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Synthesis of new bioactive β-lactam compounds

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ABSTRACT

New biologically active β-lactams were designed and synthesized, developing novel antibiotics and enzymatic inhibitors directed toward specific targets.

Within a work directed to the synthesis of mimetics for RGD (Arg-Gly-Asp) sequence able to interact with $\alpha_v\beta_3$ and $\alpha_5\beta_1$-type integrins, new activators were developed and their Structure-Activity Relationships (SAR) analysis deepened, enhancing their activity range towards the $\alpha_4\beta_1$ isoform. Moreover, to synthesize novel compounds active both against bacterial infections and pulmonary conditions of cystic fibrosis patients, new β-lactam candidates were studied. Among the abundant library of β-lactams prepared, mainly with antioxidant and antibacterial double activities, it was identified a single lead to be pharmacologically tested *in vivo*. Its synthesis was optimized up to the gram-scale, and pretreatment method and HPLC-MS/MS analytical protocol for sub-nanomolar quantifications were developed. Furthermore, replacement of acetoxy group in 4-acetoxy-azetidinone derivatives was studied with different nucleophiles and in aqueous media. A phosphate group was introduced and the reactivity exploited using different hydroxyapatites, obtaining biomaterials with multiple biological activities. Following the same kind of reactivity, a small series of molecules with a β-lactam and retinoic hybrid structure was synthesized as epigenetic regulators. Interacting with HDACs, two compounds were respectively identified as an inhibitor of cell proliferation and a differentiating agent on steam cells. Additionally, in collaboration with Professor L. De Cola at ISIS, University of Strasbourg, some new photochemically active β-lactam Pt (II) complexes were designed and synthesized to be used as bioprobes or theranostics.

Finally, it was set up and optimized the preparation of new chiral proline-derived α-aminonitriles through an enantioselective Strecker reaction, and it was developed a chemo-enzymatic oxidative method for converting alcohols to aldehydes or acid in a selective manner, and amines to relative aldehydes, amides or imines. Moreover, enzymes and other green chemistry methodologies were used to prepare Active Pharmaceutical Ingredients (APIs).

1. INTRODUCTION

1.1 β-Lactams

1.1.1 Definition

In organic chemistry, cyclic amides are defined lactams. They are formally produced from an intramolecular condensation of species characterized by the presence of both an acid and an amino function. Their structures have therefore a cyclic system of 1-azacycloalcan-2-one. Among the compounds belonging to this family the number of carbon atoms and consequently the amplitude of the ring can vary. Moreover, unsaturations, heteroatoms, substitutions or derivatizations of various types could be present.[1]

To indicate the size of the ring are used in alphabetical order Greek letters as prefixes. Thus, β-lactams have two atoms in addition to the amide function and an overall tetra-atomic cycle, γ-lactams are cycles with five atoms, δ-lactams are six-membered and ε-lactams seven-membered rings. Concerning the IUPAC nomenclature, β-lactams would be considered azacyclobutan-2-ones, as well as γ-lactams areazacyclopentan-2-ones, while δ-lactams and ε-lactams are azacycloesan-2-ones and azacycloeptan-2-ones, respectively.[2]

The importance of these heterocycles is related to their industrial and pharmaceutical applications: nylon production uses ε-caprolactam (cyclic amide with 7 terms, Figure 1.1.1), some natural products such as polycyclic alkaloids are five or six membered amides and, above all, β-lactam antibiotics are important in pharmaceutical field. As a matter of facts, the four membered amide (β-lactam) occurs in widely used antibiotics such as penicillins, cephalosporins, carbapenems and monobactams.

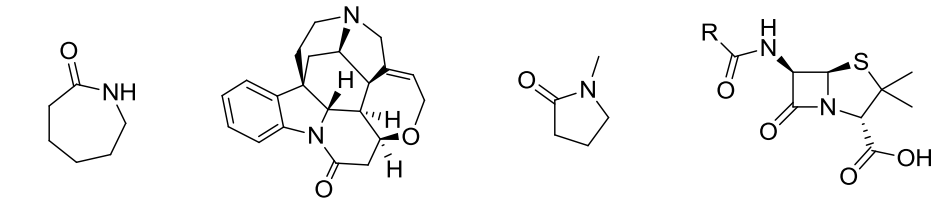


Figure 1.1.1 Some lactam compounds

1.1.2 Chemical characteristics and reactivity

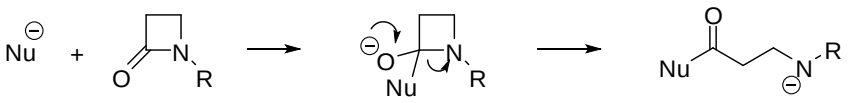
Analyzing in details the chemical characteristics of β-lactams, we can notice a remarkable reactivity of the amide group. This is mainly due to an higher Bayer strength, caused by the deformation of the

effective bond angles than those of the hybridized orbitals involved, and an high Pfizer strength, due to bond eclipsations and increasingly important with the increase of the substituent size.

Although β-lactams are amides, the resonance that involves the lone-pair of the nitrogen atom and the next carboxy group is hampered by the cyclic structure. In fact, in the charge separation resonance structure, the presence of two sp^2 hybridized atoms on the four membered ring would have a strong destabilizing effect (Scheme 1.1.1). The carbonyl group of the β-lactam amide thus maintains the electrophilicity determined by inductive effects of the next nitrogen atom, so that the electrophilicity and therefore the reactivity of this amide increase.

Scheme 1.1.1 Amide resonance

The major reactivity of these molecules is therefore due to the electrophilicity of the amidic C=O. Reactions with nucleophiles were favored with the consequent opening of the cyclic system, following a classic mechanism of the nucleophilic acyl substitution. It consists in an initial addition to the C=O and the subsequent ring opening (Scheme 1.1.2).



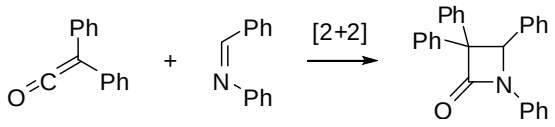
Scheme 1.1.2 β-Lactam system opening

This mechanism is particularly important because it is involved in the action of β-lactam as antibiotics and in other biological activities that these compounds showed.

However, the chemical characteristics described necessarily involve some difficulties at the experimental level; it is necessary, for instance, to make use of mild reaction conditions, inert atmosphere and low temperatures. Acidic or basic conditions, by means of Lewis or Brønsted-Lowry acids or bases, are not tollerated. Nucleophiles, such as water or primary alcohols, poses problems especially under acid/base conditions, or even with dipolar aprotic solvents, known to be excellent for S_N2 reactions. Finally, rather less attention can be paid to oxidative phenomena, at least avoiding overheating's (40 to 50)°C, except for short periods or under a inert atmosphere,

1.1.3 Synthesis

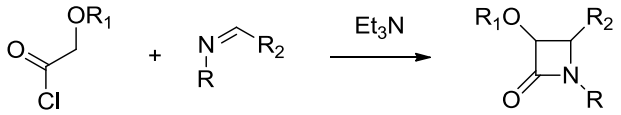
The first synthesis of a β-lactam derivative was realized in 1907 by Hermann Staudinger, who exploited a [2+2] cycloaddition between the Schiff base of benzaldehyde with aniline,[3] and diphenylketene (Scheme 1.1.3).[4]



Scheme 1.1.3 Staudinger synthesis

A [2+2] reaction can be also conducted between an alkene and an isocyanate, as in the case of large-scale synthesis of the 3-(1-*t*butyldimethylsilyloxy-ethyl)-4-acetoxy azetidin-2-one (an important commercially available β-lactam) and of other industrial applications.[5]

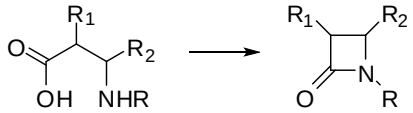
Of the many routes explored, the use of acyl chlorides or related species in the presence of tertiary amines is considered worthy of note, but also the photochemical route from metalcarbenes or the thermal way are relevant. Other intermolecular solutions require cycloaddition reactions between imines and oxygen-protected hydroxyacetylchlorides or, in few cases, corresponding carboxylic acids (Scheme 1.1.4). Moreover, the same reaction has applications, although in specific and therefore not general conditions, even if the acid chloride do not have heteroatoms as substituens or halides in α position.



Scheme 1.1.4

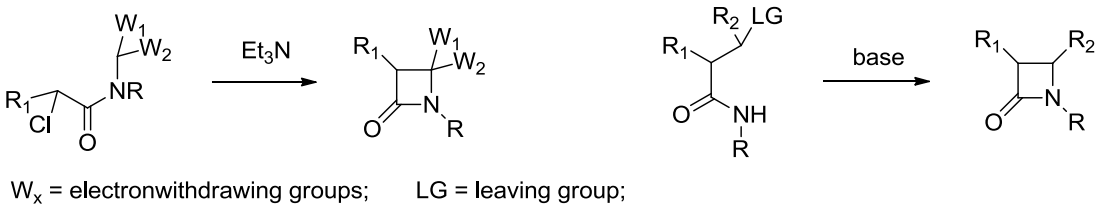
Although the applicability of these synthesis presents limits, the relief consists in the existence of stereoselective and enantioselectives variants. Some of them were recently introduced where ionic proline derivatives or chiral ferrocene complexes were used as catalysts.[6]

Classical preparations for the formation of two bonds with the nitrogen atom exists, as well as intramolecular reactions for the construction of each of the four bonds of the ring. In general, the more supported way provides for the formation of the amide bond and requires precursors such as β-amino acids or their activated derivatives, for example by the use of carbodiimides method or by amine deprotonation (Scheme 1.1.5), whereas less interesting is concerning the C2-C3 bond



Scheme 1.1.5

The formation of the C3-C4 carbon-carbon bond requires by electronwithdrawing groups in the α position respect to N, while the C-N bond could be formed by a bimolecular nucleophilic substitution reactions of the nitrogen on halides (Scheme 1.1.6).



W_x = electronwithdrawing groups; LG = leaving group;

Scheme 1.1.6

The existence of a significant number of synthetic strategies for the preparation of bicyclic β-lactams compounds deserves at least a hint, due to their great importance in the pharmaceutical field, in particular as antibiotics.

To conclude on synthetic aspects it is finally necessary to mention the *β-Lactam Synthon Method*, developed by Prof. Ojima as a technique for the preparation of oligopeptides and obviously optically active α and β-amino acids starting from chiral β-lactams. Recently, the use of enantiopure azetidin-2-onic scaffolds as versatile intermediates underwent a considerable boost, especially to meet the needs of structural variability and innovation of the pharmaceutical β-lactam based antibiotic class.

Moreover, easy ring opening reactions together with new methodologies to exploit their chirality via asymmetric induction, even in more subsequent functionalization steps, allowed a fair development both in the chemistry of natural compounds and in peptide synthesis, especially in the field of peptidomimetics.[7]

1.2 Enzyme inhibitors

1.2.1 Enzymes and enzymatic activity regulation

Enzymes are defined as biological catalysts of chemical reactions. The term was introduced in 1878 by the physiologist Wilhelm Kühne: the etymology is Greek and it means "in yeast".[8] Considering only protein enzymes, we can define the active site as that portion, very small compared to the entire enzyme, which consists of the catalytic site, where the biochemical reaction actually takes place, and of the positioning site, to which are essentially delegated recognition functions usually based on hydrophobic and no covalent interactions.[9]

To present a model for rationalizing the interactions between an enzyme and its substrate, the first model introduced was the "key-lock". It was characterized by a perfect complementarity of the two partners, explaining the specificity that distinguishes enzymes, and was proposed in 1894 by Hermann Emil Fischer.[10] The evolution of this first idea was made in 1958 by Daniel Koshland, who accepted the existence of some flexibility for the enzymatic structures proposed. Today it is known as the "induced adaptation model" of Koshland. According to it, ligands and targets are usually not exactly complementary, thus takes place a reciprocal arrangement that maximizes the interactions.[11] Although

directed at first to enzymes, such considerations established an applicability also in the pharmaceutical field. Current models indeed consider their general lines and can therefore be considered an evolution of this model. In this regard, the current theory of constitutively activated receptors always considering conformational changes, but also describes the existence of equilibria between different conformers. It introduces definitions for different ligands, examined in more details in the next section. However, the catalytic activity is the main function of enzymes but not the only one. Indeed, active sites generally occupied small portions of the whole protein. In addition to the action of the active site and the ability to ensure the positioning of themselves, for example on cell membranes if they are trans-membrane, enzymes also take part in recognition functions, always describable with the models introduced above, at least articulated on three levels:

- recognition of small molecules, called ligands;
- recognition of other proteins;
- recognition of the DNA;

which we must refer in the attempt of rationalizing the regulation mechanisms of their activities.[12]

The enzymatic efficiency can be regulated by the temperature, the acidity in term of pH and, for the sake of completeness despite irrelevant for our purposes, by phenomena of activation related to the protection from degradation of essentially an oxidative nature. Turning then to a more biochemical level, cells seem to be able to control and hence to regulate the enzymatic activities according to at least five main ways, starting from the production stage. Transcription and protein synthesis implemented are effectively managed through the classic biochemical mechanisms that govern the regulation of gene expression, including extracellular stimuli. A second control system is associated to the partitioning of enzymes, in some cases adopted to allow completely selective metabolic pathways, different and potentially all available for the same substrate. Another regulatory mechanism is based on the biosynthesis of enzymes characterized by the request of drastically different conditions (for example of pH) from those of the environment of production to become active, thus providing a certain analogy with those species defined as zymogens, biosynthesized in inactive forms. Some similarities can be highlighted even with the regulatory mechanism which provides the realization of post-transduction modifications, such as phosphorylation and glycosylation, or even the cutting of entire protein sections, typical for the zymogen activation. The last adjustment way, at least among those considerable as the mains, is related to the negative feedback action exerted by the products themselves of an enzymatic transformation or by the activity of a whole enzyme complex.[13]

It remains to be considered the so-called effectors, molecules not necessarily always endogenous but able to act on enzymatic efficiencies as activators or orthosteric inhibitors, or as allosteric modulators, thus targeting sites defined allosteric, if any. In these cases it is mandatory that there is a correlation between the subunit, or the domain, with the active site and the one, or ones, on which the allosteric interaction occurs. Depending on whether or not the allosteric effector is the same endogenous substrate, it can be labeled as homotropic or heterotropic, and the resulting modulation generally consists in conformational

deformations such as re-orientations the of enzyme-composing domains, making more or less accessible the active site or other regulatory areas. Although the allosteric sites may be the target of a drug treatment, with some advantages over classical orthosteric ligands, most of the cases concerns today the G-Protein Coupled Receptors (GPCRs), however here of less importance.

To introduce now orthosteric effectors, it must first be noticed that the differences between activators and inhibitors are not always so sharp. At the biological level, the same ligand can have both behaviors depending on various boundary conditions. Effects such as the concentration of the ligand itself or of other species, the possible interactions of the enzyme with other molecules, or even its phosphorylation state should be remembered. However, because the main interest is usually in inhibitors, at least in pharmaceutical fields, the discussion will be now limited to them. We define inhibitors those compounds able to decrease, so to modulate or even to cancel, the catalytic action of an enzyme, in a reversible or irreversible way, that is by binding covalently or non-covalently to the enzyme. As subsequently deepened, reversible inhibitors are usually considered to be of greater pharmaceutical importance. They are in turn further classified into:

- competitive: it is set in a competition between the inhibitor and the substrate for the same catalytic site, with the only difference that in the case where there is interaction with the effector this does not lead to the formation of the product. Usually characterized by structural similarity, the inhibition is highly dependent on the concentrations and the relative affinities of the species contenders against the active site of the enzyme;
- not competitive: forming a complex with the enzyme, binary or ternary based on the absence or the presence of the substrate, the site of interest is now different. The action may therefore be rationalized thinking to a decrease of the enzyme amount available for conversion of the endogenous substrate, which concentration has therefore no influence on the inhibition;
- acompetitive: also known as inhibitors of the ternary complex, they acting at the enzyme-substrate complex level. It intervenes in the mechanism by which enzymes catalyze biochemical reactions, bonding and blocking the complex in this condition.[14]

However, irreversible inhibitors have a certain prestige and the entire class of β-lactam antibiotics is indeed one of the best known examples. Other members of this family, famous in their own way, are the nerve gases, irreversible inhibitors of acetylcholine esterase, which is involved in the process that ultimately leads to muscle movement. Even the so-called suicide inhibitors, transformed into irreversible inhibitors from chemical reactions in the active site, are irreversible inhibitors.

1.2.2 Enzyme inhibitors

Drugs are defined as any compound that by interaction with biological systems produces an effect, such as in the case of enzyme inhibitors. Ideally, drugs should be easy to take and of course should ensure the

expected action without presenting side effects. It is not accidental that the term derives from the Greek word *pharmakon*, that means remedy and poison at the same time.

A first classification of drugs concern the targets and subdivides them depending if are nucleic acids, lipids (and particularly membrane phospholipids), carbohydrates or proteins. The latter are the target of most of the now available drugs, but being able to treat enzymatic or receptorial proteins, are in turn distinguished in intracellular and membrane, on the basis of the biological localization sector. Accessible not only to ligands able of entering in cells, membrane receptors basically transduce informations and are further divided into six families:

- channel;
- G-protein coupled, i.e. the before mentioned GPCRs against which is directed the action of about 70% of drugs active against to receptors;
- with intrinsic tyrosine-kinase activity;
- with intrinsic guanylate-cyclase activity;
- adhesion;
- cytokine;

where the first two alone account for about the 90% of the targets which drugs active on receptors are directed.[15]

Still limiting to the compounds having proteins as targets of their pharmacological actions, it is initially possible to put them together in a single treatment without distinguishing between those directed at enzymes and those at receptors. Molecules active on proteins, either receptors or enzymes, can act as agonists or antagonists depending if they reduce or potentiate proteins' activity.

In the case of receptor proteins, the drug can activate them by binding to the active site and mimicking the natural ligand, or preventing their binding with the messenger, the endogenous transmitter, interacting with the same site but without initiate their cascades of biological events, thus behaving as antagonist. With reference to the theory of constitutively activated receptors, agonists are seen as those compounds capable of binding to the active conformer, stabilizing it and shifting the equilibrium towards it, while inverse agonists are capable of binding to the inactive form, depressing the receptor action. In this same discussion, antagonists are species able to bind to both the forms, still inhibiting the activity.

As regards enzymes, the inhibition can occur in a similar manner but obviously preventing the bond with the natural substrate, while for the activation is necessary to consider allosteric sites. In fact, as examples of allosteric agonism also existing in relation to receptor proteins, enzymatic orthosteric agonists should be different from the natural substrate, consequently leading not to reinforcing the desired action but to biological conversion products different from those obtained under normal physiological conditions. Finally, there are for both protein targets examples of allosteric antagonism, often due to conformational deformations such as to disadvantage interactions with the endogenous ligand, but in some cases are also based on the so-called "umbrella effect", hindering the access simply providing a spatial hindrance.

Thus, compounds with an inhibitory action tend to benefit of the most relevance, at least in the pharmaceutical field. Their activities are currently expressed by the inhibitory concentration (IC_{50}), defined as that drug concentration capable of inhibit 50% of its target.

Therefore, when enzymes are involved, drugs are usually inhibitors, and with regard to their action method enzyme inhibitors are still generally rationalized into three main categories:

- competitive reversible: establish with the enzyme non-covalent interactions, so as to prevent access from the endogenous substrate only temporarily. To a rising concentration of the ligand, the drug is therefore likely to be displaced;
- non-competitive irreversible: forming a covalent-nature bond with the enzyme active sites, any increase in the concentration of endogenous substrate is irrelevant;
- non-competitive allosteric reversible: the contradiction in terms is explained by the existence of an accessory site, allosteric, where the drug can reversibly bind the enzyme, inducing conformational changes such as rendering the active site no longer suitable or at least less inclined to accept the usual substrate;

which can be reinforced with other mechanisms of action, somewhat intermediate.

Among the advantages of reversible inhibition as medical cure must surely count the best controllability, management and security of the treatment. As a matter of facts, if with the target enzymes are involved more substrates, for example under certain boundary conditions such as the location or the concentration of other biological effectors, the disturbance caused to the biochemical processes related to those of no interest will be only partial and potentially modulated by playing on the relative affinities of the ligands.

As a further detriment of irreversible inhibitors, the phenomena of tolerance and addiction are here more evident and rapid in the establishment. They lead to a gradual increase in the administered dose of medication to obtain the same effect, due to a reduction in efficacy caused by the prolonged repetition of assumptions. Finally, reversible inhibitors are also easily removable for simple dilution or dialysis, when it should be necessary, while irreversible inhibition is not neutralizable. This is instead a considerable advantage for β-lactam antibiotics, because they are directed to bacterial enzymes not present in humans, with the declared intent to exterminate them.[16]

To conclude on the design of enzyme inhibitors, or more generally of drugs, the pharmacophore is defined as the portion of a drug characterized by the ability to interact with its target and which removal therefore results in the loss of the biological activity. Isosteres are chemical groups structurally similar and potentially interchangeable even if components of the pharmacophore. Peptidomimetics is the intermediate branch between chemistry and pharmacology interested in peptidomimetic compounds, molecules that due to structural similarities can mimick natural peptides and thus endogenous ligands. These exogenous peptides are delicate because of the usually rapid biological degradation by proteases that they may experience. Thus, the isostere often regards the amide bonds. Peptidomimetics precisely modified on the peptide bond are called pseudopeptides. In depsipeptides the isostere is an ester bond, while if the substitution is related to the lateral chains compounds are peptidoanalogues. All those

compounds that mimick or inhibit, but without being structurally related, the biological effect of a peptide are known as non-peptidomimetics. Finally, drugs, as commonly defined, are different from the endogenous species due to the need of performing different tasks, for example in terms of bioavailability, stability and ease of administration. A better specificity and thus a less number of possible interaction partners, potentially cause of side effects, is also an aspect not to be overlooked in designing drugs.[17]

1.2.3 β-Lactam based enzyme inhibitors

β-Lactam compounds are often potent irreversible inhibitors of enzymes based on a mechanism of covalent modification of the target enzyme and in particular on the selective acylation on the OH of the serine which is part of the active site. Obviously, they do not act only for the reactivity of the ring but also thanks to appropriate geometries for the site on which they must interact and to the structure subsequently adopted from the enzyme-inhibitor adduct, which became no longer divisible. Also other inhibitors, specific for β-lactamase and often administered in combination with azetidin-2-onic antibiotics to avoid their inactivation, work with the same mechanism of acylation. β-Lactamase are enzymes that can destroy β-lactams with a rate close to the limit of diffusion control. The formation of covalent bonds can therefore be a successful strategy for the identification of new enzyme inhibitors, and typically the β-lactam scaffold constitute a rather versatile starting point, although nothing prohibits the use of γ-lactams duly activated, in order to compensate, in terms of reactivity, the lower Bayer and Pfizer strengths that characterize them.[18] Indeed, since the 80s, following the discovery of the first monobactam antibiotic, studies relating to the inhibitory activity of β-lactams have risen sharply, so that countless structural variants of the first natural compounds are today directed to the antimicrobial action, so antibacterial, antifungal and antiviral too. However, azetidin-2-ones have subsequently demonstrated biological activities as irreversible inhibitors with different pharmaceutical effects, not exclusively related to the antibacterial properties, and have been therefore exploited as acylating agents for the covalent inhibition of a wide variety of targets. Among them, we can list elastases, trombines, cathepsin K, calpain, prostate specific antigen, low-density lipoproteo-phospholipases, transacylases, cholesterol-acyltransferases and proteases of cytomegalovirus. Moreover, are today known compounds with anti-cancer and anti-tuberculosis properties, anti-HFAAH, HDACs, proteasomes, cathepsins and vasopressin, and even cholesterol absorption inhibitors, analgesics and anti-inflammatories compounds. Furthermore, are known molecules processed by enzymes themselves in irreversible inhibitors due to reactions that take place in the active sites, defined for this mechanism of action suicide inhibitors. In addition to the antimicrobial activity was possible to associate an antioxidant effects by introducing of polyphenolic moieties, and are finally worth of mentioning the compounds active in DNA cleavage.[19] The biological activity, however, is always related to the type of derivatization present on the β-lactam structures, but the great relevance in the pharmaceutical field is also due to the aspect of being often well tolerated by the organism. In fact, allergies related to β-lactam antibiotics are almost the unique side reaction associated to them. Therefore

taken here as a reference, these molecules offer a therapeutic index (TI) always very high. TI is defined in animals as the ratio between the lethal dose (toxic, for studies on humans) on 50% of the treated subjects (LD_{50}) and that effective on the same percentage (ED_{50}), but it now disused for ethical and economical problems. When taken in appropriate cases and in compliance with the correct dosages, the only noteworthy side effect is the possibility to affect the intestinal flora. Thus, they can cause a consequent change in the bowel habit, however easily compensated by taking lactic acid bacteria.[20]

1.3 Organic chemistry and pharmacology

1.3.1 Drug design

The design of biologically active molecules is a transverse aspect ranging from molecular biology to microbiology, organic chemistry and the synthesis of molecules. The identification of biological targets on which designing the synthesis of a new molecule (lead) is only the beginning of the path that leads to the "drug". Some basic steps must be rationally followed:

- identification and selection of the drug target;
- study of the active receptorial site through direct or indirect methods, of the interactions and of the molecular scaffold which better fit;
- synthesis of the molecule.

At first, you need to identify the biological target, usually an enzyme or a protein, and build a molecule able to interact with it in order to block, inhibit or more generally alter its activity. The interaction between a molecule and an enzyme was first hypothesized by Hermann Emil Fischer in 1894, and according to it enzyme and substrate possess forms that allow a perfect complementary interlock.[21] This interaction was defined as the "key-lock" model and suggested that a molecule, and/or classes of related compounds, have the unique ability to "open" the enzyme, thus interacting in a very specific way with the protein, exerting a particular function.

However, the interpretation of interaction between enzymes and substrates has evolved over time. Enzymes does not behave in a so rigid manner towards their substrates, and their own active sites were modified by binding molecules. Thus, was defined a new model called "hand-glove" or even "adaptation induced" model.[22] Enzymes were not more treated as rigid structures, but rather with a certain grade of flexibility, a feature that allowed them to be modelled according to the presence of substrates or not. Then substrates binds to the active site on an enzyme, induceing changes due to the adaptation of the enzyme itself.

The active site of an enzyme is the part that binds the substrate and which induces the enzyme to exert its activity. From a theoretical point of view, an enzyme is divided into the binding site (where binding takes place with the substrate) and the catalytic site (where really occurs the catalytic function). Some enzymes

also possess allosteric sites, which interact with molecules called allosteric effectors. In general, these molecules can modify the activity of enzymes by inducing changes in their structures.

Active and/or allosteric sites must be identified in order to understand the structure of the molecule able to interact. A direct methodology is surely the use of X-rays on the target protein bounded to its substrate. If is not known the receptor structure, is possible to use computational techniques, among which one of the more important is QSAR (Quantitative Structure-Activity Relationship). This method establishes a relationship between an overall molecular structure and the biological activities of a series of compounds active against the target protein. It predicts the activity and the affinity of not yet known compounds from the analysis of their structural similarities and differences, providing information about the structural requirements of the active receptorial site. Thus, when the structural requirements of the molecule, or of the class of molecules, with a biological activity were identified, it is possible to reach a synthetic approach. The synthesis of biologically active molecules, when the pharmacophore model was identified, can be oriented towards different approaches:

- Target Oriented Synthesis (TOS);
- construction of combinatorial library;
- Diversity Oriented Synthesis (DOS).

TOS is a methodology that leads to the production of a single molecule, with a set of substituents and usually with a very specific synthetic pathway. This methodology was one of the first to be used, but in general is chosen when the molecular target has a strong and demonstrated biological activity or a considerable complexity.[23] However, this approach severely limits the possibility to vary the molecular scaffold, due to the nature of the mentioned specific synthetic pathway (which usually has a retrosynthetic strategy).

The combinatorial synthesis, which has become very popular especially thanks to the solid-phase peptide synthesis of Merrifield in the '70s,[24] allows to obtain a series of scaffolds in a single synthetic process, thus reaching a great substrates diversity. From the synthetic and the operating points of view, the combinatorial approach allows to expand and enrich the number of similar molecules by random varying the substituents, therefore obtaining different combinations. Through this scheme, starting from a unique molecule recognized as the founder compound, can be achieved an high number of new compounds generated by combinations of different substituents. Combinatorial chemistry has been particularly widespread and has led to techniques such as the so-called "split and mix" and many others. The huge number of molecules obtained through combinatorial chemistry can extend what is known as "chemical space". It is a virtual zone of n dimensions where the dimensions are descriptors of structural features or chemical-physical properties (e.g. empirical formula, table of molecular connections, polarity in terms of logP, molar volume, numbers of bonds free to rotate, etc.). The chemical space gives an idea of the molecules diversity. It is estimated that the number of possible molecules is 10^{200}, among which 10^{60} may be active from the biological point of view. However, from the literature, over the year have been synthesized only 10^{7} different compounds. Comparing the chemical space of a target oriented and a

combinatorial synthesis, is immediately clear how the second method leads to a greater variability and therefore to a greater success rate in identifying new active molecules (Figure 1.3.1).

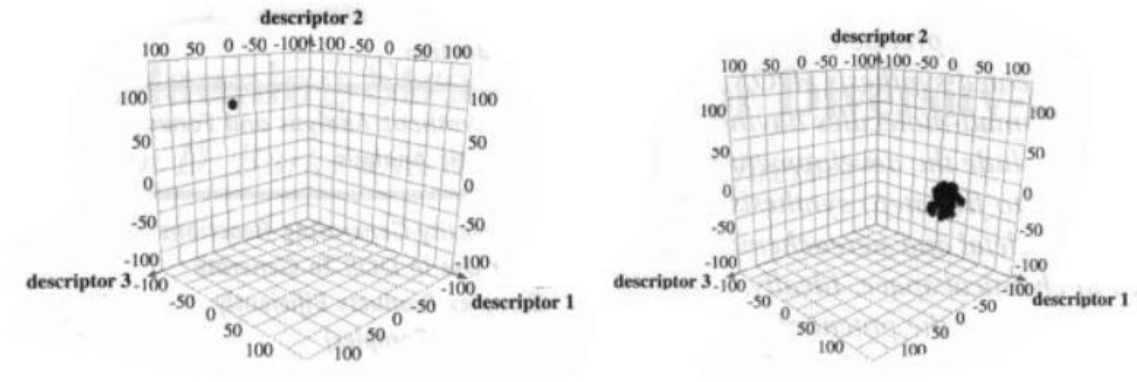


Figure 1.3.1 TO (left) and combinatorial (right) Synthesis chemical spaces

The advantage of the combinatorial synthesis is remarkable if considering that a target molecule can often be able to exert its biological activity, but also results toxic at the same time, or leading to other problems (in terms of absorption, excretion, simultaneously interaction with other proteins, ..). The possibility of having the other n-1 structures similar but slightly different can lead to the discovery of molecules with probably the same biological activity, although maybe slightly decreased, but not toxic.

In 2004 Professor Schreiber proposed a synthetic approach that leads to a further expansion of the chemical space: the "diversity oriented synthesis".[25] This methodology goes beyond the combinatorial approach and aims to develop strategies to synthesize very different molecules starting from similar molecules.

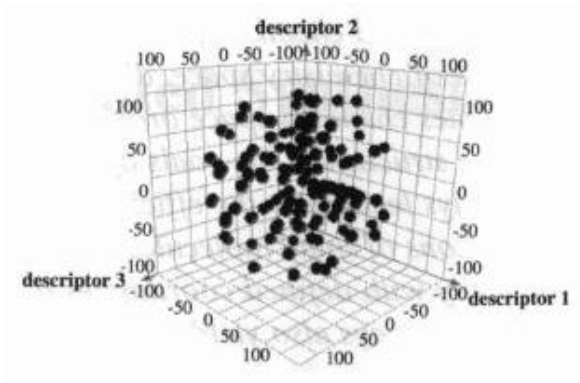


Figure 1.3.2 Diversity Oriented Synthesis chemical space

In the chemical space, this strategy results in the obtainment of a larger number of potential lead compounds, with more n variables than what offered by combinatorial chemistry (Figure 1.3.2). If we consider that the increasing of the chemical space is related to the success of a particular lead, then the diversity oriented synthesis, also called divergent synthesis, probably will offer great benefits. The divergent synthesis opens the door to the concept of "forward analysis", the opposite of the retrosynthetic

analysis. If in retrosynthesis you wonder how a complex molecule can be synthesized from simple elements, in a forward analysis you must wonder how a collection of simple molecules and other similar compounds can be turned into a library of complex products.

1.3.2 Multi-Target Directed Ligands (MTDL)

However, many diseases do not allow to focus on a single biological target for the development of specifically designed drugs: multifactorial diseases depend on multiple genetic factors, and sometimes also on environmental aspects. The multifactorial character may be:

- continuous: different phenotypes of the same chromosomal character cannot be perfectly distinguished and should be measured. They are more similar between relatives, because they have had similar environmental influences and have the same genotype;
- discontinuous: i.e. phenotypes clearly distinguishable from each other without transmission according to Mendel's laws. They are also called character with threshold, and indeed the phenotype occurs when genetic and environmental factors exceed a threshold.

The most common multifactorial diseases fall into Alzheimer's and Cystic Fibrosis diseases. In these cases, it became necessary to take more drugs simultaneously. With the development of chemical, pharmaceutical and medical sciences, the assumption that until now has characterized the pharmaceutical research "one drug, one target" is now giving way to a new approach called Multi Target Directed Ligands (MTDL). MTDLs design has its roots on the new hire "one drug, more targets". In fact, in diseases with different biological processes altered, the modulation of only one of them may not be sufficient to put under control the clinical situation. The MTDL approach is here particularly suitable for the design and development of new bioactive molecules able to more efficiently treat these diseases, but philosophy of such drugs is so recent that commercially available examples can not yet be cited. However, the number of research projects in progress realizes the very high confidence placed in this new perspective of modern pharmacology. Of course, there are pros and cons,[26] and among the advantages can be considered that:

- these drugs allow a single metabolic pathway, reducing the number of drugs and derived species circulating in the patient's body and thus decreasing the side effects related to treatments with more compounds;
- through the unification of two activities in one molecule the risk of potentially harmful interactions between drugs is prevented;
- being an only molecule, will also be sufficient a single route of administration for the treatment of multiple symptoms, making the drug more easily in its intake;
- the presence of a drug with a structure different from the ones of its two individual components disadvantages the occurrence of the bacterial resistance phenomena, for compounds having antibiotic activity.

However, there are some complications:

- the drug specifically designed and synthesized will be used only for specific symptoms, as opposed to a relatively non-specific classic compound, usable in a plurality of similar situations;
- the molecule will be more difficult to design, as it will be necessary to optimize the structure relating to the modulation of all its activities;
- for the same reasons, the synthesis will be as well more difficult.

1.3.3 Green chemistry and biocatalysis

In recent years, the world trend of organic synthesis and chemistry in general was strongly directed to reinvent itself through a new philosophy called "Green Chemistry", which consist in the research of a sustainable development. This means also to begin the replacement of polluting and high environmental impact methods with more environmentally friendly processes. Professor Anastas, in 1991, set out the basis of sustainable chemistry listing 12 principles:

1. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. Chemical products should be designed to preserve efficacy of function while reducing toxicity.
5. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6. Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
7. A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.
8. Reduce derivatives - Unnecessary derivatization (blocking group, temporary modification, protection and deprotection) should be avoided whenever possible.
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.
11. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. Substances and the form of a substance used in a chemical process should be chosen to minimize potential for chemical accidents, including releases, explosions, and fires.[27]

All these principles summarize the sense of green chemisty: it looks for the development of processes that use less material and energy as possible and therefore that produce less pollution, all in compliance with

the safety of operators. In general, the application of the principles is based on the acquisition of ethics by chemists and humanity, based on awareness of the environment as a limited resource that needs protection and security. In this sense, green chemistry become a discipline which is transversal respect to chemistry.

In organic synthesis, it takes more specific meanings:

- limit of solvents use; develop reactions in solid state or without solvent, or use solvents with lower environmental impact, such as water;
- development of more efficient catalysts; exploit simple catalysts that may be recoverable, or biocatalytic methods;
- limit the number of synthetic steps; if possible avoid the use of protecting groups and try to incorporate the greater number of reagents used into the final product;
- if possible, favor reactions that take place at room temperature; if you cannot, take advantage of alternative energy sources;

Therefore, in recent years have been developed several innovative methodologies which are necessarily part of a new vision of organic synthesis. Overall, the introduction of these methodologies is gradually revolutionizing the approach to organic synthesis, giving access to molecules with remarkable complexity for various uses but in respect of environment health. In particular, two aspects have been recognized as crucial for the development of processes more adherent to the green chemistry principles: the reaction medium choice and the use of catalytic processes in opposition to stoichiometric processes. As regards the problem of using solvents, are known processes that occur even without dissolution in organic solvent, and sometimes even two solids are able to react, with high yields and selectivities. Obviously, a process without solvent lowers the cost, if you can transfer it to an industrial level. An alternative to organic solvents that is gradually increase its spreading is the ability to carry out the reaction in water. Water has never attracted much attention, because of the difficulty of dissolving lipophilic compounds. However, the possibility of using mixtures of water and organic solvents is often enough for overcoming insolubility problems.

In addition, if reagents can be only partially solubilized, the reaction can still proceed shifting the equilibrium towards the products.

However, precisely the difficult solubility of the reactants in water can sometimes lead to “melt into one another” by forcing them in reverse micelles, for hydrophobic effect. This has the result of accelerating the reactions and it is therefore a significant and important kinetic advantage.

The use of water and/or the optimization of a process operating in these conditions has many advantages:

- water is cheap, much more than any other organic solvent;
- water is safe, it is not flammable, and it is neither toxic nor carcinogenic;
- water allows you to carry out the reaction without an inert atmosphere;
- water allows a better control of the temperature because of its high thermal capacity;
- water in some cases facilitates reactions for organic hydrophobic effect.

The research for always best catalytic methods is closely related to the principles of Green Chemistry. The best results in terms of environmental sustainability can be achieved by using catalysts able to work in water and/or in salt solutions: this is the case of enzymes, biocatalysts fundamental and ubiquitous which are gradually replacing chemical catalysts and innovating organic chemistry.

Biocatalysis[28] is known to men for a long time, and its applications were already found in ancient Mesopotamia, in China and Japan, in the production of food and beverage. A more scientific approach to biocatalysis only began in '800; in 1814 Kirchoff observed that the wheat gluten could convert starch into sugar. Subsequently, Payen and Persoz observed that the extracts of barley seeds were able to hydrolyze the starch furnishing dextrin's and other sugars.[29] These extracts were called "diastase" and were nothing else then mixtures of amylases; in 1935 Berzelius recognized the catalytic power of these extracts and from those years dextrin began to be produced very easily and commonly.[30] It is only from 1894 that first theories of catalysis of these extracts began to be interpreted and explained by Emil Fisher. The scientist was convinced that enzymes were proteins (their true nature was certainly elucidated only after 20 years) able to work with a "key-lock" mechanism. A further step on enzymology was made by Buchner, who said that the enzyme catalysis does not necessarily require the presence and action of living cells.[31] Nowadays, "biocatalysis" means all the chemical processes that are mediated by enzymes or whole cells; it is therefore to indicate the use of techniques for the biocatalytic synthesis of "fine chemicals", and the fermentations for "bulk" production of molecules such as ethanol and acetic acid. Inside the biocatalysis are also present biotechnology and genetic engineering, i.e. those sciences that aim to build enzymes and/or microorganisms ad hoc designed to perform specific functions.

Between advantages, the first and positive aspect of biocatalysis is certainly linked to the nature of enzymes. Enzymes are regioselective, chemoselective and stereoselective catalysts particularly efficient. This means that polyfunctional molecules can be transformed in a very selective manner and without the use of protecting groups, which is usually impossible the normal organic synthesis. This means a smaller number of synthetic steps, with money and time gains, and the possibility of carrying out the synthesis in aqueous medium avoiding, or reducing, the use of organic solvents must also be added. Moreover, was intensively studied the use of enzymatic catalyst in organic solvents. The chirality of the molecules can thus be introduced without the use of chiral catalysts, expensive and often very specific, and/or chiral auxiliaries via kinetic resolutions, kinetic dynamics, desymmetryzations and other processes. The use of biocatalytic systems allows compliance with many "rules" related to the principles of "green chemistry" enunciated by Anastas.

The disadvantages associated with the biocatalysis are generally due to the nature of the enzyme and/or of the whole cell. Enzymes can be very expensive, because they have to be produced, extracted and purified from bacterial cells progenitor. They often need to be designed ex-novo, with developments very long and expensive. They can be easily re-used only if supported, but up to date a general technique doesn't exist and each enzyme immobilization should be treated as a special case.

The immobilization in some cases may be even impossible, especially very complex for enzymes such as dehydrogenases. Therefore, the structure of the protein must often be changed to fit immobilization. If the cost of the enzyme is not a problem, you have to keep in mind that many of them work with cofactors such as NADH and FADH. Their costs must be considered, as well as the possibility to overcome the problem by building a system for recycling the cofactor. In some cases, the enzymes may also not be stable, preventing a long-term storage and decreasing the availability.

If using enzymes can often be far from easy, it is not the case of working with whole cells; microorganisms in fact are generally very resistant, cheaper and can be cultivated in order to never exhausting them. A further advantage is related to the fact that cells do not need cofactors to operate.

2. INTEGRIN LIGANDS

2.1 β-Lactams as integrin ligands

2.1.1 Cell Adhesion Molecules

Cell Adhesion Molecules (CAMs) are trans-membrane proteins located on cell surfaces. CAMs are composed of an intracellular, an extracellular and a trans-membrane domain. They take part in the so-called process of cellular adhesion, essential in ensuring the maintenance of a multicellular structure both from the point of view of the three-dimensional shape and as far as concerning the arrangement and the different tissues organization. This mechanism can obviously affect the adhesion with other cells, through junctions therefore called direct, or even with a surface or with the extracellular matrix. Thus, it is so involved in the connection between cells, between cells and the cytoplasm, or even in signal transduction. Clearly, the importance of cell adhesion varies from a tissue to the other, or from a species to another.

Adhesion molecules involved in the just considered mechanisms are divided into at least four different families, and as regards human cells, in addition to integrins also selectins, cadherins and molecules belonging to the so-called immuneglobulin superfamily of adhesion (IgSF CAMs) exist. CAMs are always described according to their nature and to the links that can establish, homophilic if directed to proteins of the same type or heterophilic if directed to the extracellular matrix or to adhesion molecules belonging to a different family. IgSF CAMs are an adhesion molecules family known since a long time and characterized by structural similarities with immunoglobulins. IgSF CAMs are able to make both homophilic and heterophilic bonds; they contain immuneglobulin-like extracellular domains, which perform important roles also for the immune system. Some members of this family worthy of consideration are Intercellular CAMs (ICAMs), Vascular CAMs (VCAMs) and Neural CAMs (NCAMs), frequently expressed from different cell types and involved in the fine adjustment of development and regeneration the processes.

Cadherins are often expressed with them from the same cells and are characterized by a much stronger mediated adhesion, mainly maintaining the cells together and isolated in discrete tissues, and preserving the integrity of these tissues. Cadherins are trans-membrane omophilic glycoproteins involved in adhesion between cells and appear to be the most common adhesion molecules. Their name results from the contraction of “calcium” and “adhesion”, thus already defining their Ca^{2+} ions mediated mechanism of action. As a matter of facts, extracellular domains usually have flexibility points stiffened by interactions with calcium ions. Conformations can then match with that of cadherins, equal or of a closely related subtype, expressed by adjacent cells. The frequently poor affinity of these interactions has to be associated with a weakness of the adhesion itself. However, this limit is solved by the formation of the so-called anchored junctions, consisting in a relatively high number of parallel formed bonds, always characterized by a mutual recognition of the partners.

Selectins are calcium dependent glycoproteins all made by a single protein chain and often considered to be a type of lectin, proteins specifically interacting with carbohydrates. Even selectins bind oligosaccharide portions, ensuring adhesion functions essentially between cells in the bloodstream, therefore transients and characterized by mild interactions.

The management of white blood cells, in addition to an implication in the inflammatory phenomen, is among the main roles of these molecules. However, selectins does not work alone but often in collaboration with integrins, as regards both the inflammation and the passage of white blood cells from blood to tissues. Selectins, thus obviously heterophilic, in this process act from endothelial cells of blood vessel walls causing the rolling of these white blood cells, which may eventually activate an integrin. Finally, thanks to a conformational variation induced on integrins, which commonly allow the interaction of cells with the matrix, white blood cells can reach tissues.[32]

2.1.2 Integrins

Integrins are glycoproteic membrane receptors involved both in the mechanisms of adhesion between cells and the extracellular matrix (ECM), and in the signals transduction from ECM to cells. ECM is formed, organized and decomposed by cells. It act on them through cell adhesion proteins like, mainly, integrins, at least in animal cells. Integrins are characterized by a surprising ability to transmit signals across cell membranes in both the directions. Interaction with molecules present in the matrix allows the transport of a message inside as well as an internal condition can control interactions with the matrix. In cases such as for the interaction of white blood cells with the endothelium and a few others, internal conditions can even act on surface molecules expressed on different cells. Moreover, in addition to molecular or mechanical signals transmission, it has been recently discovered the way by which integrins can convert a signal type into another. Among the functions of integrins, are present also biological activities related to cellular migration and immune system, and the mechanism that prevents certain virus to bind to cells.

Nowadays are known twenty-four human integrins. These integrins are all formed by two glycoproteic subunits non-covalently linked and usually indicated by the Greek letters α and β. These subunits both cross the cell membrane, and while the outer portions bind specific aminoacid sequences of proteins, or in some cases even to molecules on surfaces of other cells, the inner ones are connected to a protein complex attached to the cytoskeleton. From suh a connection derivied the not yet cited control function on shape and cell mobility (Figure 2.1.1).

A remarkable property of integrins is given by the possibility of assuming an active or inactive conformation in response to a specific interaction. It can happen by replicating to the action of certain ligands on allosteric sites. The activation consists, with regard to the outer portion, in an extension reflected in a mutual eversion of the subunits α and β, both in the trans-membrane area and in intracellular domains.

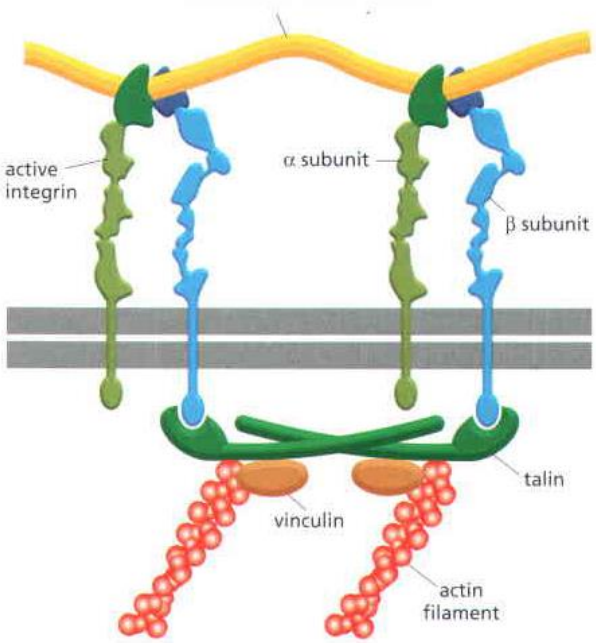


Figure 2.1.1

The "outside-in activation" process ends with the establishment of a strong interaction between the β subunit and an anchor protein, which seems to be the key component of the connection between the internal regions and ECM. The protein is known as Talin and is thus in competition with the protein chain α (Figure 2.1.2). It follows the possibility of a reverse mechanism, an "inside-out activation" already observed and intracellular regulated by molecules whose signal is thus transferred to the matrix.

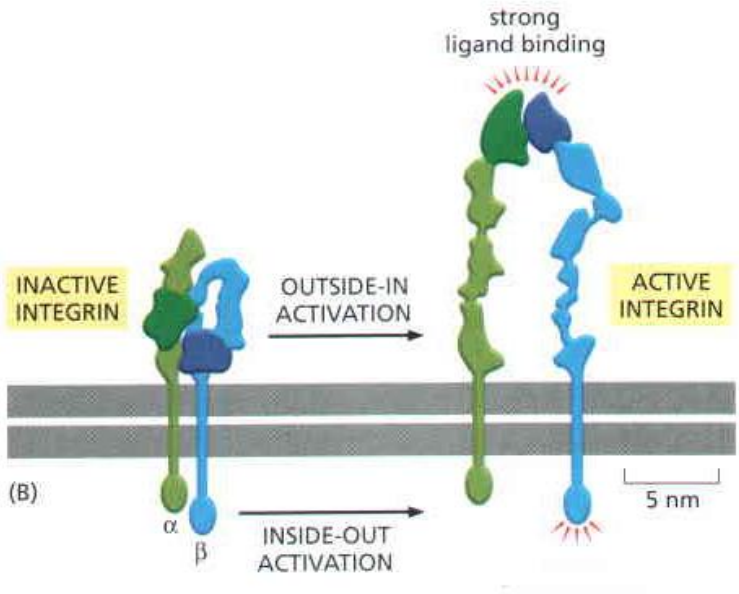


Figure 2.1.2

At different dimerizations, associable with one of the eighteen subunits α and one of the eight β chains nowadays identified, can be correlated both properties and various functions, as well as different specificities in respect of the ligands involved. The concentrations of Ca^{2+} and Mg^{2+} also affect these interactions, in terms of specificity and affinity, and obviously overlaps exist. However, activities of integrins have high variability depending on the protein chains. Just to list a few examples, mutations may be involved in bleeding, favored by lack of platelet aggregation, severe vesicular cutaneous manifestations, some forms of muscular dystrophy and even in embryos death.

As previously mentioned for other CAMs, integrins as well exert additional actions to the simple adhesion. For example, in vitro studies showed how a lot of cell types tend not to grow or proliferate, albeit with wide availability of nutrients, if the expression of integrins that determine their membership with the ECM was inhibited. Moreover, but only for certain types of cells, the loss of contact with the ECM induces apoptosis, the process of programmed cell death. A mutation involving integrins can determines the loss of cell dependence from the control system of growth and proliferation, and thus easily degenerate into cancerous diseases. Furthermore, in vitro and in vivo studies demonstrated that the molecular signals associated with integrin systems are crucial for proliferation and cell survival.[33]

Signals managed by integrins and received from cells are therefore related survival, growth, division, differentiation and apoptosis of themselves. Furthermore, were highlighted involvements in the migration of embryonic cells, in maintaining the integrity of tissues and in the process of blood coagulation. Finally, some integrins has an important role even in the angiogenesis mechanism. It consists in the construction of new blood vessels from existing ones, and also endothelial cells are involved.

Concerning this, worthy of attention is the $\alpha_v\beta_3$ type integrin, the first crystallized and characterized among the α_v and the first shown to be active in regulating the angiogenesis process. On one hand, angiogenesis is biologically essential for embryonic growth and tissue survival and repair. On the other hand is involved in inflammatory processes becoming chronic, in tumor growth and in metastatic developments. While in normal physiological conditions it is strictly controlled and finely adjusted, in cases of hyperactivity the transition of a latent tumor to an evil one is favored.[34] The development of angiogenesis inhibitors has so begun, studying proteins such as fibronectin, fibrinogen, vibronectin and osteopontin, ECM components which interact with integrins. Obviously, the aim was to identify the pharmacophore and develop inhibitor drugs.

Moreover, also $\alpha_4\beta_1$-type integrin has been shown to play important roles in many diseases such as embryogenesis and pathogenesis, which involve both cell adhesion and cell migration. Integrin $\alpha_4\beta_1$ can mediate cell-cell and cell-extracellular matrix adhesion by binding to either fibronectin or Vascular Cell Adhesion Molecule 1 (VCAM-1). Both interactions are important for extravasation of leukocytes from blood. Thus, rationally designed inhibitors of $\alpha_4\beta_1$ function may be useful for treating a various inflammatory conditions.[35] Ligand-binding sites are contained within the HepII/IIICS domain of fibronectin and within the homologous immunoglobulin domains 1 and 4 of VCAM-1. Previous studies have shown that the binding of each ligand to $\alpha_4\beta_1$ is mutually exclusive, suggesting that they may

employ similar mechanisms to bind receptor. Fibronectin contains at least three distinct peptide sequences that are active sites for $\alpha_4\beta_1$ binding, two homologous sequences Leu-Asp-Val-Pro (LDVP) and Ile-Asp-Ala-Pro (IDAP), and a third related to Arg-Gly-Asp (RGD).[36] Small peptide and non-peptide $\alpha_4\beta_1$ antagonists have already shown efficacy in disease models of inflammation (BIO12118[37] and TR-140359[38]).

2.1.3 Integrin inhibitors

Fibrinogen is a plasmatic glycoprotein essential in the process of blood coagulation and perhaps the best known integrin inhibitor. Studies on it and on other related proteins have led to identificate the RGD peptide sequence (Arg-Gly-Asp) like the key portion for the recognition of ligands operated by integrins (Figure 2.1.3).

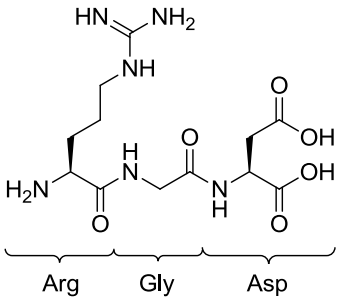


Figure 2.1.3 RGD peptide sequence

Studies conducted by x-rays on $\alpha_v\beta_3$ type integrins have shown that interactions established with their ligands were mainly electrostatic. A positive charge was seen to be delocalized by the guanidine group of the arginine, while an opposite sign charge is carried by the aspartic acid residue, at physiological pH. These charges interact with integrins on two other aspartic units in α chains and on a metal cation of the β parts, respectively. These observations has also been extended to $\alpha_5\beta_1$ and, recently, $\alpha_4\beta_1$ type integrins.

Thus, integrins become a promising target for the development of angiogenesis treating agents. Nowadays many ligands, also with significantly different scaffolds, are able to mimic the RGD sequence showing affinities in some cases very high. Structures of these compounds varied during the time, from cyclic oligopeptides therefore conformationally rigid to derivatives partially and completely retro-inverse, i.e. made in part or in whole by amino acids of the non-natural (D) series.

New peptidomimetics of the RGD sequence have recently been proposed. They are always cyclic, but contains a β-lactam ring as a further reduction of the molecular flexibility. Results furnished are comparable to the one of Cilengitide, a ciclopentapeptide endowed of an high activity against the $\alpha_v\beta_3$ type integrin, so with a very low concentration able to inhibit the activity of the 50% of targets (IC_{50}).

The key role of the cationic and anionic dual functionality, originally due to an arginine and an aspartic residues and left unscathed in first peptide analogues and pseudopeptides developed, always remained,

albeit if in very diverse structures. Acidity and basicity of the two functions, as well as the relative distances, immediately appeared to be irreplaceable. First peptidomimetic inhibitors with high activity and therefore with a certain therapeutic potential presented however bioavailability problems. These necessities prompted the science researchers towards non-peptidomimetic inhibitors (some examples in Figure 2.1.4).

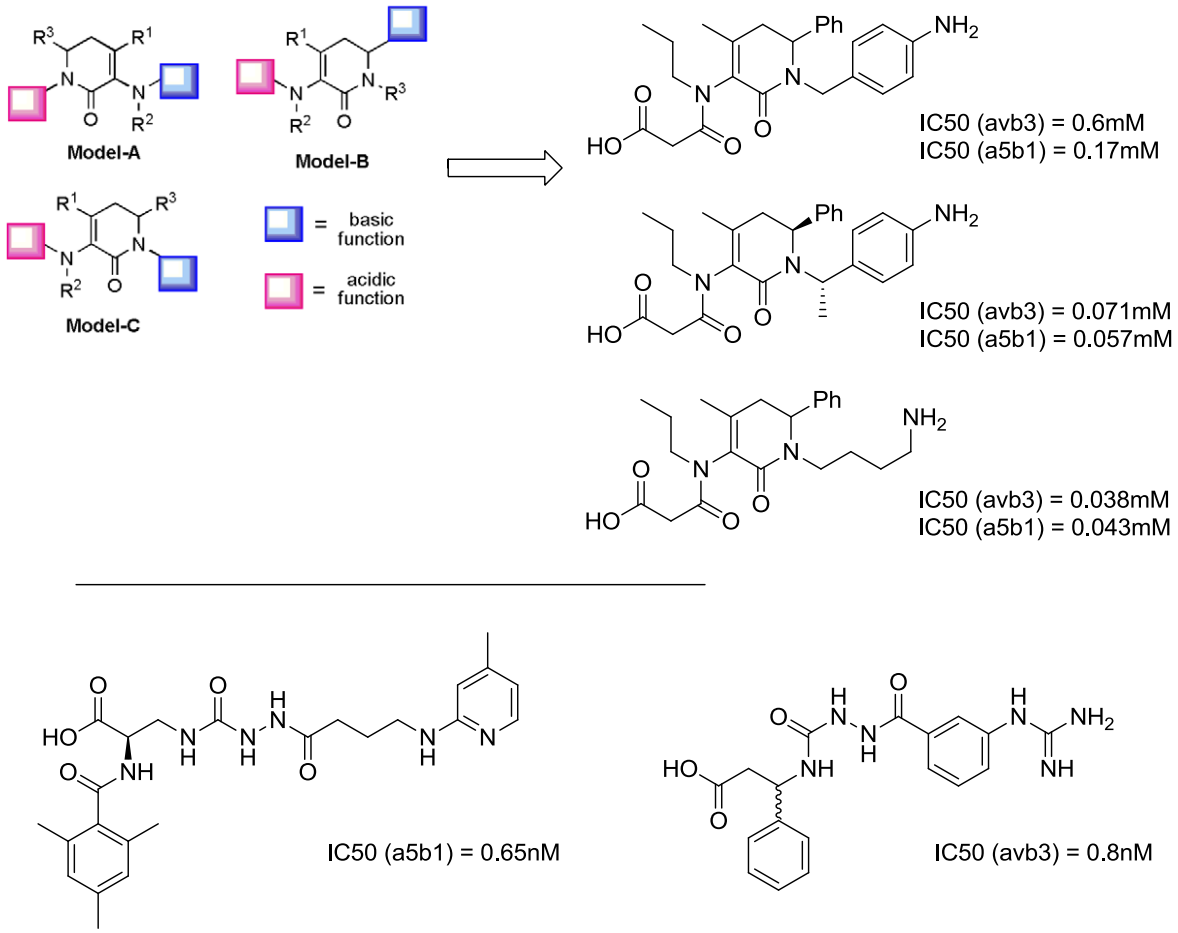


Figure 2.1.4 Specific integrin inhibitors with high activities

However, among these antagonists no one has an actual use, and none is still entered into clinical trials, not because of an affinity lack or a low target selectivity but for times and costs of the discovery process itself.[39]

It is evident that also remarkable structural variations compared to the RGD sequence are possible. In particular, as common in the design of new compounds for potential pharmacological uses and as already seen in the cyclic compounds shown, scaffolds exploited introduce a conformational rigidity which is often an added value for similar applications. Reduction of molecular weights, increase of metabolic stabilities and bioavailabilities, and reduction of the number of potential interaction partners, avoiding the consequent side effects, are always the main advantages.

2.1.4 β-Lactam $\alpha_v\beta_3$ and $\alpha_5\beta_1$-type integrin agonists

As previously mentioned, among the integrin superfamily $\alpha_v\beta_3$ and $\alpha_5\beta_1$ type integrins play a pivotal role in the formation of new blood vessels and are overexpressed on activated endothelial cells in physiological and pathological angiogenesis.[40] The $\alpha_v\beta_3$ integrin is extensively expressed on tumor cells. There are some evidences that this receptor is present in late stage glioblastomas, ovarian carcinoma, melanomas; it also is an endothelial cell marker in breast cancer and regulates melanoma cell proliferation, survival and metastases.[41] The involvement of integrin $\alpha_v\beta_3$ in highly important pathologies induced the development of potential candidates able to inhibit the functions of this receptor, but the mode of action of these molecules has not been elucidated. This means that they could be full or partial agonists, inverse agonists or antagonists; they probably engage the integrin altering its conformation as response, preventing intramolecular shape change or dragging a conformational equilibrium towards the inactive form of the receptor.[42]

The activation of integrin-mediated cell adhesion by antibodies or small molecules has been recently reported: Vanderslice et al. demonstrated that agonists enhanced the effects of stem cell-based therapies by improving cell retention and engraftment; Yea et al. found that a single agonist antibody against the alpha chains of integrins can induce human stem cells to become dendritic cells.[43] It was thus recognized that integrin agonists could open up novel opportunities for therapeutics which gain benefits to increase rather than decrease integrin-dependent adhesion. For instance, a significant factor in chemoresistance in melanoma is a loss of integrin-mediated adhesion; in this case, stimulation of integrin signaling by agonists significantly improved the response to chemotherapy. Gupta et al. have reported that small molecule mediated activation of integrins, rather than inhibition, reduced leukocyte migration, tissue accumulation and inflammatory injury.[44]

Up to now, a large number of peptide and non-peptide ligands for the $\alpha_v\beta_3$ receptor have been developed, which are all related to the minimal recognition RGD motif present on $\alpha_v\beta_3$ integrin ligands of the ECM such as fibronectin and vitronectin. As an example, Cilengitide is a small RGD-containing cyclic pentapeptide currently in clinical phase III for glioblastoma multiform and in phase II for other types of cancers due to its capability to antagonize $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins (Figure 2.1.5).[45] Recent research efforts have focused on improving the pharmacological parameters mainly by altering the polarity and rigidity of the scaffold and the nature of the basic moiety. Among several heterocyclic structures able to antagonize integrins, lactam derivatives found their own niche. In Figure 2.1.5 are reported some lactam derivatives active as integrin ligands and some of the ligands include the RGD tripeptide framework. Lactams **D** and **E** demonstrated that a phenylamine portion could successfully replace the arginine residue.[46] In previous papers, some of us have identified a series of $\alpha_v\beta_3/\alpha_5\beta_1$ integrin ligands with unsaturated β-amino acid fragment: was known that incorporating a distinct β-amino acid into a RGD-containing peptide resulted in the stabilization of specific conformations of the ligand.[47] It was argued that a restricted conformation introduced by unsaturated β-amino acid and of a cyclic structure could give a

favorable alignment of both the basic and carboxylate moieties on the ligand, thus meeting the crucial requirements for integrin affinity and selectivity. The β-lactam ring constitutes by itself a site of conformational restriction with a β-amino acid moiety in a cyclic structure and constrained features.

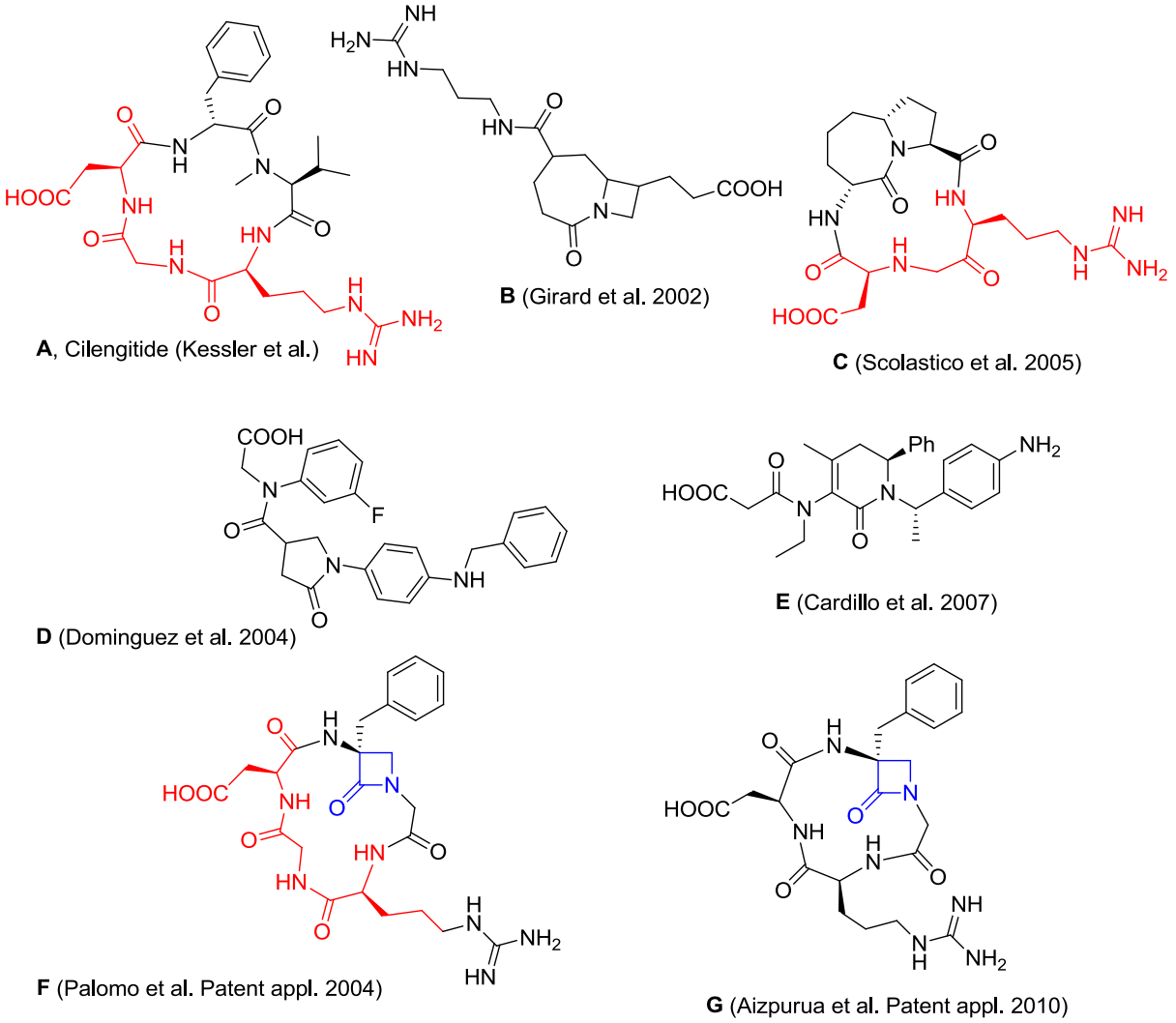


Figure 2.1.5 Cilengitide and other integrin ligands, with in red the RGD sequence

As a part of our studies on design and synthesis of new β-lactam derivatives and of an on-going interdisciplinary project,[48] we would like to evaluate the ability of azetidinones, not inserted into cyclopeptides, to target integrins. We report the synthesis and the preliminary biological results on K562 (human erythroleukemia expressing $\alpha_5\beta_1$ integrin), SK-MEL-24 (human malignant melanoma expressing $\alpha_v\beta_3$ integrin), and Jurkat E6.1 human T cells (expressing $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins) cells of some new β-lactam derivatives. The approach for the design of the new molecules was based on rationalization of known ligands structures: we set out to explore molecules containing the azetidinone as a rigid cyclic framework, armed with carboxylic acid and amine terminus spaced from 9 to 14 atoms to activate a conceivable recognition by integrins (Figure 2.1.6).

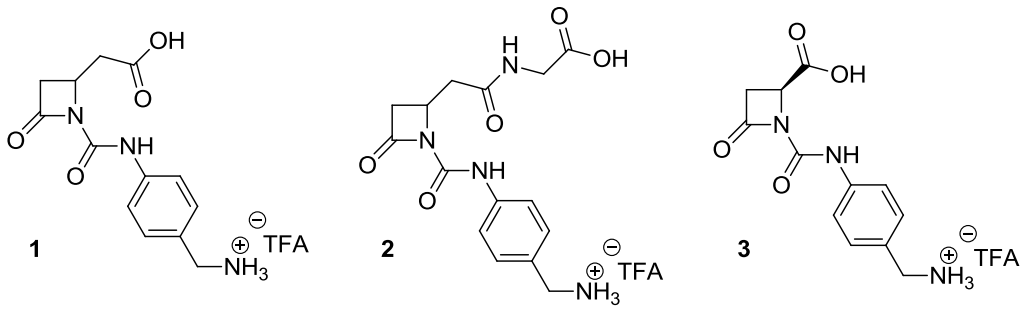


Figure 2.1.6 β-Lactams synthesized and tested as trifluoroacetic acid (TFA) salts

A 4-amidobenzylamine residue was chosen as the basic terminus directly linked to the β-lactam nitrogen atom thus resulting as imido function. The carboxylic acid was on the C-4 side chain of the azetidinone and differently spaced from the ring: in compound **1** the β-lactam ring together with the C-4 side chain could be considered a sort of β-glutamic acid derivative, which was elongated with a glycine residue in compound **2**, while in the azetidinone **3** the carboxylic acid is directly linked to the β-lactam ring thus resembling an aspartic cyclic amide. The C-3 position was not substituted in order to mimic the methylene residue of glycine in the RGD peptide. Herein I report our discovery of azetidinones as new cell adhesion ligands and their characterization as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ agonists.

