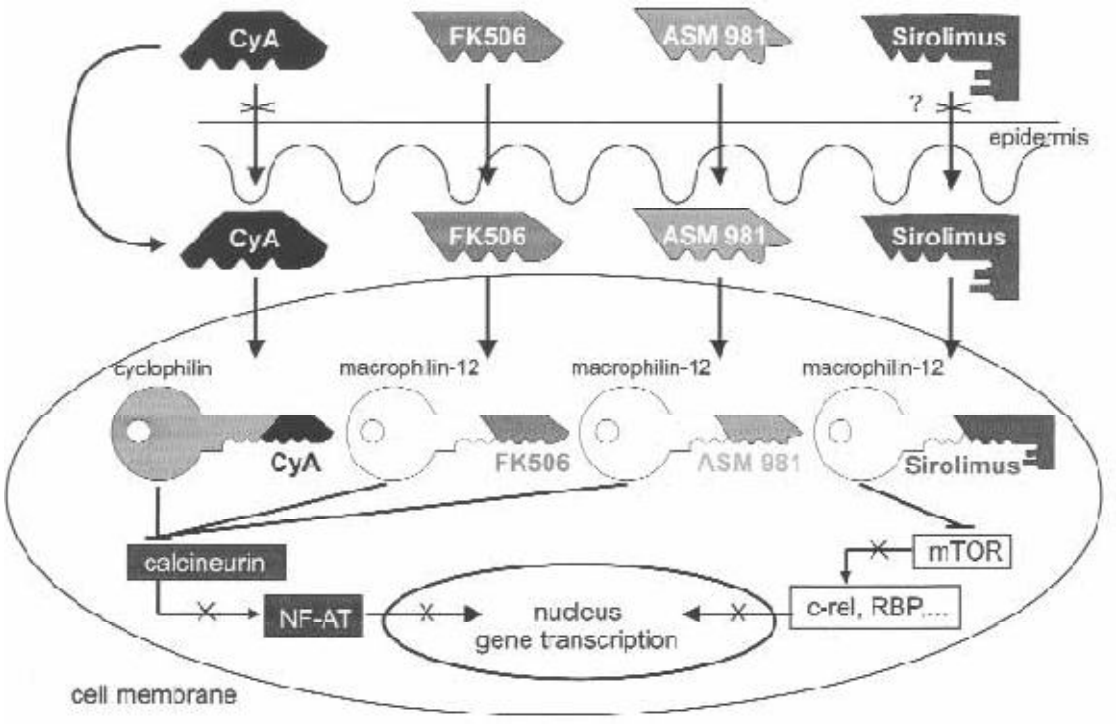


Figure 2.6. Mechanisms of action of macrolactams immunomodulators. *CyA*, Cyclosporine; *mTOR*, mammalian target of rapamycines*; *NF-AT*, nuclear factor of activated T-cell; *RBP*, rapamycin binding protein. *Among the family of macrolactam immunosupressor, there is also sirolimus, also known with the name of rapamycin, a 31 member macrocyclic polyketide first isolated from *Streptomyces hygroscopicus* as

antifungal agent and later shown to be a immunosuppressive and antiproliferative agent. The biological activities of r. are dependent on the binding to FKBP12 and the subsequent formation of a tertiary complex with mTor (mammalian target-of-rapamycin). The compound is currently approved for use in renal transplantation and shows promise as a coating for coronary stents to prevent restenosis following angioplastic but is not suitable for topical application.[7]

By inhibiting the action of calcineurin, the pimecrolimus-macrophilin complex prevents the dephosphorylation of cytoplasmatic component of the nuclear factors of activated T cells (NF-AT). NF-AT regulates the mRNA transcription of a number of inflammatory cytokines; therefore, pimecrolimus blocks this transcription, especially Th1 (IL-2, IFN-γ) and Th-2 (IL-4, IL-10) type cytokines (Figure 2.6). Other cytokines, including IL-5, IL-10 and TNF-α, are decreased in production by pimecrolimus in a dose-dependent manner. It doesn't appear to cause systemic immunodeficiency. Pimecrolimus also targets mast cells which play an important role to anti-inflammatory activities. Pimecrolimus inhibits not only the transcription and synthesis of cytokines from mast cells, induced by antigen/IgE, but also the released of preformed mediators serotonin and β-hexosaminidase by the inhibition of Fcϵ-RI (hight affinity IgE-receptor)-mediated degranulation and secretion. Moreover, studies of the gene expression of blood cells in patients treated with oral pimecrolimus, showed that pimecrolimus caused a strong down-regulation of the expression of mRNA for genes associated with the macrolactam target pathway and inflammation. However, no changes were found in the mRNA for genes which generally reflect drug related side effect like those associated with apoptosis, stress and enzymatic induction. [4], [8]

From a pharmacokinetics point of view, the systemic adsorption of pimecrolimus showed to be negligible. In adult patients being treated for AD, with an involvement between 13% and 62% of Body Surface Area, for periods up to a year, a maximum pimecrolimus concentration of 1.4 ng/ml was observed among those subjects with detectable blood levels and in the majority of samples in adult (91% of the subjects), blood concentration of pimecrolimus were below 0.5 ng/ml.

Laboratory in vitro plasma protein binding studies using equilibrium gel filtration have shown that 99.5% of pimecrolimus in plasma is bound to proteins over the p. concentration range of 2-100 ng/ml tested. The major fraction of p. in plasma appears to be bound to various lipoproteins. As with other topical calcineurine inhibitors, it is not know whether p. is absorbed into cutaneous lymphatic vessels or regional lymph nodes.

The metabolism of pimecrolimus has been studied in human subjects by using the radiolabeled drug. After a single oral dose in healthy subjects, unalterated pimecrolimus was the major drug-derived component found in blood. Numerous minor metabolites of moderate polarity, which appeared to be products of o-demethylations and oxygenation of the parent compound, were identified. Drug-related radioactivity was excreted principally in feces (78.4%) and only a small fraction (2.5%) was recovered in urine.[5]

2.4 Clinical applications

Pimecrolimus was developed for the treatment of inflammatory skin diseases. Several studies have evaluated its effectiveness. The systemic immunosoppressive potential of pimecrolimus is low and pharmacokinetic studies have shown very low blood level of pimecrolimus after topical application, with no accumulation after repeated application. The cream Elidel, containing pimecrolimus in the concentration of 1% was approved by the FDA in December of 2001 for the treatment of atopic dermatitis. It is indicated as second-line therapy for the short term and non continuous chronic treatment of mild to moderate AD in non-immunocompromised adults and children 2 years of age and older, who have failed to respond adequately to other topical prescription treatment or when those treatment are not advisable. They are indicated for a short-term or intermittent treatment.[9]

Atopic dermatitis, or eczema, is a common skin disease that is often associated with other atopic disorders, such as allergic rhinitis and asthma. The clinical manifestation of atopic dermatitis vary with age; three stages can often be identified. In infancy, the first eczematous lesion usually emerges on the cheeks and the scalp. Scratching, which frequently starts a few week later, causes crusted erosions. During childhood, lesions involve flexures, the nape, and the dorsal aspect of the limbs. In adolescents and adulthood, lichenified plaques affect the flexures, head and neck. In each stage, itching that continues throughout the day and worsens at night causes sleep loss and substantially impair patient's quality of life.

The hallmark of atopic dermatitis are a chronic, relapsing form of skin inflammation, a disturbance of epidermal-barrier function that culminates in dry skin, and IgE-mediated sensitization to food and environmental allergens. The histological features of acute eczematous patches and plaques are epidermal intracellular edema (spongisis) and prominent perivascular infiltrate of lymphocytes, monocytes macrophages, dendrites cells,

and few eosinofils in the dermis. In subacute and chronic lichenified and excoriated plaques, the epidermis is thickened and its upper layer is ipertrophied.

Two hypotheses concerning the mechanisms of atopic dermatitis have been proposed. One holds that the primary defect resides in an immunologic disturbance that causes Ig-E mediated sensitization, which epithelial-barrier dysfunction regarded as a consequence of the local inflammation. The other proposes that an intrinsic defect in the epithelial cells leads to the barrier dysfunction; the immunologic aspects are considered to be an epiphenomenon. Several candidates genes have been identified in atopic dermatitis, notably on chromosome 5q31-33. All of them encode cytokines involved in the regulation of IgE synthesis: interleukine-4, interleukine-5, interleukine-12, interleukine-13 and granulocyte-macrophage colony stimulating factor (GM-CSF). These and other cytochines are produced by two main types of T lymphocytes. Type 2 helper T cells (Th2) produce interleukine-4 as well as interleukine-5 and interleukine-13, two cytokines that up-regulate the production of IgE and stimulates production of IgG antibodies. Type 1 helper T cells (Th1) produce mainly interleukin-12 and interferon-γ, which suppresses production of IgE and stimulates production of IgG antibodies.

Epidemiologic studies indicates that the worldwide incidence of AD has increased in recent years, especially in industrialized countries; in the United States, AD has become a common condition in children with a prevalence of anywhere from 7% to 17%.

The initial mechanisms that induce skin inflammation in patients with AD are unknown. They could entail neuropeptide-induced, irritation-induced, or pruritus-induced scratching, which releases pro-inflammatory cytokines from keratinocytes, or they could be T-cell-mediated but IgE-independent reactions to allergens present in the disturbed epidermal barrier or in food (so-called food-sensitive atopic dermatitis). Allergen-specific IgE is not a prerequisite, however, because the atopy patch test can show that aeroallergens applied under occluded skin induce a positive reaction in the absence of allergen-specific IgE.

By resuming, barrier dysfunction and chronic inflammation are characteristic of atopic dermatitis; therefore, a long-term clinical management should emphasize prevention and reduction of bacterial colonization by means of local application of lotions containing antiseptic such as triclosan and chlorexidine and, most important, the control of inflammation by the regular use of topical corticosteroids or topical calcineurine inhibitors.
[10]

Although at present, pimecrolimus has been approved only for the treatment of AD, in the course of the last 10 years, have also been beneficial in numerous other dermatological diseases. Among the several dermatitis, best evidence of its effectiveness are achieved for seborrhoieic dermatitis, a common problem in parkinson's disease and perianal dermatitis, in which pimecrolimus acts faster and with a longer effect, if compared with the topical corticosteroids: within 1 week of treatment, a complete clinical remission could be obtained. There are promising results from small series and case reports on oral and genital lichen planus and anogenital lichen sclerosus. Other possible indications include allergic and irritant contact dermatitis, cutaneous lupus erythematosus, rosacea and vitiligo. [11], [12]

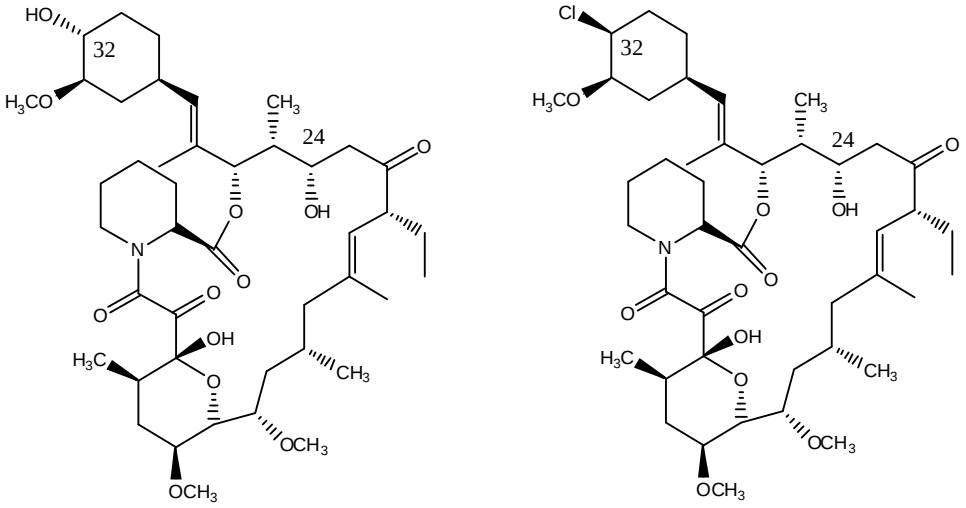
The well known effect of inactivation of T-limphocytes proliferation, may also be related to cancer development. On March 2005, the FDA issued an advisory to healthcare providers urging caution in prescribing topical t. and p., particularly for use in children, because of a possible increased risk of cancer. This advisory was made on the recommendations stemming from the FDA Pediatric Advisor Meeting on February 15, 2005. The concern was based on information from animal studies, a small number of case reports, and the pharmacology of the drugs. The small number of cases reported to the FDA med watch program were lymphomas, melanoma and squamous cell carcinoma. On January 2006, a labeling change was approved for both agents. The new labeling included a boxed warning about the possible risk of cancer: it issued that human studies to correlate the risk of cancers with the use of p. and t. it may take many years and it clarifies that these drugs are second-line treatments for AD, that should be used only for patient who have failed treatment with other therapies and that they are not indicated for use in children younger than 2 years old.

Nowdays, there is no causal proof that TCI, pimecrolimus and tacrolimus, cause limphoma or nonmelanoma skin cancer. Treatment with systemic calcineurine inhibitors as a part of an immunosuppressive regimen increases the risk of sun-induced non-melanoma skin cancer and melanoma.

The most frequent adverse effect observed with topical pimecrolimus in most studies was skin burning. However, this was a transient phenomenon with no further consequences in contrast to skin atrophy and the hypotalamic-pituitary- adrenal axis suppression that the exaggerated use of corticosteroids may cause. [13], [14]

2.5 Aim of the work

As clearly described above, Pimecrolimus is a recent compound of very high value, widely used for systemic antiplatelet therapy and very promising for the treatment of several dermatological diseases. For this reason, its preparation has elicited great interest among medicinal chemistry.



Ascomycin, **1** *Pimecrolimus,* **2**

Figure 2.7. Structure similarity between ascomycin and pimecrolimus.

Pimecrolimus is a semisynthetic product that could be prepared starting from a product of fermentation, ascomycin: in fact, as underlines in Figure 2.7, the two macrolactams differ only for the substituent present at position 32, an oxydryl or a chlorine of inverted configuration respectively for **1** and **2**. The presence of two equivalent secondary hydroxy groups at position 24 and 32 makes difficult the directly introduction of the chlorine on the nucleus of ascomicin. Therefore, till now, the preparation of pimecrolimus was accomplished by the previous protection of the 24-alcohol, usually carried out by silylation of both the 2 hydroxy group, followed by a partially selective deprotection at position 32, with fluoridric acid at low temperature.[15]

The disadvantages of this protocol of reaction are the large amount of silylating agent required for the reaction of protection (3- 4 eq) and the need to purify by chromatography every intermediate for both the protection and the deprotection step. Moreover the complexity of the compound, bearing many different functional groups, makes every chemical reaction susceptible of the formation of byproducts: it has been shown that ascomycin, upon treatment with nucleophiles or strong bases, undergoes a wide variety of

rearrangement-, cyclization-, clevage-, and epimerization reactions.[16] In fact this is a very complex molecule, containing a pipecolate residue linked by an amide and an allylic ester moiety (C26-29) to a polyketide backbone. Most notably, the macrolactam features the unusual pattern of three adjacent carbonyl groups within the binding domain (C8-C10, tricarbonyl portion, α, β diketoamide moiety, whereby one carbonyl group (C 10) is involved in emiketal formation with the secondary hydroxyl group at C 14 to form the tetrahydropyrane unit (C 10- 14). Although the structure at the binding domain of ascomycin shown by formula in Figure 2.8 A is the main isomeric form adopted in organic solution, the close proximity of the tricarbonyl portion to the hydroxyl group at C-14 could potentially lead to the formation of numerous alternative isomers (Figure 2.8 B-T-E-F). Moreover, each potential equilibria product may exibit a mixture of rotamers with respect to the geometry of the amide bond. [6], [17]

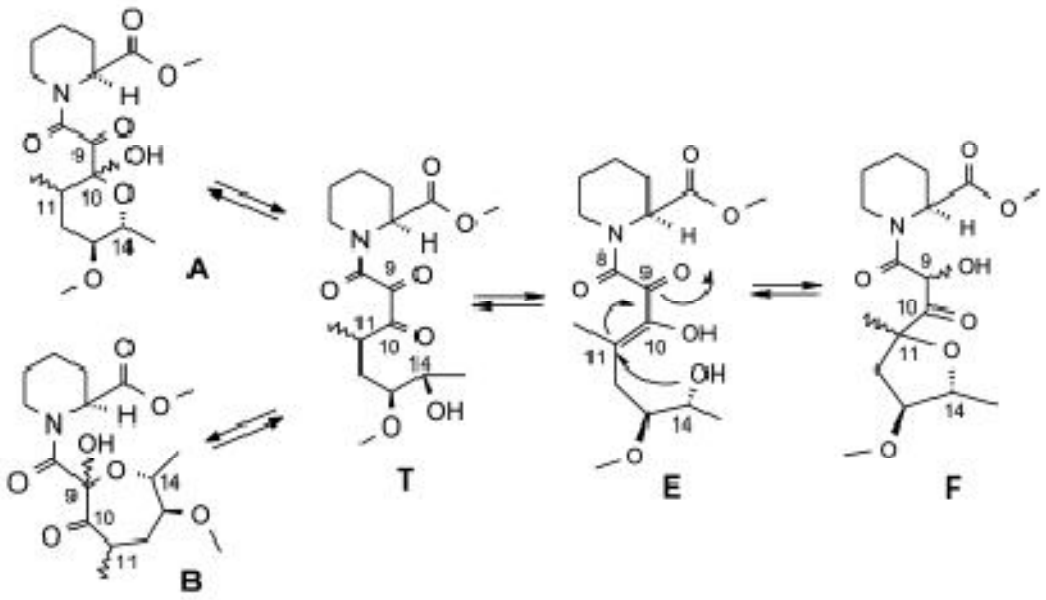
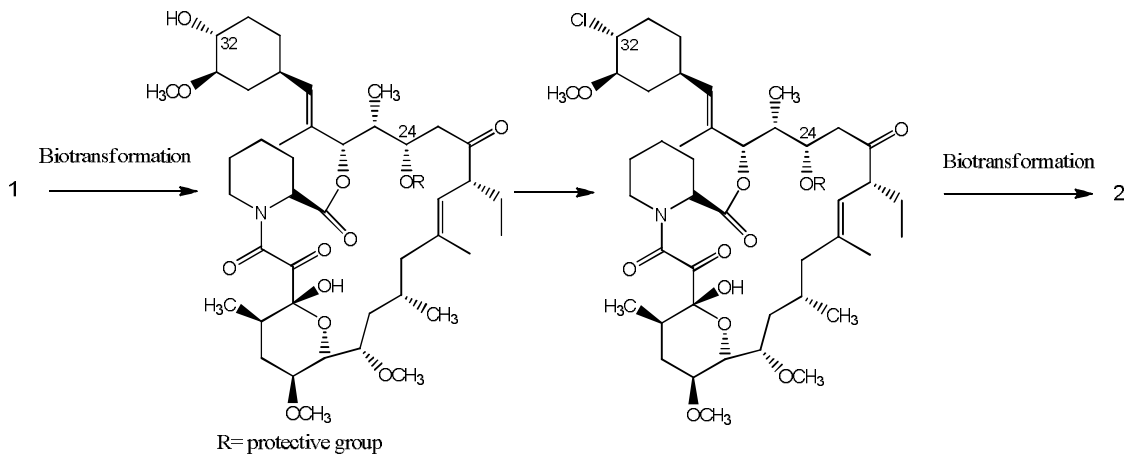


Figure 2.8: Rearrangements at the tri-carbonylic portion of the binding domain of ascomycin.

For these reasons mainly, could be interesting and useful to employ biocatalysis to prepare, through a selective transformation, the 24-protected key intermediate to perceive the conversion of ascomycin into pimecrolimus.

More in detail, the main objective of the work, will be the regioselective protection of the hydroxy group bounded to the carbon 24, by acylation, realized by a suitable enzyme in presence of an acyl donor. Then, the so obtained 24-protected intermediate should be transformed into the correspondent 32-*epi*-chloro and finally converted in the final

compound **2** by the same enzyme catalyzed regioselective hydrolysis of the 24-protected group (Scheme 2.1).

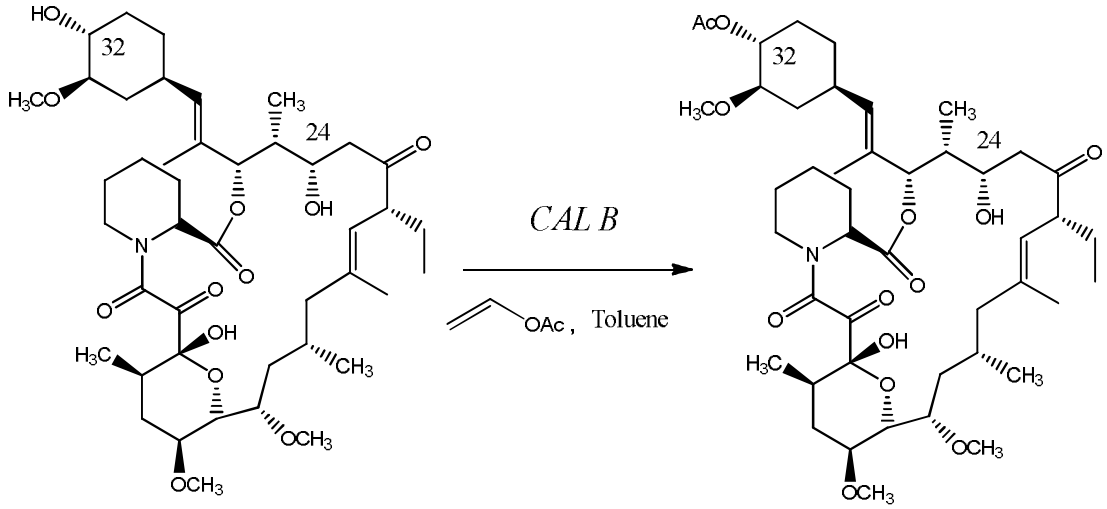


Scheme 2.1. General enzymatic pathway.

As enzymes, we choose to study lipases as potential catalysts, first of all because they are the most suitable and used enzymes for the reaction of transesterification, the reaction required for the protection of the hydroxyl function, more over because this class of hydrolases accept substates poorly soluble in water, as for the macrolactams, being their catalytic activity in organic solvents very well documented.

2.6 Results and discussion

In order to indagate the selectivity of lipases, at first we treated ascomycin with several of them among the most common and available, in irreversible transesterification conditions using vinyl acetate as acyl donor in an organic solvents.[18, 19] This preliminary screening allowed us to conclude that ascomycin was not substrate for *Pseudomonas fluorescens* (PFL), porcine pancreas (PPL) and *Candida cylindracea* (CCL) lipases, whereas *Candida antarctica* lipase (CAL B, Novozym, 435), as revealed by NMR analysis, regioselectively afforded the 32-monoacetate, as unique product, with a percent conversion depending on the chosen solvent (Table 1).



1 **3**

Scheme 2.2. Regioselective *Candida antartica* lipase B-catalyzed transformations of ascomycin **1** to its 32-monoacetate **3**.

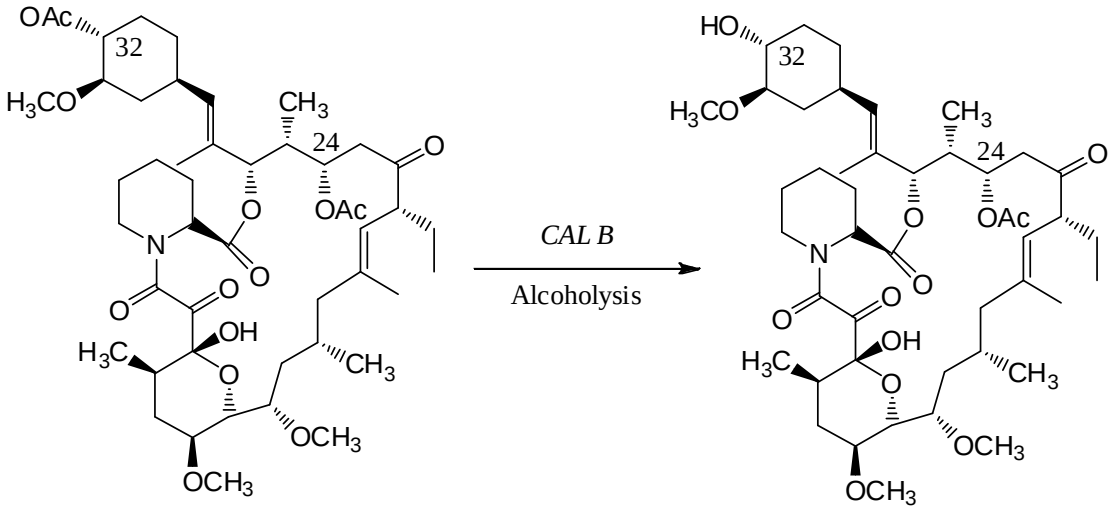
Lipase	Solvent	Time (h)	Conversion (%)[a]
PFL	Chloroform	100	0
PPL	Toluene	94	0
CCL	Toluene	92	0
CAL B	Acetonitrile	53	30
CAL B	*tert*Butylmethylether	53	10
CAL B	Toluene	80	100

[a] from TLC and ^{1}H-NMR

Table 2.1. Lipases-catalyzed transesterification of **1** to **3** with vinyl acetate.

Evidences about enzymatic reactions outcomes were preliminary obtained by means of a ^{1}H-NMR analyses comparison with the literature data of ascomycin **1**[20] and 24,32-di-O-formyl-ascomycin .[6] In fact ^{1}H-NMR data of acetates **3-5** are not reported. Ascomycin **1** presents two signals, at 3.40 and 3.92 ppm, assigned to H-32 and H-24, respectively. Whereas the former is overlapped to the OCH_3 signals the latter (*i.e.* H-24 signal) and the H-32 signal (at 4.71 ppm) of 24,32-diformate **,** are more predictive for our purposes. The presence of a signal at 4.70 ppm, beside the signal at 3.92 ppm, allowed to conclude that the 32-monoacetate **3** was the unique product of CAL B-catalyzed transesterification (Scheme 2.2). ^{1}H-NMR analyses showed also that the catalytic action of the enzyme was directed on both the two rotamers of **3**.

This result was surprising, not only considering the big size of the compound, that is however accepted as substrate but more over because the lipase showed further chemoselectivity leaving inalterated the cyclic lactone present in the structure of the compound, that, if opened, should be difficult to cyclize again in the same position. The result is in agreement with the reported selective lipase-catalyzed 32-acylation of tacrolimus and sirolimus (42-position) type macrolides [21], [22] and is probably imputable at the high hindrance that surrounds the 24 hydroxy group that makes this position not accessible at the active site of the enzyme. Even if the regioselectivity of CAL B was opposite than the desiderate, we decided, however, to take advantage of the discovered regiopreference of the enzyme working on the substrate 24, 32-diacetate **4** in hydrolytic conditions. Following this second protocol, reported in the Scheme 2.3, we adapted the substrate to the selectivity of the enzyme in order to obtain the desiderate 24- protected ascomycin **5**.



24, 32 diacetyl-ascomycin **4**

24, monoacetyl-ascomycin **5**

Scheme 2.3. Regioselective *Candida antartica* lipase B-catalyzed transformations of 22, 32 diacetyl ascomycin **4** to its 24-monoacetate **5**.

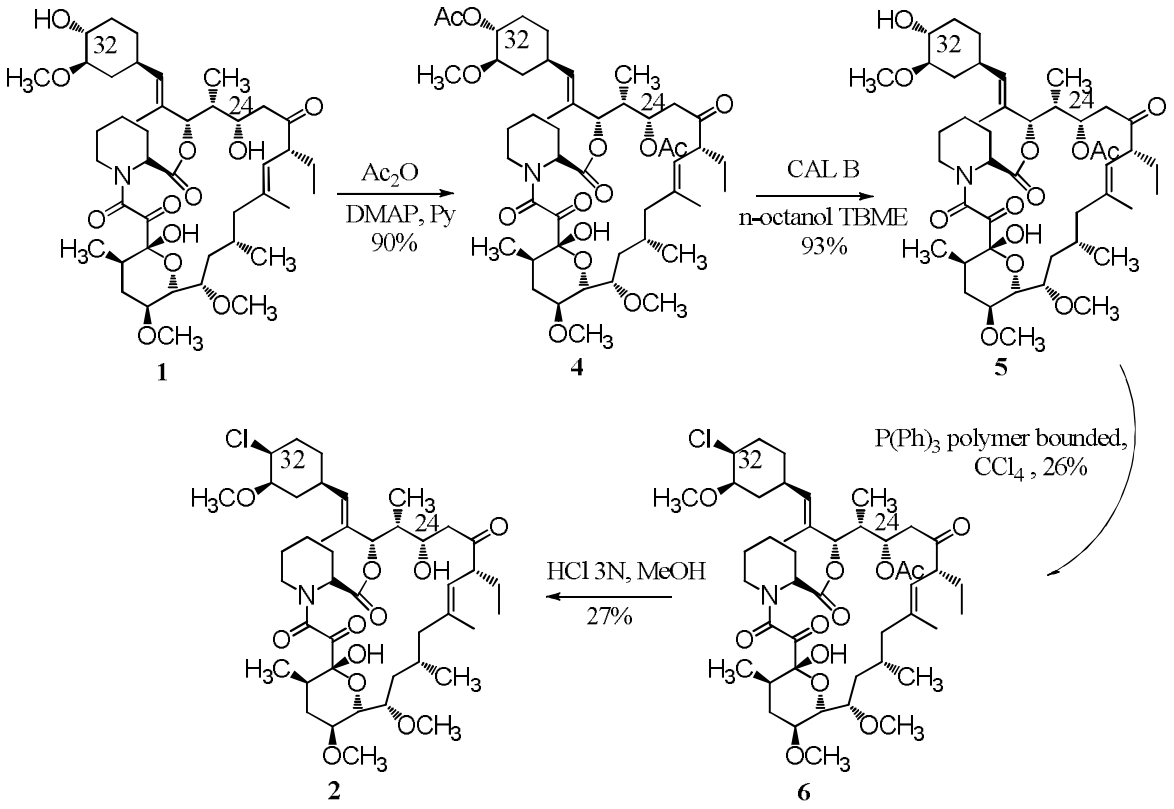
Solvent	Acyl acceptor	Time (h)	Conversion (%)[a]
Toluene	H_2O	80	0
Toluene	Methanol	48	0
Toluene	Ethanol	100	0
Toluene	*n*-Butanol	120	30
*tert*Butylmethylether	*n*-Octanol	100	100

Table 2.2. CALB-catalyzed hydrolysis or alcoholysis of **4** to **5** [a] from TLC and ^{1}H-NMR

Again NMR analysis allowed us to assign the structure of 24-monoacetate **5** to the product obtained from alcoholysis: the H-32 signal at 4.71 ppm was absent, indicating that the hydroxy group was not engaged in an ester bond; in addition, also the resonance due to the H-24 of 24-alcohol, at 3.92 ppm was absent whereas the region between 5.0 and 5.4 ppm, where usually is present the H-24 of esterified ascomycin (5.22 ppm for diformate), was modified.

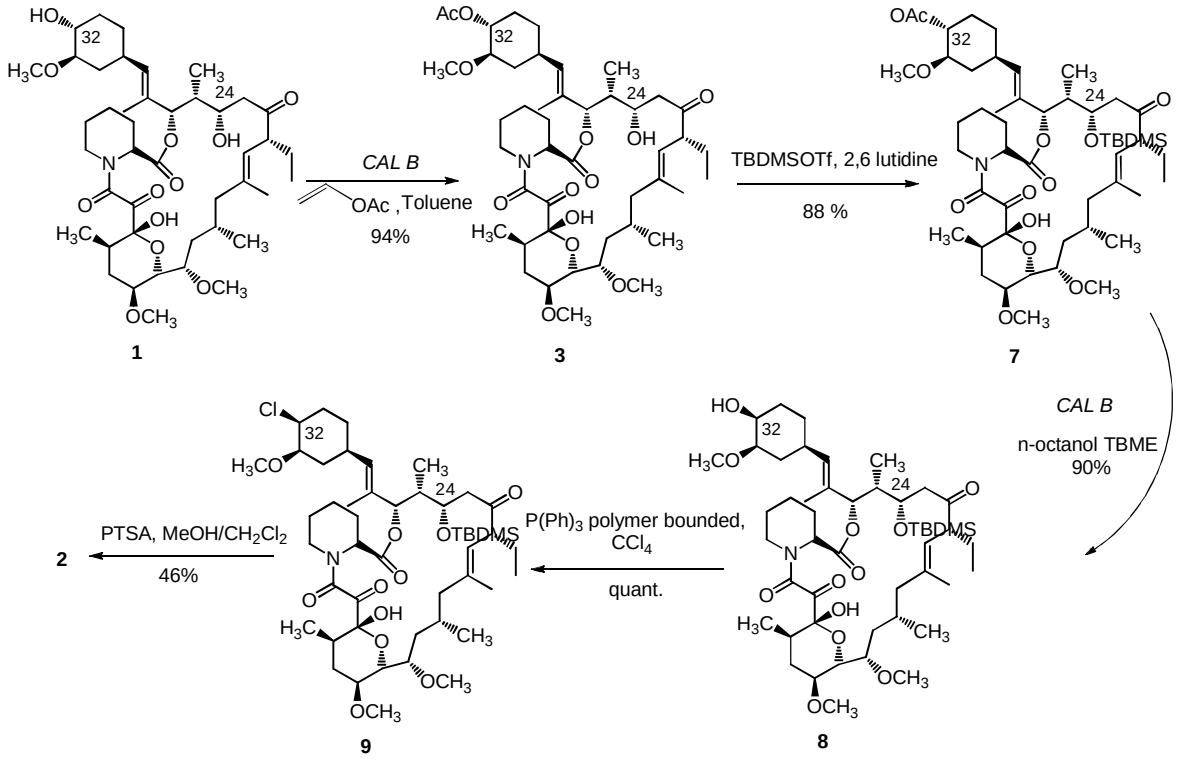
As summarized in the Scheme 2.4, the diacetate **4** was easily prepared from ascomycin by treatment with acetic anhydride and dimethylaminopyridine (DMAP) in pyridine[23] and then selectively deacetylated at position 32, by means of CAL B-catalyzed alcoholysis, with tertbutyl methyl ethr (TBME) as the solvent and *n*-octanol as the acyl acceptor.