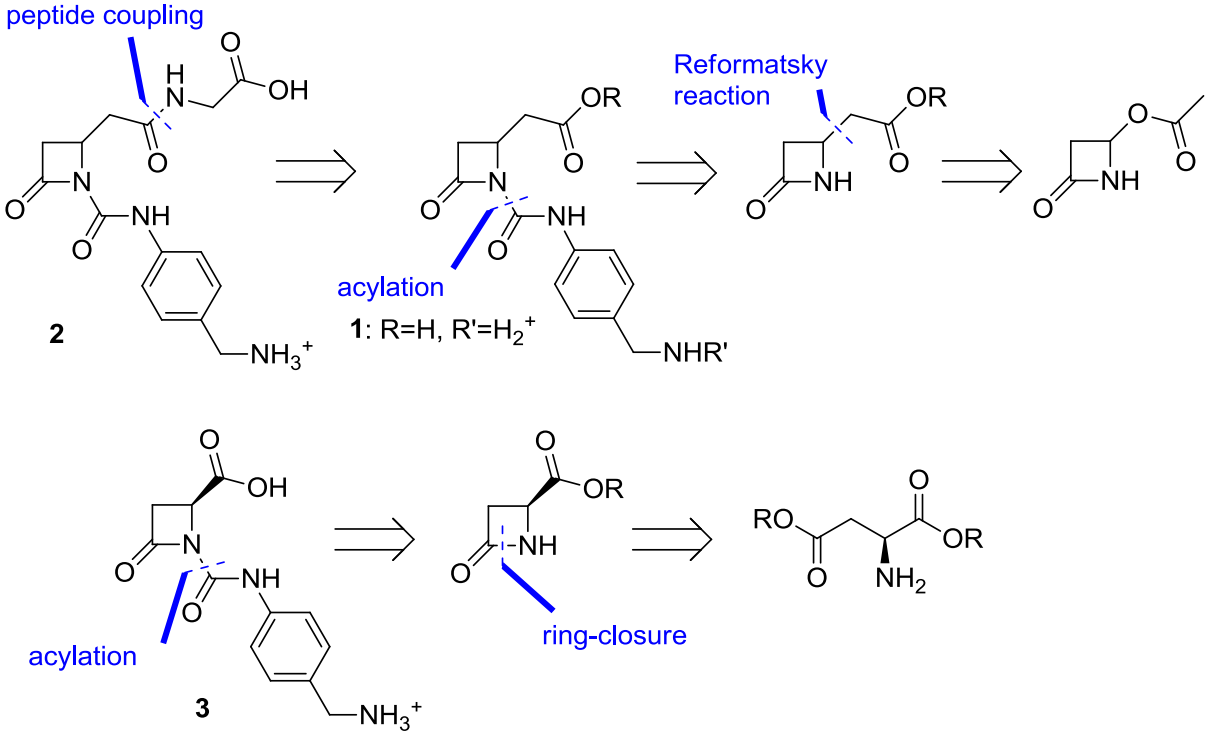
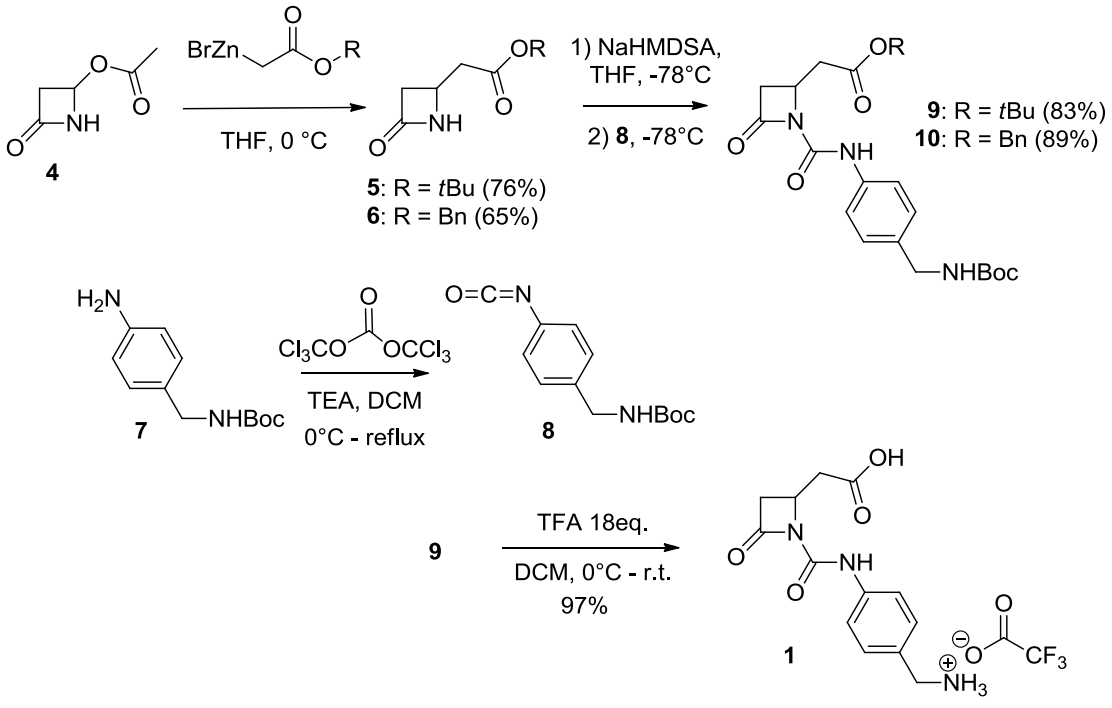


Figure 2.1.7 Synthetic strategies for compounds **1**, **2** and **3**

Compounds **1** and **2** were both obtained starting from the commercially available 4-acetoxy-azetidin-2-one. The introduction of the carboxylic function in the C-4 side chain was achieved through a Reformatsky reaction followed by N-acylation of the β-lactam ring to give compound **1**. The C-4 side

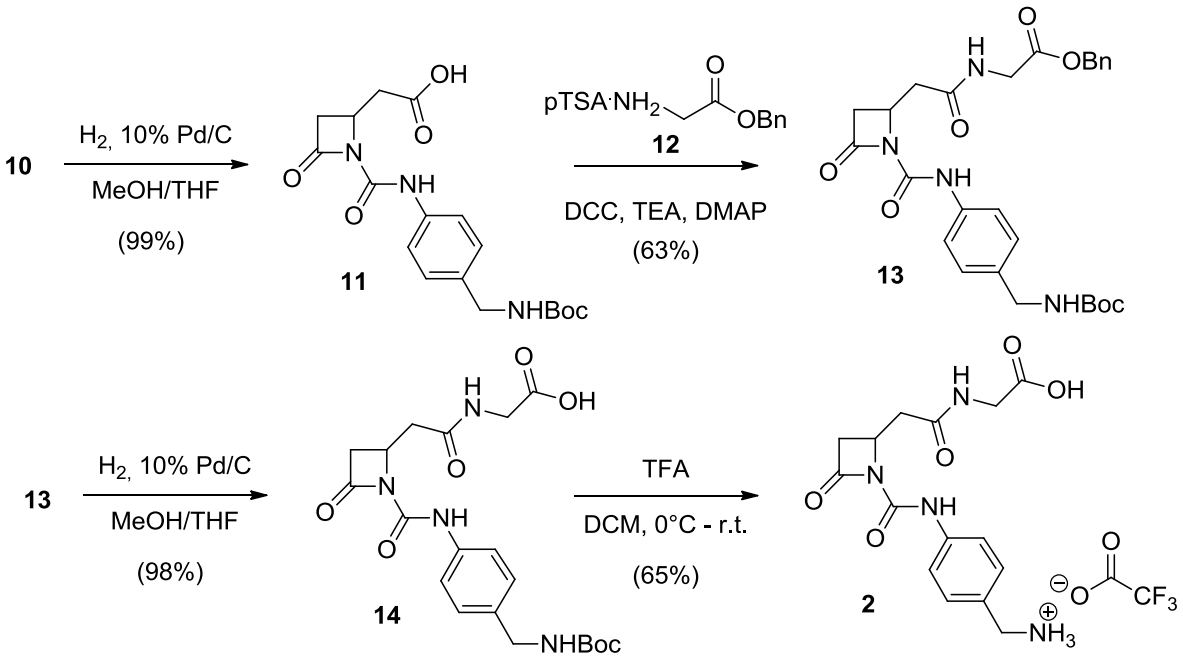
chain was eventually converted into a longer peptide chain through insertion of a glycine unit to give **2**, while **3** was prepared from 4-carboxylic-azetidin-2-one, easily obtained in enantiomerically pure form by ring-closure of an L-aspartic acid diester (Figure 2.1.7). A careful protecting group strategy for the C-4 and N-1 side chains of azetidinones was developed to preserve the β-lactam ring throughout the synthesis and to enable specific combination of temporary or permanent protecting groups to achieve a full or partial deprotection depending on the synthetic strategy requirements.

In Schemes 2.1.1-3, the synthetic steps are described in details. As outlined, *t*-butylbromoacetate and benzylbromoacetate were treated with an excess of metallic Zn in THF to furnish the corresponding Reformatsky reagents, which were then coupled with 4-acetoxyazetidin-2-one **4** to give 4-acetate-azetidin-2-one esters **5** and **6** in good overall yields after purification by flash column chromatography. The N-1 side chain was constructed starting from 4-aminobenzylamine **7** selectively protected with a Boc group on the benzyl amine and then transformed into the corresponding isocyanate **8** with triphosgene. Treatment of azetidinones **5** and **6** with NaHMDSA (Sodium bis-trimethylsilyl-amide) followed by addition of isocyanate **8** furnished compound **9** and **10** in good yields after purification by flash column chromatography. Compound **9** was then deprotected by treatment with trifluoroacetic acid both on the C-4 and the N-1 side chains to give compound **1** as trifluoroacetate salt (Scheme 2.1.1).



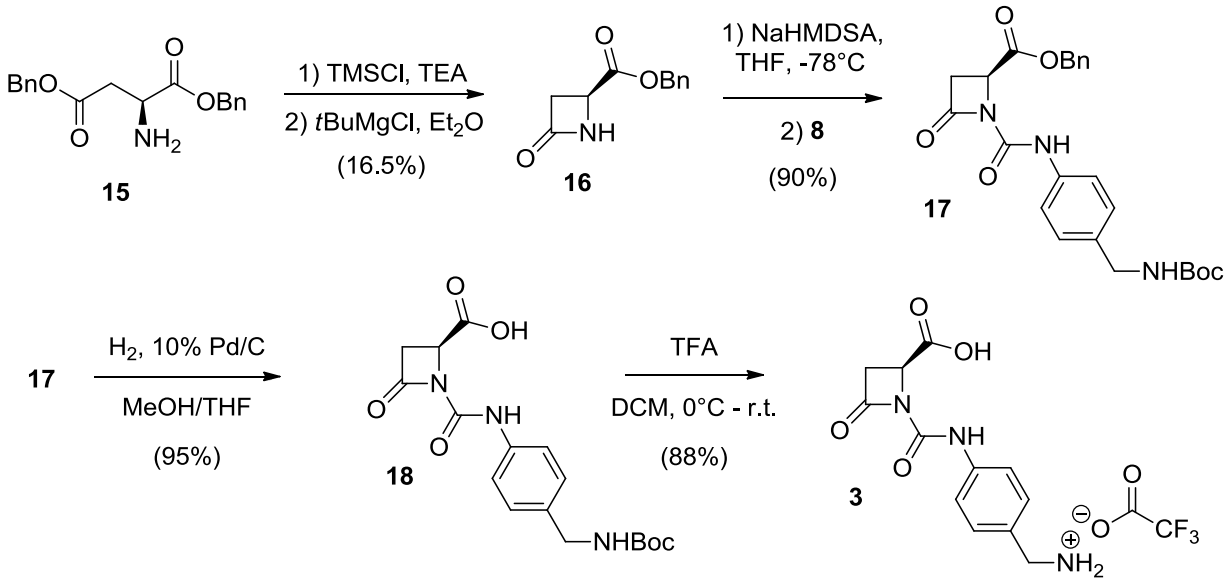
Scheme 2.1.1 Synthesis of compound **1**

The benzyl ester of compound **10** was in turn selectively deprotected to give acid **11** so that a DCC mediated coupling with benzylglycine **12** could be accomplished to deliver compound **13**. Compound **13** was subsequently deprotected in a two steps procedure furnishing **14** by hydrogenolysis and then azetidinone **2** as trifluoroacetate salt treating with TFA (Scheme 2.1.2).



Scheme 2.1.2 Synthesis of compound **2**

As to the synthesis of **3**, the starting 4-carboxylic-azetidin-2-one **16** was obtained by a Grignard mediated cyclization of L-aspartic acid dibenzylester **15** Enantiomerically pure **16** was then N-acylated to give **17** and deprotected in two steps with the same procedures exploited above furnishing **18** and, finally, **3**, isolated as trifluoroacetate salt (Scheme 2.1.3).



Scheme 2.1.3 Synthesis of compound **3**

Pharmacology

The ability of **1**, **2** and **3** to modulate the adhesion of K562 (expressing $\alpha_5\beta_1$ integrin) or SK-MEL-24 (expressing $\alpha_v\beta_3$ integrin) cells to immobilized fibronectin (10mg/mL) was evaluated. These cell models

are widely used to investigate potential ligands capable of influencing cell adhesion mediated by the above mentioned integrins. Interestingly, β-lactams **1**, **2** and **3** showed a concentration dependent enhancement in fibronectin-mediated adhesion of K562 and SK-MEL-24 cells (in Figure 2.1.8, the concentration response curves showing the effects of **1**, **2** and **3** on cell adhesion; cells were incubated for 30min at room temperature with each compound or with the vehicle as described in the experimental section).

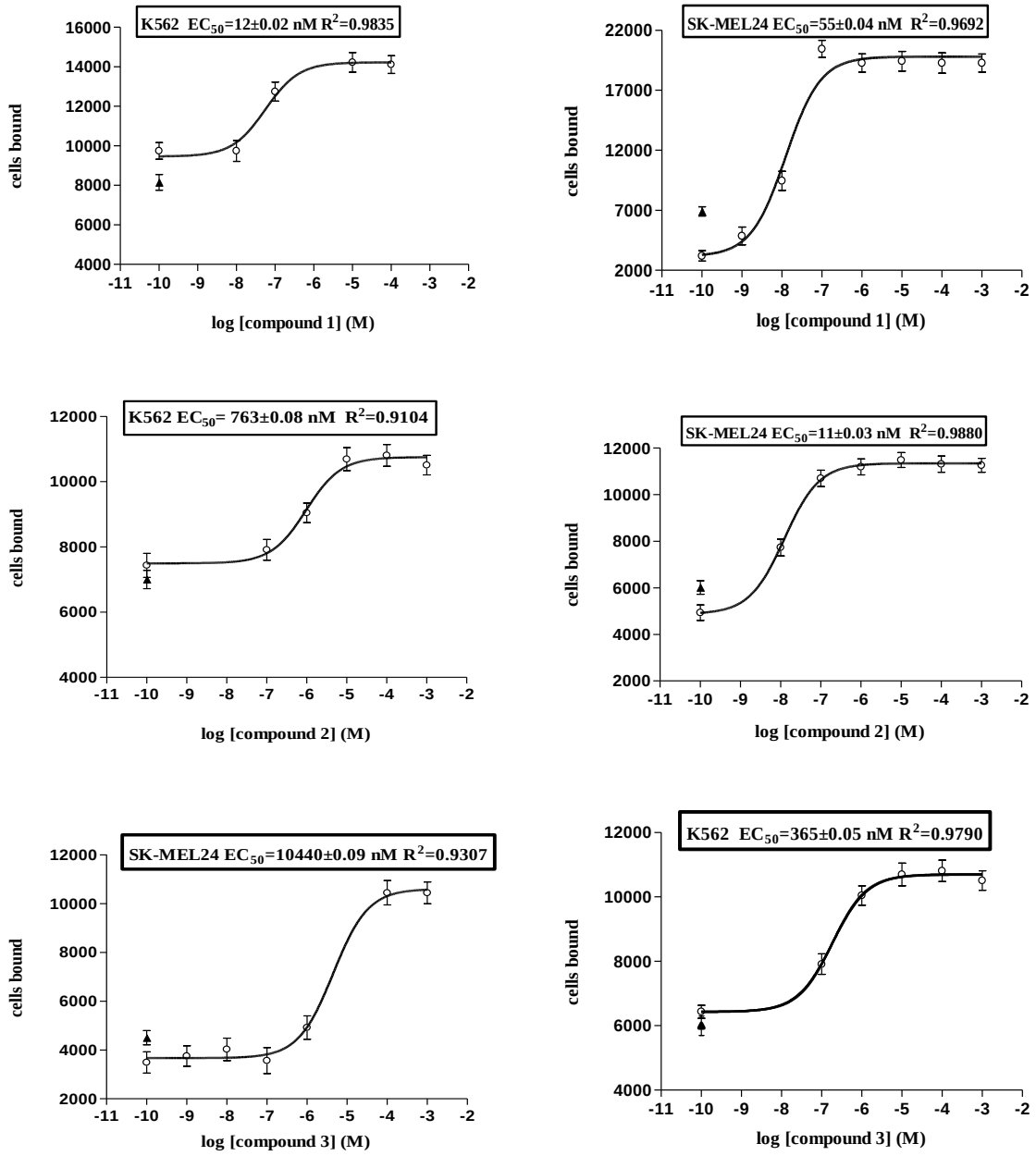


Figure 2.1.8 1, **2** and **3** enhancement of fibronectin-mediated adhesion

With regards to $\alpha_5\beta_1$ integrin expressing cells, **1** was the most potent in enhancing cell adhesion with an EC_{50} of 12nM; it was more or less five times less potent toward $\alpha_v\beta_3$ integrin. β-Lactam **2** was the most

effective toward $\alpha_v\beta_3$ integrin (EC_{50} = 11nM) and **3** was 1000 less effective. These cell models were validated demonstrating that the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ agonist fibronectin produces a concentration-dependent elevation of cell adhesion in SK-MEL-24 and K562 cells (data not shown).

Reference antagonists were capable to displace cell adhesion in SK-MEL-24 cell line (Ac-Asp-Arg-Leu-Asp-Ser-OH, IC_{50} = 25nM) and in K562 cell line (cyclo-Arg-Gly-Asp-D-Phe-Val, IC_{50} = 34.7mM). In another set of cell adhesion assays, **1** and **2** were ineffective to modify Jurkat E6.1 cell adhesion mediated by $\alpha_4\beta_1$ - (toward vascular cell adhesion molecule-1, abbreviated in VCAM-1) and $\alpha_L\beta_2$ - (toward intercellular adhesion molecule-1, ICAM-1) integrin ($IC_{50} \geq 10$ mM, data not shown), thus demonstrating selective interactions by the new β-lactam ligands. The ability of the new β-lactams to increase cell adhesion was then tested in the absence of fibronectin. In a second set of experiments, adhesion of K562 and SK-MEL-24 cells to wells previously coated by passive adsorption with the most active β-lactams **1** and **2**, fibronectin as comparison, and BSA as a control, were tested (in Figure 2.1.9, K562 and SK-MEL-24 cell adhesions to wells coated with 10μg/mL of fibronectin FN, compound **1** and **2**. Controls were cells plated in wells coated with 10μg/mL of bovine serum albumin BSA. Each value is the mean ± S.E.M. from four separate experiments carried out in duplicate).

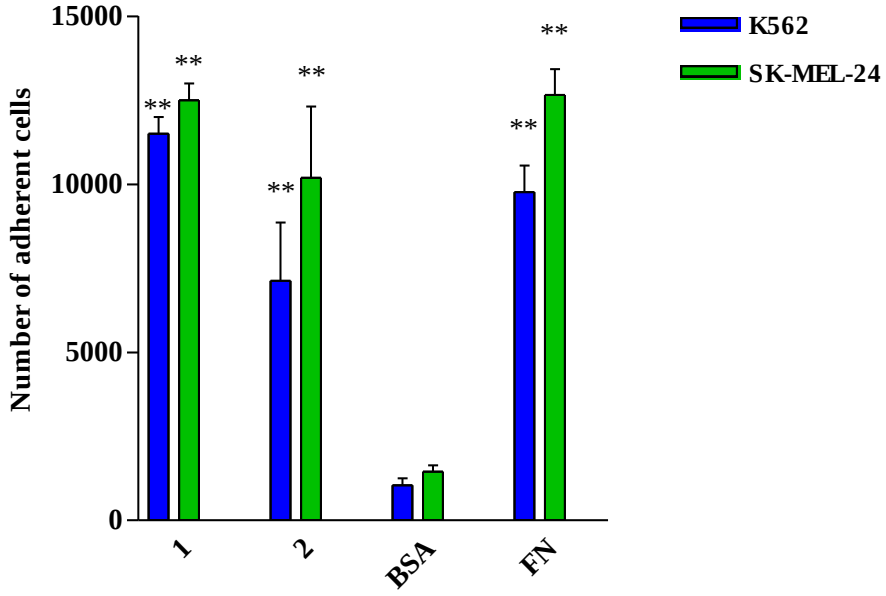


Figure 2.1.9 K562 and SK-MEL-24 cell adhesions

Both β-lactams **1** and **2** produced a significant adhesion, comparable to fibronectin, of K562 and SK-MEL-24 cells, while on the contrary both cell lines did not adhere to wells coated with bovine serum albumin alone. Neutralizing antibodies to the β_1 or α_v integrin subunit (10mg/mL), added to the cells 10 min in advance, blocked the adhesion mediated by compounds **1** and **2** (10mg/mL) to K562 and SK-MEL- 24 cells, respectively (data not shown). This result strengthened the evidence that the cell adhesion was effectively and specifically mediated on $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins by the new β-lactam ligands.

Wandzik et al. have reported that the protein kinase C (PKC) activator PMA (Phorbol Myristate Acetate) induces megakaryocytic differentiation of K562 cells upregulating $\alpha_{IIb}\beta_3$ integrin expression, which may represent a target of the assayed β-lactams.[49] We ascertained, by flow cytometry analysis, that K562 cells exposed to PMA (25 /mL for 40h) express this integrin subtype, in addition
to $\alpha_5\beta_1$. Interestingly, cell adhesion of K562 cells mediated by compounds **1** and **2** was partially blocked by the selective $\alpha_{IIb}\beta_3$ antagonist tirofiban (in Figure 2.1.10, integrin $\alpha_{IIb}\beta_3$ partially mediates K562 cells adhesion to compound **1** and **2**. **A**: PMA, 25ng/mL for 40h, induces upregulation of $\alpha_{IIb}\beta_3$ integrin expression on K562 cell surface in comparison to untreated cells. A representative experiment repeated three times with the same result is shown. **B**: the selective $\alpha_{IIb}\beta_3$ antagonist tirofiban TF at 5, 10 or 50mM partially blocks K562 cell adhesion to wells coated with **1** or **2**. Each value is the mean ±S.E.M. from four separate experiments carried out in duplicate).[50]

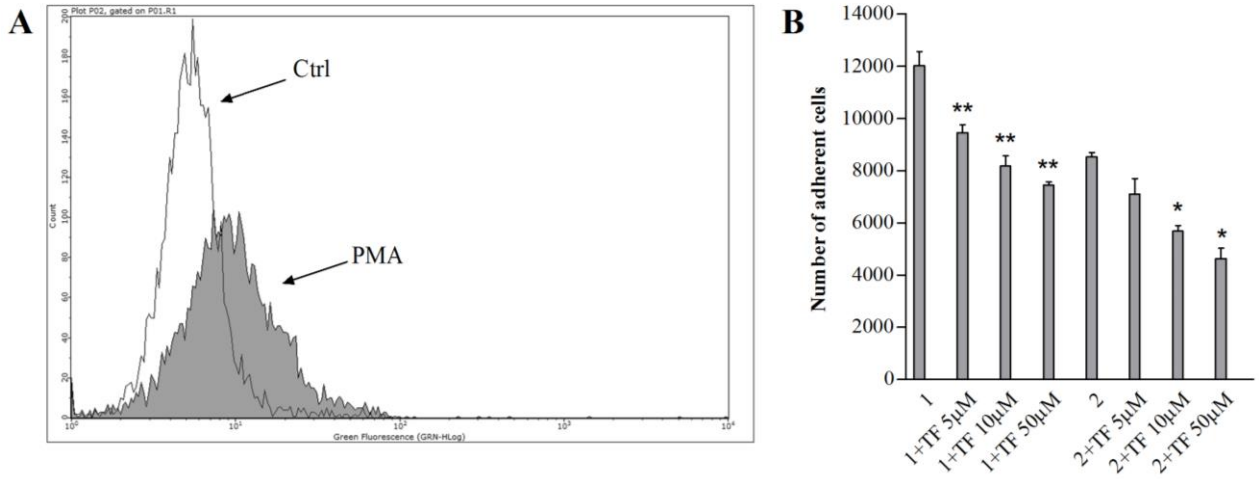


Figure 2.1.10 cytometry analysis of αIIbβ3 integrin expression on K562

These data seem to be indicative that both $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins may contribute to mediate cell adhesion induced by **1** and **2** in K562 cells. These data support the hypothesis that the novel β -lactam derivatives **1** and **2** possess an interesting activity as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin agonists. Compound **1** displays an higher affinity toward $\alpha_5\beta_1$ integrin whereas **2** is more selective for integrin $\alpha_v\beta_3$. Both integrins are targeted by the RGD sequence and it is conceivable to presume that these novel compounds could mimic the sequence. Interestingly, Aizpurua et al. have already described cyclic peptides bearing a β-lactam moiety (as already shown in Figure 2.1.5) that bind to $\alpha_v\beta_3$ integrin: the cyclic tetrapeptide with a deleted glycine residue behaves as an agonist and displays opposite angiogenic gene-regulation activity of the peptidomimetic RGD parent.[51]

Agonists have also been described for the β_2 family of integrins and for $\alpha_M\beta_2$.[52] These latter compounds are thought to stabilize the high affinity conformation of $\alpha_M\beta_2$ integrin and may function as anti-inflammatory drugs through a novel mechanism of action (perturbation of integrin de-adhesion).

Recently, Vanderslice et al. have reported that a small molecule agonist of $\alpha_4\beta_1$ integrin induces progenitor cell adhesion and may be an adjunct to cell-based therapy.

Until now, small molecules acting as integrin antagonists have been developed and proposed as novel drugs. However, it would be advisable to develop novel small molecules that behave as integrin agonists and increase rather than decrease integrin dependent cell adhesion. Further studies will better address the mechanism of agonism of these ligands and how they may influence cell signaling.

2.2 Other integrin ligands

2.2.1 Developing β-lactam $\alpha_v\beta_3$ and $\alpha_5\beta_1$-type integrin antagonists

The activating effect shown by compounds **1**, **2** and **3** is particularly of interest when compared with other mimetics of the RGD sequence which carry the same *p*-aminobenzylamine moiety. There are abundant examples that showing how this portion, as mimetic for the basic group of arginine, determines an inhibitory activity against the same $\alpha_v\beta_3$ and $\alpha_5\beta_1$ type integrins.From a first Structure-Activity Relationship analysis, this inconsistency seemed to depend only from the connectivity of the aminobenzylamine portion of the molecule.

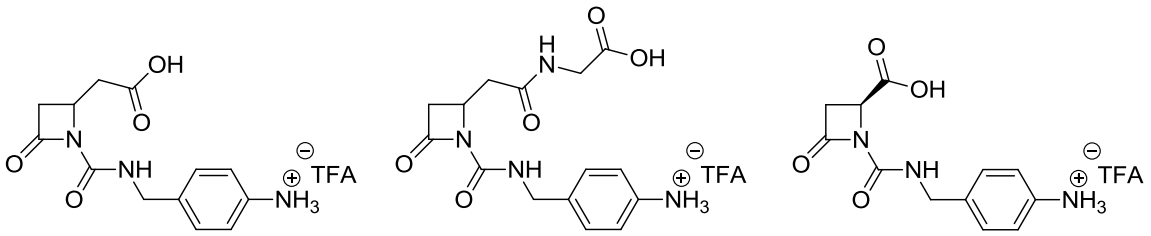


Figure 2.2.1 β-Lactams synthesized and tested as TFA salts **19**, **20** and **21**

Notwithstanding the need for a separation between the two functions of 9-14 carbon atoms, we therefore designed three new compounds to be tested as inhibitors for the same $\alpha_v\beta_3$ and $\alpha_5\beta_1$ type integrins. Therefore, these structures are very similar to those of compounds **1**, **2** and **3**, although only **1** and **2** have proven to be interestingly active as agonists in the assays conducted by us. The only difference was the aminobenzylamine connectivity, as shown in Figure 2.2.1.

Regarding the retrosynthetic analysis of these three new β-lactam based molecules, we designed a synthesis based on the previously shown strategy in Figure 2.1.7. The chain on the C-4 of β-lactam ring can follow the same synthetic sequence, with or without the elongation steps previously shown, as well as also the Reformatsky reaction can be maintained.

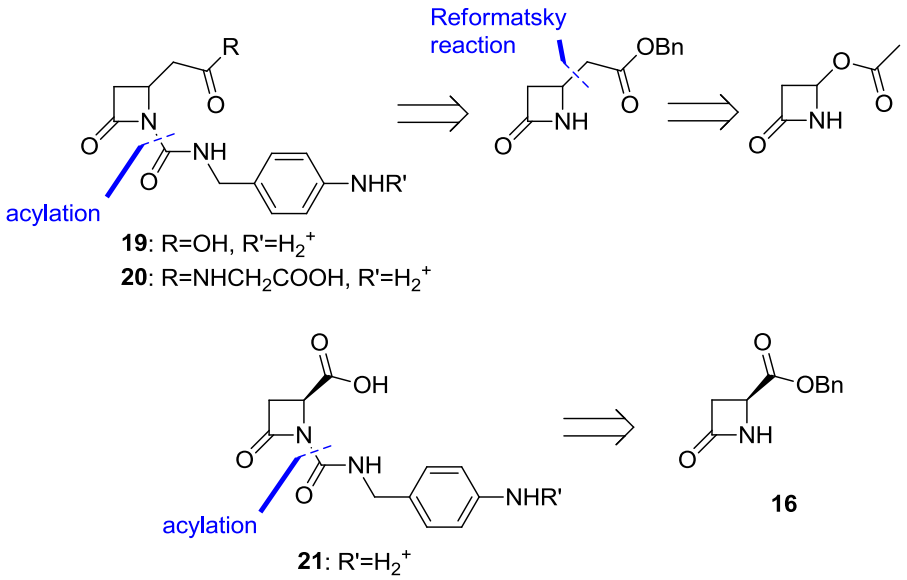
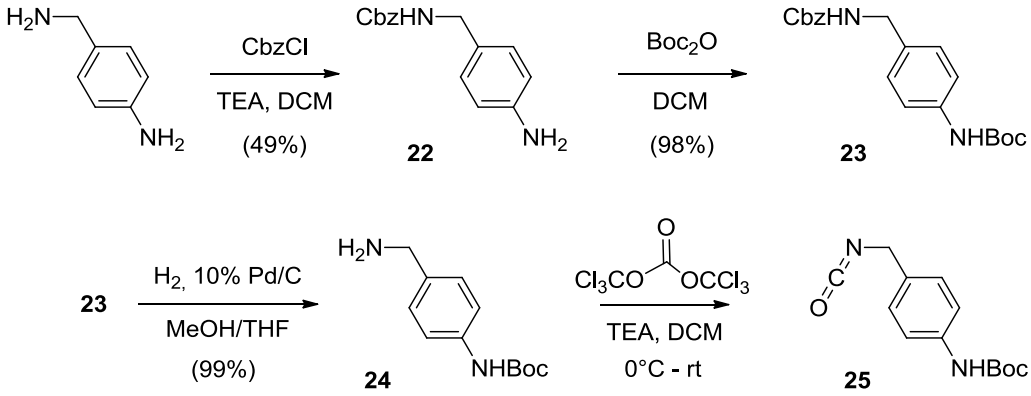


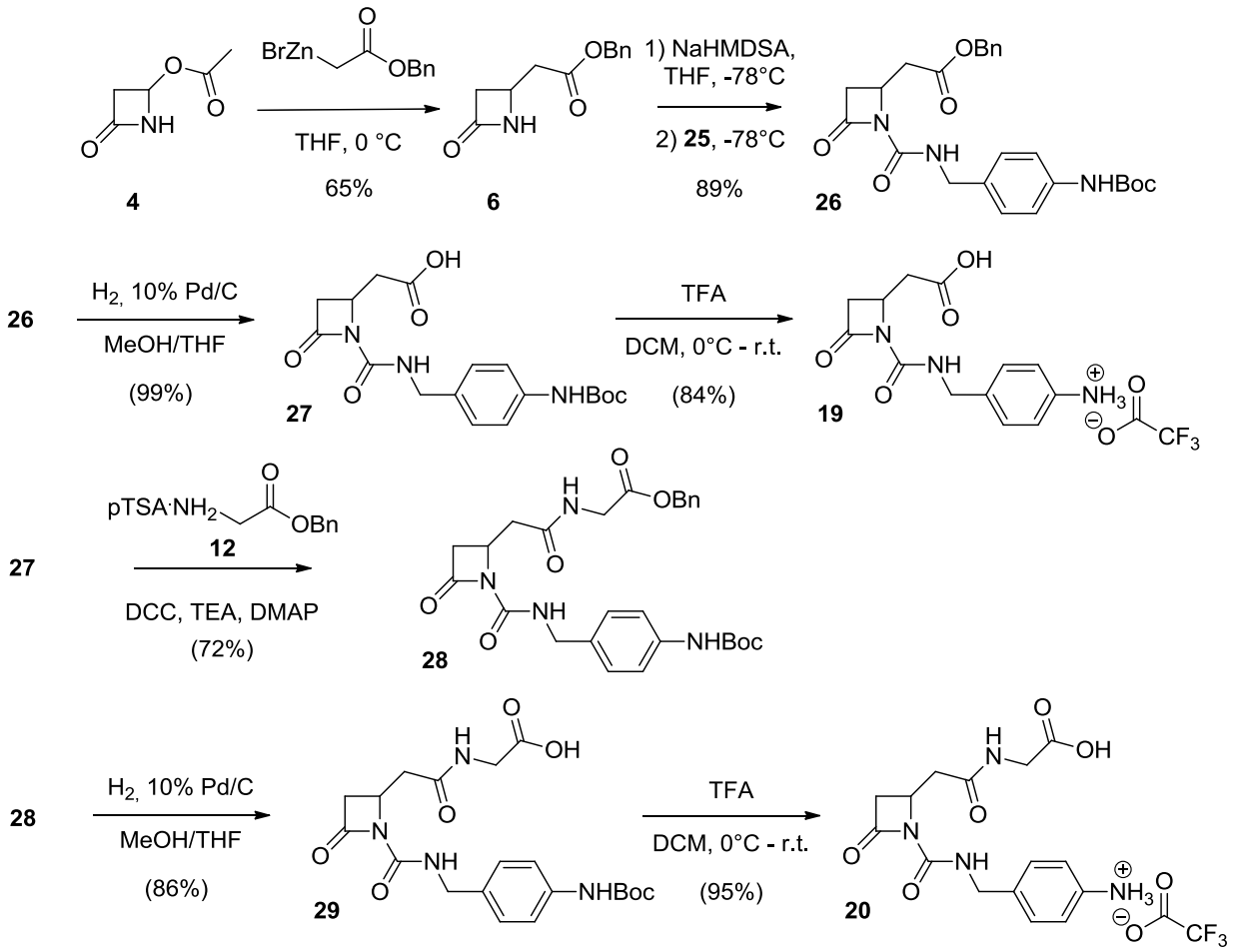
Figure 2.2.2 Synthetic strategies for compounds **19**, **20** and **21**

The amine used for the formation of its corresponding isocyanate and the subsequent β-lactam acylation can as well be prepared from the 4-aminobenzylamine, but it must be instead protected on the aniline group (Scheme 2.2.1).



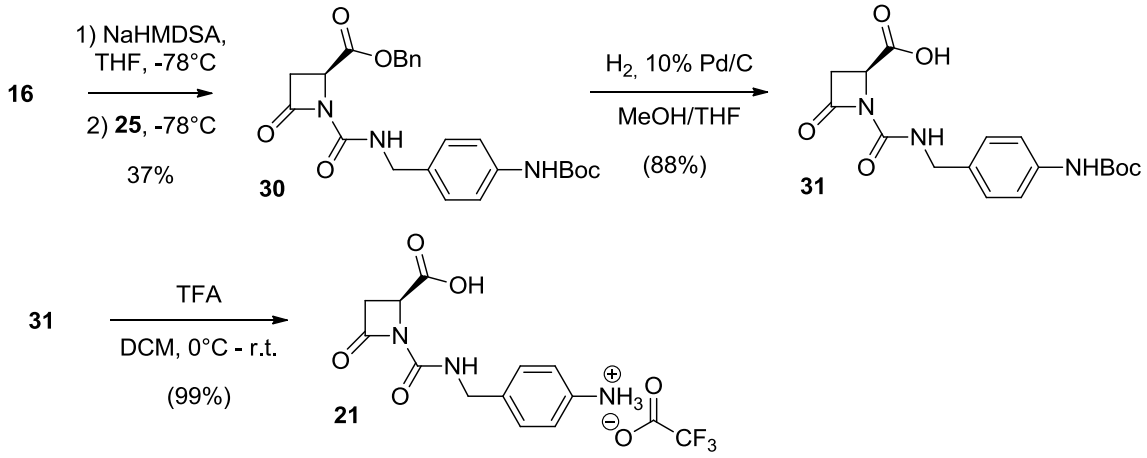
Scheme 2.2.1 Synthesis of 4-(Boc-amino)-benzylamine **24** and of its isocyanate **25**

The protecting groups strategy was kept unchanged as well. Moreover, we chose the more efficient vstrategy with two orthogonal protecting groups for the acid and the amine, to be removed at the end in two steps, an hydrogenolysis and a subsequent trifluoroacetic acid treatment.It allowed to obtain the two targets as their trifluoroacetic acid salts (Scheme 2.2.2).



Scheme 2.2.2 Synthesis of compounds **19** and **20**

Compound **21** was prepared starting from the already shown intermediate **16**. It requires an acylation of the β-lactam nitrogen and then the two steps deprotection (Scheme 2.2.3).



Scheme 2.2.3 Synthesis of compound **21**

β-Lactams **19** and **21** showed interestingly and opposite results in fibronectin-mediated adhesion on K562 cells (in Figure 2.2.3, the concentration response curves showing effects of **19** and **21** on cell adhesion;

cells were incubated for 30min at room temperature with each compound or with the vehicle, as described in the experimental section).

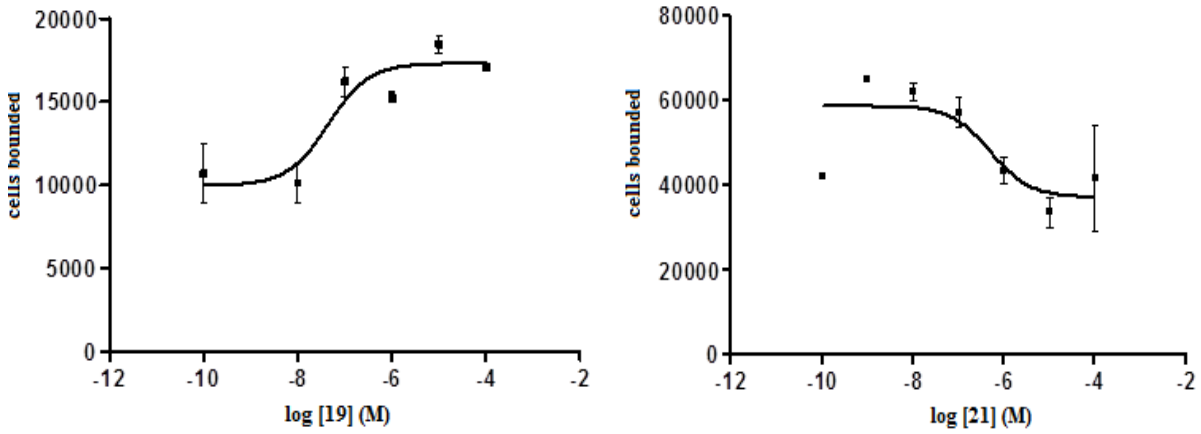


Figure 2.2.3 19 and **21** influence on fibronectin-mediated adhesion

With regards to $\alpha_5\beta_1$ integrin expressing cells, **19** was quite potent in enhancing cell adhesion with an IC_{50} of 44.5nM. Indeed, β-lactam **21** was an antagonist toward the same $\alpha_5\beta_1$ integrins (IC_{50} = 525nM). These cell models were validated demonstrating that the $\alpha_5\beta_1$ agonist fibronectin produces a concentration-dependent variation of cell adhesion in K562 cells (data not shown).

2.2.2 $\alpha_4\beta_1$-type integrin inhibitors

As mentioned before, $\alpha_4\beta_1$-type integrin are involved in many diseases such as embryogenesis and pathogenesis. Antagonism of $\alpha_4\beta_1$ represents a well validated target for the treatment of inflammatory disorders, and the *o*-tolyl-ureido moiety seems to be fundamental for interactions between ligands and these integrins. Comparing to the already developed compounds, we designed new β-lactam molecules that must present the same acid function, and an *o*-tolyl-moiety in place of the amine group.

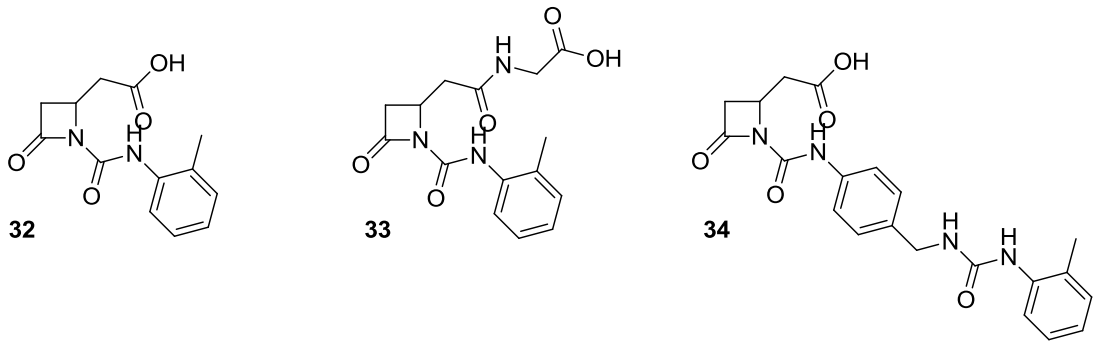


Figure 2.2.4 β-Lactams synthesized and tested **32**, **33** and **34**

The distance between these two functionality must be fixed, as before considered, from nine to fourteen atoms. Thus, we started a new study with three β-lactam molecules specifically designed for interacting with $\alpha_4\beta_1$-type integrins, in order to initiate a preliminary SAR evaluation (Figure 2.2.4).

For the synthesis, we thought to work on the chain in position 4 of the ring by introducing it with the same Reformatsky reaction previously optimized, elongating it where necessary by peptide coupling between the deprotected acid group and the O-benzyl protected glycine (Figure 2.2.5).

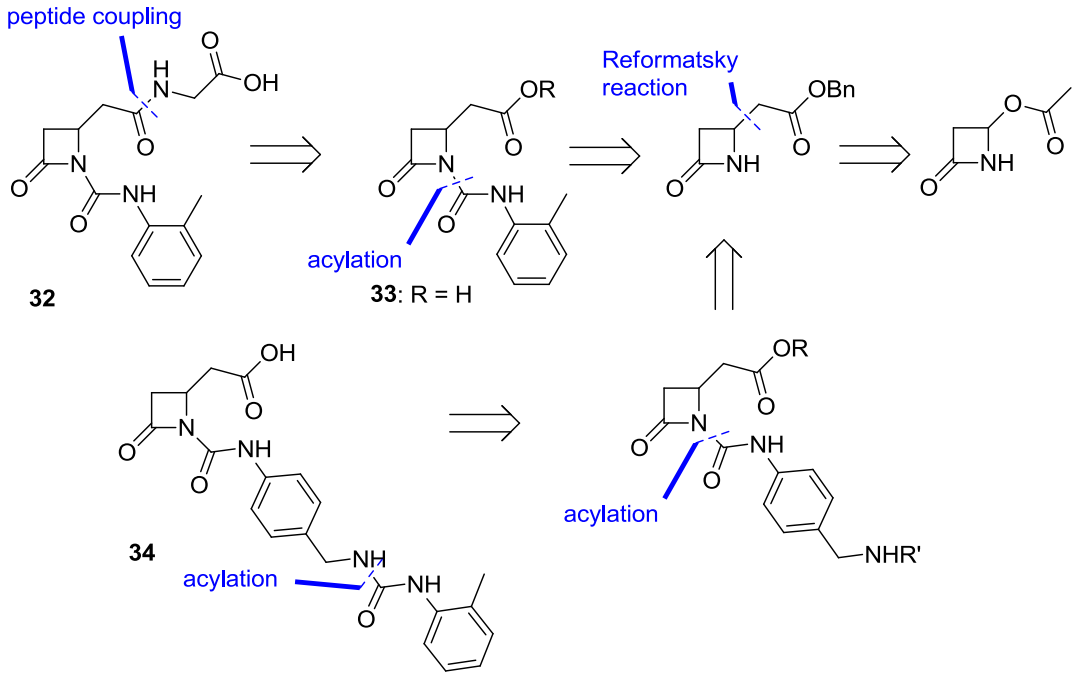
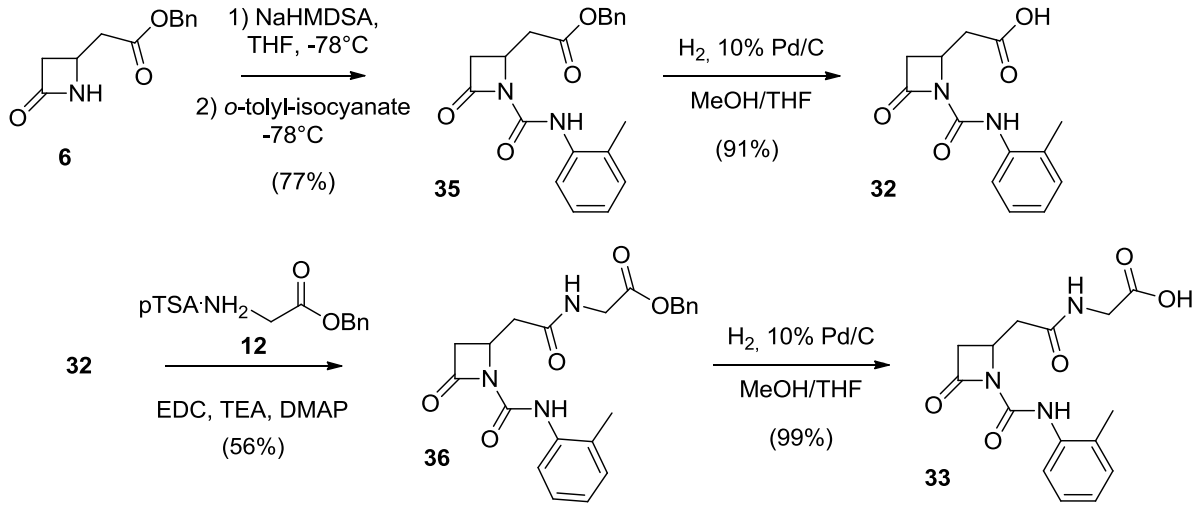


Figure 2.2.5 Synthetic strategies for compounds **29**, **30** and **31**

Preparations of compounds **32** and **33** differ only in the length of the C-4 chain. Therefore, reaching **33** from **32** requires two additional synthetic steps (Scheme 2.2.4).

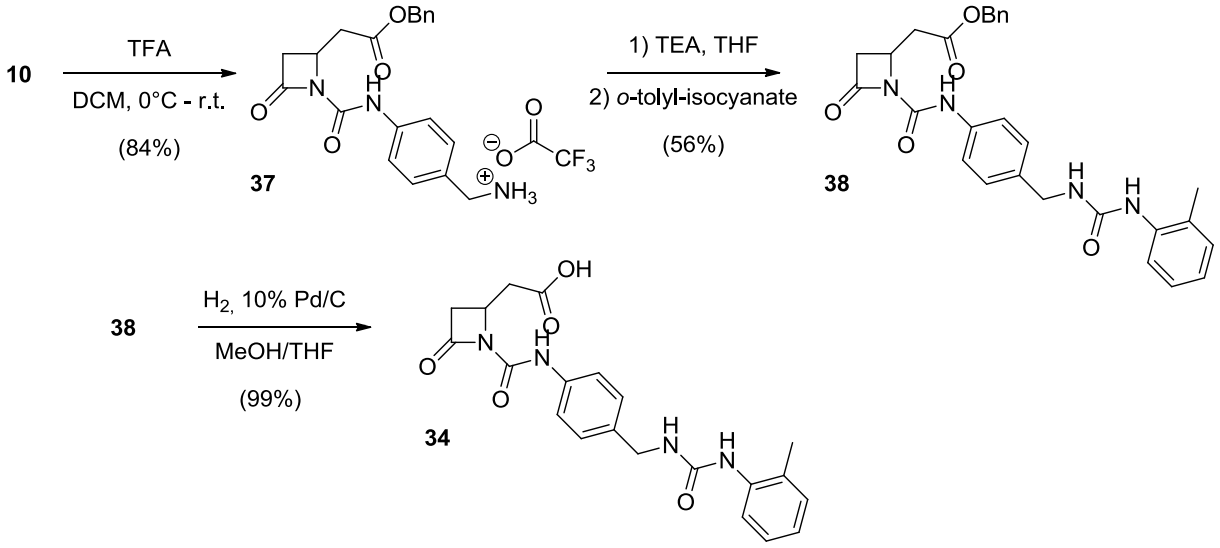
As regards the portion in 1, even in this case we conducted the acylation reactions of the nitrogen with appropriate isocyanates. Indeed, a good comfort about that was provided by the commercial availability of the *o*-tolylisocyanate. Furthermore, to lengthen this portion of the molecule, we evaluated two different synthetic pathways, both however converging on the benzyl ester **6** obtained by Reformatsky reaction (Figure 2.2.5).

To lengthen the chain on the β-lactam nitrogen atom we exploited the protecting groups orthogonality on compound **10**, already shown, selectively removing the Boc moiety by treatment with trifluoroacetic acid. The corresponding free amine in the corresponding TFA salt form was so obtained. After a desalting treatment with triethylamine, conducted under nitrogen athmosphere, we acylated the N-chain with the same *o*-tolylisocyanate. The reaction was carried out under basic conditions and without any metalation.



Scheme 2.2.4 Synthesis of compounds **32** and **33**

Target compound **34** was finally obtained liberating the acid functionality by removal of the benzyl protecting group, via Pd-catalyzed hydrogenolysis (Scheme 2.2.5).



Scheme 2.2.5 Synthesis of compound **34**

Preliminary biological results, obtained in collaboration with prof. S. Spampinato of the University of Bologna, showed for the last derivative an activity roughly nothing, or at least with a scarce relevance. Compounds **32** and **33** appeared to be much more encouraging. The first one has mainly shown an agonist activity against $\alpha_4\beta_1$ type integrin, whereas the latter appeared to have an antagonist behavior.

Although these data derived from essays that still being completed and fully investigated, we decided to continue the study exacerbating the structural characteristics of these two compounds. We shortened the portion bringing the acid in one case, designing compound **39**, and we lengthened the same chain while designing compound **40** (Figure 2.2.6).

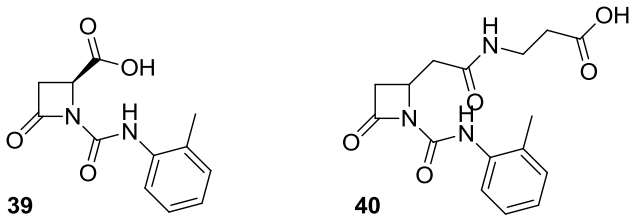
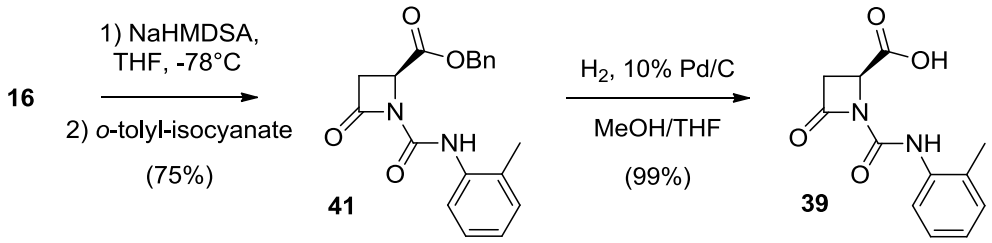


Figure 2.2.6 β-Lactams synthesized and tested as TFA salts **39** and **40**

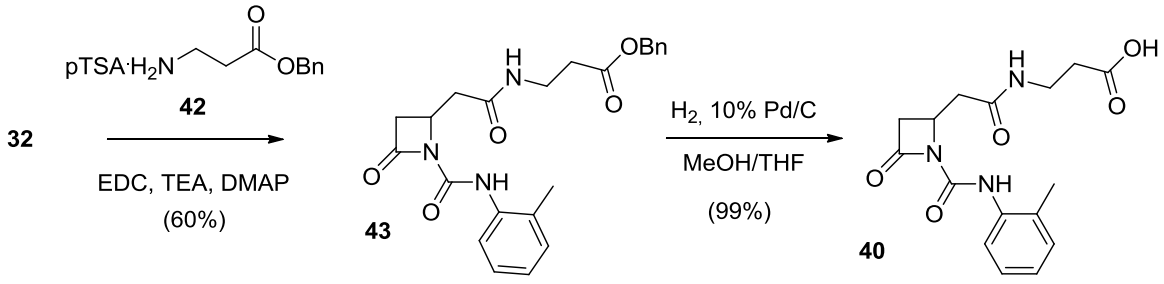
These modifications took account of past experiencse in the synthesis of related compounds and were by a formal removal, in the case of compound **39**, and an introduction, for **37**, of only one methylene residue. Such a small modification should theoretically be able to provide sufficient indications on the conjectured SAR considerations.

For the synthesis of compound **39** we followed the acylation pathway. It was conducted using the commercially available *o*-tolylisocyanate on the benzyl ester **16**, then deprotecting the acid functionality by hydrogenolysis (Scheme 2.2.6).



Scheme 2.2.6 Synthesis of compound **39**

For the preparation of compound **40** we followed the synthesis of intermediate **36**, but for the peptide coupling was employed the corresponding protected amino acid with one more methylene, i.e. the benzyl ester of β-alanine. It was obtained by treating, in the presence of *p*-toluenesulfonic acid, β-alanine with benzyl alcohol (Scheme 2.2.7). At the end, the acid functionality was at the end obtained by hydrogenolysis, reaching the desired target **40**.



Scheme 2.2.7 Synthesis of compound **40**

Preliminary results showed what we expected. Shortening the chain on C-3 of the β-lactam ring, antagonist activity resulting accentuated, as shown by the data for compound **39**, while lengthening it, as in **40**, a sharp increase in adhesion and thus in agonism was observed (in Figure 2.2.7, the concentration

response curves showing the effects of **39** and **40** on cell adhesion; cells were incubated for 30min at room temperature with each compound or with the vehicle as described in the experimental section).

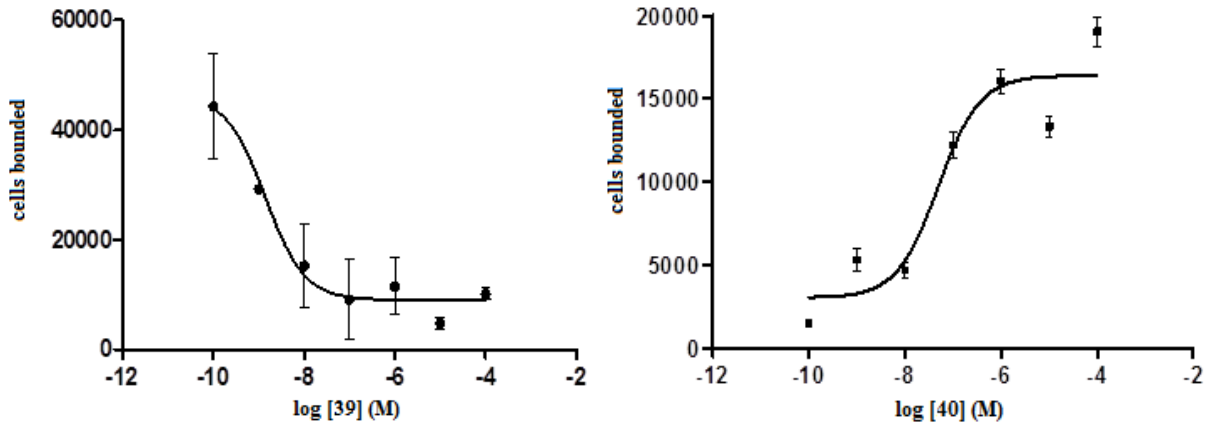


Figure 2.2.7 39 and **40** influence on fibronectin-mediated adhesion

With regards to $\alpha_4\beta_1$ integrin expressing cells, **39** was very potent in reducing adhesion with an IC_{50} of 1.39nM. Indeed, **40** was an agonist toward the same $\alpha_4\beta_1$ integrins (IC_{50} = 49.0nM). These cell models were validated demonstrating a concentration-dependent variation of cell adhesion in K562 cells (data not shown).

2.3 Experimental section

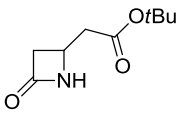
2.3.1 General informations

Commercial reagents were used as received without additional purification. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with an INOVA 400 or a GEMINI 200 instrument with a 5mm probe. All chemical shifts are quoted relative to deuterated solvent signals (δ in ppm and J in Hz). Polarimetric Analyses were conducted on Unipol L 1000 "Shcmidt&Haensch" Polarimeter at 598nm. FTIR spectra: Thermo Nicolet 380 instrument, measured as films between NaCl plates; wave numbers are reported in cm^{-1}. TLC: Merck 60 F254plates. Column chromatography: Merck silica gel 200-300mesh. HPLC-MS: Agilent Technologies HP1100 instrument, equipped with a ZOBRAX-Eclipse XDB-C8 Agilent Technologies column, mobile phase: H_2O/CH_3CN, 0.4 mL/min, gradient from 30 to 80% of CH_3CN in 8min, 80% of CH_3CN until 25min, coupled with an Agilent Technologies MSD1100 single-quadrupole mass spectrometer: full scan mode from m/z = 50 to 2600, scan time 0.1s in positive ion mode, ESI spray voltage 4500V, nitrogen gas 35psi, drying gas flow 11.5mL/min, fragmentor voltage 20V. Elemental analysis were performed on a Thermo Flash 2000 CHNS/O Analyzer. A Shimadzu UV-1601 PC spectrophotometer was used for spectrophotometric measurements.

2.3.2 Synthesis

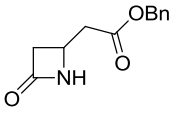
Azetidinone **4** is commercial, compounds **7**[53], **12**[54], and **15**[55] were synthesized following already reported procedures, while for **42** was followed the same procedure indicated for **12**.

_t_Butyl 2-(4-oxo-azetidin-2-yl) acetate (**5**)



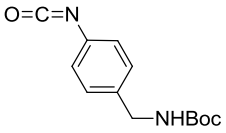
In a 50mL 3-neck flask under nitrogen, Zn powder (2.0g, 31mmol) and THF (10mL) were introduced followed by TMSCl (200mL, 1.55mmol). After 30 min of stirring the temperature was raised to 30÷35°C and a solution of tbutylbromoacetate (1.43mL, 15.5mmol) in THF (20mL) was slowly added in 30min. After 30min of stirring the mixture was cooled to rt and decanted, providing a limpid grey supernatant that was slowly added dropwise to a 100mL flask under nitrogen containing a solution of **4** (500mg, 3.88mmol) in anhydrous THF (22mL) at 0°C. The mixture was stirred at rt for 3h, quenched with ice and a saturated Seignette salt solution and extracted with EtOAc. The organic layers were dried on Na_2SO_4, filtered and concentrated in vacuum. Flash-chromatography (cyclohexane/EtOAc, 6/4) gave 5 (545mg) as a white solid in 76% yield.
M.p. 83-84°C; Rf 0.34 (cyclohexane/EtOAc, 1/4)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.49 (s, 9H, *t*Bu), 2.50 (dd, J = 8.8, 16.0Hz, 1H, CH*H*CO_2*t*Bu), 2.67 (dd, J = 4.8, 16.0Hz, 1H, C*H*HCO_2*t*Bu), 2.69 (ddd, J = 1.2, 2.4, 14.8Hz, 1H, CH*H*CHCH_2CO_2*t*Bu), 3.19 (ddd, J = 2.4, 4.8, 14.8Hz, 1H, C*H*HCHCH_2CO_2*t*Bu), 3.95 (dddd, J = 2.4, 4.8, 4.8, 8.8Hz, 1H, CHHC*H*CH_2CO_2*t*Bu), 6.40 (bs, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 28.0, 41.0, 43.3, 43.9, 81.4, 167.2, 170.1 ppm
IR: $\tilde{\nu}$ = 3238, 2964, 1763, 1735, 1398, 1261 cm^{-1}
GC-MS: Rt = 13.5min, m/z (%) = 170 (7), 129 (13), 112 (13), 101 (10), 86 (12), 70 (36), 57 (100)

Benzyl 2-(4-oxoazetidin-2-yl) acetate (**6**)



In a 50mL 3-neck flask under nitrogen, Zn powder (2.0g, 31mmol) and THF (10mL) were introduced followed by TMSCl (200mL, 1.55mmol). After 30min of stirring the temperature was raised to 30÷35°C and a solution of benzylbromoacetate (2.46mL, 15.52mmol) in THF (19.4mL) was slowly added in 30min. After 30min of stirring the mixture was cooled to rt and decanted, providing a limpid grey supernatant that was slowly added dropwise to a 100mL flask under nitrogen containing a solution of **4** (500mg, 3.88mmol) in anhydrous THF (22mL) at 0°C. The mixture was stirred at rt for 3h, quenched with ice and a saturated Seignette salt (potassium sodium tartrate) solution and extracted with EtOAc. The organic layers were dried on Na_2SO_4, filtered and concentrated in vacuum. FC (cyclohexane/EtOAc, 1/1) gave **6** (552mg) as a white solid in 65% yield.
M.p. 92-95°C; Rf 0.42 (cyclohexane/EtOAc, 1/4)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.64 (dd, J = 9.2, 16.8Hz, 1H, CH*H*CO_2Bn), 2.66 (ddd, J = 1.2, 2.4, 15.2Hz, 1H, CH*H*CHCH_2CO_2Bn), 2.78 (dd, J = 4.8, 16.8Hz, 1H, C*H*HCO_2Bn), 3.15 (ddd, J = 2.4, 4.8, 15.2Hz, 1H, C*H*HCHCH_2CO_2Bn), 3.96 (dddd, J = 2.4, 4.8, 4.8, 9.2Hz, 1H, CHHC*H*CH_2CO_2Bn), 5.15 (s, 2H, CH_2Ph), 6.22 (bs, 1H, NH), 7.34-7.39 (m, 5H, Ph) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 39.8, 43.4, 43.8, 66.8, 128.3, 128.5, 128.7, 135.3, 166.9, 170.7 ppm
IR: $\tilde{\nu}$ = 3238, 2961, 1738, 1460, 1372, 1262 cm^{-1}
HPLC-MS: Rt = 4.82min, m/z = 220 $[M+H]^+$, 237 $[M+H_2O]^+$, 439 $[2M+H]^+$

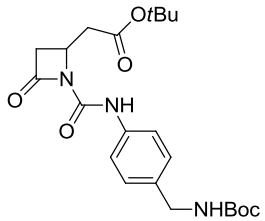
_t_Butyl 4-isocyanatobenzylcarbamate (**8**)



In a 50mL 2-neck flask with a reflux condenser under nitrogen atmosphere, a solution of (**7**) (149mg, 0.67mmol) and TEA (188mL, 1.34mmol) in anhydrous DCM (7.0mL) was cooled to 0°C and after was added with bis(trichloromethyl)-

carbonate (398mg, 1.34mmol) in one portion. The mixture was warmed to rt and then refluxed for 3-4h. The reaction was monitored through the increase of isocyanate IR signal (found at 2274cm^{-1}). The solvent was evaporated under vacuum avoiding any air exposure. The crude was extracted five times with anhydrous Et_2O under nitrogen, concentrated in vacuum, stored under nitrogen atmosphere and immediately used.

_t_Butyl 2-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl acetate (**9**)



In a 25mL 2-neck flask under nitrogen at -78°C, a solution of NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (500µL, 0.5mmol) was added dropwise to a solution of **5** (83mg, 0.45mmol) in anhydrous THF (4mL). After 15min of stirring, a solution of isocyanate **8** (166mg, 0.67mmol) in THF (2mL) was added dropwise. After completion (TLC monitoring, 30min) the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and then DCM. The combined organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (DCM/Et_2O, 95/5) affording **9** (162mg) as a white solid in 83% yield.

Rf 0.67 (cyclohexane/EtOAc, 2/3)

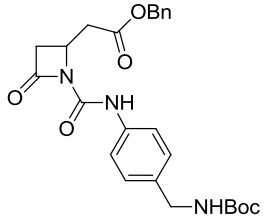
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.45 (s, 9H, *t*Bu), 1.46 (s, 9H, *t*Bu), 2.68 (dd, J = 8.8, 16.0Hz, 1H, CHCH*H*CO_2*t*Bu), 2.96 (dd, J = 2.8, 16.0Hz, 1H, CHC*H*HCO_2tBu), 3.24 (dd, J = 4.0, 16.4Hz, 1H, CH*H*CHCHHCO_2*t*Bu), 3.35 (dd, J = 5.6, 16.4Hz, 1H, C*H*HCHCHHCO_2*t*Bu), 4.28 (d, J = 5.6 Hz, 2H, C*H*$_2$NHCO_2tBu), 4.39 (m, 1H, CHHC*H*CHHCO_2*t*Bu), 4.80 (bs, 1H, NH), 7.25 (d, J = 8.8Hz, 2H, arom), 7.43 (d, J = 8.8Hz, 2H, arom), 8.47 (bs, 1H, NH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 28.0, 28.3, 37.9, 42.6, 44.2, 47.7, 81.7 (2 C), 119.8, 128.2, 134.9, 136.1, 140.3, 147.7, 166.9, 169.0 ppm

IR: ṽ = 3337, 2976, 2926, 2852, 1769, 1712, 1603, 1543, 1367, 1162 cm^{-1}

HPLC-MS: Rt = 9.71min, m/z = 456 $[M+Na]^+$, 472 $[M+K]^+$

Benzyl 2-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl acetate (**10**)



In a 2 mL 2-neck flask under nitrogen at -78°C, a solution of NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (500µL, 0.5mmol) was added dropwise to a solution of **6** (100mg, 0.45mmol) in anhydrous THF (4mL). After 15 min of stirring at room temperature, a solution of **8** (166mg, 0.67mmol) in THF (2mL) was added dropwise. After completion (TLC monitoring, 30min) the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and then DCM. The combined organic extracts were dried over Na_2SO_4, filtered, concentrated in vacuum and purified by flash-chromatography (FC, with DCM/Et_2O 95/5) affording **10** (187mg) in 89% yield as a colorless oil.

Rf 0.73 (cyclohexane/EtOAc, 2/3)

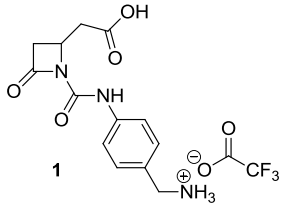
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.47 (s, 9H, tBu), 2.80 (dd, J = 8.8, 16.4 Hz, 1H, CHCH*H*CO_2Bn), 2.94 (dd, J = 3.2, 16.4 Hz, 1H, CHC*H*HCO_2Bn), 3.34 (dd, J = 2.8, 16.4Hz, 1H, CH*H*CHCH_2CO_2Bn), 3.35 (dd, J = 4.0, 16.4Hz, 1H, C*H*HCHCH_2CO_2Bn), 4.27 (d, J = 5.2Hz, 2H, C*H*$_2$NHCO_2*t*Bu), 4.44 (dddd, J = 2.8, 3.2, 4.0, 8.8Hz, 1H, CH_2C*H*CH_2CO_2Bn), 4.88 (bs, 1H, NHCO_2*t*Bu), 5.14 (d, J_{AB} = 12.4Hz, 1H, PhCH*H*), 5.18 (d, J_{AB} = 12.4Hz, 1H, PhC*H*H), 7.23e7.43 (m, 9H, arom), 8.42 (s, 1H, NCONH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 28.3, 36.8, 42.5, 44.0, 47.4, 66.7, 79.3, 119.7, 128.1, 128.2, 128.3, 128.5, 134.9, 135.2, 135.9, 147.6, 155.8, 166.5, 169.6 ppm

IR: ṽ = 3337, 2976, 1769, 1732, 1709, 1603, 1543, 1244, 1168 cm^{-1}

HPLC-MS: Rt = 9.84 min, m/z = 412 $[M-t\text{Bu}+2H]^+$, 485 $[M+H_2O]^+$

4-(2-Carboxymethyl-4-oxoazetidine-carboxamido) phenylmethanaminium trifluoroacetate (1)



In a 10mL 2-neck flask under nitrogen at 0°C, TFA (34µL, 457µmol) was added dropwise to a solution of **9** (33mg, 76µmol) in anhydrous DCM (1.4mL). The mixture was stirred at rt for 21h, cooled at 0°C and then treated with another aliquot of TFA (68µL, 910µmol). At starting material disappearing, the mixture was evaporated under vacuum yielding **1** (29mg) in 97% yield as a light yellow waxy oil.

^{1}H NMR (400MHz, CD_3OD): δ = 2.85 (dd, J = 8.8, 16.4Hz, 1H, CHCH*H*CO_2H), 3.05 (dd, J = 3.2, 16.4Hz, 1H, CHC*H*HCO_2H), 3.22 (dd, J = 3.2, 16.4Hz, 1H, CH*H*CHCHHCO_2H), 3.40 (dd, J = 5.6, 16.4Hz, 1H, C*H*HCHCHHCO_2H), 4.12 (s, 2H, CH_2N), 4.45 (m, 1H, CHHC*H*CHHCO_2H), 7.45 (d, J = 8.8Hz, 2H, arom), 7.63 (d, J = 8.8Hz, 2H, arom) ppm

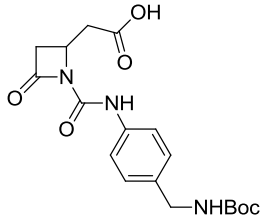
^{13}C NMR (50MHz, D_2O): δ = 35.9, 41.4, 42.2, 47.4, 115.9 (q, J_{1CF} = 290Hz), 121.7, 129.0, 129.4, 136.3, 149.1, 162.5 (q, J_{2CF} = 35Hz), 168.4, 174.0 ppm

IR: $\tilde{\nu}$ = 3334, 2958, 2921, 1769, 1677, 1610, 1544, 1422, 1335, 1203 cm^{-1}

HPLC-MS: Rt = 1.10min, m/z = 261 [M-TFA-NH_3+H]$^+$, 296 [M-TFA+H_2O+H]$^+$, 555 [2M-2TFA+H]$^+$, 577 [2M-2TFA+Na]$^+$

Found C, 46.32; H, 4.21; N, 10.43%; $C_{15}H_{16}F_3N_3O_6$ requires C, 46.04; H, 4.12; N, 10.74%

2-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl acetic acid (11)



In a 25mL 2-neck flask a mixture of **10** (100mg, 0.214mmol), anhydrous THF (2.5mL), MeOH (2.5mL) and Pd/C 10%$_{w/w}$ (10mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **11** was obtained as a light yellow solid (76mg) in 94% yield after titration with DCM.