Preparation of pimecrolimus was then accomplished starting from compound **5** that was treated with polymer bound triphenylphosphine in carbon tetrachloride to afford 32-chloroderivative **6** (40%); removal of 24-acetate was performed in acidic conditions (3N HCl, 40% yields) to avoid the lactone hydrolysis in presence of bases, [24, 25] affording pimecrolimus **2** in only 14% overall yield (Scheme 2.4).



Scheme 2.4. First chemoenzymatic approach using O-Ac as 24-protecting group.

Since the presence of 24-acetate seemed to be crucial for the lowering of the yields of two final steps, we planned to switch the acetyl group into a silyl one, more easy to idrolyze; so we prepared, through the regioselective enzymatic approach, 24-silyl derivative **8**, *i.e.* the key intermediate of traditional syntheses of pimecrolimus **2** (Scheme 2.5).



Scheme 2.5. Final chemoenzymatic approach to obtain pimecrolimus **2** using a silyl ether as 24-protecting group.

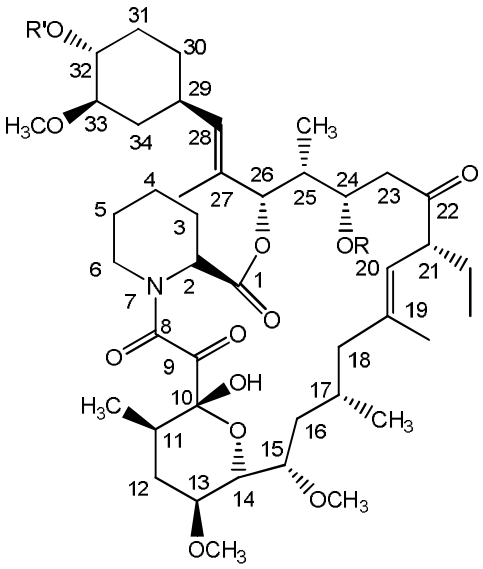
Starting from the previously enzymatically obtained 32-monoacetate **3**, by silylation with TBDMSOTf (5 equiv), in presence of 2,6-lutidine, 24-O-TBDMS, 32-O-acetyl derivative **7** was obtained; removal of 32-acetyl group was achieved by means of a CAL B-catalyzed alcoholysis,[19] affording intermediate **8** in good yields (80%). In these mild conditions, typical of enzyme-catalyzed transformations, problems related to acidic or basic treatments of ascomycin family compounds were avoided. In the biocatalyzed alcoholysis step it is possible to recycle the same enzyme sample used in the previous 32-acylation step, without significant loss of activity; in fact, if fresh CAL B is used, instead of the recycled enzyme, a comparable time is required to achieve the same conversion percent of **7** to **8** (about quantitative). Again, in order to introduce the chlorine at position 32, polymer bound triphenyl phosphine[26] was used, leading to **9**. We observed that when, after a 60-70% transformation, the polymer is removed and replaced with fresh reagent a

nearly complete conversion is achieved and the 32-chloro derivative **9** can be used in the next step without any further purification. The same transformation was successfully (73%) realized also with dichlorotriphenylphosphorane not applicable, on the contrary, in the case of 24-monoacetate **7** for the formation of a complex mixture.[27] PTSA in dichloromethane/methanol (1/1) at 25-30 °C, among the tested conditions (hydrochloric acid in methanol, PTSA in tetrahydrofuran/water) gave best results for 24-hydroxy group deprotection;[28] pimecrolimus[29] **2** was recovered in 46% yields, after separation, by column chromatography, from a less polar compound (30-35%), identified as 23-dehydropimecrolimus.[30] The presence of this compound is common to other reported deprotection methods, as *e.g.* hydrofluoric acid in acetonitrile,[15] in variable elevated amounts.

Overall yields (29%) of pimecrolimus **2** from ascomycin **1**, were comparable to those observed when intermediate **8** is prepared through the reported bissilylation-monodesilylation process.[15]

2.7 ^{1}H NMR analysis of compounds 3, 4 and 5.

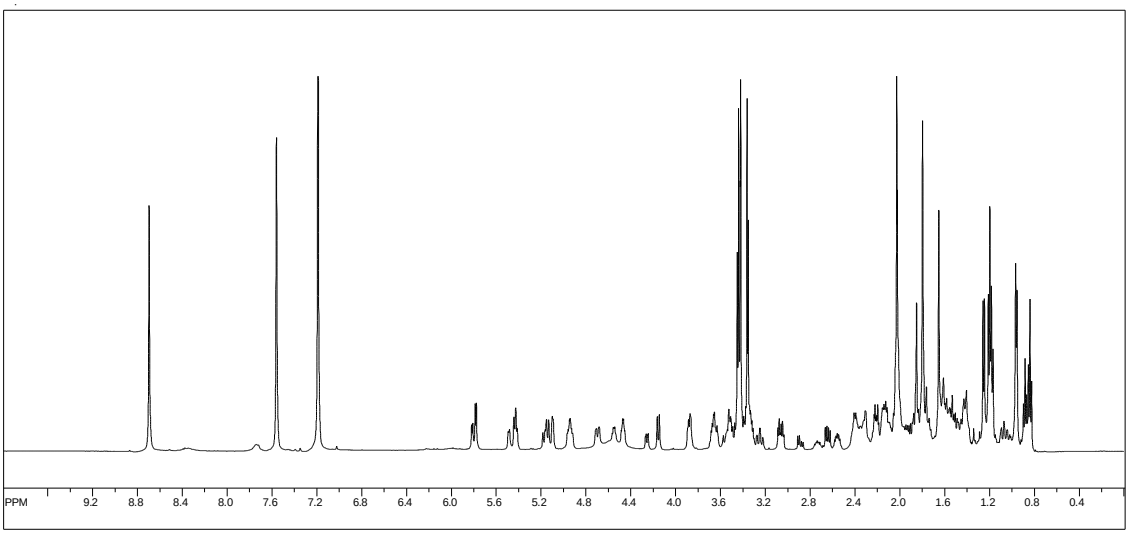
To ascertain the structure of acetates **3** and **4** a more accurate NMR study carried on by Prof. Diego Colombo. ^{1}H-NMR analysis were performed at 500 MHz with a Bruker FT-NMR AVANCE^{TM} DRX500 spectrometer using a 5 mm z-PFG (pulsed field gradient) broadband reverse probe, and ^{13}C-NMR spectra were collected at 125.76 MHz at 323K. Through 1D and 2D COSY, HSQC and HMBC experiments, it was possible to unambiguously establish the acetyl positions in these compounds. The experiments were carried out in Pyd_5 at 323K. Using these experimental conditions the spectra showed a good spread of the proton resonances and, especially, of all the methyl groups. Two sets of signals are identified, since the macrolactam system exists as a mixture of two rotamers in a 60/40 ratio, as in the case of ascomycin **1**.[20] The study started with 24,32-diacetate **4** and was focused on the signals of ascomycin moiety carrying the acetyl groups. As an entry point for the study of **4**, the well resolved characteristic C-25 linked methyl doublet at 1.12 and 1.11 ppm (major and minor rotamer) was selected. Starting from that signal, H-25 (2.18 and 2.42 ppm, major and minor rotamer), H-24 (5.50 and 5.65 ppm, major and minor rotamer) and H-26 (5.47 and 5.52 ppm, major and minor rotamer) were assigned by COSY.



3, R= H; R' = Acetyl
4, R, R'=Acetyl
5 R= Acetyl; R' = H

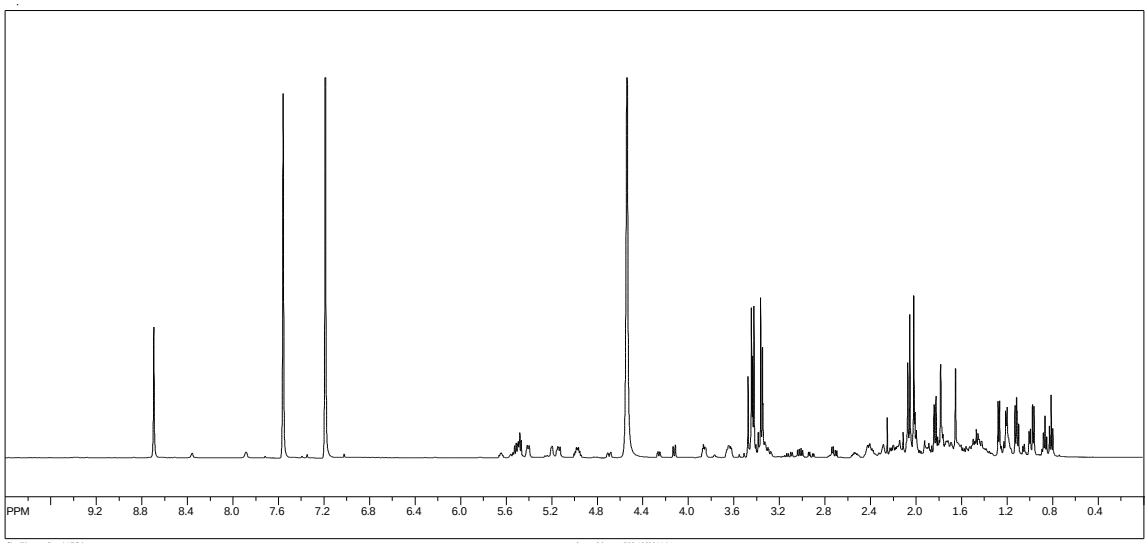
Figure 2.10 Numbered structure of macrolactams belonging to ascomycin.

- 32-Monoacetate **3**



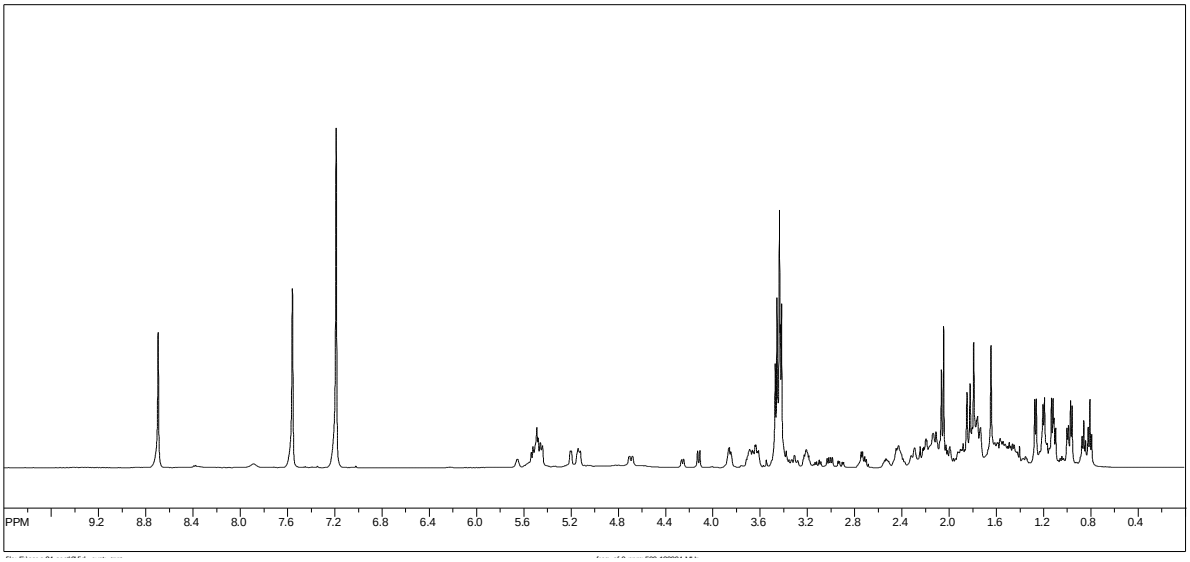
Attribution	**^{1}H (ppm)**	**Integration**
CH_3-25	1.20 and 1.18 (major and minor rotamers)	3H
H-25	2.10 and 2.24 (major and minor rotamers)	1H
H-24	4.47 and 4.55 (major and minor rotamers)	1H
H-26	5.78 and 5.81 (major and minor rotamers)	1H
H-28	5.43 and 5.42 (major and minor rotamers)	1H
H-29	2.40 (major and minor rotamers)	1H
H-30a	1.18 (major and minor rotamers)	1H
H-30b	2.15 (major and minor rotamers)	1H
H-31	3.33 (major and minor rotamers)	1H
H-32	4.94 (major and minor rotamers)	1H
OCH_3-31	3.36-3.35 (major and minor rotamers)	3H
O=C-$\underline{CH_3}$-32	2.03-2.02 (major and minor rotamers)	3H

- 24, 32 Diacetate **4**.



Attribution	**^{1}H (ppm)**	**Integration**
CH_3-25	1.12 and 1.11 (major and minor rotamers)	3H
H-25	2.18 and 2.42 (major and minor rotamers)	1H
H-24	5.50 and 5.65 (major and minor rotamers)	1H
H-26	5.47 and 5.52 (major and minor rotamers)	1H
O=C-$\underline{CH_3}$-24	2.05 and 2.07 (major and minor rotamers)	3H
H-28	5.40 and 5.51 (major and minor rotamers)	1H
H-29	2.42 (major and minor rotamers)	1H
H-30a	1.20 (major and minor rotamers)	1H
H-30b	2.15 (major and minor rotamers)	1H
H-31	3.34 (major and minor rotamers)	1H
H-32	4.97 (major and minor rotamers)	1H
OCH_3-31	3.36-3.35 (major and minor rotamers)	3H
O=C-$\underline{CH_3}$-32	2.02 (major and minor rotamers)	3H

- 24-Monoacetate **5**



Attribution	**^{1}H (ppm)**	**Integration**
CH_3-25	1.13 and 1.11 (major and minor rotamers)	3H
H-25	2.19 and 2.42 (major and minor rotamers)	1H
H-24	5.50 and 5.65 (major and minor rotamers)	1H
O=C-$\underline{CH_3}$-24	2.05 and 2.07 (major and minor rotamers)	3H
H-26	5.48 and 5.52 (major and minor rotamers)	1H
H-28	5.44 and 5.45 (major and minor rotamers)	1H
H-29	2.44 (major and minor rotamers)	1H
H-30a	1.18 (major and minor rotamers)	1H
H-30b	2.15 (major and minor rotamers)	1H
H-31	3.21 (major and minor rotamers)	1H
H-32	3.68 (major and minor rotamers)	1H
OCH_3-31	3.46-3.44 (major and minor rotamers)	3H

2.8.References

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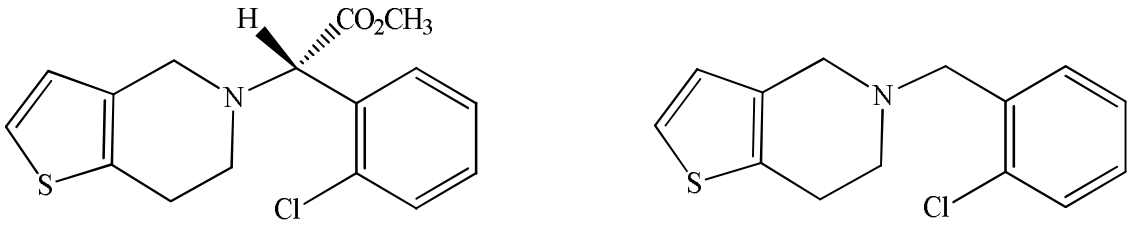
3. THE ANTITHROMBOTIC CLOPIDOGREL.

3.1 Introduction

Despite many recent advancements in the treatment of coronary hearth disease, it continues to be an important source of patients morbidity and mortality as well as a significant source of healthcare-related expenditures. Clopidogrel **1**, Figure 3.1**,** an antiplatelet thienopyridine, has become an integral part of the management of acute coronary syndromes (ACS) and, consequently, has become one of the world's best selling drugs. Numerous trial have demonstrated the clinical efficacy of clopidogrel in revuscalized and unrevuscalized ACS including unstable angina and myocardial infarction. As a results of trial, the use of clopidogrel has been incorporated into the American College of cardiology/American Hearth Association guidelines.[1]



1. *Clopidogrel* **2**. *Ticlopidine*

Figure 3.1. Antiplatelet thienopiryridines.

3.2 Platelet activation

In order to better understand the correlation between clopidogrel's mechanism of action and the final cardioprotective role, is essential to think back over the ethiopatology of the coronary manifestation. The role of platelets in the pathophysiology of atherosclerosis and atherothrombosis that always evolves in artherial thrombosis is well-established; artherial thrombosis frequently causes acute and irreversible damage or infarction of target organs, most notably the heart and the brain, leading to death or permanent disability. Arterial thrombi are composed predominantly of plateles and relatively little fibrin or red cells and for this are called "white thrombi". It has been established that antiplatelet agents, rather than anticoagulants should be the primary strategy for the treatment and prevention of arterial thrombosis. Actually, anticoagulants are effective in preventing acute myocardial infarction in patients with unstable angina or previous myocardial infarction and, to a lesser extent, in preventing stroke in patients with cerebrovascular disease.[2]

As shown in Figure 3.2, the monolayer of endotelian cells that normally lines the intima of the entire circulatory tree is a thromboresistant surface that secrets potent, locally-acting platelet inhibitory products. The two best characterized endothelium-derived platelet inhibitors are prostacyclin (PGI_2) and nitric oxide (NO), which inactivate platelets by raising intraplatelet cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels, respectively. The physiological role of these autacoids is to preserve blood fluidity and, moreover, to relax smooth muscle of the vessel wall.

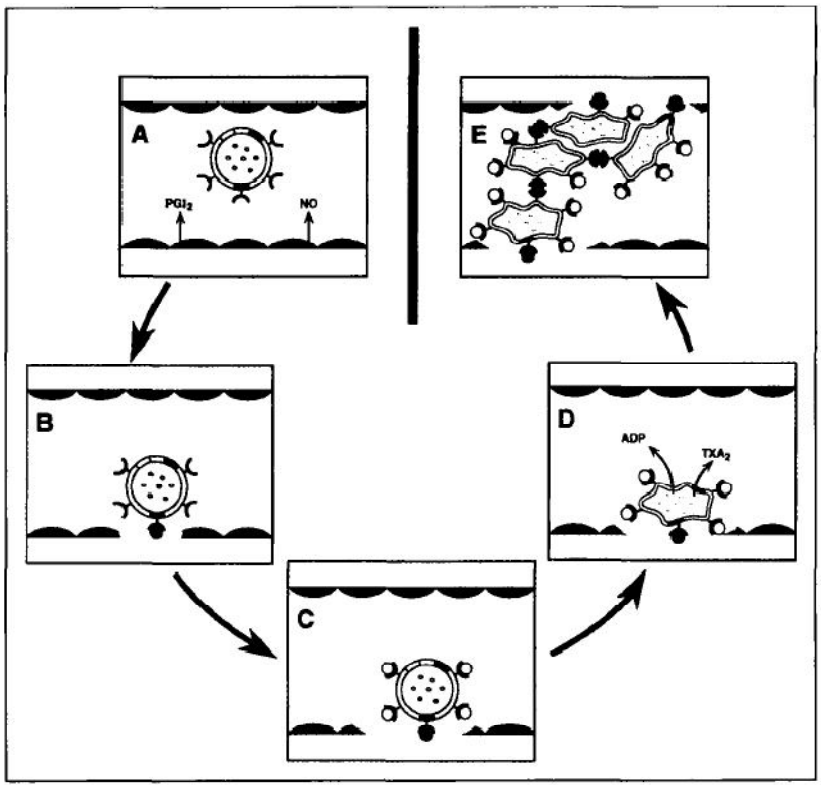


Figure 3.2. Mechanism of platelet activation at a site of vascular injury. (A) Release of platelet inhibitory products of endotelial cells, including prostacyclin (PGI_2) and nitric oxide (NO), maintains quiescent state of platelets traversing intact vessels. (B) Disruption of endothelium exposes thrombogenic subendothelial vessels wall costituents (eg, collagen); platelets adesion to this site is mediate by von Willebrand factor (vWf) binding to its platelet receptors localized in membrane glycoprotein (Gp)lb. (C) Other platelet activators (eg, thrombin, epinephrine) bind to their specific platelet receptors. (D) Platelets activated by these stimuli degranulate and undergo the release reaction, releasing ADP and thromboxane A_2 (TXA_2); ADP and TXA_2 bind to their respective platelet receptors to further amplify the platelet activation process. (E) Platelet aggregation is mediated by fibrinogen or vWf binding to platelet membrane Gp IIb/IIIa, a functional heterodimeric receptor which is expressed on the surfaces of only activated platelets.

When occurs a vascular injury, as shown in Figure 3.2B thromboresistant endothelium is disrupted and prothrombotic subendothelian vessel wall costituents (eg, collagen) are exposed to blood. Circulating platelets recognize this intimal breaks and undergo the process of “adhesion”, in which a carpet of platelets become anchored to the vessel wall. The ligand of adesion is von Willebran

factor (vWf), which binds to specific platelet receptors localized in membrane glycoprotein (Gp) Ib.

A variety of other platelet stimuli (eg, collagen, thrombin, epinephrine, serotonin) can simultaneously bind to their specific platelet surface receptors (Figure 3.2C) and they function in concert to trigger a cascade of intracellular reactions that lead to the activation and subsequent aggregation of platelets. Fluids shear stress can also directly activate platelets. Activated platelets undergo the release reaction (Figure 3.2D), secreting preformed substances (eg, adenosine diphosphate (ADP) from their intracellular storage granules.[3] Platelet activation also induces phospholipase A_2 (PLA_2)-mediated hydrolysis of free arachidonic acid (AA) from membrane phospholipid pools (Figure 3.3). Free AA is then rapidly metabolyzed by cyclooxygenase (COX) to the labile prostaglandin endoperoxides, PGG_2 and PGH_2 and then by thromboxane synthase (TxS) to thromboxane A_2 (TXA_2). Released ADP and PGH_2/TXA_2 bind to their respective platelet receptors to further amplify the platelet activation process. Platelet activation (and vasoconstriction) by TXA_2 is antagonized in the presence of intact endothelium by platelet inhibitory and vasodilatory PGI_2, which is produced in endotelial cells through prostacyclin synthase (PS) from either endothelium-derived or platelet-derived endoperoxides.

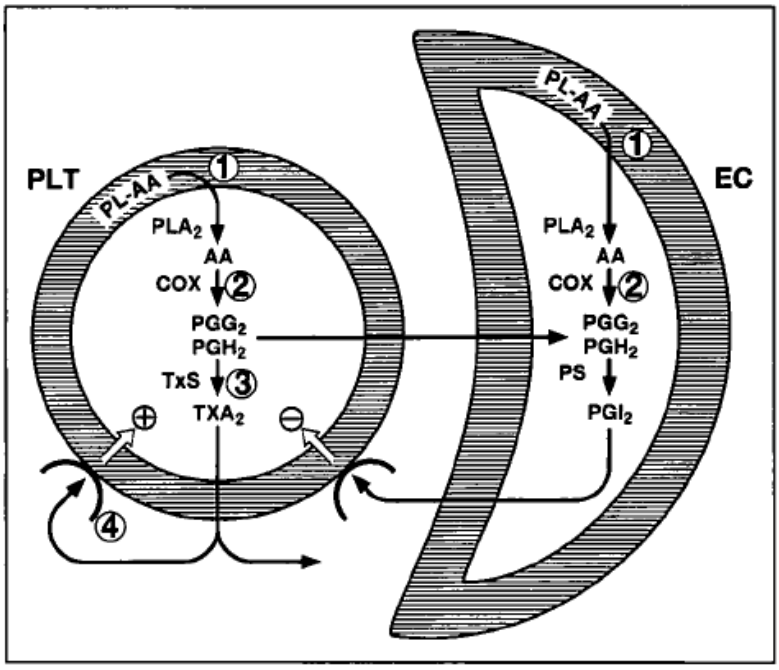


Figure 3.3 Platelet (PLT)-endothelial cell (EC) interactions mediated by arachidonic acid (AA) metabolites. Activators of each cell type induce the phospholipase A_2 (PLA_2)-mediated hydrolysis of the AA from the membrane phospholipid (PL) pools (1) . Arachidonic acid is converted in each cell type by cyclooxygenase (COX) to prostaglandine endoperoxides, PGG_2 and PGH_2 (2). Endoperoxides are metabolized to thromboxane A_2 (TXA_2) by thromboxane synthase (TxS) in platelets and to PGI_2 by prostacyclin synthase and (PS) in endothelial cells (3). TXA_2 binds to platelet receptors to stimulate platelet activation (4) ; PGI_2

binds to separate platelet receptors to inhibit platelet activation. Endothelial cells can also utilized platelet-derived endoperoxides as substrates for PS.

Finally, activated and degranulated platelets attach to each other in the process of aggregation to form an occlusive thrombus at the site of vascular damage (Figure 3.2E). The ligand for platelet aggregation is fibrinogen (or vWf in higher shear stress regions of the circulation). The platelet receptor for these aggregating ligands is exposed only on the surfaces of activated platelets by the complexing of GpIIb and GpIIIa to form the GpIIb/IIIa complex. The binding of fibrinogen or vWf to their platel receptor GpIIb/GpIIIa receptor is mediated by the tripeptide amino acid sequence, arginine-glycine-aspartic acid (abbreviated "RGD"), a motif which is present on both of these adhesive molecules. [4]

3.3 Antiplatelet agents

Each of the steps described above has been targeted in the development of antiplatelet agents.

In order to maintain high level of platelet cAMP and cGMP, intravenous infusion of prostacyclin may have transient beneficial effects in coronary artery diseases but its systemic use is limited by its potent vasoactive effects and extreme lability.

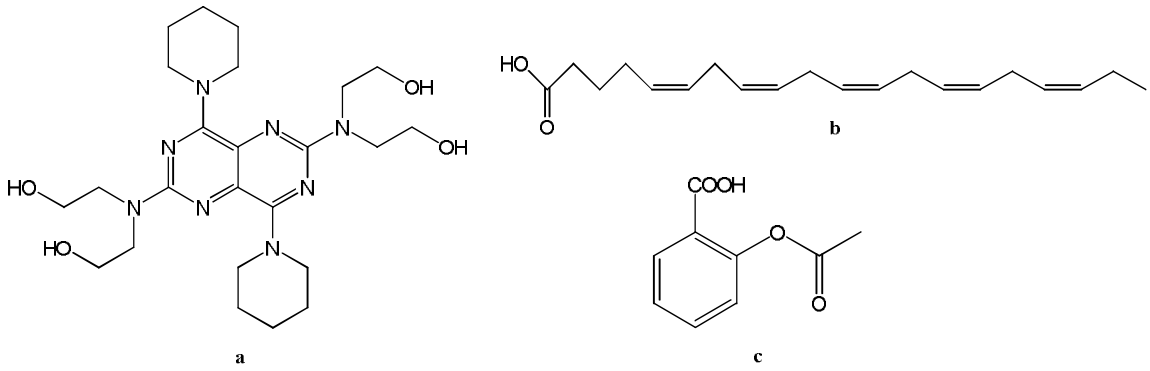


Figure 3.4. Compounds used in antiplatelet theraphy.

Dipyridamole, (compound a in Figure 3.4), a coronaric vasodilatator, has been suggested to act as an antiplatelet drug by several possible mechanisms. It directly stimulates prostacyclin synthesis, potentiates the platelet inhibitory action of prostacyclin, inhibits phosphodiesterase to raise platelet cyclic AMP levels and blocks the uptake of adenosine into vascular and blood cells, thereby causing accumulation of this platelet-inhibitory and vasodilatory compound in the thrombotic environment. Its side effects, include dose-related gastrointestinal symphtoms, headache, together with its variable absorption kinetics, does not support the use of dipyridamole as an antiplatelet agent.

Several antiplatelet strategies have been based on interruption of platelet TXA_2 synthesis and/or action. Drugs can block platelet arachidonic acid (AA) mobilization, AA oxygenation by COX, thrombaxane synthase and TXA_2 receptor.

Evidences about reduced incidence of atherosclerotic in population eating marine lipids-rich diets, such as Greenland Eskimos, pointed out the antiplatelet activity of omega-3 fatty acid. Therefore, the major omega-3 fatty acid in fish oils is eicosapentaenoic acid (EPA)(compound b in Figure 3.4). The antithrombotic alterations in platelet-vascular interaction attributed to fish oils are due to incorporation of EPA into cell-membrane phospholipids, leading to competition between EPA and AA as substrates of COX. The COX methabolism of AA in platelets produces the platelet-activating and vasoconstrictor TXA_2, whereas the product of EPA metabolism is TXA_3, biologically inert.

The most extensively studied antiplatelet agent for stroke prevention certainly is Aspirin, (compound c in Figure 3.4), acetyl salycic acid, that irreversibly inactivates COX by acetylating serine residues at position 529, leading to inhibition of platelet TXA_2 synthesis. Moreover aspirin blocks the production of prostacycline, the major COX metabolite of AA in vascular endothelial cells. Therefore the half-life of aspirin is 20 minutes, its antiplatelet effect go on for 4-7 days, as the life time of the irreversible acetylated platelet. Aspirine is effective for primary and secondary prevention of myocardial infarction, stable and unstable angina, coronary arthery bybass graft patency, mechanical or high-risk tissue valves (when added to warfarin) and prevention of stroke or recurrent TIA. Aspirin is also possible effective in peripheral vascular disease and to prevent shunt thrombosis and spontaneous abortion in patients with lupus anticoagulant.

Another important approach developed to achieve an antiplatelet effect is the use of compound able to interact and to inhibit platelet agonist-receptor. A theoretical limitation of inhibitors of individual platelet agonist-receptor interactions is their ability to block the initiation of only one of several pathways of platelet activation. Perhaps surprisingly, therefore, some of these agents have proven to be experimentally and clinically effective as antiplatel therapy.

Of the platelet-derived agonists, trombaxane A_2 receptor antagonist and ADP receptor blocker have received the most attention. ADP is present in high concentration in the dense granules within platelets and can initiate and reinforce aggregation after secretion of these granules. Ticlopidine **2**, and its analog, clopidogrel **1**, are thienopyridine derivatives that exert their antiplatelet action by inhibiting the binding of ADP to its platelet receptor and thereby inhibiting ADP-induced platelet aggregation.

3.4 From ticlopidine to clopidogrel

Ticlopidine **2** was discovered in 1972 through a screening tests performed in vivo while looking for anti-inflammatory compounds. The discovery of ticlopidine's platelet antiaggregant properties led to its development as an antithrombotic drug some years later. In 1978 ticlopidine was introduced into the market in a very narrow therapeutic indication: prevention of thrombosis during extracorporeal circulation. Besides the wanted therapeutical effect, ticlopidine's adverse effect profile include nausea, dyspepsia e diarrhea in more than 20% of patients; 1% of the treated patients was affected by neutropenia and thrombocytopenia.[5]

Clopidogrel **1** is a second generation thienopyridine antiplatelet agents and was first filed in 1987. In its molecule, the presence of a methoxycarbonyl group on the benzylic position provides an increased pharmacological activity and better safety and tolerability profile compared with those seen with ticlopidine.[6]

3.5 Mechanism of action and pharmacokinetics.

Both ticlopidine **2** and clopidogrel **1** exhibit a platelet antiaggregant effect only "in vivo", while "in vitro" they are inactive. In particular for clopidogrel, this effect occurs only after two hours after a single oral or intravenous administration. This behavior suggested that clopidogrel is, really, a prodrug that acts only after its metabolic activation. After its oral absumption, an amount of clopidogrel is rapidly converted to an inactive carboxylic acid metabolite after absorption from the gastrointestinal tract. Citocrome YP-mediated hepatic metabolism is required to produce the acting antiaggregating moiety, i.e. the acid **4a** showed in the Figure 3.5.[7]

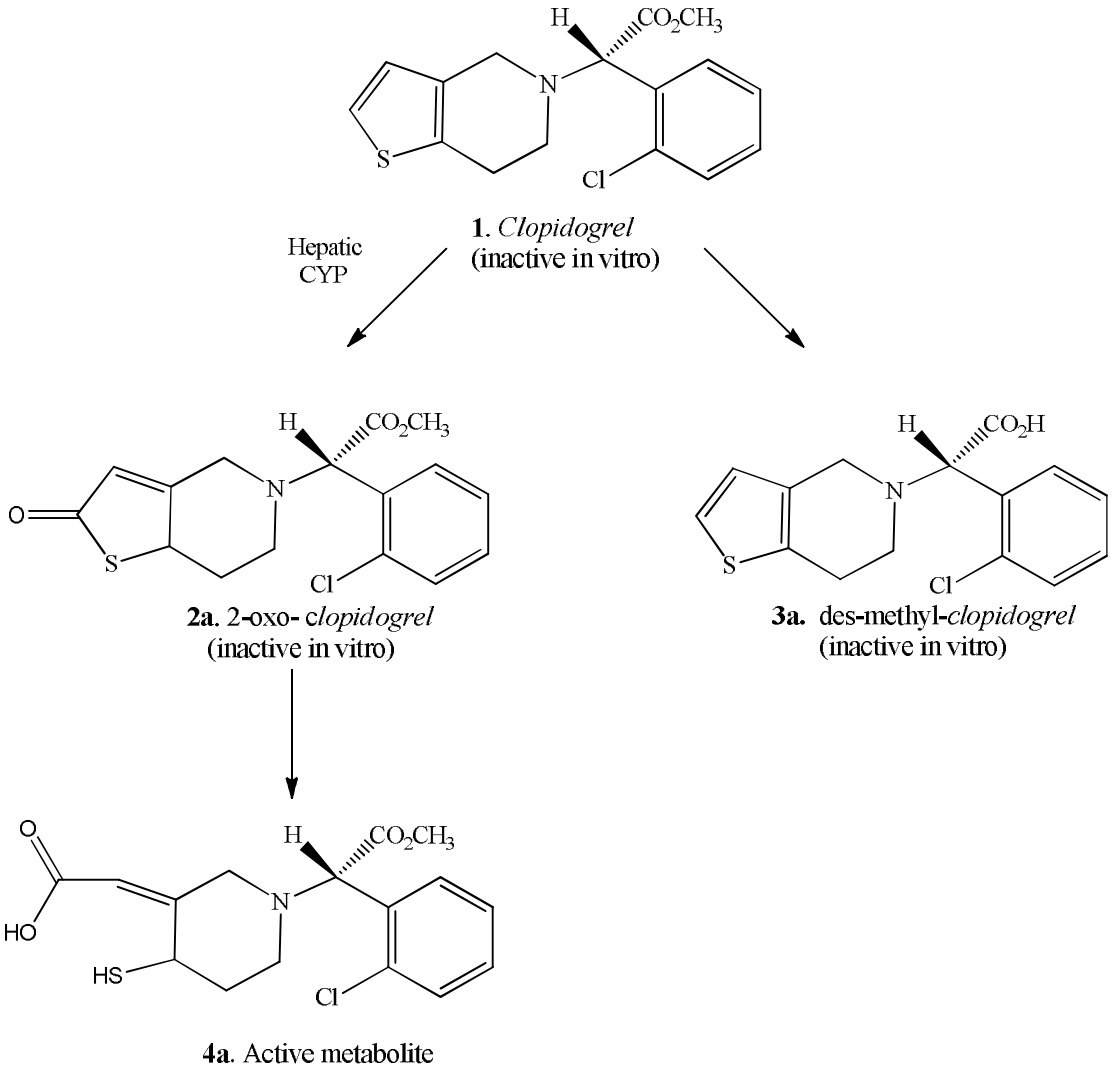


Figure 3.5. Metabolism of clopidogrel in vivo.

Because of the requirement for *in vivo* activation the pharmacodynamic properties of the drug have been characterized in *ex vivo* studies in animals and humans. After metabolic activation, the pharmacological effect of clopidogrel are mediated by P2-purino-ceptors located on the surface of platelets. Different subtype of P2- purinoceptors are located on the surface of platelet's membrane and involved in the mechanism of platelet aggregation, $P2Y_1$ and $P2Y_{12}$, that act in synergy to trigger platelet activation, being ineffective alone. The putative clopidogrel-sensitive purinoceptors is $P2Y_{12}$ and is thought to be linked to adenylyl cyclase through an inhibitory G-protein. As mentioned above, ADP is a platelet activator that is relased from damaged endothelial cells, red blood cells, and activated platelets that induce platelet aggregation adhesion. The binding of the active metabolite of clopidogrel, the thiol derivative, to the $P2Y_{12}$ purineceptor subtype, lead to inhibition of downregulation of adenylyl-cyclase through a protein G_i-dependent pathway and a resultant inhibition in the fall in intracellular cyclic adenosine monophosphate (AMP) level. Clopidogrel also inhibits other intracellular pathways including phosphatidylinhositol triphosphate (PI3) kinase and RhoA/Rho-kinase downstream of $P2Y_{12}$ activation.

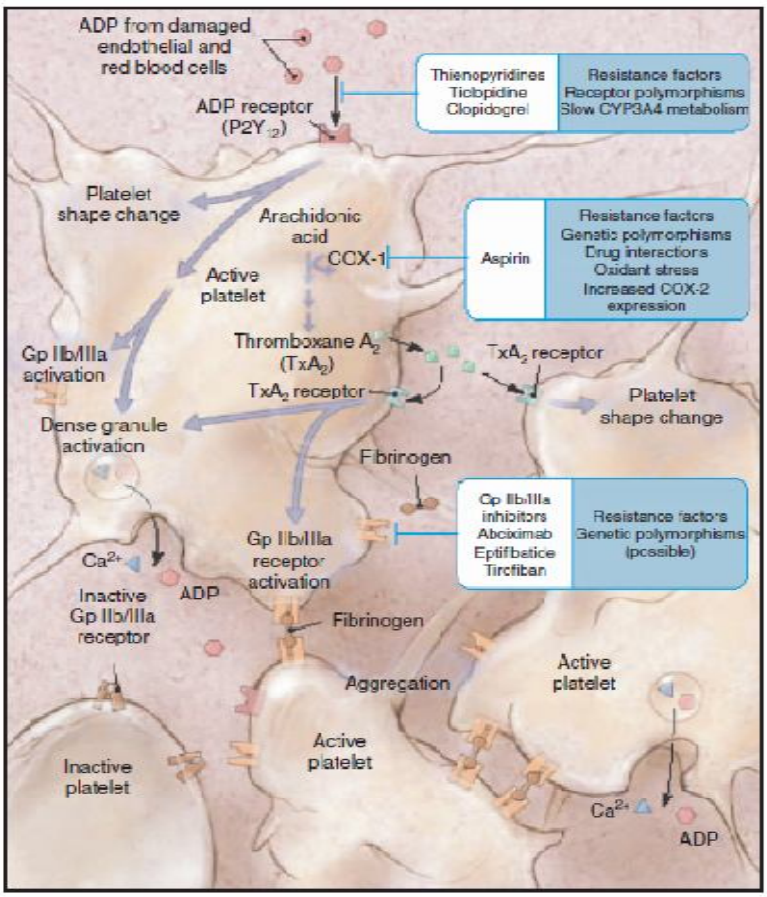


Figure 3.6. Mechanism of action of different antiplatelet agents. Theinopyridines inhibits the stimulation of the adenosine diphosphate (ADP) receptor, a G-protein-coupled purinergic receptor ($P2Y_{12}$). Aspirin (acetylsalicylic acid) inhibits cyclo-oxygenase (COX-1) and therefore thrombaxane A_2 (TxA_2) production with resultant decreased stimulation of the TxA_2 receptor. Glycoprotein (Gp)IIb/IIIa inhibitors directly inhibits platelet aggregation at the receptor which thereby inhibits the final event in the pathway of platelet aggregation, the finding of fibrinogen by activating the (Gp)IIb/IIIa receptor. The boxes to the right of the drugs indicate potential mechanisms of limited drug effectiveness.

Additionally, clopidogrel also has other important effects on several proinflammatory events. It inhibits release of CD40 ligand from platelets and reduces the expression of P-selectine, which is a membrane glycoprotein that is stored in platelet granules and promotes adhesion between platelets and leukocytes. Finally, clopidogrel inhibits the ADP-mediated effects of amplifier of other agonists of platelet aggregation including collagen and thrombin. [8]

The pharmacokinetics of the drug was studied by determining the fate of radiolabel after administration of [^{14}C]-clopidogrel in healthy young male volunteers and showed that the inactive carboxylic acid is the major circulating methabolite that could be detected in the plasma less than 1 hour after oral administration of clopidogrel while the inactive parent methylester, is below the limit of quantification (0.0025 mg/L) beyond 2 hours after administration of a repeating oral dose of 75 mg. The adsorption of clopidogrel dose occurres for the 50% from the gastrointestinal tract.

The thieno-active methabolite of clopidogrel was isolated after exposure of clopidogrel or 2-oxo clopidogrel to human epatic mycrosomes; clopidogrel does not share any common metabolites which ticlopidine. Clopidogrel and its de-esterified metabolite are reversibly and highly (98 and 94%, respectively) bound in an non saturable manner (at concentration ≤ 100 mg/L) to human plasma proteins *in vitro*. The half life of clopidogrel is 8 hours with excretion via urine and feces. Administration of clopidogrel with food or antiacids does not significantly alter the bioavailability of the drug and a low propensity to drug-drug interaction was found.

3.6 Clinical applications

Clopidogrel is one of the most widely prescribed drugs in the world, for the sodministration to patient that need antiplatelet theraphy and when drug-eluting stents for which dual antiplatelet theraphy may be needed for a number of years.

Clopidogrel was US FDA approved in 1996 primarily based on data from the CAPRIE trial (Clopidogrel versus Aspirin in patients at Risk of Ischaemic Events). At that time, clopidogrel represented a new thienopyridine which was similar to ticlopidine, whose efficacy when used alone or with aspirin was widely demonstrated. However, its use was limited by potentially unfavorable adverse effects including diarrhea, nausea, vomiting, skin rush and neutropenia which did result in several fatalities. The CAPRIE trial enrolled 19 185 patients and compared the efficacy of clopidogrel 75 mg/day versus aspirin 325 mg/day in patients with atherosclerotic vascular disease: recent ischemic stroke, recent myocardial infart, and symphtomatic peripheral arterial disease. Clopidogrel showed to be superior to Aspirin in all this deseases, for all the population treated.

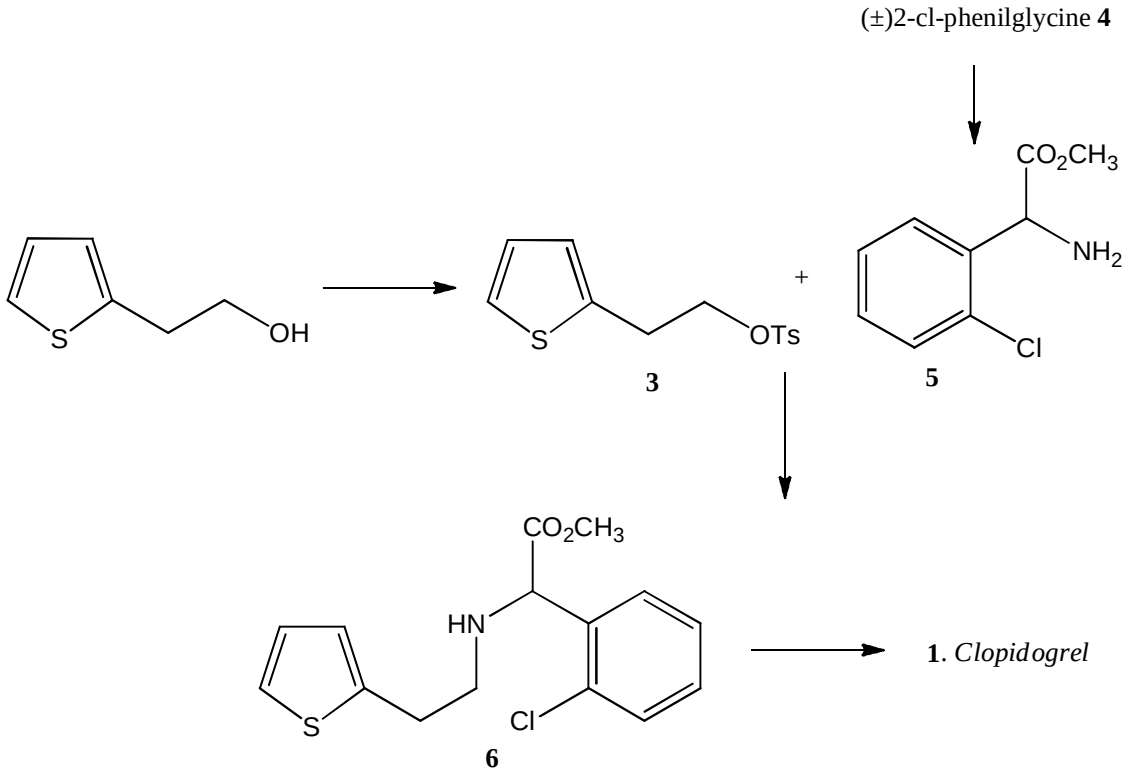
In their article about the issue of the *American Journal of Cardiovascular Drugs*, Collins and Gurm review many of the clinical trials that have demonstrated the clinical efficacy of clopidogrel in patients with acute coronary syndromes, especially in myocardial infarction and unstable angina. Nowadays Clopidogrel in sominister ed in association with aspirin after placement of drug eluiting stent and for the treatment of patients with acute coronary syndromes.[9]

Clopidogrel is being further evaluated in a range of atherothrombotic clinical settings such as the treatment of high-risk patients with recent transient ischemic attack or ischemic stroke.

3.7 Aim of the work

The structure of clopidogrel, as shown in the Figure 3.1, contains a stereocenter whose (S) - configuration is important to preserve in order to obtain the desiderated pharmacological effect. In view of this, in a preparative approach is important to focus on the different methods we have to introduce this stereogenic carbon, that is nowadays object of studies and justifies the critical issue of cost. The most common methods to achieve the product enantiopurity are the fractional

crystallizations of the precursor (RS) 2-chlorophenylglycine **4** [10], [11] or the final compound (RS) **1**, with an optically pure acid, such as (1R)-camphor sulfonic acid or L(+) tartaric acid. The so-obtained diastereomeric salts were then subjected to a series tedious and time consuming fractional crystallizations to increase the enantiopurity of the recovered precipitate; then, the removal of the acidic component by a basic extraction, afforded often the desiderate stereoisomer with unsatisfied factory yields. [12], [13]



Scheme 3.1. One of reported pathways to obtain clopidogrel.

The main objective of this work, was to obtain (S)- clopidogrel **1**, in a fast, economic manner, that could afford the expensive thienopyridine with a very high enantiomeric excess, by the use of a biocatalyzed resolution. Analyzing the clopidogrel structure, two moiety can be easily recognized: the thiophen-ethanol and the 2-chloro phenyl-glycine; a nucleophylic substitution between tosilate **3** and methylester **5** should, then, be a fast and easy way to obtain the clopidogrel skeleton being both the molecules easily prepared from commercially available, not expensive compounds. The obtained intermediate **6** could be then transformed into the final target by treatment with a formaldehyde source. (±)

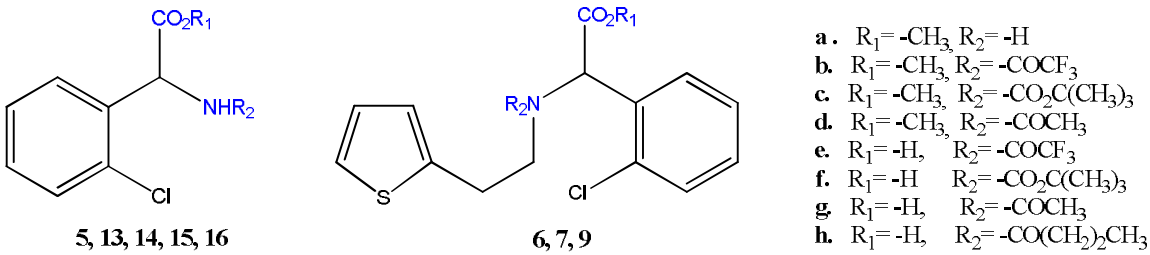


Figure 3.7. Substrates of enzymatic resolution.

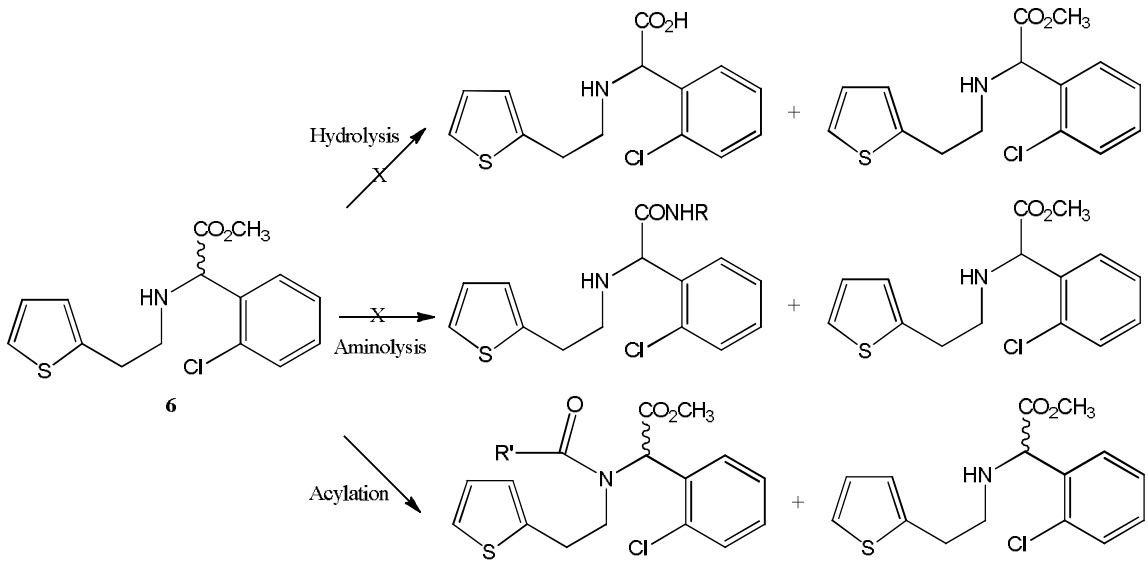
Since in the course of an enzymatic resolution usually 50% of substrate has to be discarded, we decided to focalize our attention on the less precious precursor (±) 2-chloro-phenylglycine methyl ester **5**, easily prepared from the commercially available **4**, and on the key intermediate ***(±)***-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate **6**, appropriately derivatized as showed in the Figure 3.7. In fact, both the compound are bifunctional molecules, providing two possible site of enzymatic attach: the methyl ester and the primary or secondary aminic group. Among the enzymes, were selected proteases, whose proteins and aminoacids are the natural substrates, esterase and chymotrypsin, for their ability to recognize ester moiety and lipases because their versatility to adapt to a wide range of different functional groups. Beside the screening of the enzymatic reaction, including the selecting of substrate, and the setting of the biocatalysts in the best conditions for they activity, the second critical aspect of the work will be the checking of the enantiopurity of all the intermediates. These because the presence of a benzylic proton at the α-position makes aryl-glycines recemization prone.[14]

3.8 Results and Discussion

a) Enzymatic screening on derivatives of compound 6

From the intermediate **6**, we started the enzymatic screening, mainly because provided a new substrate to explore the behaviour of the different classes of enzymes. The first attempts were done directly on the intermediate **6**, avoiding time consuming derivatization.

The bi-functionality of the compound, allowed us to test both the hydrolysis of the methyl-ester moiety and the acylation of the secondary aminic function (Scheme 3.2).



Scheme 3.2. Enzymatic screening on intermediate **6**.

Entry	Enzyme	Solvent	Time(h)	Conversion (%)*
a	CAL B	*tert*-butylmethylether, H_2O 8 eq	25	0
b	CAL B	THF, H_2O 8 eq	48	0
c	CAL B	*tert*-butylmethylether, 20% buffer pH 7.6	48	0
d	α-Chymotripsin	buffer phosphate pH 7.6	48	10
e	α-Chymotripsin	buffer phosphate pH 7.6, DMF 20%	120	Traces
f	α-Chymotripsin	Acetonitrile, H_2O 4 eq	48	0
g	CCL	Toluene , H_2O 4 eq	48	0
h	PFL	THF, H_2O 4 eq	48	0
i	PPL	buffer pH 7.6	48	Traces
l	Papain	buffer phosphate pH 7.6, DMF 20%	60	0
m	Protease from *Bacillus licheniformis*	Acetonitrile, H_2O, TEA (9:1:0.8)	60	0
n	CAL A	Dioxane, butylamine, 1 eq.	70	0
o	CAL B	Toluene, butylamine, 1 eq.	70	0
p	CCL	Hexane, butylamine, 1 eq.	70	0

Table 3.1. Condition for methylester hydrolysis on substrate **6**.

*By tlc analysis

A wide range of enzymes are reported to hydrolyze an ester: first of all the lipases, for whom the triacylglycerols are the natural substrates and then the esterases, and α-chymotripsin and proteases endowed with esterase activity. Considering the low polarity of the substrate, all the reaction were carried on in a mixture of water, necessary to act as the nucleophilic compound that accept the acyl moiety, and a co-solvent.The choice of the cited cosolvents, arises from the previous results reported in literature; the use of only slightly alkaline medium is suggested in order to prevent the non-enzymatic ester hydrolysis.[15] As described in Table 3.1 no one enzyme accepted the ester as substrate, with the exception of α-chymotripsin and PPL. The slowness of α-chymotripsin hydrolysis was in agreement with the reported resolution of (±) *threo*-methylphenidate, structurally similar to compound **6**.[16] Further attemps to increase the rate of reaction by solubilization of the starting material with a cosolvent failed.

We also attempted to carry on the hydrolysis of the ester employing as nucleofile acceptor buthylamine, with lipases in the most suitable solvent for one of them, following the protocols reported by Gotor.[17] No activity was detected by tlc and NMR analyses. (entry n-p, Table 3.1)

The negative outcomes obtained working on the methyl-ester moiety, suggested us to select the acylation of the secondary aminic function as biocatalyzed reaction. This project was supported by a wide range of successful examples of resolution of secondary amines by acylation with lipases, in particular by lipase from *Candida antarctica* that seems to prefer hindered amine and amino esters.[18] Our results are in agreement with these note knowledge: the acylation was obtained only with CAL A, but in a long time and without enantioselectivity (see Table 3.2).

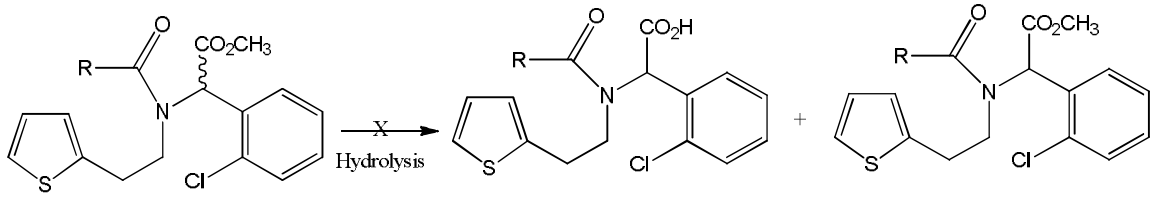
Entry	Enzyme	Solvents	Time (h)	Conversion* (%)	ee*%
a	CAL B	Vinylacetate, *tert*-butylmethylethere	90	0	-
b	CAL B	Vinylacetate, toluene	48	0	-
c	CAL B	Trifluoro-ethylbutyrate DIPE	120	0	-
d	CAL B	Trifluoro-ethylbutyrate *tert*-butylmethylether	120	0	-
e	CAL B	ACOEt	120	0	-
f	CAL A	ACOEt	120	20	0

g	CAL A	Vinylacetate	120	30	0
h	CAL A	Trifluoro-ethylbutyrate *tert*-butylmethylether	120	56	0
i	CAL A (supported on celite and saccharosium)	Vinyl-butyrate	120	0	-

Table 3.2. Condition for acylation on substrate **5**. *By chiral Hplc analysis

Thinking that these discouraging results on the intermediate **6**, were probably imputable to the presence of two functional groups in the compound that could interact, we decide to protect the aminic function as acetyl amide and tert-butoxycarbonyl, respectively compounds **7** [19] and **9**, and to screen again some of the above mentioned hydrolases.

Again no hydrolysis was observed.



R = - CH_3 **7**
R = -$OC(CH_3)_3$ **9**

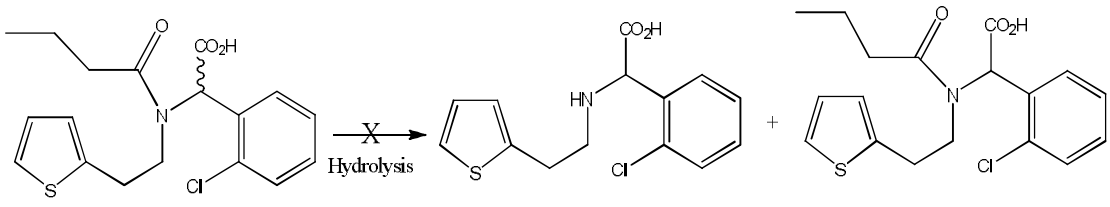
Scheme 3.3 Enzymatic screening in hydrolytic conditions on compounds **7** and **9.**

Compound	**Enzyme**	**Solvent**	**Time (h)**	**Conversion * (%)**
7	α-chimotrypsin	buffer pH 7.6	48	0
7	PPL	buffer pH 7.6	48	0
9	Protease from *Bacillus licheniformis*	buffer pH 8, 10%v/v acetonitrile	120	0
9	Protease Carlsberg	buffer pH 8, 20% TBME	120	0
9	PLE	buffer pH 8, 20% acetonitrile	120	0
9	PPL	buffer pH 8, hexane 20%	120	0

Table 3.3. Condition of hydrolysis on substrate **7** and **9**. *By chiral Hplc analysis

As possible explanation for the results exposed till now, we proposed that the flexible structure of the intermediate **6** and of all its derivatives **7, 9** didn't fit into the 2 hydrophobic pockets present in the active site of the hydrolases employed.

As last approach, we carried on the hydrolysis of the butyric amide on the derivate **10** employing acylases from different microorganisms.



10

Scheme 3.4. Enzyme-catalyzed hydrolysis on derivative **10**

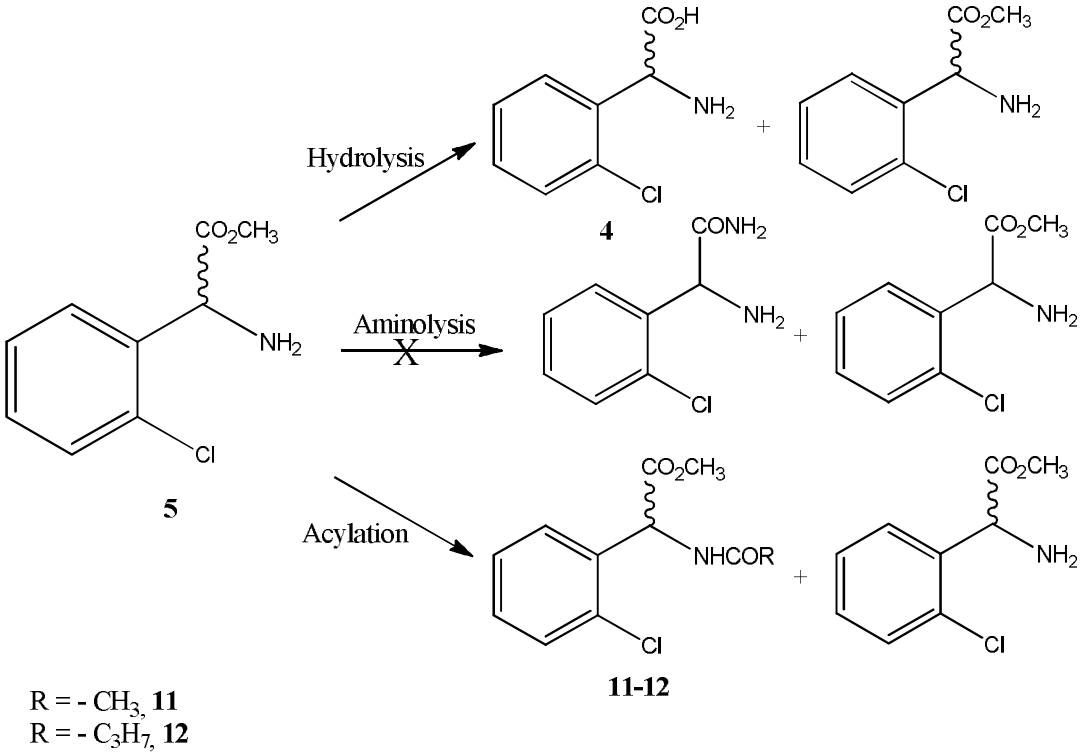
Entry	Enzyme	Solvent	Time (h)	Conversion* (%)
a	Acylase from *Aspergillus melleus*	buffer pH 7.5	120	0
b	Acylase from *Streptomyces toyocaensis*	buffer pH 7.5	120	0
c	Acylase I from porcine kidney	buffer pH 7.5	120	0

Table 3.4. Aminolysis on substrate **10**. *By TLC analysis

b) Enzymatic screening on derivatives of compound 5

The study on derivatives **5** started, fist of all, examining the numerous publications dealing with the enzymatic resolution on amino acids, considering that their are important building blocks in the pharmaceutical field for the production of semisynthetic antibiotics, peptides and pesticides[20]. In particular, our analysis selected the works involving phenylglycine, the non proteogenic aminoacid sharing the most similarity with our substrate **4**. Only a few cases of 2-chlorophenylglycines resolutions are described: for example the resolution of the tioester of the racemic 2-chlorophenylglycine, protected on the aminic function as *tert*-butoxycarbonyl, by subtilisin Carlsberg [21] and the successful work, reported by Fadnavis et al, realyzed by employing immobilized penicillin G acylase.[22] The value of this attempt of resolution, is underlined not only by the possibility to obtain an optically pure compound in a previous step of synthesis of clopidogrel, with a gain on the final yield, but also because the comparison of these results with those regarding phenylglycine will be useful to better understand the influence of the chlorine ortho-substituent on the activity and selectivity of the enzymes. As in the case of compound **6**,

again we started from 2-chlorophenylglycine methylesther **5**, at first,in the same conditions tested above (Scheme 3.5).



Scheme 3.5. Enzymatic screening on derivative **5**.

Differently from what observed for the compound **6**, all the enzymes transformed the methyl ester into the free acid, even if with different rate and enantioselectivity. As described in the Table 3.5, all the reaction were done at room temperature, at first with only buffer phosphate, pH 7.5, and later in presence of 20% of co-solvent, always to increase the substrate solubility and hopefully the selectivity of the enzyme. As we expected, lipases were no enantioselective, probably for the well documented preference of organic medium: for this reason, following how reported for the same reaction on D,L-phenylglycine, the reaction was carried on also in the presence of proper concentration of the ionic liquid IL BMIM BF_4, capable of dissolving a wide range of substrates, expecially those high polar and sparingly soluble in common organic ones. As evident from the result, this ionic cosolvent could boost the activity and the enantioselectivity of the lipase, probably for its interaction with charge group of the enzyme's structure and making the enzyme more preferential for the preponderant enantiomer.[23] The result was different when α-chymotripsin was used: in agreement with the results obtained for the compound **6**, the enzyme afforded the best result with the only aqueous medium. Considering the global low degree of enantiopreference, a blance was done to verify that no chemical hydrolysis occurred.

Entry	Enzyme	Solvents	Time (h)	Conversion * (%)	ee %*(*S*)-**9**
a	CAL B	buffer pH 7.5, BMIM BF_4 (20% v/v)	12.5	60	ee_s = 30
b	CAL B	buffer pH 7.5	12.5	44	ee_p = 11
c	α-chymotripsin	buffer pH 7.5	4	42	ee_s = 67
c	α-chymotripsin	buffer pH 7.5 CH_3CN 20%	12	10	ee_p = 0
d	α-chymotripsin	buffer pH 7.5 EtOH 20%	6	13	ee_p = 60
e	α-chymotripsin	buffer pH 7.5 MeOH 20%	4	22	ee_p = 50
f	α-chymotripsin	buffer pH 7.5 BMIM BF_4 20%	4	17	ee_p = 12
g	α-chymotripsin	buffer pH 7.5 BuOH 20%	27	46	ee_p = 16
h	α-chymotripsin	buffer pH 7.5 propanol 20%	27	58	ee_s = 0
i	Protease from *Bacillus licheniformis*	buffer pH 7.5	12.5	88	ee_s = 27[a]
l	subtilisin	buffer pH 7.5	12.5	81	ee_s = 23[a]
m	PPL	buffer pH 7.5	12.5	77	ee_s = 24
n	CCL	buffer pH 7.5	12.5	56	ee_s = 0
o	PFL	buffer pH 7.5	12.5	66	ee_s = 17
p	Papain	buffer pH 7.5	12.5 h	39	ee_p = 0

Table 3.5. Enzymatic-hydrolysis on compound **5**.

*By chiral Hplc analysis. The enantiopreference in favour of the enantiomer *(S)* of the acid **4** was obtained by comparison with HPLC of enanantiomerically pure *(S)* **4** and *(S)* **5**.

[a] The *(S)* configuration belongs to unreacted ester **5**.

The HPLC analysis of hydrolysis on the compound **5**, showed that the enantiopreference of the screened subtilisins, was opposite to the one of all the above cited lipases. More in detail, while the lipases showed the expected prefential hydrolysis of the *S* enantiomer of the racemic ester, even if

with a low selectivity, for both the two tested subtilisins the weak enantiopreference was directed against the *R* enantiomer, as reported in the Table 3.5. This outcome could be explained by the reported observation that subtilisin-protease have the catalytic site arranged as the mirror image of lipase's catalytic site.[24]

Going on with the reactions on compound **5**, we screened the ability of lipases, CAL A, CAL B, CCL, to perform the ammoniolysis of the methyl ester using, as source of ammonia, ammonium carbamate (8 eq): this reactive replaces the role of ammonia that if used in excess could be detrimental for the enzyme. Again we followed the reported conditions for phenylglycine methyl ester.[25, 26] As described in the Scheme 3.5, the ammoniolysis didn't occur but was detected a chemical hydrolysis of the methyl ester that led to recover the free amino acid **4**.

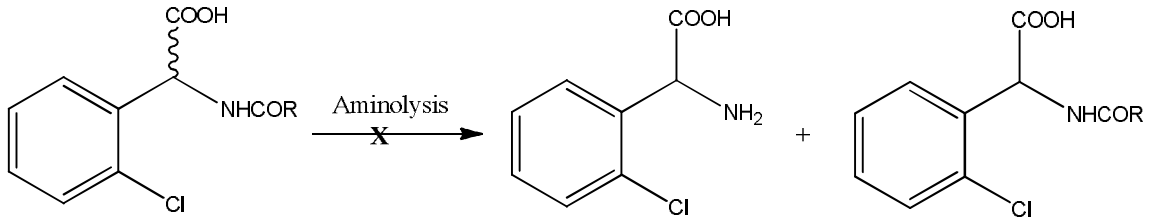
In the Table 3.6, the conditions of N-acylation on substrate **5**, using different acyl donor, from the most blande ethyl acetate to the activated trifluoroethyl butirrate, are collected. CAL A and CAL B were able to acylate the aminic function of the methylester **6** in organic medium, at room temperature, but, as reported for phenylglycine methylester with PPL and PFL, their action was no selective.