M.p. 162-164°C, dec.; Rf 0.69 (DCM/MeOH/NH_4OH, 30/10/1)

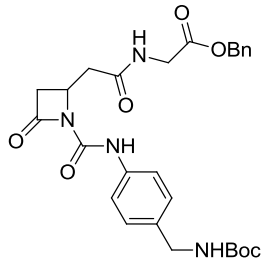
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.47 (s, 9H, *t*Bu), 2.81 (dd, J = 8.4, 16.8Hz, 1H, CHCH*H*CO_2H), 2.98 (dd, J = 3.2, 16.8Hz, 1H, CHC*H*HCO_2H), 3.33e3.42 (m, 2H, CH_2CHCH_2CO_2H), 4.28 (m, 2H, CH_2N), 4.46 (m, 1H, CH_2C*H*CH_2CO_2H), 4.83 (bs, 1H, NHCO_2*t*Bu), 7.25 (d, J = 8.4Hz, 2H, arom), 7.43 (d, J = 8.8Hz, 2H, arom), 8.46 (s, 1H, NH) ppm

^{13}C NMR (50MHz, CD_3OD): δ = 29.6, 38.6, 44.2, 45.4, 46.9, 81.0, 122.0, 129.7, 137.8, 138.3, 150.7, 159.4, 169.5, 179.7 ppm

IR: $\tilde{\nu}$ = 3338, 2977, 2929, 1767, 1699, 1650, 1539, 1333, 1246, 1168 cm^{-1}

HPLC-MS: Rt = 4.77min, m/z = 395 [M+H_2O]$^+$, 400 [M+Na]$^+$

Benzyl 2-(2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl)acetamido) acetate (13)



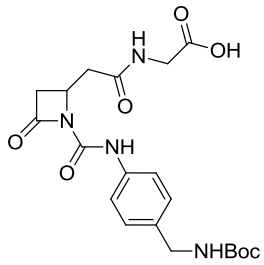
In a 25mL 2-neck flask, dicyclohexylcarbodiimide (DCC) (45.4mg, 0.22mmol) was added to a solution of **11** (51mg, 0.135mmol) in anhydrous DCM (2.5mL) and CH_3CN (0.5mL) at 0°C. Then a previously prepared solution of **12** (106.6 mg, 0.3mmol) and stoichiometric TEA in DCM (2mL) was immediately added dropwise, followed by DMAP (4.9mg, 0.04mmol). The solution was warmed to rt and after complete consumption of the starting material (16h) the mixture was quenched with H_2O (10mL) and extract with DCM (3x10mL). The organic layers were dried on Na_2SO_4 and filtered. The crude was suspended in EtOAc, the residual urea by-product of DCC remained solid and was eliminated by filtration, the organic layer was concentrated in vacuum and purified by FC (DCM/CH_3CN, 90/10) to afford **13** (45mg) in 63% yield.

Light yellow waxy solid. Rf 0.75 (EtOAc), 0.79 (DCM/CH_3CN, 1/1)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.46 (s, 9H, *t*Bu), 2.74 (dd, J = 8.4, 15.2Hz, 1H, CHCH*H*CONH), 3.16 (m, 2H, CHC*H*HCONH, CH*H*CHCHHCONH), 3.32 (dd, J = 5.6, 16.4Hz, 1H, C*H*HCHCHHCONH), 4.02 (dd, J = 5.2, 18.0Hz, 1H, NCH*H*CO_2), 4.10 (dd, J = 5.6, 18.4Hz, 1H, NC*H*HCO_2), 4.27 (d, J =

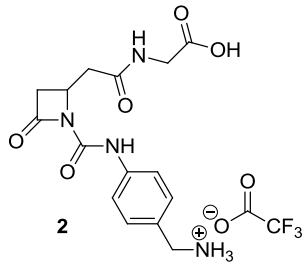
5.6Hz, 2H, tBuOCOCH_2N), 4.42 (m, 1H, CHHC*H*CHHCONH), 4.85 (bs, 1H, NHCO$_2$*t*Bu), 5.16 (s, 2H, CH_2Ph), 6.50 (t, J = 5.2 Hz, CONH), 7.22-7.42 (m, 9H, arom), 8.51 (s, 1H, NCONH) ppm
^{13}C NMR (50MHz, CD_3OD): δ = 28.3, 38.1, 41.2, 42.6, 44.1, 48.3, 67.1, 79.5, 120.0, 128.1, 128.2, 128.3, 128.5, 128.6, 135.0, 135.9, 148.2, 155.9, 167.0, 169.2, 169.4 ppm
IR: ṽ = 3326, 2978, 2921, 1761, 1741, 1708, 1687, 1663, 1601, 1548, 1413, 1319, 1246, 1164, 1050 cm^{-1}
HPLC-MS: Rt = 10.51min, m/z = 525 $[M+H]^+$, 542 $[M+H_2O]^+$, 547 $[M+Na]^+$

2-(2-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl acetamido) acetic acid (**14**)



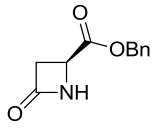
In a 10mL 2-neck flask, Pd on C (10%$_{w/w}$) (3mg) was added to a solution of **13** (31mg, 59mmol) in anhydrous THF (350mL) and MeOH (350mL). The mixture was stirred under H_2 atmosphere at rt (1atm) and after complete SM consumption (2h) it was filtered and concentrated. The crude was then titrated with chloroform to afford **14** after evaporation as a white solid (25mg) in 98% yield.
Rf 0.12 (CH_3CN/MeOH, 4/1)
^{1}H NMR (400MHz, CD_3OD): δ = 1.48 (s, 9H, tBu), 2.75 (dd, J = 8.4, 14.8 Hz; 1H, CHCH*H*CONH), 3.10 (dd, J = 3.2, 16.0Hz, 1H, CH*H*CHCHHCONH), 3.17 (dd, J = 4.4, 14.8Hz, 1H, CHC*H*HCONH), 3.34 (dd, J = 5.6, 16.0Hz, 1H, C*H*HCHCHHCONH), 3.91 (d, J = 17.6Hz, 1H, NCH*H*CO$_2$), 3.97 (d, J = 17.6 Hz, 1H, NC*H*HCO$_2$), 4.21 (s, 2H, CH_2NHCO$_2$*t*Bu), 4.45 (m, 1H, CHHC*H*CHHCONH), 7.26 (d, J = 8.4Hz, 2H, arom), 7.46 (d, J = 8.4 Hz, 2H, arom), 8.39 (bs, 1H, NH), 8.77 (s, 1H, NH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 29.6, 39.7, 42.6, 43.9, 45.4, 50.5, 81.0, 122.0, 129.7, 137.8, 138.3, 150.6, 159.4, 169.4, 173.2,173.7 ppm
IR: ṽ = 3314, 2974, 2925, 1757, 1712, 1683, 1638, 1609, 1556, 1422, 1319, 1172 cm^{-1}
HPLC-MS: Rt = 2.16min, m/z = 457 $[M+Na]^+$, 473 $[M+K]^+$

4-(2-(2-(Carboxymethylamino)-2-oxoethyl)-4-oxoazetidine-1-carboxamido) phenylmethanaminium trifluoroacetate (**2**)



In a 10 mL 2-neck flask under nitrogen at 0°C, TFA (12μL, 165μmol) was added dropwise to a solution of **14** (24mg, 55μmol) in anhydrous DCM (1.4mL). The mixture was stirred at rt for 21h, cooled at 0°C and then treated with another aliquot of TFA (16μL, 220μmol). At starting material disappearing, the mixture was evaporated under vacuum, the crude was triturated with a drop of MeOH and pentane to provide **2** as a white solid (16mg) in 65% yield.
^{1}H NMR (400MHz, CD_3OD): δ = 2.78 (dd, J = 8.4, 14.8Hz, 1H, CHCH*H*CONH), 3.13 (m, 2H, CHC*H*HCONH + CH*H*CHCH2CONH), 3.35 (m, 1H, C*H*HCHCH$_2$CONH), 3.90-3.98 (m, 2H, NHCH_2COOH), 4.11 (s, 2H, $CH_2NH_3^+$), 4.46 (m, 1H, CH2CHCH2CONH), 7.44 (d, J = 8.8Hz, 2H, arom), 7.61 (d, J = 8.8Hz, 2H, arom), 8.90 (s, 1H, NH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 39.6, 42.6, 43.9, 44.7, 50.5, 116.8 (q, J_{1CF} = 286Hz), 122.2, 130.8, 131.7, 140.5, 150.5, 162.3 (q), 169.4, 173.2, 173.7 ppm
IR: ṽ = 3387, 2949, 2921, 2839, 1772, 1707, 1678, 1654, 1621, 1560, 1458, 1417, 1020 cm^{-1}
HPLC-MS: Rt = 1.13min, m/z = 318 $[M\text{-}TFA\text{-}NH_3+H]^+$, 357 $[M\text{-}TFA+Na]^+$, 373 $[M\text{-}TFA+K]^+$, 669 $[2M\text{-}2TFA+H]^+$, 691 $[2M\text{-}2TFA+Na]^+$
Found C, 45.86; H, 4.34; N, 12.38%; $C_{17}H_{19}F_3N_4O_7$ requires C, 45.54; H, 4.27; N, 12.50%

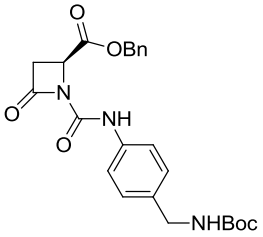
(S)-Benzyl 4-oxoazetidine-2-carboxylate (**16**)



Commercially available, it has also been prepared starting from dibenzylester of L-aspartic acid **15** according to the procedure reported in literature.[56]

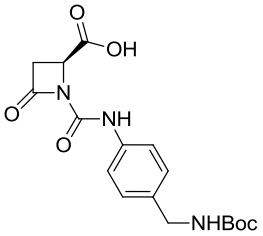
M.p. 137-140°C; Rf 0.6 (cyclohexane/EtOAc, 1/4)
^{1}H NMR (400MHz, $CDCl_3$): δ = 3.05-3.11 (m, 1H, CHCH*H*), 3.33 (dd, J = 6.0, 14.8Hz, 1H, CHC*H*H), 4.22 (dd, J = 2.8, 6.0Hz, 1H, C*H*CHH), 5.21 (s, 2H, CH_2Ph), 6.32 (bs, NH), 7.36e7.38 (m, 5H, Ph) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 43.5, 47.3, 67.4, 128.5, 128.6, 128.7, 134.8, 166.3, 170.8 ppm
HPLC-MS: Rt = 4.29min, m/z = 206 $[M+H]^+$, 223 $[M+H_2O]^+$, 228 $[M+Na]^+$, 433 $[2M+Na]^+$

(S)-Benzyl (4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidine-2-carboxylate (**17**)



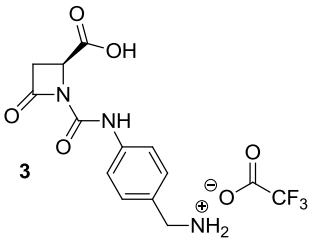
In a 10mL 2-neck flask under inert atmosphere at -78°C, NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (190µL, 0.190mmol) was added dropwise to a solution of **16** (35mg, 0.17mmol) in anhydrous THF (1.5mL) followed after 30 min by a solution of isocyanate **8** (64mg, 0.26mmol) in THF (1mL); after starting material disappearing (30min), the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and DCM. The organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (DCM to DCM/Et_2O 95/5) to afford **17** as a colorless viscous oil (69 mg) in 90% yield.
Rf 0.6 (cyclohexane/EtOAc, 4/1)
$[\alpha]_D$ = -64.1° (c = 0.7, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.47 (s, 9H, *t*Bu), 3.10 (dd, J = 2.8, 16.0Hz, 1H, CH*H*CH), 3.40 (dd, J = 6.0, 16.0Hz, 1H, C*H*HCH), 4.28 (d, J = 5.2Hz, 2H, C*H*$_2$NH), 4.60 (dd, J = 2.8, 6.4Hz, 1H, CHHC*H*), 4.85 (bs, 1H, NHCO$_2$*t*Bu), 5.24 (d, J_{AB} = 12.4Hz, 1H, PhCH*H*), 5.29 (d, J_{AB} = 12.4Hz, 1H, PhC*H*H), 7.25 (d, J = 8.4Hz, 2H, arom), 7.35-7.38 (m, 5H, arom), 7.43 (d, J = 8.4Hz, 2H, arom), 8.28 (s, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 28.3, 41.2, 44.1, 48.9, 67.8, 79.4, 119.9, 128.2, 128.3, 128.6, 128.7, 134.7, 135.1, 135.8, 146.6, 155.8, 165.0, 168.8 ppm
IR: ν̃ = 3424, 3342, 3060, 2974, 2921, 1777, 1744, 1708, 1683, 1601 cm^{-1}
HPLC-MS: Rt = 11.14min, m/z = 471 $[M+H_2O]^+$, 476 $[M+Na]^+$

(S)-1-(4-Boc-aminomethyl-phenylcarbamoyl)-4- oxoazetidine-2-carboxylic acid (**18**)



In a 10mL 2-neck flask, Pd/C (10%$_{w/w}$) (3mg) was added to a solution of **17** (33mg, 73µmol) in anhydrous THF (400mL) and MeOH (400mL). The mixture was stirred under H_2 atmosphere (1atm) at room temperature and after starting material complete consumption (2h), it was filtered and concentrated in vacuum. The crude was then triturated with a few drops of DCM and pentane to afford the desired product as a white lumpy solid (25mg) in 95% yield.
Rf 0.03 (cyclohexane/EtOAc, 1/4)
$[\alpha]_D$ = -51.5° (c = 0.4, CH_3OH)
^{1}H NMR (200MHz, CD_3OD): δ = 1,48 (s, 9H, *t*Bu), 3.13 (dd, J = 3.2, 16. Hz, 1H CH*H*CH), 3.52 (dd, J = 6.2, 16.0Hz, 1H, C*H*HCH), 4.21 (s, 2H, CH_2N), 4.56 (dd, J = 3.2, 6.2Hz, 1H, CHHC*H*), 7.27 (d, J = 8.4Hz, 2H, arom), 7.47 (d, J = 8.4Hz, 2H, arom), 8.69 (bs, 1H, NH) ppm
^{13}C NMR (50MHz, CD3OD): δ = 29.6, 42.9, 45.4, 51.1, 81.1, 122.2, 129.7, 138.0, 138.1, 149.8, 159.4, 167.9, 173.6 ppm
IR: ν̃ = 3342, 2978, 2929, 1773,1704,1667, 1605,1540,1417,1319,1242,1164 cm^{-1}
HPLC-MS: Rt = 2.03min, m/z = 280 $[M-CO_2tBu+H_2O]^+$

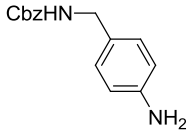
(S) - (2-carboxy-4-oxoazetidine-1-carboxamido) phenylmethanaminium trifluoroacetate (**3**)



In a 10mL 2-neck flask under nitrogen at 0°C a, TFA (13µL, 165µmol) was added dropwise to a solution of **18** (24µg, 66µmol) in anhydrous DCM (1.2mL). After stirring at rt for 15h, the mixture was again cooled to 0°C and treated with another aliquot of TFA (20mL, 265mmol). After starting material complete disappearing (24h), the crude was evaporated under

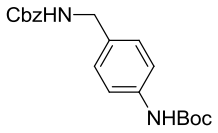
vacuum and triturated with a drop of DCM and pentane to provide **3** as a white solid (22mg) in 88% yield.
$[\alpha]_D$ = -41.8° (c = 0.25, DCM)
^{1}H NMR (200MHz, CD_3OD): δ = 3.15 (dd, J = 3.0, 16.2Hz, 1H, CH*H*CH), 3.54 (dd, J = 6.4, 16.2Hz, 1H, C*H*HCH), 4.11 (s, 2H, $CH_2NH_3^+$), 4.58 (dd, J = 3.0, 6.4Hz, 1H, CHHC*H*), 7.45 (d, J = 8.4Hz, 2H, arom), 7.63 (d, J = 8.4Hz, 2H, arom) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 43.0, 44.7, 51.3, 116.8 (q), 122.4, 131.0, 131.7, 140.3, 149.7, 163.7 (q), 167.9, 173.6 ppm
IR: ṽ = 3411, 2954, 2921, 2851, 1777, 1691, 1625, 1552, 1462, 1323, 1204 cm^{-1}
HPLC-MS: Rt = 1.07min, m/z = 247 $[M\text{-}TFA\text{-}NH3+H]^+$, 527 $[2M\text{-}2TFA+H]^+$, 549 $[2M\text{-}2TFA+Na]^+$
Found C, 44.78; H, 3.95; N, 10.88%; $C_{14}H_{14}F_3N_3O_6$ requires C, 44.57; H, 3.74; N, 11.14%

Benzyl 4-aminobenzylcarbamate (**22**)



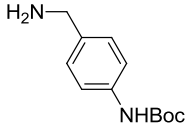
In a 25mL 2-neck flask under nitrogen, in 3.25mL of anhydrous DCM were dissolved 244mg (2.0mmol) of 4-amino-benzylamine and, at 0°C, were added 253µL (1.8mmol) of TEA and, dropwise, 257µL (1.8mmol) of CbzCl. The mixture was stirred at rt and follow by TLC (cyclohexane/EtOAc 4/6), then filtered on cotton at, and concentrated under vacuum. The slurry was then treated with water, checking pH < 7, extracted with EtOAc (3 times), dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (EtOAc/cyclohexane 25/75, then 40/60). Yield: 49%.

Benzyl 4-Boc-aminobenzylcarbamate (**23**)



In a 25mL 2-neck flask under nitrogen, 243mg (0.95mmol) of amine **22** were dissolved in 3.75mL of anhydrous THF and, at 0°C, were slowly added 229mg (1.05mmol) of Boc_2O. The reaction was stirred at rt for 40h, following by TLC (cyclohexane/EtOAc 4/6) the starting material disappearance, and then directly concentrated under vacuum and finally purified by flash-chromatography (EtOAc/cyclohexane 25/75, then 40/60). Yield: 96%.
M.p. 134-135°C; Rf 0.78 (cyclohexane/EtOAc, 4/6)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.52 (s, 9H, *t*Bu), 4.33 (d, J = 5.6Hz, 2H, CH_2N), 5.01 (bs, 1H, NH), 5.14 (s, 1H, CH_2O), 6.47 (bs, 1H, NH), 7.21 (d, J = 8.0Hz, 2H, Ar), 7.32 (d, J = 8.4Hz, 2H, Ar) 7.34-7.37 (m, 5H, Bn) ppm.
^{13}C NMR (100MHz, $CDCl_3$): δ = 28.2, 44.5, 66.7, 80.4, 118.7, 128.0, 128.1, 128.3 128.4, 132.8, 136.4, 137.7, 152.7, 156.4 ppm
IR: ṽ = 3331, 2978, 2932, 2851, 1702, 1698, 1526, 1242, 1160 cm^{-1}
HPLC-MS: Rt = 9.88min, m/z = 374 (100) $[M+H_2O]^+$, 379 (89) $[M+Na]^+$, 395 (10) $[M+K]^+$

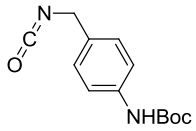
***t*Butyl (4-(aminomethyl)phenyl)carbamate** (**24**)



In a 10mL 2-neck flask, Pd/C ($10\%_{w/w}$) (34mg) was added to a solution of **23** (325mg, 0.91mmol) in anhydrous THF (11mL) and MeOH (11mL). The mixture was stirred under H_2 atmosphere (1atm) at room temperature and after starting material complete consumption (2h), it was filtered and concentrated in vacuum. The crude was then triturated with a few drops of DCM to afford the desired product as a white lumpy solid (202mg) in 100% yield.
M.p. waxy solid; Rf 0.01 (cyclohexane/EtOAc, 40/60)
^{1}H NMR (400MHz, CD_3OD): δ = 1.51 (s, 9H, *t*Bu), 3.78 (s, 2H, C*H*$_2$NH$_2$), 7.24 (d, J = 8.4Hz, 2H, Ar), 7.37 (d, J = 8.0Hz, 2H, Ar) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 28.7, 45.5, 80.8, 119.9, 129.4, 130.3, 140.0, 155.2 ppm
IR: ṽ = 3309, 2980, 2932, 1715, 1599, 1524, 1316, 1244, 1163, 738 cm^{-1}

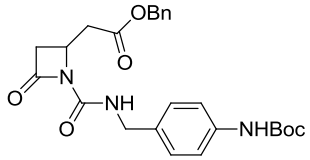
HPLC-MS: Rt = 1.28min, m/z = 206 (100) $[M\text{-}NH_3+H]^+$, 245 (8) $[M+ Na]^+$, 445 (54) $[2M+H]^+$

_t_butyl (4-isocyanatomethylphenyl)carbamate (**25**)



In a 50mL 2-neck flask with a reflux condenser under nitrogen atmosphere, a solution of (**24**) (87mg, 0.39mmol) and TEA (109µL, 0.78mmol) in anhydrous DCM (3.76mL) was cooled to 0°C; was then added in one portion with 231.5mg (0.78mmol) of bis(trichloromethyl)-carbonate. The mixture was warmed to rt and monitored through the increase of isocyanate IR signal ($2274cm^{-1}$). After 1hthe solvent was evaporated under vacuum avoiding any air exposure. The crude was extracted five times with anhydrous Et_2O (5x4mL) under nitrogen, concentrated in vacuum, stored under nitrogen atmosphere and immediately used.

benzyl 2-(1-(4-_t_butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidin-2-yl acetate (**26**)



In a 25mL 2-neck flask under nitrogen at -78°C, a solution of NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (325µL, 0.325mmol) was added dropwise to a solution of **6** (57mg, 0.26mmol) in anhydrous THF (2.2mL). After 15min of stirring, a solution of isocyanate **25** (157mg, 0.39mmol) in THF (1.15mL) was added dropwise. After completion (TLC monitoring, 30min) the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and then DCM. The combined organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (DCM/Et_2O, from 95/5 to 80/20) affording **26** (70mg) as a colorless oil in 62% yield.

Rf 0.88 (cyclohexane/EtOAc, 20/80)

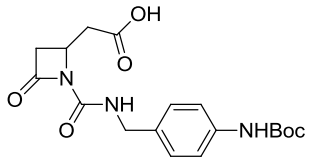
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.52 (s, 9H, *t*Bu), 2.72 (dd, J = 9.2, 16.4Hz, 1H), 2.85 (dd, J = 2.4, 16.0Hz, 1H), 3.27 (dd, J = 5.6, 16.4Hz, 1H), 3.35 (dd, J = 3.6, 16.4Hz, 1H), 4.33-4.40 (m, 1H), 4.34 (dd, J = 6.0, 9.6Hz, 1H, CH*H*N), 4.41 (dd, J = 6.0, 14.8Hz, 1H, C*H*HN), 5.15 (d, J_{AB} = 12.0Hz, 1H, CH*H*O), 5.17 (d, J_{AB} = 12.4Hz, 1H, C*H*HO), 6.47 (bs, 1H, BocNH), 6.78 (t, J = 6.0Hz, NH) 7.21 (d, J = 8.4Hz, 2H, Ar), 7.32 (d, J = 8.8Hz, 2H, Ar), 7.31-7.40 (m, 5H, Bn) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 28.2, 37.0, 42.5, 43.0, 47.0, 66.6, 80.4, 118.7, 128.2, 128.3, 128.4, 128.5, 132.2, 135.3, 137.8, 150.2, 152.7, 166.1, 169.7 ppm

IR: $\tilde{\nu}$ = 3365, 2979, 2930, 1769, 1730, 1703, 1697, 1530, 1315, 1161 cm^{-1}

HPLC-MS: Rt = 10.18min, m/z = 468 (16) $[M+H]^+$, 485 (85) $[M+H_2O]^+$, 490 (100) $[M+Na]^+$, 506 $[M+K]^+$

2-(1-(4-_t_butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidin-2-yl acetic acid (**27**)



In a 25mL 2-neck flask a mixture of **26** (70mg, 0.15mmol), anhydrous THF (1.74mL), MeOH (1.74mL) and Pd/C 10%$_{w/w}$ (7mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **27** was obtained as a white solid (76mg) in 84% yield after trituration with DCM.

M.p. 78-81°C; Rf 0.69 (cyclohexane/EtOAc, 40/60)

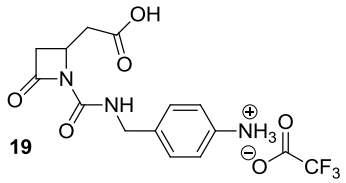
^{1}H NMR (400MHz, CD_3OD): δ = 1.51 (s, 9H, *t*Bu), 2.73 (dd, J = 8.8, 16.4Hz, 1H), 2.91 (dd, J = 2.8, 16.0Hz, 1H), 3.13 (dd, J = 4.0, 16.8Hz, 1H), 3.27 (dd, J = 5.2, 16.0Hz, 1H), 4.27- 4.32 (m, 1H), 4.35 (s, 2H, CH_2NH), 7.20 (d, J = 8.4Hz; 2H, Ar), 7.35 (d, J = 8.8Hz; 2H, Ar), 8.85 (bs, 1H, ArNH) ppm

^{13}C NMR (50MHz, CD_3OD): δ = 28.7, 37.3, 43.2, 43.7, 43.8, 80.8, 119.9, 128.9, 133.9, 139.7, 152.4, 155.2, 168.1, 173.5 ppm

IR: $\tilde{\nu}$ = 3355, 2977, 2927, 1769, 1731, 1707, 1527, 1318, 1160 cm^{-1}

HPLC-MS: Rt = 1.92min, m/z = 412 (95) $[M-t\text{Bu}+2H]^+$, 485 (100) $[M+Na]^+$

4-(2-(carboxymethyl)-4-oxoazetidine-1-carboxamidomethyl) benzenaminium trifluoroacetate (**19**)

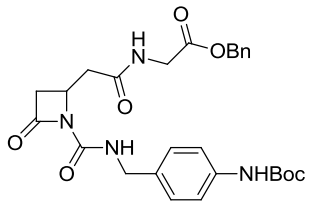


19

In a 10mL 2-neck flask under nitrogen at 0°C, TFA (8.3µL, 111µmol) was added dropwise to a solution of **27** (14mg, 37µmol) in anhydrous DCM (0.68mL). The mixture was stirred at rt for 21h, cooled at 0°C and treated with other TFA (11µL, 148µmol). At SM disappearing, the mixture was evaporated and the crude triturated with MeOH and pentane to provide **2** as a yellow oil in 99% yield.

Rf = 0.01 (EtOAc);
^{1}H NMR (400MHz, CD_3OD): δ = 2.75 (dd, J = 8.8, 16.4Hz, 1H), 2.94 (dd, J = 2.8, 16Hz, 1H), 3.11 (dd, J = 3.6, 16.4Hz, 1H), 3.29 (dd, J = 5.6, 16.0Hz, 1H), 4.31 (m, 1H), 4.47 (s, 2H, CH_2NH), 7.35 (d, J = 8.4Hz, 2H, Ar), 7.49 (d, J = 8.8Hz; 2H, Ar) ppm
^{13}C NMR (100MHz): δ = 30.7, 37.3, 43.2, 43.5, 124.0, 130.1, 131.5, 141.2, 152.6, 168.1, 173.5 ppm
^{19}F NMR (400MHz, CD_3OD): -77.0 ppm
IR: $\tilde{\nu}$ = 3365, 2924, 2854, 2626, 1771, 1703, 1679, 1539, 1201 cm^{-1}
HPLC-MS: Rt = 4.21min, m/z = 278 (100) $[M\text{-}TFA+H]^+$

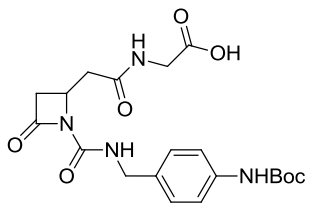
Benzyl 2-(2-(4-*t*butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidin-2-yl acetamido) acetate (**28**)



In a 25mL 2-neck flask, dicyclohexylcarbodiimide (DCC) (45.4mg, 0.22mmol) was added to a solution of **27** (27mg, 72µmol) in anhydrous DCM (0.85mL) and CH_3CN (0.5mL) at 0°C. Then a previously prepared solution of **12** (38mg, 0.11mmol) and stoichiometric TEA in DCM (0.91mL) was immediately added dropwise, followed by DMAP (4.9mg, 0.04mmol). The solution was warmed to rt and after complete consumption of the starting material (16h) the mixture was quenched with H_2O (10mL) and extract with DCM (3x10mL). The organic layers were dried on Na_2SO_4 and filtered. The crude was suspended in EtOAc, the residual urea by-product of DCC remained solid and was eliminated by filtration, the organic layer was concentrated in vacuum and purified by FC (DCM/CH_3CN, from 95/5to 80/20) to afford **28** as a light yellow waxy solid in 72% yield.

M.p. 64-66°C; Rf 0.68 (EtOAc)
^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.51 (s, 9H, *t*Bu), 2.70 (dd, J = 8.0, 14.8Hz, 1H), 3.06 (dd, J = 2.8, 16.0Hz, 1H), 3.11 (dd, J = 4.0, 14.8Hz, 1H), 3.23 (dd, J = 5.6, 16.0Hz, 1H), 4.00 (dd, J = 4.8, 18.0Hz, 1H, NHCH*H*COO), 4.06 (dd, J = 5.6Hz, 18.0Hz; 1H, NHC*H*HCOO), 4.31-4.33 (m, 1H), 4.33-4.38 (m, 2H, CH_2N), 5.17 (s, 2H, CH_2Bn), 6.55 (bs, 1H, BocNH), 6.67 (bs, 1H, NH), 6.91 (t, J = 6.8Hz, 1H, NHCH_2Ar), 7.20 (d, J = 8.0Hz, 2H, Ar), 7.28 (d, J = 8.4Hz, 2H, Ar), 7.30-7.37 (m, 5H, Bn) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ = 28.3, 38.4, 41.3, 42.5, 43.1, 48.0, 67.2, 80.6, 118.8, 128.3, 128.4, 128.5, 128.6, 132.2, 135.1, 137.8, 150.9, 152.7, 166.6, 169.3, 169.5 ppm
IR: $\tilde{\nu}$ = 3355, 2976, 1766, 1724, 1707, 1691, 1671, 1598, 1161 cm^{-1}
HPLC-MS: Rt = 8.98min, m/z = 525 (55) $[M+H]^+$, 542 (40) $[M+H_2O]^+$, 547 (100) $[M+Na]^+$, 563 $[M+K]^+$

2-(2-(1-(4-*t*butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidin-2-yl) acetamido) acetic acid (**29**)

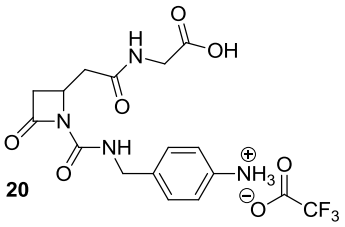


In a 10mL 2-neck flask a mixture of **28** (22mg, 42µmol), anhydrous THF (0.49mL), MeOH (0.49mL) and Pd/C 10%$_{w/w}$ (3mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. TM **27** was obtained as a white solid in 86% yield after trituration with DCM.

M.p. 100-103°C; Rf 0.01 (cyclohexane/EtOAc, 40/60)
^{1}H NMR (400MHz, CD_3OD): δ = 1.51 (s, 9H, *t*Bu), 2.65 (dd, J = 8.4, 14.4Hz, 1H), 2.99 (d, J = 16.0Hz, 1H), 3.10 (d, J = 14.4Hz, 1H), 3.24 (dd, J = 5.2, 16.0Hz, 1H), 3.86 (d, J = 20.0Hz, 1H, NHCH*H*COOH),

3.91 (d, J = 20.0Hz, 1H, NHC*H*HCOOH), 4.35 (m, 3H, NHCH_2Ar + NC*H*CH_2), 7.21 (d, J = 7.6Hz, 2H, Ar), 7.35 (d, J = 8.0Hz, 2H, Ar), 8.87 (bs, 1H, ArCH_2N*H*) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 28.7, 30.7, 39.1, 43.0, 43.8, 49.3, 80.8, 119.9, 129.0, 129.0, 134.0, 139.8, 152.5, 155.3, 168.1, 172.4 ppm
IR: ν̃ = 3350, 2927, 2855, 1768, 1717, 1694, 1682, 1530, 1317, 1239, 1162, 1056 cm^{-1}
HPLC-MS: Rt = 2.50min, m/z = 412 (95) [M-*t*Bu+2H]$^+$, 485 (100) [M+Na]$^+$

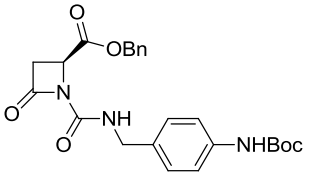
4-(2-(1-carboxymethyl-amidomethyl)-4-oxoazetidine-1-carboxamidomethyl) benzenaminium 2,2,2-trifluoroacetate (**20**)



20

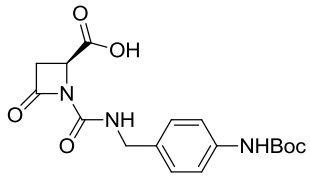
In a 10mL 2-neck flask under nitrogen at 0°C, TFA (9.7µL, 131µmol) was added dropwise to a solution of **29** (19mg, 44µmol) in anhydrous DCM (0.81mL). The mixture was stirred at rt for 24h, cooled at 0°C and then treated with another aliquot of TFA (13µL, 175µmol). At starting material disappearing, monitored by TLC (MeCN/MeOH 80/20) and by HPLC-MS (sample preparation in MeCN and pH = 7.5 phosphate buffer 1/1), the mixture was evaporated under vacuum, and the crude was triturated with a drop of MeOH and pentane to provide **20** as a yellow oil in 95% yield.
Rf = 0.01 (cyclohexane/EtOAc 40/60);
^{1}H NMR (400MHz, CD_3OD): δ = 2.68 (dd, J = 8.4, 15.2Hz, 1H), 3.01 (dd, J = 3.2, 16.4Hz, 1H), 3.05 (dd, J = 4.0Hz, 14.8Hz, 1H), 3.25 (dd, J = 6.0, 16.4Hz, 1H), 3.89 (t, J =10.0Hz, 2H), 4.36-4.31 (m, 1H), 4.47 (t, J = 15.2Hz, 2H), 7.35 (d, J = 8.8Hz, 2H, Ar), 7.49 (d, J = 8.4Hz, 2H, Ar), 2.75 (dd, J = 8.8, 16.4Hz, 1H), 2.94 (dd, J = 2.8, 16Hz, 1H), 3.11 (dd, J = 3.6, 16.4Hz, 1H), 3.29 (dd, J = 5.6, 16.0Hz, 1H), 4.31 (m, 1H), 4.47 (s, 2H, CH_2NH), 7.35 (d, J = 8.4Hz, 2H, Ar), 7.49 (d, J = 8.8Hz, 2H, Ar) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 30.7, 38.9, 41.7, 42.9, 43.5, 123.9, 130.1, 131.7, 141.0, 152.6, 168.1, 172.4, 172.8 ppm
^{19}F NMR (400MHz, CD_3OD): -77.0 ppm
IR: ν̃ = 3356, 2925, 2854, 1793, 1782, 1736, 1712, 1698, 1465, 1286, 1170 cm^{-1}
HPLC-MS: Rt = 4.57min, m/z = 334 [M-TFA+H]$^+$, 485 [M+Na]$^+$

(S)-benzyl 1-(4-*t*butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidine-2-carboxylate (**30**)



In a 10mL 2-neck flask under inert atmosphere at -78°C, NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (375µL, 0.375mmol) was added dropwise to a solution of **16** (61.5mg, 0.3mmol) in anhydrous THF (2.5mL) followed after 30min by a solution of isocyanate **25** (106mg, 0.45mmol) in THF (1.3mL); after starting material disappearing (30min), the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and DCM. The organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (DCM, then DCM/Et_2O 99/1 to 80/20) to afford **30** as a colorless oil (51mg) in 37% yield.
Rf 0.92 (cyclohexane/EtOAc, 20/80), 0.45 (DCM/cyclohexane/Et_2O, 60/20/20)
$[\alpha]_D$ = -37.77° (c = 9.27, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.52 (s, 9H, *t*Bu), 3.01 (dd, J = 2.8, 16.0Hz, 1H, CH*H*CH), 3.31 (dd, J = 6.0, 15.6Hz, 1H, C*H*HCH), 4.38 (dd, J = 6.0, 15.2Hz, 1H, CH*H*NH), 4.46 (dd, J = 5.6, 14.8Hz, 1H, C*H*HNH), 4.54 (dd, J = 2.4, 6.4Hz, 1H, CHHC*H*), 5.23 (d, J_{AB} = 17.2Hz, 1H, PhCH*H*), 5.27 (d, J_{AB} = 17.2Hz, 1H, PhC*H*H), 6.57 (s, 1H, $NHCO_2$*t*Bu), 6.67 (bt, J = 5.2Hz, 1H, N*H*CH_2), 7.21 (d, J = 8.0Hz, 2H, arom), 7.28-7.39 (m, 7H, arom + Ph) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 28.2, 41.0, 43.3, 48.7, 67.6, 80.4, 118.7, 128.2, 128.3, 128.5, 129.9, 132.0, 134.7, 137.8, 149.3, 152.6, 164.5, 169.0 ppm
IR: ν̃ = 3358, 2976, 2927, 1780, 1744, 1731, 1709, 1700, 1527, 1159 cm^{-1}
HPLC-MS: Rt = 10.01min, m/z = 471 [M+H_2O]$^+$, 476 [M+Na]$^+$

(S)-1-(4-*t*butoxycarbonylaminobenzylcarbamoyl)-4-oxoazetidine-2-carboxylic acid (**31**)



In a 25mL 2-neck flask a mixture of **30** (51mg, 110µmol), anhydrous THF (1.3mL), MeOH (1.3mL) and Pd/C 10%w/w (5mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material complete consumption (2h, as by TLC monitoring) the mixture was directly filtered and concentrated in vacuum. The desired product **31** was obtained as a waxy white solid in 88% yield after flash-chromatography purification (MeCN, then MeCN/MeOH 90/10) and repetead DCM titration and evaporation.

Rf 0.01 (cyclohexane/EtOAc, 40/60)

$[\alpha]_D$ = -46.57° (c = 6.36, MeOH)

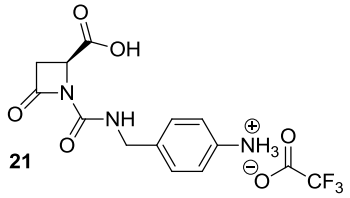
^{1}H NMR (400MHz, CD_3OD): δ = 1.50 (s, 9H, *t*Bu), 2.90 (dd, J = 2.8, 15.6Hz, 1H, C*H*HCHCOOH), 3.27 (dd, J = 6.0, 15.6Hz, 1H, C*H*HCHCOOH), 4.30-4.41 (m, 3H, NHCH_2Ar + NC*H*COOH), 7.22 (d, J = 8.4Hz, 2H, Ar), 7.32 (d, J = 8.8Hz, 2H, Ar) ppm

^{13}C NMR (100MHz, CD_3OD): δ = 28.7, 30.7, 42.0, 43.8, 80.8, 119.8, 129.0, 133.9, 139.7, 152.4, 155.2, 168.5, 176.8 ppm

IR: $\tilde{\nu}$ = 3363, 2979, 2931, 1770, 1699, 1694, 1692, 1600, 1327, 1162, 1068 cm^{-1}

HPLC-MS: Rt = 6.50min, m/z = 362 $[M-H]^-$

(S)-4-(2-carboxy-4-oxoazetidine-1-carboxamidomethyl) benzenaminium 2,2,2-trifluoroacetate (**21**)



In a 25mL 2-neck flask under nitrogen at 0°C, TFA (21µL, 288µmol) was added dropwise to a solution of **31** (35mg, 96µmol) in anhydrous DCM (1.8mL). The mixture was stirred at rt for 24h, cooled at 0°C and then treated with other aliquots of TFA (116µL, overall). At starting material disappearing, monitored by TLC (MeCN/MeOH 90/10) and by HPLC-MS (sample preparation in MeCN and pH = 7.5 aqueous phosphate buffer 1/1), the mixture was directly evaporated under vacuum. The resulting crude was triturated with a drop of MeCN and pentane to provide **21** as a sticky solid in 99% yield.

Rf = 0.04 (MeCN/MeOH 90/10)

$[\alpha]_D$ = -28.22° (c = 8.545, MeOH)

^{1}H NMR (400MHz, CD_3OD): δ = 3.04 (dd, J = 2.8, 15.6Hz, 1H), 3.44 (dd, J = 6.0, 15.6Hz, 1H), 4.45-4.48 (m, 3H, C*H*COOH + CH_2NH), 7.35 (d, J = 7.2Hz; 2H, Ar) 7.49 (d, J = 8.4Hz, 2H, Ar), ppm

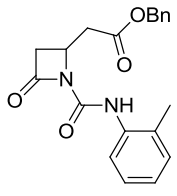
^{13}C NMR (100MHz, CD_3OD): δ = 42.0, 43.6, 50.2, 124.2, 129.8 (q, J = 21.2Hz), 130.1, 131.1, 141.2, 151.8, 162.4 (q, J = 35.6Hz), 166.6, 172.9 ppm

^{19}F NMR (400MHz, CD_3OD): -77.1 ppm

IR: $\tilde{\nu}$ = 3362, 2924, 1780, 1698, 1677, 1543, 1202, 1139, 800 cm^{-1}

HPLC-MS: Rt = 1.52min, m/z = 264 $[M-TFA+H]^+$, 281 $[M-TFA+H_2O]^+$

Benzyl 2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl) acetate (**35**)

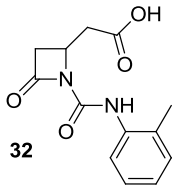


In a 10mL 2-neck flask under inert atmosphere at -78°C, NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (360µL, 0.36mmol) was added dropwise to a solution of **6** (88mg, 0.4mmol) in anhydrous THF (3.51mL). After 30 minutes stirring at -78°C a solution of *o*-tolyl-isocyanate (59.2µL, 0.48mmol) in THF (1.43mL) was added dropwise.At starting material disappearing (30min), the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and DCM. The organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuum. The crude was purified by flash-chromatography (cyclohexane/EtOAc 80/20) to finally afford **35** as a white solid in 77% yield.

M.p. 74-76°C; Rf 0.81 (cyclohexane/EtOAc, 40/60)

^{1}H NMR (400MHz, CD_3OD): δ = 2,30 (s, 3H, Me), 2.82 (dd, J = 8.8, 16.8Hz, 1H), 2.96 (dd, J = 2.8, 16.4Hz, 1H), 3.37 (m, 2H), 4.48 (m, 1H, C*H*NH), 7.06 (t, J = 7.2Hz, 1H), 7.19 (d, J = 6.8Hz, 1H), 7.22 (t, J = 7.6Hz, 1H), 7.30-7.40 (m, 5H, Bn), 7.93 (d, J = 8.4Hz, 1H), 8.43 (s, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 17.6, 36.9, 42.6, 47.5, 66.8, 120.9, 124.4, 126.8, 127.5, 128.3, 128.4, 128.6, 130.4, 135.2, 135.3, 147.8, 166.7, 169.7 ppm
IR: $\tilde{\nu}$ = 3338, 1767, 1733, 1713, 1614, 1593, 1549, 1459, 1334, 1307, 1253, 1171, 755, 698 cm^{-1}
HPLC-MS: Rt = 10.35min, m/z = 353 $[M+H]^+$, 375 $[M+Na]^+$, 727 $[2M+Na]^+$

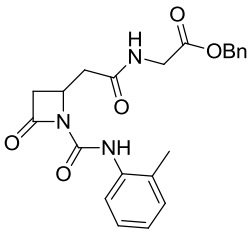
2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl) acetic acid (**32**)



32

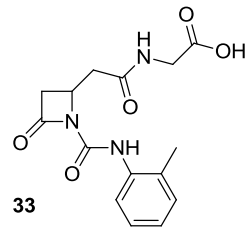
In a 25mL 2-neck flask **35** (103mg, 292μmol) was dissolved in a mixture of anhydrous THF (3.5mL) and MeOH (3.5mL), then Pd/C 10%$_{w/w}$ (10mg) was added and the reaction was stirred under H_2 atmosphere (1atm) at room temperature. At starting material complete consumption (2h, as by TLC monitoring) the mixture was directly filtered with celite, washed with MeOH and concentrated in vacuum. The desired product **27** was finally obtained as a white solid in 91% yield after repeated titration with MeOH and DCM.
M.p. 114-117°C; Rf 0.19 (cyclohexane/EtOAc, 40/60)
^{1}H NMR (400MHz, CD_3OD): δ = 2.27 (s, 3H, Me), 2.90 (dd, J = 9,2, 16.8Hz,1H), 3.08 (dd, J = 2.8, 16.0Hz, 1H), 3.26 (dd, J = 3.6, 16.8Hz, 1H), 3.42 (dd, J = 5.6, 16.0Hz, 1H), 4.45 (ddt, J = 3.2, 6.0, 8.8Hz, 1H), 7.02 (t, J = 7.6Hz, 1H), 7.17 (t, J = 8.0Hz, 1H), 7.22 (d, J = 7.6Hz, 1H), 7.99 (d, J = 8.4Hz, 1H, NH), 8.55 (s,1H, NH), 10.98 (bs, 1H, COOH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 17.6, 36.6, 43.3, 48.4, 121.2, 124.7, 127.4, 127.7, 131.2, 136.9, 148.8, 168.4, 171.7 ppm
IR: $\tilde{\nu}$ = 3344, 2922, 1766, 1708, 1614, 1593, 1552, 1460, 1331, 1307, 1253, 1124 cm^{-1}
HPLC-MS: Rt = 5.06min, m/z = 263 $[M+H]^+$, 285 (100) $[M+Na]^+$, 547 $[2M+Na]^+$

Benzyl 2-(2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl) acetamido) acetate (**36**)



In a 25mL 2-neck flask, to a solution of **32** (98mg, 374μmol) in anhydrous DCM (5.4mL) was added dropwise a previously prepared solution of **12** (189mg, 0.56mmol) and stoichiometric TEA in DCM (4.7mL). Then was immediately introduced 45.0mg (374μmol) of DMAP and, at 0°C, 72mg (374μmol) of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The solution was warmed to rt and after complete consumption of the starting material (16h) the mixture was quenched with H_2O (10mL) and extracted with DCM (3x10mL). The organic layers were dried on Na_2SO_4, filtered, concentrated in vacuum and purified by flash-chromatography (DCM/MeCN, from 95/5to 80/20) to afford **36** as a light yellow waxy solid in 56% yield.
M.p. 174-176°C; Rf 0.61 (DCM/MeCN 80/20)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.31 (s, 3H, Me), 2.79 (dd, J = 8.0, 14.8Hz, 1H), 3.17 (dd, J = 2.8, 15.2Hz, 1H), 3.19 (dd, J = 2.4, 16.4Hz, 1H), 3.35 (dd, J = 5.2, 16.4Hz, 1H), 4.03 (dd, J = 5.2, 18.0Hz, 1H, CH*H*NH), 4.12 (dd, J = 5.6, 18.4Hz, 1H, C*H*HNH), 4.47 (m, 1H), 5.17 (s, 2H, Bn), 6.44 (bs, 1H,NH), 7.06 (t, J = 7.6Hz, 1H, Tol), 7.20 (d, J = 7.6Hz, 1H, Tol), 7.22 (t, J = 7.6Hz, 1H, Tol), 7.32-7.39 (m, 5H, Bn), 7.90 (d, J = 7.6Hz, 1H, Tol), 8.52 (s, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 38.2, 41.3, 42.6, 48.3, 67.2, 121.2, 124.6, 126.7, 127.8, 128.4, 128.5, 128.6, 130.5, 135.0, 135.1, 148.5, 167.2, 169.2, 169.4 ppm
IR: $\tilde{\nu}$ = 3321, 2923, 1767, 1708, 1645, 1614, 1593, 1550, 1458, 1308 cm^{-1}
HPLC-MS: Rt = 8.49min, m/z = 410 (100) $[M+H]^+$, 432 (44) $[M+Na]^+$

2-(2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl) acetamido) acetic acid (**33**)



33

In a 25mL 2-neck flask a mixture of **36** (85mg, 207µmol), anhydrous THF (2.4mL), MeOH (2.4mL) and Pd/C 10%$_{w/w}$ (9mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **27** was obtained as a white solid in 99% yield after trituration with DCM.

M.p. 182-185°C; Rf 0.03 (DCM/MeCN 80/20)

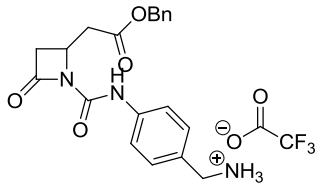
^{1}H NMR (400MHz, CD_3CN): δ = 2.27 (s, 3H, Me), 2.70 (dd, J = 8.0, 15.2Hz, 1H), 3.01 (dd, J = 4.0, 6.0Hz, 1H), 3.05 (dd, J = 3.6, 6.4Hz, 1H), 3.25 (dd, J = 6.0, 16.4Hz ,1H), 3.86 (d, J = 5.6Hz, 2H, C*H*$_2$COOH), 4.35 (m, 1H), 6.86 (bs, 1H, NH), 7.05 (t, J = 7.6Hz, 1H), 7.18-7.24 (m, 2H), 7.86 (d, J = 8.0Hz, 1H), 8.47 (bs, 1H, NH) ppm

^{13}C NMR (100MHz, CD_3OD): δ = 18.6, 39.7, 42.6, 43.9, 55.7, 123.7, 126.7, 128.5, 130.7, 132.3, 137.5, 150.9, 169.8, 173.1, 173.7 ppm

IR: ν̃ = 3300, 2924, 2853, 2464, 1764, 1720, 1649, 1617, 1595, 1556, 1458, 1390, 1367, 1345, 1308, 1284, 1256, 1209 cm^{-1}

HPLC-MS: Rt = 2.51min, m/z = 318 $[M-H]^-$, 637 $[2M-H]^-$

4-(2-(2-(benzyloxy)-2-oxoethyl)-4-oxoazetidine-carboxamido)phenyl methanaminium 2,2,2-trifluoroacetate (**37**)



In a 25mL 2-neck flask under nitrogen at 0°C, TFA (160µL, 2.16mmol) was added dropwise to a solution of **10** (133mg, 284µmol) in anhydrous DCM (3.98mL). The mixture was stirred at rt for 24h, cooled at 0°C and then treated with another aliquot of TFA (160µL, 2.16mmol). At SM disappearing (as by TLC cyclohexane/EtOAc 40/60) the mixture was evaporated under vacuum, and the crude was triturated with a drop of MeOH and pentane to provide **37** as a waxy white solid in 99% yield.

Rf = 0.01 (cyclohexane/EtOAc 40/60)

^{1}H NMR (400MHz, CD_3OD): δ = 2.72 (dd, J = 7.6, 16.0Hz, 1H), 2.88 (d, J = 16.0Hz, 1H), 3.20 (d, J = 12.8Hz, 1H), 3.29 (d, J =10.4Hz, 1H), 3.90 (bs, 2H, C*H*$_2$NH$_3$), 4.36 (m, 1H, C*H*CH$_2$COOBn), 5.12 (s, 2H, Bn), 7.21-7.35 (9H, Ar), 7.97(bs, 3H, NH_3), 8.39 (s, 1H,NH) ppm

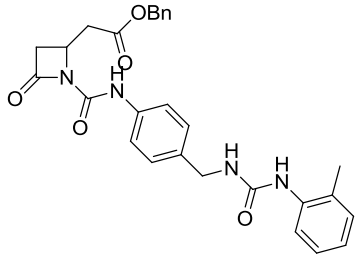
^{13}C NMR (100MHz, CD_3OD): δ = 36.7, 42.5, 43.0, 47.6, 66.8, 120.4, 127.9, 128.2, 128.4, 128.5, 129.8, 135.2, 137.3, 147.9, 166.8, 169.9 ppm

^{19}F NMR (400MHz, CD_3OD): -75.8 ppm

IR: ν̃ = 3336, 3037, 2957, 2923, 1771, 1680, 1605, 1546, 1422, 1385, 1319, 1298, 1246, 1203, 1139, 723, 700 cm^{-1}

HPLC-MS: Rt = 1.43min, m/z = 351 (100) $[M-TFA-NH_3+H]^+$, 735 (52) $[2M-2TFA+H]^+$

Benzyl 2-(4-oxo-(4-(3-*o*-tolylureidomethyl)phenylcarbamoyl)azetidin-2-yl) acetate (**38**)

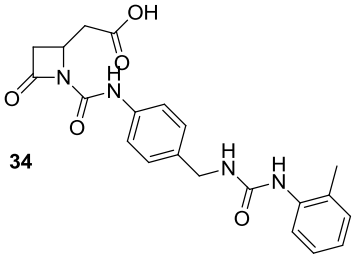


In a 25mL 2-neck flask under inert atmosphere, to a solution of **37** (136mg, 0.28mmol) in dry THF (1.6mL) were added dropwise 69µL (0,49mmol) of TEA. The conversion was followed the by TLC (cyclohexane/EtOAc 20/80), and after 30min the mixture was cooled at 0°C and a solution of *o*-tolyl-isocyanate (43µL, 0.35mmol) in THF (0.8mL) was introduced. After starting material disappearing (1h30min), the mixture was concentrated under vacuum and the resulting crude purified by flash-chromatography (DCM/EtOAc, from 90/10 to 70/30) to afford **38** as a white solid in 56% yield.

M.p. 184-187°C; Rf 0.70 (cyclohexane/EtOAc 20/80)

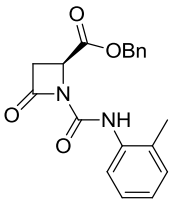
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.25 (s, 3H, Me), 2.79 (dd, J = 9.2, 16.8Hz, 1H), 2.95 (dd, J = 2.8, 16.4Hz, 1H), 3.35 (m, 2H), 4.40 (s, 2H), 4.46 (m, 1H), 5.15 (d, J = 12.8Hz, 1H), 5.18 (d, J = 12.8Hz, 1H), 6.19 (bs, 1H, NH), 7.12-7.41 (m, 14H, Ar+NH), 8.41 (s, 1H, NH) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ = 17.8, 36.9, 42.6, 43.7, 47.5, 66.8, 119.9, 125.2, 125.8, 127.0, 128.1, 128.3, 128.4, 128.5, 128.6, 130.9, 132.3, 135.1, 135.3, 136.0, 147.7, 157.5, 166.5, 169.6 ppm
IR: $\tilde{\nu}$ = 3319, 2924, 2362, 1771 1712, 1631, 1604, 1588, 1545, 1457, 1418, 1308, 1242, 1193, 1120, 750 cm^{-1}
HPLC-MS: Rt = 9.20 min, m/z = 501 $[M+H]^+$, 523 $[M+Na]^+$, 1001 $[2M+H]^+$

2-(4-oxo-(4-(3-*o*-tolylureidomethyl)phenylcarbamoyl)azetidin-2-yl) acetic acid (**34**)


34

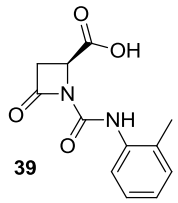
In a 25mL 2-neck flask a mixture of **38** (79mg, 157μmol), anhydrous THF (1.8mL), MeOH (1.8mL) and Pd/C 10%$_{w/w}$ (8mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **34** was obtained as a white solid in 99% yield after trituration with DCM.
M.p. 160-162°C; Rf 0.06 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3CN): δ = 2,21 (s, 3H, Me), 2.76 (dd, J = 8.0, 16.4Hz, 1H), 2.93 (d, J = 16.0Hz, 1H), 3.13 (d, J = 16.4Hz, 1H), 3.26 (d, J = 16.0Hz, 1H), 4.32 (m, 3H, C*H*$_2$NH, C*H*CH$_2$COOH), 5.75 (bs, 1H, NH), 6.68 (bs, 1H, NH), 6.98 (m, 1H, Tol), 7.14 (m, 2H, Tol), 7.28 (d, J = 7.2Hz, 2H, Ar), 7.43 (d, J = 7.6Hz, 2H, Ar), 7.67 (d, J = 8.0Hz, 1H, Tol), 8.45(s, 1H, NH), 9.36 (bs, 1H, COOH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 18.9, 38.1, 44.2, 45.0, 49.9, 122.0, 125.7, 126.3, 128.3, 129.9, 132.3, 132.6, 137.9, 138.4, 139.0, 150.6, 159.7, 169.4, 174.5ppm
IR: $\tilde{\nu}$ = 3319, 3054, 2926, 1774, 1707, 1629, 1603, 1545, 1440, 1420, 1332,
1264, 737, 702 cm^{-1}
HPLC-MS: Rt = 13.61min, m/z = 411 $[M+H]^+$, 433 $[M+Na]^+$, 821.6 $[2M+H]^+$

(S)-benzyl 4-oxo-(*o*-tolylcarbamoyl)azetidine-2-carboxylate (**41**)



In a 25mL 2-neck flask under inert atmosphere at -78°C, NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0M in THF (600μL, 0.6mmol) was added dropwise to a solution of **16** (103mg, 05) in anhydrous THF (4.3mL) followed after 30min by a solution of *o*-tolyl-isocyanate (74μL, 0.6mmol) in THF (1.79mL); after starting material disappearing (30min), the mixture was quenched with a saturated solution of NH_4Cl and extracted with EtOAc and DCM. The organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (cyclohexane/EtOAc 80/20) to afford **41** as a slightly brown oil solid in 75% yield.
Rf 0.85 (cyclohexane/EtOAc, 40/60)
$[\alpha]_D$ = -86.15° (c = 11.49, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2,31 (s, 3H, Me), 3.12 (dd, J = 2.8, 15.6Hz, 1H), 3.42 (dd, J = 6.0, 15.6Hz, 1H), 4.63 (dd, J = 2.8, 6.4Hz, 1H, C*H*COOBn), 5.25 (d, J_{AB} = 12.0Hz, 1H, Bn), 5.30 (d, J_{AB} = 12.0Hz, 1H, Bn), 7.07 (t, J = 7.6Hz, 1H, Ar), 7.20 (d, J = 6.8Hz, 1H, Ar), 7.22 (t, J = 8.0Hz, 1H, Ar), 7.34-7.39 (m, 5H, Bn), 7.93 (d, J = 8.4Hz, 1H, Ar), 8.29 (s, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 17.3, 41.0, 48.7, 67.6, 120.9, 124.4, 126.6, 127.4, 128.1, 128.4, 128.4, 130.2, 134.6, 134.9, 146.6, 165.1, 168.7 ppm
IR: $\tilde{\nu}$ = 3348, 3064, 3033, 2964, 1776, 1751, 1718, 1594, 1459, 1549, 1307, 1253, 1188, 755 cm^{-1}
HPLC-MS: Rt = 9.96min, m/z = 339 $[M+H]^+$, 361 $[M+Na]^+$, 699 $[2M+Na]^+$

(S)-4-oxo-(*o*-tolylcarbamoyl)azetidine-2-carboxylic acid (**39**)



39

In a 25mL 2-neck flask a mixture of **41** (115mg, 0.34mmol), anhydrous THF (1.88mL), MeOH (1.88mL) and Pd/C 10%$_{w/w}$ (12mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **39** was obtained as a white solid in 99% yield.

M.p. 121-124°C; Rf 0.01 (cyclohexane/EtOAc 40/60)

$[\alpha]_D$ = -151.04° (c = 10.0, DCM)

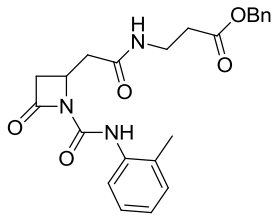
^{1}H NMR (400MHz, $CDCl_3$): δ = 2,33 (s, 3H, Me), 3.34-3.41 (m, 1H), 3.45-3.51 (m, 1H), 4.32 (m, 1H, C*H*COOH), 7.11 (t, J = 7.6Hz, 2H, Ar), 7.22 (d, J = 8.4Hz, 1H, Tol), 7.23 (t, J = 8.4Hz, 2H, Ar), 7.87 (d, J = 8.0Hz, 1H, Tol), 8.38 (s, 1H, NH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 17.5, 41.2, 49.3, 121.5, 125.0, 126.8, 128.1, 130.5, 134.5, 147.7, 165.6, 171.8 ppm

IR: ṽ = 3338, 1787, 1755, 173, 1690, 1590, 1461, 1327, 1311, 1250, 1136, 1061, 759 cm^{-1}

HPLC-MS: Rt = 1.61min, m/z = 247 $[M-H]^-$

Benzyl 3-(2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl)acetamido) propanoate (**43**)



In a 25mL 2-neck flask, to a solution of **32** (63mg, 240μmol) in anhydrous DCM (3.47mL) was added dropwise a previously prepared solution of **42** (127mg, 0.36mmol) and stoichiometric TEA in DCM (3.0mL). Then was immediately introduced 29.3mg (240μmol) of DMAP and, at 0°C, 46mg (240μmol) of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The solution was warmed to rt and after complete consumption of the starting material (18h) the mixture was quenched with H_2O (10mL) and extracted with DCM (3x10mL). The organic layers were dried on Na_2SO_4, filtered, concentrated in vacuum and purified by flash-chromatography (DCM/EtOAc 80/20, then 70/30) to afford 61mg (yield = 60%) of **43** as a white waxy solid.

Rf 0.74 (EtOAc)

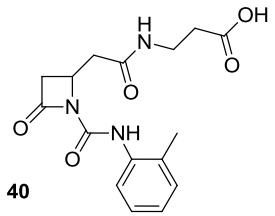
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.30 (s, 3H, Me), 2.58 (t, J = 6.0Hz, 2H, $NHCH_2CH_2$), 2.63 (dd, J = 5.6, 16.0Hz, 1H), 3.08 (dd, J = 4.0, 15.2Hz, 1H), 3.13 (dd, J = 3.2, 16.8Hz, 1H), 3.33 (dd, J = 5.6, 16.4Hz, 1H), 3.53 (ddd, J = 2.0, 6.8, 12.8Hz, 2H, $NHCH_2CH_2$), 4.40 (m, 1H), 5.07 (d, J_{AB} = 12.0Hz, 1H, C*H*HPh), 5.10 (d, J_{AB} = 12.4Hz, 1H, CH*H*Ph), 6.45 (bs, 1H, NH) 7.05 (t, J = 7.6Hz, 1H, Tol), 7.18-7.23 (m, 2H, Tol), 7.32-7.40 (m, 5H, Ph), 7.93 (d, J = 8.0Hz, 1H, Tol), 8.51 (s, 1H, NH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 17.5, 33.8, 34.8, 38.4, 42.6, 48.3, 66.4, 120.9,124.4, 126.7, 127.5, 128.1, 128.3, 128.5, 130.4, 135.1, 135.5, 148.1, 167.2, 168.8, 172.0 ppm

IR: ṽ = 3334, 3065, 2951, 1765, 1732, 1712, 1656, 1594, 1549, 1382, 1173, 1065, 755 cm^{-1}

HPLC-MS: Rt = 8.73 min, m/z = 423 (85) $[M+H]^+$, 446 (100) $[M+Na]^+$

3-(2-(4-oxo-(*o*-tolylcarbamoyl)azetidin-2-yl)acetamido) propanoic acid (**40**)



40

In a 25mL 2-neck flask a mixture of **43** (61mg, 0.144μmol), anhydrous THF (1.7mL), MeOH (1.7mL) and Pd/C 10%$_{w/w}$ (6mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (2h) the mixture was filtered and concentrated in vacuum. The desired product **39** was obtained as a white solid in 99% yield after trituration with DCM and pentane.

M.p. 175-178°C; Rf 0.01 (EtOAc)

^{1}H NMR (400MHz, $CDCl_3$): δ = 2.28 (s, 3H, Me), 2.52 (t, J = 6.4Hz, 2H, CH_2CH_2COOH), 2.64 (dd, J = 8.4, 14.8Hz, 1H), 3.05 (t, J = 14.4Hz, 2H), 3.34 (dd, J = 6.0, 14.8Hz, 1H), 3.43 (t, J = 6.4Hz, 2H,

C*H*$_2$CH$_2$COOH), 4.41 (m, 1H), 5.17 (s, 2H, Bn), 7.05 (t, J = 7.6Hz, 1H, Tol), 7.18 (t, J = 7.6Hz, 1H, Tol), 7.21 (d, J = 8.0Hz, 1H, Tol), 7.81 (d, J = 7.6Hz, 1H, Tol), 8.19 (s, 1H, NH) 8.64 (s, 1H, NH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 17.8, 34.6, 36.4, 39.3, 43.1, 49.8, 122.8, 125.8, 127.6, 129.7, 131.5, 136.6, 150.0, 159.1, 169.0, 172.0 ppm
IR: $\tilde{\nu}$ = 3300, 2923, 2854, 1769, 1700, 1638, 1614, 1592, 1309, 1125, 758 cm^{-1}
HPLC-MS: Rt = 3.87min, m/z = 332 (100) $[M-H]^-$, 665 (44) $[2M-H]^-$.

3. ANTIBIOTICS AND DUAL ACTIVE COMPOUNDS

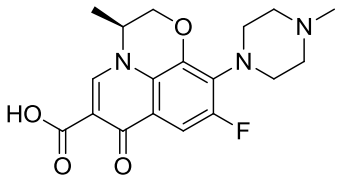
3.1 Synthesis of new β-lactam antibiotics

3.1.1 Antibiotics

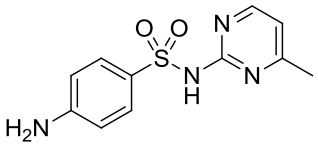
Antimicrobial compounds are defined as molecules capable of suppressing infections caused by microorganisms such as fungi and protozoa. Antibiotics, such as β-lactam derivatives, appear to be an antimicrobial subgroup. The term antibiotic derives from the Greek *anti*, against, and *bios*, life. It was introduced in 1942 by Selman Waksman to indicate substances produced by microorganisms and characterized by an antagonistic effect against the growth of other bacterial species, thus excluding compounds with non-microbial origin, such as the gastric juices, or synthetic molecules.[57]

Currently, also thanking the progress of pharmaceutical chemistry, the definition includes semi-synthetic compounds, such as molecules found in nature and chemically implemented with the aim to enhance the desired effects. Moreover, exclusively synthetic molecules are now included. Classified according to their chemical structures or referring to spectra of action, antibiotics are today commonly distinguished in relation to the different mechanisms of their antimicrobial activities:

- molecules that act on essential bacterial enzymes, such as quinolones and sulfonamides;

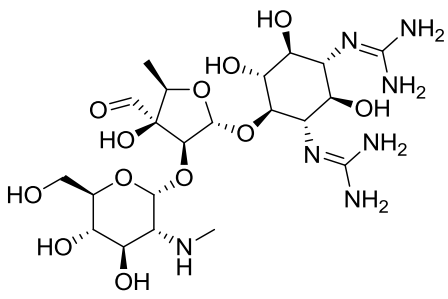


Levofloxacine, a quinolone

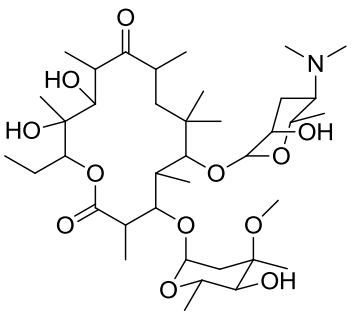


Sulfamerazine, a sulfonamide

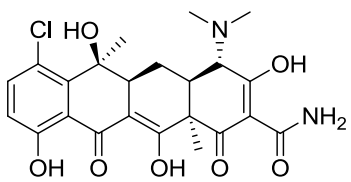
- molecules which interfere with protein synthesis, so aminoglycosides, macrolides and tetracyclines. - They are usually bacteriostatic, since only able to inhibit or limit bacterial replication without really kill microorganisms;



Streptomycin

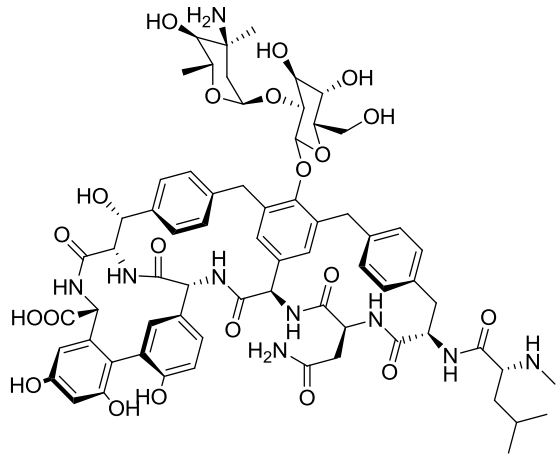


Erythromycin

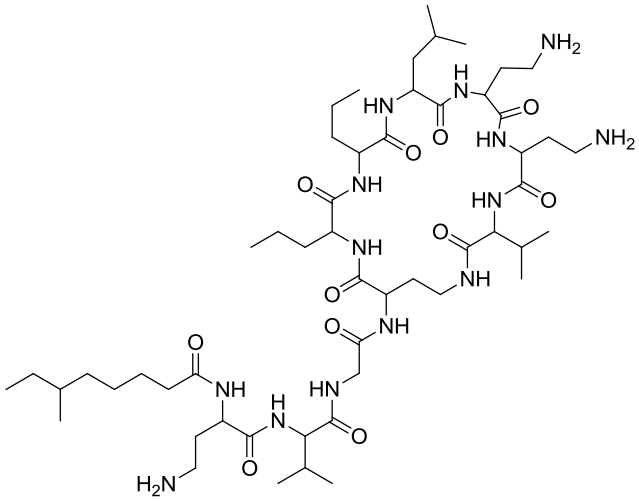


Chlortetracycline

- molecules that interfere with the bacterial cell walls construction, as the cases of penicillins, cephalosporins and carbapenems, or with that of cell membranes (glycopeptides such as vancomycin and polymyxins);



Vancomycin



Colystin, a polymixin

although there is a limited number of examples which outside this division, including daptomycin, natural and newly discovered,[58] and others. However, in most cases antibiotics are small semisynthetic compounds characterized by a molecular mass lower than 2000 units,[59] while first antibiotics entered in the pharmacopoeia were exclusively natural.

In a reverse chronological order, are herein listed the most important antibiotics. Thus, as the most recent achievements should be cited daptomycin, a lipopeptide characterized by a working mechanism different from those previously studied and exploited. Known since the '80 but approved by the FDA (acronym of Food & Drug Administration, the US government agency that deals with the regulation of food and pharmaceuticals) only in 2003, it seems to act by binding cell membranes causing rapid depolarization and subsequent inhibition of protein, RNA and DNA synthesis.[60] Erythromycin was the founder of macrolides: it was isolated in 1953.[61] Aureomycine (a chlortetracycline), the first tetracycline antibiotic, was discovered in 1945,[62] while Streptomycin, the first aminoglycoside compound, was achieved in 1943.[63]

Discovery of penicillin G by Alexander Fleming dates back to 1928. He has the merit of having realized the phenomenon, supposing a fungal contamination to produce an antibacterial agent. Due to the time needed to identify and isolate the fungus, *Penicillum notatum*, and to identificate and reproduce the agent, clinical evidences on the effectiveness of penicillin came only in 1941. For their penicillin in batch production, Chain and Florey earned the Nobel Prize together , with Fleming in 1945.[64]

However, the first commercially available antibiotic was a sulfonamide named Prontosil. It was discovered in 1932 and it was obtained after five years of tests on thousands of compounds structurally related to azoic dyes. Prontosil earned to Gerhard Domagk the medicine Nobel Prize of 1939.[65]

Salvarsan is a synthetic antibacterial based on arsenic and used for the treatment of syphilis and trypanosomiasis. As it dates back to 1910, it is considerable as the first modern chemotherapeutic agent. It is derived from the screening of hundreds of synthetic dyes implemented by Paul Ehrlich. After realizing the selectivity in coloring bacterial cells, animals or human, he hypothesized the existence of a species able to bind and kill only bacterial cells without damaging the human ones.[66]

All our actual chemotherapy agents can be considered as originated by the development of this selective toxicity concept.