Entry	**Enzyme**	**Solvent**	**Time (h)**	**Conversion*(%)**	**ee*%**
a	CAL B	ACOEt	120	35	$ee_p = 0$
b	CAL B	Trifluoroethylbutyrate, DIPE	22	50	$ee_p = 0$
c	CAL A	Trifluoroethylbutyrate, DIPE	10	70	$ee_s = 0$
d	CAL A	ACOEt	78	20	$ee_p = 0$
e	Subtilisin from *Bacillus licheniformis*	Trifluoroethylbutyrate *tert*-BuOH	74	10	$ee_p = 0$
6	Subtilisin from *Bacillus subtilis*	Trifluoroethylbutyrate tert-BuOH	74	30	$ee_p = 0$

Table 3.6. Condition of acylation on substrate **5**.*By chiral Hplc analysis

The same acylation realized with protease from bacillus subtilis and licheniformis, didn't afforded better results on compound **5**, while, the same reaction has permitted to obtain the correspondent natural amino acids phenylglycine with an ee of 65%.[27]

Further investigations on the resolution starting from the amidic functions were conduct on the acetyl- and trifluoroacetyl derivatives **13** and **14** respectively. The most suitable enzymes to operate the hydrolysis of these two amide are acylases from *Aspergillus melleus, Streptomyces toyocaensis*, and porcine kidney: the hydrolytic reaction were performed in aqueous solution at pH 7.5 , which is close to the maximum catalytic activity of aminoacylase I.[28] In the medium was also added 1 mM of $CoCl_2$, an additive that seems to enhance the rate of reaction[29]. All the acylases didn't recognize the N-acetyl derivative **13** and, concerning the derivative **14**, the low hydrolysis of its N-trifluoroacetyl amide, 10% after 24 h, as reported in the Table 3.7, was probably due to a spontaneous chemical hydrolysis, as confirmed by the assence of enantiopurity in the recovered free amino acid. Then, we can conclude that neither amides **13** and **14** were not substrate of the examined enzymes.



R = CH_3, compound **13**
R = CF_3, compound **14**

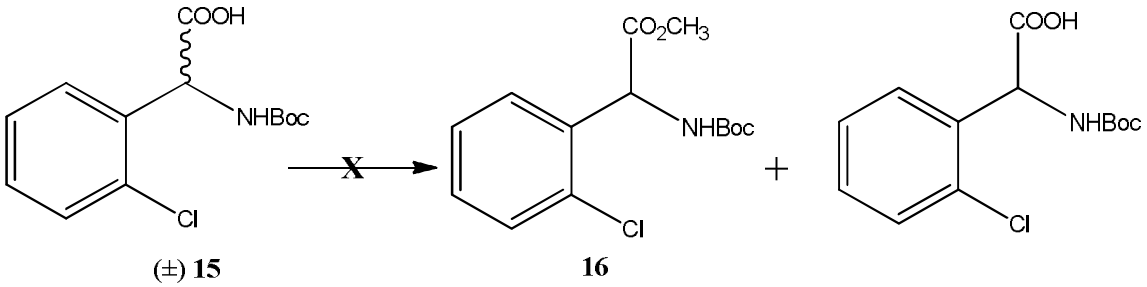
Scheme 3.6. Aminolysis on derivatives **13** and **14**

Compound	Enzyme	Solvents	Time (h)	Conversion* (%)	ee_p* %
13	Acylase from *Aspergillus Melleus*	buffer pH 7.5 $CoCl_2$	24	0	-
13	Acylase from *Streptomyces toyocaensys*	buffer pH 7.5 $CoCl_2$	24	0	-
13	Acylase I from PKA	buffer pH 7.5 $CoCl_2$	24	0	-
14	Acylase from *Aspergillus Melleus*	buffer pH 7.5 $CoCl_2$	24	10	0
14	Acylase from *Streptomyces toyocaensys*	buffer pH 7.5 $CoCl_2$	24	10	0
14	Acylase I from PKA	buffer pH 7.5 $CoCl_2$	24	10	0

Table 3.7. Conditions of aminolysis on derivatives **13** and **14**.*By chiral Hplc analysis

These preliminary negative results prompted us to go back again on the hydrolysis of the ester moiety, tha gave the best results in term of activity of the enzymes but in order to enhance their stereoselectivity we decided to protect the aminic function as a tert-butoxycarbonyl (Boc)

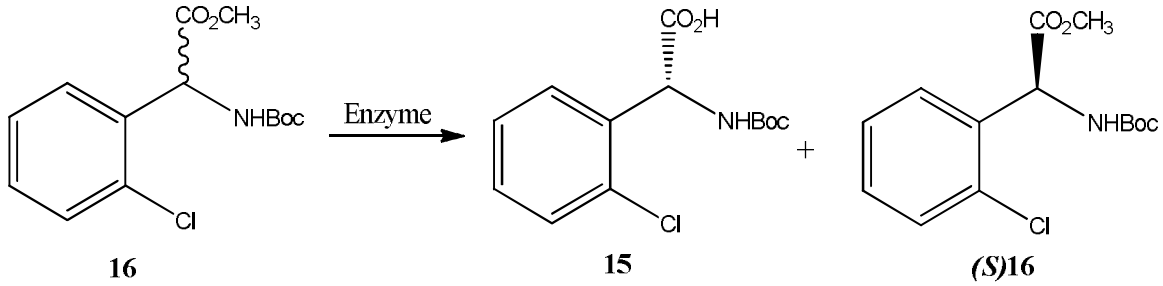
derivative. The choice of this protective group was due to its easy cleavage and reported wide acceptability by hydrolytic enzyme. Since for the clopidogrel synthesis we needed the (S)-methyl ester **5** and not the (S)-acid, as first approach, we tried to use subtilisin as biocatalyst of the opposite reaction *i.e.* the esterification of the racemic acid **15**: in fact, in this case, due to the enzyme (S)-enantiopreference, (S)-ester **16** should be obtained. Several examples of subtilisin-catalyzed esterification of aminoacids[30] or peptides[31] are reported but in our case only the starting material was recovered in all the tested conditions. Also papain and α-chymotripsin were tested, using dichloromethane as organic medium and MeOH as alcohol donor. A low activity was detected, 20 % of methyl ester formed after 5 days but without any enantiopreference. Since these results could be due to the negative effects of methanol on the enzyme[25] we tried to use a known more stable preparation of subtilisin, the cross-linked enzyme aggregation (CLEA) commercialised (Novozymes) with the Alcalase-CLEA® name [26-28] but also in this case no esterification of **15** to **16** was observed.



(±) **15** **16**

Scheme 3.7. Enzyme catalyzed esterification of derivative **15**.

However, the experimental advantages offered by the CLEA-subtilisin (*e.g.* the insoluble aggregate can be removed by simple paper filtration, avoiding tedious work-up of the obtained emulsions, when the free enzyme was used) prompted us to test this enzymatic preparation in hydrolytic conditions.



16 **15** ***(S)*16**

Scheme 3.8. Enzyme-catalyzed hydrolysis on derivative **16**.

Entry	Enzyme	Solvent	Time (h)	Conversion* (%)	ee* %
a	CAL B	buffer pH 7.5/TBME (2:1)	96	0	-
b	CCL	buffer pH 7.5 toluene 20%	96	0	
c	CAL A	buffer pH 7.5 hexane 20%	120	10	ee_p = 0*
d	α chymotripsin	buffer pH 7.5CH_3CN 20%	96	0	-
e	Protease from *Bacillus licheniformis*	buffer pH 7.5/TBME (2:1)	65	40	ee_p = 97.6
f	Protease from *Bacillus subtilis*	buffer pH 7.5/TBME (2:1)	65	40	ee_p = 96
g	PPL	buffer pH 7.5 hexane 20%	120	10	ee_p = 54
h	PFL	buffer pH 7.5 THF 20%	12	66	ee_s = 17
i	Papaina	buffer pH 7.5 hexane 20%	120	0	-
l	PLE	buffer pH 7.5 CH_3CN 20%	120	10	ee_p = 0
m	Alcalase CLEA	pH 8 buffer/CH_3CN 10/1	15	40	79
n	Alcalase CLEA	pH 8 buffer/DMF 10/1	16	40	65
o	Alcalase CLEA	pH 8 buffer/THF 10/1	14	40	98

Table 3.8. Condition of hydrolysis on derivative **16**.

*By chiral Hplc analysis

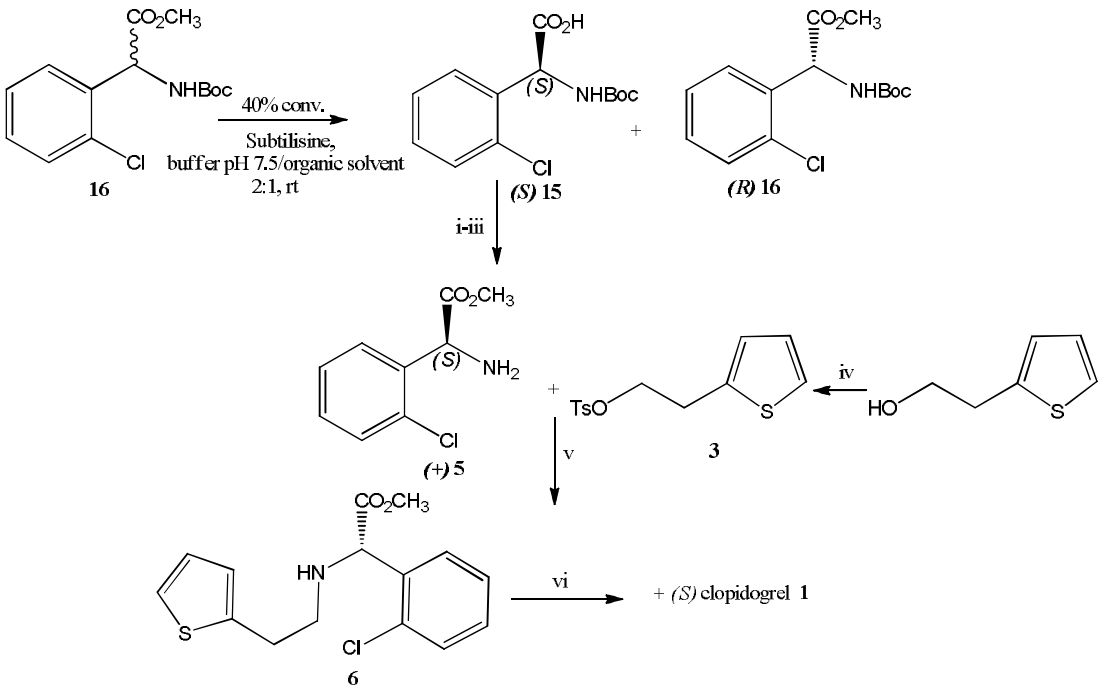
As showed in Table 3.8, among the tested lipases, only PPL showed a weak activity to hydrolyze the methylester **16** into the acid **15** while, surprisingly, CAL A, CAL B and CCL were no active: these results could be explained assuming that the bulk of the Boc compromise the access of the substrate in the active site of the enzyme. Also α-chymotripsin, which was the more promising enzyme in the case of unprotected methylester **5**, did not transform at all the derivative **16**. In agreement with how published for 2-chlorophenilglycine thioester and for several other amino acids, proteases succeeded to resolve the racemic **16**, affording the desidered acid (S)+ **15** in 40% of yield and an enantiomeric excess higher than 98%.

These satisfactory results were in agreement with the reported ones, by Arosio *et al.*, in a detailed study of the proteases-catalyzed hydrolysis of a series of arylglycines thioesters.[21] The ethylthioester, instead of a carboxyester, was chosen by the authors since suitable for a complete substrate deracemization through an *in situ* base-catalyzed continued racemization of R-isomer.

Use of bad smelling ethantiol was judged not suitable for large scale preparation purposes and, on the other hand, considering the low cost of (R,S)-2-chlorophenylglycin toward the final product value, the complete deracemization process was not so mandatory; in any case the (R)-isomer could be later racemized and recycled. The same reaction was also carried on with a known more stable preparation of subtilisin, the cross-linked enzyme aggregation (CLEA) commercialised (Novozymes) with the Alcalase-CLEA® name [32,33,34]. However, the experimental advantages offered by the CLEA-subtilisin (*e.g.* the insoluble aggregate can be removed by simple paper filtration, avoiding tedious work-up of the obtained emulsions, when the free enzyme was used) prompted us to test this enzymatic preparation in hydrolytic conditions. Among the examined organic solvents tetrahydrofuran afforded best results (>98% ee) while in acetonitrile and in dimethylformamide only 79% and 65% ee, respectively, were observed (Table 3.8, entries m-o). Use of cross-linked enzyme in THF/H_2O shortened also reaction time, the desired 40% conversion to (S)-acid 1**5** being reached after 14 h, instead of 63 h required in the case of free subtilisin in TBME/H_2O. The (S)-1**5** isomer was converted, by esterification (methanol, DCC, DMAP in dichloromethane) and removal of protecting group (TFA), into (S)-methyl ester **5** (90% yield), the suitable precursor for the preparation of clopidogrel **1**.

c) Preparation of *(S)*-clopidogrel 1

The (S)-**5** prepared by the CLEA enzymatic hydrolysis, we accomplished the preparation of the final compound following a reported pathway, explained in the Scheme **3.9**. Chemo-enzymatically prepared (S)-**5** was treated with tosylate **3** in acetonitrile in presence of sodium hydrogen carbonate to afford intermediate **6** (70%).[13] The best results (50% yield) for the heterocyclic ring formation were obtained with paraformaldehyde,[35] as formaldehyde source, formalin[13] or 1,3-dioxolane affording lower yields and more complex final reaction mixture. The enantiopurity of the final clopidogrel (S)-**1** was checked by HPLC analysis on a chiral column (see below).



Scheme 3.9. Final pathway followed for the preparation of *(S)*+ **1**. Reagents and conditions: (i) CH_3OH, DCC, DMAP, CH_2Cl_2; (ii) TFA, CH_2Cl_2; (iii) 20% NH_4OH; (iv) TsCl, $(iPr)_2O$, Et_3N; (v) $NaHCO_3$, KI, CH_3CN; (vi) paraformaldehyde, $ClCH_2CH_2Cl$, HCl in DMF.

3.9 HPLC analyses

In the case of N-unprotected 2-chlorophenylglycine methylester **4** the enzymatic reaction progress could be monitored, contemporary to the ee evaluation, using a chiral column.

In the case of N-Boc derivatives **14** and **15** a chiral column screening showed some difficulties (very long retention times and broad peaks) in performing the two evaluations at the same time; we decided, then, to control the reaction progress by means of a C18 column, delaying the ee evaluation after the Boc group removal. In this way we could detect not only the stereochemical outcome of the biotransformation but also to verify if some racemization eventually occurred during the deprotection step. The known arylglycines tendency of racemization suggested to check the ee, not only of final product **1**, but also of all intermediates. In fact, for example, when we tried to prepare (S)-**4** as free base (necessary for the nucleofilic substitution on tosilate **3**) from the trifluoroacetic salt obtained from Boc removal, use of 0.5M sodium hydrogen carbonate, caused the formation of a 10 % (R)-isomer. On the contrary, by treatment with 20% ammonium hydroxide no racemization was observed. Using chiral stationary phases optical purity of intermediate **5** and final product **1** were also determined. Chosen columns, mobile phases and retention times are collected in Table 3.9.

Compound[a]	Column	Mobile phase[b]	Rt(min)- (R)- (S) isomers
4	Daicel Crownpack CR (+) 0.4 cm X 15 cm	10^{-2} M $HClO_4$	2.71-5.02
5	Daicel Crownpack CR (+) 0.4 cm X 15 cm	10^{-2} M $HClO_4$	12.49-21.35
15	Water spherisorb 5 µm OD 0.46 cm X 25 cm	$H_2O/CH_3CN/HClO_4$ 1:1:0.1	10.90
(R) 16	Water spherisorb 5 µm OD 0.46 cm X 25 cm	$H_2O/CH_3CN/HClO_4$ 1:1:0.1	5.71
6	Daicel Chiralpack IA 0.46 cm X 25 cm	Hexane/AcOEt/Et_3N 95:5:0.1	17.28-15.00
1	Agilent Lichrocart 250-4 Chiradex 5 µ	CH_3CN/CH_3OH/0.01 M KH_2PO_4 15:5:80	32.42-26.40

Table 3.9. Hplc conditions

[a]Detector wavelength was set at 220 nm for all compounds, with exception of **6** (250 nm); [b]Flow rate was of 1ml/min, with exception of **9** (0.7 ml/min).

3.10 References

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4. AMINOACYL-tRNA

AMIDOTRANSFERASE INHIBITORS RELATED TO CHLORAMPHENICOL

4.1 Introduction

Biochemical investigations of the last few years selected the enzyme amidotransferase, involved in the translation process, as possible target for the development of new antibacterial compound.[1]

In most organisms the translation of the genetic information into new proteins synthesis proceeds through a direct pathway involving 20 aminoacyl tRNA synthetases (aaRS) that produce the required set of 20 different aminoacyl tRNAs. As shown in the Figure 4.1, this process starts with the reaction of the aminoacid with ATP to form an enzyme-bound aminoacyl adenylate intermediate (aa-AMP) with displacement of pyrophosphate (PPi). In the second step, the aminoacyl group is transferred to a OH of the ribose at one end of the tRNA generating aminoacyl-tRNA (aa-tRNA) and adenosine monophosphate (AMP). [2]

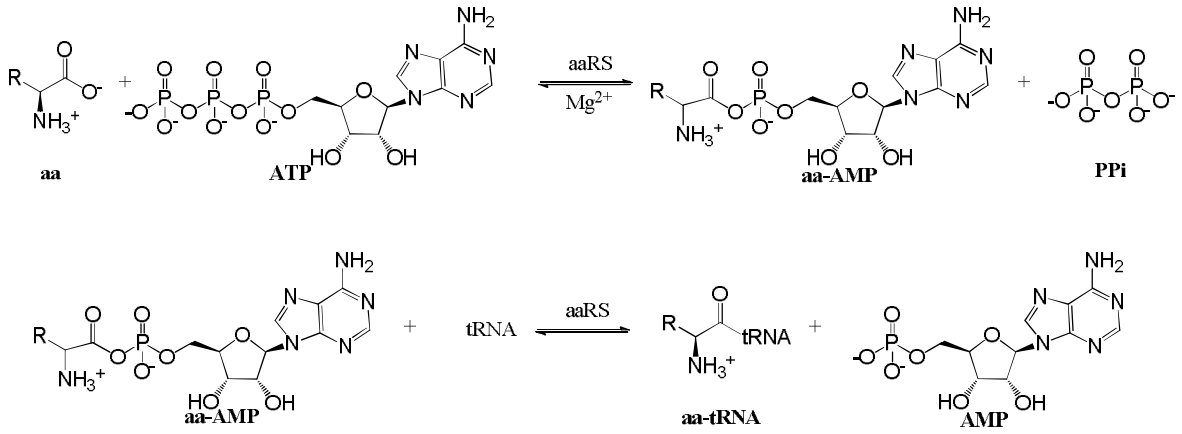


Figure 4.1: Direct pathway of tRNA aminoacylation: aaRS = aminoacyl-tRNA synthetase.

Recent genomic studies revealed the absence of glutaminyl-tRNA synthetase (GlnRS) and/or asparaginyl-tRNA synthetase (AsnRS) in archaeabacteria, Gram-positive eubacteria and many Gram-negative eubacteria.[3] The survival of microorganisms missing one or both of these essential enzymes implies an alternative pathway for the formation of Gln-tRNA^{Gln} and Asn-tRNA^{Asn}

(Figure 4.2). This indirect pathway involves the misacylation of tRNA of $tRNA^{Gln}$ with Glu (or $tRNA^{Asn}$ with Asp) by a non discriminating aminoacyl tRNA synthetase (ND-aaRS) and the subsequent transamidation of the misacylated aa-tRNA by an amidotransferase (adT).

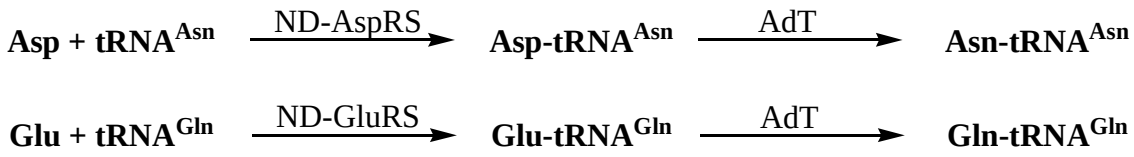
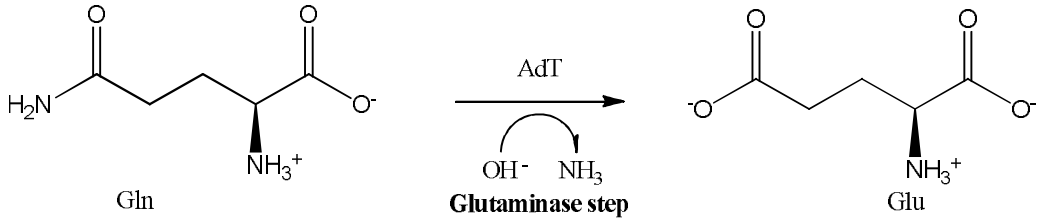


Figure 4.2: Indirect pathway for Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$ biosynthesis: ND-AspRS = non discriminating aspartyl-tRNA synthetase; ND-GluRS = non discriminating glutamyl-tRNA synthetase; AdT = aminoacyl-tRNA amidotransferase.

The proposed mechanism for the transamidation reaction catalysed by amidotransferase is a three step event (Figure 4.3). First, the hydrolysis of the amino donor, glutamine, provide enzyme bound NH_3 (glutaminase step) (Figure 4.3 a). The second step is the activation of the side-chain carboxylic group of the amino acid fixed on the tRNA (Glu-$tRNA^{Gln}$ or Asp-$tRNA^{Asn}$) resulting from the reaction of this carboxyl group with ATP to form a mixed anhydride (kinase step) (Figure 4.3 b). In this intermediate, the high-energy anhydride bond activates the carboxyl group. Finally the aminolysis of the activated amino acceptor by enzyme-bound NH_3 (transamidase step) forms the final product (Gln-$tRNA^{Gln}$ or Asn-$tRNA^{Asn}$). The overall reaction is the simple conversion of the side chain carboxilyc acid (Glu or Asp) into an amide (Gln o Asn) while the amino acid is still attached to a tRNA (pretranslational modification). [4]

a)



b)

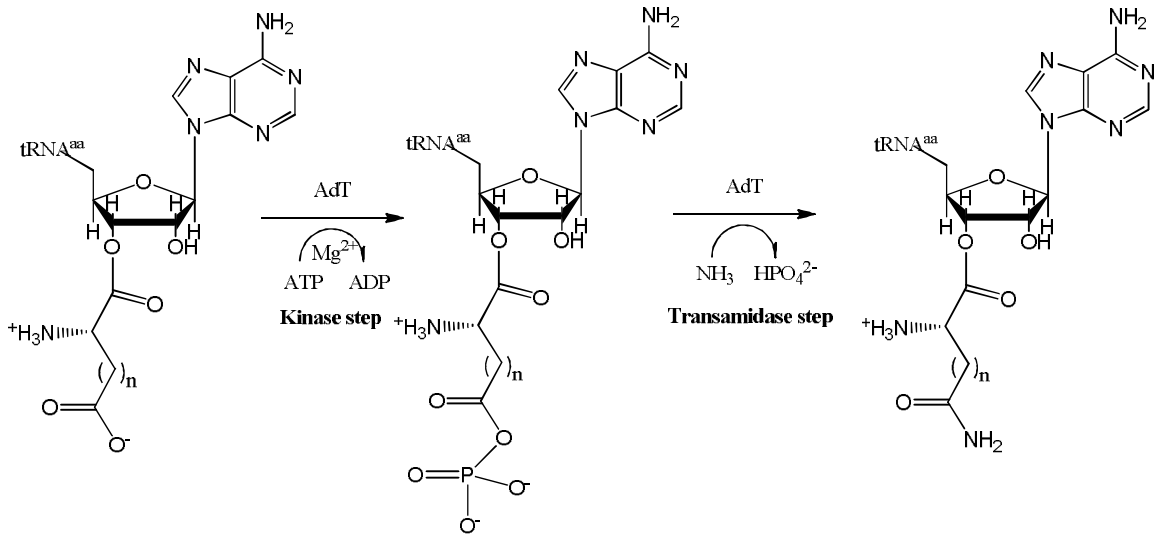


Figure 4.3: Reaction mechanism of aminoacyl-tRNA amidotransferase. For Asp-tRNA^{Asn} amidotransferase (AspAdT), n = 1, tRNA^{aa} =tRNA^{Asn}; for Glu-tRNA^{Gln} amidotransferase (GluAdT), n = 2, tRNA^{aa} =tRNA^{Gln}.

Two types of amidotransferases have been identified in nature: a trimeric one (GatCAB), present in most bacteria and some archea, can use both Glu-tRNA^{Gln} and Asp-tRNA^{Gln} as substrates, whereas a dimeric GatDE, present only in archaea, uses only Glu-tRNA^{Gln}. [5] Crystal structures of GatCAB and GatDE have been determined recently.[6]

4.2 Characterization of GatCAB from the hyperthermophilic bacterium *Aquifex aecolicus.*

Last year, Smith *et al.* reported the crystal structure of GatCAB from the hyperthermophilic bacterium *Aquifex aeolicus* complexed with glutamine, asparagine, aspartate, ADP or ATP and unreact ATP with Mn^{2+} ions.[6(d)] As shown in the Figure 4.4 the enzyme is made by three units, colored differently in the stereo ribbon diagram, blue for the A subunit, green for the B and magenta for the C.

The *A. aeolicus* A-subunit, which includes 478 amino acid residues, contains a conserved amidase signature sequence (62-192) that forms the enzymatic core composed of 11-stranded β-sheet surrounded by 12 α-helices.

The B-subunit (478 amino acids) is composed of an N-terminal "cradle" domain (3-293), followed sequentially by a helical domain (294-412). The C-subunit (94 amino acid) wraps around the interface of the A and B subunits. The A-subunit of GatCAB functions to liberate ammonia from an amide donor and belongs to the amidase family of enzymes.[7] Amidases are characterized by a

Gly/Ser-rich sequence motif, which folds to assemble a Ser-*cis*Ser-Lys "catalytic scissor" (Ser171, *cis*Ser147 and Lys72 in the *A.aeolicus* A-subunit).[8]

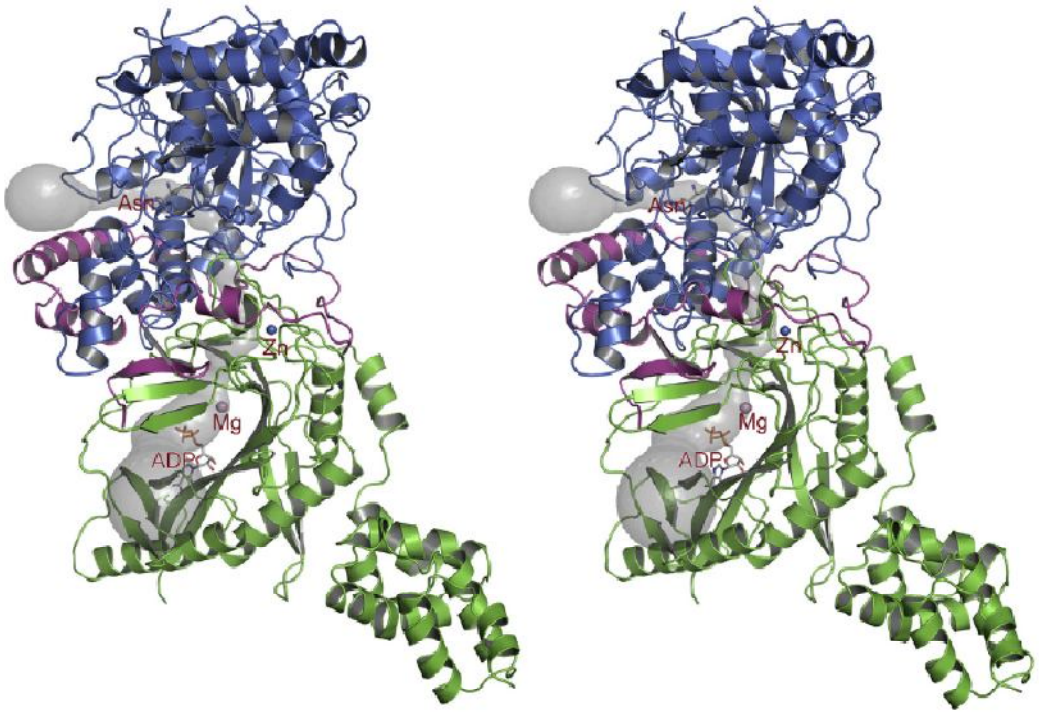


Figure 4.4: Overall structure of the *A. aeolicus* GatCAB-Asn/ADP complex.

Co-crystal with Gln and Asn and kinetic data revealed that GatCAB is able to efficiently use both amino acids as amide donors (K_{cat}/ K_{cat} of 9.7 and 11.1 s^{-1} and mM^{-1}, respectively). This subunit recognize both Gln and Asn in an identical manner using two invariant "anchor" side chain: an Arg for the substrate α-carboxyl group and a Asp for the α-amino group. The ability to form an acyl-enzyme intermediate at the nucleophilic Ser171 depends on the distance of Ser-*cis*Ser-Lys catalytic scissor from the Arg-Asp anchor residues.

The B-subunit is responsible of the ATP-dependent activation of substrate aa-tRNA (Glu-$tRNA^{Gln}$ or Asp-$tRNA^{Asn}$) that leads to the formation of a phosphoryl-aa-tRNA intermediate, then amidated with the ammonia liberated by the A-subunit. The hydrolysis product ADP binds the B-subunit active site: the adenine base fits into an hydrophobic pocket formed by Val8, Phe208 and Pro158 with hydrogen bonds from N1 and N6 of the adenine to the conserved Ser199 side chain. The ADP α- and β- phosphates interact with bound water molecules. There are no bound metal ion associated with ADP.

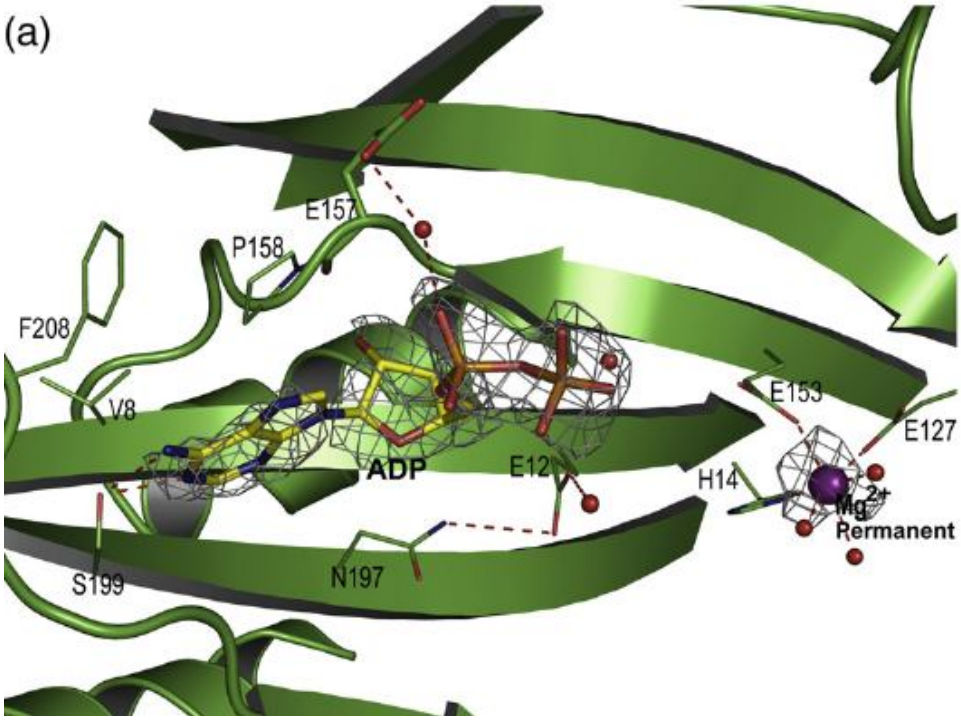


Figure 4.5 (a): Synthetase activity site in the B-subunit: ADP/Mg^{2+} complex.

Crystallization of the protein with the complex ATP/Mg^{2+} showed that the γ-phosphate of ATP, together with the side chains of Glu12 and Glu213 and a molecule of water, coordinate the "transient" metal site of Mg^{2+} that probably assists phosphoryl transfer (Figure 4.5 b). In the B-subunit there is also a permanent metal site: here the metal is coordinated by His14, Glu127 and Glu153.(Figure 4.5 a) The permanent metal is too far from the nucleotide to participate directly in the phosphoryl-transfer reaction but mutation of residues in this area lead to inactive mutant enzymes suggesting a critical role for this site.

Furthermore soaked with Asp, as a mimic of the aminoacyl end of Asp-$tRNA^{Asn}$, showed that one of the Asp carboxyl groups coordinate the metal ion in the permanent site and faces the ATP γ-phosphate (Figure 4.5 b).

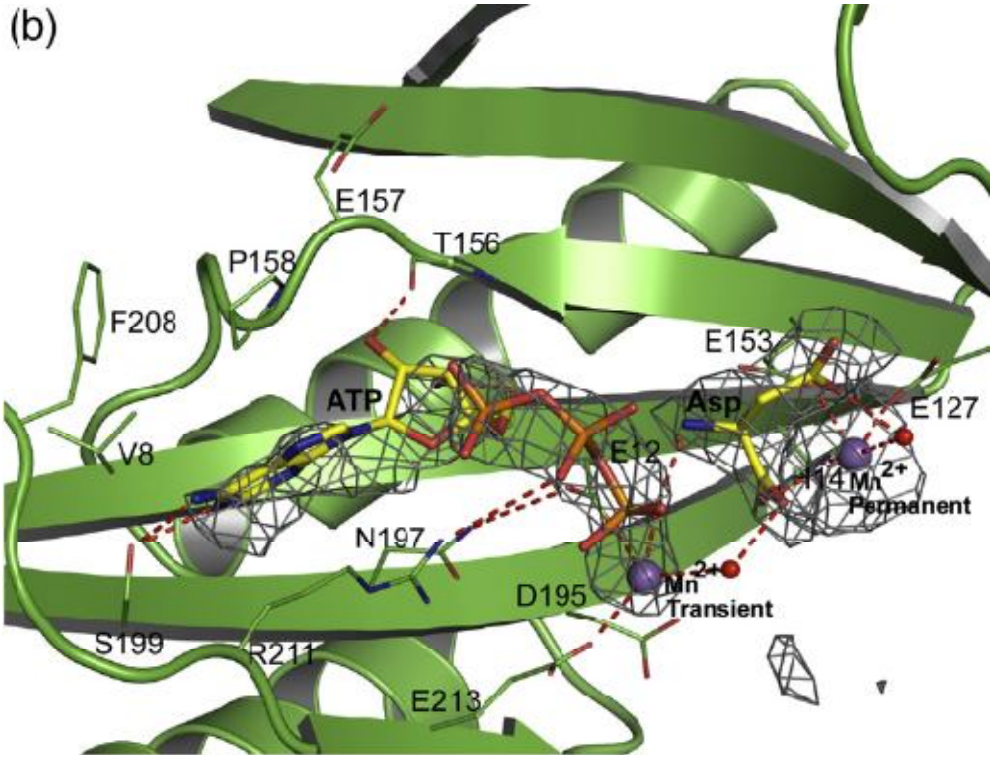


Figure 4.5(b): Synthetase activity site in the B-subunit: ATP/Mg^{2+} complex .

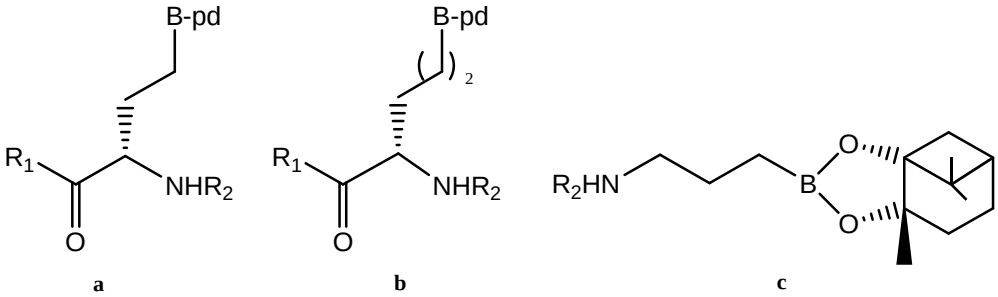
The active sites of the A and B subunits are connected by a continuous hydrophobic tunnel 35-Å long that allows the channelling of ammonia from A to B subunits. In the B-subunit, between the NH_3 channel the C subunit there is a Zn^{2+} binding site with tetrahedral coordination by Cys25, Cys27, Cys40, Cys43. The presence of the Zn $(Cys)_4$ site stabilises a B-subunit loop (residues 25-40) that forms several hydrogen bonds with the C-subunit including a direct hydrogen bound from Ser68 in the C-subunit to the Cys27 Zn^{2+} ligand. In addition, residues Val42, Cys43 and Leu44 form part of the wall of the NH_3 channel. Thus, Zn $(Cys)_4$ is an important motif for maintenance of the channel and for the binding between the B- and C-subunits.

4.3 Aim of the work

The presence of a trimeric AdT in most bacteria, and the absence of either dimeric or trimeric AdT in the cytoplasm of eukariotes, identify GatCAB as a promising target for the development of new types of antiobiotics. The new AdT inhibitors will target protein biosynthesis in the above mentioned bacteria, without affecting it in mammalian cytoplasm or mitocondria.

Up to recently, only a few AdT inhibitors have been reported in part because of the absence of a convenient assay. So far, AdT inhibitors are essentially synthetic analogues of glutamine, ATP and aminoacyl-tRNA. In a mechanism-based approach, glutamyl-γ-boronate derivatives were evaluated as substrate-based (Gln) inhibitors acting as serine inactivators in the AdT-glutaminase active site (Figure 4.6). [9]

Compound	R_1	R_2	IC_{50}(μM) GLA	IC_{50}(μM) TRA
a	-OH	- H	1.6	0.10
a	$-OCH_3$	-H	1.3	0.05
a	$-OCH_3$	-H	1.5	0.07



a **b** **c**

Figure 4.6: Glutamyl- γ-boronate derivatives and best values of activity against the glutaminase (GLA) and transferase (TRA) activities of Glu-AdT.

In general, a serine inhibitor contains an electrophilic centre that is reactive toward an hydroxyl nucleofile and boronic-acid have been shown to be effective serine "traps" through the formation of a reversible serine-boronate-acetal.[10] Some of these inhibitors, as shown in the Figure 4.6, provided potent inhibition *in vitro* and displayed also antibacterial activities for many AdT-dependent bacteria. In another study, ATP analogues have been useful to characterize the reaction coupling between glutaminase and kinase-transamidase active sites in AdT .[11] However, as these analogues will likely to interfere with several other enzymatic processes within mammalian cells, they cannot be lead compounds for the design of novel antibiotics.

In the laboratory where I worked during the third year of my PhD, the development of amidotransferases inhibitors is one of the research topics under investigation. In searching for inhibitors specific for AdT, at first, they focalized their attention on analogues of 3' ends of its aa-tRNA substrates; [16] these are asparticin and glutamycin (Figure 4.7 b), the second of which is a

puromycine analogue that shows a weak inhibition of glutamyl-tRNA^{Glu} reductase, the first enzyme of the tRNA dependent tetrapyrrole biosynthesis pathway.

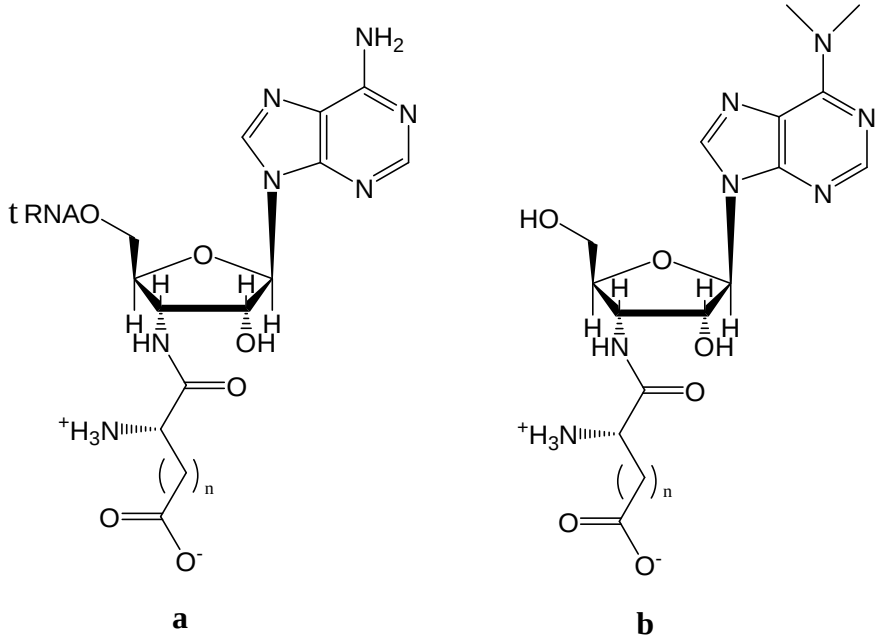


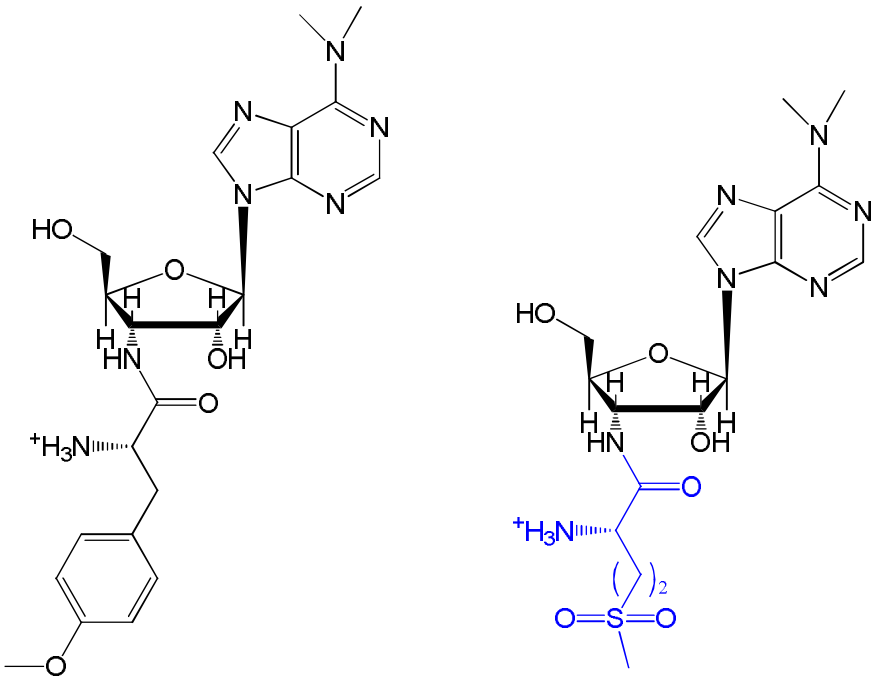
Figure 4.7: (a) Asp-tRNA^{Asn} when n = 1 and Glu-tRNA^{Gln} when n = 2; (b) Aspartycin and Glutamycin analogues of Asp-tRNA and Glu-tRNA , respectively.

Both compounds revealed to be competitive inhibitors of the transamidase activity of *Helicobacter pylori* GatCAB with respect to Asp-tRNA^{Asn} with K_i values of 134 μM for aspartycine and 105 μM for glutamycine. This result is compatible with the fact that GatCAB has two natural aminoacyl-tRNA substrates whose nucleotide sequences differ significantly allowing more interactions with the 3'- terminal features.

Aspartycin, even if it presents the same aminoacid portion as the natural Asp-tRNA^{Asn} is not a substrate for Adt, which indicates that its β-carboxyl group is not positionated in the active site exactly as for the natural substrate.

More recently, going forward with this project to discover inhibitors of *Helicobacter pylori* GatCAB aminoacyl-tRNA amidotransferase, they reported a series of puromycin **1** (Figure 4.8) derivatives.[12] In fact puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger* that cause premature chain termination in the process of translation. Although its structure resembles the 3'-terminal of aminoacyl tRNA, puromycin is a very weak inibitor for Adt (k_i = 4 mM). This is probably due to the amino acid chain, related to tyrosine, different from the

aspartic and glutamic side chain transformed by Adt. Starting from this consideration, they evaluated the inhibiton activity of a serie of compounds obtained by the replacement of the methoxyphenyl moiety by carboxylic acid derivatives. This substitution considerably enhances the ability to inhibit Adt and the compound **1** represented in the Figure 4.8, was found to have the most potent enzyme activity (K_i = 4 µM with respect to Asp-tRNA^{Asn}) with the L-methionyl-sulfone moiety mimicking the transition state in the transamidation process.



1 **2**

Figure 4.8: (1) Puromycin; (2) Most active AdT inhibitor related to the structure of puromycin.

With this results in hand, they decided to prepare a new serie of compounds, maintaining this promising L-methionyl-sulfone moiety binding to a simplified nucleoside. The antibiotic chloramphenicol **3** (Figure 4.9) inhibits protein synthesis by binding to the peptidyl transferase region of the ribosome, and overlaps the binding site of puromycin.[13] Moreover its nucleus of amino 1,3 propan-diol bounded to the p-nitrophenyl ring shows a similarity and a structural relationship with the sugar of puromycin. At first they assayed the activity of the pure chloramphenicol who is a very weak inhibitor of GatCAB ($K_i \approx 1.9$ mM). Replacement of the dichloroacetyl moiety of chloramphenicol **3** by L-methionyl-sulfone considerably enhances the activity and compound **4** was identified as a very active inhibitor with a K_i value of 30 µM.

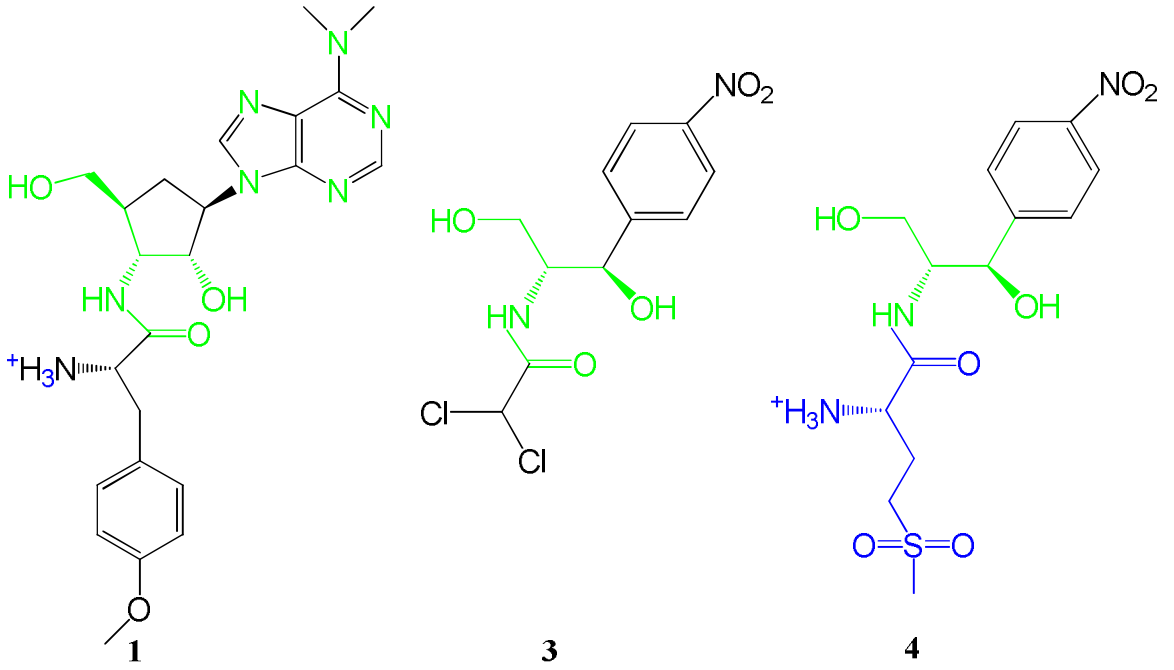


Figure 4.9: Structural similarity between (1) Puromycin; (3) Chloramphenicol, (4) AdT inhibitor related to the structure of chloramphenicol.

In order to complete this structure-activity relationship study, we wanted to evaluated the effect of the configurations at C-1 and C-2 stereogenic centers on the activity and to assay the activity of the diastereoisomers (1S, 2R)-**5** and (1R, 2S)-**6**.

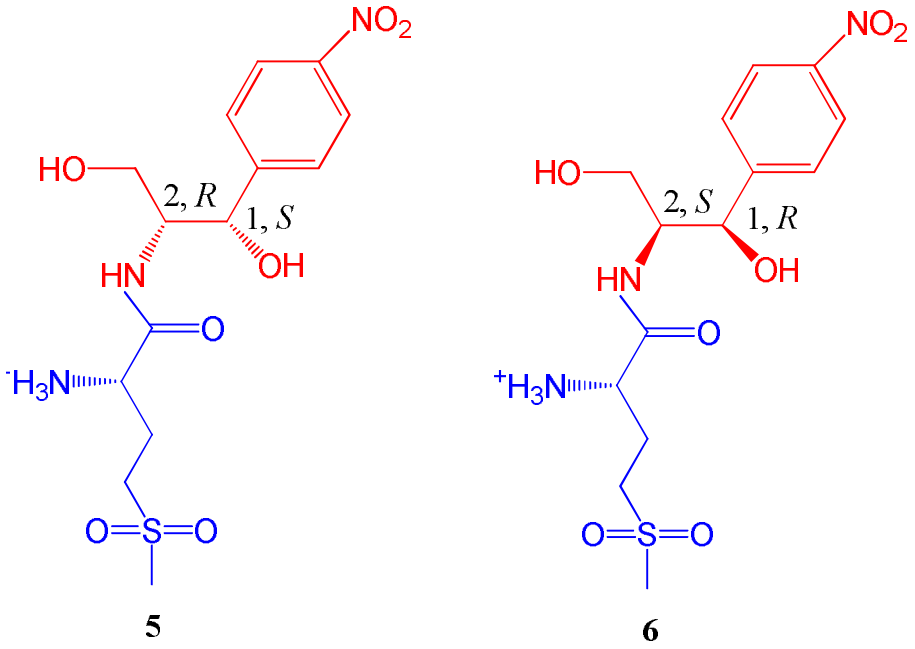
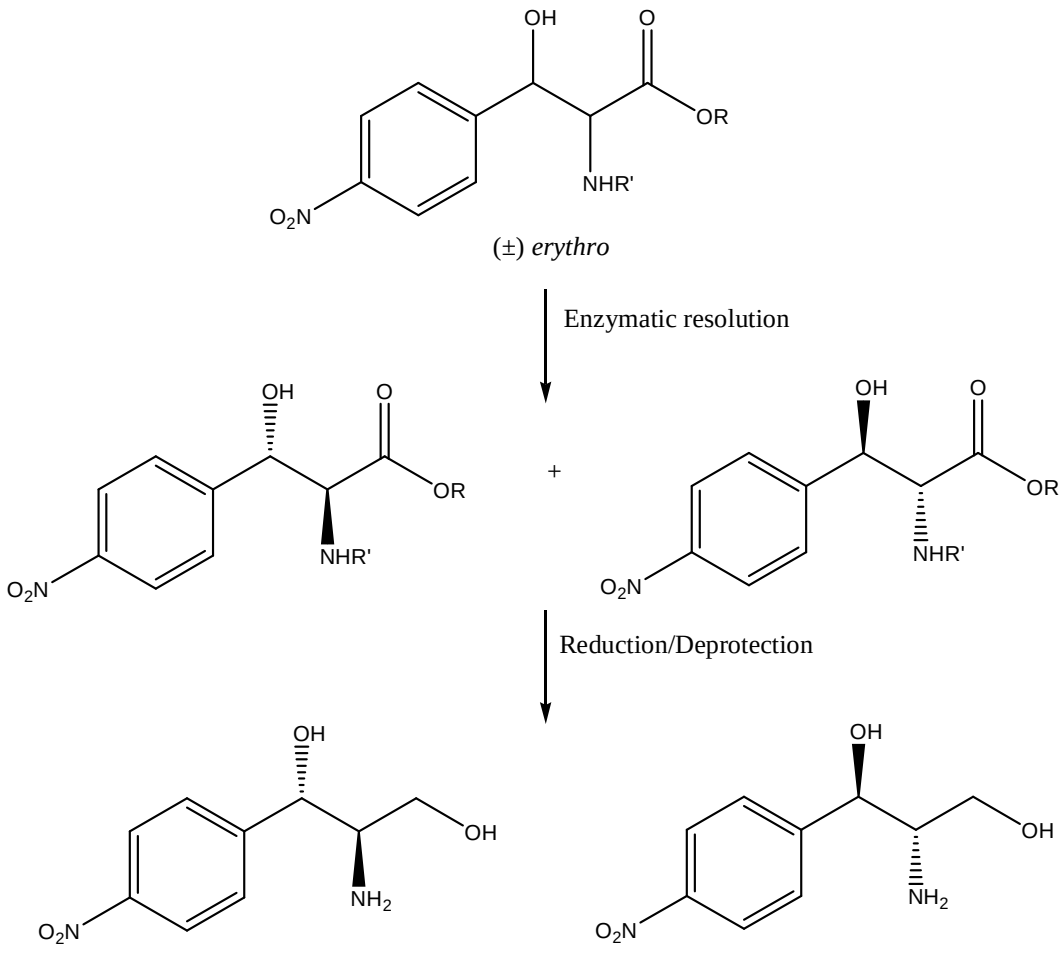


Figure 4.10: Structure of the two *threo* diastereoisomers analogues of chloramphenicol.

As in the structures of the diastereoisomers **5** and **6** two moieties can be recognized, (Figure 4.10), these compounds can be prepared by a coupling reaction between the commercially available L-methionine-sulfone (represented in blue) and the aminic function present in the structure of the two enantiomeric forms of *p*-nitrophenylserinol (red portion of the structure), not commercially available.

The goal of my research was to prepare these two enantiomers, with an high optical purity.

In order to reach this objective following the fastest approach, I decided to start from the racemic mixture of *erythro*-p-nitrophenylserine, the compound that share with the final products the most structural similarity, easily converted to the latter by a reduction at the carbonyl function (Scheme 4.1).



Scheme 4.1: General synthetic pathway to obtain the 2 enantiomers of *p*-nitrophenyl-serinol. R/ R' = suitable protective groups to carry on the enzymatic reaction.

In 1954, Moersch and co-workers reported the synthesis of (1R, 2S) *erythro*-p-nitrophenylserine, achieved with a long procedure and low yield.[14]

The well known enantioselectivity of enzymes could be useful to obtain these products with high optical purity and good yields overcoming the disadvantages of fractional crystallization and, moreover, could be interesting to explore the behaviour of enzymes towards these diasteroisomers.

Indeed the β-hydroxy aminoacids are very attractive and versatile molecules, included in the structure of many bioactive compounds.[15] L-Threonine is an essential amino acid, the threo-D-phenyl serine is active against some microorganisms of Influenza A, some type of *E.Coli* and some lactobacilles. The structure of *threo*-L-phenyl serine is included in the molecule of bradikynine, a nonapeptide endowed with vasodilatant action, and is essential for its terapeutic activity.

Also the β-hydroxy aminoacids are chiral scaffold/building block very interesting for the preparation of enzymatic inhibitors and β lactam antibiotics.[16]

Due to their importance in the past, a lot of research groups accomplished the preparation of *threo* and *erythro* phenylserine using enzymes.

In 1953 Fones reported the resolution of the four optical isomers of *threo* and *erhytro* phenyl-serine through the asymmetric hydrolysis by carboxypeptidase of the N-trifluoroacetyl of the respective diastereoisomers.[17]

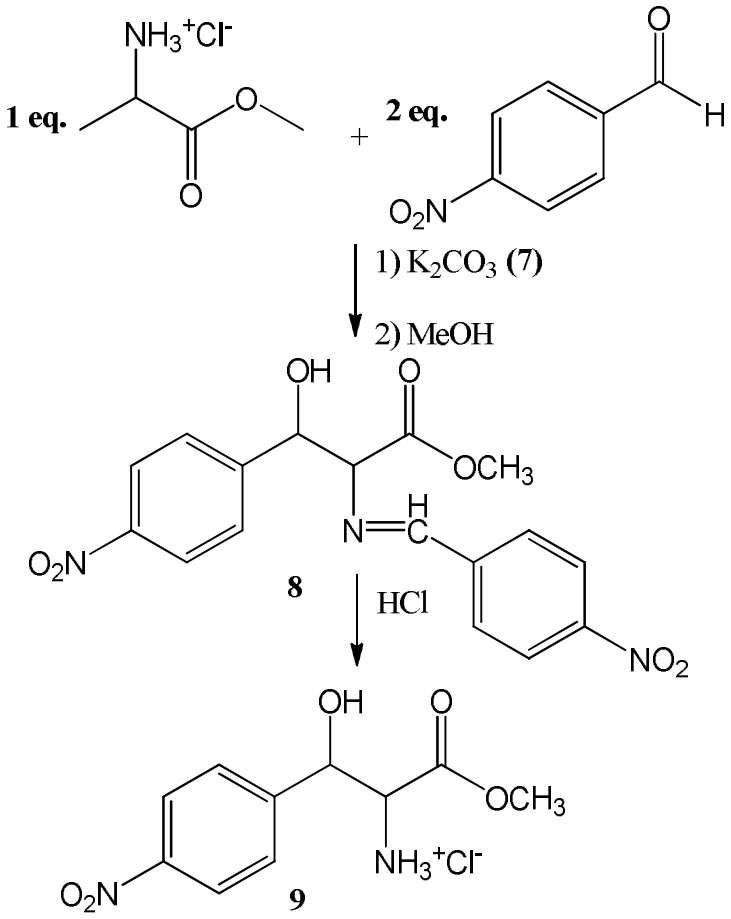
In 1989 Chênevert *et al*. realized the enantioselective hydrolysis of the esters of both *threo* and *erythro* p-nitrophenyl-serine N-acetyl derivatives and developed an efficient method for the preparation of the chloramphenicol. [18]

There are no studies on the optical resolution of the *erythro* enantiomers of p-nitrophenyl-serines with the free aminic group.

4.4 Results and discussion

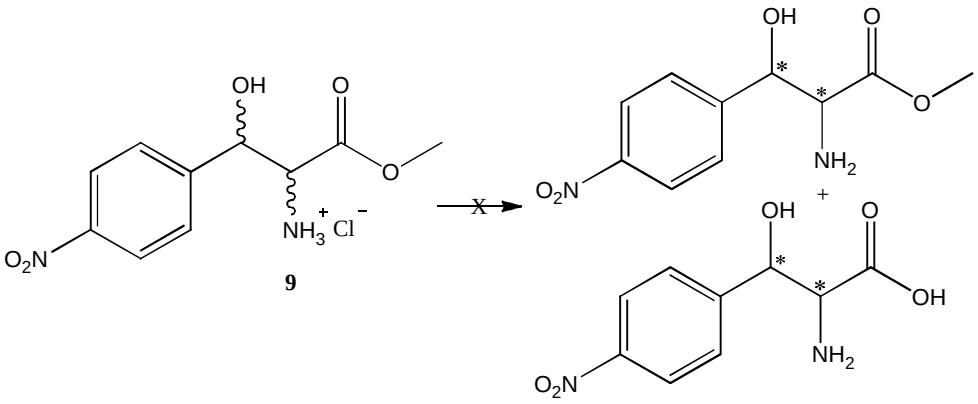
The preparation of the racemic substrate **9** was realized following the procedure of Elphimoff-Felkin that allows to obtain the only *erythro* isomer (Scheme 4.2). [19]

Methyl glycinate was condensed with 2 equivalent of p-nitrobenzaldeide to give the erythrobenzylidene **8**.



Scheme4. 2: Synthetic pathway to obtain the *(±) erythro p*-nitrophenilserine methylester hydrochlorhyde **9**.

At the beginning of our work, in order to discover a new enzymatic approach for the resolution of *erythro* p-nitrophenyl-serine we tried to achieve this objective working on the β-hydroxy aminoacid ester **9** without any N-protection, in order to simplify the synthetic pathway (Scheme 4.3). Lipases and proteases have been widely used for the enantioselective hydrolysis of both natural and unnatural amino acid esters[20]: following the same procedure reported for florfenicol[21] we screened several of them in hydrolytic conditions. As shown in the Table 4.1 below, using an aqueous medium, no enzyme exhibited any enantioselectivity even if, as is clearly visible by the times of reaction reported, proteases were the most active.



(±) erythro *(±) erythro*

Scheme 4.4: Enzymatic hydrolysis on derivative **9**.

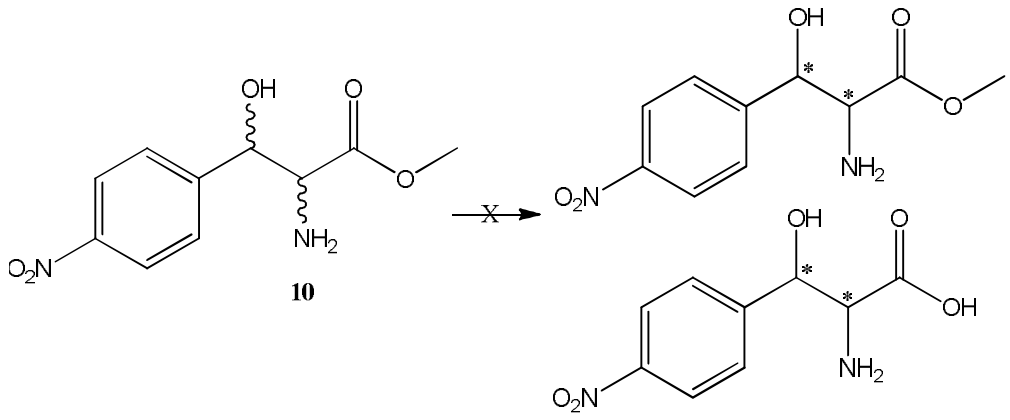
Enzyme	**Conditions**	**Time (h)**	**Conversion***	**ee %****
Protease from *Aspergillus oryzae*	buffer pH 7.00, 30°C	3	40	$ee_s = 0$
Protease from Subtilisin Carlsberg	buffer pH 7.00, 30°C	5.5	40	$ee_s = 0$
α Chymotrypsin	buffer pH 7.00, 30°C	45 h	30	$ee_s = 0$
Chymopapain	buffer pH 7.00, 30°C	45 h	30	$ee_s = 0$
Protease from *Streptomyces griseus*	buffer pH 7.00, 30°C	48 h	30	$ee_s = 0$
CAL B	*i*-Pr_2O H_2O (5-10 eq), 30°C	40 h	-	-
CCL	*i*-Pr_2O H_2O (5-10 eq), 30°C	40 h	-	-
CRL	*i*-Pr_2O H_2O (5-10 eq), 30°C	40 h	-	-
Lipase from *Burkholderia cepacia*	*i*-Pr_2O H_2O (5-10 eq), 30°C	40 h	-	-

Table 1: Screening of hydrolytic condition on **3**. *By TLC analysis;**By chiral HPLC analysis.

The probable reason of this low enantioselectivity is due to the non-enzymatic spontaneous hydrolysis as was confirmed by the blank. The same results were obtained with the ethyl-ester derivative.

In order to overcome this problem, the aqueous solution was replaced by organic solvents, *i*-PrOH and toluene, in presence of only 5-10 equivalent of water acting as nucleofile but no hydrolysis occurred, probably because of the low solubility of the starting material in the medium of reaction.

Later, following the protocol reported by Tasnàdi *et al.* [22] for the resolution of β amino esters, with the objective to increase the solubility of the starting material in the organic solvent, known to be the best medium for lipases, I tested the same enzymes on the free base of *erythro* p-nitrophenyl-serine methyl ester **10**.



Scheme 4.5: Enzymatic hydrolysis on derivative **10**.

As reported in the table 4.2, the amino ester **10** was substrate for CAL B, PLE and lipase from *Pseudomona sp.* but only CAL B showed some enantioselectivity.

Enzyme	Conditions	Time(h)	Conversion*	ee %**
CAL B	*i-Pr_2O* *H_2O (5-10 eq), 30°C*	*48*	*≥50*	*ee_s =67*
CCL	*i*-Pr_2O H_2O (5-10 eq), 30°C	94	-	-
CRL	*i*-Pr_2O H_2O (5-10 eq), 30°C	94	traces	-
Lipase from *Burkholderia cepacia*	*i*-Pr_2O H_2O (5-10 eq), 30°C	40	traces	-
PLE	*i*-Pr_2O H_2O (5-10 eq), 30°C	120	≥50	ee_s = 0
Protease from *Streptomyces griseus*	*i*-Pr_2O H_2O (5-10 eq), 30°C	48	-	-
Lipase from *Pseudomonas Sp.*	*i*-Pr_2O H_2O (5-10 eq), 30°C	120	≥50	ee_s = 0
PPL	*i*-Pr_2O H_2O (5-10 eq), 30°C	94	-	-

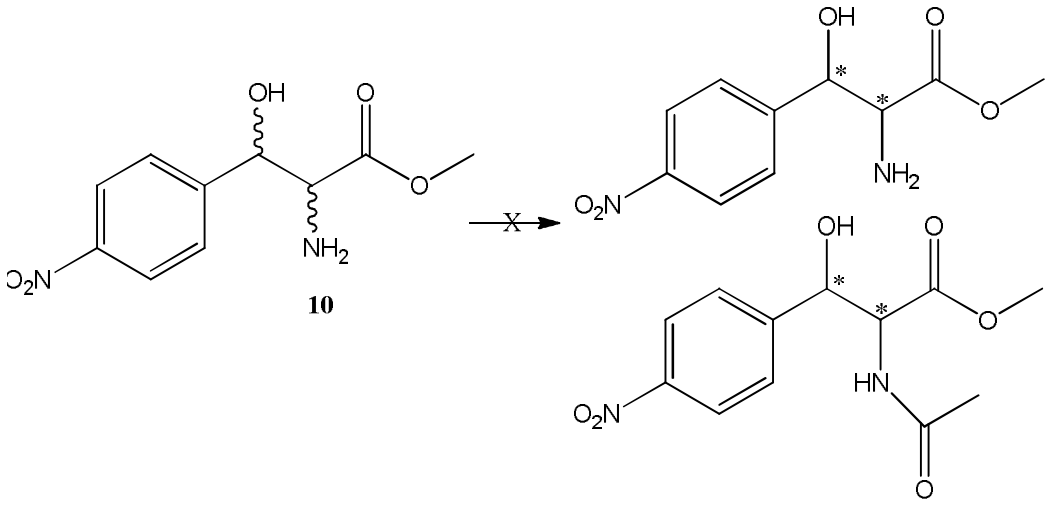
Table 4.2: Screening of hydrolytic condition on **10**. *By TLC analysis;**By chiral HPLC analysis.

Further investigations with CAL B in the usual preferred solvents didn't afford better results (Table 4.3).

Conditions	Time	Conversion*	ee %*
i-Pr_2O H_2O (1-7 eq*), 30°C	7 days	50	ee_s=15
Toluene H_2O (1-7 eq), 30°C	7 days	50	*ee_s = 0*
Et_2O H_2O (1-7 eq), 30°C	100 h	50	*ee_s = 0*

Table 4.3: Screening of hydrolytic condition on **10** with CAL B lipase. *By TLC analysis;**By chiral HPLC analysis.

Going on with the intention to obtain the resolution on the free amino ester, we investigated the well known ability of lipases and proteases to enantioselectively acylate the amino group on compound **10**. [23]



(±) erythro *(±) erythro*

Scheme 4.6: Enzymatic acylation on derivative **10**.

As reported in the Table 4.4, the reactions were carried on using ethyl acetate as acyl donor and solvent: inspection of the results obtained in this reaction revealed that all the screened enzymes are ineffective catalysts.