β-Lactam antibiotics

Starting from the most common and celebrated penicillins and cephalosporins, all their semi-synthetic variants have today the 6-aminopenicillanic acid and the 7-aminocephalosporanic acid as precursors. The β-lactam ring represent the reference structures of many families of molecules with an antibiotic activity: penams, penems, clavams and clavems (Figure 3.1.1), as well as the most modern carbapenams, carbapenems and the so-called monobactams, all belong to the β-lactam family.

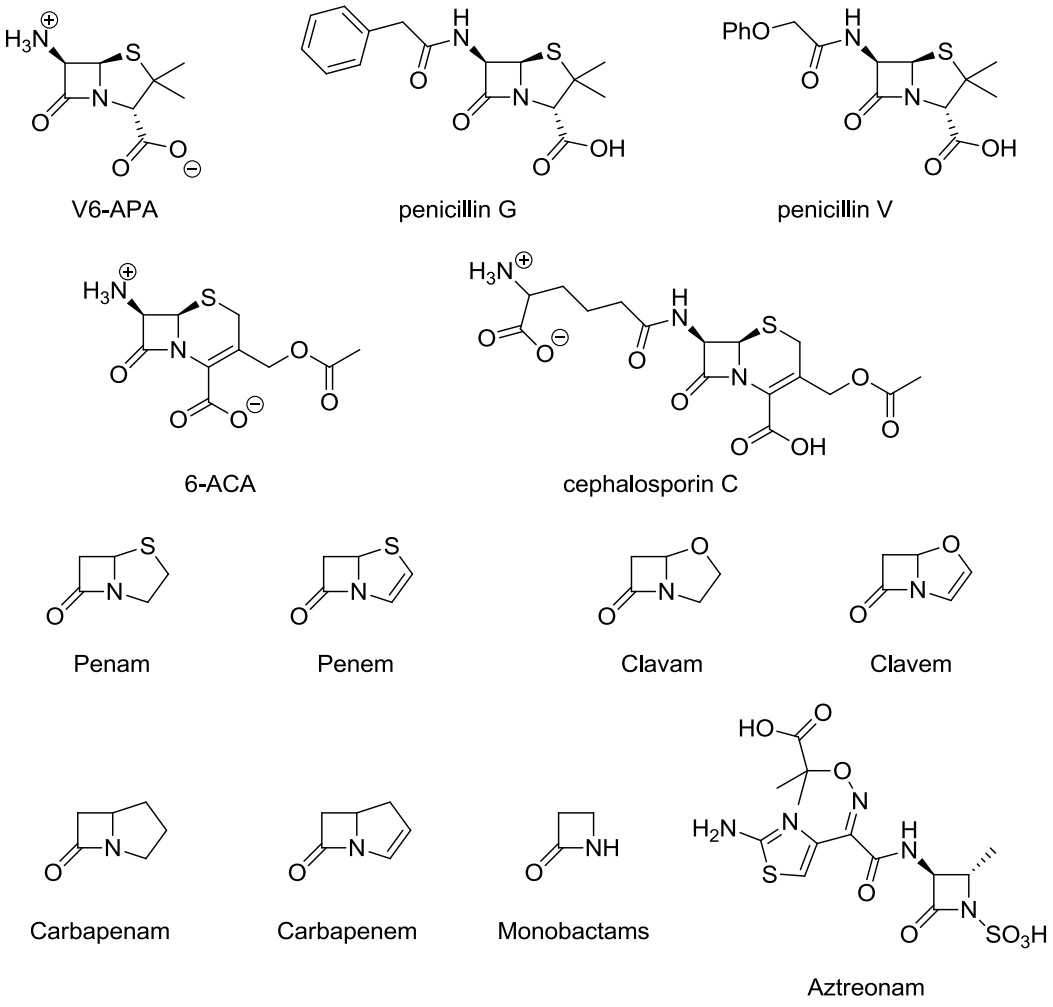


Figure 3.1.1 Reference structures for β-lactam antibiotics

The mechanism by which these molecules exert the antibiotic action is quite specific and related to the enzymes responsible for cross-linking of the bacterial cell walls, constituted by the peptidoglycan. It is a polymer formed by alternate units of NAG (N-AcetylGlucosamine) and NAM (N-AcetylMuramic acid) which is anchored by an (L)-Ala to a (L)Ala-(D)Glu-(L)Cys-(D)Ala-(D)Ala pentapeptide derivatized on the cysteine residue with a moiety consists of five (L)-Gly units. The enzyme involved in the crosslinking is a transpeptidase and catalyzes the formation of a pentaglycine cross-link through the replacement of the (D)Ala-(D)Ala bond with a (D)Ala-(L)Gly bond, where the glycine unit will be the last of one of the adjacent pendants (Figure 3.1.2).

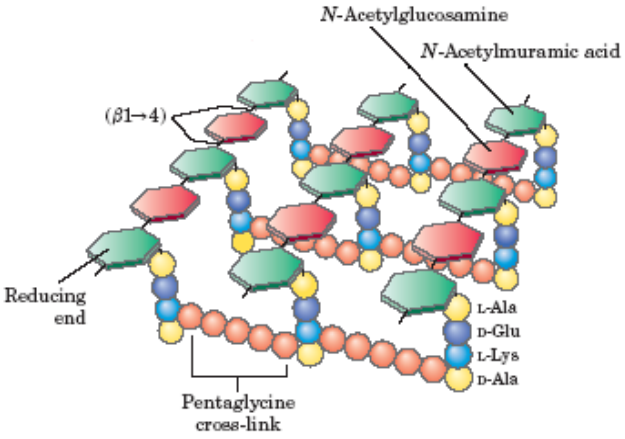


Figure 3.1.2 Cross-linked peptidoglycan

A non-crosslinkage leaves the cell wall not sufficiently resistant and, due to the usually high internal salt concentration and to the water permeability, the cell will tend to increase the internal pressure by osmosis, until lysis and consequent cell death.

The transpeptidase enzyme is usually serine-dependent. The mechanism by which β-lactams intervene on it passes to their easy opening by attack nucleophilic of serine OH in the transpeptidase active syte. This reaction form an irreversible covalent bond. Indeed, because of the different hindrances of antibiotics with respect to the natural substrate, the bond formed is no longer divisible. Thus the enzyme resulted irreversible inhibitio. Of course, the process is favoured by the how easy azetidin-2-one ring tends to undergo opening.

Although can always occur allergic phenomena, inhibiting an enzyme specific for bacteria these molecules do not exhibit toxicity to animal cells, which lack of the transpeptidase. However, even the bacterial mature cells, already developed, do not suffer the azetidin-2-one derivatives action. To be an efficient therapy and to avoid favoring the occurrence of phenomena of resistance, and therefore the development of resistant strains derived from this aspect, is really high the importance of always carrying out the entire cycle of each antibiotic treatment, the duration of which depends also on the generations replacement time of bacterial species treated.

At this point, it should consider the aspect of bacterial resistance, commonly defined as the ability of certain bacterial species to survive and multiply in the presence of molecules having antibiotic activity. It is then appropriate to distinguish the natural resistance, for example due to the absence of the specific target on which an antibiotic is active, from that triggered by prior exposure to the antibiotic. Induced resistance phenomena usually occur at the level of drug target changes, or through the development of systems to inactivate or even to expel the antibiotic before it exerts its action. In the case of β-lactam compounds, enzyme systems known as β-lactamase generally intervene for inactivating the antibiotic with a ring opening mechanism.[67] The phenomenon is typical of antibiotics use, because of natural spontaneous mutations that occur every 10^7-10^8 replications and which are transmitted, if favorable, to subsequent generations in vertical mode, encouraging their growth and multiplication. Moreover, the resistance may also result from genetic horizontal exchange between different strains and also different species, which in turn may be selected from others contexts of massive antibiotics presence, such as hospital environments. Although this is not the only case, the abuse and the inappropriate use has nevertheless contributed to the phenomenon.[68] Clearly, now "the first rule of antibiotics is to try not to use them, and the second is try not to use too many",[69] but in addition to guidelines for rationalizing and limingt the administration, where possible, is important as well as developing new drugs with new mechanisms of action. In recent years, pharmacology introduced different solutions to solve or at least try to stem his problem, starting from marketing preparations containing combinations of the antibiotic and, for cases in question, of a β-lactamase inhibitor. Nowadays are known three serine-dependent (A, C, D) β-lactamases and one metal-dependent (B) β-lactamase. The actual β-lactamase inhibitors present in pharmaceutical preparations are clavulanic acid, sulbactam and tazobactam, active against the A class. (Figure 3.1.3).[70]

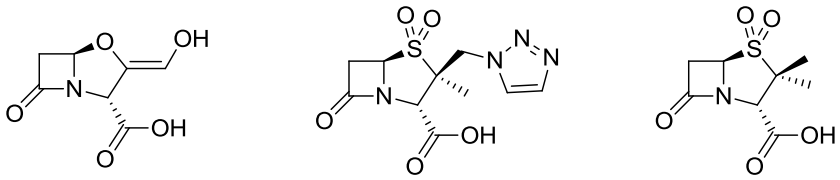


Figure 3.1.3 β-Lactamase A inhibitors

Despite the existence of precautions and tricks, antibiotic resistance is an overall proportion phenomenon, soaring and not negligible also because, as already seen, even judicious uses determine a selective pressure, which generates resistance.

The spectrum of the spread of bacterial strains resistant to carbapenems, detected in some hospitalized patients, leaves little antibiotic solutions usable. New molecules such as Tigecycline, first of a new class of compounds called glycylcyclines, and Colystin, a polymyxin, despite very active are still far from being pharmacologically applicable.[71]

However, some hope is associated to the class of monobactams: Aztreonam is the only commercially available member of this family. It is naturally resistant to β-lactamases and it is active against Gram-negative bacteria, usually the most difficult to be killed. Due to their antibiotic potentialities, a significant number of other monobactams exists: they are synthetic and semisynthetic, currently in development and in some cases already in clinical phase, such as for Tigemonam.[72]

3.1.2 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disease, thus a genetic disorder caused by recessive allelic form of a defective gene on an autosome (a non-sexual chromosome). It is fully manifested only in the homozygotes recessive for that gene, with no clinical symptoms in heterozygotes. Individuals with CF present a genotype rr, where "r" is the recessive allele that codes for the disease, while heterozygotes have a genotype Rr, where "R" is the dominant allele, resulting in an asymptomatic course of the disease. In the case of heterozygotes, the presence of only one recessive allele makes individuals immune to the effects of typhoid fever.

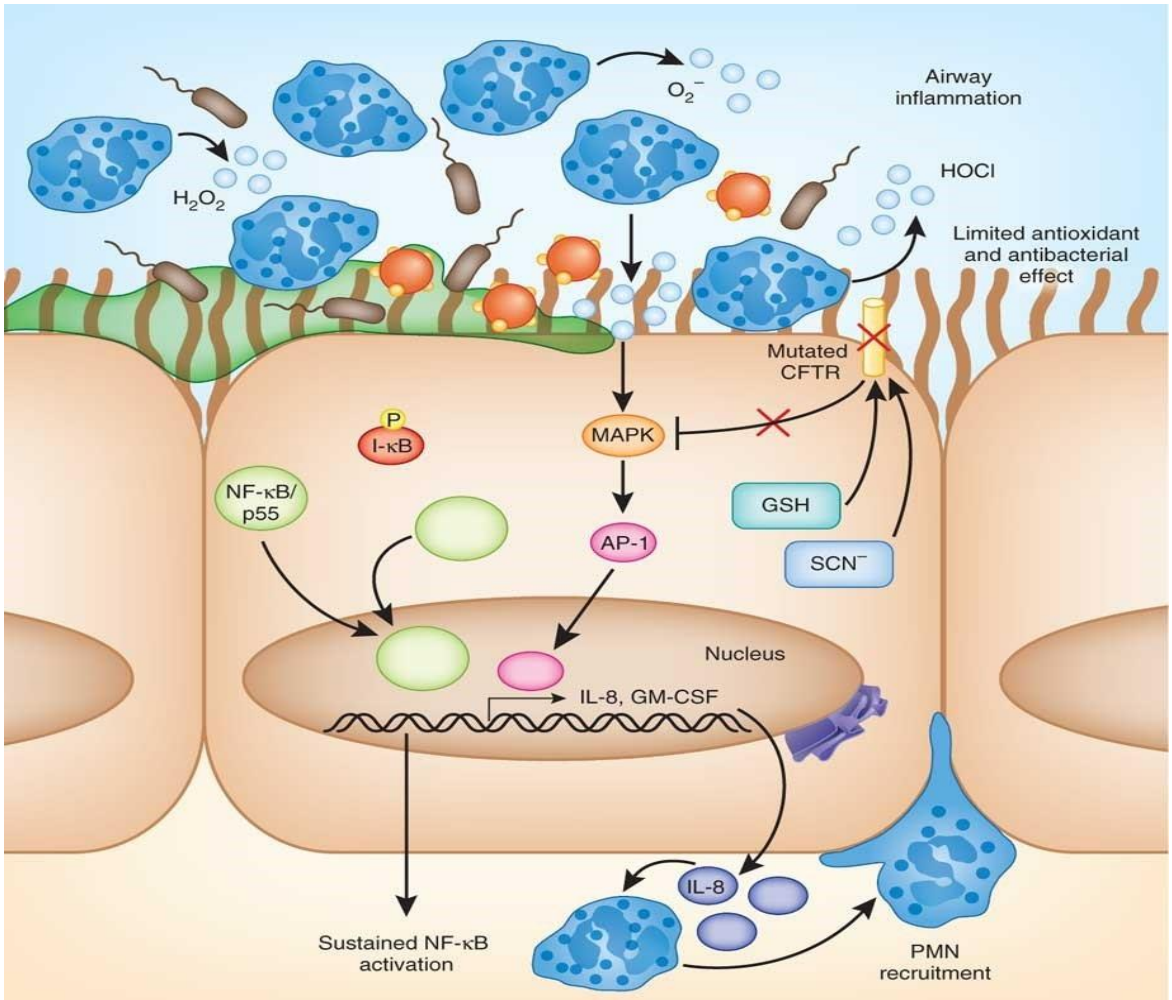


Figure 3.1.4 Biochemical processes related to the CFTCR gene mutation

This phenomenon takes the name of "advantage in favor of heterozygotes" and makes healthy carriers frequency very high (1 in 25) and the disease widespread. In CF, the mutated gene is the CFTCR (Cystic Fibrosis Transmembrane Conductance Regulator), which encodes for a protein of 1480 aminoacids located on the cell membrane of epithelial cells. These cells are present in lungs and upper airways, pancreas, intestines, sweat glands, salivary glands and deferens vases. Their function is to transport chlorine ions in the apical membrane, i.e. the membrane exposed on the cavity of the organ in question.[73] CF is therefore characterized by a fault in chlorine transport at cellular membrane level, resulting in the formation of an ionic imbalance which also causes an increased reabsorption of sodium and water. Because of this ionic stress, glands secrete a thick and sticky mucous (Figure 3.1.4). In affected organs, these abnormally slimy mucous secretions determine an obstruction of the main ducts causing the most typical clinical manifestations of the disease, such as recurrent pulmonary infections, pancreatic insufficiency, steatorrhea, malnutrition states, liver cirrhosis, intestinal obstruction and male infertility.[74]
By focusing on lungs, airways obstruction caused by mucous accumulation generates various issues including the onset of inflammations and infections. As a matter of facts, the lung conditions so determined, allow bacteria that normally inhabit the mucous membranes to grow out of control. This growth can cause pneumonia, and *S. aureus*, *P. aeruginosa* and *Haemophilus influenzae* are the three most common organisms in cystic fibrosis patients (Figure 3.1.5).

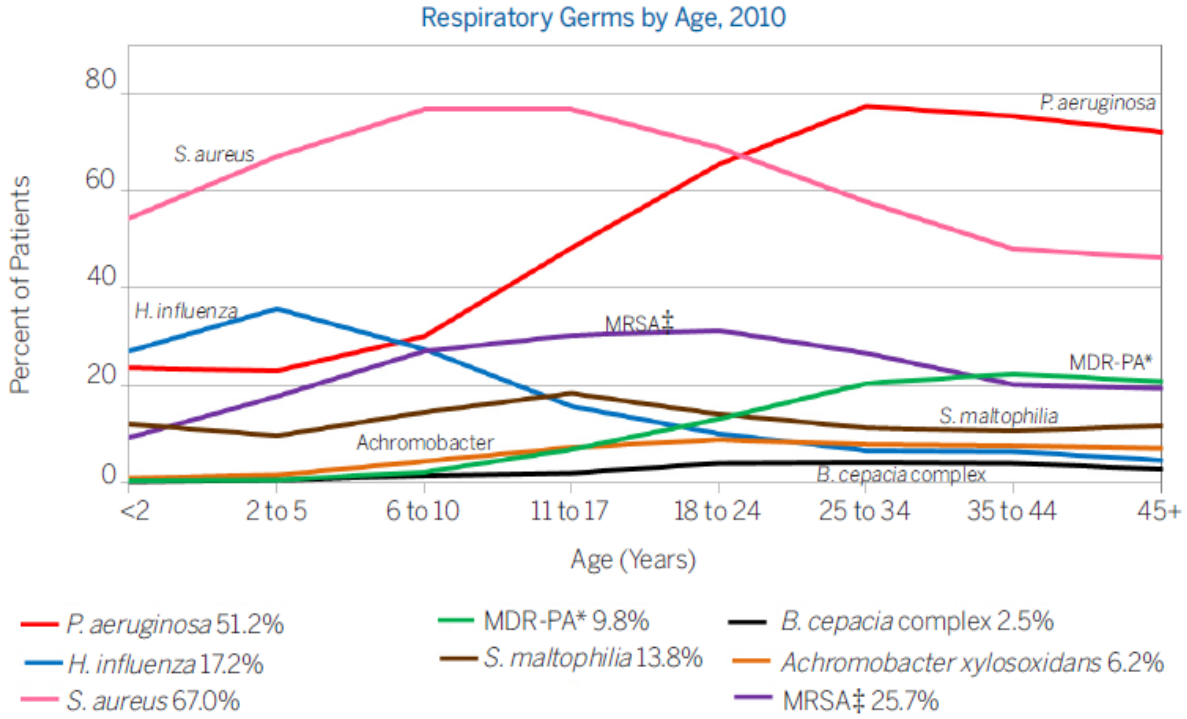


Figure 3.1.5 Infections incidence related to patients' ages

However, less common pathogens can not be easily neglected. For example, bacteria such as *B. Cepacia* often determined drastic living conditions worsening of CF patients. Moreover, since the average life expectancy is around 37 years and CF patients are subjects of frequent and long hospitalizations,

infections appear to be often by resistant bacterial strains, further complicating the treatment of the disease.

Inflammation phenomena is a physiological response to infections. In CF patients' lungs, epithelial cells release highly reactive molecules (ROS and RNS, Reactive Oxygen and Nitrogen Species) in the extracellular space to counteract pathogens. This is the so-called oxidative stress phenomenon.[75]

However, several studies showed the inefficient these defenses, especially against infections by resistant strains. In CF patients, the production of these chemical species increases during years, probably due to a progressive dysfunction of cells. Moreover, not only the chlorine transport is affected by the CF, but it is also altered the transport of other small molecules, including glutathione, an endogenous antioxidant. All these aspects promoted a ROS and RNS accumulation in extracellular spaces, attacking the patient tissues and thus increasing symptoms.

3.1.3 New β-lactam antibiotics

The group with whom I did my Ph.D. is working since a long time on the synthesis and design of potential β-lactam leads able to counteract resistant bacterial strains. In particular, a completely new class of β-lactams called 4-alkylidene β-lactams was designed and synthesized.[76] Their structures were designed with the objective of increasing the electrophilicity of the amide carbon, promoting the nucleophilic attack of bacterial transpeptidases (Figure 3.1.6). The C-4 double bond allows the stabilization of the ring opening product through delocalization by resonance on the unsaturated system. Other functionalization evaluated from our group regarded the derivatization of nitrogen atom[77] and the introduction of particular functions on the C-3 β-lactam position.[78]

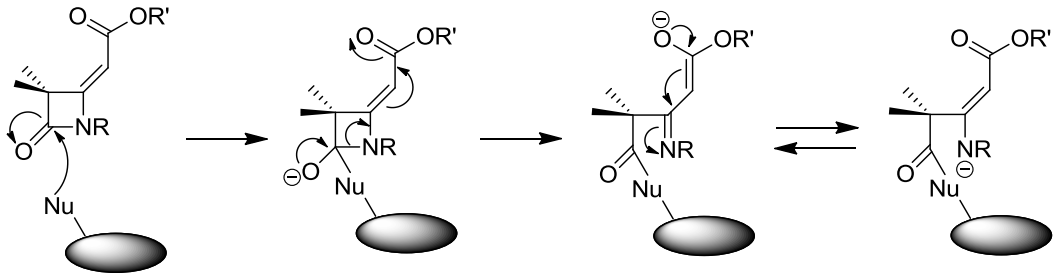


Figure 3.1.6 4-Alkylidene β-lactams reactivity towards transpeptidases

The antibacterial activity of 4-alkylidene β-lactams was assessed by the research group of Prof. Cocuzza, University of Milan-Bicocca. The evaluation allows to determine the antibiotic activity in vitro and to calculate MICs (Minimum Inhibitory Concentration, expressed as mg/L) on Gram-positive and Gram-negative bacterial strains, with high resistance and β-lactamase producing strains and not (*Streptococcus pyogenes* and p*neumococci*, methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*,

Staphylococcus epidermis and *Haemophilus influenzae*). The best results were obtained from the molecules in Table 3.1.1, showed with the relative MIC values, expressed in mg/L.

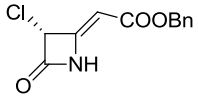
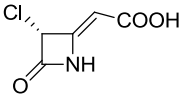
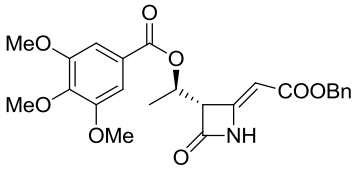
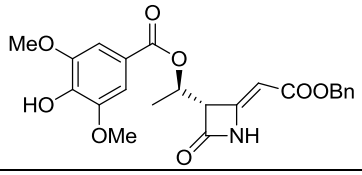
Compound	S. Pyog.	S. Pneum.	MRSA	MSSA	S. Epiderm.	H. Infl
	32	128	16	8	8	1
	16	128	128	32	64	64
	128	>128	128	8	4	>128
	128	128	32	16	2	64

Table 3.1.1 MICs for some 4-alkylidene β-lactams

The encouraging results obtained led to further modifications on the 4-alkylidene structures, in particular synthesizing 4-alkylidene β-lactams characterized by a particular substituent on the nitrogen atom: the thiomethyl group (-SMe). This allowed to synthesize a library of 4-alkylidene β-lactams characterized by a stronger lipophilicity, induced by that group, and interesting biological activities.

As a matter of facts, *N*-thiomethyl β-lactams were already studied for their biological activity against resistant bacterial strains such as MRSA and *Bacillus* species.[79] Moreover, the interest towards this class of biomolecules has evolved due to the biological activity shown especially against *Staphylococcus* strains.

The research group of Professor Turos focused his attention on synthesis and antibacterial properties of monobactam *N*-thiomethylated derivatives,[80] as well as on bicyclic derivatives. Chemical properties that this group gave to β-lactams were also highlighted:

- the entire substrate resistance against many of the nucleophiles used in organic synthesis (-CN, -N_3, alcohols) and biomolecules (such as glutathione and penicillanases, proteins responsible for penicillins degradation);
- highly lipophilic character;
- stability during the processes of chromatographic purification;

Furthermore, the idea is that the 4-alkylidenic structure favors not only the ring opening reaction, which is the typical reactivity for β-lactams antibiotic action, but also the thioalkylation of biological targets (nucleophiles with a sulfhydryl -SH, i.e. cysteine of enzyme active sites or other substrates bearing the same function).

4-Alkylidene β-lactams provide an increased reactivity due to the presence of a good electron-withdrawing group. We wondered that the same reactivity can be reached if in C-4 position were alternately present a good leaving group. An example could be the acetate group already present in some commercially available azetidinones commonly used in the synthesis of β-lactam derivatives. These substitution could allow an easier synthetic pathway of molecules which can be thought to present activities comparable to the ones of 4-alkylidene derivatives. Moreover, potencies could be increased by thiomethyl derivatization.

In every case, the comparison could allow to understand if on C-4 position an electron-withdrawing group able to delocalize an ionic charge is more or less important than the presence of a leaving group, thus defining an important structure-activity relationship for β-lactams.

3.1.4 New dual active β-lactams

As part of an interdisciplinary research project, involving the synthesis of new monocyclic β-lactams specifically designed to target resistant Gram-positive bacterial strains, a series of new azetidinones with polyphenolic side chains were evaluated for both their antibacterial[81] and antioxidant activities.[82] As a general trend, to activate the antioxidant potency of the tested compounds to a significant extent the presence of phenolic OH resulted necessary, with the exception of 4-alkylidene-thiophenol derivative (**1**) which had no phenolic residues but retained a certain antioxidant activity. More recently a series of *N*-methylthio-azetidinones were evaluated in vitro against Gram-positive and Gram-negative clinical isolates and in particular on MRSA and MSSA strains isolated from patients with cystic fibrosis.[83] The combination of an N-SMe group and a benzyl ester on the 4-alkylidene-side chain, or electronwithdrawing groups, such as OAc, on the C-4 position of the β-lactam ring, appeared to strengthen the potency against Gram-positive bacteria.

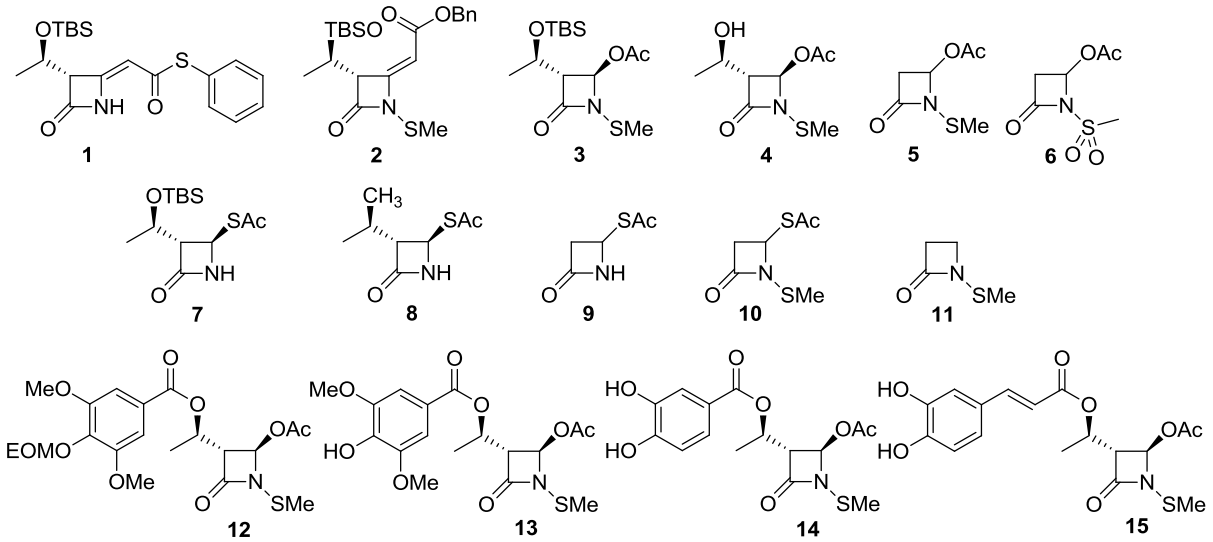
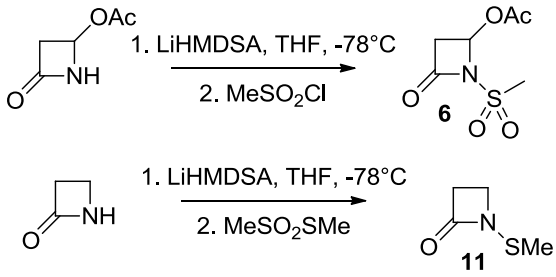


Figure 3.1.7 β-Lactam compounds tested for the antioxidant activity

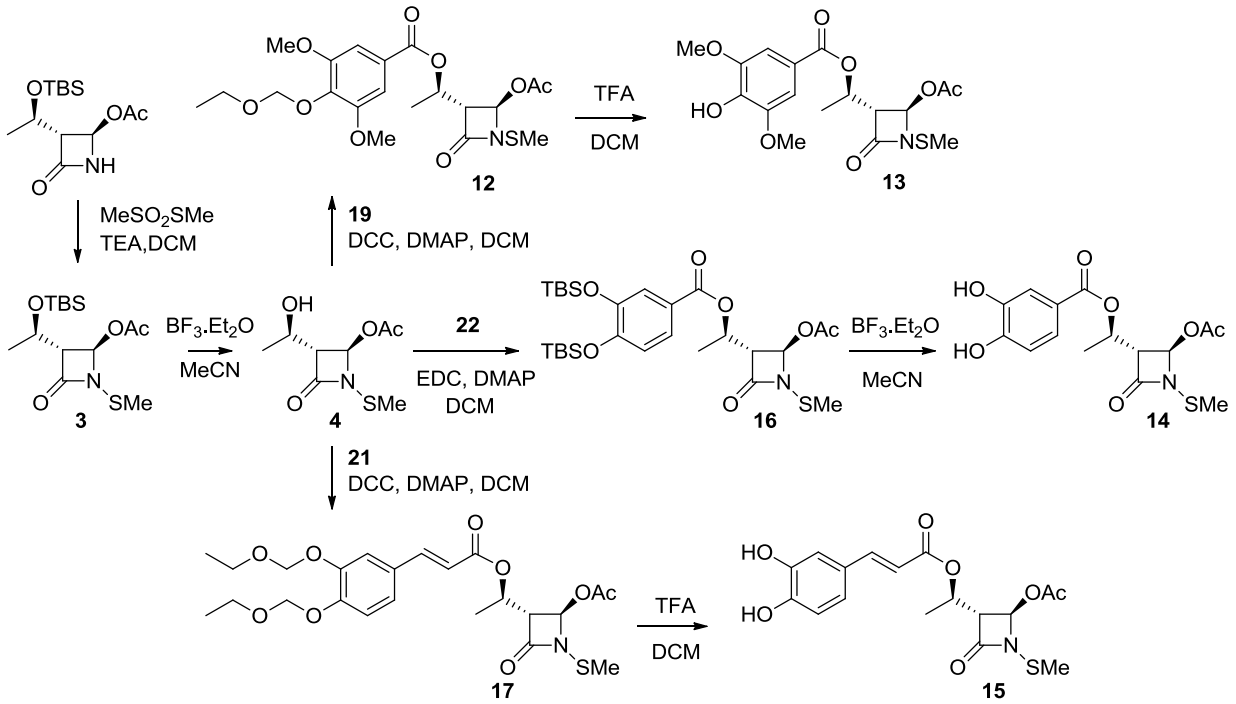
This study reports the evaluation of physicochemical parameters, such as the antioxidant activity and redox potentials, of some *N*-methylthio-azetidinones with antibacterial potency. The aim of this ongoing project was in fact to develop dual targeting molecules with elements incorporated in their structure that would allow dual antibacterial and antioxidant activities in order to contrast unfavorable clinical conditions, such as those resulting from bacterial colonization of the lung associated with extensive epithelial damage brought about by chronic pulmonary oxidative stress.

All 15 monocyclic β-lactams investigated here are depicted in Figure 3.1.7. Compounds **1**, **2**, **3**, **4**, **5**, **7**, **8**, **9**, and **10** were prepared according to previously reported procedures.[84] *N*-Methylsulfoxide-4-acetoxy-azetidin-2-one **6** (Scheme 3.1.1) was prepared starting from commercial 4-acetoxy-azetidin-2-one which was treated with LiHMDSA followed by addition of mesylchloride.



Scheme 3.1.1 Synthesis of β-lactams **6** and **11**

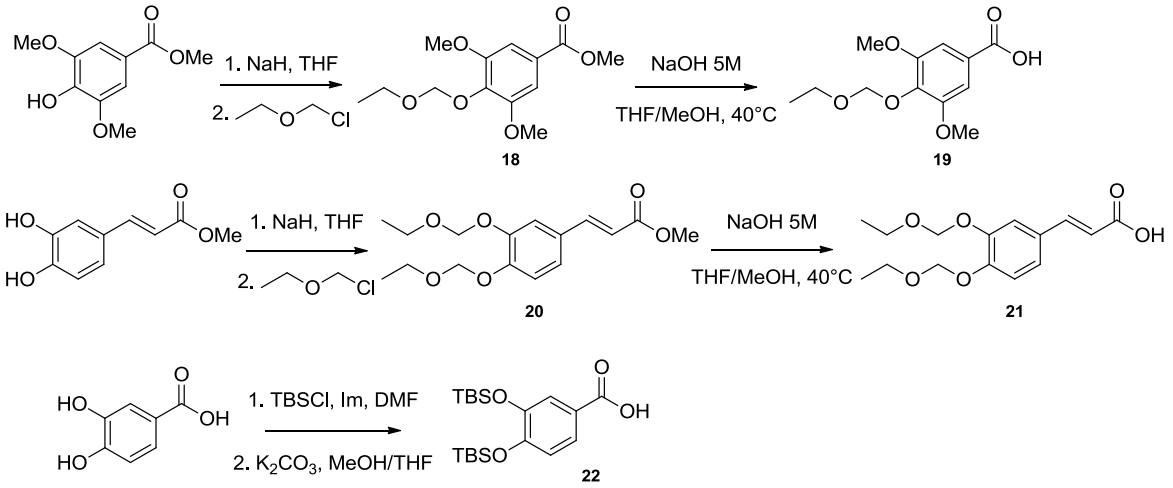
Analogously, N-methylthio-azetidin-2-one **11** was obtained treating commercial azetidin-2-one with LiHMDSA and then S-methyl methanethiosulfonate (Scheme 3.1.1).



Scheme 3.1.2 Synthesis of β-lactams **13**, **14**, **15**, **16**, **17** and **19**

Compounds **12**-**15** were prepared starting from the commercial available (3R,4R)-4-acetoxy-3-[(1R)-*t*butyldimethylsilyloxy-ethyl]-azetidin-2-one. The starting compound was *N*-thiomethylated to afford compound **3** using Et_3N as a base because LiHMDSA or LDA led to the formation of significant amounts of by-products. Compound **3** was then deprotected using $BF_3 \cdot Et_2O$ in MeCN on the 3-O-(*t*butyldimethylsilyl)-ethyl side chain to give the corresponding alcohol **4** (Scheme 3.1.2).

Treatment of **4** with the appropriate O-protected benzoic acid **19**, **21** or **22**, DCC or EDC as coupling reagents and in the presence of a catalytic amount of DMAP in dichloromethane, gave compounds **12**, **16** and **17**.



Scheme 3.1.3 Synthesis of acids **6**, **21** and **22**

As regards the benzoic acids, methyl 4-(ethoxymethoxy)-3,5- dimethoxybenzoic acid **19** and (E)-3-(3,4-bis(ethoxymethoxy)phenyl)acrylic acid **21** were prepared starting from syringic methyl ester or caffeic methyl ester, respectively through protection of the phenolic oxygen atoms with chloromethylethylether followed by alkaline hydrolysis of methyl esters **18** and **20** (Scheme 3.1.3).

Finally, compounds **12** and **17** were treated with trifluoroacetic acid (TFA) to eliminate the phenolic protection and to give compounds **13** and **15**. The ethoxymethylether as protective group was not effective in the synthesis of 3,4-dihydroxybenzoic ester **14** which was successfully obtained using TBS to protect the phenolic positions in compound **16**.

Antioxidant activity

Antioxidant activity determination based on different approaches was carried out, in light of the importance of a multidimensional evaluation of the antioxidant activity.[85] The β-lactams were then subjected to four antioxidant assays: the Briggse Rauscher (BR) oscillating reaction,[86] the Trolox Equivalent Antioxidant Capacity (TEAC) assay,[87] the DPPH (2,2-diphenyl-1-picrylhydrazyl DPPH· stable radical) test[88] and the Ferric Reducing Antioxidant Power (FRAP) method.[89] The majority of the synthesized β-lactams did not show appreciable antioxidant activity with BR, TEAC, DPPH, or FRAP

methods; moreover compounds **2**, **3** and **10** were also tested with the Foline Ciocalteu (FC) reagent[90] to check if the *N*-methylthio-β-lactam skeleton has some reductive properties, but the result was negative. N-unsubstituted compounds **8** and **9** gave also negative results to antioxidant tests. Only the *N*-methylthio-β-lactams **12-15** with phenolic moieties were active and showed values in Table 3.1.2 (a: *S. aureus* American Type Culture Collection ATCC 29213 and ATCC 43300 were used as control strains. b: na = no activity, nd = not determined. c: See reference [84]. d: Result of only one measure at 14.7μM in mixture. e: FOT = cefotaxime and CTX = ceftriaxone were used as reference antibiotics). TEAC values for compounds **14** and **15** are in line with those of some polyphenolic-β-lactams we previously reported, ranging from 0.4 to 2.0mM eq.Trolox. The DPPH and FRAP values are in satisfactory agreement with the TEAC ones taking into account the different chemistry of the methods. For comparison, the TEAC values for ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are 1.05 and 0.97 respectively, the DPPH values are 1.1 and 0.5 respectively, and the FRAP values is 2.0 for the two vitamins. As expected, compound **12** doesn't shows antioxidant activity with these three methods because it does not contain phenolic OH groups in the molecule (negative control). Compound **13** is a very low active antioxidant than the other two phenolic substituted molecules, a possible interpretation is given in next paragraph. The BR values for all compounds are surprisingly higher than those of some polyphenolic-β-lactams previously reported, ranging from 0.021 to 0.28μM eq. Re. We suspect that these unusual results are due to parallel reaction of oxidation of sulphur in the NSMe group by acidic iodate that compete with the HOO· scavenging reaction. On the contrary this parallel reaction doesn't occur with the usual *N*-unsubstituted β-lactams.

Compd.	BR (μM equiv Resorcinol)	TEAC (mmol equiv TROLOX)	DPPH (mmol equiv TROLOX)	FRAP (mmol equiv Fe)	Antimicrobial activity on MRSA from CF[a]		
					MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
4	na[b]	na	na	na	32->128[c]	64[c]	>128[c]
5	na	na	na	na	32−64[c]	32[c]	64[c]
8	na	na	na	na	16−128[c]	64[c]	128[c]
9	na	na	na	na	16−64[c]	32[c]	64[c]
10	na	na	na	na	16−32[c]	16[c]	32[c]
12	0.70[d]	na	Negligible	na	nd[b]	nd	nd
13	1.8 ± 0.3	0.037 ± 0.001	0.0091 ± 0.0002	0.19 ± 0.02	16−128	64	128
14	0.38 ± 0.02	1.23 ± 0.02	1.96 ± 0.13	1.91 ± 0.07	32 to >128	128	>128
15	1.95 ± 0.06	0.98 ± 0.03	1.23 ± 0.03	1.98 ± 0.04	16−128	64	64
FOT[e]					1 to >128	128	>128
CTX[e]					1 to >128[c]	8[c]	>128[c]

Table 3.1.2 Results of antioxidant and antibacterial tests

Structure-activity relationships

As a general trend, to activate the antioxidant potency of the tested compounds, the presence of two or three phenolic OH groups are necessary.[91] A theoretical method to calculate the Bond Dissociation Enthalpies (BDE) for molecules belonging to the class of polyphenols and to correlate them with their free radical scavenging activities has been reported by Wright et al.;[92] these authors also proposed empirical additivity rules that take into account the electronic, H-bond and conjugation effects of substituents in the phenol parent molecule to evaluate the BDE of a given phenolic OH group. In this way

a value of BDE or DBDE = (BDE_{comp} - $BDE_{\Phi\text{-}OH}$) can easily be calculated from the data reported in reference [93]. It was found that the number of phenolic OH is not so important and that it is the strategic placing of these groups with respect to other substituents that determine the strength of the OH group and then the antioxidant activity. From the data reported in reference [93], we calculated the DBDE values for two model compounds A and B in comparison with the free radical scavenging activity for compounds **13** and **14** (Figure 3.1.8).

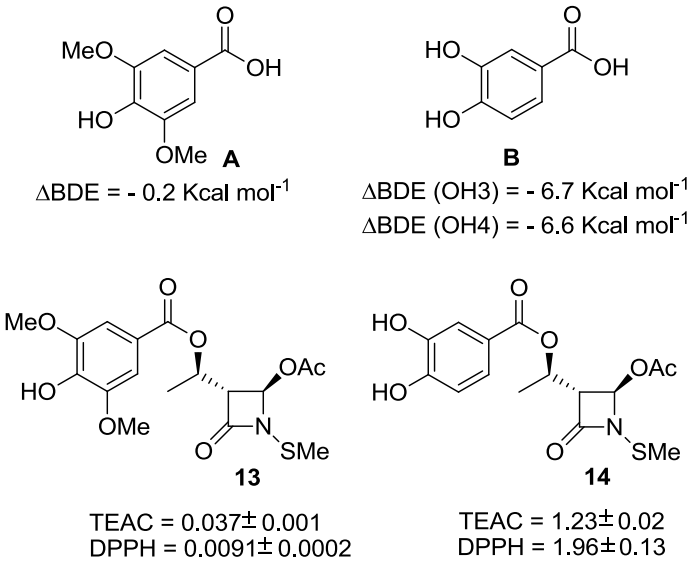


Figure 3.1.8 ΔBDE for and B in comparison with the for **13** and **14**

As can be seen, results for the model match well with the experimental data, thus justifying the very low free radical scavenger potency of compound **13** with respect to that of **14**.

Voltammetry

It is well known that thioethers can be oxidized to sulfoxides and further to sulfones by H_2O_2 or by many other oxidizing agents.[93] The negative results of the antioxidant assays gave evidence that in the experimental conditions the N-methylthio group was not prone to be oxidized. To interpret these results we decided to perform some cyclic voltammetry experiments on the two molecules **9** and **11** chosen as models. In the first molecule the bivalent sulphur atom is bonded to C-4 atom of the ring while in the second molecule the bivalent sulphur atom is bonded to the nitrogen atom of the β-lactam ring. Aim of the investigation was to measure the ability of such model compounds to undergo oxidation to evaluate their antioxidant activity through the measure of the corresponding standard potentials. We also submitted to cyclic voltammetry the thioester **1**, which has no antibiotic activity and that in a previous work [83] showed some antioxidant activity both in the BR or TEAC tests (acidic and neutral pH method, respectively), and explained with a possible generation of an intermediate radical cation $R_2S\cdot^+$. The voltammograms are reported in Figure 3.1.9. All species displayed in acetonitrile a very similar CV behavior showing, in the positive potential region, a main anodic peak at about 0.9V attributed to the

reversible oxidation of the pristine species. Weak-to-strong adsorption effects were observed in the CV pattern, leading in most cases to fouling of the electrode surface and thus to a highly irreversible behavior. On the basis of such results, and assuming that potentials determined under the aprotic and relatively apolar conditions of CV (Figure 3.1.9) may be directly used for comparisons in the aqueous media, all the investigated compounds should not show appreciable antioxidant activity towards TEAC, DPPH or FRAP antioxidant methods.

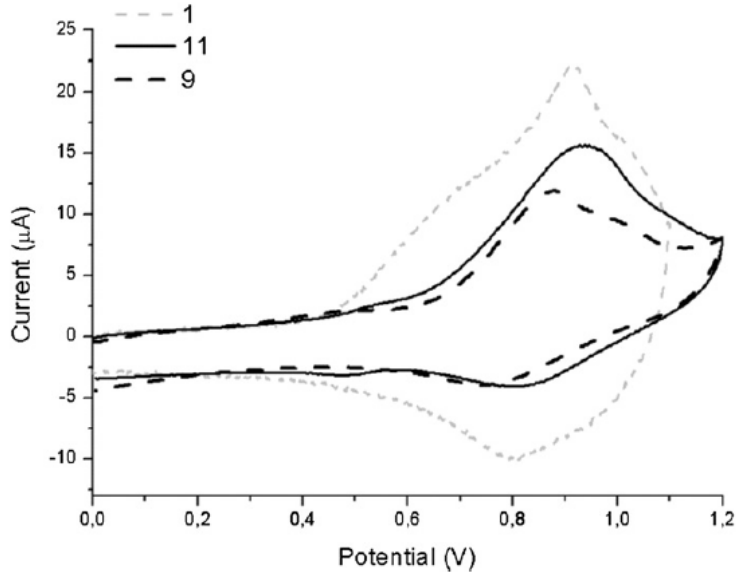


Figure 3.1.9 Current potential diagrams for compounds **1**, **9** and **11**

The redox potential of the couples ABTS·$^{+}$/ABTS (TEAC); DPPH·/ DPPH, $(TPTZ)_2Fe^{3+}$/$(TPTZ)_2$/Fe^{2+} (FRAP) are in fact 0.68V, 0.28V,[94] and 0.77V,[95] respectively, then ABTS·$^{+}$, DPPH·, $(TPTZ)_2Fe^{3+}$ can thermodynamically be reduced by compounds with lower reduction potential. This is likely applicable to all N-methylthio-β-lactams synthesized here, except for compounds **13**, **14** and **15**. These compounds in fact contain phenolic moiety in the side chain, and many phenolic compounds have lower oxidation potential thus reacting with ABTS·$^{+}$, DPPH· and $(TPTZ)_2Fe^{3+}$.

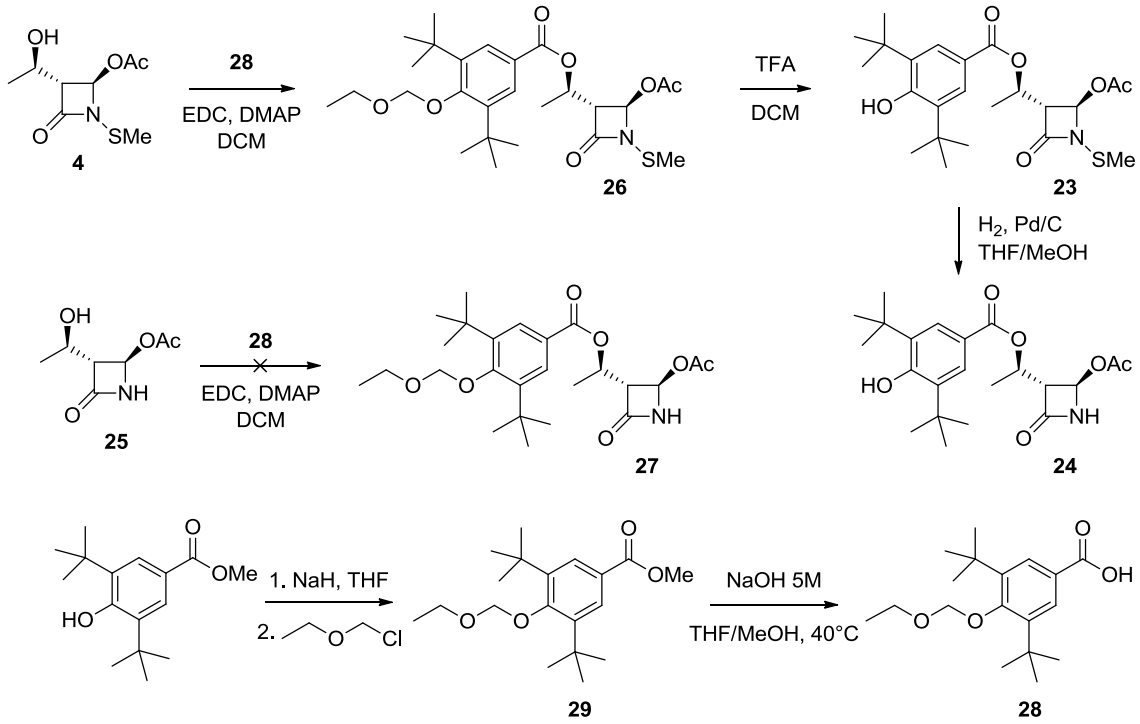
Antibacterial activity

In order to evaluate the potential antibacterial activity of the new β-lactam derivatives with anti-oxidant properties, the minimum inhibitory concentrations (MICs) of compounds **13-15** were determined in vitro against 45 clinical strains of MRSA isolated from CF patients. Preliminary results (see Table 3.1.2) on the antibacterial activity demonstrated compounds **13** and **15** to be the most active with MIC values ranging from 16 to 128 mg/L against the tested isolates; in particular compound **15** showed MIC_{50} and MIC_{90} values equal to 64mg/L. Table 3.1.2 summarizes the antioxidant and antibacterial data of exemplificative new β-lactams. Compounds **4**, **5**, **8**, **9** and **10** notwithstanding the potency against resistant S. aureus strains and the presence of a sulphur atom in the structure did not present a sufficient antioxidant activity. The dual activity is present in case of compounds **13**, **14**, **15** in which the presence of phenolic residues on

the hydroxyethyl-side chain switched on the antioxidant potency thus conjugated with an encouraging result of antibacterial activity.

3.1.5 Other β-lactam antibiotics and dual active compounds

To further investigate the potentialities of this interesting family of antioxidant and antibacterial β-lactams, more easy to synthesize if compared to the previous developed 4-alkylidenes, we designed two other β-lactam molecules in which the antioxidant moiety is the same of BHT. We reached the two desired targets by final deprotection of the phenolic moieties as well as described before (Scheme 3.1.4).



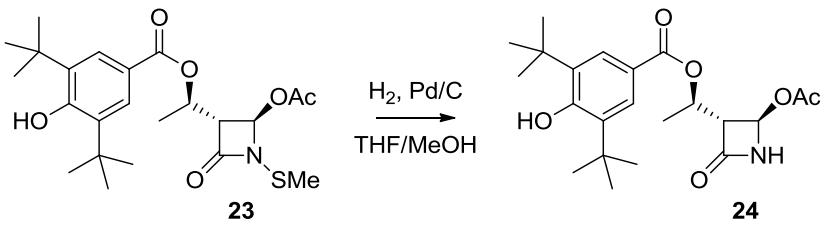
Scheme 3.1.4 Synthesis of dual active β-lactam derivatives **23** and **24**

As shown in the previous paragraph, the synthetic pathway to obtain the new lactams was maintained, only the benzoic acid used for the esterification step was changed. The carboxylic acid was prepared following the previously reported procedure with a methyl esterification followed by phenolic group protection and finally saponification of the acid function to reach intermediate **28**, as reported in Scheme 3.1.4.

Compound **24** was synthesized by hydrogenolysis from **23** A direct comparison between antibacterial potencies of **23** and **24** should account for the efficacy of N-thiomethyl group to the specific activity.. Synthesis of these *N*-thiomethylated derivatives, as shown in Scheme 3.1.4, requires alcohol **25** as β-lactam starting material. It corresponds to compound **4** unless for thiomethyl functionalization, and it was directly obtainable from the same commercially available substrate.

Unfortunately, the yield of all the esterification processes wereunsatisfactory, in particular, in this case it was not possible to get the β-lactam **27**.

However, we found an interesting reaction which was able to give the free NH converting an *N*-thiomethylated β-lactam by hydrogen treatment in the presence of Pd/C (Scheme 3.1.5). This observation was particularly interesting because it opens the route to NH derivatives poorly obtained fromesterification reactions.



Scheme 3.1.5 Synthesis of β-lactam **24** by de-thiomethylation of **23**

As will shown in the next paragraph, this couple of molecules already confirmed what we thought about the thiomethylation: it improves antibacterial activities of β-lactams, particularly bring a bacteriostatic effect and not properly an antibiotic action (Table 3.1.4).

To further investigate this effect, we designed other compounds in which acetoxy group was not present (Figure 3.1.10). In this way we tried to demonstrate both the importance of this leaving group in the position C-4 of the β-lactam ring and the value of antibacterial action bring by the thiomethyl moiety.

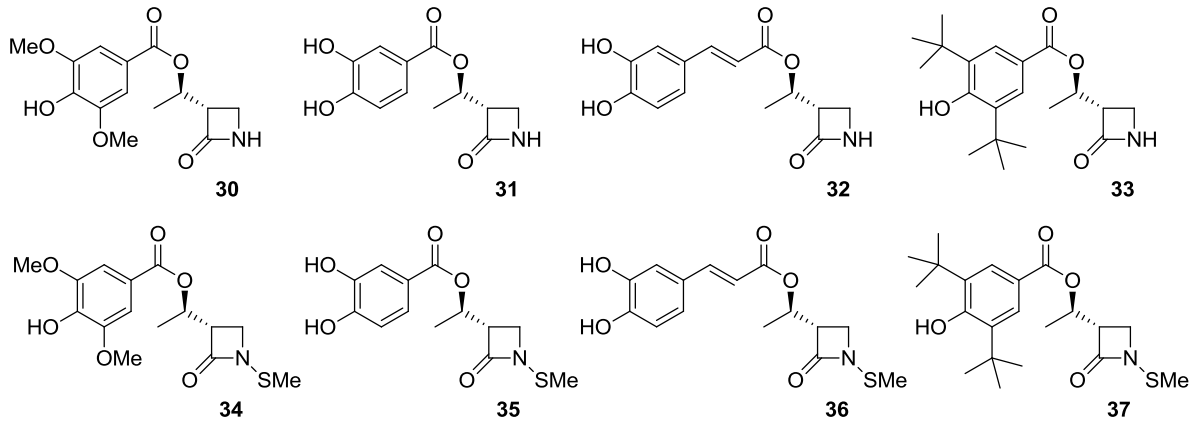
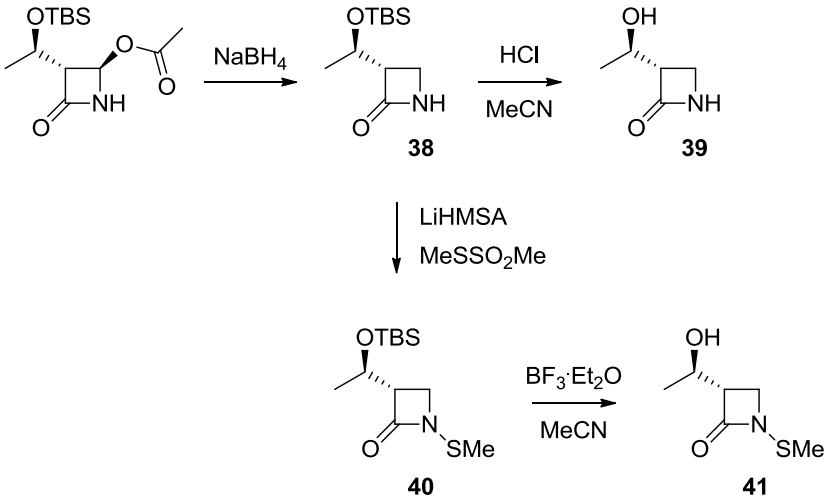


Figure 3.1.10 New β-lactams synthesized to demonstrate SAR hypothesis

Fortunately, we observed that treating our 4-acetoxy-β-lactam derivatives with a reducing agent such as sodium borohydride was possible to selectively remove the acetoxy group, thus obtaining a methylene group on the C-4 position (Scheme 3.1.6).

Thus, β-lactam intermediate **38** way directly obtained. The corresponding thiomethylated compound **40** was reached by the same thiomethylation reaction conditions mentioned above. The two intermediate

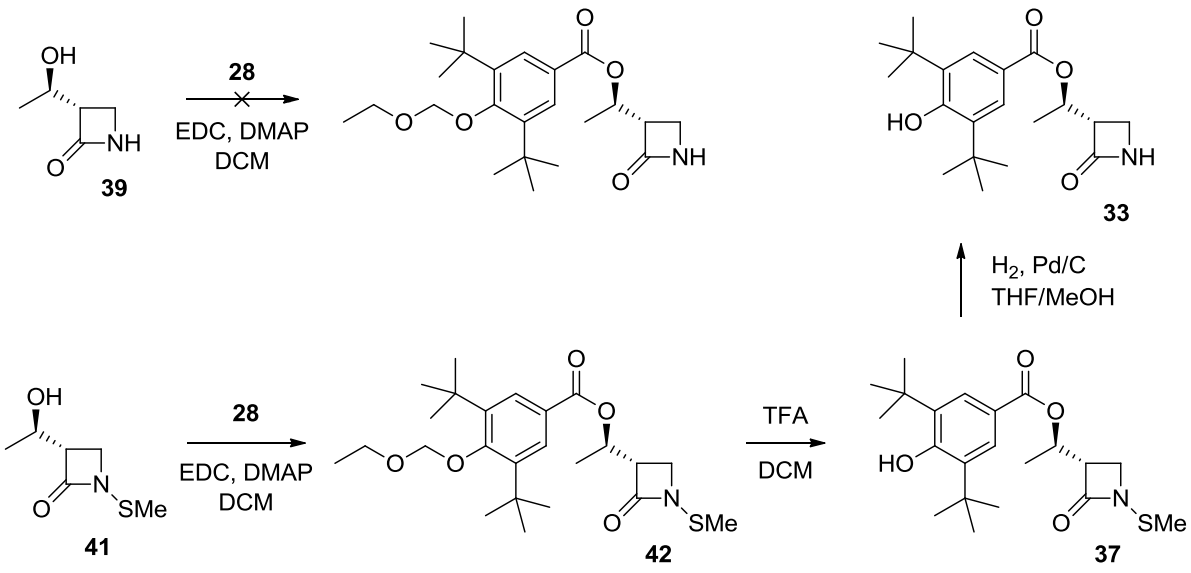
were both deprotected, with an acid treatment by HCl to reach **39**, and using BF_3 as Lewis acid to obtained **41**.



Scheme 3.1.6 Synthesis of β-lactams precursors **39** and **41**

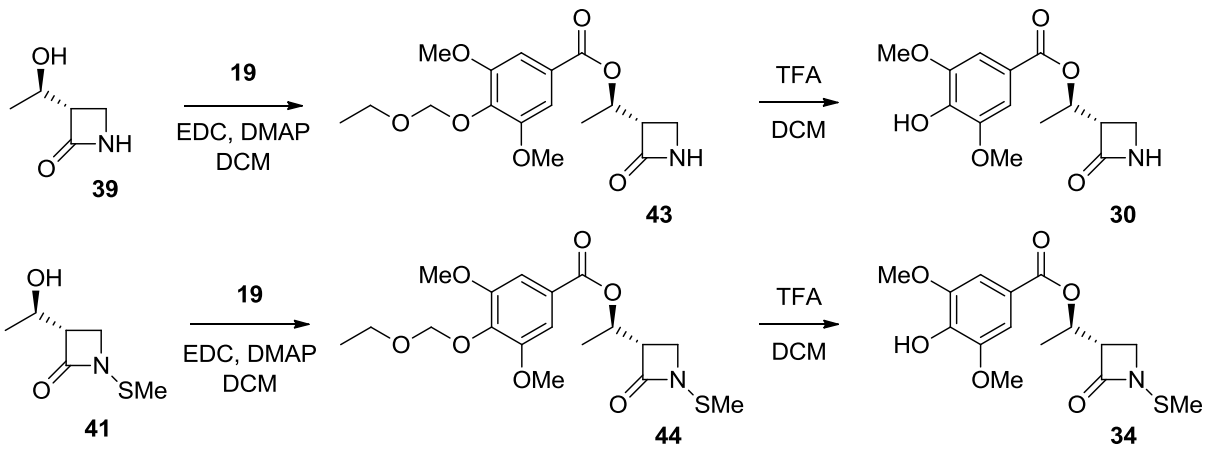
Using the appropriate protecting groups strategy we prepared alibrary of new molecules showed in Figure 3.1.7, tested for both antibiotic and antioxidant activities.

As well as for compounds **23** and **24**, the β-lactam **33** was prepared by hydrogenolysis treatment to remove the thiomethyl moiety from compound **37** (Scheme 3.1.7).



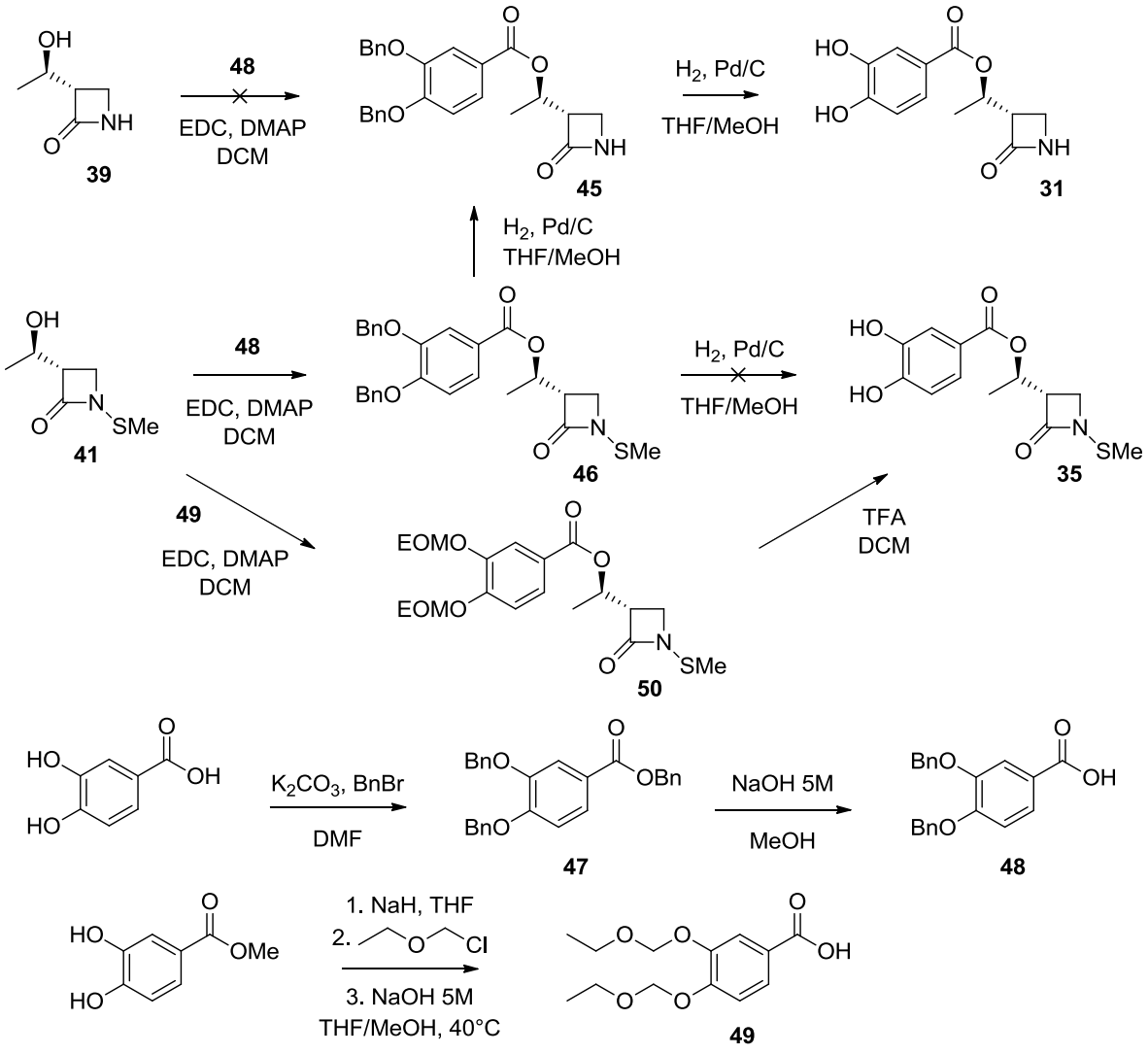
Scheme 3.1.7 Synthesis of compounds **33** and **37**

For compounds **30** and **34** we were able to maintain the synthetic pathway equal to what shown in the previous paragraph, modifying the esterification process just changing the β-lactam substrate (Scheme 3.1.8).



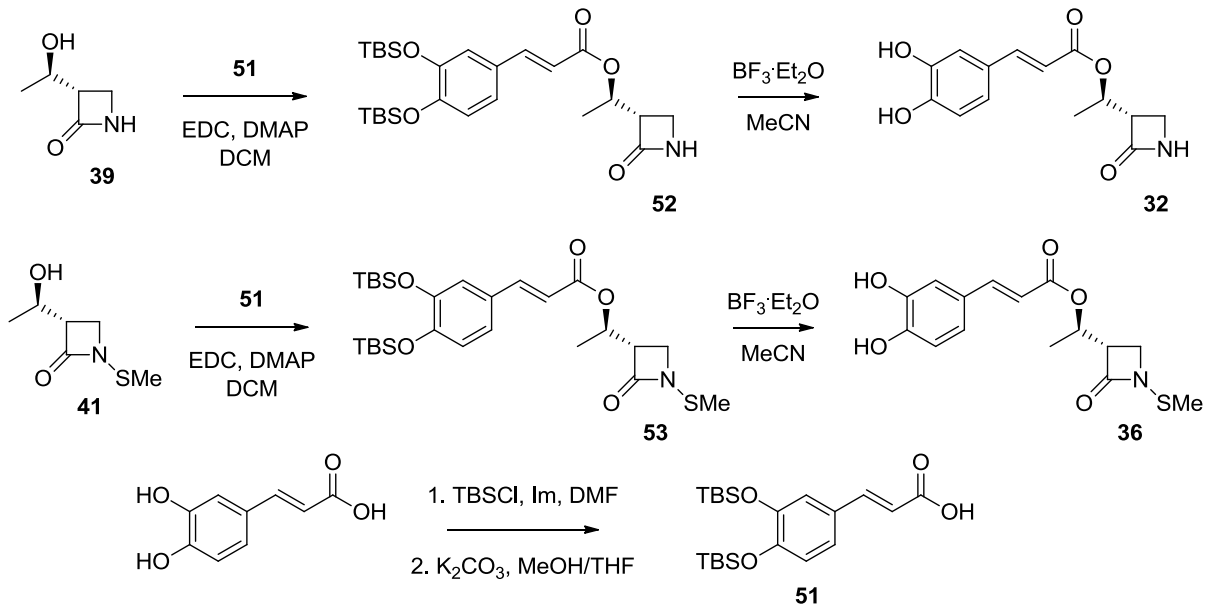
Scheme 3.1.8 Synthesis of compounds **30** and **34**

In case of compounds **31** and **35** it was necessary to differentiate the protecting group strategy due to the inefficiency of esterification processes or difficulties in deprotection steps (Scheme 3.1.9).



Scheme 3.1.9 Synthesis of compounds **31** and **35**, and of acids **48** and **49**

The preparation of compounds **32** and **36** required a different protecting group strategy.



Scheme 3.1.10 Synthesis of compounds **32** and **36**, and of acid **51**

In this case, we found in the *t*butyldimethylsilyl ether (OTBS)the best choice for protecting phenolic groups inthe esterification. The TBS could be removed by Lewis acid treatment, such as boron trifluoride (Scheme 3.1.10).

Antibacterial and antioxidant activities

All the new compounds showed an interesting antioxidant activity, as shown in Table 3.1.3 for the more antioxidant molecules however only N-thiomethylated derivatives had a certain antibacterial activity and, in particular, it was established that they were bacteriostatic agents.

	BR, µM eq. Resorcinol	TEAC, mM eq. Trolox	DPPH, mM eq. Trolox	FRAP, mM eq. $FeSO_4$
23	only > 37.2 in mix	0.10 ± 0.01	negligible	negligible
24	0.99 ± 0.04	1.26 ± 0.02	0.8 ± 0.2	2.05 ± 0.03
30	only > 46.5 in mix	negligible	negligible	negligible
34	only > 41.9 in mix	negligible	negligible	negligible

Table 3.1.3 Antioxidant activities evaluation for β-lactams **23**, **24**, **30** and **34**

This demonstrated that an electron-withdrawing or a leaving group, on the C-4 position, was fundamental for this kind of biological activity (Table 3.1.4, with activities evaluated over more than twenty different clinical bacterial strains isolated from CF patients and compared with of Linezolid, Ciprofloxacin, and Cefuroxime as reference antibiotics). Only selected antibiotic activities are herein reported, but it clearly appered that the best MIC value belongs to compound **23**, which has both Nthiomethyl- and the acetoxy group on the C-4 position of the β-lactam ring.

Due to the interesting enhancement of antibacterial activity brought from introduction of the thiomethyl group, we took some C-3 halogenated β-lactams already known from our group and we prepared their

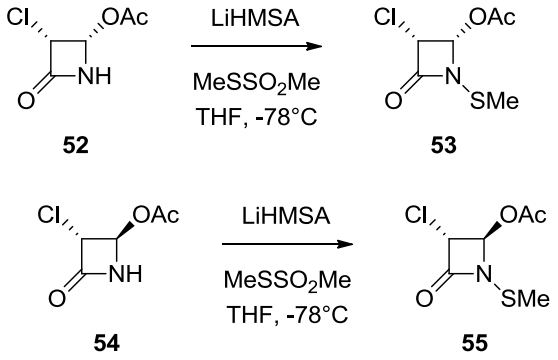
corresponding *N*-thiomethyl derivatives (Scheme 3.1.11). Being the synthesis of the chlorinated molecules not stereoselective, the two diastereoisomeric precursors were separated by flash chromatography and derivatized.

	S. aureus strains	S. faecalis strains	S. hominis strains	S. epidermidis strains
23	4 - 128			
24	> 128	> 128	> 128	> 128
30	> 128	> 128	> 128	> 128
34	> 128	> 128	> 128	> 128
36	32 - 128	128	128	32 - 64
Linezolid	0.5 - 2	1	32	8 - 16
Ciprofloxacin	1 - 128	4	> 128	> 128
Cefuroxime	2 - > 128	> 128	8	16 - > 128

Table 3.1.4 Antibiotic activities for β-lactams **23**, **24**, **30**, **34** and **36**

Unfortunately, as for their precursors, the new β-lactam **53** and **55** showed a certain cytotoxicity when tested in vitro and were therefore left apart.