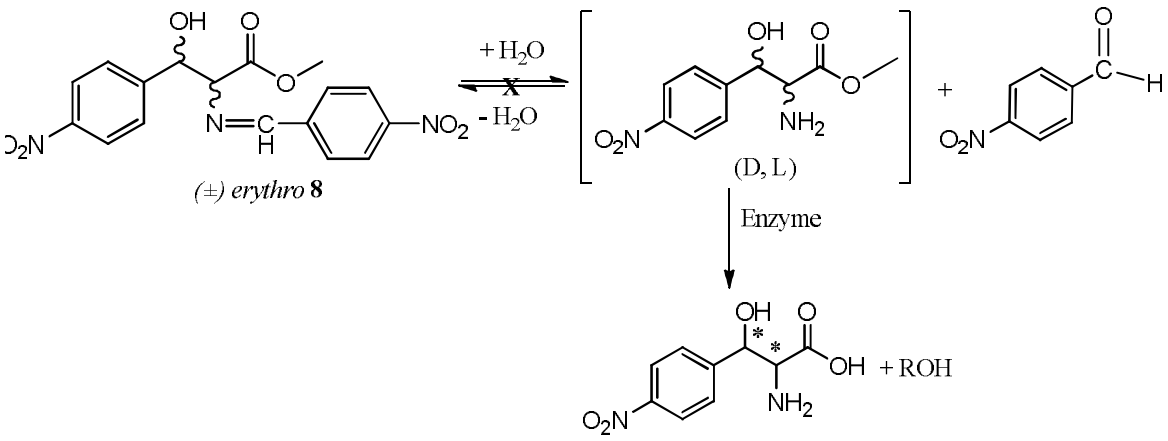
Enzyme	**Conditions**	**Time(h)**	**Conversion***	**ee %****
CAL B	AcOEt, 30°C	44	60	$ee_p = 0$
Protease from *Subtilisin Carlsberg*	AcOEt, 30°C	63 h	60	$ee_p = 0$
CCL	AcOEt, 30°C	65 h	60	$ee_p = 0$
PPL	AcOEt, 30°C	64 h	60	$ee_p = 0$
Lipase from *Burkholderia cepacia*	AcOEt, 30°C	63 h	60	$ee_p = 0$
CRL	AcOEt, 30°C	42 h	60	$ee_p = 0$

Table 4.4: Screening of acylation with diffeents lipases and proteases on substrate **4**. *By TLC analysis;**By chiral HPLC analysis.

Continuing with this study and taking into account that the presence of the free amino group favourises the spontaneous chemical hydrolysis of the ester moiety, we decided to protect the amino function and to work on substrates more similar to the natural ones of the enzymes.

Following this approach, we focalized our attention on the hydrolysis of the methyl ester product by α chymotrypsin and protease from subtilisin Carlsberg, since their activity has already been stressed on *erythro* N-acetyl p-nitrophenyl-serine methyl-esters.

In searching for new N-protective groups, more easy to hydrolyze than the acetyl amide, at first we proposed, as possible substrate the (±) *erythro* nitrobenzyl-iden-*p*-nitrophenylserinate methyl ester **8**, an intermediate of the synthesis of final β-hydroxy amino acid. [24]



Scheme 4.7: Enzymatic hydrolysis on derivative **8**.

In fact, the advantages of this substrate, are the lability of the N-protective group and their increased solubility in aqueous and aqueous-organic solvents.

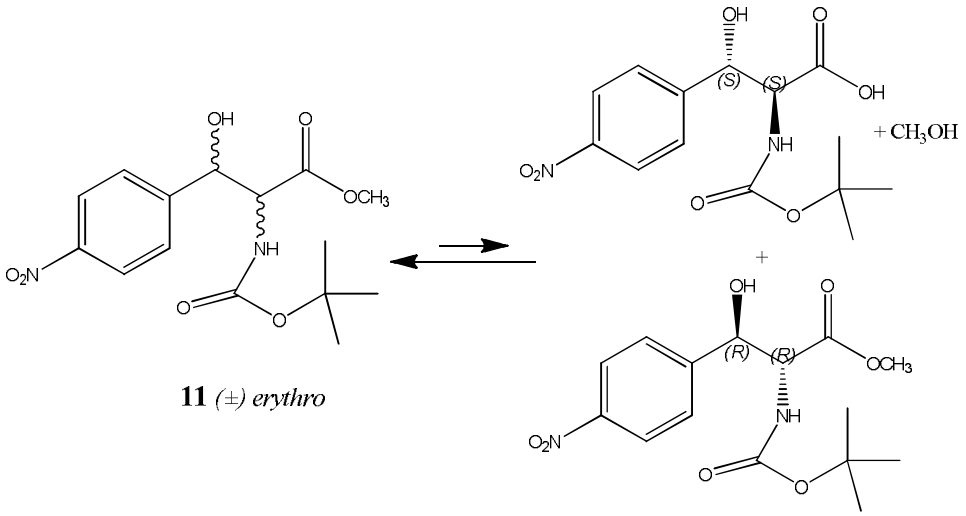
The real substrate is the free amino ester that was liberated from the Schiff bases by reaction with water and consequently the dissociation of the Schiff base is the rate-limiting process (Scheme 4.7). The reaction was carried out at room temperature in acetonitrile with 10 equivalents of water but after 10 days there was no detectable hydrolysis of the Schiff base; the possible explanation of the phenomenon might be the formation of the oxazolidine ring via the interaction of the C=N bond with the OH group of the side chain. [25]

This failure suggest us to test the usual amine protective groups and then we protected the *(±) erythro* p-nitrophenyl-serine methyl ester **9** as *tert*-butoxycarbonyl amide, one of the most used for the resolution of N-protected amino acids. [26]

Among the screened enzymes, both subtilisin Carlsberg and α-chymotripsyn recognized as substrate **11**, as reported for the N-acetyl derivative.

Differently, due to the low polarity of the *tert*-butoxycarbonyl moiety, the addition of a co-solvent was required and the best results were obtained with the mixture buffer pH 7.7/*i*-ProH (2:1) (Table 4.5). Even with the co-solvent the reaction time was very long, compared with the one reported for

the N-acetyl derivative, and was not possible to achieve the desiderated conversion of 50%, necessary to recover both the acid and the unhydrolyzed ester with an high enantiomeric excess (Scheme 4.8). When the reaction was done on 2 g of racemic ester **11**, the enzyme spent 3 weeks to hydrolyze the 40% of compound; at this time the reaction stopped and further additions of enzyme were not useful. Probably the hindrance of the *tert*-butoxycarbonyl group makes difficult the access of the ester moiety to the active site of the enzyme.



Scheme 4.8: Enzymatic hydrolysis on derivative **11**.

Enzyme	**Conditions**	**Time (h)**	**Conversion***	**ee %***
α Chymotripsin	buffer pH 7.7, 23°C	50	< 5	-
α Chymotripsin	buffer pH 7.7/CH_3CN (2:1), 23°C	100	< 5	-
α Chymotripsin	buffer pH 7.7/*tert*-BuOH (2:1), 23°C	100	< 5	-
α Chymotripsin	buffer pH 7.7/TBME (2:1), 23°C	100	30	$ee_p = 93$
α Chymotripsin	buffer pH 7.7/THF (2:1), 23°C	100	> 5	-
α Chymotripsin	*buffer pH 7.7/ i-Pr_2O (2:1), 23°C i-Pr_2O*	*100*	*45*	$ee_p = >95$
CAL B	buffer pH 7.7/THF (2:1), 23°C	20	0	-

Lipase from *Burkholderia cepacia*	Buffer pH 7.7/THF (2:1), 23°C	20 h	0	-
Protease from *Aspergillus oryzae*	Buffer pH 7.7/THF (2:1), 23°C	20 h	0	-
Protease from *Streptomyces griseus*	Buffer pH 7.7/ *i*-Pr_2O (2:1), 23°C	20 h	0	-
Protease from subtilisin Carlsberg	Buffer pH 7.7 23°C	70 h	25	ee_p = *73*
Protease from subtilisin Carlsberg	Buffer pH 7.7/TBME (2:1), 23°C	26 h	7	-

Table 4.5: Screening on hydrolysis of compound **11**. *By chiral HPLC analysis.

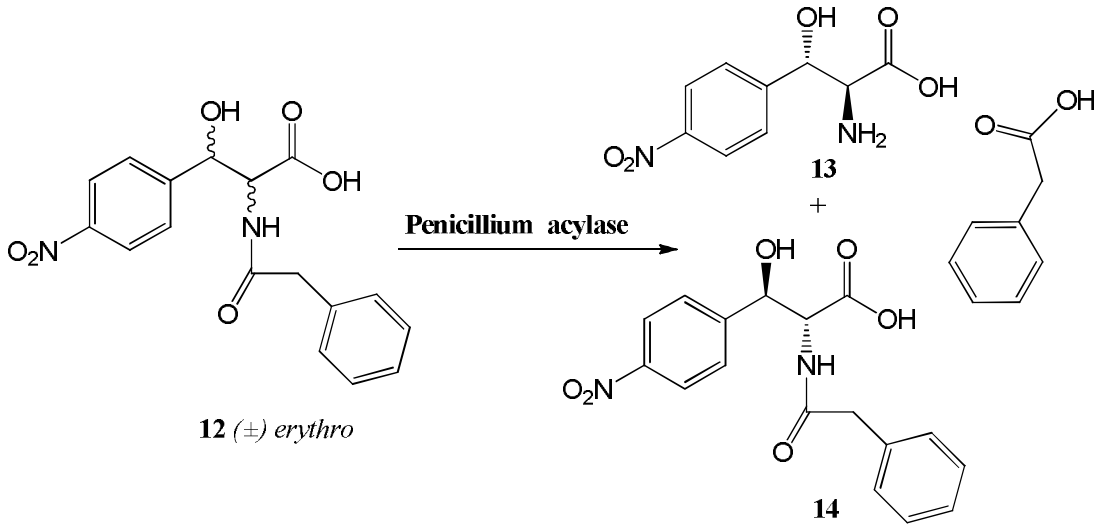
So, we decided to test, as N-protective group, the trifluoro-acetyl amide, smaller in size and easy to hydrolyze under mild conditions. Unfortunately this derivative was not stable under the reaction conditions and its spontaneous hydrolysis occurred in both the product and the starting material.

With this results in hand, we decided to change approach and to investigate the possibility to achieve the resolution by the amino function.

As mentioned above, good results were obtained with carboxypeptidase A on both *threo* and *erythro* N-trifluoroacetyl phenyl-serine while acylase I was found to be ineffective toward either N-acetyl or N-chloroacetyl derivatives of β-phenyl-serines. For this reason, we decided to perform the enzymatic deacylation of the N-amide not with the common acylases but to test another enzyme useful for the resolution of chiral amines: penicillin acylase from *E.coli*. [27]

As deeply described in the introduction, this enzyme (E.C. 3.5.1.11) selectively transfers the phenylacetyl-moiety to water and one of its largest application was the production of 6-aminopenicillanic acid, the basic raw starting material for the industrial production of semisynthetic penicillins, from penicillin G. [28] The enzymatic activity of Pen G Acylase has been associated with the phenylacetyl moiety, even if the phenoxyacetyl derivatives are also accepted as substrates, and a wide variety of phenylacetyl derivatives of primary amines and alcohols has been selectively hydrolyzed. [29] The well known L-directed stereochemical preference in hydrolyzing N-phenylacetylamino acids, makes this enzyme useful in the field of resolution of racemates and of configurational correlations[30]: Fadnavis *et alt.* reported the penicillin acylase catalyzed resolution of hydroxyleucine[31] and in 1997, an Italian research group reported the resolution of a series of aminoacids, among them serine, the natural compound most similar to **9**, by hydrolysis of the L-N-

acetylphenylderivative. [30] On the basis of these previous works it seems interesting to examine the potentiality of this enzyme towards this unnatural substrate; the presence of an additional chiral carbon atom poses the question about the stereoselectivity of the amidase.



Scheme 4.9: Enzymatic resolution on derivative **12**.

Conditions	Time (h)	Conversion*	ee %**
Buffer pH 7.8, 25°C	5	> 50	**14**-ee_s = > 99
Buffer pH 7.8, 25°C	3	< 50	**13**-ee_p = > 99

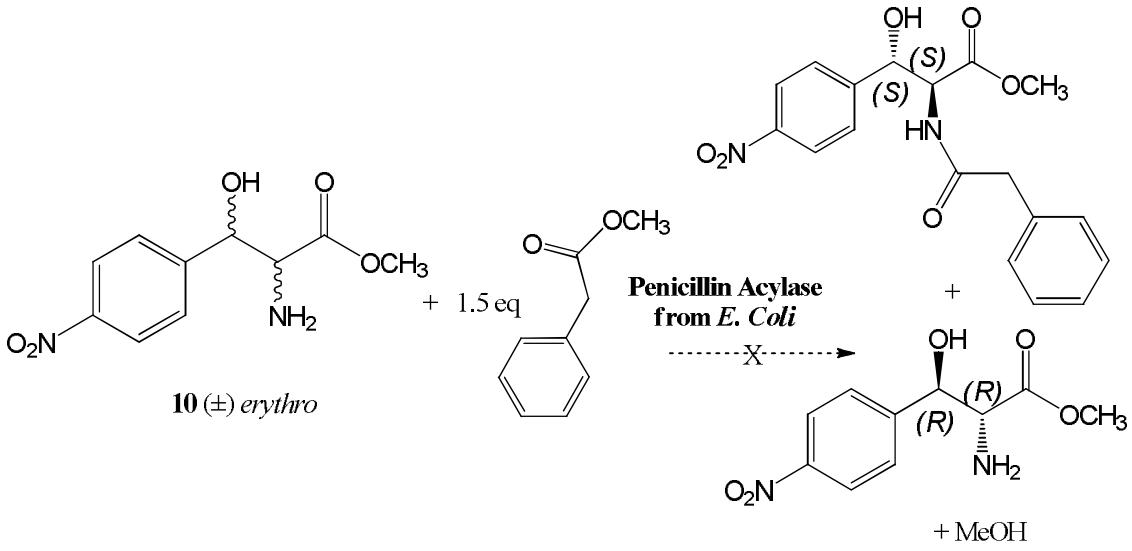
Table 4.6: Screening on hydrolysis of compound **12**. *By reverse phase HPLC; **By chiral HPLC

We prepared the suitable N-phenylacetyl-derivative of *erythro* p-nitrophenyl-serine **12** following a known method and incubated it with the enzyme (80 U/ mmol substrate) in buffer phosphate at pH 7.8; no cosolvent was required thanks to the good solubility of the substrate in the basic aqueous medium (Scheme 8). In preliminarys studies, we decided to analyzed the enantioselectivity of the enzyme being in the best conditions for the enantiomeric excess: with the help of reverse phase Hplc we stopped the reaction around 40% and 60% and checked the enantiopurity of the product and of the starting material, respectively. The >99 % enantiomeric excesses, evaluated by analysis with a chiral Hplc, reported in Table 4.6 , show that the hydrolytic activity of penicillin acylase toward the substrate **12** is very enantioselective. In fact with the aim to obtain both the two enantiomers **13** and **14** with high value of optical purity, by the same reaction, in further experiments, the hydrolysis was stopped around the 48-50% of conversion.

The unreacted starting material **14**, together with the phenyl-acetic acid, were separated from the free amino acid **13** by the usual work up and their enantiomeric excess was determined by a chiral hplc analysis. Both the compound **13** and **14** showed an enantiopurity superior to 95%.

The very high enantiomeric excess for both the product and the starting material, makes penicillin acylase the best enzyme to achieve the preparation of the precursors of the two amino alcohols **5** and **6** analogues of chloramphenicol.

In order to simplify the synthetic pathway and to avoid the delicate reduction of the free amino acid, we investigated also the ability of the enzyme to selectively acylate the free amino group of the *erythro* p-nitrophenyl-serine methylester **10** using methyl phenyl-acetate as acyl donor (Scheme 4.10). As shown in the table 4.7, the reaction was carried on in the suitable conditions of pH and employing as medium different organic solvents, with a different polarity. We observed the formation of the amide in aqueous solution but the reaction was very slow and didn't overcome the 15-20% conversion: the reason of this is probably due, as seen previously, the formation of the free amino acid produced by the spontaneous hydrolysis of the methyl ester.



Scheme 4.10: Enzymatic acylation on derivative **10**.

Conditions	**Time (h)**	**Conversion* %**	**ee_s(Unreact substrate)****	**ee_p(Product of acylation)****
Buffer pH 6 [a], 20°C	60	10-15	-	*92*
THF	24	-	-	-
CH_2Cl_2	48	-	-	-
DIPE	48	traces	-	-

Toluene	69	25	76	-
Hexane	48	40	*70*	*45*
buffer pH 6/Toluene(1:1), 20°C	60 h	10-15	*92*	-
Toluene	40 h	-	-	-
iPrOH	24h	-	-	-
BuOH	24 h	-	-	-

Table 4.7: Screening on acylation of compound **10** with penicillium amidase. [a] Using penicillium acylase supported on Celite. *By reverse phase HPLC; **By chiral HPLC

In the case of the organic solvents, the enzyme, as reported for phenylglycine methyl ester and *p*-hydroxy-phenylglycine methyl ester[32], was able to acylate the poorly soluble free amino group, present in the reaction medium mainly as a suspension , only in toluene or hexane, even if the enantioselectivity was not satisfacient. More polar solvents such as dichloromethane and tetrahydrofuran seemed to have a negative effect on the enzymatic activity, even if they solubilize the starting material. Since the penicillin acylase used for all the enzymatic reaction described in the Scheme 4.10 is a solution in buffer phosphate at pH 7.5, that is the best form only for the hydrolytic reaction, we repeated the reaction in the same conditions, using a sample of penicillin acylase supported on Celite, prepared according to the De Martin *et al* method.[33] . No reaction occurred using toluene as solvent. No reaction wa also observed when the free amino acid (±) **13** was submitted to the enzyme-catalyzed reaction.

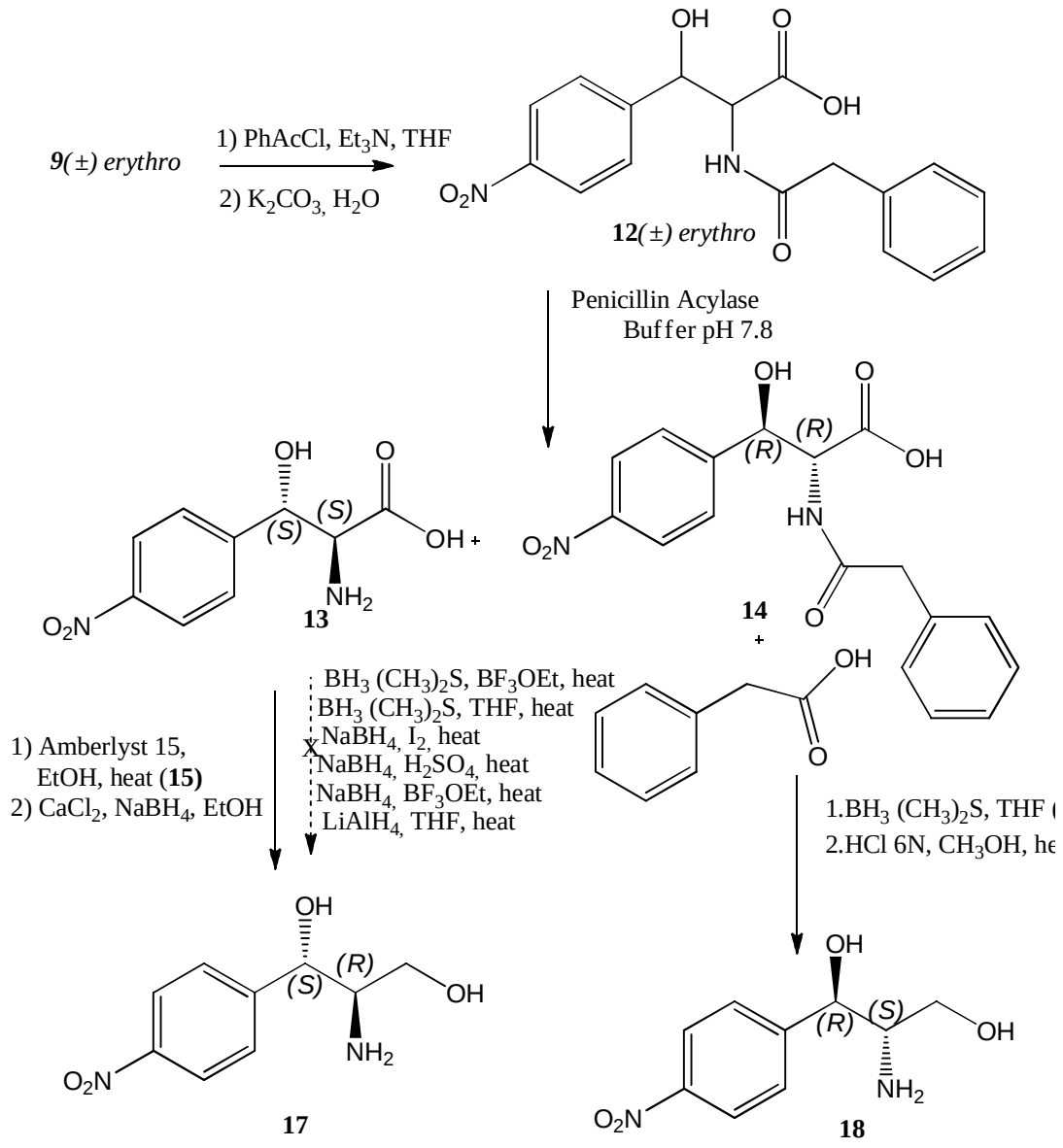
The penicillin acylase- catalyzed hydrolysis of (±)-*erhytro*-**12** is, in conclusion, the best way to obtain enantiomerically pure (S, S)- and (R, R)- desired diasteroisomers, necessary as starting materials for our preparative purposes.

The final preparative pathway is summarized in the scheme 4.11.

Starting from two diastereoisomers of *erythro* p-nitrophenyl-serine methyl-ester hydrochloride **9**, the racemic N-acetyl-derivate **12**, substrate of the enzymatic reaction, was obtained in 81% yields by treatment with PhAcCl (1.1 eq) in presence of Et_3N (1.5 eq) in THF. The product was purified by crystallization from Acetone/Et_2O and after the treatment with aqueous K_2CO_3 afforded the desiderated acid, recovered after acidification of the aqueous layer (83%).

The enzymatic reaction was carried on (±) **12**, as previous described, at room temperature in buffer solution at pH 7.8, using a solution of penicillium acylase in the same buffer at pH 7.5. The progress of reaction was followed by a reverse phase Hplc (Zorbax C-8) eluted with the mixture of

$H_2O/CH_3CN/TFA$ (80:20:0.1), with a flow rate of 0.8 ml/min and it was stopped approximately near the 50% of conversion. The reaction mixture was then cooled and acidified and phenyl-acetic acid and the enantiomerically pure starting material **14** were recovered by an extraction with dichloromethane and separated by a chromatography on Silica gel employing as eluent the mixture Hexane/AcOEt 70:30 (46%). The pH of aqueous solution containing the L-free amino acid **13** was adjusted near its isoelectric point (pH 5.5); water was removed by evaporation at reduce pressure until crystals appeared (36%). The evaluation of the enantiomeric excess (≥ 98%) for both the product **13** and the remaining substrate **14** were determined by using a Chiral OD-H column (Daicel Chemical Industriee, LTD) eluted with hexane/i-PrOH/TFA (80:20:0.1), with a flow rate of 0.7 ml. Peaks were detected at 210 and 254 nm. The Hplc analysis of the free amino acid **13** was done after its derivatization as N-acetyl derivative, in the mixture acetic acid/acetic anhydride.

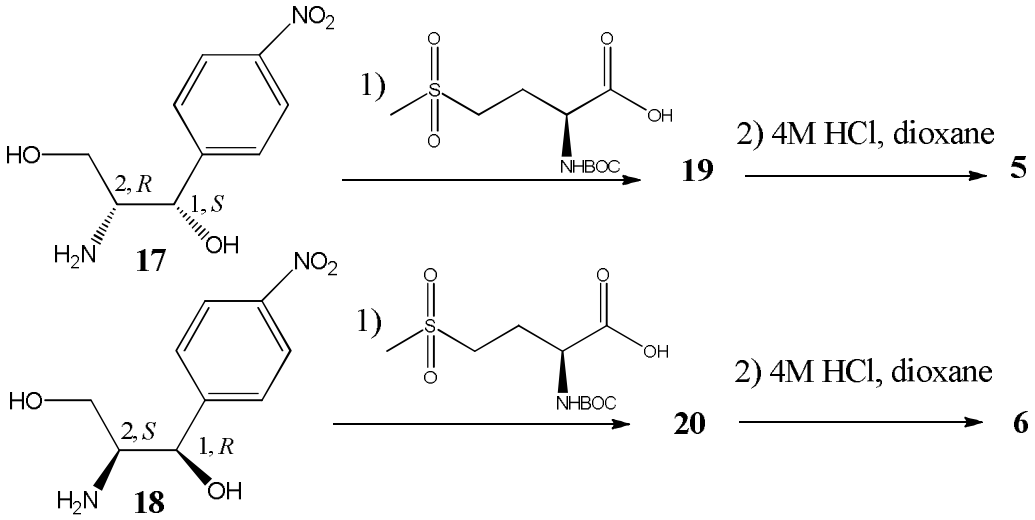


Scheme 4.11: Final synthetic pathway to obtain enantiomers **17** and **18**.

In order to complete the preparation of the enantiomers of p-nitrophenylserinol a reaction of reduction was required.

For the free amino acid **13**, the methods reported in literature showed to be ineffective[34] so it was reduced after its previous esterification employing the mild acidic conditions of the resin Amberlyst 15.[35] The reaction was accomplished in EtOH after a 16 h reflux, due to the low solubility of the starting material in the solvent of reaction. To be sure that these conditions didn't affect the enantiopurity of the compound, a little sample of the ester **15** was withdrawed and, after its conversion to the N-acetyl derivative, analyzed then with the chiral HPLC that confermed its purity (≥ 98%). The so obtained ethyl-ester **15** was then transformed into the wanted amino alcohol **17** by treatment with $CaCl_2$ and $NaBH_4$ (60%).[21]

In the case of the unreact substrate **14** the reduction was obtained with the complex $BH_3.SMe_2$ in THF (70% after purification by chromatography) to give the alcohol **16** and the subsequent hydrolysis of the phenylacetic amide in HCl 6N, at reflux, afforded the enantiomer **18**. The high enantiopurity of the two amino alcohols **17** and **18** was confirmed by comparison with the values of optical rotation reported in literature[36] and shows that, in agreement with the results reported on literature for analogues substrates, penicillin acylase hydrolized only the the (S)-enantiomer of the racemic substrate **12**.



Scheme 4.12: Coupling reactions between amino-alcohols **17** and **18** and n-Boc-L-methionine-sulfone.

The conversion of the amino-alcohols **17** and **18** into the final amides **5** and **6** is explained in the Scheme 4.12.

Commercially available *N*-Boc-methionine-sulfone was condensed with enantiomerically pure amines **5** and **6** under standard conditions using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide

hydrochloride (EDC)/*N*-hydroxysuccinimide in DMF; treatment with 4 M HCl/dioxane resulted in cleavage of the *N*-tert-butoxycarbonyl group provided the corresponding amides **5** and **6** (70%).

4.5 Enzymatic assays

The chemo-enzymatically obtained compounds **5** and **6** were evaluated for *in vitro* inhibitory activity against *H. pylori* GatCAB aminoacyl-tRNA amidotransferase.

This investigation was done at the Département de Biochimie et de Microbiologie, PROTEO, Faculté des Sciences et de Génie, Université Laval in Québec.

The transamidases activity assays were done in presence of the natural substrates L-glutamine and Asp-tRNA^{Asn}

Differents concentrations of inhibitors were used: 0, 62.5, 125, 250, 500 and 1000 µM.

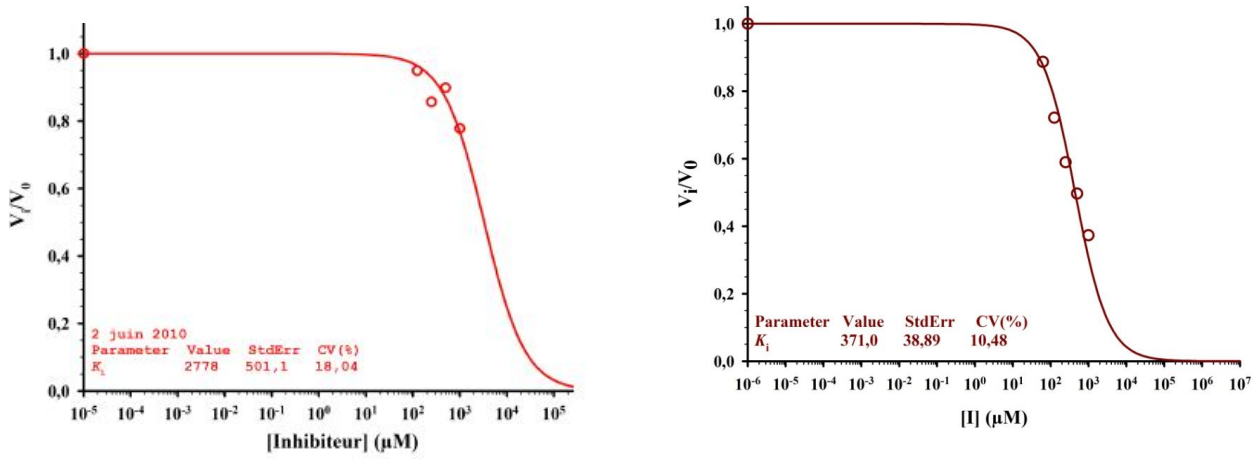
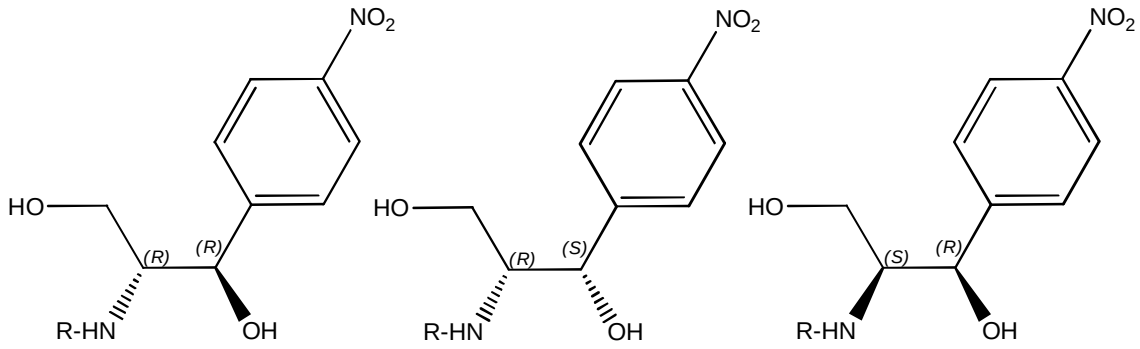


Figure 4.11: Graphics of the kinetic of inhibition for the compound **5** and **6** respectively.

Competitive inhibition with respect to Asp-tRNA^{Asn} was observed for both the two chloramphenicol derivatives. The value of K_i was calculated with the following equation:

$$\frac{v_i}{v_0} = \frac{[S] + K_m}{[S] + K_m(1 + (\frac{[I]}{K_i})}$$ and were 2.8 mM and 371 µM, respectively, for the diastereoisomers (1*S*,2*R*)-**5** and (1*R*,2*S*)-**6.**

Compared with the Ki of the derivative maintaining the same stereochemistry of chloramphenicol **4**, **5** and **6** are less active and, in particular, the loss in potency was more marked for (1*S*,2*R*) **5** than for the (1*R*,2*S*) **6** isomer. As explanation for these results, we can assume that (1*R*,2*R*) stereochemistry of chloramphenicol combined with the L-methionyl-sulfone moiety appeared to be optimal in this series.



R = L-methionine sulfone

4 k_i = 30 µM **5** $\mathbf{k_i}$ = 2.8 mM **6** k_i = 371 µM

Figure 4.12: Three new amidotransferase inhibitors related to the structure of chloramphenicol .

Compound **4** can be considered as a stable analog of the transition state in the last step of the transamidation process, where the carbonyl to be attacked by ammonia is replaced by a tetrahedral sulfur atom with a methyl group mimicking ammonia. It is noteworthy that **4** has an affinity for GatCAB comparable to the substrate Asp-tRNA^{Asn} ($K_m \approx 2$ µM) considering its relative lower size.

Chloramphenicol analogues **4** and **5** and **6** represent a new class of AdT inhibitors and may provide the basis for the design of other low-molecular weight inhibitors.

4.6 References

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5.1. Materials and Methods

General for chapter 2 and 3.

All the reagents and enzymes are purchased from Sigma-Aldrich. All reactions were monitored by TLC on silica gel 60 F_{254} precoated plates with a fluorescent indicator (Merck). For the detection of the products described in the first chapter was employed an ethanolic solution of phosphomolibdic acid (10%); for the compound of chapter two a ninydrin solution (0.3g in butanol, 100mL, and acetic acid, 3 mL) and heating at 110 °C. TLC eluant was prepared mixing water, butanol and acetic acid (5:4:1), and separating the organic phase, after vigorous stirring. Column chromatography were performed on silica gel 60 (0.063-0.200 mm) (Merck). ^{1}H-NMR spectra were recorded on a Bruker-Avance 500 MHz spectrometer. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1 dm cell at 25 °C. HPLC analyses were performed with a Merck-Hitachi L-6200. Mass spectra were recorded on a Thermo Quest Finnigan LCQTM DECA ion trap mass spectrometer. Differential scanning calorimetry (DSC) was performed on a Perkin Elmer DSC-7 instrument. A detailed description of the commercial characteristics of the enzymes utilized is reported in the Table 5.1.1.

Table 5.1.1 Main features of enzymes employed for the experimental parts related to chapters 2-3-4.

Name	**Abbrevation**	**Commercial source**	**U for mg of protein (U/mg)**	**U of enzyme for mmol of substrate (U/mmol)**
Lipase immobilized from *Candida antarctica* A	CAL A	Fluka	3	100-600
Lipase from *Candida antarctica* B	CAL B Novozym 435	Fluka	2.5	50-400
Lipase from *Candida cilindracea*	CCL	Fluka	5.18	90-400
Lipase from *Candida rugosa*	CRL	Sigma	1.14	100
Lipase from *Burkholderia cepacia*	BKL	Sigma	30	100
Lipase from *Pseudomonas fluorescens*	PFL	fluka	42.5	200
Lipase from hog pancreas	PPL	Fluka	23.9	300
Hog liver esterase suspension in 3.2 ammonium sulphate solution	PLE	Fluka	1273	100
α-chymotripsin	α-Chy	Fluka	50	300

Papain	-	Sigma	1.2	300
Protease from *Bacillus subtilis*	-	Biochemika	20	75
Protease from *Bacillus licheniformis (subtilisin Carlsberg)*	STC	Fluka	10.7	75
ALCALASE CLEA®	-	Sigma	5	76
Acylase from porcine kidney, Grade II	PKA	Sigma	500-1.500	5
Acylase from *Aspergyllus melleus*	CAL A	Fluka	1.06	5
Acylase from *Streptomyces toyocaensis*	-	Fluka	46	5
Penicillin acylase from *E. coli*, buffer solution pH 7.5	Pen G	Fluka	37; (56 mg enzyme/ml sol)	80

5.1. Materials and methods chapter 2

5.1.2 32-O-acetyl-ascomycin (3).