To conclude the behavior analysis of *N*-thiomethyl-β-lactam compounds, we found MIC ranges of 0.5-32mg/L for **54** and of 2-32mg/L for **52**, to be compared with very variable and worse range for compounds **53** and **55** (8->128mg/L).



Scheme 3.1.11 Synthesis of compounds **53** and **55**

The antibacterial data obtained let us to conjectured that this group maybe determines a different mechanism of action which need to be associated with other structure features for a giving a lead antibiotic drug.

3.1.6 Thio-methylation reaction improvements

Because of the interest in the N-thiomethylation reaction, some efforts were dedicated to its optimization. We worked on the two commercially available starting materials depicted in Figure 3.1.11 as model substrate.

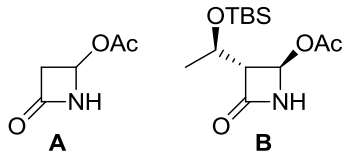
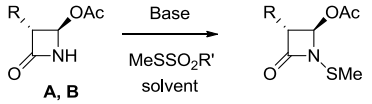


Figure 3.1.11 Compounds used for optimizing thiomethylation reaction

As reported in the Table 3.1.5, despite the variation of a lot of parameters, such as the base used and its equivalents, the amount of the thiomethylating agent and the reaction medium, it was never possible to reach high yields. obtained after flash chromatography purification and in other cases evaluated from ^{1}H-NMR

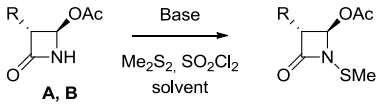


SM, [mmol]	base, eq.	$MeSSO_2R'$, eq.	solvent	y%
B, 1.0	LDA, 1.0	Me, 2.5	THF	35
B, 0.25	LDA, 1.0	MeC_6H_4, 2.5	THF	38
B, 0.25	TEA, 2.7; DMAP, 0.1	Me, 2.5	DCM	20
B, 0.25	TEA, 2.0; DMAP, 0.1	Me, 1.5	DCM	38
B, 0.25	TEA, 2.0; DMAP, 0.1	MeC_6H_4, 1.5	DCM	11
B, 0.25	TEA, 2.0; DMAP, 0.1	Me, 1.5	THF	~0
B, 0.25	TEA, 0.2	Me, 2.5	DCM	10
B, 0.25	DiPEA, 2.0; DMAP, 0.1	Me, 1.5	DCM	4
A, 0.25	DBU, 2.0	Me, 1.5	DCM	not determined
B, 0.25	NaH, 1.1	Me, 2.5	THF	8
B, 0.25	K_2CO_3, 2.0	Me, 1.5	acetone	18
B, 0.25	Imidazole, 2.0	Me, 1.5	DMF	~0
B, 0.25	Pyridine, ~123.6	Me, 1.5	pyr	~0
B, 0.25	*t*BuOK, 1.1	MeC_6H_4, 1.5	THF	~0
B, 0.25	TEA, 2.0	Me, 1.5	DCM	<20

Table 3.1.5 Base, solvent and thiomethylating agent optimization

It appeared that the best conditions were obtained with LDA as base in THF as solvent, or with the couple TEA and DMAP in DCM, but the temperature of -78°C and the general conditions necessary for using LDA prompt us to prefer the second procedure.

The introduction of a more reactive thiomethylating agent appeared to be fundamental for improving the process yield. Thus, we tried to produce in situ and immediately use a form of chlorinated thiomethyl group. Using sulfuryl chloride, a chlorine source, and dimethyl disulfide, we were able to generate this reactant. Thus, we studied the conditions for its preparation and use, optimizing this promising procedure (Table 3.1.6).



SM, [mmol]	base, eq.	Me_2S_2/SO_2Cl_2 eq.	solvent	[h]	T [°C]	TM/SM
B, 0.5	TEA, 1.1	0.5/0.55	DCM	1	r.t.	10/4
B, 0.5	-	0.5/0.55	DCM	1	r.t.	1,5/10
B, 0.5	TEA, 1.1	1.0/1.1	DCM	1	r.t.	8,4/10
B, 0.5	TEA, 2.2	1.5/1.65	DCM	1	r.t.	10/6
B, 0.5	TEA, 1.1	0.5/0.55	DCM	1	r.t.	10/4,5
B, 0.5	TEA, 2.2	1.0/1.1.	DCM	1	r.t.	10/4,5
B, 0.5	TEA, 1.1	0.5+0.5/0.55+0.55	DCM	1+1	r.t.	10/5
B, 0.5	TEA, 1.1	0.5/0.55	DCM	1	reflux	10/2,5
A, 0.5	TEA, 1.1	0.5/0.55	DCM	1	r.t.	10/6
B, 0.5	TEA, 1.1	0.5/0.55	DCM	1	reflux	10/5
B, 0.5	TEA, 1.1	0.5/0.55	DCM	4	30-35	10/13
B, 0.5	TEA, 1.1	0.5/0.75	DCM	1	r.t.	10/13,5
B, 0.5	TEA, 1.1	0.75/0.5	DCM	1	r.t.	10/5,5
B, 0.5	TEA, 2.0	0.5/0.55	DCM	1	r.t.	10/8
B, 0.5	TEA, 1.1	0.5/0.55	DCM	16	r.t.	10/5,5
B, 0.5	TEA, 1.1	0.5/0.55	MeCN	1	r.t.	3,5/10
B, 0.5	TEA, 1.1	0.5/0.55	THF	1	r.t.	0/10
B, 2.0	TEA, 1.1	0.5/0.55	DCM	1	r.t.	10/6,5
B, 0.5	DiPEA, 1.1	0.5/0.55	DCM	1	r.t.	10/7,5
B, 0.5	TEA, 2.2	0.5/0.55	DCM	1	r.t.	10/13

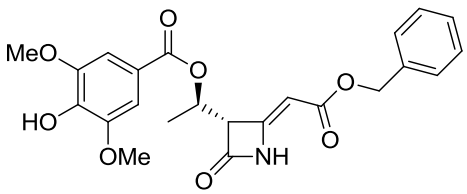
Table 3.1.6 Cl-SMe thiomethylation reaction conditions evaluation

In this way, we reached yields up to 80% with reagent very less expensive of the others previously used. Moreover, yields can be seen as conversions, thus SM could be recovered untouched and re-used.

3.2 A new lead dual active β-lactam

3.2.1 Lead β-lactam synthesis optimization

From the library of new β-lactam compounds developed one derivative emerged as a possible lead compound to testin vivo the bioavailability. The molecule identified for the treatment of CF patients as the optimal one, due to its entire set of features, had been already synthesized by our group and was previously treated in Table 4.1.1 . Lead drug **56** is herein depicted in Figure 3.2.1.

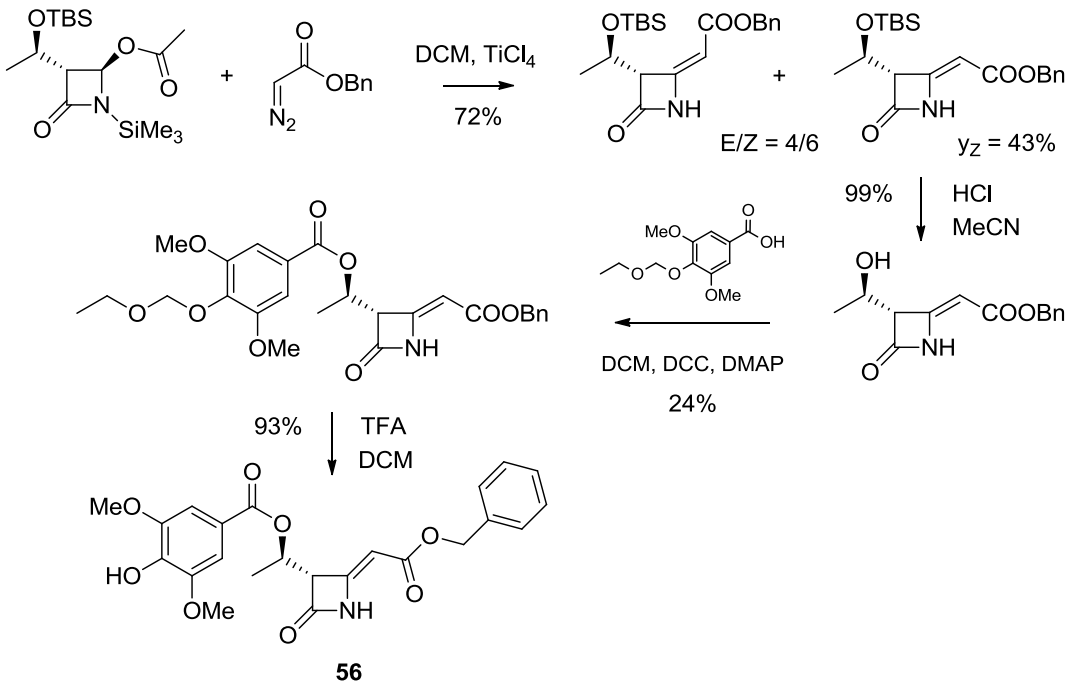


56

Figure 3.2.1 Lead dual active β-lactam **56** chosen for in vivo evaluation

At first, was necessary to optimize its synthesis up to the gram-scale. Of course, for doing that we came back to its original synthesis (Scheme 3.2.1, as previously reported from our research group): there was two main crucial steps: *i.*the formation of two diastereoisomers in the 4-alkylidene synthesis with a yield never exceeding 50%; *Ii.* the esterification, always critical for this kind of molecules.

The synthesis of 4-alkylidene had already been studied in the past: although it was proved to be possible an almost completely selective formation of E isomer, it has never been possible to reach the opposite. However only the product with the Z configuration has the higherantibacterial activity.



Scheme 3.2.1 Synthetic pathway for **56**, to be optimized

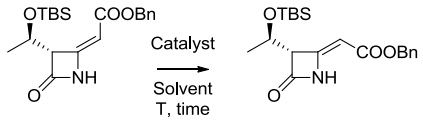
For this reason it was decided to study and develop an isomerization reaction, to convert the not useful E-isomer into the Z one. Then, we worked on the esterification reaction itself: it was made in peptide coupling condition without good yields. However, other more classical conditions for esterification were unsuccessfully tried, such as both Lewis and Brönsted bases and acids catalyzed processes, Mitsunobu reaction, Yamaguchi protocols ,and various activation methodologies.

E to Z isomerization

Previous studies have shown that the Z isomer is more thermodynamically stable than the E, due the formation of an hydrogen bond between the NH and the carbonyl group of the C-4 side chain. Despite this, it was never observed a spontaneous isomerization of the pure solid compound, even after long times at r.t. and exposed to air.

Then, it was evaluated the possibility to thermal driven isomerization. Moreover, we studied the opportunity of making the isomerization with a catalyst such as Amberlyst 15 (an acid resin), DMAP (an

organic base) and $CsCO_3$ (an inorganic base), as shown in Table 3.2.1, E/Z ratios were evaluated by ^{1}H-NMR analysis.



SM, [mmol]	Catalyst	Solvent	T [°C], t	E/Z ratio
0.05	-	MeCN	50°C, 4h	1/0.12
0.05	Amberlyst H-15	MeCN	r.t., 12h	1/0.19
0.05	DMAP	MeCN	r.t., 12h	1/0.93
0.05	$CsCO_3$	MeCN	50°C, 12h	-
0.05	DMAP	MeCN	50°C, 4h	1/ 9

Table 3.2.1 E/Z isomerization conditions optimization

As previously indicated , E/Z ratio were evaluated referring to C*H*CONH signals of ethyl side chain on the C-3s. In Figure 3.2.2 are clarified the signals used for data presented just now. Given the positive results obtained with DMAP as catalyst, the last test was performed maintaining the same conditions of the third entry (in Table 3.2.1) a part of temperature, increased to 50°C. It allowed a 90% formation of the desired Z isomer.

Thus, for isomerization we identified as optimal reaction conditions the use of MeCN as solvent, DMAP as catalyst in 10% loading, and 4h stirring at 50°C as best time and temperature setting.

To deeper analyze this conversion process, we set on a kinetic isomerization study. Working in MeCN, with a boiling point of 81.6°C (354.6K), we designed a set of experiments at different temperatures: 258K, 273K, 303K, 323K and 343K.

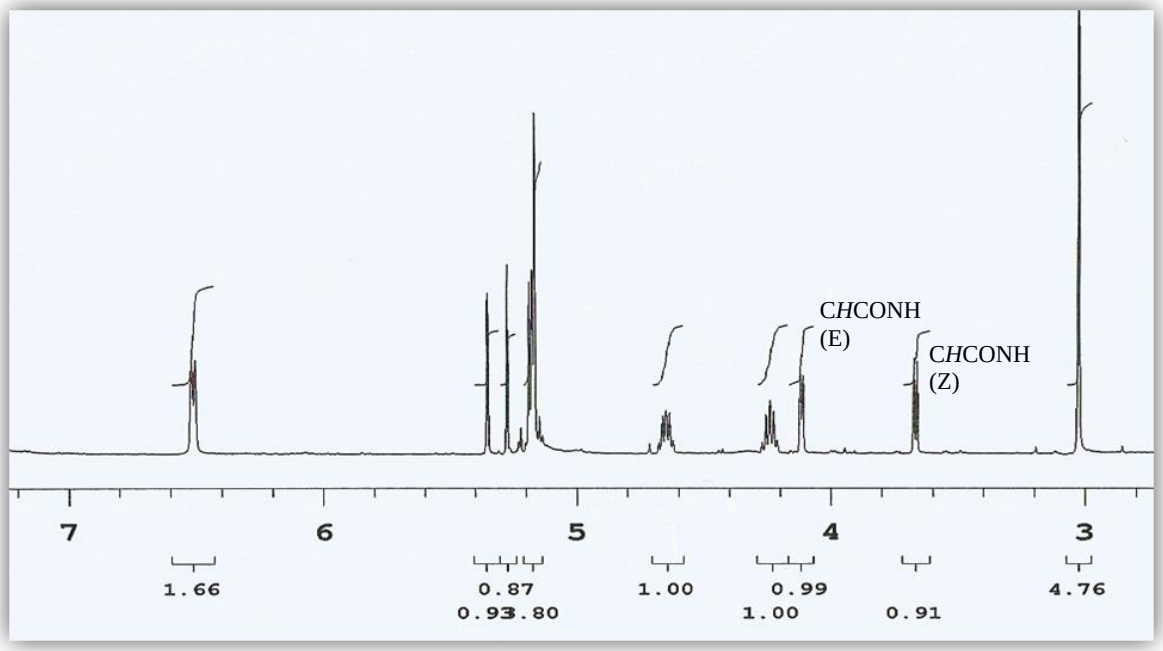


Figure 3.2.2 ^{1}H-NMR zoomed spectra for a ~1/1 E/Z mixture

Being aware that after about 4h the reaction equilibrium is completely unbalanced toward the Z isomer, and wanting to avoid this, reactions were carried out only for 1h (for further experimental details see the Experimental section).

Evaluating E/Z ratio (Table 3.2.2) at the end, we could construct a curve from which evaluate an estimate value for the $\Delta G^{\ddagger}$ between E and Z isomers, starting from the Boltzmann distribution equation:

$\ln (Z/E) = 1/T \cdot (\Delta G^{\ddagger}/k_B \cdot N_A)$

As a matter of facts, from the curve was necessary to cut out the warmer data, maybe due to experimental difficulties in keeping constant the inside reagents concentration at a temperature so near to MeCN boiling point, so herein related will not reported at all.

Z	E	Z/E	ln (Z/E)	T [K]	1/T [K^{-1}]
0,13	1	0,13	-2,040220829	258	0,003876
0,18	1	0,18	-1,714798428	273	0,003663
0,30	1	0,30	-1,203972804	303	0,003300
0,46	1	0,46	-0,776528789	323	0,003096

Table 3.2.2 Data evaluated and calculated for E-Z $\Delta G^{\ddagger}$ determination

With reference to the Boltzmann equation, we made the curve of the logarithm of Z/E ratio as a function of the temperature inverse.

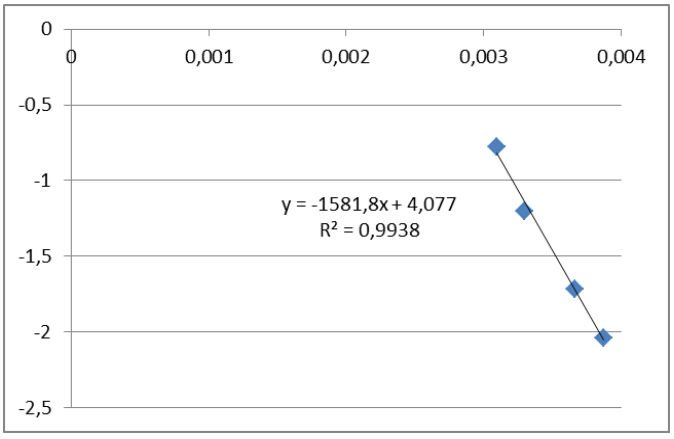
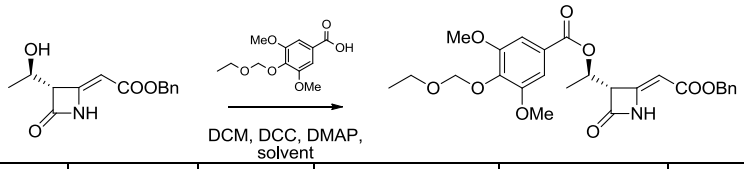


Figure 3.2.3 Curve obtained from the varying temperature experiments

The slope, reported on the graph showed in Figure 3.2.3, will be so directly related to $\Delta G^{\ddagger}$ between the two isomers. Thus, we were able to determine the value of $\Delta G^{\ddagger}$ between the two species in 3.14kcal/mol.

optimization of the Esterification reaction.

To optimize this step, we conducted different experiments varying a lot of parameters such as adding order of reagents, solvent, anhydricity of the system, base and activator, base and activator equivalents employed, scale of the process, temperature and MW irradiation. Every tentative exploited is reported in Table 3.2.3, with yield always obtained after FC purification.



SM, [mmol]	Acid	DMAP	DCC	Solvent	T or MW	Time [min]	y [%]
0.2	1.58	0.2	1.58	PhCl, 2mL	100W	10 (x4)	36
0.2	1.58	0.2	1.58	PhCl, 2mL	40W	10	36
0.2	1.58	0.2	1.58	PhCl, 2mL	40W	30	37
0.5	1.58	0.2	1.58	DMF, 2mL	40W	10	-
0.2	1.1	0.2	1.1	PhCl, 2mL	40W	10	5
0.2	1.58	0.2	1.58	PhCl, 2mL	r.t.	240	29
0.3	1.58	0.2	1.58	PhCl, 2mL	40W	10	18
0.2	1.58	0.2	1.58	PhCl, 2mL	400W	1	8
0.2	1.58	0.2	1.58	PhCl, 2mL	r.t.	240	10
0.2	1.58	0.2	1.58	PhCl, 2mL	r.t.	240	nd
0.2	1.58	0.2	1.58	PhCl, 2mL	r.t.	240	< 3
0.2	1.58	0.2	1.58	PhCl, 2mL	r.t.	240	< 3
0.2	1.58	0.5	1.58	PhCl, 2mL	r.t.	240	3
0.2	2.0	0.2	2.0	PhCl, 2mL	r.t.	240	< 3
0.3	1.58	0.2	1.58	PhCl, 2mL	50°C	120+120	34
0.2	1.58	1.0	1.58	PhCl, 2mL	r.t.	240	12
0.2	1.0	1.0	1.0, EDC	PhCl, 2mL	40W	10	< 22
0.31	1.58	0.2	1.58	PhCl, 2mL	40W	10 (x3)	63

Table 3.2.3 Esterification reaction optimization

Summarizing what we obtained, reactants introduction order must be β-lactam SM at first, followed by the solvent, then acid **19**, DMAP and DCC.

The reaction should be conducted using MW irradiation as a heat source, with a power of 40W for 10 minutes. Alternatively, the best results were obtained at r.t. for 4h stirring. Chlorobenzene was shown to be the best solvent, while as the activator was optimal the use of DCC, although it is more difficult to remove during work-up and FC purification. An increase of DCC and acid equivalents was excluded: 1.58 equivalents were sufficient in both cases, while for DMAP 0.2 equivalents seemed to be enough.

Working in solvents saturated with water and without a protecting atmosphere, comparing to the use of commercial anhydrous solvents and an inert atmosphere and anhydrous reaction conditions, appeared to furnish similar results. This allows to reduce the operating costs and to simplify the experimental procedure.

Increasing the scale up to 0.3mmol seemed to furnish an increase of the yield, but the use of non-protected syringic acid (data not reported) was shown to be impossible in these optimized conditions.

3.2.2 thermostability, lipophilicity and cytotoxicity of the Lead β-lactam

Wanting to support **56** on a nano-carrier (i.e. Solid Lipid Nanoparticles, in collaboration with Nanovector s.r.l., Turin) for its delivery, have been initially assessed its thermal stability. As a matter of facts, the conveying process necessary for the formulation requires to withstand for ten minutes at a temperatures of

about 50°C. For this reason, compound was placed in water and stirred at 60°C for 1h: HPLC-MS and 600MHz ^{1}H-NMR analysis showed no decomposition. As further confirmation, the same sample was brought to 70°C for 30min, checking again the complete absence of signals associated to degradation, either by HPLC-MS and by 600MHz ^{1}H-NMR (for further details, see Experimental section).

Lipophilicity in terms of logP and water solubility of our compound were both evaluated, in order to better set out the conveying conditions and to optimize their formulation (always in collaboration with Nanovector s.r.l.):

56: 1.58 (logP, ChemBiodraw est.), 23.044 mg/L (Wat. Sol., v1.01 est.)

In collaboration with Prof. C.E.A. Cocuzza group, University of Milan-Bicocca, potential non selective toxicities of the new antimicrobial agents (**56** and **57**, the internal standard designed and synthesized for the biodistribution analytical evaluation, as below described) were evaluated by cell viability assays performed in adenocarcinoma alveolar basal epithelial cells (A549). A549 cell line was chosen as suitable model of respiratory system for drug toxicity and targeting studies because of its high degree of morphological and functional differentiation in vitro. For each experiment cells were seeded as 30000cells/cm^2 onto 24-well plates, and after two days treated with increasing concentrations of different antimicrobial compounds:

56: 18-70µg/mL

57: 80-120µg/mL

As lead, **56** showed an increasing cytotoxic effect only reaching the LC_{50} at 70µg/mL, when administrated alone or encapsulated (Figure 3.2.4). About 20-25% mortality was observed in cells treated with SLNs alone, independently to the lipid concentrations administrated. The cytotoxic effects observed by might be due to a partial interference with the viability assay employed, indeed a different cell morphology can be observed. Thus, compound **56** by itself and as SLN formulation was completely ready for biodistribution and safety *in vivo* studies on mice.

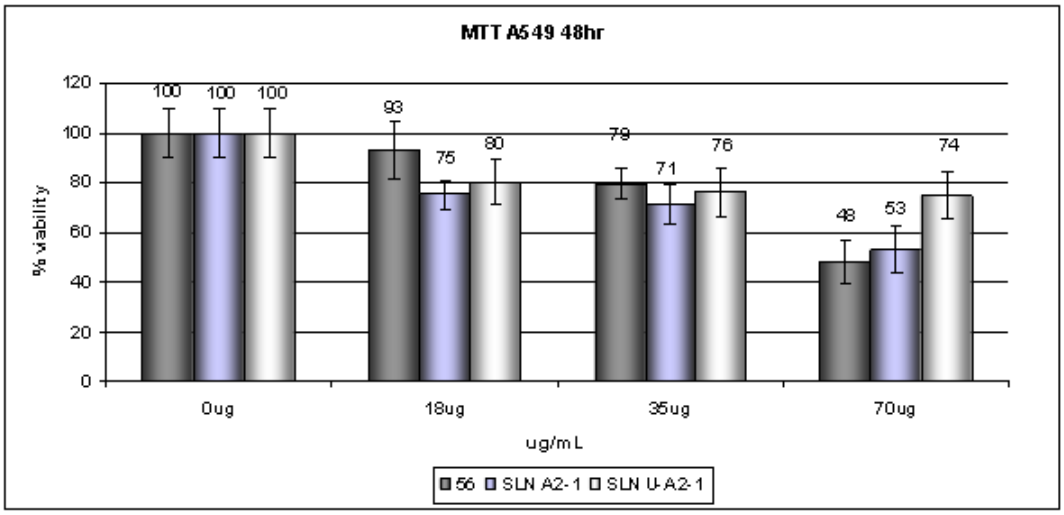


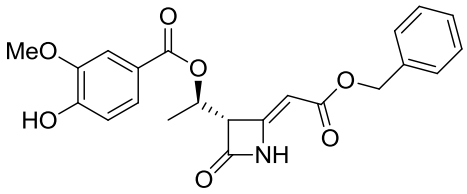
Figure 3.2.4 MTT assay results for cytotoxicity evaluation

Thus, we did the intratracheal DiR-SLN administration and the whole *in vivo* study in collaboration with Prof. C.E.A. Cocuzza, University of Milan-Bicocca.

3.2.3 Analytical evaluation of lead β-lactam for *in vivo* biodistribution

The analysis of biological samples derived from *in vivo* tests of compound **56**, administered by itself and conveyed on Solid Lipid Nanoparticles (SLN formulation), required the use a particularly specific and sensitive technique because the amount to be analyzed was in the order of nanomolar or less.

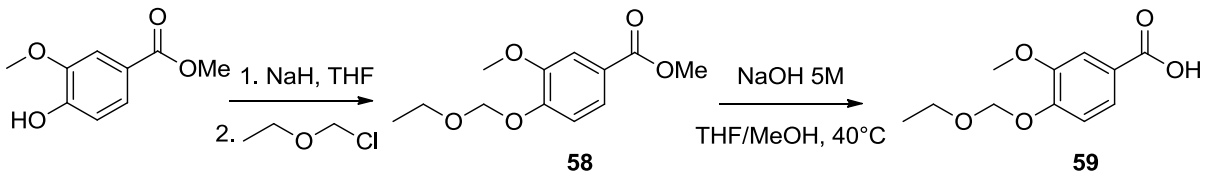
We chose HPLC/MS-MS instrumentation, working with a triple quadrupole. Moreover, given the biological origin of the samples and the consequent presence of complex matrixes, it was necessary to identify and introduce an appropriate pre-treatment method. As below specified, we optimized an SPE system using a sample of dry extract from mouse lungs, evaluating as biological matrix influenced the determination of lead compound and internal standard (matrix effect), and optimizing sample preparation and chromatographic run. Indeed, the need of a quantitative analysis required the design, the synthesis and the use of an internal standard (depicted in Figure 3.2.5), to be introduced in biological samples before the pretreatment step.



57

Figure 3.2.5 Designed internal standard **57**

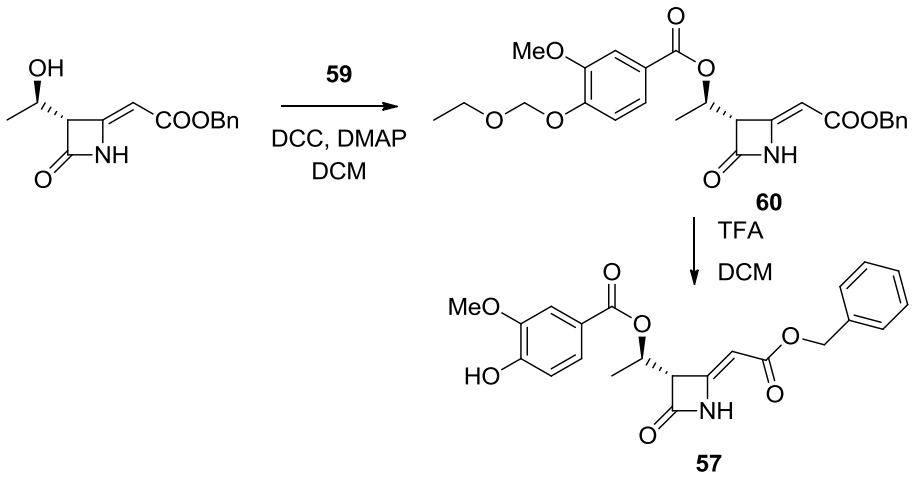
In this way, referring to the known amount of introduced standard, it was possible to relate the signals obtained for the lead **56** to those of the standard, obtaining quantitatively reliable data. We designed the internal standard as similar as possible to compound **56** (Figure 3.2.5), in order to have the most close chemical properties from every points of view. In fact, working in MS-MS it was only fundamental a significative difference in the molecular weights. For the synthesis, we followed the reliable procedure reported for the lead **56**, substituting to the syringic acid moiety the vanillic one, as in Scheme 3.2.2.



58 **59**

Scheme 3.2.2 Acid **59** preparation

Using this intermediate we applied the known procedure to the preparation of the internal standard **57**, as herein reported in Scheme 3.2.3.



Scheme 3.2.3 Synthesis of the designed internal standard **57**

Then, we set out an SPE pre-treatment method and we demonstrated that was possible to recover only one fraction (f7, see Experimental section for details) for collecting all the **56** and **57** amounts, that during HPLC run will be completely separated from matrixes residues (Figure 3.2.5, on the left). Moreover, we analyzed the matrix effect on the **56** MS-MS signal, found that it became present in the terms of a signal enhancement only after **56** and **57** elution with the adopted HPLC conditions (Figure 3.2.5, on the right). This method was validated for only the lung matrix. Thus, to analyze other samples the conditions herein found must be demonstrated in their validity. However, we verified that **56** and **57** could be simultaneously MS-MS analyzed using a negative ionization in the conditions optimized for **56** (see Experimental section for further details), furnishing significative signals for nM to also tens of pM analyte amounts.

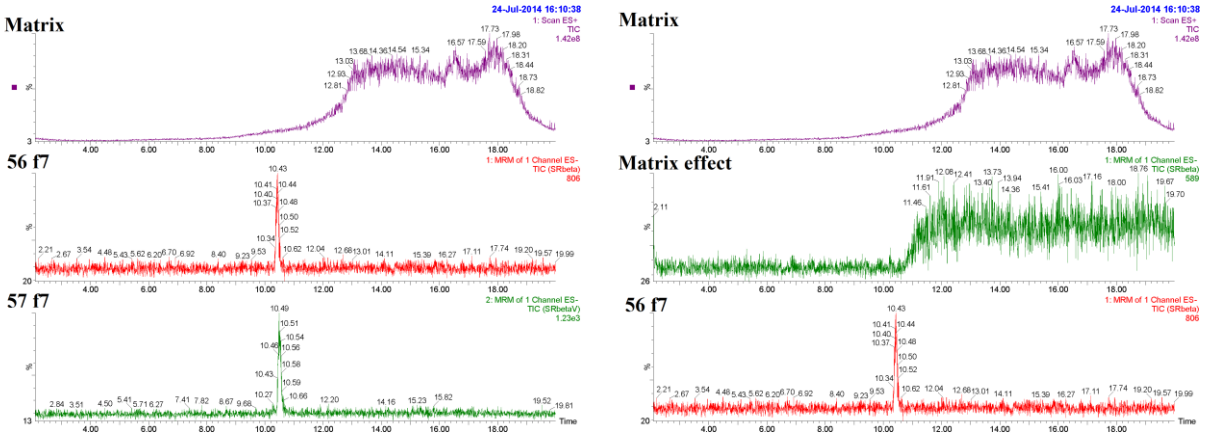


Figure 3.2.5 Matrix effect removal evaluation

From the preliminary analysis (Table 3.2.4, although the whole study still be in progress), we found no **56** in plasma samples (or amounts lower that the technique limit of detection), while the biggest amounts were recognized in brains. Moreover, in brains of mice treated with **56** without SLN formulation was found a little excess of the detected drug amounts. Livers of mice treated with the SLN formulation resulted more loaded as well. On the contrary, higher concentrations were found in lungs for the administration of the lead compound **56** by itself. Thus, it could be hazardously correlated to the use or not of the SLN nanoconveying system. To further confirm the obtained data, very close to the detection limit of the already developed technique, we also pre-treated and analyzed samples from double and triple amounts of biological samples (respectively, in Table 3.2.5 and 3.2.6).

	56 area	**57 area**	**56/57**	**56 correct area**	**56 [nM]**
Brain6	10	23358	0,00042812	26,0	0,05565
Brain7	5,07	11940	0,00042462	13,2	0,05520
Brain8	9	27164	0,00003500	23,4	0,04307
Brain9	7	13793	0,00050750	18,2	0,06597
Brain10	10	29978	0,00033358	26,0	0,04336
Liver2	20	1070	0,01869159	52,0	2,42991
Liver3	19	1599	0,01188243	49,4	1,54471
LUng8	19	4085	0,00465116	49,4	0,60465
LUng9	50	5336	0,00937031	130,0	1,21814

Table 3.2.4 Preliminary data from mice biological samples

Compared to what obtained from the first tissues amounts, no significative difference in the recorded data could be noticed analyzing doubled quantities of lungs.

	56 area	**57 area**	**56/57**	**56 correct area**	**56 [nM]**
LUng3 D	23	5789	0,00397305	59,8	0,516497
LUng6 D	43	6093	0,00705728	111,8	0,917446
LUng7 D	11	1490	0,00738255	28,6	0,959732
LUng8 D	570	1134	0,50264550	1482	65,34392
LUng9 D	160	3611	0,04430906	416	5,760177
LUng10 D	22	3966	0,00554715	57,2	0,72113

Table 3.2.5 Preliminary data from mice biological double samples

The same observation could be made while triple amounts of all the tissue analyzed.

	56 area	**57 area**	**56/57**	**56 correct area**	**56 [nM]**
Plasma3 T		814			
Brain3 T	10	19630	0,00050942	26	0,089702
Liver3 T	16	516	0,03100775	41,6	5,459989
LUng3 T	12	3511	0,00341783	31,2	0,601827
Plasma8 T		1554			
Brain8 T	6	18331	0,00032731	15,6	0,057635
Liver8 T		737			
LUng8 T	50	3782	0,01322052	130	2,32793

Table 3.2.6 Preliminary data from mice biological triple samples

The developed method is therefore confirmed in its validity for quantitative determination of **56**, compared to **57**, in biological samples at least for lung matrixes. The applicability to other matrixes samples must deepened.

3.3 Experimental section

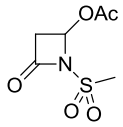
3.3.1 General informations

As previously reported (Paragraph 2.3.1).

3.3.2 Synthesis of new β-lactam dual active compounds

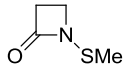
Azetidinones **1**,[96] **2**, **3**, **4**, **5**, **7**, **8**, **9** and **10** were prepared as previously reported [79].

1-Methylsulfonyl-4-oxoazetidin-2-yl acetate (**6**)



A 1M solution of LiHMDSA (1.1mL, 1.1mmol) was added to a solution of 4-acetoxy-2-azetidinone (129mg, 1.0mmol) in THF (10mL) at -78°C under inert atmosphere, followed by methanesulfonyl chloride (194µL, 2.5mmol). The solution was allowed to warm to room temperature and was monitored by TLC. After 3h the reaction was quenched with aqueous NH_4Cl (15mL) and extracted with EtOAc (3x10mL). The organic extracts were dried over Na_2SO_4 and the residue was purified by flash-chromatography (cyclohexane/EtOAc: 60/40) to afford product **6** (42 mg, 20%) as a pale yellow oil.
^{1}H (400MHz, $CDCl_3$): 2.17 (s, 3H, CH_3CO_2), 3.10 (dd, J = 2.0, 16.4Hz, 1H, CHC*H*H), 3.23 (s, 3H, SO_2CH_3), 3.54 (dd, J = 4.8, 16.4Hz, 1H, CHCH*H*), 6.47 (dd, J = 2.0, 4.8Hz, 1H, C*H*CHH) ppm
^{13}C (100MHz, $CDCl_3$): 20.7, 42.5, 45.7, 75.6, 161.0, 169.3 ppm
IR: $\tilde{\nu}$ = 2917,1757, 1161 cm^{-1}
HPLC-MS: Rt = 2.47min, m/z = 225 $[M+H_2O]^+$, 230 $[M+Na]^+$, 246 $[M+K]^+$, 437 $[2M+Na]^+$
Found C, 34.92; H, 4.51; N, 6.90; S, 15.32%; $C_6H_9NO_5S$ requires C, 34.78; H, 4.38; N, 6.76; S, 15.48%

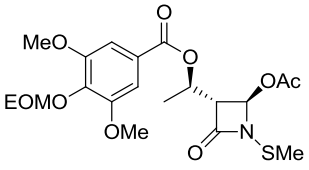
1-(Methylthio)azetidin-2-one (**11**)



LiHMDSA (2.2mL, 1M solution in THF, 2.2mmol) was added to a solution of 2-azetidinone (142mg, 2mmol) in THF (14mL) at -78°C under inert atmosphere, followed shortly by S-methyl methanethiosulfonate (514µL, 5mmol). The solution was allowed to warm to room temperature and was monitored by TLC. After 3h, the reaction was quenched with aqueous NH_4Cl (15mL) and extracted with EtOAc (3x10 mL). The organic extracts were dried over Na_2SO_4 and the residue was purified by flash-chromatography (cyclohexane/EtOAc: 75/25) to afford product **11** (187 mg, 80%) as a pale yellow oil.
^{1}H NMR (200MHz, $CDCl_3$): δ 2.41 (s, 3H, SMe), 3.04 (t, J = 4.8Hz, 2H, CH_2), 3.40 (t, J = 4.8Hz, 2H, CH_2) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 21.9, 38.7, 43.0, 170.9 ppm
IR: $\tilde{\nu}$ = 2970, 2917, 1757,1161 cm^{-1}
HPLC-MS: R_t = 1.97min, m/z: 118 $[M+H]^+$, 135 $[M+H_2O]^+$, 140 $[M+Na]^+$, 257 $[2M+Na]^+$
GC-MS: R_t = 8.04min, m/z: 117 (M), 75 (M-$COCH_2$), 60 (M-$COCH_2$-Me)

Found C, 40.83; H, 5.98; N, 11.87; S, 27.15 %; C_4H_7NOS requires C, 41.00; H, 6.02; N, 11.95; S, 27.37%

(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-4-(ethoxymethoxy)-3,5-dimethoxybenzoate (**12**)



To a solution of **4** (117mg, 0.53mmol), in DCM (20mL) at 0°C under inert atmosphere, the acid **19** (216mg, 0.84mmol), DMAP (13mg, 0.11mmol) and DCC (174mg, 0.84mmol) were added). After 10 minutes the mixture was allowed to warm to room temperature. After 70 hours the reaction was washed with cool water and extracted with DCM (3x15mL). The collected organic phases were dried on Na_2SO_4 and evaporated. The residue was treated with EtOAc and filtered. The solid was discarted whereas the solvent was evaporated and purified by flash-cromatography (cyclohexane/EtOAc: 70/30) obtaining product **12** (177mg, 73%) as a white syrup.

$[\alpha]_D$ = -3.25° (c = 1.6, $CHCl_3$)

^{1}H NMR (400MHz, $CDCl_3$): δ 1.17 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$), 1.48 (d, J = 6.4Hz, 3H, C*H*$_3$CHOCO), 2.14 (s, 3H, C*H*$_3$CO$_2$), 2.47 (s, 3H, SC*H*$_3$), 3.46 (dd, J = 1.2, 6.4Hz, 1H, C*H*CHO), 3.84 (q, J = 7.2Hz, 2H, CH$_3$C*H*$_2$), 3.87 (s, 6H, 2OC*H*$_3$), 5.20 (s, 2H, OC*H*$_2$O), 5.43 (quintet, J = 6.4 Hz, 1H, CH$_3$C*H*O), 6.21 (d, J = 1.2Hz, 1H, C*H*OAc), 7.22 (s, 2H, Ar) ppm

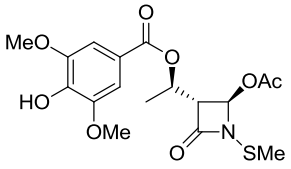
^{13}C NMR (50MHz, $CDCl_3$): δ 14.8, 18.2, 20.8, 22.8, 56.1, 64.1, 64.9, 66.9, 81.5, 96.4, 106.7, 125.1, 138.9, 153.0, 164.9, 167.3, 169.6 ppm

IR: $\tilde{\nu}$ = 3323, 2926, 2850, 1789, 1755, 1716, 1126 m^{-1}

HPLC-MS: R_t = 8.62min, m/z: 475 $[M+H_2O]^+$

Found C, 52.58; H, 5.94; N, 3.11; S, 6.89%; $C_{20}H_{27}NO_9S$ requires C, 52.51; H, 5.95; N, 3.06; S, 7.01%

(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-4-hydroxy-3,5-dimethoxy benzoate (**13**)



A stirred solution of **12** (76mg, 0.17mmol) in DCM under inert atmosphere was treated with aliquots of trifluoroacetic acid (12µL, 0.17mmol) every 15 minutes until the disappearing of the starting material. After the completion of the reaction, the solvent and the trifluoroacetic acid were evaporated to obtain **13** (55mg, 81%) as a yellow oil.

$[\alpha]_D$ = -36.3° (c = 0.52, $CHCl_3$)

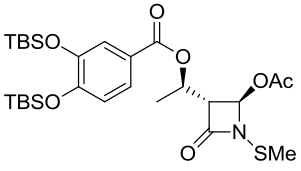
^{1}H NMR (400MHz, $CDCl_3$): δ 1.50 (d, J = 6.4Hz, 3H, C*H*$_3$CHOCO), 2.16 (s, 3H, C*H*$_3$CO$_2$), 2.49 (s, 3H, SC*H*$_3$), 3.48 (dd, J = 1.2, 6.0Hz, 1H, C*H*CHOCO), 3.94 (s, 6H, 2OC*H*$_3$), 5.44 (quintet, J = 6.4Hz, 1H, CH$_3$C*H*O), 5.97 (s, 1H, OH), 6.23 (d, J = 1.2Hz, 1H, C*H*OAc), 7.26 (s, 2H, Ar) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ 18.3, 20.8, 22.9, 56.4, 64.2, 66.7, 81.5, 106.7, 120.6, 139.5, 146.6, 165.2, 167.5, 169.7 ppm

IR: $\tilde{\nu}$ = 3412, 2938, 2850, 1783, 1756, 1711, 1116 cm^{-1}

HPLC-MS: R_t = 5.68min, m/z: 417 $[M+H_2O]^+$, 422 $[M+Na]^+$, 821 $[2M+Na]^+$

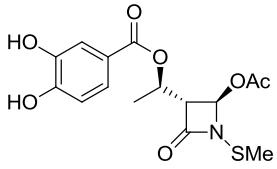
(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3,4-bis(tbutyldimethylsilyloxy)benzoate (**16**)



To a solution of **4** (121mg, 0.55mmol) in DCM (8.2mL), compound **22** (210mg, 0.55mmol) and DMAP (67mg, 0.55mmol) were added. The mixture was then cooled to 0°C, EDC (105mg, 0.55mmol) was added and the system was allowed to reach rt in 10 minutes. After 30h, the reaction was quenched with water and some drops of HCl (1M), extracted with DCM, dried on Na_2SO_4 and evaporated. The sylilated intermediate **16** was obtained after FC (DCM/Et_2O 99/1) in 23% yield (73mg) as an oil.

$[\alpha]_D$ = -13.8° (c = 0.5, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 0.22 (s, 6H, SitBu*Me*$_2$), 0.23 (s, 6H, SitBu*Me*$_2$), 0.99 (s, 9H, Si*t-Bu*Me$_2$), 1.00 (s, 9H, Si*t-Bu*Me$_2$), 1.47 (d, J = 6.0Hz, 3H, C*H*$_3$CHOCO), 2.17 (s, 3H, C*H*$_3$CO$_2$), 2.47 (s, 3H, SC*H*$_3$), 3.47 (dd, J = 1.6, 6.0Hz, 1H, C*H*CHO), 5.45 (quintet, J = 6.0Hz, 1H, CH$_3$C*H*O), 6.26 (d, J = 1.2Hz, 1H, CHOAc), 6.82-6.84 (m, 1H, Ar), 7.46-7.49 (m, 2H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ -4.2, -4.1, -4.1, -4.0, 18.3, 18.4, 18.5, 20.9, 22.8, 25.8, 25.9, 64.1, 66.3, 81.0, 120.4, 122.3, 122.9, 123.6, 146.8, 152.0, 165.0, 167.5, 169.8 ppm
IR: ν̃ = 3427, 2930, 2858, 1793, 1758, 1719, 1120 cm^{-1}
HPLC-MS: R_t = 26.60min, m/z: 601 $[M+H_2O]^+$, 606 $[M+Na]^+$, 1190 $[2M+Na]^+$

(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl3,4-dihydroxy benzoate (**14**)

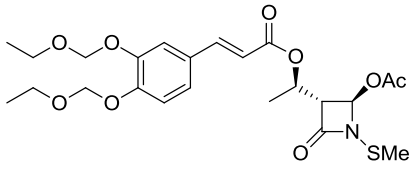


14

To a solution of **16** (47mg, 0.08mmol) in anhydrous MeCN (1.64mL) under inert atmosphere and at 0°C $BF_3{\cdot}Et_2O$ (23µL, 0.182mmol) was added dropwise. After 30 minutes the ice-bath was removed, and after 45 minutes at rt the reaction was quenched with a pH = 6 phosphate buffer solution 0.1M, extracted with DCM, dried on Na_2SO_4 and evaporated. The crude was finally triturated to afford the desired product **14** in 46%yield (13mg) as a light yellow oil.

$[\alpha]_D$ = -13.7° (c = 0.25, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.73 (d, J = 6.0Hz, 3H, C*H*$_3$CHOCO), 2.18 (s, 3H, C*H*$_3$CO$_2$), 2.47 (s, 3H, SC*H*$_3$), 3.49 (dd, J = 1.2, 4.8Hz, 1H, C*H*CHO), 5.46 (quintet, J = 6.0Hz, 1H, CH$_3$C*H*O), 6.28 (d, J = 1.2Hz, 1H, CHOAc), 6.86 (d, J = 1.2Hz, 1H, Ar), 7.44 (dd, J = 2.0, 8.4Hz, 1H, Ar), 7.50 (d, J = 2.0Hz, 1H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 18.3, 20.9, 22.7, 64.0, 66.3, 80.8, 114.8, 116.5, 121.8, 123.5, 143.4, 149.2, 165.3, 168.2, 170.1 ppm
IR: ν̃ = 3371, 2983, 2929, 1781, 1761, 1713, 1602, 1294 cm^{-1}
HPLC-MS: R_t = 5.00min, m/z: 373 $[M+H_2O]^+$, 378 $[M+Na]^+$, 733 $[2M+Na]^+$
Found C, 50.96; H, 4.91; N, 3.96; S, 8.95 %. $C_{15}H_{17}NO_7S$ requires C, 50.70; H, 4.82; N, 3.94; S, 9.02%

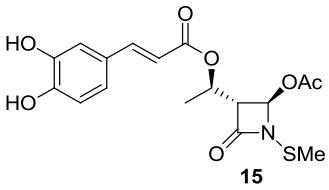
(E)-(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3-(3,4-bis(ethoxymethoxy)phenyl)acrylate (**17**)



To a solution of **4** (68mg, 0.31mmol) in DCM (11.6mL), compound **21** (145mg, 0.49mmol) and DMAP (7.6mg, 0.06mmol) were added. The mixture was then cooled to 0°C, DCC (101mg, 0.49mmol) was added and the system was allowed to reach r.t. in 15 minutes. After 65h, the reaction was quenched with water, extracted with DCM, dried on Na_2SO_4, evaporated and triturated with EtOAc to separate dicycloexylurea precipitate. The solution was then evaporated and purified by FC (cyclohexane/EtOAc 90/10 to 80/20) to afford the product in 61% yield (94mg) as an oil.

$[\alpha]_D$ = -7.4° (c = 0.84, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.21-1.28 (m, 6H, 2OCH$_2$C*H*$_3$), 1.44 (d, J = 6.4Hz, 3H, C*H*$_3$CHO), 2.17 (s, 3H, C*H*$_3$CO$_2$), 2.51 (s, 3H, SC*H*$_3$), 3.42 (dd, J = 1.6, 6.0Hz, 1H, C*H*CHO), 3.74-3.81 (m, 4H, 2OC*H*$_2$CH$_3$), 5.30 (s, 2H, OCH$_2$O), 5.31 (s, 2H, OCH$_2$O), 5.37 (quintet, J = 6.0Hz, 1H, CH$_3$C*H*O), 6.22 (d, J = 1.6Hz, 1H, CHOAc), 6.25 (d, J = 16.0Hz, 1H, CH=C*H*CO), 7.13 (dd, J = 2.0, 8.0Hz, 1H, arom), 7.19 (d, J = 8.0Hz, 1H, Ar), 7.37 (d, J = 2.0Hz, 1H, Ar), 7.53 (d, J = 16.0Hz, 1H, C*H*=CHCO) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 15.0, 15.1, 18.3, 20.9, 22.7, 64.1, 64.5, 64.6, 66.1, 80.9, 93.8, 94.1, 115.5, 115.7, 116.0, 123.4, 128.4, 145.3, 147.5, 149.5, 165.9, 167.5, 169.8 ppm
IR: ν̃ = 3327, 2977, 2930, 1789, 1757, 1711, 1635, 1599, 1510, 1437, 1251, 1159 cm^{-1}
HPLC-MS: R_t = 9.90min, m/z: 515 $[M+H_2O]^+$, 520 $[M+Na]^+$, 1017 $[2M+Na]^+$
Found C, 55.78; H, 6.32; N, 2.68; S, 6.30 %; $C_{23}H_{31}NO_9S$ requires C, 55.52; H, 6.28; N, 2.82; S, 6.44%

(E)-(R)-1-((2R,3R)-2-acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3-(3,4-dihydroxyphenyl) acrylate (**15**)



15

To a solution of **17** (94mg, 0.19mmol) in DCM (23mL) under inert atmosphere aliquots of TFA (64.5µL, 0.869mmol) were added every 15 minutes until the disappearing of the starting material (TLC monitoring). The solution was evaporated and the crude was purified by flash-chromatography (cyclohexane/EtOAc 40/60). The product obtained from the collected fractions was finally triturated with pentane to afford the desired product **15** as a white solid in 90% yield (65mg).

$[\alpha]_D$ = -19.3° (c = 1.11, DCM)

^{1}H NMR (400MHz, $CDCl_3$): δ 1.44 (d, J = 6.0Hz, 3H, C*H*$_3$CHOCO), 2.19 (s, 3H, C*H*$_3$CO$_2$), 2.52 (s, 3H, SC*H*$_3$), 3.44 (dd, J = 1.2, 5.2Hz, 1H, C*H*CHO), 5.38 (quintet, J = 6.0Hz, 1H, CH$_3$C*H*O), 6.18 (d, J = 16.0Hz, 1H, C*H*=CHCO), 6.23 (d, J = 1.2Hz, 1H, CHOAc), 6.87 (d, J = 8.0Hz, 1H, Ar), 6.97 (dd, J = 2.0, 8.4Hz, 1H, Ar), 7.06 (d, J = 1.6Hz, 1H, Ar), 7.54 (d, J = 16.0Hz, 1H, CH=C*H*CO) ppm

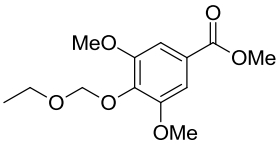
^{13}C NMR (50MHz, $CDCl_3$): δ 18.2, 20.9, 22.7, 63.8, 65.9, 80.8, 114.1, 114.3, 115.4, 122.4, 126.9, 144.2, 146.1, 147.0, 166.5, 168.5, 170.1 ppm

IR: $\tilde{\nu}$ = 3391, 2933, 1755, 1716, 1605, 1515, 1445, 1397, 1260, 1056 cm^{-1}

HPLC-MS: R_t = 5.60min, m/z: 399 $[M+H_2O]^+$, 404 $[M+Na]^+$, 785 $[2M+Na]^+$

Found C, 53.89; H, 5.13, N 3.55; S, 8.37 %; $C_{17}H_{19}NO_7S$ requires C, 53.53; H, 5.02; N, 3.67; S, 8.41%

Methyl 4-(ethoxymethoxy)-3,5-dimethoxybenzoate (**18**)



A solution of methyl 4-hydroxy-3,5-dimethoxybenzoate (1.24g, 5.9mmol) in THF (10mL) was added dropwise to a suspension of NaH (60% in mineral oil, 306mg, 7.6mmol) in THF (20mL) at 0°C under inert atmosphere. After 10 minutes a solution of chloromethylethylether (1.1mL, 11.75mmol) in THF (7mL) was added dropwise and the reaction was allowed to warm to room temperature and monitored by TLC. After 2h the reaction was quenched with aqueous NH_4Cl (30mL) and extracted with EtOAc (3x20mL). The organic extracts were dried over Na_2SO_4 and concentrated to obtain product **18** (1.46g, 92%) as pale yellow solid.

M.p. 53-56°C

$[\alpha]_D$ = -19.3° (c = 1.11, DCM)

^{1}H NMR (200MHz, $CDCl_3$): δ 1.21 (t, J = 7.0Hz, 3H, C*H*$_3$CH$_2$), 3.88 (q, J = 7.0Hz, 2H, CH$_3$C*H*$_2$), 3.90 (s, 6H, 2OC*H*$_3$), 3.92 (s, 3H, CO$_2$C*H*$_3$), 5.23 (s, 2H, OC*H*$_2$O), 7.31 (s, 2H, Ar) ppm

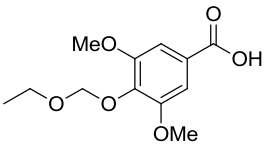
^{13}C NMR (50MHz, $CDCl_3$): δ 14.6, 51.8, 55.8, 64.6, 96.2, 106.4, 125.3, 138.4, 152.8, 166.3 ppm

IR: $\tilde{\nu}$ = 2926, 1720, 1592, 1128 cm^{-1}

HPLC-MS: R_t = 7.81min, m/z: 271 $[M+H]^+$, 293 $[M+Na]^+$, 309 $[M+K]^+$

Found C, 57.62; H, 6.80 %; $C_{13}H_{18}O_6$ requires C, 57.77; H, 6.71%

Methyl 4-(ethoxymethoxy)-3,5-dimethoxy benzoic acid (**19**)

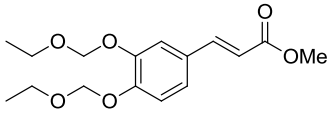


A stirred solution of **18** (730mg, 2.7mmol) in 10mL of a mixture of THF/MeOH: 90/10 was treated with 5M NaOH (2.7mL). The reaction was heated at 40°C for 4 hours and monitored by TLC. At completion, EtOAc (10mL) was added and the organic phase was separated and discarded. The aqueous phase was then cooled to 0°C and adjusted to pH = 6 with aqueous NH_4Cl. The mixture was extracted with EtOAc (3x15mL), dried on Na_2SO_4 and concentrated to afford **19** (432mg, 63%) as a white solid.

M.p. 108-109°C

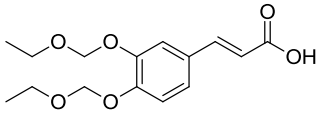
^{1}H NMR (400MHz, $CDCl_3$): δ 1.22 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$), 3.89 (q, J = 7.2Hz, 2H, CH$_3$C*H*$_2$), 3.92 (s, 6H, 2OC*H*$_3$), 5.26 (s, 2H, OC*H*$_2$O), 7.37 (s, 2H, Ar) ppm
^{13}C NMR (75MHz, $CDCl_3$): δ 14.8, 56.0, 64.9, 96.3, 107.1, 124.5, 139.3, 153.0, 171.5 ppm
IR: ν̃ = 3398, 2924, 1716, 1589, 1120 cm^{-1}
HPLC-MS: R_t = 1.53min, m/z: 279 [M+Na]$^+$, 535 [2M+Na]$^+$
Found C, 56.18; H, 6.34 %; $C_{12}H_{16}O_6$ requires C, 56.24; H, 6.29%

(E)-methyl 3-(3,4-bis(ethoxymethoxy)phenyl)acrylate (**20**)



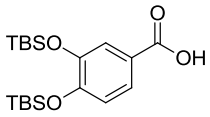
A solution of caffeic acid methyl ester (540mg, 2.78mmol) in THF (6mL) was added dropwise into a solution of NaH (60% dispersion in oil, 289mg, 7.2mmol) in THF (15mL) under inert atmosphere and at 0°C. After 10 minutes a solution of chloromethyl ethyl ether (1.01mL, 11.1mmol) in THF (7mL) was added dropwise. After 2h the solution was allowed to rt and quenched by adding a saturated NH_4Cl solution, extracted with DCM, dried over Na_2SO_4 and evaporated. The product **20** was obtained in 90% yield (776mg) after flash-chromatography (cyclohexane/EtOAc 90/10) as a colorless oil.
M.p. 108-109°C
$[\alpha]_D$ = -19.3° (c = 1.11, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.21-1.27 (m, 6H, 2OCH$_2$C*H*$_3$), 3.74-3.81 (m, 4H, 2OC*H*$_2$CH$_3$), 3.81 (s, 3H, OCH$_3$), 5.30 (s, 1H, OCH$_2$O), 5.31 (s, 1H, OCH$_2$O), 6.33 (d, J = 16.0Hz, 1H, C*H*=CHCO), 7.15 (dd, J = 2.0, 8.0Hz, 1H, Ar), 7.19 (d, J = 8.4Hz, 1H, Ar), 7.39 (d, J = 1.6Hz, 1H, Ar), 7.63 (d, J = 16.0Hz, 1H, CH=C*H*CO) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 14.9, 15.0, 51.4, 64.3, 64.4, 93.6, 94.0, 115.5, 115.9, 116.0, 123.1, 128.5, 144.4, 147.4, 149.2, 167.4 ppm
IR: ν̃ = 2977, 2900, 1717, 1634, 1600, 1582, 1511, 1436, 1393, 1105 cm^{-1}
HPLC-MS: R_t = 9.20min, m/z: 311 [M+H]$^+$, 333 [M+Na]$^+$, 349 [M+K]$^+$
Found C, 62.12; H, 7.38 %; $C_{16}H_{22}O_6$ requires C, 61.92; H, 7.15%

(E)-3-(3,4-bis(ethoxymethoxy)phenyl)acrylic acid (**21**)



A stirred solution of **20** (310mg, 1mmol) in 3.8mL of a mixture of THF/MeOH 90/10 was treated with 1mL NaOH 5M, warmed at 40°C and stirred for 4h. After completion, EtOAc (6mL) was added and discharged. The aqueous phase was treated with HCl (1M), extracted with EtOAc, the organic phase was dried over Na_2SO_4 and evaporated to afford the desired product in 96% yield (284mg) as a white solid.
M.p. 101-103°C
^{1}H NMR (400MHz, $CDCl_3$): δ 1.22-1.29 (m, 6H, 2OC*H*$_2$CH$_3$), 3.75-3.85 (m, 4H, 2CH$_3$C*H*$_2$O), 5.31 (1H, s, OCH$_2$O), 5.33 (s, 1H, OCH$_2$O), 6.35 (d, J = 16.0Hz, 1H, C*H*=CHCO), 7.18 (dd, J = 1.6, 8.8Hz, 1H, Ar), 7.22 (d, J = 8.8Hz, 1H, Ar), 7.42 (d, J = 1.6Hz, 1H, Ar), 7.73 (d, J = 16.0Hz, 1H, CH=C*H*CO) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 15.0, 15.1, 64.5, 64.6, 93.8, 94.2, 115.5, 115.8, 116.0, 123.7, 128.3, 146.8, 147.5, 149.7, 172.1 ppm
IR: ν̃ = 2976, 1683, 1626, 1596, 1514, 1418, 1244, 1114 cm^{-1}
HPLC-MS: R_t = 1.90min, m/z: 297 [M+H]$^+$, 319 [M+Na]$^+$, 335 [M+K]$^+$,
Found C, 60.52; H, 6.95 %; $C_{15}H_{20}O_6$ requires C, 60.80; H, 6.80%

3,4-bis(*t*butyldimethylsilyloxy)benzoic acid (**22**)



To a solution of 3,4-dihydroxy-benzoic acid (500mg, 3.24mmol) in DMF (5.8mL) under inert atmosphere, imidazole (1.983g, 29.16mmol) and then TBSCl (2.2g, 14.6mmol) were added. After 66h the reaction was quenched at 0°C by adding water, and then extracted with Et_2O (10x4mL). The organic phases were washed twice with

water and brine, dried over Na_2SO_4 and evaporated. To the persilylated intermediate (2.049g, 4.12mmol) in a 6mL MeOH/THF 40/60, a solution of K_2CO_3 (231mg, 1.67mmol) in water (2.2mL) was added. After 12h the mixture was quenched a 0°C by adding a saturated solution of citric acid and extracted with Et_2O. The organic phases were washed with water and brine, dried over Na_2SO_4 and evaporated. The product was finally obtained by flash-chromatography (DCM/MeCN: 90/10) purification in 75% yield (930mg) as a white solid.
M.p. 152-154°C
^{1}H NMR (400MHz, $CDCl_3$): δ 0.24 (s, 6H, Si*t*Bu*Me*$_2$), 0.25 (s, 6H, Si*t*Bu*Me*$_2$), 1.00 (s, 9H, Si*tBu*Me$_2$), 1.01 (s, 9H, Si*tBu*Me$_2$), 6.88 (d, J = 8.0Hz, 1H, Ar), 7.60 (d, J = 2.0Hz, 1H, Ar), 7.63 (dd, J = 1.6, 8.8Hz, 1H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ -4.1, -4.0, 18.4, 18.5, 25.8, 25.9, 120.5, 122.4, 122.7, 124.4, 146.8, 152.5, 171.2 ppm
IR: $\tilde{\nu}$ = 2970, 2917, 1757,1161 cm^{-1}
HPLC-MS: R_t = 1.97min, m/z: 118 $[M+H]^+$, 135 $[M+H_2O]^+$, 140 $[M+Na]^+$, 257 $[2M+Na]^+$
GC-MS: R_t = 8.04min, m/z: 117 (M), 75 (M-$COCH_2$), 60 (M-$COCH_2$-Me)
Found C, 59.64; H, 8.96 %. $C_{19}H_{34}OSi_2$ requires C, 60.80; H, 6.80%.

3.3.3 Antioxidant potencies, voltammetry and antibacterial activities

Antioxidant activity evaluation methods, electrochemical experiment conditions, bacterial strains selection and antibacterial activities evaluation method follow.

BR assay

Relative antioxidant activity (r.a.c.) with respect to a substance chosen as standard, resorcinol (Re) in our case, is determined on the basis of concentrations of sample and resorcinol that give the same t_{inhib}; r.a.c. is expressed as mM resorcinol equivalents.

Oscillatory behaviors' V(Pt) were followed potentiometrically by using the couple bright platinum electrode (Hamilton 238945)-Ag/AgCl double junction electrode (Ingold 373-90-WTE-ISE-S7) connected to a multimeter (WTW pH 540 GLP), controlled by a PC. Reacting mixtures were thermostated at 25.0±0.1°C. The straight line of the standard was checked before each series of measurements.

TEAC assay

The radical cation is preformed by reaction between ABTS and $K_2S_2O_8$ in PBS medium, pH = 7.4; the mixture was kept in the dark until the reaction was complete and absorbance at 734nm stable. Spectroscopic measurements were performed in triplicate at four concentrations of the β-lactams in DMSO. Suitably diluted solutions of the standard (Trolox) were treated in the same way. In brief, 3.0mL of diluted $ABTS^{\cdot+}$ solution and 30mL of sample were mixed in a photometric cuvette and absorbance was measured at 734nm at exactly 6min after the mixing of the reagents (T = 30.0±0.1°C). A blank with DMSO was measured in the same way. The difference between the absorbance of the blank and the sample gave ΔE6 (E6blank - E6sample = ΔE6). Data (ΔE6 vs conc. in mM) are well fitted by straight

lines through the origin (following the Lambert&Beer law). Then, the relative antioxidant activity with respect to Trolox (TEAC) was obtained by the ratio:

TEAC = m(smp)/m(Trolox)

where m(smp) and m(Trolox) are the slopes of the straight lines of the sample and the standard respectively. Standard error of this ratio is calculated in the usual way. Here also the straight line of the standard was always checked before each series of measurements.

DPPH assay

The principle of this method is the decolorization of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) by antioxidants. The DPPH· is intensely purple colored (λ_{max} = 512-530nm) due to the presence of the picric group (1,3,5-nitrobenzene) and is stabilized by either the steric hindrance of the aromatic substituents and the delocalization of the unpaired electron on the whole structure. The disappearance of this radical due to the addition of an antioxidant H-donor, may be evaluated spectrophotometrically, at its absorption maximum in methanol (515nm). The measured decrease in absorbance at 515nm is an expression of the antioxidant capacity of a sample.[97]

The extent of the decolorization is a function of concentration and time. Trolox was used as the standard. In practice 2.9mL of DPPH· methanolic solution was placed in a cuvette and the initial absorbance, A_0, was measured. Then 100mL was added and mixed; after 15min the final absorbance, A_f, was measured. The percentage of inhibition was calculated according to the following equation (%):

Inhibition = $(1-A_f/A_0)x100$.

The same procedure was repeated with the standard. Data (%inhib vs conc. in mM) are well fitted by straight lines through the origin (following the Lambert&Beer law).

Then, the relative antioxidant activity with respect to Trolox was obtained by the ratio:

DPPH = m(smp)/m(Trolox).

FRAP assay

2,4,6-Tris(2-pyridyl)-s-triazine-Fe^{3+} (FRAP reagent) was prepared by mixing suitable amounts of TPTZ and $FeCl_3$ solutions in aqueous acetate buffer. In brief, 3.0mL of FRAP reagent and 100mL of suitably diluted sample were mixed in a photometric cuvette and absorbance was measured after exactly 4min after the mixing(T = 25.0±0.1°C).

The absorbance of the blue Fe^{2+} complex is measured at 593nm (Shimadzu UV-1601 PC spectrophotometer). Four or five different sample concentrations were tested and the straight line Abs vs. conc. was then compared with that of the standard ($FeSO_4$). The Lambert&Beer law is followed, then the ratio:

FRAP = m(smp)/m(Fe^{2+})

gives the relative ferric reducing activity (mmol equiv Fe^{2+}).

Cyclic voltammetry

All electrochemical experiments were conducted in acetonitrile from Sigma-Aldrich (>99.5% over molecular sieves) after careful de-aeration of the reaction mixture with argon; in all instances a silver wire was used as a pseudo-reference electrode, while a platinum coil was used as a counter electrode. Voltammetric analysis was performed with platinum, gold and glassy carbon working electrodes (Ø = 2mm) using a function generator AMEL mod 568 equipped with a digital oscilloscope.

Bacterial isolates

A collection of 45 recently isolated and well characterized for the antimicrobial susceptibility phenotype MRSA strains recovered from CF patients was tested. All the strains were isolated at the Department of Pediatrics, CF Center, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milan.

The in vitro susceptibility of methicillin-resistant isolates of S. aureus to new antimicrobial compounds was evaluated by means of the broth microdilution method for the determination of the minimum inhibitory concentrations (MIC), in accordance with the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) guidelines.[98] Briefly, serial two-fold dilutions were made of the compounds in 96-well plates in order to obtain concentrations ranging from of 0.06-128mg/L in cation-adjusted MullereHinton broth (DIFCO). An equal volume of $1 \cdot 10^6$CFU/mL (Colony Forming Unit/mL) bacterial inoculum was added to each well of the microtitre plate containing 0.05 mL of the serial antibiotic concentrations. The microtitre plate was then incubated overnight at 37°C and subsequently analyzed for the presence of visible bacterial growth. MIC was defined as the lowest concentration of the tested compound able to inhibit visible growth of the microorganism after overnight incubation.

All the tested compounds were solubilized in 100% DMSO and appropriate dilutions were set up to prevent its toxicity.

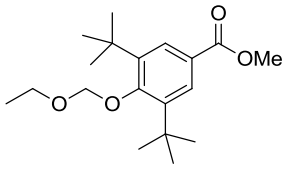
Positive strain controls without antimicrobial compounds, controls with DMSO and uninoculated media were run parallel to the tested compounds under the same conditions.

S. aureus American Type Culture Collection (ATCC) 29213 (MSSA) and ATCC 43300 (MRSA) were used as control strains. Cefotaxime sodium salt was used as the reference antibiotic for MIC experiments validation. Breakpoints for resistance were those recommended by the CLSI.[99]

3.3.4 Synthesis of other antibiotic and dual active β-lactam compounds

Azetidinone **25**, **52**, **54** and **56**, and all the intermediate for its preparation from not optimized procedures, were prepared as previously reported [19, 79].

Methyl 3,5-di-*t*butyl-4-(ethoxymethoxy) benzoate (**29**)



In a 50mL three-necks flask were suspended under nitrogen atmosphere 177mg of NaH (95%, 7.38mmol) in THF (11.0mL) and, at 0°C, a THF solution (6mL) of methyl 3,5-di-*t*butyl benzoate (650mg, 2.46mmol)was dropped, followed by 1 hour stirring at room temperature. Were then added, at 0°C, 450μL of ethoxymethyl chloride dissolved in 6mL of THF, and after 12h stirring at r.t. the reaction was quenched with a NH_4Cl saturated solution, then extracted with EtOAc (3x10mL), dried over Na_2SO_4 and concentrated in vacuum. After FC purification **29** was obtained in 95% yield (760mg) as a colorless oil.

Rf 0.60 (cyclohexane/Et_2O, 90/10)

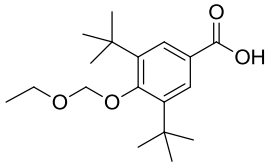
^{1}H NMR (400MHz, $CDCl_3$): δ 1.32 (t, J = 7.2Hz, 3H, EtO) 1.47 (s, 18H, *t*Bu), 3.89 (s, 3H, OMe), 3.87(q, J = 6.8Hz, 2H, EtO), 4.95 (s, 2H, OCH_2O), 7.90 (s, 2H, Ar) ppm

^{13}C NMR (50MHz, $CDCl_3$): δ 15.2, 31.8, 35.8, 51.9, 65.6, 99.6, 124.7, 128.1, 144.7, 158.9, 167.4 ppm

IR: ν̃ = 3427, 2961, 1721, 1597, 1430, 1389, 1363, 1302, 1237, 1134, 1026, 996, 952, 885, 770 cm^{-1}

HPLC-MS: R_t = 15.40min, m/z: 60 (100) $[M\text{-}EOM\text{-}2t\text{Bu-}Ph]^+$, 323 (40) $[M+H]^+$

3,5-di-*t*butyl-4-(ethoxymethoxy)benzoic acid (**28**)



In a one-neck flask 2.3mL of 5.6M NaOH aqueous solution were added to 760mg of **29** dissolved in a THF/MeOH mixture (8.3mL/0.96mL) and the temperature was then increased to 40°C. After 3 days stirring EtOAc was added and water used for washing 3 times; the aqueous phases were then acidified to pH = 6 with HCl and extracted with EtOAc (3x3mL). The organics were dried Na_2SO_4 and concentrated in vacuum affording 533mg of **28** (y = 95%) as a white solid.

M.p. (170-172)°C; Rf 0.71 (cyclohexane/EtOAc 90/10)

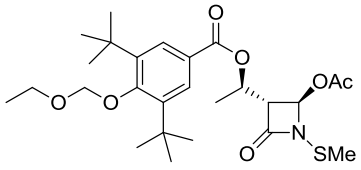
^{1}H NMR (400MHz, $CDCl_3$): δ 1.33 (t, J = 7.2Hz, 3H, EtO), 1.48 (s, 18H, *t*Bu), 3.90 (q, J = 6.8Hz, 2H, EtO), 4.95 (s, 2H, OCH_2O), 7.90 (s, 2H, Ar) ppm

^{13}C NMR (50MHz, $CDCl_3$): δ 15.2, 31.8, 35.8, 65.7, 99.7, 123.7, 128.9, 144.9, 159.9, 172.2 ppm

IR: ν̃ = 3415, 2956, 1683, 1414, 1385, 1303, 1253, 1158, 1141, 1114, 942 cm^{-1}

HPLC-MS: R_t = 13.61min, m/z: 307 (100) $[M-H]^+$, 634 (10) $[2M-2H+Na]^+$

(R)-((2R,3R)-2-acetoxy-methylthio-4-oxoazetidin-3-yl)ethyl-3,5-di-*t*butyl-4-(ethoxymethoxy) benzoate (**26**)



In a 25mL two-necks flask were dissolved under nitrogen atmosphere 75mg of **4** (0.34mmol) in 5.0mL of anhydrous DCM, followed by addition of **28** (105mg, 0.34mmol), DMAP (124mg, 0.34mmol) and, at 0°C, EDC (192mg, 0.34mmol). The solution was stirred at r.t. 3 days, following by TLC monitoring, and was finally quenched at 0°C with water, extracted with EtOAc (3x5mL), dried over Na_2SO_4 and concentrated in vacuum. After treatment by flash-chromatography (cyclohexane/EtOAc 85/15) **26** was obtained in 36% yield (63mg) as a colorless oil.

Rf 0.33 (cyclohexane/EtOAc 60/40)

$[\alpha]_D$ = -37.2°

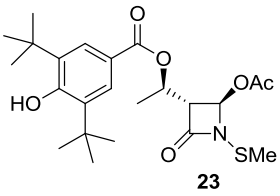
^{1}H NMR (400MHz, $CDCl_3$): δ 1.33 (t, J = 7.2Hz, 3H, EtO), 1.45 (s, 18H, *t*Bu), 1.50 (d, J = 6.0Hz, 3H, Me) 2.18 (s, 3H, OAc), 2.49 (s, 3H, SMe), 3.50 (dd, H, J = 1.6 Hz), 3.90 (quartet, J = 6.8 Hz, 2H, EtO), 4.96 (s, 2H, OCH_2O), 5.44 (quintet, J = 6.4Hz, 1H,), 6.26 (d, J = 1.6Hz, 1H) 7.91 (s, 2H, Ar) ppm

^{13}C NMR (50MHz, $CDCl_3$): δ 15.2, 18.4, 20.87, 22.9, 31.8, 35.8, 64.1, 65.6, 66.5, 81.3, 99.6, 124.2 , 128.1, 144.8, 159.3, 165.6, 169.7 ppm

IR: ν̃ = 3427, 2962, 1789, 1760, 1717, 1597, 1363, 1233, 945, 888, 736 cm^{-1}

HPLC-MS: R_t = 15.19min, m/z: 142 (100) $[M\text{-}RCOOH\text{-}OAc]^+$, 532 (90) $[M+Na]^+$, 1041 (85) $[2M+Na]^+$

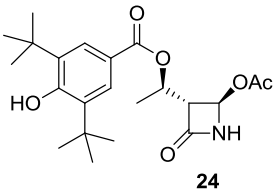
(R)-((2R,3R)-2-acetoxy-methylthio-4-oxoazetidin-3-yl)ethyl 3,5-di-*t*butyl-4-hydroxybenzoate (**23**)



23

In a 25mL two-necks flask were dissolved under nitrogen atmosphere 44mg of **26** (0.086mmol) in 10.7mL of anhydrous DCM, followed by TFA addition at 0°C (27µL) and TLC monitoring. At SM disappearance the reaction was directly concentrated to afford **23** in 97% yield (38mg) as a white solid.
M.p. (144-147)°C; Rf 0.48 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = -15.6°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.46 (s, 9H, *t*Bu), 1.47 (s, 9H, *t*Bu), 1.50 (d, J = 6.4Hz, 3H, Me), 2.18 (s, 3H, OAc), 2.48 (s, 3H, SMe), 3.50 (dd, J = 1.2, 4.4Hz, 1H,), 5.43 (quintet, J = 6.0, 6.4Hz, 1H,), 5.70 (bs, OH), 6.28 (d, 1H), 7.85 (s, 2H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 18.5, 20.9, 22,9, 30.1, 34.3, 64.2, 66.2, 81.3, 120.7, 127.1, 135.7, 158.4, 165.8, 167.6, 169.7 ppm
IR: $\tilde{\nu}$ = 3551, 2961, 2876, 1789, 1711, 1599, 1432, 1359, 1219, 944, 915, 886, 768, 738 cm^{-1}
HPLC-MS: R_t = 11.98min, m/z: 469 (100) $[M+H_2O]^+$, 474 (27) $[M+Na]^+$, 925 (89) $[2M+Na]^+$

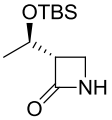
(R)-((2R,3R)-2-acetoxy-4-oxoazetidin-3-yl)ethyl 3,5-di-*t*butyl-4-hydroxy benzoate (**24**)



24

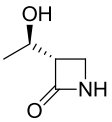
In a 10mL 2-neck flask a mixture of **27** (10mg, 22µmol), anhydrous THF (0.27mL), MeOH (0.27mL) and Pd/C 10%$_{w/w}$ (1.2mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (16h) the mixture was filtered and concentrated in vacuum. The desired product **24** was obtained as a yellow waxy solid in 95% yield (8mg) after purification via flash-chromatography (cyclohexane/EtOAc 50/50).
Rf 0.57 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = -29.9°
^{1}H NMR (400MHz, CD_3CN): δ = 1.43 (s, 9H, *t*Bu), 1.45 (s, 9H, *t*Bu), 1.50 (d, J = 6.8Hz, 3H, Me), 2.10 (s, 3H, OAc), 3.46 (dd, J = 1.2, 5.2Hz, 1H,), 5.40 (quintet, J = 6.8Hz, 1H,), 5.68 (s, H), 5.87 (d, J = 1.2Hz, 1H,), 6.53 (bs , NH), 7.85 (s, 2H, Ar) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 9.9, 18.5, 20.8, 25.7, 29.7, 30.1, 32.2, 34.3, 62.8, 66.6, 76.0, 120.8, 127.1, 135.8, 158.4, 164.6, 165.9, 171.0 ppm
IR: $\tilde{\nu}$ = 3311, 2959, 2874, 1782, 1755, 1709, 1599, 1429, 1369, 1300, 1232, 1131, 1062, 1034, 769 cm^{-1}
HPLC-MS: Rt = 10.41min, m/z = 346 (95) $[M\text{-}t\text{Bu}]^+$, 428 (40) $[M+Na]^+$, 833 (100) $[2M+Na]^+$

(S)-3-((R)-*t*butyldimethylsilyloxyethyl)azetidin-2-one (**38**)



In a 100mL two-necks flask under nitrogen atmosphere 1.0g of (2R,3R)-3-((R)-*t*butyldimethylsilyloxyethyl)-4-oxoazetidin-2-yl acetate were dissolved in 30.0mL of anhydrous THF before adding, at 0°C, 1.58g of $NaBH_4$ (4eq.) and leaving under agitation at room temperature for one day, following by TLC monitoring (cyclohexane/EtOAc 50/50 and $KMnO_4$ stain). At SM disappearance the reaction was extracted with DCM, dried over Na_2SO_4 and concentrated in vacuum to reach **38** in 99% yield (796mg) as a white solid.

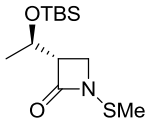
(S)-3-((R)-1-hydroxyethyl)azetidin-2-one (**39**)



In a 50mL two-necks flask under nitrogen atmosphere 796mg (3.74mmol) of **38** were dissolved in 18.2mL of MeCN, then 3.74mL of 1M aqueous HCl were added and the reaction followed by TLC monitoring (DCM/MeOH 95/5, $KMnO_4$ stain). At SM disappearance the reaction was quenched with 1M aqueous NaOH until neutral pH, then evaporated, dissolved

in a MeCN/MeOH 1/1 solution, filtered and concentrated under vacuum to afford 345mg (y = 80%) of **39** as white solid.

(S)-3-((R)-*t*butyldimethylsilyloxyethyl)-1-(methylthio)azetidin-2-one (**40**)



In a 50mL two-necks flask under nitrogen atmosphere 417mg (1.82mmol) of **38** were dissolved in 14.3mL of anhydrous THF before slowly adding, at -78°C, 2.18mL of a 1M LiHMDSA solution in THF (2.18mmol) and, after some minutes, 0.43mL of $MeSSO_2Me$ (4.55mmol). After 1h stirring at -78°C and monitoring by TLC (cyclohexane/EtOAc 70/30), the reaction was quenched with a saturated water solution of NH_4Cl, extracted with DCM, dried over Na_2SO_4 and concentrated in vacuum to reach **38** as a yellow oil in 82% yield (411mg) after FC purification.

Rf 0.71 (cyclohexane/EtOAc 70/30)

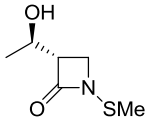
^{1}H NMR (400MHz, $CDCl_3$): δ 0.06 (s, 3H, Me), 0.07 (s, 3H, Me), 0.86 (s, 9H, *t*Bu), 1.61 (d, J = 6.4Hz, 3H, Me), 2.42 (s, 1H, SMe), 3.26 (dt, J = 4.0, 5.6Hz, 1H, CH), 3.37 (t, J = 5.2Hz, 1H, CH_2), 3.52 (dd, J = 2.0, 2.8Hz, 1H, CH_2), 4.27 (dq, J = 2.4, 4.0Hz, 1H, CHMe) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ -4.5, -4.9, 17.9, 22.0, 22.6, 25.7, 45.1, 59.3, 64.9, 171.9 ppm

IR: $\tilde{\nu}$ = 3518, 2956, 1766, 1471, 1374, 1256, 1172, 1015, 960, 778, 722 cm^{-1}

HPLC-MS: R_t = 12.5min, m/z: 276 (50) $[M+H]^+$, 298 (100) $[M+Na]^+$

3-hydroxyethyl-1-methylthioazetidin-2-one (**41**)



In a 50mL two-necks flask under nitrogen atmosphere 320mg (1.16mmol) of **40** were dissolved in 23.0mL of anhydrous MeCN before slowly adding, at 0°C, 166μL (1.32mmol) of $BF_3{\cdot}Et_2O$. After 1h at 0°C (TLC monitoring, cyclohexane/EtOAc 70/30), the reaction was quenched with a pH = 6 phosphate buffer solution, then allowed to reach r.t., extracted with DCM, dried over Na_2SO_4 and concentrated to reach **41** as a yellow oil in 71% yield (137mg).

Rf 0.35 (cyclohexane/EtOAc 50/50)

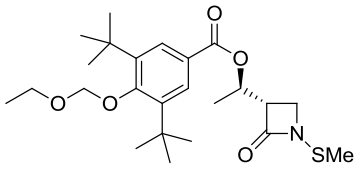
^{1}H NMR (400MHz, $CDCl_3$): δ 1.28 (d, J = 6.4Hz, 3H), 1.80 (bs, 1H), 2.45 (s, 1H), 3.34 (dt, J = 2.0, 3.6Hz, 1H), 3.47 (t, J = 5.6Hz, 1H), 3.50 (dd, J = 2.4, 3.2Hz, 1H), 4.21 (dq, J = 6.0, 6.4Hz, 1H) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ 21.2, 21.5, 45.5, 58.5, 64.4, 172.3 ppm

IR: $\tilde{\nu}$ = 3423, 2969, 2920, 1740, 1418, 1309, 1185, 1058, 962, 903 cm^{-1}

HPLC-MS: R_t = 0.38min, m/z: 162 (100) $[M+H]^+$, 184 (100) $[M+Na]^+$, 345 (35) $[2M+Na]^+$

(R)-1-((S)-methylthio-2-oxoazetidin-3-yl)ethyl 3,5-di-*t*butyl-4-(ethoxymethoxy) benzoate (**42**)



In a 50mL two-necks flask under nitrogen atmosphere 161mg (1.0mmol) of **41** were dissolved in 23.0mL of anhydrous DCM before adding 308mg (1.0mmol) of acid **28**, 124mg (1.0mmol) of DMAP and, at 0°C, 192mg (1.0mmol) of EDC. The reaction was monitored by TLC and quenched at 0°C with HCl until neutrality, then extracted with EtOAc, dried over Na_2SO_4 and concentrated in vacuum to reach **42** as a white solid in 17% yield (77mg) after purification via FC (cyclohexane/EtOAc 85/15).

M.p. (137-140)°C; Rf 0.54 (cyclohexane/EtOAc 60/40)

$[\alpha]_D$ = -50.9°

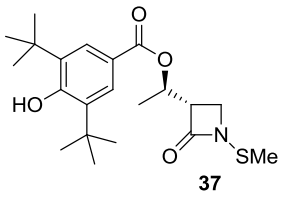
^{1}H NMR (400MHz, $CDCl_3$): δ 1.30 (t, J = 6.8 Hz, 3H, EtO), 1.44 (s, 18H, *t*Bu), 1.46 (d, J = 6.8Hz, 3H, Me), 2.42 (s, 3H, SMe), 3.49 (t, J = 1.6Hz, 1H), 3.54 (dd, J = 1.6 Hz, 1H), 3.86 (quartet, J = 6.8Hz, 2H, EtO), 4.93(s, 2H, OCH_2O), 5.44 (quintet, J = 6.4Hz, 1H), 7.90 (s, 2H, Ar) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ 15.2, 18.7, 21.9, 29.6, 31.8, 35.8, 46.9, 56.9, 65.6, 68.3, 99.6, 124.4 , 128.1, 144.8, 159.3, 165.8, 169.9 ppm

IR: $\tilde{\nu}$ = 3419, 2964, 1761, 1718, 1597, 1431, 1387, 1365, 1333, 1293, 1236, 1135, 1181, 1113, 1083, 1058, 942, 767 cm^{-1}

HPLC-MS: R_t = 14.48min, m/z: 452 (100) $[M+H]^+$, 469 (14) $[M+H_2O]^+$, 925 (13) $[2M+Na]^+$

(R)-1-((S)-methylthio-2-oxoazetidin-3-yl)ethyl 3,5-di-*t*butyl-4-hydroxybenzoate (**37**)



37

In a 50mL two-necks flask were dissolved under nitrogen atmosphere 92mg of **42** (0.17mmol) in 22.0mL of anhydrous DCM, followed by TFA addition at 0°C (54µL) and TLC monitoring. At SM disappearance the reaction was directly concentrated to afford **37** in 95% yield (67mg) as a white solid after pentane titration.

M.p. (138-140)°C; Rf 0.49 (cyclohexane/EtOAc 50/50)

$[\alpha]_D$ = -58.8°

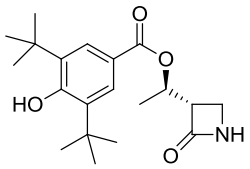
^{1}H NMR (400MHz, $CDCl_3$): δ 1.44 (s, 18H, tBu), 1.45 (d, J = 6.0Hz, 3H, Me), 2.42 (s, 3H, SMe), 3.51 (t, J = 6.4Hz, 1H), 3.57 (dd, J = 2.4, 5.6Hz, 1H), J = 6.8Hz), 5.43 (quintet, J = 6.4Hz, 1H), 5.70 (bs, OH), 7.83 (s, 2H, Ar) ppm

^{13}C NMR (50MHz, $CDCl_3$): δ 18.6, 21.9, 30.1, 34.3, 47.1, 56.7, 68.1, 120.6 , 127.1, 135.8, 158.5, 166.3, 171.1 ppm

IR: $\tilde{\nu}$ = 3563, 2960. 1758, 1709, 1600, 1428, 1361, 1300, 1233, 1188, 1124, 1083, 1054, 1030, 890, 768, 737 cm^{-1}

HPLC-MS: R_t = 11.47min, m/z: 394 (100) $[M+H]^+$, 416 (10) $[M+Na]^+$, 809 (30) $[2M+Na]^+$

(R)-1-((2R,3R)-2-acetoxy-4-oxoazetidin-3-yl)ethyl 3,5-di-*t*butyl-4-hydroxybenzoate (**33**)



33

In a 10mL 2-neck flask a mixture of **37** (55mg, 0.14mmol), anhydrous THF (1.7mL), MeOH (1.7mL) and Pd/C 10%$_{w/w}$ (8.0mg) was stirred under H_2 atmosphere (1atm) at rt overnight. At starting material consumption (16h) the mixture was filtered and concentrated in vacuum. The desired product **33** was obtained as a white solid in 41% yield (20mg) after purification via FC (cyclohexane/EtOAc 50/50).

Rf 0.31 (cyclohexane/EtOAc 50/50)

$[\alpha]_D$ = -28.1°

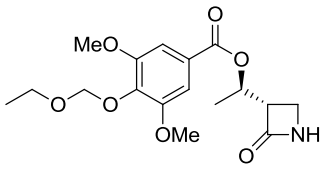
^{1}H NMR (400MHz, CD_3CN): δ = 1.43 (s, 18H, *t*Bu), 1.46 (d, J = 6.0Hz, 3H, Me), 3.36 (dd, J = 2.8, 3.2Hz, 1H), 3.44 (t, J = 5.2Hz, 1H), 3.50 (dd, J = 1.2Hz, 1H), 5.43 (quintet, J = 6.4Hz, 1H), 5.83 (bs, OH), 7.86 (s, 2H, Ar) ppm

^{13}C NMR (100MHz, CD_3OD): δ = 18.7, 30.1, 34.3, 39.4, 56.9, 68.5, 121.1 , 127.1, 135.7, 158.3, 166.1, 167.7 ppm

IR: $\tilde{\nu}$ = 3311, 2959, 2873, 1756, 1705, 1599, 1428, 1361, 1301, 1234, 1192, 1131, 1067, 1030 cm^{-1}

HPLC-MS: Rt = 9.77min, m/z = 348 (100) $[M+H]^+$, 717 (40) $[2M+Na]^+$,

(S)-((R)-2-oxoazetidin-3-yl)ethyl 4-(ethoxymethoxy)-3,5-dimethoxybenzoate (**43**)



In a 50mL two-necks flask under nitrogen atmosphere 47mg (0.41mmol) of **39** were dissolved in 8.76mL of anhydrous DCM before adding 150mg (0.58mmol) of acid **19**, 72mg (0.58mmol) of DMAP and, at 0°C, 111mg (0.58mmol) of EDC. The reaction was monitored by TLC and quenched at 0°C with HCl until neutrality, then extracted with EtOAc, dried over Na_2SO_4 and concentrated in vacuum to reach **43** as a colorless oil in 29% yield (41mg) after purification via FC (cyclohexane/EtOAc 80/20 to 30/70).

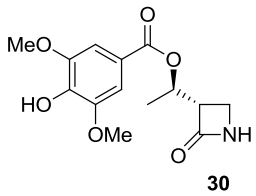
Rf 0.42 (EtOAc)

$[\alpha]_D$ = -29.0°

^{1}H NMR (400MHz, $CDCl_3$): δ 1.19 (m, 3H), 1.49 (d, J = 8.0Hz, 3H), 3.33 (dd, J = 4.0, 8.0Hz, 1H), 3.46 (dd, J = 4.0, 8.0Hz, 1H), 3.54 (m, 1H), 3.85 (m, 2H), 3.88 (s, 6H), 5.21 (s, 2H), 5.95 (bs, 1H, NH) 7.25 (s, 2H) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ 14.9, 18.5, 39.5, 56.2, 56.4, 65.0, 69.4, 96.5, 106.8, 125.5, 138.9, 153.1, 165.2, 167.4 ppm
IR (KBr): ṽ = 3416, 2976, 2938, 1747, 1591, 1338, 1128 cm^{-1}
HPLC-MS: R_t = 6.25min, m/z: 354 (100) $[M+H]^+$, 371 (15) $[M+H_2O]^+$, 729 (30) $[2M+Na]^+$

(R)-1-((S)-2-oxoazetidin-3-yl)ethyl 4-hydroxy-3,5-dimethoxybenzoate (**30**)

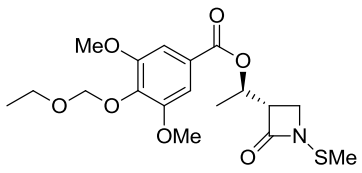


30

In a 25mL two-necks flask were dissolved under nitrogen atmosphere 27mg of **43** (0.17mmol) in 9.27mL of anhydrous DCM, followed by TFA addition at 0°C (28µL) and TLC monitoring (cyclohexane/EtOAc 10/90). At SM disappearance the reaction was directly concentrated to afford **30** in 22% yield (5mg) as a white solid after FC purification (DCM/acetone 98/2 to 90/10).
Rf 0.40 (DCM/acetone 70/30)

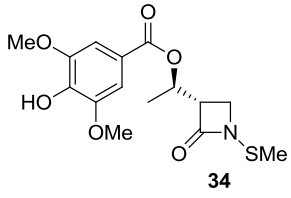
$[\alpha]_D$ = -58.8°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.28 (d, J = 16.0Hz, 3H), 3.35 (dd, J = 4.0, 8.0Hz, 1H), 3.39 (dd, J = 4.0, 8.0Hz, 1H), 3.55 (m, 1H), 3.95 (s, 6H), 5.58 (m, 1H), 5.72 (bs, 1H, NH), 5.93 (s, 1H, OH), 7.25 (s, 2H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 18.2, 47.1,56.5, 56.6, 68.7, 106.8, 119.1, 139.8, 146.5, 165.8, 168.1 ppm
IR (KBr): ṽ = 3399, 2922, 2849, 1740, 1607, 1515, 1462, 1338, 1230, 1116 cm^{-1}
HPLC-MS: R_t = 2.49min, m/z: 296 (100) $[M+H]^+$

(R)-1-((S)-1-(methylthio)-2-oxoazetidin-3-yl)ethyl 4-(ethoxymethoxy)-3,5-dimethoxybenzoate (**44**)



In a 50mL two-necks flask under nitrogen atmosphere 66mg (0.41mmol) of **41** were dissolved in 8.76mL of anhydrous DCM before adding 150mg (0.58mmol) of acid **19**, 72mg (0.58mmol) of DMAP and, at 0°C, 111mg (0.58mmol) of EDC. The reaction was monitored by TLC and quenched at 0°C with a saturated NH_4Cl solution and 1M HCl until neutrality, then extracted with DCM, dried over Na_2SO_4 and concentrated in vacuum to reach **44** as a colorless oil in 27% yield (44mg) after purification via FC (cyclohexane/EtOAc 85/15 to 70/30).
Rf 0.45 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = -53.5°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.2 (dd, J = 4.0, 12.0Hz, 3H), 1.49 (d, J = 4.0Hz, 3H), 2.45 (s, 3H), 3.47 (dd, J = 4.0, 8.0Hz, 1H), 3.57 (dd, J = 4.0, 8.0Hz, 2H), 3.85 (m, 2H), 3.87 (s, 6H), 5.22 (s, 2H), 5.44 (m, 1H), 7.25 (s, 2H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 14.9, 18.5, 22.0, 46.9, 56.1, 56.8, 65.0, 68.9, 96.5, 106.7, 125.3, 139.0, 153.1, 165.1, 169.8 ppm
IR (KBr): ṽ = 2976, 2934, 1767, 1592, 1416, 1330, 1127 cm^{-1}
HPLC-MS: R_t = 8.54, m/z: 400 (100) $[M+H]^+$, 417 (75) $[M+H_2O]^+$, 821 (35) $[2M+Na]^+$

(R)-1-((S)-1-(methylthio)-2-oxoazetidin-3-yl)ethyl 4-hydroxy-3,5-dimethoxybenzoate (**34**)



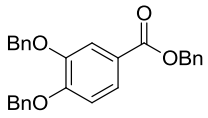
34

In a 25mL two-necks flask were dissolved under nitrogen atmosphere 42mg of **44** (0.1mmol) in 12.2mL of anhydrous DCM, followed by TFA addition at 0°C (37µL, 0.5mmol) and TLC monitoring (cyclohexane/EtOAc 40/60). At SM disappearance the reaction was directly concentrated to afford **34** in 32% yield (11mg) as a white solid after pentane titration.
Rf 0.35 (cyclohexane/EtOAc 40/60)

$[\alpha]_D$ = -12.7°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.28 1.46 (d, J = 8.0Hz, 3H), 2.42 (s, 3H), 3.46 (dd, J = 8.0, 12.0Hz, 1H), 3.55 (dd, J = 8.0, 12.0Hz, 2H), 3.88 (s, 6H), 5.41 (m, 1H), 5.92 (bs, 1H, OH), 7.24 (s, 2H) ppm

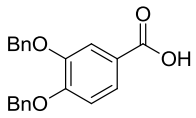
^{13}C NMR (100MHz, $CDCl_3$): δ 18.5, 22.0, 47.0,56.5, 56.7, 68.7, 106.7, 120.3, 139.5, 146.6, 165.3, 169.9 ppm
IR (KBr): ṽ = 3423, 2923, 1749, 1638, 1458, 1116 cm^{-1}
HPLC-MS: R_t = 5.34min, m/z: 342 (100) $[M+H]^+$, 359 (10) $[M+H_2O]^+$, 705 (10) $[2M+Na]^+$

methyl 3,4-bis(benzyloxy) benzoate (**47**)



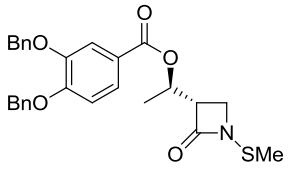
In a 25mL two-necks flask under nitrogen atmosphere 500mg (3.24mmol) of 3,4-dihydroxy benzoic acid were dissolved in 6.5mL of anhydrous DMF before adding 2.76g (20.0mmol) of K_2CO_3 and 1.8mL (15.0mmol) of BnBr. The reaction was monitored by TLC (cyclohexane/EtOAc 90/10) and quenched with ice, extracted with EtOAc, washed with a saturated aqueous NH_4Cl solution (2 times) and brine (1 time), then dried over Na_2SO_4 and concentrated in vacuum to reach **47** as a white solid in 97% yield (1.332g) after purification via FC (cyclohexane/EtOAc 95/5).

3,4-bis(benzyloxy)benzoic acid (**48**)



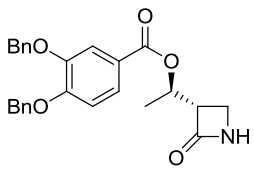
In a 50mL flask 1.125g (2.65mmol) of **47** were dissolved in 25.0mL of MeOH and 4.9mL of 5M aqueous NaOH (24.6mmol)were added. After three day stirring at reflux and following by TLC monitoring (Et2O/EtOAc 90/10) half of the solvent was evaporate under vacuum, water was added and the crude treated with petroleum ether before acidifying the aqueous phase with 1M HCl and extracting with EtOAc. The organic phase was then dried over Na_2SO_4, filtered and concentrated to reach **48** as a white solid in 99% yield (0.879g).

(R)-1-((S)-1-(methylthio)-2-oxoazetidin-3-yl)ethyl 3,4-bis(benzyloxy) benzoate (**46**)



In a 100mL two-necks flask under nitrogen atmosphere 150mg (0.93mmol) of **41** were dissolved in 21.0mL of anhydrous DCM before adding 311mg (0.93mmol) of acid **48**, 115mg (0.93mmol) of DMAP and, at 0°C, 178mg (0.93mmol) of EDC. The reaction was monitored by TLC and quenched at 0°C with HCl until neutrality, then extracted with EtOAc, dried over Na_2SO_4 and concentrated in vacuum to reach **46** as a yellow oil in 18% yield (81mg) after purification via FC (cyclohexane/EtOAc 85/15).
Rf 0.49 (cyclohexane/EtOAc 60/40)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.45 (d, J = 6.0Hz, 3H, Me), 2.43 (s, 3H, SMe), 3.45 (dt, J = 2.4, 4.0Hz, 1H, CH), 3.52 (t, J = 5.6Hz, 1H, CH_2), 3.54 (m, 1H, CH_2), 5.22 (m, 2H, CH_2Bn), 5.40 (dq, J = 3.2, 3.6Hz, 1H, CHMe), 6.92 (d, J = 8.0Hz, 1H, Ar), 7.41 (m, 5H, Bn), 7.49 (m, 5H, Bn), 7.59 (m, 2H, Ar) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 18.5, 21.8, 46.8, 56.8, 68.5, 70.7, 71.2,113.1, 115.6, 122.7, 123.9, 127.0, 127.3, 127.8, 127.9, 128.4, 128.5, 136.4, 136.7, 148.3, 153.1, 165.1, 169.8 ppm
IR: ṽ = 3064, 3032, 2928, 1766, 1709, 1599, 1510, 1454, 1427, 1380, 1270, 1206, 1130, 1024, 875,735, 697 cm^{-1}
HPLC-MS: R_t = 11.50min, m/z: 478 (60) $[M+H]^+$, 495 (100) $[M+H_2O]^+$, 500 (40) $[M+Na]^+$, 979 (70) $[2M+Na]^+$

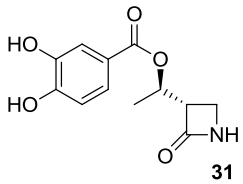
(R)-1-((S)-2-oxoazetidin-3-yl)ethyl 3,4-bis(benzyloxy)benzoate (**45**)



In a 10mL 2-neck flask a mixture of **46** (106mg, 0.22mmol), anhydrous THF (1.5mL), MeOH (1.5mL) and Pd/C 10%$_{w/w}$ (14mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (16h) the mixture was filtered and concentrated in vacuum. The desired product **24** was obtained as a white waxy solid in 27% yield (26mg) after purification via flash-chromatography (cyclohexane/EtOAc 40/60).

Rf 0.60 (cyclohexane/EtOAc 40/60)
$[\alpha]_D$ = -39.9°
^{1}H NMR (400MHz, CD_3CN): δ = 1.44 (d, J = 6.4Hz, 3H, Me), 3.26 (dd, J = 1.6, 4.0Hz, 1H), 3.39 (t, J = 5.6Hz, 1H), 3.57 (dd, J = 7.2, 6 .0Hz, 1H), 5.40 (quintet, J = 6.4Hz, 1H), 5.71 (bs, NH), 6.90 (d, J = 9.2Hz, 2H, Ar), 7.35 (m, 10H, Bn), 7.57 (d, J = 6.8Hz, 1H, Ar) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 18.6, 22.3, 25.8, 29.7, 35.1, 39.3, 40.3, 46.0, 51.9, 55.9, 56.7, 61.1, 63.9, 68.9, 70.8, 71.3, 76.6, 101.4, 113.2, 115.7, 123.0, 127.1, 127.3, 127.9, 128.0, 128.5, 128.6, 136.4, 136.8, 148.3, 152.9, 153.2, 165.2, 167.4 ppm
IR: $\tilde{\nu}$ = 3332, 2974, 2930, 2872, 1755, 1710, 1600, 1510, 1454, 1427, 1380, 1343, 1270, 1206, 1130, 1064, 1023, 763, 738, 697 cm^{-1}
HPLC-MS: Rt = 10.01min, m/z = 449 (100) $[M+H_2O]^+$, 454 (10) $[M+Na]^+$, 885 (45) $[2M+Na]^+$

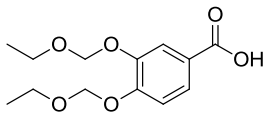
(R)-1-((S)-2-oxoazetidin-3-yl)ethyl 3,4-dihydroxybenzoate (**31**)


31

In a 10mL 2-neck flask a mixture of **45** (26mg, 61μmol), anhydrous THF (0.43mL), MeOH (0.43mL) and Pd/C 10%$_{w/w}$ (4mg) was stirred under H_2 atmosphere (1atm) at room temperature. At starting material consumption (16h) the mixture was filtered and concentrated in vacuum. The desired product **31** was obtained as a white waxy solid in 59% yield (9mg) after purification via flash-chromatography (cyclohexane/EtOAc 50/50).

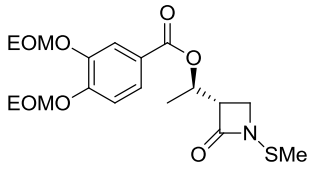
Rf 0.33 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = -22.6°
^{1}H NMR (400MHz, CD_3CN): δ = 1.39 (d, J = 6.0Hz, 3H, Me), 3.31 (dd, J = 2.8Hz, 1H), 3.41 (t, J = 5.6Hz, 1H), 3.50 (dd, J = 2.4, 2.8Hz, 1H), 5.31 (quintet, J = 6.4Hz, 1H), 7.01 (bs, NH), 7.44 (m, 2H, Ar,), 7.48 (s, 1H, Ar), 8.27 (bs, OH), 8.62 (bs, OH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 18.7, 30.1, 34.3, 39.4, 56.9, 68.5, 121.1 , 127.1, 135.7, 158.3, 166.1, 167.7 ppm
IR: $\tilde{\nu}$ = 3380, 2925, 1734, 1701, 1603, 1522, 1443, 1363, 1295, 1228, 1114, 1092, 1064, 952, 766 cm^{-1}
HPLC-MS: Rt = 1.95min, m/z = 252 (85) $[M+H]^+$, 274 (50) $[M+Na]^+$, 525 (100) $[2M+Na]^+$

3,4-bis(ethoxymethoxy)benzoic acid (**49**)



A solution of methyl 3,4-dihydroxybenzoate (495g, 2.95mmol) in THF (5.9mL) was added dropwise to a suspension of NaH (60% in mineral oil, 354mg, 8.85mmol) in THF (17.7mL) at 0°C under inert atmosphere. After 10 minutes a solution of chloromethylethylether (808μL, 8.85mmol) in THF (6.4mL) was added dropwise and the reaction was allowed to warm to room temperature and monitored by TLC. After 2h the reaction was quenched with aqueous NH_4Cl (30mL) and extracted with EtOAc (3x20mL). The organic extracts were dried over Na_2SO_4 and concentrated to obtain methyl 3,4-bis(ethoxymethoxy) benzoate (733mg, 87%) as pale yellow oil after flash-chromatography purification (cyclohexane/EtOAc 50/50). It was directly dissolved in a mixture of THF/MeOH (7.25/0.87mL) and treated with 5M NaOH (2.14mL). The reaction was heated at 40°C for 4 hours and monitored by TLC. At completion, EtOAc (2x10mL) was added and the organic phase was separated and discarded. The aqueous phase was then cooled to 0°C and adjusted to pH = 6 with aqueous 1M HCl. The mixture was extracted with EtOAc (3x15mL), dried on Na_2SO_4 and concentrated to afford **49** (571mg, 99%) as a white solid.

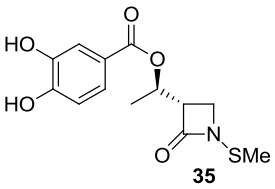
(R)-1-((S)-1-(methylthio)-2-oxoazetidin-3-yl)ethyl 3,4-bis(ethoxymethoxy)benzoate (**50**)



In a 100mL two-necks flask under nitrogen atmosphere 95mg (0.59mmol) of **41** were dissolved in 13.6mL of anhydrous DCM before adding 224mg (0.83mmol) of acid **49**, 101mg (0.83mmol) of DMAP and, at 0°C, 159mg (0.83mmol) of EDC. The reaction was monitored by TLC and quenched at

0°C with HCl until neutrality, then extracted with DCM, dried over Na_2SO_4, filtered and concentrated in vacuum to reach **50** as a colorless oil in 32% yield (78mg) after purification via FC (cyclohexane/EtOAc 80/20).
Rf 0.73 (cyclohexane/EtOAc 40/40)
$[\alpha]_D$ = -36.1°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.15-1.27 (m, 6H, EtO), 1.45 (d, J = 6.0Hz, 3H, Me), 2.43 (s, 3H, SMe), 3.37-3.38 (m, 1H), 3.47-3.48 (m, 1H), 3.52-3.54 (m, 1H), 3.70-3.80 (m, 4H, EtO), 5.28 (s, 2H, OCH_2O), , 5.32 (s, 2H, OCH_2O), 5.41 (quintet, J = 6.4Hz, 1H, CHO), 7.18 (d, J = 8.4Hz, 1H, Ar), 7.62 (d, J = 8.4Hz, 1H, Ar) 7.77 (s, 1H, Ar) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 15.0, 18.3, 18.7, 21.9, 46.9, 56.9, 64.6, 68.0, 68.6, 93.5, 94.2, 115.0, 118.0, 123.6, 124.6, 146.7, 151.2, 165.0, 169.9 ppm
IR: $\tilde{\nu}$ = 3503, 2977, 2922, 1768, 1715, 1601, 1510, 1434, 1263, 1170, 1112, 981, 765 cm^{-1}
HPLC-MS: R_t = 9.13min, m/z: 414 (100) $[M+H]^+$, 431 (85) $[M+H_2O]^+$, 436 (25) $[M+Na]^+$, 849 (37) $[2M+Na]^+$

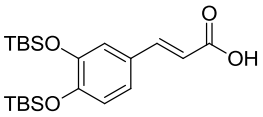
(R)-1-((S)-1-methylthio-2-oxoazetidin-3-yl)ethyl 3,4-dihydroxybenzoate (**35**)



35

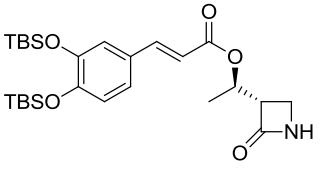
In a 25mL two-necks flask were dissolved under nitrogen atmosphere 78mg of **50** (0.19mmol) in 23.0mL of anhydrous DCM, followed by TFA repeated addition, every 30min, at 0°C (139µL, 0.5mmol, repeated for 7 times) and with TLC monitoring (cyclohexane/EtOAc 40/60). At SM disappearance the reaction was directly concentrated to afford **35** in 41% yield (23mg) as a yellow oil after FC purification (DCM/acetone 90/10).
Rf 0.34 (cyclohexane/EtOAc 40/60), 0.33 (DCM/acetone 80/20)
$[\alpha]_D$ = -164.4°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.40 (d, J = 6.4Hz, 3H, Me), 2.41 (s, 3H, SMe),
3.55-3.59 (m, 3H), 5.43 (quintet, J = 5.6Hz, 1H, CHO), 6.84 (d, J = 8.4Hz, 1H, Ar), 7.39 (dd, J = 1.2, 8.4Hz, 1H, Ar), 7.50 (d, J = 1.2Hz, 1H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 18.3, 21.9, 46.6, 56.5, 67.7, 114.7, 116.5, 121.7, 123.3, 143.7, 149.4, 165.7, 171.5 ppm
IR (KBr): $\tilde{\nu}$ = 3441, 1701, 1685, 1637, 625 cm^{-1}
HPLC-MS: R_t = 4.02min, m/z: 298 (100) $[M+H]^+$, 315 (35) $[M+H_2O]^+$, 617 (13) $[2M+Na]^+$

(E)-3-(3,4-bis(*t*butyldimethylsilyloxy)phenyl)acrylic acid (**51**)



To a solution of caffeic acid (450mg, 2.5mmol) in DMF (4.5mL) under inert atmosphere, imidazole (1.53g, 22.5mmol) and then TBSCl (11.25mmol) were added. After 40h the reaction was quenched at 0°C by a citric acid saturated solution and then extracted with Et_2O. The organic phases were washed twice with water and brine, dried over Na_2SO_4 and evaporated. To the persilylated intermediate (1.798g) in 1.86mL MeOH and 3.10mL of THF, a solution of K_2CO_3 (187mg, 1.35mmol) in water (1.86mL) was added. After 24h the mixture was quenched a 0°C by adding a saturated solution of citric acid and extracting with Et_2O. The organic phases were washed with water and brine, dried over Na_2SO_4 and evaporated. The product was finally obtained by FC purification (DCM/MeCN 95/5) in 70% yield (984mg) as a white solid.

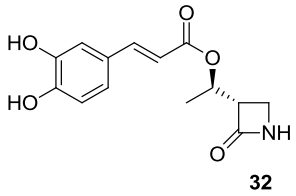
(E)-(R)-1-((S)-2-oxoazetidin-3-yl)ethyl3-(3,4-bis((*t*butyldimethylsilyl)oxy) phenyl)acrylate (**52**)



In a 50mL two-necks flask under nitrogen atmosphere 127mg (1.1mmol) of **39** were dissolved in 25.0mL of anhydrous DCM before adding 629mg (1.54mmol) of acid **51**, 191mg (1.54mmol) of DMAP and, at 0°C, 296mg (1.54mmol) of EDC. The reaction was monitored by TLC and quenched at

0°C with 1M HCl until neutrality, then extracted with DCM, dried over Na_2SO_4 and concentrated in vacuum to reach **52** as a colorless oil in 36% yield (215mg) after purification via two FCs (DCM/Et_2O 80/20, then cyclohexane/EtOAc 90/10).

(E)-(R)-1-((S)-2-oxoazetidin-3-yl)ethyl 3-(3,4-dihydroxyphenyl)acrylate (**32**)



32

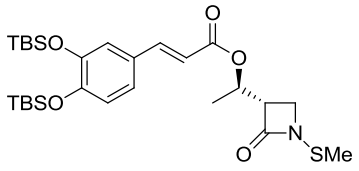
In a 50mL three-necks flask under nitrogen atmosphere 101mg (0.2mmol) of **52** were dissolved in 4.1mL of anhydrous MeCN before slowly adding, at 0°C, 58µL of $BF_3 \cdot Et_2O$. After 30 minutes stirring at 0°C the reaction was allowed to reach room temperature and was then quenched at 0°C with a pH = 7.5 aqueous phosphate buffer solution at starting material disappearance, extracted with EtOAc, dried over Na_2SO_4, filtered and concentrated in vacuum to reach **32** as a yellow oil in 14% yield (8mg) after pentane titration.

Rf 0.27 (cyclohexane/EtOAc 40/60)

^{1}H NMR (400MHz, CD_3OD): δ 1.69 (d, J = 6.4Hz, 3H, Me), 3.00 (dd, J = 7.2, 13.2Hz, 1H), 3.82 (dd, J = 8.0, 14.8Hz, 1H), 3.88 (dd, J = 5.6, 14.8Hz, 1H), 5.03 (quintet, J = 7.2Hz, 1H, CHO), 6.46 (d, J = 15.6Hz, 1H, C_{sp2}H), 6.87 (d, J = 8.4Hz, 1H, Ar), 7.09 (dd, J = 2.0, 8.4Hz, 1H, Ar), 7.16 (d, J = 2.0, 1H, Ar), 7.76 (d, J = 15.6, 1H, C_{sp2}H) ppm

HPLC-MS: R_t = 1.502min, m/z: 278 (100) $[M+H]^+$

(E)-(R)-((S)-methylthio-2-oxoazetidin-3-yl)ethyl 3-(3,4-bis(*t*butyldimethylsilyloxy)phenyl)acrylate (**53**)



In a 100mL two-necks flask under nitrogen atmosphere 150mg (0.93mmol) of **41** were dissolved in 21.0mL of anhydrous DCM before adding 380mg (0.93mmol) of acid **51**, 115mg (0.93mmol) of DMAP and, at 0°C, 178mg (0.93mmol) of EDC. The reaction was monitored by TLC and quenched at 0°C with HCl until neutrality, then extracted with EtOAc, dried over Na_2SO_4, filtered and concentrated in vacuum to reach **53** as a yellow oil in 55% yield (280mg) after purification via flash-chromatography (cyclohexane/EtOAc 85/15).

Rf 0.37 (cyclohexane/EtOAc 60/40)

$[\alpha]_D$ = -41.5°

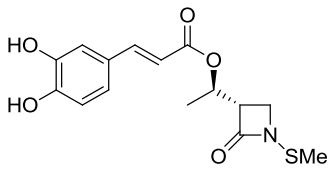
^{1}H NMR (400MHz, $CDCl_3$): δ 0.22 (s, 2H, Me_2Si of TBS), 0.23 (s, 2H, Me_2Si of TBS), 0.99 (s, 9H, Me_3C of TBS), 1.01 (s, 9H, 3Me), 1.45 (d, J = 6.4Hz, 3H, Me), 2.47 (s, 3H, SMe), 3.45 (dt, J = 2.4, 4.0Hz, 1H, CH), 3.53 (t, J = 5.6Hz, 1H, CH_2), 3.54 (dd, J = 2.4, 4.0Hz, 1H, CH_2), 5.35 (dq, J = 6.0, 6.4Hz , 1H, CHMe), 6.19 (d, J = 16.0Hz, 1H, =CH), 6.82 (d, J = 8.8Hz, 2H, Ar), 7.01 (m, 1H, Ar), 7.57 (d, J = 16.0Hz, 1H, =CH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ -4.2, -4.1, 18.3, 18.4, 18.5, 21.8, 25.7, 25.8, 46.8, 56.7, 68.0, 115.1, 120.3, 121.0, 122,3 127.6, 145.2, 147.1, 149.5, 166.1, 169.9 ppm

IR: $\tilde{\nu}$ = 3411, 2930, 2858, 1770, 1709, 1633, 1595, 1567, 1508, 1423, 1289, 1163, 1029, 906, 840, 782, 735, 696 cm^{-1}

HPLC-MS: R_t = 32.94min, m/z: 574 (100) $[M+Na]^+$, 1125 (15) $[2M+Na]^+$

(E)-(R)-1-((S)-1-(methylthio)-2-oxoazetidin-3-yl)ethyl 3-(3,4-dihydroxyphenyl)acrylate (**36**)

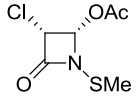


36

In a 50mL three-necks flask under nitrogen atmosphere 115mg (0.21mmol) of **53** were dissolved in 4.3mL of anhydrous MeCN before slowly adding, at 0°C, 60µL of $BF_3 \cdot Et_2O$. After 30 minutes stirring at 0°C the reaction was allowed to reach room temperature and was quenched at 0°C with a pH = 6 phosphate buffer aqueous solution at starting material disappearance, then

allowed to r.t., extracted with EtOAc, dried over Na_2SO_4, filtered and concentrated in vacuum to reach **36** as a yellow waxy solid in 95% yield (64mg) after purification via flash-chromatography (cyclohexane/EtOAc 80/20).
Rf 0.45 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = -44.8°
^{1}H NMR (400MHz, $CDCl_3$): δ 1.33 (d, J = 6.4Hz, 3H, Me), 2.44 (s, 3H, SMe), 3.52 (dt, J = 2.4, 4.0Hz, 1H, CH), 3.54 (t, J = 5.6Hz, 1H, CH_2), 3.58 (dd, J = 2.0, 2.4Hz, 1H ,CH_2), 5.35 (dq, J = 6.0, 6.4Hz, 1H, CHMe), 6.22 (d, J = 16.0Hz, 1H, =CH), 6.85 (d, J = 8Hz, 1H, Ar), 7.08 (d, J = 8Hz, 1H, Ar), 7.13 (s, 1H, Ar), 7.52 (d, J = 16.0Hz, 1H, =CH), 8.16 (bs, OH), 8.45 (bs, OH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 17.8, 20.8, 45.9, 56.6, 67.4, 114.3, 114.5, 115.5, 121.7, 126.6, 145.2, 145.4, 147.9, 165.6, 169.5, 205.4 ppm
IR: $\tilde{\nu}$ = 3365, 2926, 1735, 1601, 1515, 1445, 1363, 1263, 1179, 1055, 983, 856, 816 cm^{-1}
HPLC-MS: R_t = 5.33min, m/z: 324 (100) $[M+H]^+$, 346 (15) $[M+Na]^+$, 669 (80) $[2M+Na]^+$

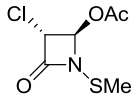
(2S,3R)-3-chloro-1-(methylthio)-4-oxoazetidin-2-yl acetate (**53**)



53

A 1M solution of LiHMDSA (605µL, 0.605mmol) was added to a solution of **52** (70mg, 0.55mmol) in THF (5.5mL) at -78°C under inert atmosphere, followed by methanesulfonyl chloride (130µL, 2.5mmol) added drop by drop. The solution was allowed to warm at room temperature and was monitored by TLC (cyclohexane/EtOAc 50/50). After 3h the reaction was quenched with aqueous NH_4Cl and extracted with EtOAc (x2) and DCM (x3). The organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuum. The residue was then recovered and purified by flash-chromatography (cyclohexane/EtOAc 70/30) to afford product **53** in an overall 16.5% yield.
Rf 0.64 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ 25 = +226.0° (c = 3.1, DCM)
^{1}H (400MHz, $CDCl_3$): 2.24 (s, 3H, MeCO), 2.57 (s, 3H, SMe), 5.09 (d, J = 4.0Hz, 1H, $ClCH_ACH_B$), 6.12 (d, J = 3.2Hz, $ClCH_ACH_B$) ppm
^{13}C (100MHz, $CDCl_3$): 20.7, 22.9, 61.0, 76.7, 77.0, 77.3, 165.4, 169.6 ppm
IR: $\tilde{\nu}$ = 2923, 2359, 1791, 1756, 1699, 1221, 1162, 1090 cm^{-1}
HPLC-MS: Rt = 4.91min, m/z = 168 $[M\text{-}AcO+H_2O]^+$, 210 $[M+H]^+$, 227 $[M+H_2O]^+$, 229 $[M(^{37}Cl)+H_2O]^+$, 232 $[M+Na]^+$

(2R,3R)-3-chloro-1-(methylthio)-4-oxoazetidin-2-yl acetate (**55**)



55

A 1M solution of LiHMDSA (605µL, 0.605mmol) was added to a solution of **52** (70mg, 0.55mmol) in THF (5.5mL) at -78°C under inert atmosphere, followed by methanesulfonyl chloride (130µL, 2.5mmol). The solution was allowed to warm to room temperature and was monitored by TLC (cyclohexane/EtOAc 50/50). After 3h the reaction was quenched with aqueous NH_4Cl and extracted with EtOAc and DCM. The organic extracts were dried over Na_2SO_4 and the residue was purified by flash-chromatography (cyclohexane/EtOAc 70/30) to afford product **55** in 5% yield.
Rf 0.78 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = +13.0° (c = 0.9, DCM)
^{1}H (400MHz, $CDCl_3$): 2.21 (s, 3H, MeCO), 2.55 (s, 3H, SMe), 4.74 (d, J = 0.8Hz, 1H, $ClCH_ACH_B$), 6.03 (d, J = 0.4Hz, $ClCH_ACH_B$) ppm
^{13}C (100MHz, $CDCl_3$): 20.7, 22.6, 61.5, 76.7, 77.0, 77.3, 164.1, 169.4 ppm
IR: $\tilde{\nu}$ = 2924, 2853, 1799, 1759, 1216, 1156, 1048cm^{-1}
HPLC-MS: Rt = 4.77min, m/z = 168 $[M\text{-}AcO+H_2O]^+$, 227 $[M+H_2O]^+$, 229 $[M(^{37}Cl)+H_2O]^+$, 232 $[M+Na]^+$

Thiomethylation optimized general procedure

In a 10mL two-necks flask under nitrogen atmosphere 22.5μL (0.25mmol) of dimethyl disulfide were dissolved in 0.45mL of anhydrous THF before slowly adding, at 0°C, 22.3μL (0.275mmol)of sulfuryl chloride dissolved in 0.55mL of THF. After 5-10 minutes stirring at 0°C β-lactam substrate (0.55mmol, 143mg for **B**) were added, followed by slowly dropped TEA (77μL, 0.55mmol). The reaction was allowed to reach r.t. after 15min and quenched with saturated aqueous NH_4Cl solution after 1h stirring, then extracted with DCM, dried over Na_2SO_4 and concentrated in vacuum to reach the desired product after FC purification (cyclohexane/EtOAc 80/20 for **B**).

3.3.5 Lead β-lactam synthesis optimization

For the isomerization step, we identified as optimal reaction conditions the use of MeCN as solvent, DMAP as catalyst in 10% loading, and 4h stirring at 50°C as best time and temperature conditions.

For the kinetic study of isomerization process, 0.4mL of an E-isomer MeCN mother solution (0.125M, 0.05mmol) were put in five different reaction vessels already brought at the working temperature. Were then added a 0.1mL of a 0.05M DMAP solution in MeCN (0.005mmol). After 1h stirring the reactions were directly evaporated, dissolved in $CDCl_3$ and analyzed at ^{1}H-NMR for E/Z ratio determination. For the esterification process, in a 10mL flask 132mg (0.3mmol) of (Z)-benzyl 2-((S)-3-(R)-1-hydroxyethyl-4-oxoazetidin-2-ylidene) acetate (the β-lactam intermediate) were dissolved in 2.0mL of chlorobenzene before adding 121mg (0.474mmol) of acid **19**, 7mg (0.06mmol) of DMAP and 98mg (0.474) of DCC. The reaction was then treated for 10 minutes with MW irradiation at 40W, monitored by TLC (cyclohexane/EtOAc 50/50) and finally purified by FC (DCM/MeCN 97/3) to reach the target compound in a 67% yield (100mg), in the best case.

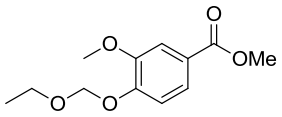
3.3.6 Thermostability

For thermostability evaluation, in 0.6mL of milliq. water were suspended and sonicated 0,63mg of **56**. First HPLC-MS analysis were made after one week in these conditions, then after 30min stirring at 60°C, and finally after 60min stirring at 70°C. After both the thermal treatments, water was evaporated and 0.6mL of $CDCl_3$ were added to record 600MHz ^{1}H-NMR spectra. The same thermostability analysis was performed on 0.64mg of compound **57**, chose as internal standard, (see below) in 0.6mL of milliq. water.

3.3.7 Analysis method development

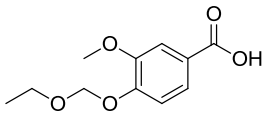
(Z)-Benzyl 2-((S)-3-(R)-1-hydroxyethyl-4-oxoazetidin-2-ylidene) acetate (the free alcohol β-lactam intermediate) was prepared as previously reported [19].

Methyl 4-(ethoxymethoxy)-3-methoxybenzoate (58)



In a 100mL 3-necks flask with an addition funnel, to a suspension of NaH (60% in oil, 256mg, 6.4mmol) in 1.05mL of anhydrous THF was added dropwise at 0°C a solution of methyl 4-hydroxy-3-methoxybenzoate (898mg, 4.93mmol) in THF (8.0mL) and, after 10min, a solution of EOM-chloride in 7.0mL of THF. The reaction was allowed to stir at r.t. and was followed by TLC monitoring (cyclohexane/EtOAc 50/50). At the disappearance of SM (2h) it was quenched with a saturated NH_4Cl solution until pH = 6, then extracted with EtOAc (3x15mL) and evaporated to afford only **58** as a yellow oil (1.183g, y = 99%).
Rf 0.75 (cyclohexane/EtOAc 50/50)
^{1}H (400MHz, $CDCl_3$): 1.23 (t, J = 14.4Hz, 3H, Et), 3.78 (quartet, J = 14.0Hz, 2H, Et), 3.90 (s, 3H, COOMe), 3.94 (s, 3H, OMe), 5.35 (s, 2H, OCH_2O), 7.21 (d, J = 17.2Hz, 1H, Ar)), 7.57 (d, J = 4.0Hz, 1H, Ar)), 7.65 (dd, J = 3.6, 16.8Hz, 1H, Ar) ppm
^{13}C (100MHz, $CDCl_3$): 14.7, 51.5, 55.6, 64.2, 93.3, 112.2, 114.4, 123.0, 123.5, 148.8, 150.4, 166.3 ppm
IR: $\tilde{\nu}$ = 3409, 2952, 1712, 1598, 1513, 1435, 1293, 1112, 765 cm^{-1}
HPLC-MS: Rt = 7.37min, m/z = 241 (100) $[M+H]^+$, 263 (14) $[M+Na]^+$

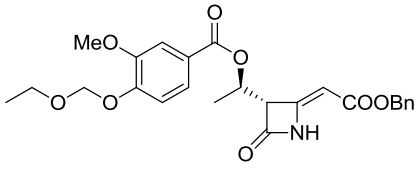
4-(ethoxymethoxy)-3-methoxybenzoic acid (**59**)



In a 100mL flask **58** (1.183g, 4.93mmol) was dissolved in 9.86mL of THF and 4.93mL of MeOH, then a solution of NaOH (986mg, 24.64mmol) in water (4.93mL) was added and the reaction left stirring at r.t. until SM disappearance (18h). EtOAc was then added (2x10mL), the aqueous phases recovered and treated at 0°C with an aqueous HCl solution (1M) to reach pH = 7-8, then extracted again with EtOAc (1x10mL), and finally was added HCl (1M) until pH = 5 to allow EtOAc (3x10mL) extraction, Na_2SO_4 anhydrification, filtration and in vacuum concentration of the desired product. **59** was obtained as white solid (568mg) in 51% yield, eventually recrystallizing from MeCN.
M.p. (133-135)°C; Rf 0.21 (cyclohexane/EtOAc 50/50)
^{1}H NMR (400MHz, CD_3OD): δ 1.24 (t, J = 7.2Hz, 3H, Et), 3.79 (quartet, J = 6.8Hz, 2H, Et), 3.96 (s, 3H, OMe), 5.38 (s, 2H, OCH_2O), 7.25 (d, J = 8.4Hz, 1H, Ar), 7.63 (d, J = 1.6Hz, 1H, Ar), 7.75 (dd, J = 1.6, 8.4Hz, 1H, Ar) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 15.0, 56.0, 64.7, 93.7, 112.8, 114.6, 122.9, 124.3, 149.1, 151.4, 171.9 ppm
IR: $\tilde{\nu}$ = 3396, 3048, 2962, 2901, 1681, 1590, 1458, 1426, 1279, 1116, 737 cm^{-1}
HPLC-MS: Rt = 11.07min, m/z: 225 (100) $[M-H]^-$

(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl

4-(ethoxymethoxy)-3-methoxybenzoate (**60**)

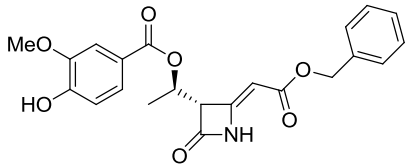


In a 50mL 2-neck flask, to a solution of (Z)-benzyl 2-((S)-3-(R)-1-hydroxyethyl-4-oxoazetidin-2-ylidene) acetate (the 4-alkylidene β-lactam intermediate, 83mg, 0.32mmol) in DCM (12.0mL) was added **59** (115mg, 0.51mmol), DMAP (8mg, 0.064mmol) and, at 0°C, DCC (105mg, 0.51mmol), allowing to reach r.t. after 15 minutes and then to stir 40 hours before quenching the reaction with water, extracting with DCM (3x10mL), evaporating and recovering five times with EtOAc to remove the insoluble DC-Urea. The mixture was concentrated in vacuum and the crude was finally purified by flash-chromatography (DCM/MeCN 98/2 to 95/5) to afford **60** in 16% yield (24mg) as a colorless oil.
Rf 0.81 (cyclohexane/EtOAc 30/70), 0.58 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = +13.6° (c = 4.36mg/mL, DCM)
^{1}H NMR (400MHz, CD_3OD): δ 1.22 (t, J = 7.2Hz, 3H, Et), 1.52 (d, J = 6.4Hz, 2H, Me), 3.77 (quartet, J = 7.2Hz, 2H, Et), 3.90 (s, 3H, OMe), 4.02 (d, J = 6.4Hz, 1H, MeCHC*H*), 5.15 (d, J_{AB} = 12.4Hz, 1H, Bn),

5.19 (d, J_{AB} = 12.0Hz, 1H, Bn), 5.28 (s, 1H, CHCOOBn), 5.34 (s, 2H, $_{OCH2O}$), 5.48 (quintet, J = 6.4Hz, 1H, MeC*H*CH) 7.20 (d, J = 8.0Hz, 1H, Ar), 7.32-7.37 (m, 5H, Bn), 7.54 (d, J = 2.0Hz, 1H, Ar), 7.61 (dd, J = 2.0, 8.8Hz, 1H, Ar), 8.70 (bs, 1H, NH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ 15.0, 18.1, 55.9, 61.6, 64.6, 66.1, 67.1, 90.6, 93.6, 112.4, 114.7, 123.2, 123.4, 128.3, 128.5, 135.7, 149.2, 151.0, 152.2, 164.6, 165.0, 166.6 ppm
IR: $\tilde{\nu}$ = 3295, 2980, 1822, 1704, 1658, 1601, 1511, 1268, 1217, 1139, 1109, 736 cm^{-1}
HPLC-MS: Rt = 9.99min, m/z: 487 (100) $[M+H_2O]^+$, 961 (13) $[2M+Na]^+$

(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)

ethyl-4-hydroxy-3-methoxybenzoate (**57**)



57

In a 25mL two-necks flask were dissolved under nitrogen atmosphere 22mg of **60** (46.9µmol) in 5.8mL of anhydrous DCM, followed by 23.1 TFA equivalents distributed, every 30min, in 5 hours and always added at 0°C. At starting material disappearance the reaction was directly concentrated to afford **57** in 99% yield (19mg) as a sticky-waxy solid after pentane titration.

Rf 0.01 (cyclohexane/EtOAc 40/60)
$[\alpha]_D$ = +11.3°(c = 3.45mg/mL, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.52 (d, J = 6.4Hz, 2H, Me), 3.91 (s, 3H, OMe), 4.02 (d, J = 6.0Hz, 1H, MeCHC*H*), 5.15 (d, J_{AB} = 12.0Hz, 1H, Bn), 5.19 (d, J_{AB} = 12.0Hz, 1H, Bn), 5.28 (s, 1H, CHCOOBn), 5.48 (quintet, J = 6.0Hz, 1H, MeC*H*CH) 6.94 (d, J = 8.4Hz, 1H, Ar), 7.33-7.37 (m, 5H, Bn), 7.51 (s, 1H, Ar), 7.60 (d, J = 8.4Hz, 1H, Ar) 8.73 (bs, 1H, NH) ppm
^{13}C NMR (50MHz, $CDCl_3$): δ 18.1, 56.0, 61.7, 66.2, 67.1, 90.6, 111.8, 114.2, 121.7, 124.3, 128.2, 128.3, 128.6, 135.7, 146.4, 150.4, 152.2, 164.7, 165.0, 166.7 ppm
IR: $\tilde{\nu}$ = 3379, 2933, 2852, 1819, 1699, 1659, 1597, 1514, 1282, 1220, 1144, 1029 cm^{-1}
HPLC-MS: Rt = 8.32min, m/z: 409 (100) $[M-H]^-$; 429 (100) $[M+H_2O]^+$, 845 (36) $[2M+Na]^+$

SPE pre-treatment and HPLC/MS-MS analysis

All solvents employed were LC-MS grade and were used with 0,1% of LC-MS grade HCOOH when specified.

Was used an SPE Waters Oasis HLB 3cc column, preconditioned with 3mL MeCN + 0,1% HCOOH followed by 3mL H_20 + 0,1% HCOOH and then loaded with the dried sample recovered in 150µL of a 95/5 mixture of H_20 + 0,1% HCOOH and MeCN + 0,1% HCOOH: the first fraction (f1) was so collected. Three 1mL H_20 + 0,1% HCOOH fractions were then eluted, followed by a sequence of 1mL 80/20 of H_20 + 0,1% HCOOH and MeCN + 0,1% HCOOH (f5), 1mL of a 60/40 mixture (f6), 1mL of 40/60 (f7) and 1mL of 20/80 (f8).

The HPLC column Waters XSelect HSS T3 3.5µm (2.1x150mm) was conditioned at 0.3mL/min with two cycles H_2O/MeCN 80/20 → 20/80 (10min), then 5min → 80/20 (10min), then 5min → 40/60.

Were always injected 20µL of samples via a 250µL Hamilton syringe, washed every time with a 1/1 mixture of H_20 + 0,1% HCOOH and MeCN + 0,1% HCOOH.

The analysis was performed at 0.3mL/min, with H_2O/MeCN 90/10 → 10/90 (10min), then 5min → 90/10 (5min), for a 20min overall running time.

Rt (**56**, **57**): 12.3-12.6min (due to manual injection and analysis starts)

The MS-MS analysis was made on a WatersMicromassQuattroPremier XE, with parameters optimized for **56** (440.16 $[M-H]^-$ → 196.77 $[Syringic-H]^-$). To monitoring **57** were employed the same conditions, but working on different ions (410.06 $[M-H]^-$ → 166.84 $[Vanillic-H]^-$).

4. NUCLEOPHILIC SUBSTITUTIONS IN WATER

4.1 Green synthesis of new antioxidant β-lactams

4.1.1 Water as solvent

Unlike what occurs today, water was used as reaction solvent in the development of the first organic chemistry industrial processes. In 1828, F.Wohler developed urea synthesis by heating an aqueous solution of ammonium isocyanate. During years, water has been substituted by hydrocarbon solvents and its use decreased to very few processes. This was due to the improvement of petrol industries and organometallic chemistry, which employs reagents extremely sensitive to moisture and air.[100]

Recently, the re-discovery of water as a solvent was linked to the growth of an environmental awareness in the community. Therefore, chemical transformations started to be designed with more sustainable approaches. The use of the so-called green solvents is one of the already mentioned "Green Chemistry" principles, and water is of course included in environmentally friendly solvents.

The advantages of using water are extremely interesting in terms of safety, toxicity, costs (no organic solvent is so cheap as water) and eco-compatibility. However, its use in organic reactions is not so easy to implement. A solvent must allow the dissolution of reagents without reacting with them, and it needs to be easy eliminated at the end, allowing product isolation. Many organic reagents and compounds are poorly soluble in water, and various functional groups are reactivity towards water.

It is impossible to modify intrinsic reactivity of water, but the low solubility of organic compounds could be bypassed. In this regard, some interesting strategies were developed:

- ionic derivatization, which consists in varying pH of aqueous solutions to increase the solubility of ionizable substances. For example, it was exploited the use of buffer solutions that maintain constant the pH.[101] It is often possible to recover products by precipitation, varying back the solution pH;
- use of surfactants, amphiphilic molecules characterized by a hydrophilic and a hydrophobic portion. At specific concentrations (called critical micellar concentrations CMCs), surfactants can form aggregates in water. These micelles are spheroidal structures characterized by a hydrophobic core and a hydrophilic outer surface. Thus, hydrophobic molecules can be incorporated, allowing their solubilization in water. The reaction efficiency is in some cases also increased, because of a contact promotion between reactive species inside micelles;
- use of cyclodextrins (CD), oligosaccharides formed by 6, 7 or 8 monomers of D-(+)-glucopyranose α-linked through 1-4 glucosidic bonds. CDs are characterized by a truncated conical structure with all hydroxyl groups placed in the outer region, hence resulting water-soluble. The internal cavity is hydrophobic, allowing complexes formation with suitable dimensioned hydrophobic molecules and "increasing" their water-solubility;[102]

functionalization with hydrophilic molecules, a method borrowed from medicinal chemistry to allow the transport of drugs into body. It consists in covalently binding small hydrophilic molecules to hydrophobic substances, increasing their solubility in aqueous environments;

- use of an organic cosolvent miscible with water: polar protic solvents such as low MW alcohols, and aprotic compounds such as dimethylformamide, dimethylsulfoxide, acetonitrile or acetone are commonly employed. Formation of hydrogen bonds, which usually determine the insolubility of many apolar species in water, .results decreased.

The last method is the most applied and constitutes a good compromise for many chemical transformations commonly carried out in organic solvents. The use of water as solvent in organic synthesis still be a poorly explored field, albeit highly challenging and interesting especially for large-scale processes.

4.1.2 nucleophilic substitution in water on β-Lactam compounds.

As a part of a project aimed to introduce polyphenolic antioxidant moieties on antibiotic β-lactams, we tried to exploit the reactivity of two commercially available β-lactams (Figure 4.1.1) in water. We developed and optimized a nucleophilic substitution reaction of the C-4 acetate group that can be carried out in water under mild conditions.

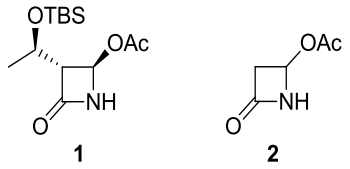
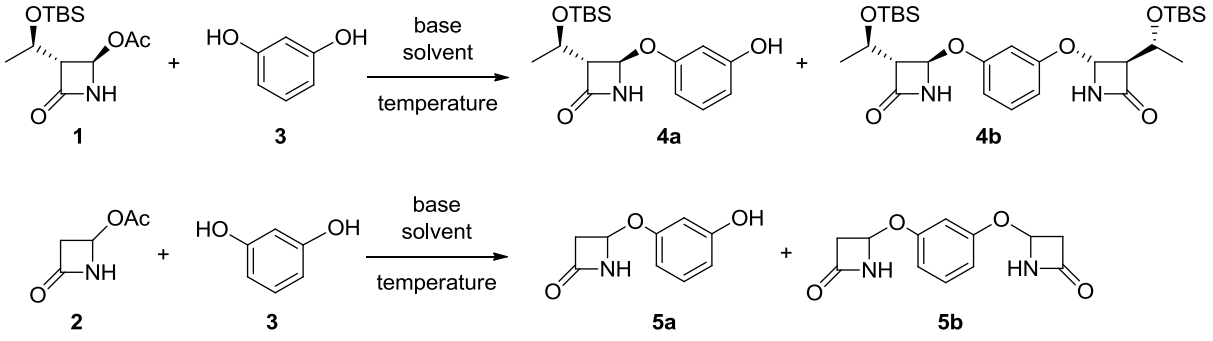


Figure 4.1.1 Commercially available β-lactams

We conducted a preliminary screening on β-lactams **1** and **2** for choosing the best combination of solvent, basic catalysts and reaction conditions, We used resorcinol as nucleophile, as in Scheme 4.1.1.



Scheme 4.1.1 Reaction of 4-acetoxy β-lactams and resorcinol

Solvent importance in nucleophilic substitutions is well known. On one hand, protic solvent such as water strongly increased monomolecular substitutions, like this process seems to be, but on the other hand organic reactions are always hard to proceed in water. Above solubility, β-lactams can undergo ring opening reactions with water as nucleophile.