To a solution of ascomycin **1** (0.1 g; 0.126 mmol) in toluene (8 mL) vinyl acetate (0.473 g, 5.5 mmoles) and CAL B, 0.240 g, were added. The reaction progress was monitored by TLC (hexane/acetone 65/35). After stirring at 40°C for 80 h the enzyme was filtered off and the recovered filtrate was concentrated under reduced pressure. The residue was crystallized from acetone/water (0.1 g; 94%).

$C_{45}H_{71}NO_{13}$: 834.05 g/mol; white solid.
Differential Scanning Calorimetry (DSC) endothermic peak of fusion 134.25 °C;
$[\alpha]_D^{20°C}$ -74.2 (*c* 0.5 $CHCl_3$);
IR λ_{max} 3484.245, 2935.287, 1735.331, 1649.741, 1450.039, 1372.278 cm^{-1};
MS (ESI+) (*m/z)* 856.4 $[M+Na]^+$.

5.1.3 24,32-di-O-acetyl- ascomycin (4).

To a stirred solution of ascomycin **1** (1 g; 1.26mmol) in pyridine (12.5 mL), kept at 0°C, DMAP (0.680 g) and acetic anhydride (0.570 g, 5.6 mmol) were added. After 1.5 h, under stirring at 0°C, the reaction mixture was diluted with water and extracted with ethyl acetate (3 x 25 mL); collected organic phases were washed with 0.5 N HCl (5 x 10 mL), dried over

Na_2SO_4 and evaporated at reduced pressure. The residue was crystallized from acetone/water (0.985 g; 90%). A sample was purified for analytical purposes by silica gel column chromatography (hexane/acetone 8/2 as eluant).

$C_{47}H_{73}NO_{14}$: 876.08 g/mol; white solid.
DSC endothermic peak of fusion 234.10 °C;
$[\alpha]_D^{20°C}$ -100.0 (*c* 0.5 $CHCl_3$);
IR λ_{max} 3462.749, 2935.824, 1734.403, 1650.739, 1449.091, 1371.079 cm^{-1};
MS (ESI+) (*m/z)* 898.4 $[M+Na]^+$.

5.1.4 24-O-acetyl-ascomycin (5).

To a solution of 24,32-di-O-acetyl-ascomycin **4** (0.5 g; 0.57 mmol) in TBME (25 mL) *n*-octanol (4.5 eq; 0.371 g) and CAL B 1.1 g were added. The reaction progress was monitored by TLC (hexane/acetone 65/35). After stirring at 40°C for 100 h the enzyme was filtered off and the recovered filtrate was concentrated under reduced pressure. The residue was crystallized from acetone/water (0.44 g; 93%). A sample was purified for analytical purposes by column chromatography on silica gel (hexane/acetone 7/3 as eluant).

$C_{45}H_{71}NO_{13}$: 834.05 g/mol; white solid.
DSC endothermic peak of fusion 134.68 °C;
$[\alpha]_D^{20°C}$ -102.7 (*c* 0.5 $CHCl_3$);
IR λ_{max} 3491.528, 2935.860, 1744.728, 1710.227, 1652.310, 1448.662, 1371.335 cm^{-1};
MS (ESI+) (*m/z)* 856.4 $[M+Na]^+$.

5.1.5 24-O-acetyl-pimecrolimus (6).

To a stirred suspension of polimer bound triphenyl phosphine (1.6 eq; 0.10 g) in anhydrous toluene (3 ml), under argon, 24-O-acetyl-ascomicin **4 (**0.2 mmol; 0.167 g) dissolved in 2 ml of toluene and anhydrous pyridine (0.54 ml) were added. The reaction was warmes at 60 °C for 1 h and then, after cooling at room temperature, H_2O (2 ml) was added.
The organic phase was recovered by extraction and washed with NaCl sat.sol. (3 X 3 ml), dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified by silica

gel column chromatography (hexane/acetone 85/15 as eluant) to afford **6** (0.05 g; 40% yield).

$C_{45}H_{70}ClNO_{12}$: 852.49 g/mol; white solid.
DSC endothermic peak of fusion 231.67 °C;
$[\alpha]_D^{20°C}$ -75.2 (*c* 0.5 $CHCl_3$);
^{1}H-NMR ($CDCl_3$) selected data of major rotamer (ppm) 2.06 (s, CH_3CO), 4.50-4.60 (m, H-2 and H-32), 5.22 (m, H-24).
IR λ_{max} 3464.941, 2934.360, 1738.993, 1650.366, 1450.424, 1371.557 cm^{-1};
MS (ESI+) (*m/z)* 874.3 $[M+Na]^+$.

5.1.6 24-O-tertbutyldimethylsilyl, 32-O-acetyl-ascomycin (7).

To a stirred solution of 32-O-acetyl-ascomycin **3** (0.1 g; 0.12 mmol) in CH_2Cl_2 (5 mL), kept at 0°C, 2,6 lutidine (15 eq; 0.2 ml) and TBDMSOTf (5 eq, 0.14 ml) were added. The reaction mixture was stirred at room temperature for 1 h, then cooled and quenched with $NaHCO_3$ sat.sol. (5 ml). The organic phase was then recovered and washed with HCl 0.5 N (3 x 5 mL) and with NaCl (3 x 5 mL), dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified for analytical purposes by silica gel column chromatography (hexane/acetone 9/1 as eluant) to afford **7**, 74%.

$C_{51}H_{85}NO_{13}Si$: 852.49 g/mol; white solid.
DSC endothermic peak of fusion 236.43 °C;
$[\alpha]_D^{20°C}$ -81.4 (*c* 0.5 $CHCl_3$);
^{1}H-NMR ($CDCl_3$) selected data of major rotamer δ (ppm) 0.05,(s, CH_3Si), 0.06 (s, CH_3Si), 0.89 (s, $(CH_3)_3C$), 2.10 (s, CH_3CO), 4.08 (m, H-24), 4.71 (m, H-32).
IR λ_{max} 3462.948, 2934.450, 1739.236, 1649.937, 1450.323, 1371.477cm^{-1};
MS (ESI+) (*m/z)* 970.5 $[M+Na]^+$.

5.1.7 24-O-tertbutyldimethylsilyl-ascomycin (8).

Removal of 32-acetate was realized in the same conditions utilized for preparation of 24-O-acetyl-ascomycin **5** from corresponding 24,32-diacetate **4**. Chemical-physical data of **8** are in agreement with those reported in literature (Ref. 6).

5.1.8 Pimecrolimus (2).

a) The chlorination of 24- O-tertbutyldimethylsiklyl-ascomycin **8** was performed following the procedure reported for **6**, yield 70%.

b) To a solution of compound **9** (1.23 g, 1.35 mmol) in dichloromethane/methanol (1/1, 11 mL) PTSA was added (0.100 g, 0.53 mmol). The mixture was kept, under stirring, at 20-25 °C for 72h, monitoring the reaction progress by TLC (hexane/acetone 8/2). A sodium dihydrogen carbonate (0.04 g) aqueous solution (6 mL) was added; the organic phase was washed with brine and water, dried over sodium sulphate. After solvents evaporation at reduced pressure crude pimecrolimus, as a foam, was recovered. Silica gel column chromatography (hexane/acetone 8/2) afforded pure pimecrolimus that was crystallized from ethyl acetate/ cyclohexane/ water (0.5 g, 46%).

$C_{43}H_{68}ClNO_{11}$: 810.45 g/mol; white solid.
DSC endothermic peak of fusion 142.58 °C;
$[\alpha]_D^{20°C}$ -52.0 (*c* 0.5 $CHCl_3$)
IR λ_{max} 3458.61, 2937.83, 1738.37, 1690.26 1634.72, 1445.60, 1445.63, 1382.72 cm^{-1};
MS (ESI+) (*m/z)* 832.5 $[M+Na]^+$.
^1H and ^{13}C-NMR data were in agreement with the reported ones (Ref.29).

5.2 Materials and Methods chapter 3

5.2.1 2-(2-Thienyl)ethyl 1-*p*-tolylsulfonate (3)

p-Toluenesulfonyl chloride (4.55 g, 23.87 mmoles) and triethylamine (3.36 mL, 24.1 mmoles) were added to a solution of 2-(2-thienyl)-ethanol **7** (3 g, 23.4 mmoles) in diisopropylether (23 mL) kept under stirring at room temperature until starting material disappearance (50 h, TLC toluene/ethyl acetate 9/1, detection with a 5% phosphomolybdic acid ethanol solution). The organic phase was washed with water (15 mL), a 30% potassium carbonate aqueous solution (10 mL) and water (2 x 10 mL) and, then, dried over sodium sulphate, filtered and evaporated under reduced pressure to afford tosylate **3** (5.85 g, 86%).

$C_9H_{10}N_2O_5$, 266.19 g/mol : yellow oil.

^{1}H-NMR ($CDCl_3$) δ 2.47 (s, 3H, CH_3Ar), 3.20 (t, 2H, CH_2CH_2O, J 7 Hz), 4.25, (t, 2H, CH_2O, J 7 Hz), 6.82 (d, 1H, H-3', J 3.4 Hz), 6.92 (m, 1H, H-4'), 7.16 (d, 1H, H-5', J 5 Hz), 7.34 (d, 2H, Ar, J 8 Hz), 7.78 (d, 2H, Ar, J 8 Hz).

5.2.2 (R,S)-2-Chlorophenylglycine methylester (5)

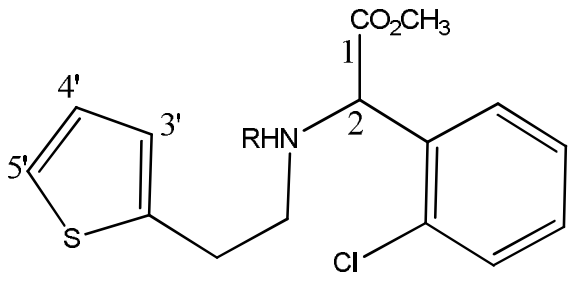
To a solution of compound **4** (3 g, 10.5 mmoles) in dry dichloromethane (80 mL), anhydrous methanol (0.85 mL), DCC (2.38 g, 11.54 mmoles) and DMAP (0.12 g, 1.05 mmoles) were sequentially added. The reaction mixture was kept under stirring at room temperature (2h). The ester formation was monitored by TLC. The white precipitate was removed by suction. The filtrate was washed with a sodium hydrogen carbonate solution at pH 8 (2 x 20mL), dried by treatment with sodium sulphate and evaporated at reduced pressure. The residue was purified by silica gel column chromatography (1/10) affording pure **6** (eluant hexane/ethyl acetate 98/2, 2.7 g, 86%).

$C_9H_{10}ClNO_2$, 199.63 g/mol : yellow oil.

^{1}H-NMR ($CDCl_3$) δ 2.3 (m, 2H, NH_2), 3.75 (s, 3H, CH_3O), 5.06 (br s, 1H, CH), 7.25-7.33 (m, 2H, Ar), 7.36-7.43 (m, 2H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 52.57, 55.95, 127.41, 128.65, 129.29, 129.96, 133.36, 137.79, 173.63.

MS(ESI +) *m/z* 199.9 $[M+H]^+$.



5.2.3 Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate (6)

To a solution of **5** (2.2 g, 11.0 mmoles) in acetonitrile (11 mL) tosylate **3** (2.8 g, 9.9 mmoles), sodium hydrogen carbonate (1.1 g, 13.2 mmoles) and potassium iodide (0.18g, 1.1 mmoles) were added. The mixture was kept under stirring at reflux (14h). At this time an additional amount of potassium iodide (0.18g) was added and the reaction was continued for other 6 h, at reflux, monitoring the reaction progress by TLC (toluene/ethyl acetate 95/5, detection with 5% phosphomolybdic acid ethanol solution). Solvent was evaporated at reduced pressure and the residue was dissolved with ethyl acetate (30 mL); the organic phase was washed with water (10 mL) and a 15% sodium chloride aqueous solution (15 mL). After drying over sodium sulphate and filtration the solvent was removed under reduced pressure. Crude residue **6** was purified by silica gel column chromatography (1/10). Elution with hexane/ ethyl acetate 9/1 afforded pure **9** (2.38g, 70%).

$C_{15}H_{16}ClNO_2S$, 309.81 g/mol : yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.21 (m, 1H, NH), 2.82 (m, 1H, CH-N), 2.96 (m, 1H, CH-N), 3.07 (t, 2H, *CH_2*-thienyl, J 7 Hz), 3.72 (s, 3H, CH_3O), 4.98 (br s, 1H, *CH*-$COOCH_3$), 6.85 (d, 1H, H-3', J 3.4 Hz), 6.94 (m, 1H, H-4'), 7.15 (d, 1H, H-5', J 5 Hz), 7.23-7.32 (m, 2H, Ar), 7.35-7.43 (m, 2H, Ar).

^{13}C NMR (120 MHz, CD_3OD) δ 30.21, 48.70, 52.17, 61.32, 123.45, 124.90, 126.65, 127.14, 128.54, 129.10, 129.84, 133.94, 135.78, 141.86, 172.27.

MS (ESI+) m/z 310 (M+1 with ^{35}Cl), 312 (M+1 with ^{37}Cl).

- General procedure for the enzyme-catalyzed acylation of (6)

To a stirred solution of **6** (100 mg; 0.32 mmol), at room temperature, in the suitable solvent (5 ml), see (Table 3.1), the selected enzyme and acyl-donor were added (2 eq.). The reaction progress was monitored by TLC (Hexane/AcOEt 90:10). After stirring at room

temperature for the times reported in the table **2**, the enzyme was filtered off and the recovered filtrate was evaporate under reduced pressure. The values of conversion and of enantiomeric excess were elaborated after analysis, of a sample of the reaction, with a chiral HPLC.

The HPLC analysis was carried on a chiral Daicel Chiralpack IA 0.46 cm x 25 cm, using as mobile phase the mixture of hexane/THF/Et_2NH (80:20:0.1) and a flow rate of 0.7 ml/min. Detection wavelenght: 220 nm.

R_T: Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate **(6)** : 6.95-7.70 min

R_T:N-Acetyl-Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate **(7)**18-22 min

R_T: N-butanoyl-Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate **(8)** : 11.18-12.67 min

The identity of the products was confirmed by the comparison with the characterizations of the compounds obtained by chemical synthesis.

-General procedure for the preparation of the substrates 7 and 8

To a stirred solution of **6** (2 g; 6.4 mmol) in pyridine (10 ml) 5 eq of the suitable anhydride (acetyc anhydride for compound **7**, butyric anhydride for compound **8**) was added, cooling the solution with a bath of ice. The reaction was then stirred at room temperature for 1 night. The reaction was diluted with water and extracted with ethyl acetate (3 x 25 mL); collected organic phases were washed with 0.5 N HCl (5 x 10 mL), dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified for analytical purposes by silica gel column chromatography (hexane/AcOEt 80/20 as eluant). Yields: 80-85 %.

5.2.4 N-Acetyl-Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate (7)

$C_{17}H_{18}ClNO_3S$, 351.85 g/mol : yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.13 (s, 3H, CH_3-CON),2.47 (m, 1H, CH-N), 2.87 (m, 1H, CH-N), 3.45 (m, 2H, *CH_2*-thienyl), 3.76 (s, 3H, CH_3O), 6.37 (br s, 1H, *CH*-$COOCH_3$), 6.59 (d, 1H, H-3', J 2.9 Hz), 6.85 (dd, 1H, J_1 = 3.4, J_2 = 5.0, H-4'), 7.07 (d, 1H, H-5', J 5.1 Hz), 7.31-7.42 (m, 2H, Ar), 7.43-7.57 (m, 2H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 21.35, 29.72, 48.75, 52.46, 59.14, 123.93, 125.33, 127.01, 127.31, 130.05, 130.33, 130.54, 132.45, 135.51, 139.91, 170.56, 171.26.

MS (ESI+) m/z 351.8 (M+1 with ^{35}Cl).

5.2.5-N-butanoyl-Methyl *(±)*-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate (8)

$C_{19}H_{22}ClNO_3S$, 379.90 g/mol : colourless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, 3H, J = 7.4, CH_3-$(CH_2)_2CO$), 1.74 (sestetto, 2H, J = 7.4, CH_2-CH_3), 2.34 (m, 2H, CH_2-CO), 2.48 (m, 1H, *CH_2*-thienyl), 2.88 (m, 1H, *CH_2*-thienyl), 3.47, (t, 2H, J = 8, CH_2-N) 3.79 (s, 3H, CH_3O), 6.38 (br s, 1H, *CH*-$COOCH_3$), 6.60 (d, 1H, H-3', J = 3.3 Hz), 6.88 (dd, 1H, H-4', J_1 = 3.4, J_2 = 5.1 Hz), 7.10 (d, 1H, H-5', J = 5.1 Hz), 7.30-7.44 (m, 3H, Ar), 7.44-7.55 (m, 2H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 13.95, 18.81, 30.08, 35.08, 48.08, 52.55, 59.40, 123.97, 123.35, 127.10, 127.38, 130.17, 130.35, 130.64, 132.76, 135.70, 140.17, 170.85, 173.90.

MS (ESI+) m/z 379.9 [M].

5.2.6 N-*tert*-butoxycarbonyl-Methyl*(±)*-2-(2-chlorophenyl)-2-[2-(thien-2 yl) ethylamino]-acetate (9)

To a stirred solution of **6** (2 g; 6.4 mmol) in CH_2Cl_2 (15 ml) tert-butoxycarbonyl anhydride (5 eq; 7 g) and Et_3N (5 eq; 4.46 ml) were added. The so obtained solution was stirred at room temperature for 1 night. The reaction was then diluted with water. The organic phase was recovered and washed with HCl 0.5 N (3 x 5 mL); dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified for analytical purposes by silica gel column chromatography (hexane/AcOEt 90/10 as eluant) to afford 2.5 g of **9**. Yield 95%.

$C_{20}H_{24}ClNO_4S$, 409.93 g/mol : colourless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.48 (s, 9H, $(CH_3)_3$-O),2.80 (m, 2H, CH-thienyl), 3.04 (t, 2H, J = 6.8, CH-N), 3.80 (s, 3H, CH_3O), 6.85 (d, 1H, J = 2.7, *CH*-$COOCH_3$), 6.92 (dd, 1H, J_1 = 3.5, J_2 = 4.8, H-3'), 7.13 (d, 1H, J = 5.0, H-4'), 7.24-7.32 (m, 3H, Ar), 7.32-7.43 (m, 1H, Ar), 7.45-7.55 (m, 1H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 27.86, 28.50, 48.75, 52.42, 52.92, 74.11, 123.63, 125.25, 126.65, 127.27, 129.44, 130.26, 131.16, 134.60, 135.65, 141.46, 167.39, 169.78.

MS (ESI+) m/z 409.8

-General procedure for the enzymatic hydrolysis of (R,S)-2-Chlorophenylglycine methylester 5

The substrate *(R,S)*-2-chlorophenylglycine methylester **5**, (100 mg; 0.5 mmol) was dissolved in the selected medium (5 ml) and the catalyst was added (see table **5**). The reaction was stirred at room temperature and periodically monitored by TLC analysis (eleuent:BuOH/AcOH 99:1 saturated of water; relevating agent: ninhydrine). At the times repoted in the Table 3.5, the reaction was stopped by filtering off the enzyme. The samples for HPLC analyses were prepared by extraction of an amount of reaction mixture, at pH 3, evaporation of solvents and dilution of the residue with methanol.

The HPLC analysis was carried on a chiral Daicel Crownpack CR(+) 0.4 cm x 15 cm, using as mobile phase a solution of $HClO_4$ 10^{-2} M and a flow rate of 1 ml/min. Detection wavelenght: 220 nm.

R_T (R,S)-2-Chlorophenylglycine **4** : 3-5 min

R_T (R,S)-2-Chlorophenylglycine methylester **5** : 18-33 min

-Enzyme-catalyzed acylation of (R,S)-2-Chlorophenylglycine methylester (**5)**

The screening of enzymes-catalyzed acylation on (R,S)-2-Chlorophenylglycine methylester **5** was carried on in the condition reported in Table 3.6 following the same procedure described for the substrate **6**. The conversion and the enantiomeric excess of both the product and the unreact substrate were evaluated with the same column ChiralPack Daicel described above.

mobile phase: Hexane/EtOH/Et_2NH (93:7:0.1)

flow rate: 0.7 ml/min, wavelength: 220 nm

R_T (R,S)-2-Chlorophenylglycine methylester **5** : 17.8-21.3 min

R_T (R,S)N-acetyl-2-Chlorophenylglycine methylester **11** : 26.9-30.4 min

R_T N-butirryl(R,S)-2-Chlorophenylglycine methylester **12** : 18.3-21.9 min

The identity of the products was confirmed by the comparison with the characterizations of the compounds obtained by chemical synthesis. For the experimental procedures see the protocols reported for compound **7**and **8**.

5.2.7 N-acetyl(R,S)-2-Chlorophenylglycine methylester (11)

$C_{11}H_{12}ClNO_3$, 241.67 g/mol : white solid.

^{1}H-NMR ($CDCl_3$) δ 2.03 (s, 3H, CH_3-CO), 3.75 (s, 3H, CH_3O), 5.94 (d, 1H, J = 7.5, CH-NH), 6.23 (d, 1H, J = 6.5 , NH), 7.24-7.31 (m, 2H, Ar), 7.36-7.43 (m, 2H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 22.94, 53.02, 54.68, 127.34, 129.81, 130.18, 130.44, 133.59, 134.82, 169.62, 170.95.

MS(ESI +) *m/z* 241.9.

5.2.8 N-butanoyl(R,S)-2-Chlorophenylglycine methylester (12)

$C_{13}H_{16}ClNO_3$, 269.72 g/mol : white solid.

^{1}H-NMR ($CDCl_3$) δ 0.93 (s, 3H, J = ,7.3, CH_3-CH_2), 1.67 (m, 2H, CH_2-CH_3), 2.23 (m, 2H, CH_2-CO), 3.74 (s, 3H, CH_3O), 5.94 (d, 1H, J = 7.4 , CH-NH), 6.63 (d, 1H, J = 6.8, NH), 7.24-7.31 (m, 2H, Ar), 7.36-7.43 (m, 2H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 13.68, 19.00, 38.25, 53.15, 57.70, 127.06, 127.76, 130.10, 130.46, 133.65, 135.63, 171.05, 172.5.

MS(ESI +) 269.9 *m/z* .

-The preparation for the substrates 13 and 14 was achieved following the procedure reported in ref.1 and 2 respectively.

5.2.9 N-acetyl-(R,S)-2-Chlorophenylglycine (13)

$C_{10}H_{10}ClNO_3$, 227.54 g/mol : white solid.

^{1}H-NMR ($CDCl_3$) δ 2.2 (s, 3H, CH_3-CO), 5.75 (d, 1H, J = 4.5, CH-NH), 7.23-7.32 (m, 2H, Ar), 7.41 (dd, J_1 = 1.7, J_2 = 7.4, 1H, Ar), 7.41 (dd, J_1 = 1.7, J_2 = 7.4, 1H, Ar), 8.3 (d, J = 4.5 , NH).

^{13}C NMR (125 MHz, $CDCl_3$) δ 27.96, 55.11, 81.97, 127.09, 127.99, 129.04, 129.51, 136.84, 156.95, 172.69.

MS(ESI -) *m/z* 227.8.

5.2.10 N-trifluoroacetyl-(R,S)-2-Chlorophenylglycine (14)

$C_{10}H_7ClF_3NO_3$, 281.62 g/mol : white solid.

^{1}H-NMR (CD_3OD) δ 6.00 (s, 1H, CH-NH), 7.31-7.41 (m, 2H, Ar), 7.43-7.52 (m, 2H Ar).

^{13}C NMR (125 MHz, CD_3OD) δ 53.38, 116.46, 126.48, 128.62, 128.94, 129.36, 132.47, 133.47, 156.46, 169.65.

MS(ESI -) *m/z* 281.8.

5.2.11 N-Boc-(R,S)-2-Chlorophenylglycine (15)

To a suspension of 2-chlorophenylglycine **4** (10 g, 0.054 mol) in water (50 mL) 1,4-dioxane (40 mL) and sodium hydroxide (2.37 g, 0.059 mol) were added. After addition of di-*tert*-butyl dicarbonate (12.4 mL, 0.054 mol) the mixture was kept under stirring at room temperature (18 h). The reaction progress was monitored in TLC. The mixture was concentrated at reduced pressure and 1M hydrochoric acid was added until pH 3. The precipitated product **15** (13.7 g, 89% yield) was recovered by suction.

$C_{13}H_{16}ClNO_4$; 285.08 g/mol: white solide

^{1}H-NMR ($CDCl_3$) δ 1.23 (s, 9H, CH_3C), 5.75 (d, 1H, CH, J 4.5 Hz), 7.22-7.34 (m, 2H, Ar), 7.41 (d, 1H, Ar, J 6.5 Hz), 7.51 (d, 1H, Ar, J 6.9 Hz), 8.23 (d, 1H, NH, J 4.5 Hz).

^{13}C NMR (125 MHz, $CDCl_3$) δ 28.02, 54.99, 82.00, 128.55, 128.84, 129.55, 130.11, 134.08, 136.99, 154.13, 172.86.

MS (ESI -) m/z 284 (M-1 with ^{35}Cl), 286 (M-1 with ^{37}Cl).

5.2.12 N-Boc-(R,S)-2-Chlorophenylglycine methylester (16)

To a solution of compound **15** (3 g, 10.5 mmoles) in dry dichloromethane (80 mL), anhydrous methanol (0.85 mL), DCC (2.38 g, 11.54 mmoles) and DMAP (0.12 g, 1.05 mmoles) were sequentially added. The reaction mixture was kept under stirring at room temperature (2h). The ester formation was monitored by TLC. The white precipitate was removed by suction. The filtrate was washed with a sodium hydrogen carbonate solution at pH 8 (2 x 20mL), dried by treatment with sodium sulphate and evaporated at reduced

pressure. The residue was purified by silica gel column chromatography (1/10) affording pure **16** (eluant hexane/ethyl acetate 98/2, 2.7 g, 86%).

$C_{14}H_{18}ClNO_4$; 299.75 g/mol: white solide

^{1}H-NMR (CD_3OD) δ 1.47 (s, 9H, CH_3C), 3.74 (s, 3H, CH_3O), 5.73 (br s, 1H, CH), 7.30-7.38 (m, 2H, Ar), 7.43 (m, 1H, Ar), 7.50 (m, 1H, Ar).

^{13}C NMR (125 MHz, $CDCl_3$) δ 28.37, 52.96, 55.70, 80.30, 127.34, 129.70, 129.90, 130.20, 133.70, 135.38, 154.95, 171.17.

MS (ESI+)m/z 321.9 [$M+Na^+$]

5.2.13 N-Boc-(S)-2-Chlorophenylglycine (15)

*a. By subtilisin-catalyzed hydrolysis of (R,S)-**(16)***

To a solution of (R,S)-1**6** (1 g, 3.34 mmoles) in TBME (18 mL), pH 7.5 buffer (0.1 M KH_2PO_4/ 0.1M NaOH 50 mL/ 40.9 mL) (36 mL) and protease from *Bacillus licheniformis* (24 mg, 255 U) were added. The reaction was kept under stirring, at 35 °C, and pH was maintained at pH 7.5 by addition of 0.5M sodium hydroxide. The reaction progress was monitored by HPLC (see Table 3). The samples for HPLC analysis were prepared as follows: an amount of aqueous and organic phase, 20 µl and 10 µl respectively, was withdrawn and after collection, TBME was removed by means of a nitrogen stream. After addition of methanol, the mixture was filtered through a 0.45 µm GHP ACRODISC and the resultant solution was analyzed. At 40% conversion (65 h) the pH was brought at 8 and the aqueous phase, after separation from the organic one, extracted with TBME (5 x 15 mL). Extraction with TBME of aqueous phase at pH 3, drying with sodium sulphate, filtration and evaporation under reduced pressure afforded (S)-acid 1**5** (0.33 g. 35%).

$[\alpha]_D$ +101 (c 1, CH_3OH).

A sample was treated with TFA, as described below for compound 1**6**, and analyzed by HPLC (Table 3.9) in order to determine the ee (>99%).

*b. By Alcalase-CLEA®-catalyzed hydrolysis of (R,S)-**(16)***

To a solution of (R,S)-**16** (1 g, 3.34 mmoles) in tetrahydrofuran (9 mL) water (90 mL) was added and the pH was adjusted to 8 with 2M sodium hydroxide. Alcalase-CLEA (10 g, 255 U) was added and the mixture was kept under stirring at 30 °C, maintaining the pH 8

by addition of 2N sodium hydroxide, monitoring the reaction progress by HPLC (see Table 3). The samples for HPLC analyses were prepared by extraction of an amount of reaction mixture, at pH 3, evaporation of solvents and dilution of the residue with methanol. At 40% conversion (14 h) the reaction mixture was extracted with TBME (3 x 80 mL); the aqueous phase was filtered by suction to remove the enzyme and after acidification to pH 3, extracted with TBME (4 x 60 mL). The organic phase was treated with sodium sulphate and, after filtration, evaporated under reduced pressure, affording (S)-acid **15** (0.32 g, 34%). A sample, after removal of protecting group to give the salt of compound **4**, was analyzed by HPLC (See table 3.9) showing a 98% ee.

5.2.14 N-Boc-(S)-2-Chlorophenylglycine methylester (16)

Esterification of (S)-acid **15** (0.6 g, 2.10 mmoles) was performed following the procedure previously described for (R,S)-**15**. The crude (S)- methylester **16** (0.6 g, 95%) was used in the next step without any further purification. A sample was purified for analytical purposes.

$[\alpha]_D$ + 119.3 (c 1, $CHCl_3$) lit[3] +117.1

5.2.15 (S)-2-Chlorophenylglycine methylester (5)

To a solution of (S)-**16** (0.5 g, 1.67 mmoles) in dichloromethane (10 mL), under stirring at room temperature, a solution of TFA (0.64 mL, 8.35 mmoles) in dichloromethane (10 mL) was added dropwise. The reaction progress was monitored by TLC until disappearance of starting material (3 h). The solvent was removed at reduced pressure. To the residue (98% ee by HPLC) dissolved in water (10 mL), 20% ammonium hydroxide was added until pH 7. Extraction with dichloromethane (3 x 20 mL), followed by treatment with sodium sulphate, filtration and evaporation of the solvent afforded title compound **5** (0.315 g, 90% from (S)-**5,** 98% ee).

$[\alpha]_D$ + 123 (c 1, CH_3OH) lit[4] +134.

^{1}H-NMR ($CDCl_3$) δ 2.3 (m, 2H, NH_2), 3.75 (s, 3H, CH_3O), 5.06 (br s, 1H, CH), 7.25-7.33 (m, 2H, Ar), 7.36-7.43 (m, 2H, Ar).

5.2.16 Methyl (S)-2-(2-chlorophenyl)-2-[2-(thien-2-yl)ethylamino]-acetate (6)

The compound was prepared with the same procedure above for the racemic mixture.

ee 98% (by HPLC, see Table 3.9).

^{1}H-NMR ($CDCl_3$) δ 2.21 (m, 1H, NH), 2.82 (m, 1H, CH-N), 2.96 (m, 1H, CH-N), 3.07 (t, 2H, *CH_2*-thienyl, J 7 Hz), 3.72 (s, 3H, CH_3O), 4.98 (br s, 1H, *CH*-$COOCH_3$), 6.85 (d, 1H, H-3', J 3.4 Hz), 6.94 (m, 1H, H-4'), 7.15 (d, 1H, H-5', J 5 Hz), 7.23-7.32 (m, 2H, Ar), 7.35-7.43 (m, 2H, Ar).

MS (ESI+) m/z 310 (M+1 with ^{35}Cl), 312 (M+1 with ^{37}Cl).

5.2.17 Clopidogrel 1

To a solution of **6** (1 g, 3.23 mmoles) in 1,2-dichloroethane (20 mL) paraformaldehyde (0.11 g, 3.67 mmoles) was added; the mixture was kept under stirring, at reflux, removing water in azeotropic conditions for 4 h. After cooling at 30 °C, a solution (10 mL) of hydrochloric acid in dimethylformamide (4 g of HCl in 100mL of DMF) was added dropwise. The mixture was heated at reflux (2 h) monitoring the reaction progress by TLC (toluene/ethyl acetate 95/5, detection with 5% phosphomolybdic acid ethanol solution). After cooling at room temperature, water (200 mL) was added and the pH was adjusted to 7.5 by means a 30% aqueous potassium carbonate solution. Extraction with ethyl acetate (3x 100mL), followed by washing with water (100 mL), drying over sodium sulphate, filtration and removal of solvent at reduced pressure gave a residue (0.67 g) that was purified by silica gel column chromatography (1/10, hexane/ethyl acetate 98/2 as eluant) affording pure **1** (0.52 g, 50%).

$C_{16}H_{16}ClNO_2S$; 321.82 g/mol: white solide

^{1}H-NMR ($CDCl_3$) δ 2.87-2.96 (m, 4H, H-6 and H-7), 3.68 (d, 1H, H-4, J 4Hz), 3.75 (s, 3H, CH_3O), 3.80 (d, 1H, H-4, J 4 Hz), 4.97 (br s, 1H, H-8), 6.68 (d, 1H, H-3, J 5 Hz), 7.08 (d, 1H, H-2, J 5 Hz), 7.24-7.36 (m, 2H, H-12 and H-13), 7.44 (d, 1H, H-14, J 7.7 Hz), 7.74 (d, 1H, H-11, J 7.7 Hz).

MS (ESI+) m/z 322 (M+1 with ^{35}Cl), 324 (M+1 with ^{37}Cl).

$[\alpha]_D$ + 45 (c 1, CH_3OH).

Ee 98% (by HPLC).

For analytical purposes a sample was transformed into the corresponding hydrogen sulphate:

$[\alpha]_D$ + 54.8 (c 1, CH_3OH) lit[31] +55

DSC (5 °C/min) endotermic peak of fusion at 178.50 °C (ΔH 75.769 J/g).