Hence, the base used became very relevant. Morover, its importance is also related to the discrimination of the two possible products derived from a mono- or a di-substitution of resorcinol alcoholic functions (Scheme 4.1.1). For our purposes, at least one phenolic group must be kept free in order to achieve an antioxidant action on the synthesized molecules.

Therefore, conditions screening was directed to yield increasing and to the optimization of the processes selectivity.

Co-solvent screening

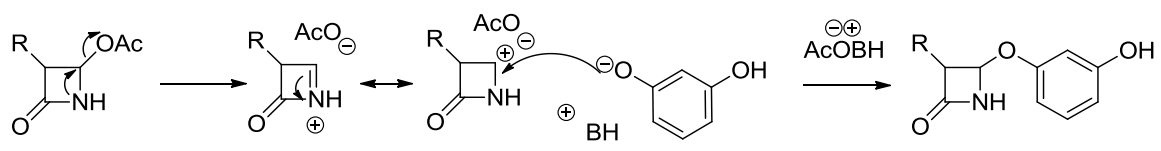
At first, we worked with triethylamine as base and we varied reaction mediums and stirring times (as summeryzed inTable 4.1.1, *: yield for flash-chromatography or Prep HPLC isolated products; **: yield calculated from ^{1}H-NMR crude spectra; ***: conversion for the two starting materials).

entry	β-Lactam	Solvent	T	t	conv*** %	y (a) %	y (b) %
1	**1**	H_2O	r.t.	24h	40%	5%**	20%**
2	**1**	H_2O	70°C	24h	35%	4% **	13%**
3	**1**	MeCN	r.t.	5d	75%	7%**	15%**
4	**1**	H_2O/MeCN(30%)	r.t.	8h	100%	14%*	25%*
5	**2**	H_2O	r.t.	4h	100%	13%**	16%**
6	**2**	H_2O/MeCN(30%)	r.t.	4h	100%	15%*	25%*

Table 4.1.1 Optimization of reaction conditions

The use of water as reaction solvent in the presence of a completely miscible organic co-solvent (MeCN at 30%) appears to be the preferred choice in terms of yields and conversions (entries 4 and 6). In water, **1** is not soluble: this lowered the conversion even conducting the reaction at higher temperatures (entries 1 and 2). For the water soluble substrate **2**, although a complete conversion was reached, yields were lower than those obtained in the presence of organic co-solvents. In only organic solvents, the reaction showed a slow kinetic, and after 5 days the reaction was not yet complete.

These observations suggested that water plays a key role in the nucleophilic substitution mechanism of the reaction. As a matter of facts, a polar protic solvent can stabilize intermediate carbocation of the monomolecular substitution mechanism, with a leaving group solvation. Moreover, the activation energy for the rate determining step was lowered, speeding up the reaction kinetic (Scheme 4.1.2).



Scheme 4.1.2 Monomolecular nucleophilic substitution mechanism

The monomolecular mechanism hypothesis was further corroborated by the obtainment of a 3,4-trans azetidinone diastereoisomer starting from enantiomerically pure **1**, with a complete configuration retention at C-4, as shown by NMR analysis of coupling constants.

As last observation, reaction times resulted shorter for **2**, which disappeared in 3-4 hours, than for **1** (total conversion in 8-10h).

Regioselectivity and base screening

The possibility of a double etherification on both the phenolic groups is related to the different acidity of resorcinol itself, which pk_a is 9.15, and **a** derivatives, which can be assimilated to *m*-methoxyphenol, with a slightly higher pk_a of 9.6544.

To try optimizing this aspect and the whole process, mainly in terms of yield, different organic and inorganic bases were tried (as summarized Table 4.1.2 and Table 4.1.3, respectively for substrate **1** and **2**; *: yields evaluated on products isolated from FC or Prep HPLC; **: yields calculated from ^{1}H-NMR crude spectra).

Stoichiometric amounts of the base resulted necessary. Moreover, for both substrates NaOH gave the best results in term of conversion and **a**/**b** ratio. NaOH, a strong inorganic base, was therefore chosen as reference base for all the subsequent studies and synthesis of these derivatives.

entry	Base	Base eq.	Conv** %	y (a) %	y (b) %	t
1	none	-	0%	-	-	24h
2a 2b	Et_3N	1 0.1 (10%)	100% 17%	24%* 9%**	20%* -	8h 8h
3	Et_2NH	1	85%	17%**	<2%**	10h
4	NaOH	1	100%	38%*	18%*	8h
5	$NaHCO_3$	1	100%	15%**	23%**	10h
6	AcONa	1	0%	-	-	48h

Table 4.1.2 Base screening on **1**

entry	Base	Base eq.	Conv** %	y (a) %	y (b) %	t
1	none	-	0%	-	-	8h
2	Et_3N	1	90%	12%**	19%**	3h
3	Et_2NH	1	100%	4%*	-	4h
4	NaOH	1	100%	29%**	16%**	3h
5	$NaHCO_3$	1	70%	7%*	13%*	3h

Table 4.1.3 Base screening on **2**

Conditions screening

Reaction conditions screening was made in order to optimize the synthesis of both products **a** and **b**.

For **a** products, an excess of resorcinol was use (Table 4.1.4, entry 1). It does not lead to the expected results. Trying to maintain β-lactam SM always in a low concentration comparing to the one of phenol, was evaluated a biphasic system (Table 4.1.4, entry 2): unfortunately **a** product, when formed, was able to be distributed in both phases and react giving **b**.

entry	SM	**3**	Conditions	Conv %	y (a) %	y (b) %
1	**1**, 1eq.	2eq.	excess of **3**	100%	16%	24%
2	**1**, 1eq.	1eq.	cHex/H2O	50%	7%	13%
3	**1**, 1eq.	1eq.	**1** dropped in **3**	100%	38%	18%
4	**2**, 1eq.	1eq.	**2** dropped in **3**	100%	29%	17%
5	**2**, 1eq.	1eq.	**3** dropped in **2**	100%	25%	13%
6	**1**, 1eq.	1eq.	**3** dropped in **1**	100%	-	68%
7	**2**, 1eq.	1eq.	**3** dropped in **2**	100%	-	59%

Table 4.1.4 Optimization of reaction conditions

The reaction was then conducted slowly dropping a H_2O/MeCN solution of SM on a basic solution of **3**, reaching the best results although without a complete selectivity (Table 4.1.4, entries 3 and 4).

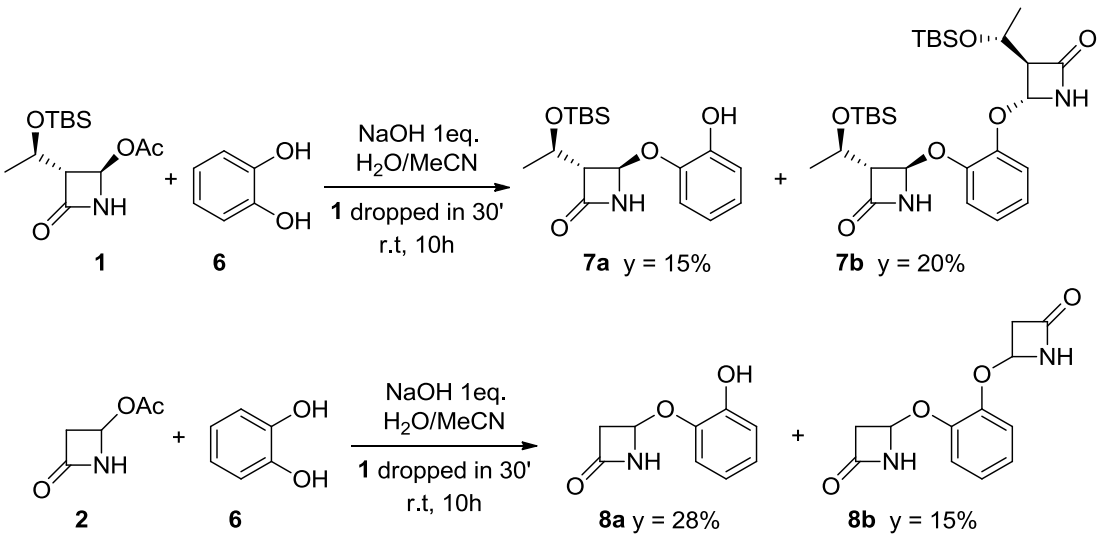
On the contrary, the obtainment of **b** products required a slowly addition of the aqueous NaOH solution to SM dissolved in H_2O/MeCN (Table 4.1.4, entries 6 and 7), allowing yields up to 60-70%.

Nucleophilic substitution with other poly-hydroxylated phenols

Once setup and optimized the procedure on both the substrates **1** and **2**, it was applied to synthesize a small library of products working with a series of poly-hydroxylated phenols. Pyrocatechol was the first nucleophile employed (Scheme 4.1.2, *: yields after flash-chromatography for **1** and after or Prep HPLC for **2**).

Reaction with pyrocatechol and **2** gave product **8a** and **6b** in relative ratio and yield fully comparable with the results obtained using resorcinol. Thus, hindrance resulting from the hydroxyl group in ortho does not disfavor the formation of the dimer.

With substrate **1**, ratio of product **a** and **b** favors the latter.

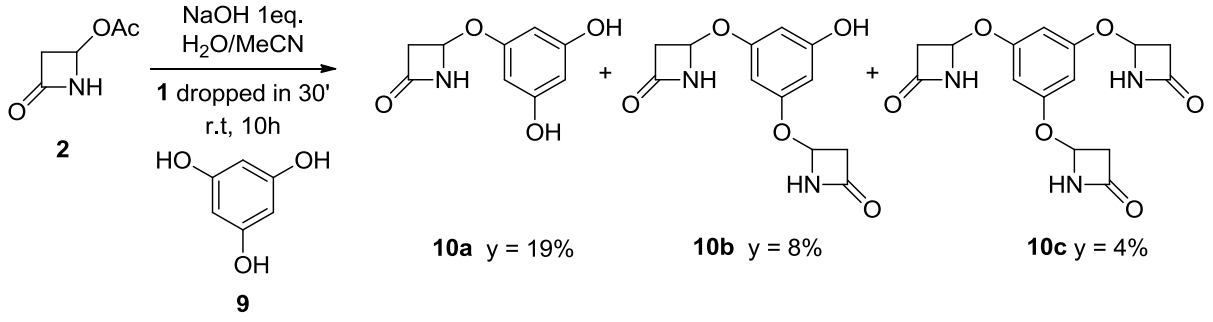


Scheme 4.1.2 Substitutions with pyrocatechol

Considering what stated before, it can be assumed that monomer **7a**, once deprotonated and due to a mesomeric +m effect of -OR substituent in *o* position, constitutes a stronger nucleophile of the monomer **4a**, which has instead the substituent -OR in *m*.

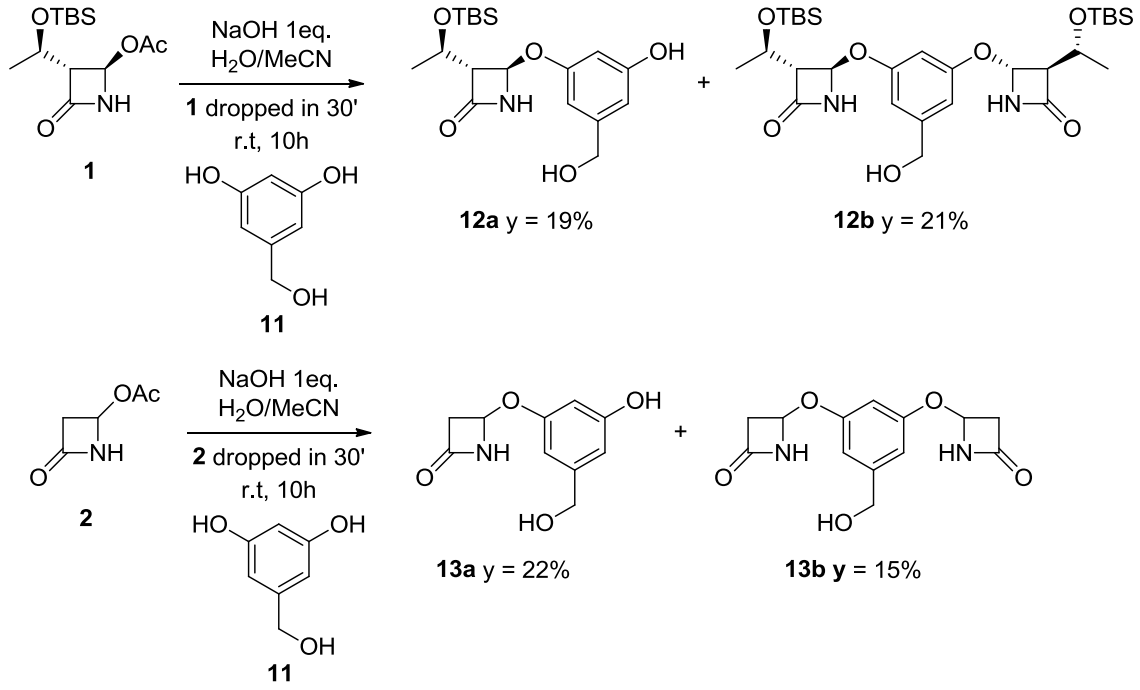
As regarding nucleophilic substitution with phloroglucinol on substrate **2**, all three possible products were isolated (Scheme 4.1.4, *: yields after flash-chromatography). Even in this case, steric hindrance on the three positions played no role in favoring less substituted products.

Moreover, the three products have ^{1}H-NMR signals belonging to other minor β-lactam species, which could be derived from C-coupling, due to electronic activation of phloroglucinol ring by enolic positions. However, eing present in very small amounts and having retention factors almost identical to those of majors species, were not isolated.



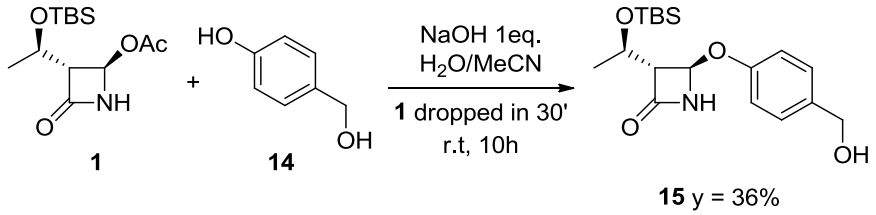
Scheme 4.1.3 Substitutions with phloroglucinol

Nucleophilic substitutions with 3,5-dihydroxybenzyl alcohol showed on **2** the same behavior of what observed with resorcinol, both in terms of yield and **a**/**b** ratio, whereas **1** gave replacement products in almost equivalent amounts of **a** and **b** (Scheme 4.1.4, *: yields after Prep HPLC for **1**, and after flash-chromatography for **2**).



Scheme 4.1.4 Substitutions with 3,5-dihydroxybenzyl alcohol

Finally, when reacted with 4-hydroxybenzyl alcohol, **1** gave substitution only on the phenolic hydroxyl group (Scheme 4.1.5).



Scheme 4.1.5 Substitution with 4-hydroxybenzyl alcohol

Stereochemistry

An important aspect in studying the products obtained concerns the stereochemistry. Starting from substrate **1**, enantiomerically pure with 3 stereogenic centers well defined in absolute configuration R, were obtained products existing in single enantiomers. Indeed, all the reactions involved a configuration retention at C-4, due to steric effects: substitution could happen only by nucleophile attack from the less hindered side of the aciliminium intermediate.

On the contrary, reactions with 4-acetoxy-azetidinone **2** gave products **a** as racemic mixtures and **b** as different diastereoisomers, it was used as racemic mixture and there were no stereochemical controls that guides the nucleophile attacks. Considering the species **5b** obtained in the reaction with resorcinol, it is clear that two diastereoisomers can exist: one is a meso form and the other is constituted by the pair of two enantiomers (R,R) and (S,S) (Figure 4.1.2). Moreover, same considerations can be extended to every **b** product synthesized with nucleophilic substitution of the acetoxy group on substrate **2**.

We then focused our attention on two of the products obtained from substrate **2**: compound **5b** resulting from coupling with resorcinol (in Figure 4.1.2), and compound **7b** derived from reaction with phloroglucinol (Figure 4.1.3).

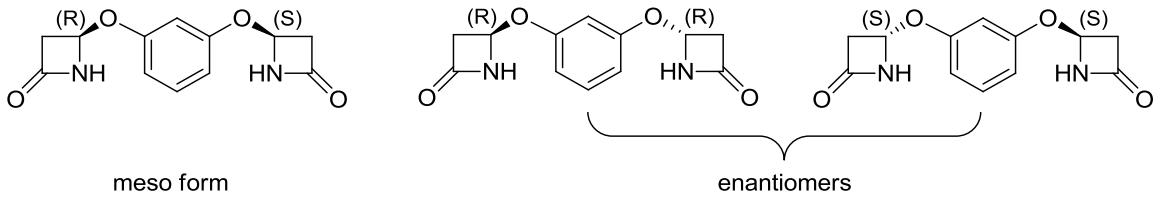


Figure 4.1.2 Analysis of **2b** products

Purification by flash-chromatography on **5b** did not allowed any separation. Then, a sample was analyzed by chiral HPLC separating three peaks of percentage areas, respectively, of 22%, 50% and 28%. These values could be related to the first enantiomer, the meso form and the second enantiomer. For peaks related to the two enantiomers would expect areas equal to 25%, given that in the reaction there was no enantioselection. Unfortunately, the separation was not optimal due to the close retention times of the two

enantiomers and of the meso form. However, it can be deduced that the two diastereoisomers were formed in equal amounts (50% - 50%) and therefore the reaction was not diastereoselective or did not proceed with a preferential formation of one diastereoisomer over the other.

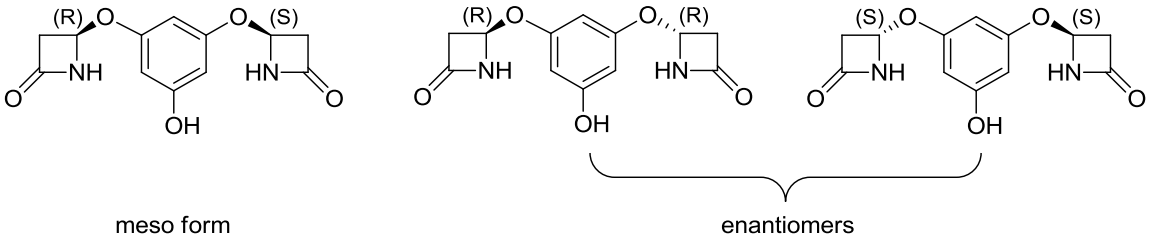
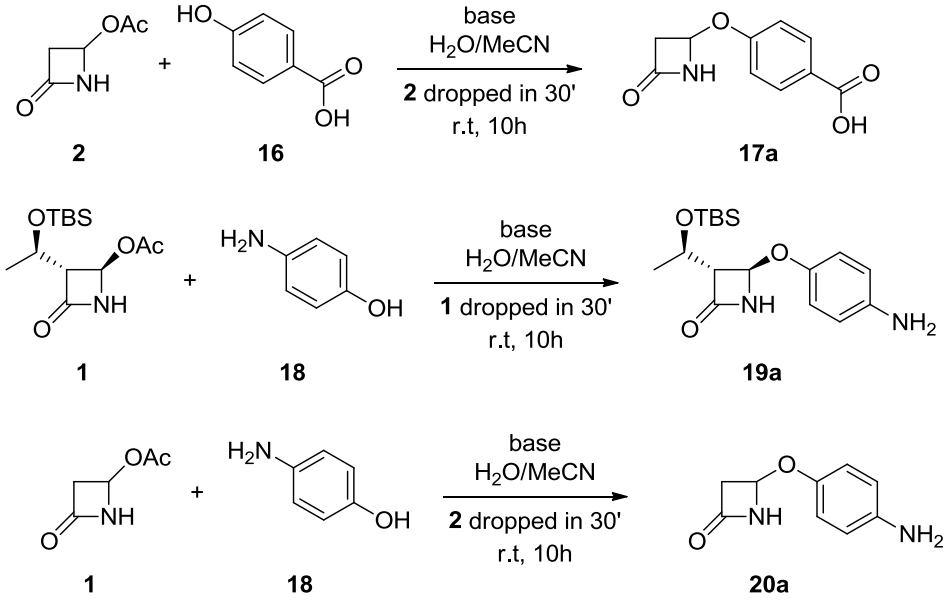


Figure 4.1.3 Analysis of **10b** products

For **10b** the ^{13}C-NMR showed two sets of signals which indicates the presence of two species not chemically equivalent. This corresponds to the two diastereoisomers represented in Figure 4.1.3. The recorded signals had relative percentage of 53% and 47%, suggesting that also in this case the reaction did not proceeded with diastereoselectivity. The mixture was then analyzed by chiral HPLC, separating two peaks whose areas were 23% and 76%. It can be assumed that the first peak belongs to one enantiomer and the second one to the overlap of the not separated meso form and second enantiomer. The hypothesis was supported by perfect match between the ratio of diastereoisomers obtained by chiral HPLC and those calculated from ^{13}C-NMR (53% to 47%).

Chemoselectivity

Chemoselectivity of reaction between substrates **1** and **2** with *p*-amino and *p*-carboxylic acid substituted phenols was finally analyzed (Scheme 4.1.6).



Scheme 4.1.6 Substitution with *p*-amino and *p*-carboxylic acid phenols

As from optimization phase, were used 2eq. of NaOH to promote deprotonation of acid hydrogens and of the phenolic OH, but a so hard basic environment was evidently not tolerated by the β-lactam, which underwent decomposition (Table 4.1.5, with * for yields evaluated on products isolated from flash-chromatography, and ** for yields calculated from ^{1}H-NMR crude spectra). Therefore, yield in the OH coupling product does not exceed 15% and the secondary product of the reaction, identified by ^{1}H-NMR, was the ester of hydroxy benzoic acid **16** with itself.

To avoid lactam decomposition, the same reaction was carried out with 1eq. of $NaHCO_3$: conversion resulted very low due to the formation of sodium carboxylate, stabilized by resonance and thus slightly nucleophilic (Table 4.1.5, entry 2). With 1eq. of TEA the conversion was as well not complete. Moreover, next to **17a** was identified by ^{1}H-NMR of the crude the presence of a compound assumed to result from coupling with the carboxylate group, index of a poor selectivity of the process.

The reaction was then conducted with 2eq. of TEA: TM yield increased, but the kinetics remained very slow (20h). It was in contrast with what observed in the previously studied reactions of poly-hydroxy phenols with **2**, which were faster (2-4h).

entry	Base	Conv %	y %
1	NaOH (2eq.)	100%	15%*
2	$NaHCO_3$ (1eq.)	10%	-
3	Et_3N (1eq.)	60%	17%**
4	Et_3N (2eq.)	100%	36%*

Table 4.1.5 Chemoselectivity with *p*-hydroxy benzoic acid on **2**

Working with aniline, without an other base was not observed any SMs conversion (Table 4.1.6, entries 1 and 2). Clearly, aniline itself was not enough reactive to attack the β-lactam and promoting the product formation without a catalyst.

Indeed, in presence of a base and with optimized conditions the reaction proceeded via nucleophilic substitution of the phenolic OH (entries 3 and 4). Products were subsequently isolated as acetic amide, after reactions with acetic anhydride, and analyzed.

entry	β-lactam SM	Base	Conv %
1	**1**	-	0%
2	**2**	-	0%
3	**1**	NaOH	100%
4	**2**	NaOH	100%

Table 4.1.6 Chemoselectivity with *p*-hydroxy aniline

Substitutions on *N*-SMe β-lactams

Reactions were conducted on two β-lactam substrates (Figure 4.1.4), using resorcinol and implementing the conditions developed in the early stages of the study.

Initially, NaOH was used as base, but since reactions did not lead to the expected products in appreciable yields a weaker base such as $NaCO_3$ was chosen, even so without positive results.

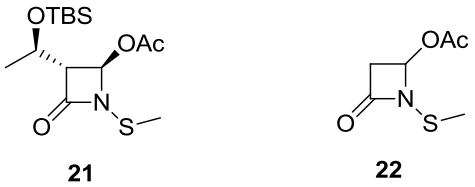


Figure 4.1.4 *N*-SMe β-lactams used as SMs

Finally, nucleophilic substitutions on the *N*-thiomethyl azetidinones were unsuccessful, a part from traces of the desired products observed by ^{1}H-NMR analysis. Therefore, the hydrogen atom bonded to nitrogen was critical to the success of the substitution on the C-4 of the cycle.

However, an effort was made to isolate by flash-chromatography purification an interesting amount of the most promising antioxidant and antibacterial compound. Derivative **23a** was obtained by reacting the substrate **21** with resorcinol (Figure 4.1.6).

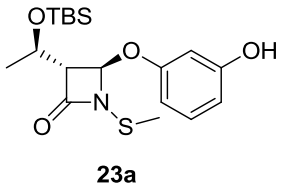


Figure 4.1.6 Product **23a** obtained in traces, isolated and characterized

4.1.3 Activities

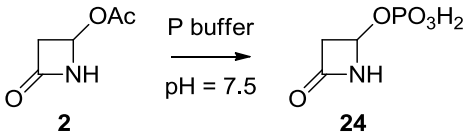
All shown compounds were tested antibacterial and antioxidant activites. Unfortunately, they seems to be completely inactive as antibiotic, while the antioxidant potencies are comparable to the ones already shown by dual active β-lactams: this kind of activity seems to be mainly related to only phenolic moieties. Although we thought that a phenolic moiety in C-4 position of the ring could act as a leaving group, thus making the same task of acetoxy group in active compounds showed in Chapter 3, this was not exact. Furthermore, it was possible that recognition by enzyme target sites of these compounds has been missed.

4.2 New β-lactam phosphate

4.2.1 Nucleophilic phosphate buffer substitution on β-lactams

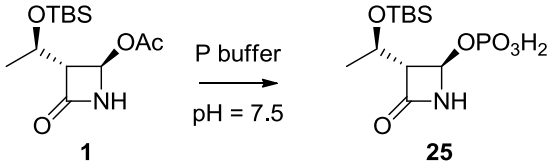
As a matter of facts, we already observed that a phosphate group could be introduce on β-lactam compounds by a nucleophilic substitution on the C-4 acetate. We deepened this reaction on the two commercially available β-lactams **1** and **2** showed in Figure 4.1.1, analyzing how it was affected by scale, phosphate buffer pH and concentration, cosolvents, temperature and time.

The reference reaction was carried out stirring compound **2**, more water soluble and reactive due to its less sterical hindrance, in 1M phosphate buffer (P buffer, pH = 7.5) for 2h, as shown in Scheme 4.2.1.



Scheme 4.2.1 Nucleophilic phosphate substitution on **2**

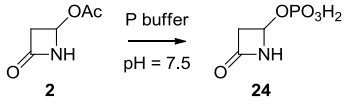
Unfortunately, an HPLC analysis was not possible on such a polar compound, thus the structure was confirmed only by ^{1}H, ^{13}C and ^{31}P NMR studies (the same also for compound **1**, which reaction is shown in Scheme 4.2.2).



Scheme 4.2.2 Nucleophilic phosphate substitution on **1**

At first, we observed the influence of scale, pH and reaction time on compound **1**, less reactive and thus easier be used in optimizations. Data collected, reported in Table 4.2.1, showed that basic pH could increase the kinetic, as demonstrated from time needed to reach SM disappearance.

However, increasing too much the pH led to side reactions and worse reaction crudes. Thus, best value identified was 7.5, with a buffer concentration of 1M. At least, the reaction scale seemed to be slightly relevant.

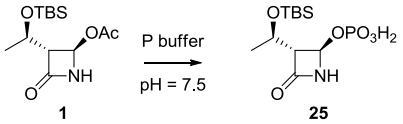


SM, [mmol]	pH, [P_{buffer}]	T [°C]	t [h]
2, 1.0	7.5, 1M	r.t.	2
2, 2.0	7.5, 1M	r.t.	2
2, 1.0	7.5, 1M	r.t.	2
2, 0.5	6.0, 1M	r.t.	65
2, 0.5	6.5, 1M	r.t.	40
2, 0.5	7.0, 1M	r.t.	16
2, 0.5	7.5, 1M	r.t.	1
2, 0.5	8.0, 1M	r.t.	1
2, 1.0	7.5, 1M	r.t.	1
2, 0.5	7.5, 1M	r.t.	1
2, 0.5	8.0, 1M	r.t.	1

Table 4.2.1 Scale, pH and time influence for **2**

Reaction on substrates **1** required harder conditions, mainly in term of temperature (Table 4.2.2). We found that 1-2h of reaction were enough only when temperature was increased up to 70°C. Probably, this

characteristic was related both to a lower reactivity, caused by the high steric hindrance, and a lower water solubility. However, the use of a cosolvent such as MeCN did not show particularly relevant yield increase, but it decreases the reaction selectivity, furnishing more complex crudes.



1 **25**

SM, [mmol]	pH, [P_{buffer}]	T [°C]	t [h]
1, 0.5	6.0, 1M	70°C	67
1, 0.5	7.5, 1M	70°C	4
1, 0.5	9.0, 1M	70°C	2
1, 1.0	7.5, 1M	50°C	14

Table 4.2.2 pH, temperature and time influence for **1**

Finally, we used the TBS-deprotected derivative of compound **1**, obtaining from the NMR spectra a 1/4 ratio between the two possible diastereoisomers. In this case, the less steric hindrance determined by the TBS absence determined a partial possibility to obtain a nucleophilic substitution also on the more hindered side of the molecule.

3.2.2 New hydroxy-apatite β-lactam bioconjugates

Hydroxyapatite is a rare mineral with chemical composition $Ca_5(PO_4)_3(OH)$. It belongs to the class of apatites and it contains an hydroxyl group.

Hydroxyapatite is also produced and absorbed by organic tissues. As a matter of facts, it is one of the main components of bones, being in form of calcium salts such as $CaCO_3$, $Ca_3(PO_4)_2$ and CaF_2.

In cases of bones substitution or regeneration necessities, the best solution would be the use of patients' own bones. Of course, this is not always possible, but the use of bones from cadavers or animals involves a certain number of risks, including rejection. Furthermore, it is difficult to overcome implications due to the use of bones with a remodeling mechanism very different from the human one, and with a lower quality because of aggressive treatments needed to minimize risks of disease transmission. For these reasons, synthetic bone substitutes are often used.

However, bone substitutes must reproduce the porosity of cancellous bones, which can vary from 50% to about 80%. Furthermore, pores of bone substitutes must have the sufficient size so that cells can migrate through them as well as blood vessels can pass. Finally, porosities must be interconnected.

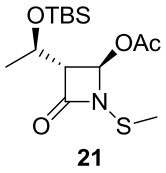
Obviously, bone substitutes must also offer sufficient mechanical properties and they must satisfy a series of biological requirements. Thus, the best match with these necessities can be achieved only using natural hydroxyapatites bones.

Zinc substituted hydroxyapatites are nowadays well known for bioactivities such as an increase in the growth of human adipose-derived mesenchymal stem cells, and antimicrobial capabilitie. Indeed, a significant decrease in the number of viable *S. aureus* bacteria was observed after Zn-hydroxyapatites

contact.[103] Moreover, it was recently shown that hydroxyapatite alternatives, such as with a certain percentage of Strontium atoms, can offer variously and interesting biological activities.[104]

Our improvement considered to enhance this materials by introducing antibiotic β-lactams via observed phosphate reaction on our 4-acetoxy derivatives. Moreover, we thought to introduce different β-lactams with specifically designed biological activities.

For the preliminary studies, we worked with commercially available compounds **1** and **2**, adding the most powerful antibiotic agent **21** (reported again in Figure 4.2.1).



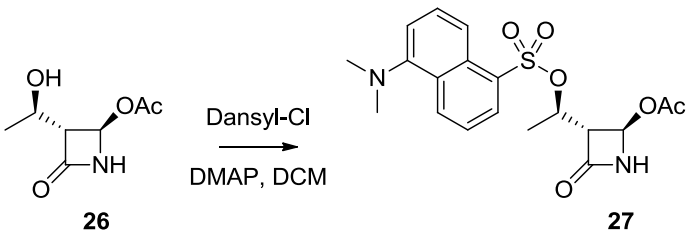
21

Figure 4.2.1 β-Lactam substrate **21**

We made different tentatives for introducing this moieties on various hydroxyapatites, prepared in collaboration with Professor A. Bigi group, University of Bologna. Ww founded that better conditions required no base presence and only water as solvent (see Experimental section for further details). Thus, Hydroxyapatites needed to be suspended in water and left stirring in presence of the β-lactams substrate, at different temperatures such as r.t. for **2** and 70°C for both **1** and **21**.

The obtained bioconjugates were recovered after centrifugation and drying. Their analysis were performed by both FT-ATR-IR and calorimetry, respectively for characterization and β-lactams contents quantification. As shown by IR spectroscopy, only β-lactam **2** was demonstrated to be chemically bonded with hydroxyapatites, while for **1** and **21** it seemed to be only a supportation. As a matter of facts, IR carbonyl signals changed in wavenumber for **2**, while the same effect was not recognized for compounds **1** and **21**. Indeed, lowest reactivity of **1** and **21** was well known for our group. By calorimetry, we found to be able of introducing β-lactams amounts up to 10% of the hydroxyapatites weights for all the three substrates evaluated, depending to the preparative reaction loadings.

Finally, we rapidly optimized the synthesis of dansyl β-lactam derivative **27** (Scheme 4.2.3).



26 **27**

Scheme 4.2.3 Dansyl derivatization to compound **27**

Luminescent derivative **27** was also supported on hydroxyapatite, thus preparing bioconjugates with interesting photochemical properties, adequate for uses as bioprobe or tracer. Moreover, it allowed a

visual immediate demonstration of its supportation, thus demonstrating the properties on our β-lactam compounds to derivatized or to be carried by hydroxyapatites (Figure 4.2.2).

Figure 4.2.2 Hydroxyapatite-**27** bioconjugate

Biological activities evaluation of these new hydroxyapatites biocojugates, at first in terms of antibiotic potencies, are currently under assessment. However, is already possible to wonder about the introduction of more active β-lactam antibiotics, such as a dual active compound also able to counteract inflammation processes, very common in bones substitutions. Moreover, due to the introduction mechanism, all the actually known β-lactam biologically activities can be successfully exploited in the preparation of other hydroxyapatites derivatives.

4.2 Experimental section

4.2.1 General informations

As previously reported (Paragraph 2.3.1).

4.2.2 Nucleophilic substitutions in water

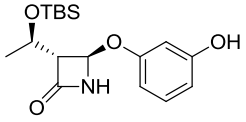
Azetidinones **1** and **2**, and all the used phenols were commercially available, while compounds **21** and **22** were prepared as previously specified.

Poly-hydroxylated β-lactam ethers synthesis

In a 50mL flask 0.5mmol of phenols were dissolved in 2mL of freshly prepared 0.25M NaOH aqueous solution. Then a H_2O/MeCN 1/1 solution of **1** or **2** (0.5mmol in 3mL) were dropped in 30 minutes, following the reaction by TLC monitoring until SM disappearance. The reaction was then quenched by

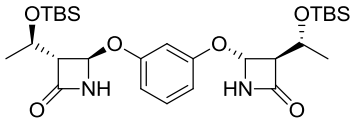
NaCl addition to separate MeCN and water, which was acidified using aqueous HCl (1M) before extracting with EtOAc (3 times). The organic phases were dried over Na_2SO_4, then filtered and concentrated in vacuum, reaching the desired product/s via flash-chromatography or Pre-HPLC purifications.

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-(3-hydroxyphenoxy) azetidin-2-one (**4a**)



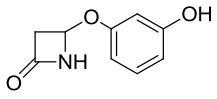
M.p. (119-124)°C, white solid; Rf 0.54 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = 71.33°
^{1}H NMR (400MHz, $CDCl_3$): δ 0.08 (d, J = 6.8Hz, 6H), 0.88 (s, 9H), 1.276 (d, J = 6.4 z, 3H), 3.31 (d, J = 3.2, 1H), 4.27 (dq, J = 3.2Hz, 6.4Hz, 1H), 5.68 (s, 1H), 6.37 (s, 1H), 6.42 (m, 1H), 6.46 (dd, J = 2.0, 8.0Hz, 1H), 6.556 (dd, J = 2.4, 8.0Hz, 1H), 6.83 (s, 1H), 7.16 (t, J = 8.0Hz, 1H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = -5.1, -4.3, 17.9, 22.4, 25.7, 64.0, 65.8, 78.4, 103.7, 108.0, 110.0, 130.6, 157.3, 157.4, 168.0 ppm
IR: $\tilde{\nu}$ = 3276, 2929, 2857, 1762, 1598, 1491, 1148, 940 cm^{-1}
HPLC-MS: Rt = 10.02min, m/z = 294 (100) $[M\text{-}NHCO]^+$, 360 (51) $[M+Na]^+$

(R,3,3'R,4,4'R)-4,4'-(1,3-phenylenebisoxy)bis(3-((R)-1-*t*butyldimethylsilyloxyethyl)azetidin-2-one) (**4b**)



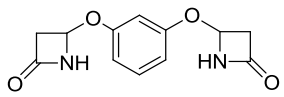
M.p. (114-116)°C, white solid; Rf 0.42 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = 74.98°
^{1}H NMR (400MHz, $CDCl_3$): δ 0.09 (d, J = 4.8Hz, 12H), 0.88 (s, 18H), 1.29 (d, J = 6.0Hz, 6H), 3.32 (dd, J = 0.8, 3.2Hz, 2H), 4.283(dq, J = 3.2, 6.0Hz, 2H), 5.63 (d, J = 0. Hz, 2H), 6.49 (m, 1H) , 6.65(dd, J = 2.4, 8.4Hz, 2H), 7.29 (t, J 8.4Hz, 1H), 7.365 (s, 2H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = -5.1, -4.4, 17.9, 22.4, 25.7, 64.1, 66.1, 79.1, 105.4, 110.9, 130.9, 157.8, 168.0 ppm
IR: $\tilde{\nu}$ = 3250, 2930, 2856, 1772, 1596, 1375, 1112, 811 cm^{-1}
HPLC-MS: Rt = 16.92min, m/z = 521 (53) $[M\text{-}NHCO]^+$, 587 (100) $[M+Na]^+$

4-(3-hydroxyphenoxy)azetidin-2-one (**5a**)



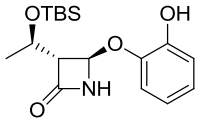
M.p. (153-165)°C, white solid; Rf 0.32 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3COCD_3): δ 2.90 (dd, J = 1.2, 14.8Hz, 1H), 3.310(ddd, J = 2.8, 3.6, 14.8Hz, 1H), 5.70 (dd, J = 1.2, 3.6Hz, 1H), 6.396 (m, 2H), 6.487 (dd, J = 2.0, 8.0Hz, 1H), 7.08 (m, 1H), 8.15 (s, 1H), 8.70 (s, 1H) ppm
^{13}C NMR (100MHz CD_3COCD_3): δ = 45.7, 76.0, 103.4, 106.3, 109.0, 130.1, 157.9, 158.6, 165.1 ppm
IR: $\tilde{\nu}$ = 3410, 1744, 1586, 1260, 1191, 1127, 1063 cm^{-1}
HPLC-MS: Rt = 2.05min, m/z = 180 (100) $[M+H]^+$, 202 (73) $[M+Na]^+$, 359 (60) $[2M+H]^+$

4,4'-(1,3-phenylenebisoxy)bis(azetidin-2-one) (**5b**)



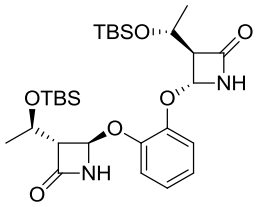
M.p. (155-160)°C, white solid; Rf 0.46 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3COCD_3): δ 2.92 (dd, J = 1.2, 14.8Hz, 2H), 3.33 (ddd, J = 2.8, 4.0, 14.8Hz, 2H), 5.78 (dd, J = 1.2, 4.0Hz, 2H), 6.52 (m, 1H), 6.61 (dd, J = 2.4, 8.0Hz,, 2H), 7.24 (t, J = 8.0Hz, 1H), 8.19 (s, 2H) ppm
^{13}C NMR (100MHz CD_3COCD_3): δ = 45.7, 76.0, 103.1, 109.1, 130.4, 157.8, 165.1.ppm
IR: $\tilde{\nu}$ = 3421, 2873, 1751, 1579, 1488, 1260 cm^{-1}
HPLC-MS: Rt = 2.17min, m/z = 247 (100) $[M+H]^+$, 264 (76) $[M+H_2O]^+$, 269 (71) $[M+Na]^+$

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-(2-hydroxyphenoxy) azetidin-2-one (**7a**)



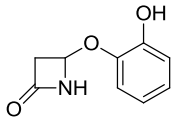
M.p. (104-106)°C, white solid; Rf 0.68 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = 41.00°
^{1}H NMR (400MHz, $CDCl_3$): δ 0.09 (s, 3H), 0.11 (s, 3H), 0.90 (s, 9H), 1.25 (d, J = 6.0Hz, 3H), 3.36 (dd, J = 0.8, 3.2Hz, 1H), 4.25 (dq, J = 3.2, 6.4Hz, 1H), 5.65 (s, 1H), 6.37(s, 1H), 6.82 (dd, J = 1.2, 2.0, 7.6 Hz, 1H), 6.88 (s, 1H), 6.92 (dd, J = 2.4, 7.6Hz, 1H), 6.96 (m, 2H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = -5.1, -4.4, 17.9, 22.5, 25.7, 64.1, 66.0, 80.4, 116.0, 116.2, 120.4, 124.2, 143.2, 147.0, 167.4 ppm
IR: ṽ = 3283, 2929, 2856, 2360, 1766, 1597, 1375, 1143, 1080, 1036 cm^{-1}
HPLC-MS: Rt = 10.52min, m/z = 294 (61) $[M-NHCO]^+$, 338 (100) $[M+H]^+$, 360 (72) $[M+Na]^+$, 675 (13) $[2M+H]^+$

(R,3,3'R,4,4'R)-4,4'-(1,2-phenylenebisoxy)bis(3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)azetidin-2-one) (**7b**)



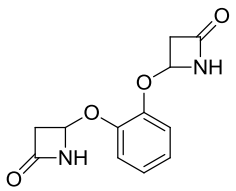
M.p. (151-154)°C, white solid; Rf 0.53 (cyclohexane/EtOAc 60/40)
$[\alpha]_D$ = 33.00°
^{1}H NMR (400MHz, $CDCl_3$): δ 0.03 (s, 6H, TBS), 0.05 (s, 6H, TBS), 0.83 (s, 18H, TBS), 1.23 (d, J = 6.0Hz, 6H, Me), 3.28 (dd, J = 0.8, 3.2Hz, 2H), 4.20 (dq, J = 3.2, 6.4Hz, 2H, C*H*OTBS), 5.61 (s, 2H, C*H*NH), 6.82 (s, 2H), 6.99 (dd, J = 1.2, 6.6Hz, 2H), 7.05 (m, 2H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = -5.1, -4.4, 17.9, 22.5, 25.7, 64.1, 66.1, 80.8, 119.6, 124.4, 124.2, 147.2, 167.1 ppm
IR: ṽ = 3256, 2929, 2856, 1771, 1375, 1253, 1141, 1079, 1033 cm^{-1}
HPLC-MS: Rt = 16.51min, m/z = 521 (100) $[M-NHCO]^+$, 582 (60) $[M+H_2O]^+$, 565 (72) $[M+H]^+$

4-(2-hydroxyphenoxy)azetidin-2-one (**8a**)



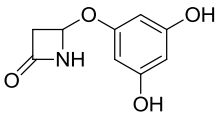
M.p. (128-130)°C, white solid; Rf 0.49 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3COCD_3): δ 3.06 (dd, J =0.8Hz, 14.8Hz, 1H, C*H*HCHNH), 3.31 (ddd, J = 2.8, 3.6, 14.8Hz, 1H, CH*H*CHNH), 5.79 (dd, J = 0.8, 3.6Hz, 1H, C*H*NH), 6.81 (m, 1H, arom), 6.94 (m, 2H, arom), 7.00 (dd, J = 1.2, 8.2Hz, 1H, arom), 7.96 (bs, 1H, OH), 8.10 (bs, 1H, NH) ppm
^{13}C NMR (100MHz, CD_3COCD_3): δ = 46.9, 79.5, 118.3, 118.8, 121.7, 125.5, 148.2, 149.8,170.2 ppm
IR: ṽ = 3235, 1803, 1596, 1351, 1270, 1212, 1194, 1135, 1049, 969, 743, 657 cm^{-1}
HPLC-MS: Rt = 2.12min, m/z = 359 (79) $[M+H]^+$, 202 (45) $[M+Na]^+$, 376 (10) $[2M+H_2O]^+$

4,4'-(1,2-phenylenebis(oxy))bis(azetidin-2-one) (**8b**)



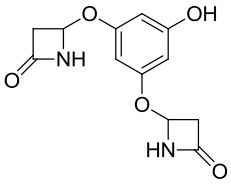
M.p. (129-131)°C, white solid; Rf 0.34 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3COCD_3): δ 3.02 (d, J = 14.8Hz, 2H, C*H*HCHNH), 3.36 (ddd, J = 1.2, 2.8, 14.8Hz, 2H, CH*H*CHNH), 5.79 (dd, J = 1.2, 3.6 Hz, 2H, C*H*NH), 7.06 (m, 4H, arom), 8.08 (s, 2H, NH) ppm
^{13}C NMR (100MHz, CD_3COCD_3): δ = 47.2, 79.6, 79.7, 119.5, 125.4, 125.4, 148.8, 148.9, 170.2 ppm
IR: ṽ = 3424, 2955, 1751, 1722, 1652, 1500, 1405, 1349,1246, 1214, 1134, 1018, 954, 746 cm^{-1}
HPLC-MS: Rt = 1.90min, m/z = 266 (100) $[M+H_2O]^+$, 519 (13) $[2M+Na]^+$

4-(3,5-dihydroxyphenoxy)azetidin-2-one (**10a**)



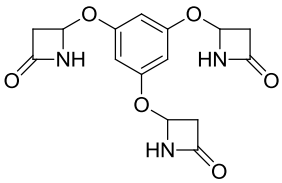
M.p. (153-165)°C, white solid; Rf 0.38 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3OD): δ 2.93 (dd, J = 0.8, 14.8Hz, 1H), 3.32 (ddd, J = 2.8, 3.6, 14.8 Hz, 1H), 5.63 (dd, J = 0.8, 3.6Hz, 1H), 5.90 (d, J = 1.2Hz, 2H), 6.00 (d, J = 1.2 Hz , 1H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 45.9, 76.0, 94.8, 96.9, 158.6, 159.5, 165.9 ppm
IR: $\tilde{\nu}$ = 3286, 1740, 1607, 1508, 1491, 1408, 1364, 1148, 1065 cm^{-1}
HPLC-MS: Rt = 1.92min, m/z = 196 (100) $[M+H]^+$, 218 (50) $[M+Na]^+$, 413 (35) $[2M+Na]^+$

4,4'-((5-hydroxy-1,3-phenylene)bis(oxy))bis(azetidin-2-one) (**10b**)



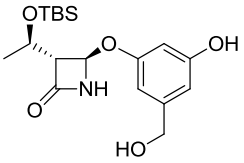
M.p. (155-160)°C, yellow solid; Rf 0.54 (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3OD): δ 2.92 (dd, J = 0.8, 14.8Hz, 2H, C*H*HCHNH), 3.34 (m, 2H, C*H*HCHNH), 5.68 (dd, J = 0.8, 3.6Hz, 2H, C*H*NH), 6.05 (d, J = 1.2Hz, 1H, arom), 6.12 (d, J = 1.2Hz, 2H, arom) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 47.3, 77.50-77.52, 95.85-96.10, 98.56-98.60, 160.1, 161.1, 166.9 ppm
HPLC-MS: Rt = 1.70min, m/z = 265 (91) $[M+H]^+$, 282 (100) $[M+H_2O]^+$, 287 (83) $[M+Na]^+$

4,4',4''-(benzene-1,3,5-triyltris(oxy))tris(azetidin-2-one) (**10c**)



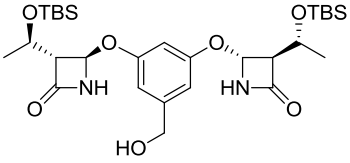
M.p. (160-167)°C, yellow solid; Rf 0. (cyclohexane/EtOAc 20/80)
^{1}H NMR (400MHz, CD_3OD): δ 2.98 (dd, J = 0.8, 14.8Hz, 3H), 3.37 (m, 3H), 5.77 (dd, J = 0.8, 3.6Hz, 3H), 6.14 (s, 3H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 46.3, 76.6, 97.3, 159.2, 165.8, ppm
HPLC-MS: Rt = 1.78min, m/z = 334 (19) $[M+H]^+$, 351 (100) $[M+H_2O]^+$, 356 (40) $[M+Na]^+$

(3R,4R)-3-((R)-1-((*t*butyldimethylsilyl)oxy)ethyl)-4-(3-hydroxy-5-hydroxymethylphenoxy) azetidin-2-one (**12a**)



Colorless oil; Rf = 0.32 (DCM/EtOAc 20/80);
$[\alpha]_D$ = 80.00°
^{1}H NMR (400MHz, CD_3OD): δ 0.11 (s, 3H), 0.14 (s, 3H), 0.93 (s, 9H), 1.30 (d, J = 6.0Hz, 3H), 3.27 (dd, J = 0.8, 3.6 Hz, 1H), 4.29 (dq, J = 3.2, 6.0Hz, 1H), 4.54 (s, 2H), 5.65 (s, 1H), 6.82 (t, J = 1.2Hz, 1H), 6.90 (dd, J = 1.2, 1.6Hz, 1H), 6.96 (dd, J = 1.2, 1.6Hz,1H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = -4.1, -3.2, 19.7, 23.6, 27.1, 65.8, 66.3, 67.5, 80.3, 104.2, 107.1, 109.9, 146.5, 160.0, 160.8, 171.4 ppm
IR: $\tilde{\nu}$ = 3274, 2930, 285, 1759, 1601, 1461, 1254, 1147, 1085, 1036 cm^{-1}
HPLC-MS: Rt = 8.65min, m/z = 324 (81) $[M-NHCO]^+$, 390 (100) $[M+Na]^+$

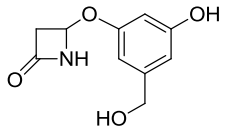
(R,3,3'R,4,4'R)-4,4'-((5-hydroxymethyl-1,3-phenylene)bisoxy) bis(3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)azetidin-2-one) (**12b**)



M.p. (94-98)°C, white solid; Rf = 0.58 (DCM/EtOAc 20/80);
$[\alpha]_D$ = 103.00°
^{1}H NMR (400MHz, CD_3OD): δ 0.11 (s, 6H), 0.14 (s, 6H), 0.93 (s, 18H), 1.31 (d, J = 6.0Hz, 6H), 3.31 (dd, J = 1.2, 3.6Hz, 2H), 4.29 (dq, J = 3.6, 6.0Hz, 2H), 4.62 (s, 2H), 5.68 (d, J = 1.2,Hz, 2H), 6.49 (d, J = 2.4Hz,

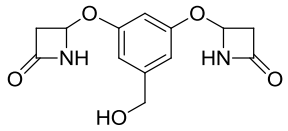
1H), 6.68 (d, J = 2.4Hz, 2H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = -4.0, -3.2, 19.7, 23.6, 27.2, 65.6, 66.4, 67.6, 80.5, 105.4, 109.8, 109.9, 147.2, 160.1, 171.2 ppm
IR: ṽ = 3204, 2926, 2853, 1758, 1598, 1374, 1138, 1082, 1085, 1032 cm^{-1}
HPLC-MS: Rt = 14.45min, m/z = 551 (100) $[M-NHCO]^+$, 612 (58) $[M+H_2O]^+$

4-(3-hydroxy-5-(hydroxymethyl)phenoxy)azetidin-2-one (**13a**)



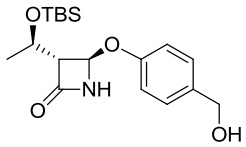
Colorless oil; Rf = 0.47 (cyclohexane/EtOAc 40/60);
^{1}H NMR (400MHz, CD_3OD): δ 2.98 (dd, J = 0.8, 14.8Hz, 1H), 3.36 (m, 1H), 4.54 (s, 2H) 5.65 (dd, J = 0.8, 3.6Hz, 1H), 6.31 (d, J = 1.2Hz, 1H), 6.40 (d, J = 1.2Hz , 1H), 6.54 (d, J = 1.2Hz , 1H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 47.0, 65.8, 80.0, 103.9, 106.7, 109.6, 246.5, 159.8, 160.8, 170.4 ppm
HPLC-MS: Rt = 1.64min, m/z = 210 (40) $[M+H]^+$, 227 (100) $[M+H_2O]^+$, 232 (40) $[M+Na]^+$

4,4'-((5-(hydroxymethyl)-1,3-phenylene)bis(oxy))bis(azetidin-2-one) (**13b**)



Colorless oil; Rf = 0.60 (cyclohexane/EtOAc 40/60);
^{1}H NMR (400MHz, CD_3OD): δ 3.01 (dd, J = 0.8, 14.8Hz, 2H), 3.98 (ddd, J = 2.4, 3.6, 14.8Hz, 2H), 4.60 (s, 2H), 5.76 (dd, J = 0.8, 3.6Hz, 2H), 6.44 (d, J = 1.2Hz, 1H), 6.63 (d, J = 1.2Hz , 2H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 47.1, 65.6, 78.0, 104.3, 109.4, 147.1, 159.87, 170.2 ppm
HPLC-MS: Rt = 1.21min, m/z = 279 (36) $[M+H]^+$, 296 (80) $[M+H_2O]^+$, 301 (100) $[M+Na]^+$

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-(4-(hydroxymethyl) phenoxy)azetidin-2-one (**15**)

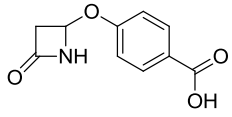


M.p. (138-140)°C, white solid; Rf 0.65 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ = 66.00°
^{1}H NMR (400MHz, $CDCl_3$): δ 0.06 (s, 3H), 0.08 (s, 3H), 0.87 (s, 9H), 1.25 (d, J = 6.4Hz, 3H), 3.10 (s, 1H), 3.23 (d, J = 3.2Hz, 1H), 4.21 (dq, J = 3.2, 6.4Hz, 1H), 4.54 (s, 2H), 5.61 (s, 1H), 6.85 (d, J = 8.4Hz, 2H), 7.25 (d, J = 8.4Hz, 2H), 7.40 (s, 1H) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = -5.1, -4.4, 17.9, 22.4, 25.7, 64.1, 64.6, 65.9, 78.8, 116.8, 128.8, 135.3, 155.7, 167.6 ppm
IR: ṽ = 3094, 2928, 2856, 1770, 1511, 1228, 1173, 1136, 1087, 1034 cm^{-1}
HPLC-MS: Rt = 9.79min, m/z = 308 (74) $[M-NHCO]^+$, 374 (100) $[M+Na]^+$

4-hydroxybenzoic acid β-lactam derivatives synthesis

In a 50mL flask 0.5mmol of 4-hydroxybenzoic acid **16** were dissolved in 3mL of a H_2O/MeCN 1/1 solution, then TEA (1mmol, 139μL) and **2** (0.5mmol in 2mL of water, dropped in 30min) were added, following the reaction by TLC monitoring until SM disappearance. The reaction was then quenched by NaCl addition to separate MeCN and water, which was acidified using HCl (1M) before extracting with EtOAc (3 times). The organic phases were dried over Na_2SO_4, then filtered and concentrated in vacuum, reaching the desired product **17a** via flash-chromatography with EtOAc.

4-(4-oxoazetidin-2-yloxy)benzoic acid (**17a**)



M.p. (123-140)°C, white solid; Rf 0.22 (cyclohexane/EtOAc 10/90)
^{1}H NMR (400MHz, CD_3OD): δ 3.03 (dd, J = 15.2Hz, 1H), 3.41 (dd, J = 3.6, 15.2Hz, 1H), 5.83 (d , J = 3.6Hz, 1H), 7.02 (d, J = 8.4Hz, 2H), 8.01 (d, J = 8.0Hz,

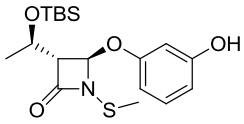
2H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 47.2, 78.1, 117.0, 126.8, 133.8, 162.3, 170.0, 170.4 ppm
IR: $\tilde{\nu}$ = 3410, 1744, 1586, 1260, 1191, 1127, 1063 cm^{-1}
HPLC-MS: Rt = 2.11min, m/z = 208 (100) $[M+H]^+$

4-hydroxyaniline β-lactam derivatives synthesis

In a 50mL flask 0.5mmol of 4-hydroxyaniline **18** were dissolved in 2mL of a freshly prepared 0.25M NaOH aqueous solution, then **1** or **2** (0.5mmol in 3mL of H_2O/MeCN 1/1 solution) were dropped in 30min, following the reaction by TLC monitoring until SM disappearance. The reaction was then quenched by NaCl addition to separate MeCN and water, which was acidified using HCl (1M) before extracting with EtOAc (3 times). The organic phases were dried over Na_2SO_4, then filtered and concentrated in vacuum, reaching the desired **19a** and **20a** products via FC with EtOAc.

Directly on the crudes obtained, the corresponding acetic amides were obtained for further analysis dissolving them in 2mL of MeCN and 4mL of 0.125M HCl aqueous solution (0.5mmol), then adding acetic anhydride (0.6mmol) and 0.5M AcONa in water (0.5mmol). Reaction s were stirred for 2 hours following the progresses by TLC and then quenched at SM disappearance by NaCl addition to separate MeCN and water, which was acidified using HCl (1M) before extracting with EtOAc (3 times). The organic phases were dried over Na_2SO_4, then filtered and concentrated in vacuum, reaching the desired acetamides of **19a** and **20a** , via flash-chromatography with EtOAc, used for evaluating the yields.

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-(3-hydroxyphenoxy)-1-methylthioazetidin-2-one (23a)



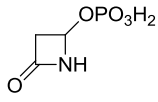
Colorless oil; Rf 0.80 (cyclohexane/EtOAc 80/20)
1H NMR (400MHz, CD3OD): δ 0.08 (d, J = 6.8Hz, 6H), 0.88 (s, 9H), 1.36 (d, J = 6.4Hz, 3H), 2.40 (s, 3H), 3.34 (d, J = 3.2Hz, 1H), 4.26 (dq, J = 3.2Hz, 6.4Hz, 1H), 5.13 (s, 1H), 5.75 (s, 1H), 6.58 (d, J = 8.0Hz, 1H), 6.71 (s, 1H), 6.81 (d, J = 8.0Hz, 1H), 7.21 (t, J = 8.0Hz, 1H) ppm
13C NMR (100MHz, CD3OD): δ = 47.2, 78.1, 117.0, 126.8, 133.8, 162.3, 170.0, 170.4 ppm
IR: $\tilde{\nu}$ = 3410, 1744, 1586, 1260, 1191, 1127, 1063 cm^{-1}
HPLC-MS: Rt = 11.48min, m/z = 406 (100) $[M+Na]^+$

4.2.3 Hydroxy-apatite derivatives

The optimized conditions required the use of 1M phosphate buffer aqueous solution (10mL every 1mmol of substrate). Substrate **2** was directly added, while for **1** the temperature was first increased to 70°c, adding **1** at 40°C to better solubilize it. At SM disappearance (TLC monitoring), the crude was evaporated using cyclohexane to form the corresponding minimum azeotrope. The resulting solid was recovered with a 1/1 MeOH/MeCN mixture, then filtered and concentrated to afford both the products as white solids.

Azetidinone **26** was prepared as previously specified.

4-oxoazetidin-2-yl phosphate (**24**)



In a 50mL one-neck flask **2** (129mg, 1mmol) was dissolved in 10mL of 1M phosphate buffer (pH = 7.5) and the reaction was followed by TLC (cyclohexane/EtOAc 20/80). At SM disappearance, water was evaporated with cyclohexane and the crude extracted with 10mL of a 1/1 MeOH/MeCN mixture, filtered and concentrated to afford **24** as a white solid in 60% yield (101mg).

Rf 0.01 (cyclohexane/EtOAc 80/20)

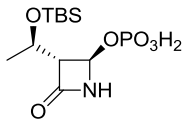
^{1}H NMR (400MHz, D_2O): δ 3.00 (d, J = 15.6Hz, 1H), 3.31 (dd, J = 3.6Hz, 15.2Hz, 1H), 5.65 (t, J = 3.6Hz, 1H) ppm

IR (ATR): ṽ = 3275, 1767, 1741, 1375, 1232, 1122, 1043, 962 cm^{-1}

^{13}C NMR (400MHz, D_2O): δ 45.6, 73.1, 170.7 ppm

^{31}P NMR (400MHz, CD_3OD): δ 0.71 ppm (while buffer is at 2.5-2.7 ppm, depending on counter ions)

(2R,3R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-oxoazetidin-2-yl phosphate (**25**)



In a 50mL one-neck flask **1** (287mg, 1mmol) was added to 10mL of 1M phosphate buffer (pH = 7.5) already warmed at 70°C. The reaction was followed by TLC monitoring (cyclohexane/EtOAc 80/20) and at SM disappearance, water was evaporated with cyclohexane and the resulting crude extracted with 10mL of a 1/1 MeOH/MeCN mixture, filtered and concentrated to afford **25** as a white solid in 62% yield (201mg).

Rf 0.01 (cyclohexane/EtOAc 60/40)

^{1}H NMR (400MHz, D_2O): δ 0.11 (s, 3H, MeSi), 0.13 (s, 3H, MeSi), 0.89 (s, 9H, *t*BuSi), 1.33 (d, J = 6.4Hz, 3H, Me), 3.28 (d, J = 3.2Hz, 1H, C*H*CONH), 4.30 (m, 1H, C*H*OTBS), 5.70 (d, J = 5.2Hz, 1H, C*H*NH) ppm

IR (ATR): ṽ = 3190, 2957, 2930, 2856, 1779, 1742, 1376, 1341,1229, 1163, 1077, 1039, 946, 837, 776 cm^{-1}

^{13}C NMR (400MHz, D_2O): δ -6.3, -5.3, 16.9, 20.9, 21.7, 48.5, 65.2, 75.1, 170.6 ppm

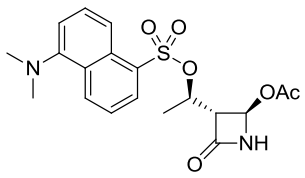
^{31}P NMR (400MHz, CD_3OD): δ 0.29 ppm (while buffer is at 2.5-2.7 ppm, depending on counter ions)

General procedure for hydroxy-apatite derivatizations

In a 10mL one-neck flask, to 200mg of hydroxyapatite suspended in 2mL of milliq. H_2O were added 50mg of the β-lactam substrates; after 4h stirring (at r.t. for **2**, or at 70°C for **1**, **21** and **27**) was highlighted by TLC that it still be present, but the mixture was however centrifuged (60s at 7.0rpm). Was performed a first extraction with H_2O (2x2mL) recovering the supernatant after each centrifugation and, over the water extracts combined, a subsequent extraction with DCM (3x2mL), which furnished after evaporation some mg of the employed lactam. The solid phase was dried at temperatures lower than 40°C to usually furnish ~220mg of the desired bioconjugate.

To prepare 5% biconjugate hydroxyapatites, initial β-lactam substrates loading must be of 20mg.

(2R,3R)-3-((R)-(((5-(dimethylamino)naphthalenyl)sulfonyl)oxy)ethyl)-4-oxoazetidin-2-yl acetate (**27**)



In a 10mL 2-necks flask, to a solution of **26** (87mg, 0.5mmol) in 5mL of anhydrous DCM were added dansyl chloride (148mg, 0.55mmol) and DMAP (32mg, 0.75mmol). The reaction was followed by TLC (cyclohexane/EtOAc 50/50) and after 24hours stirring was directly concentrated in vacuum. The crude product was purified by flash chromatography (cyclohexane/EtOAc

70/30) to afford TM in 36% yield (73mg) as a yellow-green oil.
Rf 0.32 (cyclohexane/EtOAc 50/50)
$[\alpha]_D$ 25 = -17.83° (c = 13.27, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ 1.38 (d, J = 6.0Hz, 3H, $MeCHOSO_2$), 2.00 (s, 3H, Ac), 2.93 (s, 6H, NMe_2), 3.32 (d, J = 8.8Hz, 1H, C*H*CHOAc), 4.81 (dquartet, J = 6.4, 8.4Hz, 1H, $CHOSO_2$), 5.25 (s, 1H, CHC*H*OAc), 6.34 (s, 1H, NH), 7.23 (d, J = 8.0Hz, 1H, Ar), 7.56-7.62 (m, 2H, Ar), 8.26 (d, J = 8.8Hz, 1H, Ar), 8.30 (d, J = 7.6Hz, 1H, Ar), 8.67 (d, J = 8.0Hz, 1H, Ar) ppm
^{13}C NMR (50.3MHz, $CDCl_3$): δ 19.4, 20.5, 45.3 (2C), 62.0, 75.6, 75.7, 115.5, 119.3, 123.0, 128.5, 129.6, 129.7, 130.2, 131.7, 131.8, 151.7, 163.3, 170.5 ppm
IR: $\tilde{\nu}$ = 3331, 2937, 1785, 1753, 1359, 1232, 1177, 1140 cm^{-1}
HPLC-MS: Rt = 8.74min; m/z = 407 (100) $[M+H]^+$, 813 $[2M+H]^+$, 435 (25) $[2M+Na]^+$

5. AZETIDINONE-RETINOID HYBRIDS

5.1 4-Alkylidene β-lactam retinoid hybrids

5.1.1 Design, synthesis and differentiative effects

Besides an interesting number of β-lactams in the last decades shown to be able to targeting a wide range of biological targets, usually enzymes, some bicyclic β-lactams were recently reported as inhibitors of Histone Deacetylases (HDACs), a family of proteins involved in the pattern of acetylation of chromatin proteins and thus in the regulation of gene expression.[105] We recently reported the synthesis of some azetidinone derivatives which showed good affinity and specificity towards histone deacetylases HDAC8 and HDAC6.[106]

Histone deacetylases are epigenetic regulators playing an important role in modulating gene transcription as well as protein function. Eighteen classical HDAC isoforms, divided into IV classes based on yeast deacetilases homology, are identified to date. HDAC inhibitors constitute a new class of promising anticancer drugs targeting one or more HDAC isoform. HDAC6 overexpression was found in carcinoma of oral squamous tissue,[107] although in breast cancer it correlates with better survival.[108] In lung, colon, and cervical cancer cell line the knockout of HDAC8 gene reduces cell proliferation;[109] in leukemia cell HDAC8 inhibitors leads to apoptosis.[110] The expression of HDAC8 (class I member) has been correlated with poor outcome in neuroblastoma, and in such cell line selective HDAC8 inhibitors induces differentiation and proliferation arrest.[111] Nowadays, there is great interest in the development of small molecules that regulate or modulate protein function and chemical genetic offers a valuable approach in this field.[112] Retinoids, as derivatives of Vitamin A, regulate key developmental pathways throughout life, by modulating the expression of many genes involved in cell proliferation, migration, differentiation and apoptosis, though the molecular mechanisms are not completely understood.[113] The effects of Retinoic Acid (RA) are mediated by the heterodimerization of nuclear RAR (Retinoic Acid Receptor), a family of ligand-dependent transcription factors, and RXR (Retinoid X Receptors), the 9-cis RA binding receptor. To date, retinoids are known to activate or inhibit MAPK cascade, which is involved in both proliferation and growth arrest.[114] Some synthetic RA-derived molecules exert differentiative effect rather than apoptosis, blocking tumor progression in cancer cell lines.[115] In fact, retinoids are currently used as potential chemotherapeutic drugs in the treatment of several cancer types (neuroblastoma, breast cancer, lung cancer, rhabdomyosarcoma, and many others).[116] For this reason, there is great interest in developing new synthetic molecules targeting retinoic acid receptors.

As part of an interdisciplinary project on the development of new β-lactam derivatives with a modular structure designed for specific biological activities, the effect of the azetidinone **1** (Figure 5.1.1), previously evaluated by us as specific HDAC8 inhibitor, was investigated on the proliferative activity of a neuron-like cell line.

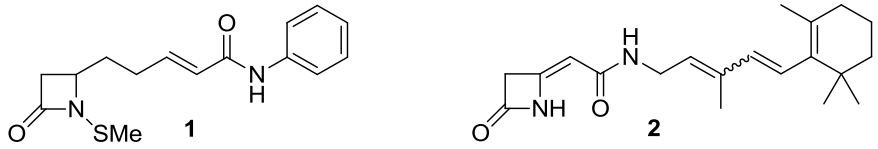


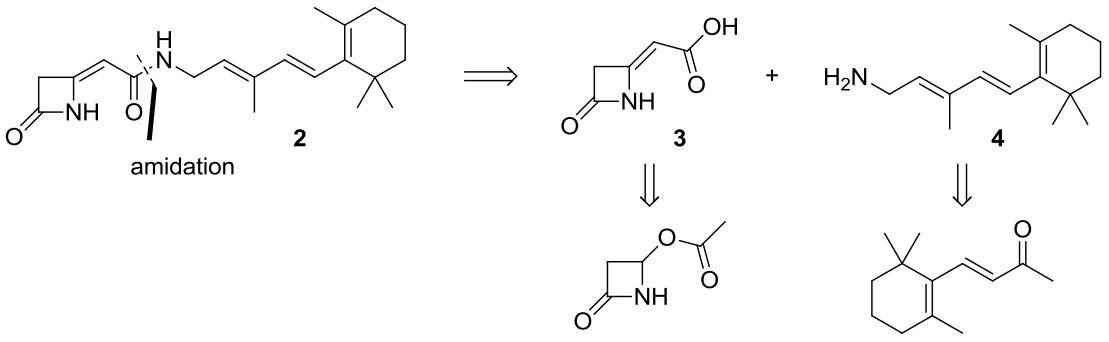
Figure 5.1.1 β-Lactam derivatives

Moreover, we developed the synthesis of new β-lactams **2** with a hybrid retinoid-azetidinone structure and we investigated the retinoic acid-dependent differentiation of a neuron-like cell line. To our knowledge, only two example of β-lactams were reported in the literature with a differentiation-inducing activity on leukemia cells.[117] We report here the synthesis of the new hybrid retinoid β-lactams and preliminary results in biological evaluation of the three molecules on neuroblastoma cell line SH-SY5Y.