5.3. Materials and Methods chapter 4

5.3.1. General

Chemical reagents were purchased from Aldrich-Sigma Chemical Company. Flash column chromatography was carried out using 40-63 µM (230-400 mesh) silica gel. Optical rotations were measured using a JASCO DIP-360 digital polarimeter (c as g of compound per 100 mL). Infrared spectra were recorded on a Bomem MB-100 spectrometer. NMR spectra were recorded on a Varian Inova AS400 spectrometer (400 MHz). Mass spectra were obtained on an Agilent 6210 mass spectrometer.

5.3.2. Glycine methyl ester (7)

To a stirred solution of glycine methyl ester hydrochloride **1** (3.00 g, 23.9 mmol), cooled in a bath of ice a cold solution of 1N NaOH (23.9 ml) was added. The aqueous phase was then extracted with CH_2Cl_2 (5 X 30 ml). The collected organic phases were dried with $MgSO_4$ and the solvent evaporated under reduced pressure to yield **7** (0.805 g, 30%).

$C_3H_7NO_2$, 89 g/mol : yellow oil.

IR(film): 3380-3320, 1735, 1600, 1200

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 2H, NH_2), 3.48 (s, 2H, CH_2), 3.77 (s, 3H, CH_3).

5.3.3 (±) – *erythro*-nitrobenzyl-idene- *p*- nitrophenylserinate methyl ester (8)

To a stirred solution of freshly prepared glycine methyl ester **7** (0.56 g, 5.09 mmol), in MeOH (9 ml), 4-nitrobenzaldehyde (1.54 g, 10.18mmol) was added. The so obtained yellow solution was warmed at 45-50 °C for 1 hour and then stirred at r.t. for 1 night. The next morning there was a suspension that was filtered under vacuum. The recovered cake was washed with MeOH (10 ml) and Et_2O (5 ml) to yield **8** (1.10 g, 58%).

$C_{17}H_{15}N_3O_7$, 373 g/mol : white solid.

mp: 144-145 °C (lit.144 °C)

IR(KBr): 3600-3140, 1720, 1643, 1600, 1515, 1435, 1380, 1342, 1230.

^{1}H NMR (400 MHz, DMSO) δ 3.73 (s, 3H, CH_3), 4.36 (d, J = 7.3 Hz, 1H), 5.32 (d, 1H, CH_3), 6.31 (d, J = 4.8 Hz, 0.38H, OH), 7.60 (d, J = 8.9 Hz, 2H),7.92 (d, J = 8.9 Hz, 2H), 8.15 (d, J = 8.6 Hz, 2H), 8.27 (d, J = 8.9 Hz, 2H), 8.30 (s, 1H).

^{13}C NMR (100 MHz, DMSO) δ 52.1, 72.9, 78.5, 122.72, 123.83, 128.45, 129.16, 140.56, 146.66, 148.77, 149.15, 163.36, 169.76.

5.3.4. (±) – *erythro*- *p*- nitrophenylserine methyl ester N-hydrochloride (9)

To a stirred suspension of **2** (0.50 g, 1.34 mmol), in MeOH (10 ml), HCl 12N (1.34 mmol) was added. After 10 minutes, the so obtained yellow solution was kept without stirring for one hour and then Et_2O (10 ml) was added. The precipitation occurres in 2 hours; after filtration under vacuum the salt **3** was recovered as a white solid. (0.36 g, 97%).

$C_{10}H_{13}ClN_2O_5$, 276.5 g/mol : white solid.

mp: 178-183 °C (dec.) (lit.183 °C, dec.)

IR(KBr): 3280, 1750, 1605, 1510, 1353, 1250.

^{1}H NMR (400 MHz, DMSO) δ 3.55 (s, 3H), 4.38 (d, J = 2.4 Hz, 1H), 5.44 (dd, J_1 = 2.4 Hz, J_2 = 4.4 Hz, 1H), 6.81 (d, J = 4.4 Hz, 0.63H, OH), 7.70 (d, J = 8.8 Hz, 2H),8.26 (d, J = 8.3 Hz, 2H), 8.95 (s, 2H, NH_3).

^{13}C NMR (100 MHz, DMSO) δ 13.72, 58.25, 61.38, 70.10, 122.99, 127.27, 146.71, 148.22, 166.21.

5.3.5 (±) – *erythro*-N-*tert*-butoxycarbonyl *p*- nitrophenylserine methyl ester (11)

To a stirred suspension of **9** (2 g, 7.62 mmol), in H_2O (50 ml), NaOH 1N (7.62 ml, 7.62 mmol) and $(Boc)_2O$ (1.83 g, 8.38 mmol), were added. The so obtained solution was refluxed for 1.5 hour and then stirred at r.t. for 1 night. The next morning there was a suspension that was filtered under vacuum. The recovered cake was crystallized from AcOEt/Hexane to yield **11** (2.10 g, 81%).

$C_{15}H_{20}N_2O_7$, 340.3 g/mol : white solid.

mp: 151-154 °C

IR(KBr): 3425, 3361, 2986, 1720, 1689, 1521, 1350, 848, 716.

^{1}H NMR (DMSO) δ 1.23 (s, 9H), 3.61 (s, 3H), 4.15 (t, J = 8.6 Hz, 1H), 4.87 (d, J = 8.0 Hz, 1H), 6.11 (sa, 1H), 7.29 (d, J = 9.2 Hz, 1H), 7.65 (d, J = 8.8 Hz, 2H), 8.19 (d, J = 8.8, 2H).

^{13}C NMR (DMSO) δ 27.96, 51.74, 59.80, 71.68, 78.61, 123.00, 128.13, 146.83, 149.94, 154.85, 171.07.

HRMS (ESI) calculated for $C_{15}H_{20}N_2NaO_7$ $[M+Na]^+$ 363.1163, found 363.1159.

5.3.6 (±) – *erythro*-N-phenylacetyl-*p*-nitrophenilserine (12)

(a) To a chilled suspension of **3** (0.6 g, 2.17 mmol), in anhydrous THF (20 ml), Et_3N (0.45 ml, 3.25 mmol) and PhAcCl (0.31 ml, 2.39 mmol) were added. The mixture of reaction was stirred at room temperature for one night, then the THF was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with $NaHCO_3$ 5% solution (3 x 10 ml) and HCl 1 N (3 x 10 ml) then anhidryfied with $MgSO_4$ and evaporated under reduced pressure. Crystallization from Acetone/Hexane afforded **12**-methylester (0.65 g, 83%).

$C_{18}H_{18}N_2O_6$, 358.35 g/mol : white solid.

mp: 130-132 °C

IR(KBr): 3452, 3281, 1754, 1646, 1519, 1345, 1203, 728.

^{1}H NMR (CD_3OD) δ 3.41 (s, 2H), 3.68 (s, 3H), 4.76 (d, J = 7.2 Hz, 1H), 5.01 (d, J = 7.2 Hz, 1H), 7.06-7.09 (m, 2H), 7.15-7.19 (m, 3H), 7.50 (d, J = 8.8 Hz, 2H), 8.04 (d, J = 8.8, 2H).

^{13}C NMR (CD_3OD) δ 43.35, 52.66, 59.35, 74.24, 124.18, 127.89, 128.84, 129.43, 130.02, 136.36, 148.80, 149.66, 171.71, 173.39.

HRMS (ESI) calculated for $C_{18}H_{19}N_2O_6$ $[M+H]^+$ 359.1238, found 359.1246.

*(b)*To a solution of **12**-methylester (0.63 g, 1.83 mmol), in MeOH (10 ml), K_2CO_3 aqueous solution was added (10 ml). After one night of stirring at room temperature MeOH was removed under reduce pressure and the aqueous residue was extracted with CH_2Cl_2 (2 x 5 ml). After the aqueous layer was chilled, acidified (pH 3) with HCl conc., and extracted with CH_2Cl_2 (3 x 10 ml). The collected organic phases were dried with $MgSO_4$ and evaporated under reduced pressure. Cromatography on silica gel with AcOEt/Hexane 70:30 as eluent afforded **5** (0.51 g, 81%).

$C_{17}H_{16}N_2O_6$, 344.32 g/mol : white solid.

mp: 98-103 °C

IR(KBr): 3465, 3282, 1712, 1524, 1350.

^{1}H NMR (CD_3OD) δ 3.44 (s, 2H), 4.75 (d, J = 6.9 Hz, 1H), 5.05 (d, J = 6.9 Hz, 1H), 7.10 (m, 2H), 7.16-7.23 (m, 3H), 7.51 (d, J = 8.8 Hz, 2H), 8.05 (d, J = 8.8, 2H).

^{13}C NMR (CD_3OD) δ 43.44, 59.33, 74.30, 124.11, 127.91, 128.89, 129.46, 130.10, 136.42, 148.78, 149.75, 172.68, 173.50.

HRMS (ESI) calculated for $C_{17}H_{17}N_2O_6$ $[M+H]^+$ 345.1086, found 345.1098.

5.3.7 Enzymatic resolution of (±) - *erythro*-N-phenylacetyl-*p*- nitrophenilserine (12)

To a stirred solution of **12** (0.75 g, 2.18 mmol), in buffer phosphate at pH 7.8 (60 ml), adjusted with NaOH 0.5 N, penicillium G acylase buffer solution (0.07 ml, 145 U) was added. The mixture was kept under stirring at 25 °C, monitoring the reaction progress by a reverse phase HPLC (Agilent Zorbax C8) using as eluent the mixture H_2O/CH_3CN/TFA (70:30:0.1). Flow rate 0.8 ml/min, detection wavelenght 254 nm (rt = 4-8-12/product, phenylacetic acid, starting material). The samples for HPLC analyses were prepared by acidification (pH 3) of an amount of reaction mixture (0.2 ml)and diluition with the mixture H_2O/CH_3CN (70:30). After 4.30 h the reaction reached approximately the 48% of conversion. The reaction was then chilled, acidified (pH 3) with HCl 2N and extracted with CH_2Cl_2 (5 x 15 ml). The collected organic phases were dried with $MgSO_4$ and evaporated under reduced pressure to afford the mixture of the (1R, 2R) *erhytro*-N-phenylacetyl-*p*-nitrophernylserine **14** and phenylacetic-acid. The enantiomerically pure starting material **14** was purified from the phenylacetic-acid by chromatography on silica gel using as eluant the mixture AcOEt/Hexane 70:30 (0.35 g, 46%). The free amino acid **13** was recovered by precipitation from the remaining aqueous solution, after adjusting the pH to 6.0 and concentration under reduced pressure (0.21 g, 36%).

13: $C_9H_{10}N_2O_5$, 266.19 g/mol : white solid.

mp: 155 °C (dec.)

IR(KBr): 3524, 3452, 3231, 1608, 1518, 1349.

^{1}H NMR (DMSO) δ 3.44 (d, J = 6.0, 1H), 5.07 (d, J = 6.0 Hz, 1H), 7.66 (d, J = 8.8 Hz, 2H), 8.18 (d, J = 8.8, 2H).

HRMS (ESI) calculated for $C_9H_{11}N_2O_5$ $[M+H]^+$ 227.0668, found 227.0672.

$[\alpha]^d_{25}$ + 66.4 (c = 1, HCl 1N)

A sample (15 mg, 0.04 mmol) was converted to the N-acetyl derivative in order to control the enantiopurity with a chiral HPLC.

The reaction was carried on in the mixture of ACOH (2 ml) and $(AC)_2O$ (1.5 eq). After two hours of strirring at room temperature the mixture was evaporated under reduced pressure; the residue was taken up in AcOEt (5 ml) and washed with H_2O (5 x 3 ml). The organic phase was then dried with $MgSO_4$ and evaporated under reduced pressure.

With the same procedure a sample of the racemic mixture was prepared.

The HPLC analysis was carried on a chiral OD-H Daicel 0.46 cm x 25 cm, using as mobile phase the mixture of hexane/*i*-PrOH/TFA (80:20:0.1) and a flow rate of 0.7 ml/min. Detection wavelenght: 254 nm.

R_T (±) N-acetyl *p*-nitrophenyl serine : 14.73-17.66

R_T (+) N-acetyl *p*-nitrophenyl serine : 17.19

14: $C_{17}H_{16}N_2O_6$, 344.32 g/mol : white solid.

$[\alpha]^d_{25}$ - 26.5 (c = 1, MeOH)

The HPLC analysis was carried on a chiral OD-H Daicel 0.46 cm x 25 cm, using as mobile phase the mixture of hexane/*i*-PrOH/TFA (80:20:0.1) and a flow rate of 0.7 ml/min. Detection wavelenght: 254 nm.

R_T (±) N-phenylacetyl *p*-nitrophenyl serine : 16.60 -19.04.

R_T (+) N-phenylacetyl *p*-nitrophenyl serine : 16.60.

5.3.8 (+) *erythro*– *p*-nitrophenyl serinolo (17)

(a) The free amino acid **13** (0.15 g, 0.56 mmol), in EtOH (10 ml), was refluxed for one night with Amberlyst 15 (200 mg). Then the reaction was cooled and the resin was removed by filtration. A sample of the filtrate was dried under reduced pressure and derivatized as N-acetyl derivative to be analyzed with a chiral HPLC. The reaction was done in H_2O with AcONa (4 eq) and Ac_2O (1.5 eq). The reaction finished in 15 minutes, then the mixture was acidified (pH 3) with HCl 1 N and the product was extracted with AcOEt (3 x 2 ml). After dring on $MgSO_4$ and removing the solvent under reduced pressure the (+) N-acetyl *p*-nitro-phenyl serine ethyl ester **15** was obtained.

With the same procedure a sample of the racemic mixture was prepared.

The HPLC analysis was carried on a chiral OD-H Daicel 0.46 cm x 25 cm, using as mobile phase the mixture of hexane/*i*-PrOH/TFA (80:20:0.1) and a flow rate of 0.5 ml/min. Detection wavelenght: 254 nm.

R_T (±) N-acetyl *p*-nitrophenyl serine ethyl ester **15** : 15.50 - 22.63.

R_T N-acetyl *p*-nitrophenyl serine ethyl ester **15** : 22.26.

(b) The remaining filtrate was chilled and treated with anhydrous $CaCl_2$ (0.086 g, 0.78 mmol) and $NaBH_4$ (0.22 g, 2.97 mmol) and stirred at room temperature for one night under argon. The reaction is cooled and H_2O (20 ml) and CH_2Cl_2 (40 ml) were added. The mixture was stirred vogorously for

1 hour. The insolubles are removed by filtration and the organic solvents were evaporated under reduced pressure. The aqueous phase was extracted with CH_2Cl_2 (5 x 10 ml) and the combined organic extracts were dried on $MgSO_4$ and the solvent was removed under reduced pressure. Compound **17** was purified by cromatography on silica gel with AcOEt/MeOH/NH_4OH 50:20:1 (0.05 g, 37%).

$C_{11}H_{18}N_2O_4$, 242.27 g/mol : white solid.

mp: 106-108 °C

IR(KBr): . 3370, 3306, 2917, 1518, 1343, 1058, 1026 cm^{-1}

^1H NMR (400 MHz, CD_3OD) δ 3.06 (q, J = 5.8, 1H), 3.55 (d, J = 6, 2H), 4.78 (d, J = 5.8, 1H), 7.63 (d, J = 8.6, 2H), 8.22 (d, J = 8.6, 2H).

^{13}C NMR (100 MHz, CD_3OD) δ 57.79, 62.11, 73.85, 123.17, 127.68, 147.59, 149.99.

HRMS (ESI) calculated for $C_9H_{12}N_2O_4$ $[M+H]^+$ 213.0870, found 213.0878.

$[\alpha]_D^{25}$ + 3.0 (c 0.5, MeOH)

5.3.9(-) -*erythro*-N-phenylacetyl-*p*- nitrophenylserinolo (16)

To a stirred solution of **14** (0.28 g, 0.81 mmol) in anhydrous THF (50 ml) cooled with a bath of ice, a 2 M THF solution of $BH_3(CH_3)_2S$ (0.6 ml, 1.2 mmol) was slowly added. The mixture was stirred at room temperature for one night. Then the reaction was chilled and MeOH (10 ml) was added. The solvents were evaporated and the resulting oil was chromatographyed on silica gel using as eluant the mixture of CH_2Cl_2/MeOH (98:2) to yield **16** (0.16 g, 59%). The unreact starting material **14** was recovered with the mixture CH_2Cl_2/MeOH (99:1), (0.1 g, 36 %).

$C_{17}H_{18}N_2O_5$, 330.34 g/mol : white solid.

mp: 130-137 °C

IR(KBr): 3542, 3407, 3291, 1657, 1524, 1355.

^1H NMR (400 MHz, CD_3OD) δ 3.33 (d, J = 2.2, 2H), 3.70 (dd, J_1 = 3.8, J_2 = 11.3, 1H), 3.83 (dd, J_1 = 5.7, J_2 = 11.2, 1H), 4.07-4.15 (m, 1H), 4.75 (d, J = 8.0, 1H), 7.00 (m, 2H), 7.11-7.16 (m, 3H), 7.47 (d, J = 8.8 Hz, 2H), 7.99 (d, J = 8.8, 2H).

^{13}C NMR (100 MHz, CD_3OD) δ 43.81, 57.55, 61.94, 73.53, 124.08, 127.74, 128.99, 129.32, 129.95, 136.66, 148.56, 151.24, 173.53.

HRMS (ESI) calculated for $C_{17}H_{19}N_2O_5$ $[M+H]^+$ 331.1288, found 331.1294.

$[\alpha]_D^{25}$ - 3.0 (c 1, MeOH)

5.3.10 (-)-*erythro-p*-nitrophenyl-serinol (18)

Compound **16** (0.12 g; 0.36 mmol) was refluxed for 1 h in HCl 6N (6 ml). After cooling of solution, the solid was removed by filtration. Then the filtrate was evaporated and the so obtained residue was taken into H_2O (5 ml) and made basic with NH_4OH. The aqueous phase was extracted with AcOEt (5 X 3 ml), the extracts were dried over $MgSO_4$ and evaporated under reduced pressure. The product was chromatographied on SiO_2 with AcOEt/MeOH/NH_4OH (50:20:1) as eluant to give a yellow oil **18** (0.07 g, 80 %). Crystallization from CH_2Cl_2 was very slow.

$C_{11}H_{18}N_2O_4$, 242.27 g/mol, yellow solid;

mp 111-112°C (lit.112-113°C).

IR (KBr) 3371, 3307, 2913, 1515, 1347, 1056, 1025 cm^{-1}.

^{1}H NMR (400 MHz, CD_3OD) δ 3.03 (q, J = 5.8, 1H), 3.49-3.62 (m, 2H), 4.76 (d, J = 5.9, 1H), 7.63 (d, J = 8.6, 2H), 8.22 (d, J = 8.6, 2H).

^{13}C NMR (100 MHz, CD_3OD) δ 59.0, 63.7, 75.3, 124.3, 128.9, 148.8, 151.3.

HRMS (ESI) calculated for $C_9H_{12}N_2O_4$ $[M+H]^+$ 213.087, found 213.0881.

$[\alpha]_D^{21}$ -2.5 (c 0.5, MeOH).

-General procedure for preparation of amides (5 and **6)**

Amines **5** (25.0 mg, 103 μmol), *N*-Boc-L-methionine-sulfone (31.7 mg, 113 μmol), *N*-hydroxysuccinimide (13 mg, 113 μmol) and EDC (23.6 mg, 123 μmol) were dissolved in anhydrous DMF (1.0 mL). The mixture was stirred at room temperature for 24 h. The solvent was coevaporated with toluene and the residue was purified by flash chromatography (7-8% MeOH/CH_2Cl_2) to yield compound **19** and **20** (70 %).

5.3.11 (1*S*,2*R*)-1-(4-Nitrophenyl)-2-(*N*$^{\alpha}$-Boc-L-methionyl-sulfone-amido)-1,3-propadiol 19.

$C_{19}H_{29}N_3O_9S$; 475.51 g/mol;

yield 84%; white solid.

mp 171-173°C (dec);

$[\alpha]_D^{21}$ -18.4 (c 0.96, CH_2Cl_2/MeOH 1:1);

IR (KBr) 3426, 3342, 2982, 1681, 1658, 1522, 1348, 1285, 1163, 1129, 1051 cm^{-1};

^{1}H NMR (400 MHz, CD_3OD) δ 1.39 (s, 9H), 1.83-1.95 (m, 1H), 1.95-2.08 (m, 1H), 2.89 (s, 3H), 3.03 (t, J = 7.8 Hz, 2H), 3.64 (dd, J = 10.9 and 2.8 Hz, 1H), 3.78 (dd, J = 10.8 and 6.4 Hz, 1H), 4.00-4.14 (m, 2H), 4.80 (d, J = 7.5 Hz, 1H), 7.60 (d, J = 8.2 Hz, 2H), 8.24 (d, J = 8.2 Hz, 2H);

^{13}C NMR (100 MHz, CD_3OD) δ 25.1, 27.4, 39.3, 50.6, 53.3, 56.6, 60.3, 72.3, 79.8, 123.0, 127.8, 147.5, 150.0, 156.4, 172.0;

HRMS (ESI) calculated for $C_{19}H_{29}N_3O_9SNa$ $[M+Na]^+$ 498.1517, found 498.1530.

5.3.12 (1*R*,2*S*)-1-(4-Nitrophenyl)-2-(*N*$^{\alpha}$-Boc-L-methionyl-sulfone-amido)-1,3-propadiol 20.

$C_{19}H_{29}N_3O_9S$; 475.51 g/mol;

yield 72%; white solid

mp 175-176°C (dec);

$[\alpha]_D^{21}$ -10.8 (c 1.7, MeOH);

IR (KBr) 3424, 3357, 2978, 1681, 1660, 1523, 1350, 1294, 1166, 1131, 1053 cm^{-1};

^{1}H NMR (400 MHz, CD_3OD) δ 1.43 (s, 9H), 1.64-1.74 (m, 1H), 1.80-1.88 (m, 1H), 2.87 (s, 3H), 2.83-2.89 (m, 2H), 3.70 (dd, J = 11.2 and 3.7 Hz, 1H), 3.86 (dd, J = 11.3 and 5.5 Hz, 1H), 4.03 (dd, J = 8.5 and 5.1, 1H), 4.15-4.19 (m, 1H), 4.82 (d, J = 8.1 Hz, 1H), 7.63 (d, J = 8.6 Hz, 2H), 8.20 (d, J = 8.8 Hz, 2H);

^{13}C NMR (100 MHz, CD_3OD) δ 26.5, 28.7, 40.6, 51.8, 54.4, 57.6, 61.9, 73.4, 81.1, 124.6, 129.3, 149.0, 151.4, 157.7, 173.1;

HRMS (ESI) calculated for $C_{19}H_{29}N_3O_9SNa$ $[M+Na]^+$ 498.1517, found 498.1505.

5.3.13 General procedure for the cleavage of the *N*-*tert*-butoxycarbonyl groups

A solution of compound **19** (40 mg, 84.1 µmol) in 4 M HCl/dioxane (2.0 mL) was stirred at room temperature for 40 min. The solvent was coevaporated under reduced pressure with MeOH. The residue was dissolved in a minimum of EtOH (2.0 mL) and the product was precipitated by the addition of Et_2O (20 mL). The product was collected by filtration and washed with Et_2O to give **5** (28 mg, 81%).

5.3.14 (1*S*,2*R*)-1-(4-Nitrophenyl)-2-(L-methionyl-sulfone-amido)-1,3-propadiol (5).

$C_{14}H_{22}ClN_3O_7S$; 411.86 g/mol;

yield 81%;white solid

mp 185-190°C (dec);

$[\alpha]_D^{21}$ 18.0 (c 0.58, MeOH);

IR (KBr) 3351, 2927, 1683, 1519, 1351, 1285, 1133 cm^{-1};

^1H NMR (400 MHz, CD_3OD) δ 2.20-2.40 (m, 2H), 2.98 (s, 3H), 3.27 (t, J = 8.0 Hz, 2H), 3.63-3.74 (m, 2H), 3.99 (t, J = 6.4 Hz, 1H), 4.15-4.22 (m, 1H), 4.88 (d, J = 6.4 Hz, 1H), 7.64 (d, J = 8.4 Hz, 2H), 8.20 (d, J = 8.8 Hz, 2H);

^{13}C NMR (100 MHz, CD_3OD) δ 24.6, 39.6, 49.1, 51.6, 57.3, 59.8, 72.4, 123.2, 127.5, 147.6, 149.9, 167.6;

HRMS (ESI) calculated for $C_{14}H_{22}N_3O_7S$ $[M+H]^+$ 376.1173, found 373.1186.

5.3.15. (1*R*,2*S*)-1-(4-Nitrophenyl)-2-(L-methionyl-sulfone-amido)-1,3-propadiol (6).

$C_{14}H_{22}ClN_3O_7S$; 411.86 g/mol;

yield 75%;white solid

mp 165-170°C (dec);

$[\alpha]_D^{21}$ -1.8 (c 1, MeOH);

IR (KBr) 3409, 2925, 1683, 1520, 1352, 1286, 1135 cm^{-1};

^1H NMR (400 MHz, CD_3OD) δ 1.88-2.09 (m, 2H), 2.70-2.90 (m, 2H), 2.92 (s, 3H), 3.72-3.86 (m, 2H), 3.90-3.99 (m, 1H), 4.23-4.33 (m, 1H), 4.84 (d, J = 8.0 Hz, 1H), 7.66 (d, J = 8.5 Hz, 2H), 8.22 (d, J = 8.5 Hz, 2H);

^{13}C NMR (100 MHz, CD_3OD) δ 25.2, 40.7, 50.2, 52.8, 57.5, 61.7, 73.1, 124.5, 129.2, 148.9, 150.9, 168.4;

HRMS (ESI) calculated for $C_{14}H_{22}N_3O_7S$ $[M+H]^+$ 376.1173, found 373.1185.

5.4 References

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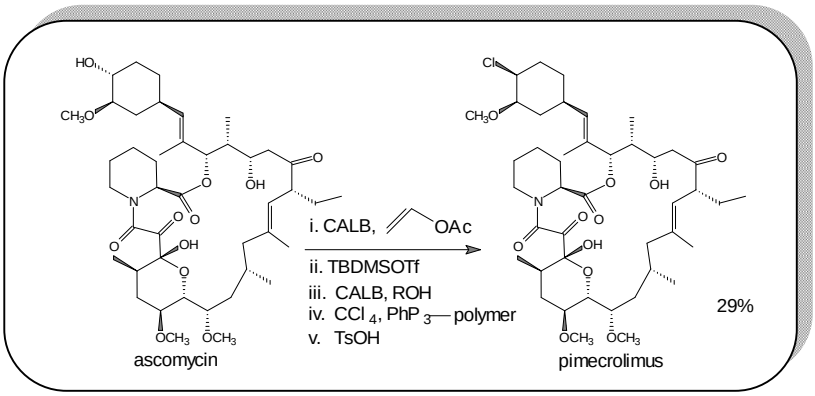
6. CONCLUSIONS

Nowadays biocatalysis is a well-assessed methodology that has moved from the original status of academic curiosity to become a widely exploited technique for the preparation of products of biological interest. Many enzymes have been so thoroughly studied that their behavior in synthesis can be predicted reliably.

The high level of chemo, regio and stereoselectivity of three of the most widespread, low cost and effective enzymes, that belong to different class of hydrolases, allowed us to achieve the preparations summarized in the schemes below.

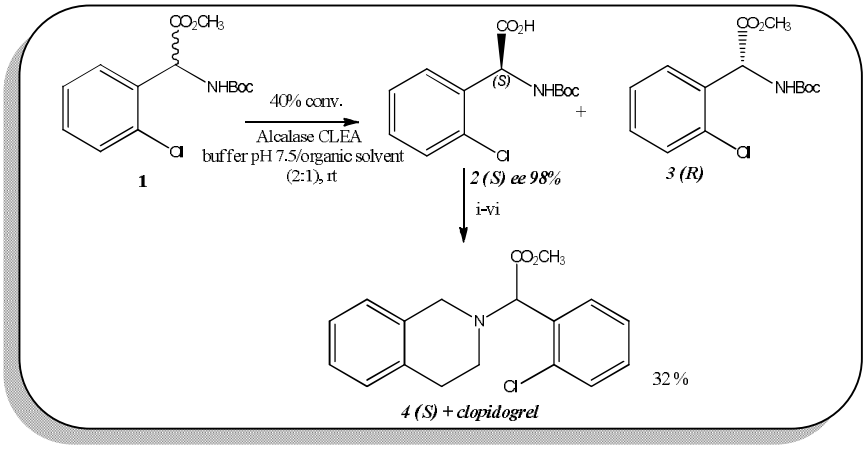
In the first project, the bacterial macrolactam ascomycin was converted into the high value immunosuppressive pimecrolimus through a regio and chemo-selective protection catalyzed by lipase from *Candida antarctica* B. Thanks to the mild conditions of this new chemoenzymatic approaches, only the desired products are obtained: the absence of rearrangements or degradations processes, very frequently observed in the case of sensitive molecules as ascomycin and related compounds, containing a large number of functionalities, afforded a gain on the final yield and on the cost of the process. In this work clearly emerges that enzymatic hydrolysis and transesterification reaction can be complementary processes for the regioselective acylation of secondary alcohol, because, the enzyme, keep its substrate selectivity unalterated in both the verses of the reaction (Scheme 6.1).



Scheme 6.1. Synthetic pathway to prepare pimecrolimus from ascomycin.

In the second project, a kinetic resolution of the non proteogenic 2-chloro phenylglycine methylester, with the aminic function protected as N-Boc **1**, was obtained with an excellent

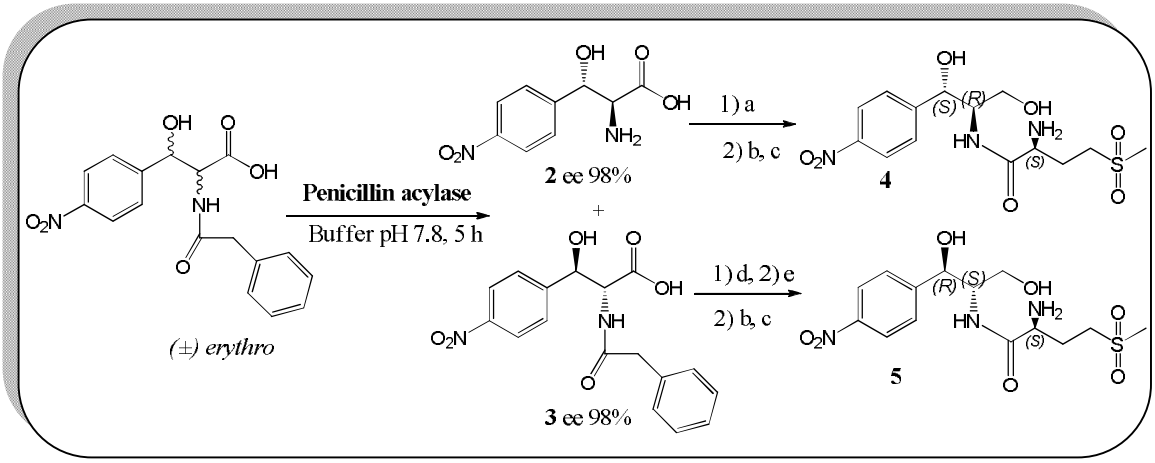
value of enantiomeric excess, by means of the Alcalase-CLEA® affording the required *(S)*-acid **2**. As shown in the second chapter, by focusing on the modulation of enzyme selectivity by medium engineering, quite simple modification of the solvent composition can really have significant effects on the performance of the biocatalyst. The optically pure acid was then converted into the *(S)*-2-chloro phenyl glycine methyl-ester, in turn used as chiral synthon in order to complete the preparation of the antiplatelet *(S)*-clopidogrel **4**. The use of a chemoenzymatic approach allowed to overcome the tedious and time consuming fractional crystallizations, till now used with the aim to obtain the optically pure *(S)*-clopidogrel (Scheme 6.2).



Scheme 6.2. Final pathway followed for the preparation of *(S)*+ **1**. Reagents and conditions: (i) CH_3OH, DCC, DMAP, CH_2Cl_2; (ii) TFA, CH_2Cl_2; (iii) 20% NH_4OH; (iv) TsCl, $(iPr)_2O$, Et_3N; (v) $NaHCO_3$, KI, CH_3CN; (vi) paraformaldehyde, $ClCH_2CH_2Cl$, HCl in DMF.

In the last project, penicillin acylase, was the best enzyme to realize the kinetic resolution of another non proteogenic amino acid, (±) *erythro* p-nitrophenylserine, important precursor of two diastereoisomers of chloramphenicol. The hydrolysis of the two amino acids, derivatized as N-phenylacetyl-aminoacids, the best substrates for the enzyme, was conducted in a very fast, and economic manner, using as medium of reaction a buffer phosphate weakly alkaline. The hydrolysis, stopped at the 50% of conversion, lead to recover the free L- amino acid **2** and the unreacted D-amide **3** both with a high value of ee. The so obtained *(S)* and *(R)* p-nitrophenylserines were later converted in the corresponding amino alcohol and N-acylated by reaction with L-sulfonilmethionine. This two compounds **4** and **5** were tested as potential inhibitors for the enzyme amidotransferase, a new target for the development of new antibacterial compounds. The importance of this project overcomes the values of the constants of inhibition found for the two compounds, but

being inserted in a structure-activity study, underlines the importance of the configuration of chloramphenicol stereocenters for the interaction with the amidotransferase.



Scheme 3. Synthetic pathway to prepare chloramphenicol derivatives **4** and **5**. a) Amberlyst 15, EtOH, heat,16h; $CaCl_2/NaBH_4$,12h; b)L-methionine sulfone N-Boc, EDC, *N*-hydroxysuccinimide, DMF, 24h (c) 4 M HCl / dioxane, 40 min; d) $BH_3(CH_3)_2S$, THF, 12h; e) HCl 6N, CH_3OH, heat, 2h.

The use of hydrolytic enzymes afforded best results either for their regio- and chemoselectivity (see preparation of pimecrolimus) either in stereoselectivity (see resolution of 2-N-Boc-chlorophenyl glycine-methylester and *erythro*-p-nitrophenyl-serine). The obtained intermediates were easily converted into known and potential biologically active compounds.

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