Design and synthesis

As mentioned above, we have previously reported an efficient synthesis of β-lactam **1**. Its design was based on the pharmacophore model of HDAC inhibitors which consists in a modular structure with a cap-group that interacts with residues in the active site of the receptor and a linker group that sits in a hydrophobic channel and positions a metal-binding group which coordinates to the zinc ion in the active site. We demonstrated that the azetidinone ring can efficiently work as the zinc-binding group. Moreover, the substituent on the nitrogen atom allowed for the specificity against HDAC8 or HDAC6 inhibition. Azetidinone **1** was therefore chosen in the present study for its specific HDAC8 activity.

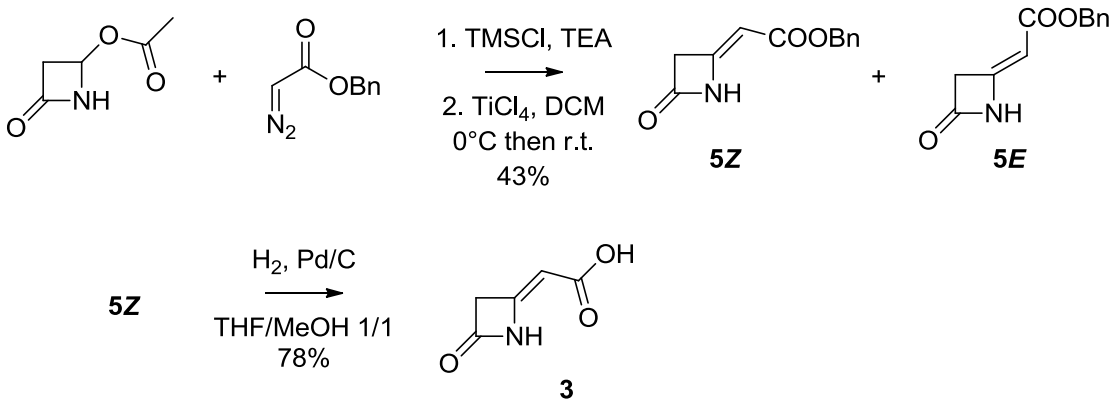
Once the β-lactam ring was confirmed as an effective zinc binding group for HDAC, we developed a new hybrid structure retaining the β-lactam scaffold to target HDACs and functionalized with a retinoic side chain which could activate differentiative processes to mimic the action of Retinoic Acid (RA). The design of β-lactam **2** was based on a 4-alkylidene-β-lactam scaffold with demonstrated enhanced reactivity in protein inhibition,[118] and functionalized with a retinoid-amide side chain.



Scheme 5.1.1 Retro-synthetic route to retinoid β-lactam **2**

The strategy is outlined in Scheme 5.1.1 and consists of a convergent synthesis with the coupling between the 4-alkylidene-azetidinone carboxyl acid **3** and the retinoid amine **4** as the key step. 4-Alkyliden-azetidin-2-ones can be obtained using an original and well established protocol developed in our laboratory starting from 4-acetoxy-azetidinones and diazoesters in the presence of Lewis acids.[119]

Given that 4-acetoxy-azetidinone is commercially available, β-lactam **3** was obtained with $TiCl_4$ and benzyldiazoacetate as outlined in Scheme 5.1.2.

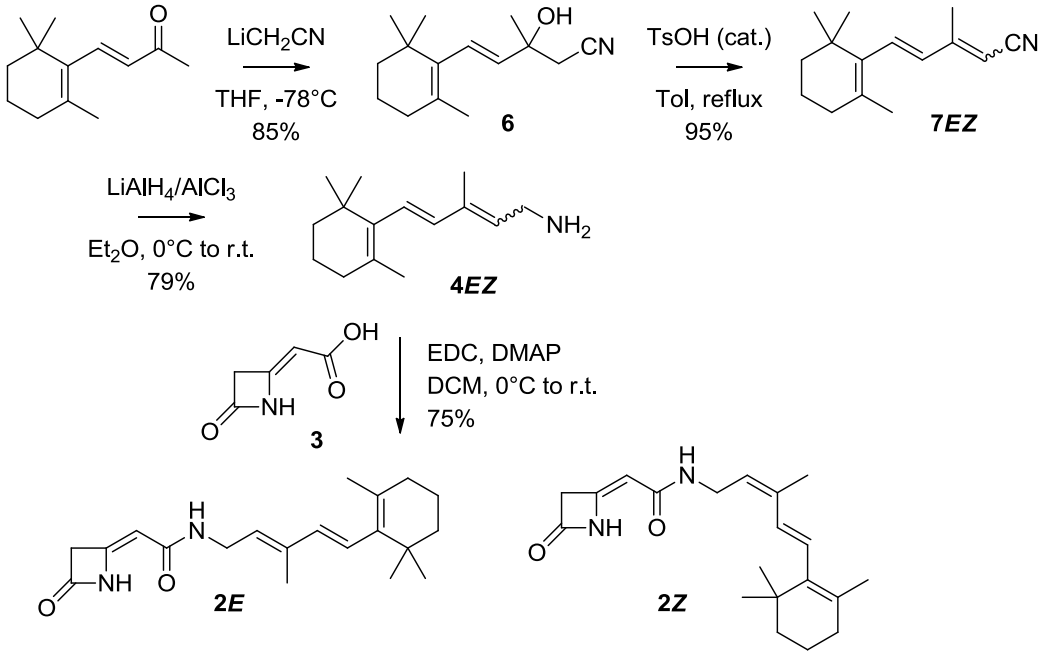


Scheme 5.1.2 Synthesis of 4-alkyliden-azetidinone **3**

The 4-alkyliden-β-lactams **5** were obtained in 43% yields and in a E/Z diastereoisomeric ratio of 41/59. The diastereoisomeric mixture **5*Z***/**5*E*** was easily separated by flash chromatography.

Hydrogenolysis of the isolated pure **5*Z*** with Pd on carbon in THF-methanol 1/1 mixture gave **3** in quantitative yield.

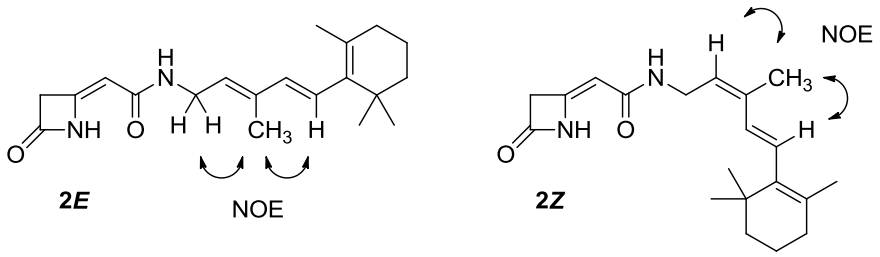
Retinoid amine **4** was obtained starting from the easily available β-ionone in a three step reaction (Scheme 5.1.3). Addition of lithium acetonitrile to β-ionone in THF at low temperature gave **6**, which underwent water elimination under *p*toluenesulfonic acid catalysis.[120]



Scheme 5.1.3 Synthesis of retinoid amine **4** and retinoid β-lactams **2*E*** and **2*Z***

The nitrile **7** obtained as a E/Z mixture was then reduced to give the amine **4*EZ*** in 79% yield. Finally, with a carbodiimide coupling method, amides **2*E*** and **2*Z*** were obtained as a mixture and easily separated by flash chromatography.

The **2*E***, **4*E*** and **2*Z***,**4*E*** configurations were assigned in the two isomers by NOE 1D analysis on the separated products **2*E*** and **2*Z*** (Scheme 5.1.4).



Scheme 5.1.4 NOE 1D correlations in new hybrids for configuration assignments

In isomer **2*E***, the methyl group in the chain, chosen as the irradiated site, showed two NOE correlations with methylene and methine, respectively, indicating a close spatial relationship whereas for β-lactam **2*Z*** NOE enhancements of the two vinyl CH were observed. The **2*E***, **4*E*** configuration for **2*E***, and the **2*Z***, **4*E*** configuration for **2*Z*** were thus attributed.

5.1.2 Biological effects

As said before, HDAC inhibitors are emerging as anti-cancer drugs. In particular, HDAC8 inhibitors have shown to regulate proliferation in neuroblastoma cells. As potential HDAC inhibitors, we evaluated the antiproliferative effect of azetidinones **1**, **2*E***, and **2*Z*** on undifferentiated SH-SY5Y. Moreover, as β-lactams **2*E*** and **2*Z*** have hybrid retinoid-azetidinone structures, we performed a set of experiments aiming to evaluate differentiative action of β-lactams **2*E*** and **2*Z*** compared to azetidinone **1** and retinoic acid treated cells.

In fact, retinoids are currently used as potential chemotherapeutic drugs in the treatment of several cancer types including neuroblastoma due to their differentiative action. In order to assess azetidinone **1**, **2*E*** and **2*Z*** cytotoxicity, we calculated first the IC_{50} (50% inhibiting concentration) using MTT assay (Figure 5.1.2, on undifferentiated SH-SY5Y; IC_{50} ranges are reported above the curves depicted. Data are shown as mean). The MTT assay is a colorimetric assay for measuring the activity of cytosolic enzymes that reduce tetrazolium dye (MTT) in formazan salt in living cells. IC_{50} values were calculated and are reported above the curves in Figure 5.1.2.

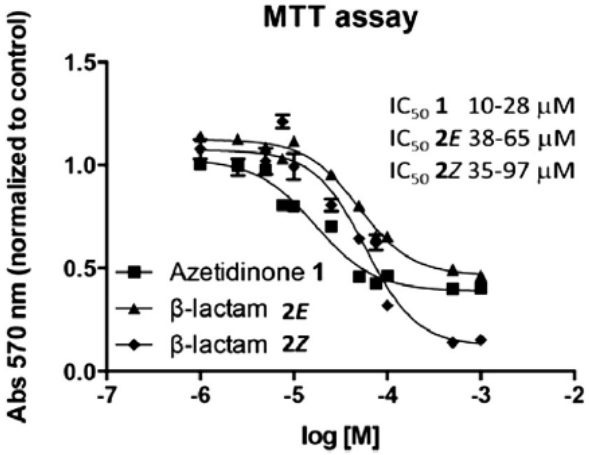


Figure 5.1.2 Cytotoxic evaluation of **1**, **2E** and **2Z**

The concentration used for subsequent experiments was chosen accordingly in order to obtain cytostatic effects rather than apoptosis or necrosis (Figure 5.1.2).

Effect on proliferation

In a preliminary analysis of biological effect on proliferation, undifferentiated SH-SY5Y cells were exposed to 7.5μM of azetidinone **1**, 10μM of β-lactams **2*E*** and **2*Z*** for 24, 48, 72 and 96h, the number of cells was counted daily and compared to control (vehicle treated). Figure 5.1.3 [The rate of cells proliferation was determined as total cell number. Cells were incubated in the presence of 7.5μM of azetidinone **1**, 10μM of **2*E*** and **2*Z*** and counted at 24, 48, 72, 96 h. Significant differences between groups were observed after 48h of treatment. Statistical analysis was performed with two-way ANOVA, Dunnet's multiple comparisons test (a: ctrl vs β-lactam **2*E***, $p < 0.05$; b: ctrl vs β-lactam **2*Z***, $p < 0.01$; c: ctrl vs azetidinone **1** and β-lactam **2*E*** $p < 0.001$, d: ctrl vs β-lactam **2*Z*** $p < 0.0001$, e: ctrl vs azetidinone **1** and β-lactam **2*E*** $p < 0.0001$, f: ctrl vs β-lactam **2*Z*** $p < 0.001$)] shows the proliferation curve of control cells (ctrl, open circle), azetidinone **1** (black square), β- lactam **2*E*** (black triangle) and **2*Z*** treated cell (black rhombus).

Significant differences were observed between the groups (twoway ANOVA, Dunnet's multiple comparisons test). β-lactams **2*E*** and **2*Z*** determine a significative reduction in cell proliferation after 48 h of treatment (a: ctrl vs β-lactam **2*E***; b: ctrl vs β-lactam **2*Z***). All the compounds tested show an antiproliferative effect compared to control from 72 to 96 h (c: ctrl vs azetidinone **1** and β-lactam **2*E***, d: ctrl vs β-lactam **2*Z***, e: ctrl vs azetidinone **1** and β-lactam **2*E*** $p < 0.0001$, f: ctrl vs β-lactam **2*Z***). Although the reduction is stable, doubling time calculated in 24-72h range (Td) is affected exclusively in cells treated with β-lactam **2*Z*** (Td_{CTRL} = 34.9h; Td_1 = 39h, Td_2 = 39.78, Td_8 = 51.25), whereas doubling time calculated in 24-96h range is not affected

These results indicated a slight effect of azetidinone **1**, **2*E*** and **2*Z*** on SH-SY5Y proliferation.

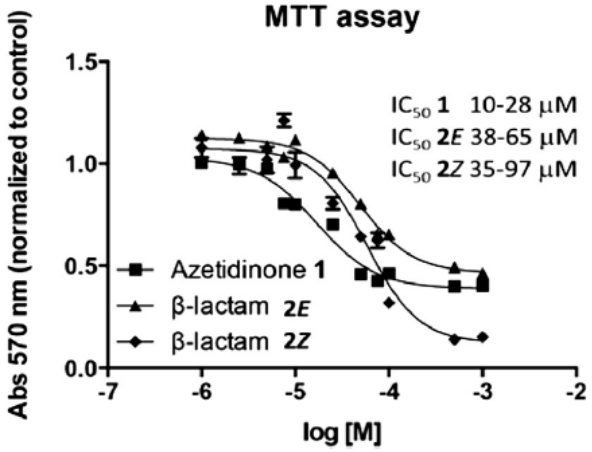


Figure 5.1.3 Effect of azetidinones **1**, **2E** and **2Z** on SH-SY5Y proliferation

Differentiation effects

As they are derived from neuroblastoma, SH-SY5Y may be differentiated toward neural-like lineage. Retinoic acid is a powerful differentiating molecule inducing growth inhibition, neurites lengthening and branching. A six-days RA treatment induces neural-like differentiation as shown by neurites outgrowth [Figure 5.1.4, βIII-tubulin (A-C) and neurofilament (D-F) were used to investigate a possible microtubule and intermediate filaments rearrangement. Panels A and F refer to vehicle treated cells; B and G to cells treated for 6 days with RA; C and F correspond to cells treated for 6 days with 7.5μM of azetidinone **1**, D and I correspond to cells treated for 6 days with 10μM of β-lactam **2*E***; E and J correspond to cells treated for 6 days with 10μM of β-lactam **2*Z*** (Scale bar = 10micron). Graphs K and L refer to βIII-tubulin and neurofilament immunoreactive area, respectively, normalized for number of cells. Statistical analysis was performed with one-way ANOVA, Tukey post test, **p < 0.01, ***p < 0.001, ****p < 0.0001] visualized by βIII-tubulin and NF200. Quantitative analysis was performed measuring the βIII-tubulin and NF200 network normalized according to the cells number. As hybrids retinoid-azetidinones, we investigated a possible differentiative effect of β-lactams **2*E*** and **2*Z*** compared to azetidinone **1** and RA treated SH-SY5Y. βIII-Tubulin is an early marker for differentiation toward neuronal lineage and the increase of βIII-tubulin area may be related to an early stage of differentiation. Neurofilament play important structural roles and influence axonal transport, neurite outgrowth and cell survival. Sample micrographs of cell SH-SY5Y exposed to the vehicle (control), RA, azetidinone **1**, and β-lactams **2*E*** and **2*Z*** are shown in Figure 5.1.4, where panels A-E refers to β-tubulin-IR, and panels F-J refers to neurofilament-IR, graph K and L to respective quantification of IR area/number of cells. While a differentiative effect of RA is evident in both βIII-tubulin and NF200 immunostaining (panels A vs panel B and panel F vs panel G, respectively) and azetidinone **1** does not display any differentiative effect (panels A vs panel C and panel F vs panel H), β-lactams **2*E*** (panels A vs panel D and panel F vs panel I) and **2*Z*** (panels A vs panel E and panel F vs panel J) induce a moderate differentiation, which is statistically significant in βIIItubulin staining (panels K and L, statistical analysis was performed with one-way ANOVA, Tukey post test).

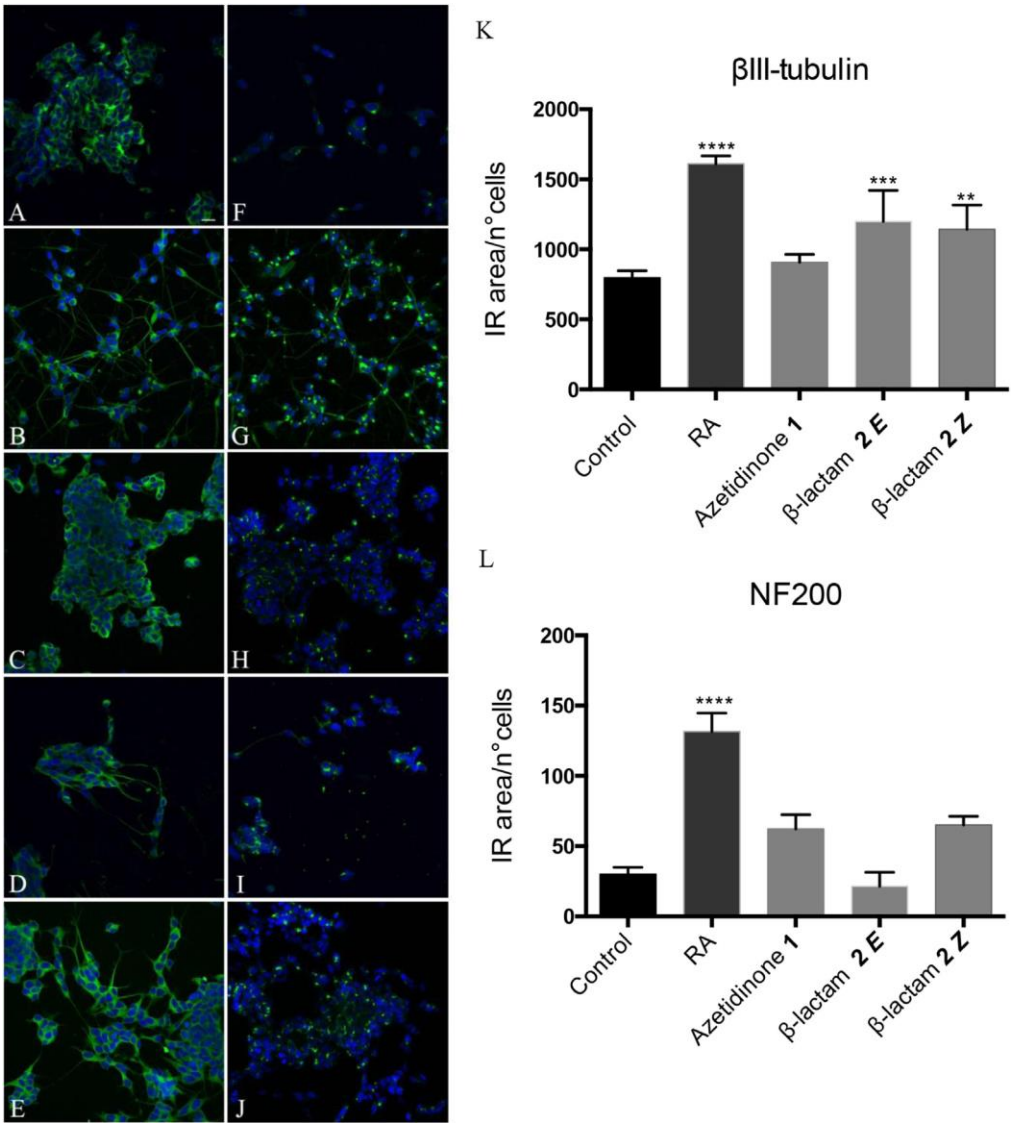


Figure 5.1.4 Effect of azetidinone **1**, **2*E*** and **2*Z*** on SH-SY5Y differentiation

This effect could be possibly related to moderate structural analogies among RA and β-lactams **2*E*** and **2*Z***. The data indicate a differentiative effect of β-lactams **2*E*** and **2*Z*** on SH-SY5Y, even though smaller when compared to RA. The absence in the β-lactams **2*E*** and **2*Z*** of an ionizable function, as like the carboxylic acid in RA, can explain the observed lower activity, as reported for some non-ionizable RA derivatives such as RA-esters or amides.[121] To bind effectively to the retinoic acid receptor (RAR), the polar terminus of the retinoid must be capable of efficient interactions, as observed in the crystal structure of RA-RAR complex.[122] The β-lactam scaffold, while it would not be considered a strong "polar terminus" in anchoring RAR, shows a positive effect on SH-SY5Y cell differentiation (β-lactams **2*E*** and **2*Z***), and it deserves further investigation.

5.2 Experimental section

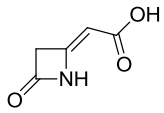
5.2.1 General informations

As previously reported (Paragraph 2.3.1).

5.2.2 Synthesis

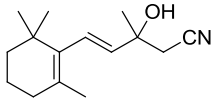
Azetidinone **5*Z*** was prepared as previously reported.[123]

(Z)-2-(4-Oxoazetidin-2-ylidene)acetic acid (**3**)



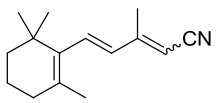
To a solution of **5*Z*** (220mg, 1.01mmol) in a 10 mL mixture of THF/MeOH 1/1 was added Pd/C 10% (44mg). The solution was treated with H_2 (1atm) and after 2h was filtered on celite and concentrated to give **3** (100mg, 78%) as a yellow solid.
M.p. 162-163°C
^{1}H NMR (400MHz, DMSO): δ = 3.57 (s, 2H), 4.98 (s, 1H), 10.47 (bs, 1H), 11.94 (bs, 1H) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 46.0, 92.2, 153.3, 169.7, 171.0 ppm
IR: ṽ = 3289, 2919, 1828, 1185 cm^{-1}
HPLC-MS: Rt = 1.66min, m/z (%) = 128 $[M+H]^+$, 150 $[M+Na]^+$, 679 $[2M+Na]^+$
$C_5H_5NO_3$: calc C 47.25, H 3.97, N 11.02%; found C 47.42, H 3.92, N 10.87%

(E)-3-Hydroxy-3-methyl-5-(2,6,6-trimethylcyclohexen-1-yl)pent-4-enenitrile (**6**)



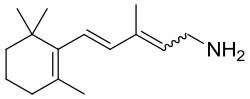
To a stirred solution of acetonitrile (330mL, 6.24mmol) in anhydrous THF (5mL) at -78°C under inert atmosphere were added dropwise nButyllithium 2.5M in THF (6.86mmol, 2.74mL) and thereupon a solution of β-ionone (1g, 5.2mmol) in dry THF (4mL) were added over a period of 10min. The solution was allowed to warm at room temperature and after 3h the reaction was quenched with aqueous NH_4Cl (10mL) and extracted with EtOAc (3x10mL). The organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuum. Flash chromatography (cyclohexane/EtOAc: 80/20) of the extracts gave **6** (1.031g, y = 85%) as a pale yellow oil.
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.92 (s, 6H, CMe_2), 1.35-1.38 (m, 2H, CMe_2CH_2), 1.41 (s, 3H, MeCOH), 1.49-1.55 (m, 2H, $CMe_2CH_2CH_2$), 1.58 (s, 3H, MeC=C), 1.88-1.91 (m, 2H, $CMe_2CH_2CH_2CH_2$), 2.56 (s, 2H, CH_2CN), 3.34 (bs, 1H, OH), 5.45 (d, 1H, J = 16.0 Hz, CH=C*H*COH), 6.11 (d, 1H, J = 16.0 Hz, C*H*=CHCOH) ppm
^{13}C NMR (100MHz, CD_3OD): δ = 18.8, 20.9, 27.4, 28.3 (2C), 31.6, 32.3, 33.6, 38.9, 70.8, 117.3, 127.1,128.5,136.0,136.6 ppm
IR: ṽ = 3444, 3023, 2925, 2256,1722, 1651, 1644, 1114 cm^{-1}
GC-MS: Rt = 16.81min, m/z (%) = 193 $[M-CH_2CN]^+$, 218 $[M-Me]^+$, 233 $[M]^+$
$C_{15}H_{23}NO$: calc C 77.21, H 9.93, N 6.00%; found C 77.01, H 9.98, N 5.92%

(2ZE,4E)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienenitrile (**7*EZ***)



A solution of **6** (400mg, 1.72mmol) and *p*toluenesulfonic acid (33mg, 0.172mmol) in toluene (17mL) was refluxed and the reaction was followed by TLC. After the completion of the reaction the solution was cooled at 0°C and washed with a solution of $NaHCO_3$ 5% and after with water. The organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuum obtaining 352mg (95%) of **7** as a E/Z: 54/46 mixture. Characterization resulted in agreement with the reported data.[124]

(2EZ,4E)-3-Methyl-5-(2,6,6-trimethylcyclohexen-1-yl) penta-2,4-dienamine (**4*EZ***)



Amine **4** was prepared as reported.[125] E/Z: 59/41, pale yellow oil

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.97 (s, 6H, CMe_2 trans), 0.98 (s, 6H, CMe_2 cis),1.41-1.48 (m, 2H, CMe_2CH_2), 1.48-1.64 (m, 2H, $CMe_2CH_2CH_2$), 1.68 (s, 3H, CH_2MeC=C trans), 1.72 (s, 3H, CH_2CH_2MeC=C cis), 1.81 (s, 3H, MeC=CH trans), 1.88 (s, 3H, MeC=CH cis), 1.95-1.97 (m, 2H, CH_2CH_2MeC=C), 2.47 (bs, 2H, NH_2), 3.40 (d, J =6.8Hz, 1H, CH_2NH_2), 5.37 (dd, 1H, J = 6.4, 7.6Hz, CH=C*H*CH_2NH_2 cis), 5.45 (dd, 1H, J = 6.4, 7.2Hz, CH=C*H*CH_2NH_2 trans),5.94-6.01 (m, 2H, CH=CH trans) 6.12 (d, J = 15.6Hz, 2H, CH=CH cis) 6.30 (d, J = 15.6Hz, 2H, CH=CH cis) ppm

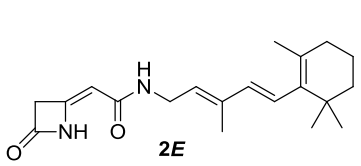
^{13}C NMR (100MHz, $CDCl_3$): δ = 12.2, 19.1, 21.6, 28.4 (2C), 32.7, 34.1, 38.4, 39.3, 126.0, 128.5, 128.6, 129.0, 129.3, 130.1,133.7,135.0,137.1,137.5,137.8 ppm

IR: $\tilde{\nu}$ = 3373, 2928, 2866, 1454, 1266 cm^{-1}

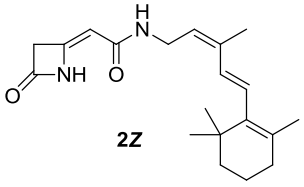
GC-MS: Rt = 16.09min (cis), 16.57min (trans), m/z (%) = 187 [M-CH_2CN]$^+$, 204 [M-Me]$^+$, 219 [M]$^+$

(2Z)-N-(2EZ,4E-3-Methyl-5-(2,6,6-trimethylcyclohexenyl)penta-2,4-dienyl)

2-(4-oxoazetidin-2-ylidene)acetamide (**2*E***, **2*Z***)



2*E*



2*Z*

To a solution of β-lactam **3** (55mg, 0.43mmol) in dry DCM (4.5mL) under inert atmosphere at 0°C were added amine 4EZ (94mg, 0.43mmol), DMAP (53mg, 0.43mmol) and after 10min EDC (82.5mg, 0.43mmol). The solution was stirred at 0°C for 45min and after was allowed to warm at room temperature. The reaction was followed by TLC and after the disappearing of the starting materials was quenched with aqueous NH_4Cl (10mL) and extracted with DCM (3x5mL). The organics were dried over Na_2SO_4, filtered and concentrated in vacuum. The E/Z mixture was separated by flash chromatography (cyclohexane/EtOAc 70/30) affording **2*E*** (62mg) and **2*Z*** (44mg) in an overall yield of 75%.

Configurations of the two diastereoisomers was assigned by NOE 1D studies.

2*E*: white syrup: Rf = 0.4 (cyclohexane/EtOAc, 50/50)

^{1}H NMR (400MHz, $CDCl_3$): δ = 1.00 (s, 6H),1.46 (m, 2H),1.62 (m, 2H),1.68 (s, 3H), 1.86 (s, 3H), 2.01 (m, 2H), 3.51 (s, 2H), 4.06 (m, 2H), 5.02 (s, 1H), 5.30 (bs, 1H, NH), 5.43 (m, 1H), 6.01 (d, J = 16.0Hz, 1H), 6.13 (d, J = 16.0Hz, 1H), 8.75 (bs, 1H, NH) ppm

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.5, 19.2, 21.6, 28.8, 29.7, 32.9, 34.2, 37.3, 39.5, 44.9, 92.2, 124.9, 127.0, 129.0, 136.6, 137.5, 137.6, 146.6, 165.3, 166.3 ppm

IR: $\tilde{\nu}$ = 3367, 3250, 2958, 2922, 2853, 1815, 1692 cm^{-1}

HPLC-MS: Rt = 12.72min, m/z (%): 329 [M+H]$^+$

$C_{20}H_{22}N_2O_2$: calc C 73.14, H 8.59, N 8.53%; found C 72.98, H 8.65, N 8.41%

2*Z*: pale yellow syrup: Rf = 0.5 (cyclohexane/EtOAc, 50/50)

^{1}H NMR (400MHz, $CDCl_3$): δ = 1.26 (s, 6H), 1.47 (m, 2H), 1.63 (m, 2H), 1.70 (s, 3H), 1.91 (s, 3H), 2.02 (m, 2H), 3.51 (m, 2H), 4.04 (m, 2H), 4.99 (s, 1H), 5.25 (bs, 1H, NH), 5.36 (m, 1H), 6.22 (d, J = 16.0Hz, 1H), 6.38 (d, J = 16.0Hz, 1H), 8.72 (bs, 1H, NH)

^{13}C NMR (100MHz, $CDCl_3$): δ = 19.2, 20.3, 21.7, 28.9, 29.7, 32.9, 34.1, 36.4, 39.5, 44.9, 92.3, 123.1, 129.0, 129.6, 129.7, 136.7, 137.7, 146.4, 165.3, 166.3 ppm

IR: $\tilde{\nu}$ = 3327, 2954, 2923, 2853, 1810, 1683 cm^{-1}

HPLC-MS: Rt = 11.21min, m/z (%): 329 (65) [M+H]$^+$, 351 (100) [M+Na]$^+$, 679 (70) [2M+Na]$^+$

$C_{20}H_{22}N_2O_2$: calc C 73.14, H 8.59, N 8.53%; found C 72.88, H 8.65, N 8.38%

6. NEW Pt(II) BIOCONJUGATES

6.1 New luminescent β-lactam Pt(II) bioconjugates

6.1.1 β-Lactam Pt (II) bioconjugates

Transition metal-bioconjugates is an active field of research which have been found to be potential therapeutics and can play a role as tracers in immunological analysis based on several analytical methods.[126] Furthermore, the possibility to combine the therapeutic properties with diagnostics tools making compounds with dual functionality, i.e. theranostic agents, recently blossomed the possibility to simultaneously use powerful imaging tool in order to unveil real-time biochemical process, compound accumulation, drug activity and interaction, and to gather information about the fate of the compounds, just to cite some.[127]

In this respect, much effort is currently devoted to the preparation of luminescent complexes based on second and third row transition metals such as Ir (III), Rh (I), Ru (II) and Pt (II), for applications in bio-imaging in vitro, in cellulo and in vivo.[128] Such increasing scientific interest is driven by the fact that judicious choice of coordinating ligands together with the presence of the heavy metal might provide enhanced physicochemical properties, such as photostability, large Stokes shifts, tunable emission colors, and high photoluminescence quantum yield.[129] Furthermore, as consequence of the formally spin-forbidden nature of the radiative processes, such Transition Metal Complexes (TMCs) possess long-lived excited states with lifetime that spans over greater orders of magnitude when compared to widely used organic fluorophores[130] (i.e., from ns to ms and from ps to ns for the formers and the latters, respectively), allowing the successful employment of time-gated microscopy techniques for imaging purposes.[131] However, TMCs typically show excitation bands in the deep-blue and ultraviolet region that severely hampers their use in real applications for in cellulo and in vivo experiments.

Among all the investigated complexes, luminescent platinum (II) derivatives possess the tendency to aggregate into self-assembled nanostructures by means of weak μ-μ and closed-shell metallophilic (d_{z2}•••d_{z2}) interactions. The establishment of such intermolecular interactions yields to the formation of lower-lying excited states, namely Metal-Metal-to-Ligand Charge Transfer (MMLCT). We have very recently demonstrated that is possible to take advantage from the formation of such aggregates to obtain luminescent TMCs based labels able to self-assemble in cellular compartments forming highly emitting aggregates as consequence of the protection from quenchers (e.g., dioxygen) in biological relevant environments. Even more interestingly, the formation of the MMLCT bands yielded sizeable bathochromic shift of both excitation and emission allowing easy excitation of the probe with visible light.

The β-lactams have been scarcely studied as ligands for metal-complexes,[132] and most of the examples deal with metallocene derivatives. Recently examples of Pd and Pt complexes with azetidinones[133] and

bio-organometallic Ir and Rh with β-lactam ligands[134] were reported. However, specifically designed metal complexes-bioconjugates should allow the possibility to couple the multifacet biological activities of β-lactams with photoactive metal complexes. This could represent an interesting combination since amongst the new strategies to combat antibiotic-resistant infections, antimicrobial photodynamic therapy appears promising for the treatment of local infections caused by both Gram-positive and Gram-negative bacteria.[135]

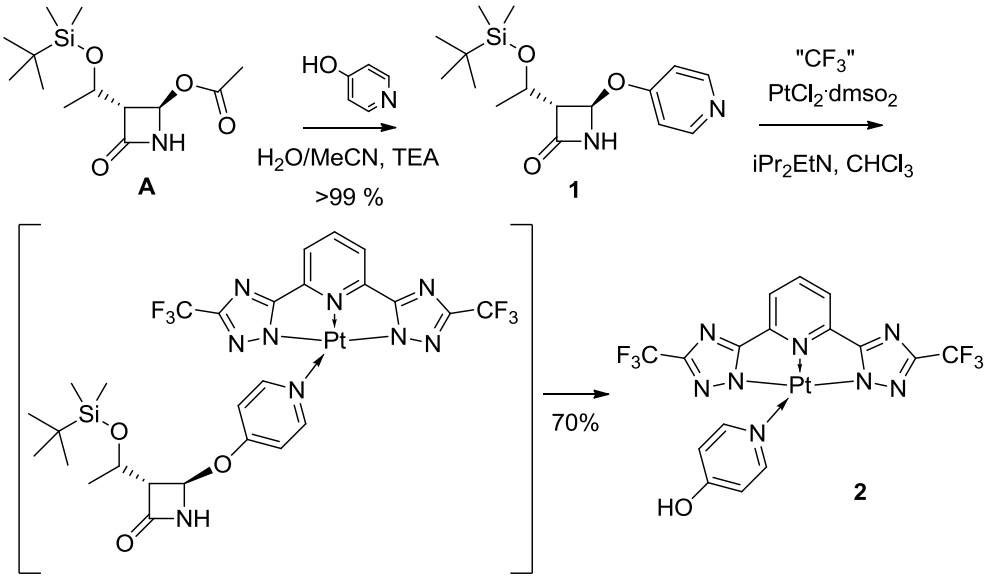
We did the first step towards the development of bio-conjugates between azetidinones and luminescent platinum complexes. We have chosen platinum compounds because of their interesting photophysics that can be tuned upon the formation of aggregates.[136] Furthermore, platinum complexes are widely used in biomedical application due to their anticancer activity, and their ability to recognize proteins. We report on the synthesis and photophysical properties of neutral luminescent platinum complexes coupled through a coordinated pyridine on the C4 side chain of the (3R,4R)-3-((R)-1-*t*butyldimethylsilyloxyethyl)-azetidin-2-one as a β-lactam model. The complexes, which bear a trifluoromethyl-substituted tridentate chromophoric ligand, are able to show high tendency towards aggregation in biological relevant environments. Furthermore, it is known in medicinal chemistry that the presence of the highly hydrophobic trifluorometyl groups would allow a certain degree of lipophilicity and cellular permeability. Many trifluoromethyl-substituted derivatives are indeed known to cross cellular and nuclear membranes.[137] We can envisage that the here reported and characterized molecules could therefore be parent compounds of a novel set of bio-imaging probes with self-assembling properties for particular biological targets, such as bacteria, proteins, or receptors.

6.1.2 Synthesis and photophysical characterization

In 1974, Clauss et al. reported that the 4-acetoxyazetidin-2-one undergoes a nucleophilic displacement of the acetoxy group with a variety of nucleophiles.[138] This observation prompted many laboratories to use 4-acyloxy-azetidinones as substrates for the synthesis of a variety of β-lactams and nowadays they are useful industrial intermediates for the synthesis of carbapenem.[139]

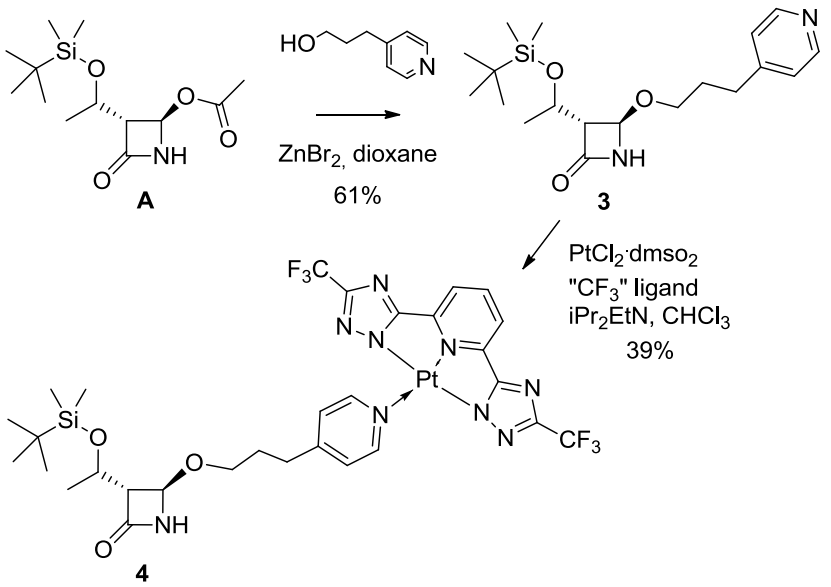
Starting from (3R,4R)-3-((R)-1-*t*butyldimethylsilyloxyethyl)-azetidin-2-one (**A**, Scheme 7.1.1), which is commercially available, we took advantage of the aptitude of 4-acetoxy group to undergo nucleophilic substitution reactions and we tried to insert on the C-4 position of the β-lactam ring a residue which will be a suitable metal ligand. On a first attempt, the 4-OH-pyridine was chosen as nucleophile and the corresponding azetidinone **1** was obtained in good yields. On attempting the complexation of **1** with $Pt(II)Cl_2(DMSO)_2$ as the platinum precursor, 2,6-bis(3-(trifluoromethyl)-1H-1,2,4-triazol-5-yl)pyridine as the tridentate ligand (py-CF_3-trzH_2) in $CHCl_3$, and in the presence of the Hünig base (iPr_2EtN) at 50°C, we obtained only the 4-OH-pyridyl-Pt(II) complex **2**. A tentative explanation is that the C-4 position of the expected β-lactam B could result activated by the direct conjugation of the phenolic moiety with the pyridine coordinated to platinum. Thus in the aqueous work-up the displacement of the ligand from the

azetidinone intermediate B occurred and only the pyridine complex **2** was isolated (Scheme 6.1.1). Compound **2** has been already characterized by De Cola and coworkers and it will not hereafter further investigated.



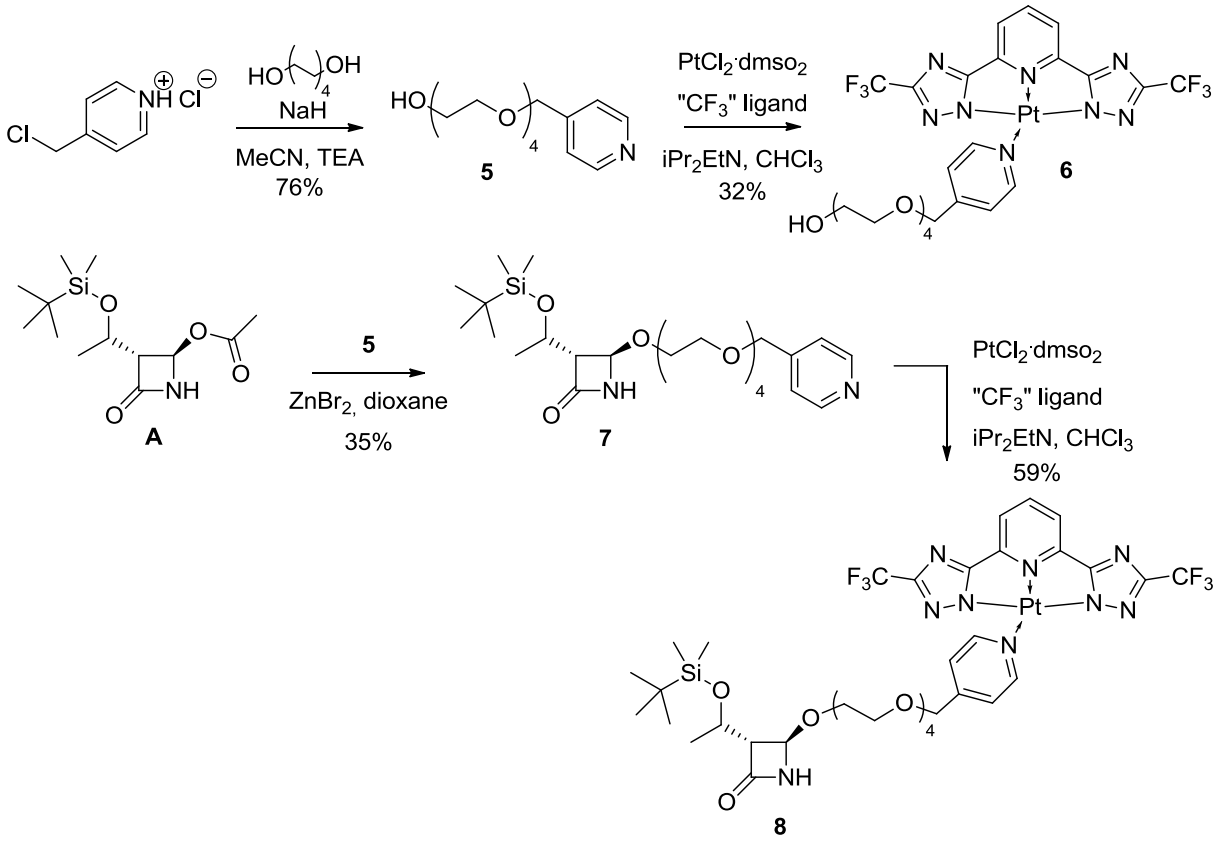
Scheme 6.1.1 Synthetic procedure for compounds **1** and **2**

To avoid the electronic coupling between the pyridine and the azetidinone moiety, a short alkyl chain was introduced as a spacer. Thus 3-pyridyl-propanol and the 4-acetoxy-azetidinone **A** were coupled (Scheme 7.1.2) to give the azetidinone **3**. Compound **3** was then treated with the platinum (II) precursor, the ligand 2,6-bis(3-(trifluoromethyl)-1H-1,2,4-triazol-5-yl)pyridine, in the presence of iPr_2EtN in $CHCl_3$ at 50°C leading to the β-lactam complex **4** (Scheme 6.1.2). The new platinum-based bio-conjugate was purified by column chromatography and fully characterized.



Scheme 6.1.2 Synthetic strategy to the new β-lactam platinum conjugate **4**

Complex **4** is however poorly soluble in organic solvents and completely insoluble in water. To improve its solubility we then inserted a short tetraethylene glycol chain as spacer between the pyridine and the β-lactam moieties. The synthetic pathway is depicted in Scheme 7.1.3 and started from the reaction of 4-chloromethylpyridine with tetraethylene glycol, to give compound **5**. The new tetraethyleneglycol-pyridyl ligand **5** was preliminarily evaluated as platinum ligand in reaction with the Pt(II) precursor obtaining complex **6** (Scheme 6.1.3) successfully. Treatment of the starting 4-acetoxyazetidinone **A** with **5** gave the β-lactam complex **7**, which was treated with the Pt(II) precursor, the py-CF_3-trzH_2, iPr_2EtN in $CHCl_3$ at 50°C to give the desired β-lactam complex **8**.



Scheme 6.1.3 Synthetic route to obtain the azetidinone-Pt(II) complex **8**

The β-lactam conjugate **8** was obtained in 59% yield after flash chromatography purification and fully characterized. The complex **8** was very soluble in several organic solvents (DCM, $CHCl_3$, THF, MeOH, ethyl acetate and acetone), and in water it rapidly formed a homogeneous colloidal suspension. The purified conjugates **4** and **8** were fully characterized by mass spectrometry (ESI), IR, and NMR spectroscopy. In Figure 7.1.1 ^{1}H NMR (400MHz) spectra of compounds **4**, **8** and the β-lactam **3** are reported in comparison. The successful complexation of the β-lactam **3** to platinum in compounds **4** and **8** was confirmed by the downfield shift of the ortho-aromatic pyridine protons of the C-4 side chain of the β-lactam, which moved from 8.5 ppm in compound **3** to 9.4 ppm for **4** and **8**.

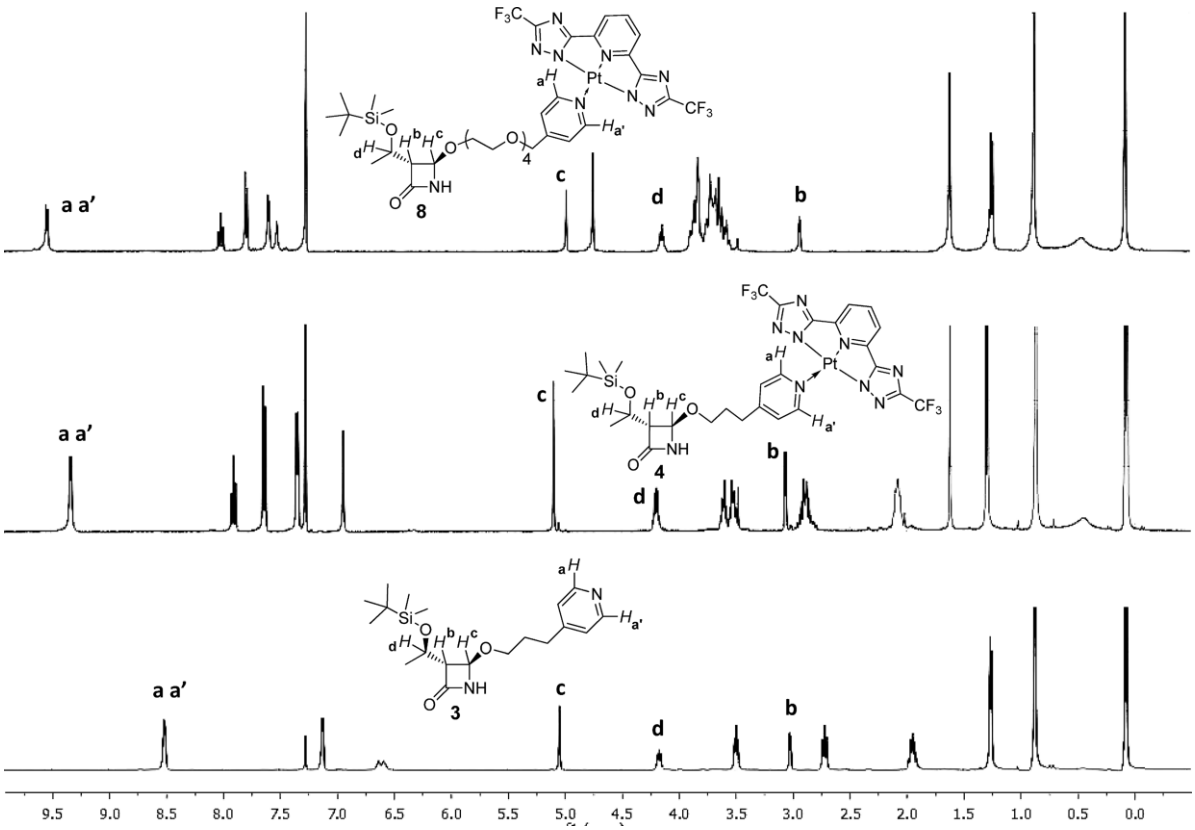


Figure 6.1.1 ^{1}H NMR (400MHz) spectra of **3**, **4** and **8** (from bottom to top) in $CDCl_3$

On the contrary, resonance peaks relative to the peculiar hydrogen atoms on the β-lactam ring b, c, and d (Figure 6.1.1) were influenced by complexation to a much lesser extent. Therefore, the β-lactam ring should not be involved in platinum complexation and it could save its own characteristics and properties. This is an important observation in view of a possible application of the conjugation described here on biologically active azetidinones.

Photophysical Characterization

The complexes **4** and **8** were also fully characterized using electronic absorption, steady state and time resolved emission spectroscopy and the corresponding data are summarized in Table 6.1.1 (where *sh* denotes a shoulder; [a]: amplitude-weighted lifetimes; [b]: recorded at 450 nm; [c]: recorded at 585 nm).

	Room temperature $CHCl_3$				77 K 2MeTHF glassy matrix		Solid state		
	λ_{abs} (ε) [nm, ($\times 10^3$ m^{-1} cm^{-1})]	λ_{em} [nm][a]	τ [ns]	PLQY (%)	λ_{em} [nm][a]	τ (<τ>[b]) [μs]	λ_{em} [nm]	τ [ns]	PLQY [%]
4	255 (22.6), 267 (19.8), 303 (15.1), 338 (2.6), 402 (0.8)	464, 492, 525, 567 (sh)	278 (77%) 13 (23%)	2	451, 480, 517, 554 (sh)	4.2, 69% 10.7, 31% (6.2)[c] 3.6, 96% 7.7, 4% (3.8)[d]	596	321	55
8	255 (24.4), 266 (22.2), 303 (16.4), 338 (3.3), 402 (1.1)	464, 492, 525, 567 (sh)	239 (66%) 14 (34%)	2	451, 483, 517, 571	4.0, 71% 9.6, 29% (5.6)[c] 3.5, 97% 7.7, 3% (3.6)[d]	589	341	56

Table 6.1.1 Photophysical data for compounds **4** and **8** at rt, 77K and in solid state

The absorption and emission spectra in dilute (concentration $1 \cdot 10^{-5}$M) $CHCl_3$ solution at room temperature (solid traces: absorption and normalized emission spectra for samples of compound **4**, in black, and **8**, in red), and at concentration of $5 \cdot 10^{-5}$M in 2-MeTHF glassy matrix at 77K (dashed traces) are displayed in Figure 6.1.2 (Emission spectra collected upon λ_{exc} at = 300nm).

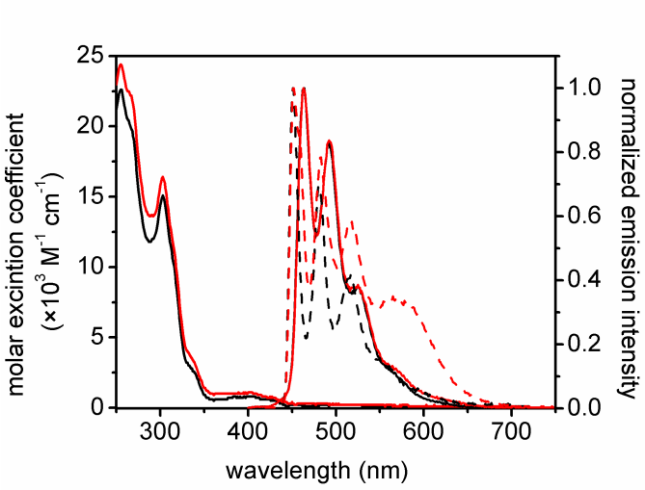


Figure 6.1.2 Absorption and emission spectra for **4** (black) and **8** (red)

As shown in the electronic absorption spectrum in dilute $CHCl_3$ fluid solution, both compounds **4** and **8** display absorption features in two main regions. At lower energy in the region 360-400 nm, a weak broad and featureless band with molar extinction coefficient, ε, of 0.8 and $1.1 \cdot 10^3 M^{-1}cm^{-1}$ (at λ_{abs} = 403nm) for compounds 4 and 8, respectively, is attributable to the overlap of energetically lower-lying spin-allowed inglet-manifold metal-to-ligand charge transfer, ^{1}MLCT, and ligand-centered, ^{1}LC, excitation processes as typical of similar reported cyclometalated platinum (II) complexes. Such excitation can be mainly described as a shift of electron density from the filled d metal-based molecular orbitals to the empty MOs of the tridentate ligand with π* character, $\pi(Pt) \rightarrow \pi^*$. On the other hand, the more intense (ε = 0.26-$2.44 \cdot 10^4 M^{-1}cm^{-1}$) and structured absorption features present at higher energy (λ_{abs} = 250-340nm) can be attributed to ^{1}LC bands. Unfortunately, the low solubility of the complexes in solvents with a wide range of polarity hampered studies on solvent dependence. As shown in Figure 6.1.2, upon photoexcitation in the range 300-400nm, diluted fluid air-equilibrated solutions in $CHCl_3$ of both complexes display moderate (PLQY = 2%) and structured emission in the blue region of the visible spectrum with a maximum at 464, and vibrational bands at 492 and 525, and a shoulder at 567nm. The observed emission band with vibronic progression is similar to other reported platinum (II) derivatives bearing N^N^N tridentate chelating motifs and is attributed to π–π* transitions involving the tridentate ligand. All the complexes has bi-exponential radiative deactivation kinetics: τ_1 = 278ns and τ_2 = 13ns for compound **4** and τ_1 = 239ns and τ_2 = 14ns for compound **8** (see Table 6.1.1 for more details).

The assembling formation for the β-lactam-platinum conjugates **4** and **8** have also been investigated in the solid state, and the corresponding normalized excitation (dotted traces) and emission (solid traces)

spectra, collected at the λ_{em} maximum and upon λ_{exc} at 350nm, for solid-state samples of **4** (black) and **8** (red) are reported in Figure 6.1.5.

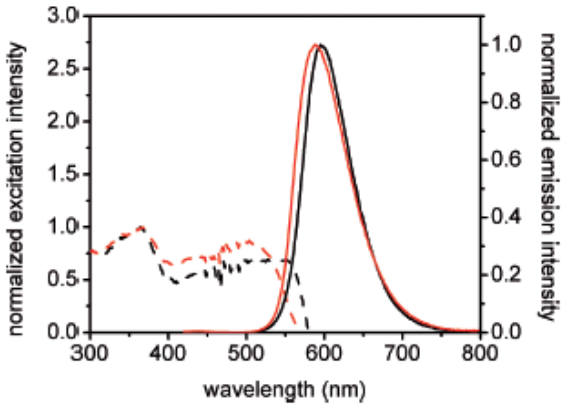


Figure 6.1.5 Normalized emission and excitation spectra for **4** and **8** in solid state

Interestingly, upon photoexcitation between 300 and 400nm, the solid state samples display a very intense emission band centered at 596 and 589nm with PLQY as high as 55 and 56% for compound **4** and **8**, respectively. The excited-state lifetimes decay with monoexponential kinetics and are 321 and 341ns for complex **4** and **8**, respectively. Similar to the 77K spectra, such bright emission is attributable to the ^{3}MMLCT emitting excited-state established upon aggregation of the complex in the solid state. Even more interestingly, the excitation spectra recorded at the maximum of emission wavelength display a typical ^{1}MMLCT band with onset wavelength as long as 580nm for compound **4**, allowing the possibility to excite such luminescent probes in their aggregated form with visible light with a wavelength that approaches the so-called therapeutic window (600-1300nm). Overall, the several nanosecond lifetime scale, the very bright emission in air-equilibrated conditions of the aggregated compounds, as well as the excellent stability of the compounds, are valuable properties that should facilitate further exploration of the behavior of such species for bioapplications and as theranostic agents.

6.2 Experimental section

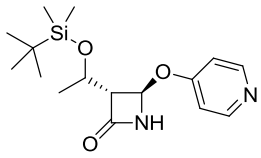
6.2.1 General informations

As previously reported (Paragraph 2.3.1).

6.2.2 Synthesis

Azetidinone **A** is commercially available and was used without any further purifications.

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl(-4-(pyridin-4-yloxy)azetidin-2-one (**1**)



In a 25mL flask, to a stirred solution of **A** (143mg, 0.5mmol) in acetonitrile (1.0mL) were slowly added H_2O (4.0mL), then 4-hydroxypyridine (95mg, 1.0mmol) and finally Et_3N (154µL, 1.1mmol). The reaction was followed by TLC (EtOAc/cyclohexane 8/2) and after 1h stirring, the mixture was extracted with DCM (3x10mL), dried with Na_2SO_4, and evaporated in vacuum. After flash-chromatography purification (acetone to acetone/MeOH 95/5), the desired product **1** was obtained in quantitative (144mg, 100%) as a white solid.

$[\alpha]_D$ = 29.8° (c = 0.62, DCM)

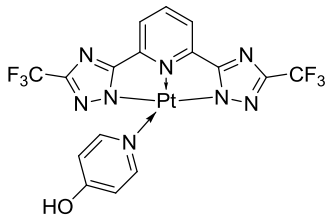
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.10 (s, 3H, MeSi), 0.12 (s, 3H, MeSi), 0.89 (s, 9H, *t*BuSi), 1.25 (d, J = 7.4Hz, 3H, Me), 3.20 (dd, J = 1.5, 4.2Hz, 1H, C*H*CONH), 4.28 (m, 1H, C*H*OTBS), 5.43 (s, 1H, C*H*OPyr), 6.37 (d, J = 7.6Hz, 2H, Ar), 7.55 (d, J = 7.6 Hz, 2H, Ar), 7.88 (s, 1H, NH) ppm

^{13}C NMR (100.6 MHz, $CDCl_3$): δ = -5.0 ,-4.3, 17.9, 22.5, 25.7, 64.4, 68.1, 68.5, 118.9, 136.3, 165.7, 179.6 ppm

IR: $\tilde{\nu}$ = 3391, 2956, 2929, 2857, 1784, 1634, 1549, 1386, 1257, 1174, 1099, 982, 836, 776 cm^{-1}

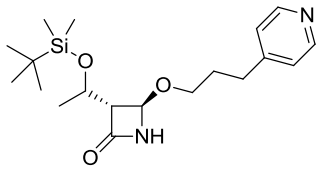
HPLC-MS: *R*t = 6.70min; *m*/*z* = 323 (100) $[M+H]^+$, 645 (59) $[2M+H]^+$, 667 (23) $[2M+Na]^+$

2,6-Bis(3-trifluoromethyl)-1,2,4-triazol-5-yl)pyridine Pt (II) pyridin-4-ol (**2**)



Compound **2** is a known compound,[140] and was prepared as follows. In a 25mL two-neck flask with a Liebig condenser under a nitrogen atmosphere, to a stirred solution of **1** (84mg, 0.23mmol) in chloroform (10.0mL) were added 80mg (0.23mmol) of 2,6-bis(3-trifluoromethyl-1*H*-1,2,4-triazol-5-yl)pyridine, 97mg (0.23mmol) of $PtCl_2{\cdot}(DMSO)_2$ and *N*-ethyl-*N*,*N*-diisopropylamine (80µL, 0.46mmol). The mixture was heated at 50°C, stirred overnight and then analyzed by TLC (EtOAc/acetone 8/2). Direct evaporation afforded a crude reaction mixture that was purified by flash-chromatography (EtOAc/acetone 9/1 to acetone) to give complex **2** (102mg, 70%) as a yellow luminescent waxy solid.

(3R,4R)-3-((R)-1-*t*butyldimethylsilyloxyethyl)-4-(3-(pyridin-4-yl)propoxy) azetidin-2-one (**3**)



In a 25mL two-neck flask dried and under a nitrogen atmosphere, to a stirred solution of **A** (143mg, 0.5mmol) in dioxane (5.0mL), 3-pyridine-propanol (274mg, 2.0mmol) and $ZnBr_2$ (450mg, 2.0mmol) were added. The reaction was followed by TLC monitoring (EtOAc/cyclohexane 8/2) and after 56h it was quenched by stirring with saturated aqueous $NaHCO_3$ (5mL), extracted with DCM (3x10mL), dried with Na_2SO_4, filtered and evaporated in vacuum. The crude was then purified by flash-chromatography (EtOAc/cyclohexane 4/6 to 8/2), and compound **3** (111mg, 61%) was so obtained as a colorless oil.

$[\alpha]_D$ = -1.9° (*c* = 0.76, DCM)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.06 (s, 3H, MeSi), 0.08 (s, 3H, MeSi), 0.87 (s, 9H, *t*BuSi), 1.25 (d, J = 6.2 Hz, 3H, Me), 1.94 (m, 2H, OCH_2CH_2), 2.71 (t, J = 7.6 Hz, 2H, $OCH_2CH_2CH_2$), 3.02 (d, J = 4.2Hz, 1H, C*H*CONH), 3.49 (t, J = 6.4Hz, 2H, OCH_2), 4.17 (m, 1H, C*H*OTBS), 5.04 (s, 1H, C*H*NH), 6.61 (bs, 1H, NH), 7.12 (d, J = 4.0Hz, 2H, arom), 8.50 (d, J = 4.0Hz, 2H, arom) ppm

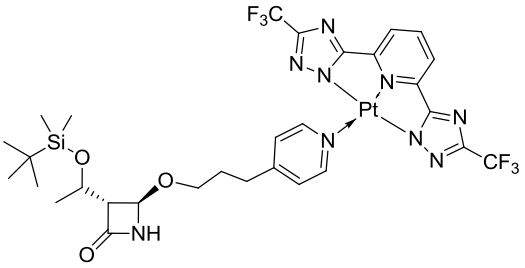
^{13}C NMR (100.6MHz, $CDCl_3$): δ = -5.1, -4.4, 17.9, 22.5, 25.7, 29.9, 31.5, 64.2, 65.5, 66.6, 80.5, 124.0, 149.3, 150.9, 167.9 ppm

IR: $\tilde{\nu}$ = 3227, 2955, 2929, 2857, 1767, 1605, 1463, 1417, 1361, 1255, 1120, 1098, 836 cm^{-1}

HPLC-MS: *R*t = 9.65min; *m*/*z* = 365 $[M+H]^+$

2,6-Bis(3-(trifluoromethyl)-1,2,4-triazol-5-yl)pyridine Pt(II)

(3R,4R)-3-((R)-1-*t*butyldimethylsilyloxyethyl)-4-(3-pyridin-4-yl propoxy)azetidin-2-one (**4**)



In a 25mL two-neck round-bottom flask with a Liebig condenser, anhydrified and put under nitrogen atmosphere, to a stirred solution of **3** (60mg, 0.165mmol) in chloroform (7.2mL), 2,6-bis[3-(trifluoromethyl)-1*H*-1,2,4-triazol-5-yl] pyridine (58mg, 0.165mmol), $PtCl_2 \cdot (DMSO)_2$ (67mg, 0.165mmol) and *N*-ethyl-*N*,*N*-diisopropylamine (69µL, 0.396mmol) were added. The mixture was heated at 50°C overnight and analyzed by TLC (EtOAc). Direct evaporation of the reaction solvent afforded a crude product that was purified by flash-chromatography (DCM/EtOAc 3/7), to obtain **4** (58mg, 39%) as a yellow to orange fluorescent waxy solid.

$[\alpha]_D$ = -2.8° (c = 0.87, DCM)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.07 (s, 3H, MeSi), 0.09 (s, 3H, MeSi), 0.87 (s, 9H, *t*BuSi), 1.29 (d, J = 6.4Hz, 3H, Me), 2.03-2.12 (m, 2 H, OCH_2CH_2), 2.75-2.99 (m, 2H, $OCH2CH_2CH_2$), 3.06 (d, J = 3.6Hz, 1H, C*H*CONH), 3.50-3.55 (m, 1H, OCH_AH_B), 3.56-3.66 (m, 1H, OCH_AH_B), 4.12-4.26 (m, 1H, C*H*OTBS), 5.09 (s, 1H, C*H*NH), 6.94 (s, 1H, NH), 7.34 (d, J = 6.2Hz, 2H, Ar), 7.63 (d, J = 8.0 Hz, 2H, Ar), 7.90 (t, J = 8.0Hz, 1H, Ar), 9.33 (d, J = 6.2Hz, 2H, Ar) ppm

^{13}C NMR (100.6MHz, $CDCl_3$): δ = -5.1, -4.3, 17.9, 22.5, 25.7, 28.9, 31.7, 64.3, 65.6, 66.0, 80.5, 118.1, 119.6 (q, $^1J_{C-F}$ = 270Hz), 121.0, 126.4, 143.0, 148.4, 152.5, 152.6 (q, $^2J_{C-F}$ = 38Hz), 155.4, 163.5, 167.8 ppm

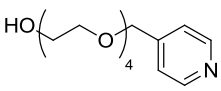
^{19}F NMR (400MHz, $CDCl_3$): δ = -64.1 ppm

IR: $\tilde{\nu}$ = 3286, 2958, 2930, 2858, 1767, 1628, 1482, 1202, 1156, 1132, 1098 cm^{-1}

MS (ESI^+): *m*/*z* = 907 $[M+H]^+$, 929 $[M+Na]^+$, 1836 $[2M+Na]^+$

$C_{30}H_{35}F_6N_9O_3PtSi$ (906.83): calc C 39.73, H 3.89, N 13.90; found C 39.65, H 4.21, N 12.44

1-(Pyridin-4-yl)-2,5,8,11-tetraoxatridecan-13-ol (**5**)



In a 100mL two-neck flask under a nitrogen atmosphere, to a stirred solution of 4-chloromethyl-pyridine hydrochloride (820mg, 5.0mmol) in acetonitrile (40.0mL) were added tetraethylene glycol (949 µL, 6.5mmol) and Et_3N (1.4mL, 10.0mmol). After 1h stirring, NaH (60% in oil, 400mg, 10.0mmol) was added and the mixture was stirred overnight at room temperature. The progress of the reaction was followed by TLC monitoring (EtOAc/cyclohexane 8/2) and after 20 hours the reaction mixture was concentrated under vacuum. The obtained crude product was then purified by flash-chromatography (acetone/MeOH 95/5) to furnish alcohol **5** in good yield (217mg, 76%) as a colorless oil.

^{1}H NMR (400MHz, $CDCl_3$): δ = 3.60-3.73 (m, 16H, OCH_2CH_2O), 4.61 (s, 2H, OCH_2Ar), 7.36 (d, J = 5.2Hz, 2H, Ar), 8.60 (d, J = 5.2Hz, 2H, Ar) ppm

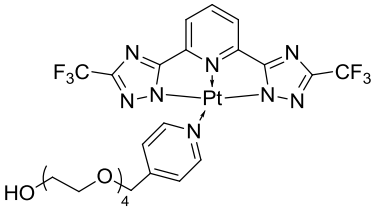
^{13}C NMR (100.6MHz, $CDCl_3$): δ = 60.8, 69.5, 69.6, 69.7, 69.9, 70.0, 70.8, 72.2, 72.3, 121.3, 147.4, 148.9 ppm

IR: $\tilde{\nu}$ = 3375, 2969, 2925, 2879, 1465, 1419, 1378, 1301, 1127, 1106, 952 cm^{-1}

HPLC-MS: *R*t = 1.28min; *m*/*z* = 286 $[M+H]^+$

2,6-Bis(3-trifluoromethyl-1,2,4-triazol-5-yl)pyridine Pt(II)

1-(pyridin-4-yl)-2,5,8,11-tetraoxatridecan-13-ol (**6**)



In a 25mL two-neck flask equipped with a Liebig condenser under a nitrogen atmosphere, to a stirred solution of **5** (48mg, 0.17mmol) in chloroform (7.4mL), 2,6-bis[3-(trifluoromethyl)-1*H*-1,2,4-triazol-5-yl]pyridine (59mg, 0.17mmol), $PtCl_2 \cdot (DMSO)_2$ (72mg, 0.17mmol) and

N-ethyl-*N*,*N*-diisopropylamine (71µL, 0.408mmol) were added. The mixture was heated at 50°C, stirred overnight and then analyzed by TLC (acetone/EtOAc 8/2). Direct solvent evaporation of the reaction mixture afforded a crude product that was purified by CombiFlash (DCM/MeOH 98/2) to obtain **6** (45mg, 32%) as a yellow to orange fluorescent waxy solid.

^{1}H NMR (400MHz, $CDCl_3$): δ = 3.56-3.87 (m, 16H, OC*H*$_2$C*H*$_2$O), 4.76 (s, 2H, OC*H*$_2$Ar), 7.68 (d, J = 6.0Hz, 2H, arom), 7.89 (d, J = 8.0Hz, 2H, arom), 8.08 (t, J = 8.0Hz, 1H, Ar), 9.62 (d, J = 6.0Hz, 2H, Ar) ppm

^{13}C NMR (100.6 MHz, CDCl3): δ = 61.6, 70.3, 70.4, 70.5, 70.6, 70.6, 70.7, 70.7, 72.5, 117.8, 119.4 (q, $^1J_{C\text{-}F}$ = 269Hz), 123.5, 142.7, 147.7, 152.0, 152.1 (q, $^2J_{C\text{-}F}$ = 38Hz), 152.6, 163.0 ppm

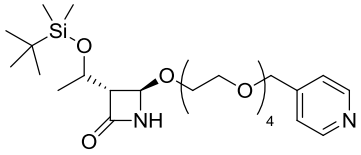
^{19}F NMR (400MHz, $CDCl_3$): δ = -64.3 ppm

IR: ṽ = 3423, 3062, 2922, 2363, 1629, 1482, 1463, 1132 cm^{-1}

MS (ESI$^+$): *m*/*z* = 850 [M+Na]+, 1677 [2M+Na]$^+$

$C_{25}H_{26}F_6N_8O_5Pt$ (827.61): calc C 36.28, H 3.17, N 13.54; found C 35.99, H 3.52, N 11.90

(3R,4R)-3-((R)-1-(*t*butyldimethylsilyloxy)ethyl)-4-((1-pyridin-4-yl-2,5,8,11-tetraoxatridecan-13-yl)oxy)azetidin-2-one (**7**)



In a 25mL two-necked flask equipped with a Liebig condenser, dried and under a nitrogen atmosphere, to a stirred solution of **A** (143mg, 0.5mmol) in dioxane (4.0mL), **5** (142mg, 0.5mmol) and $ZnBr_2$ (112mg, 0.5mmol) were added. The reaction was warmed to 50°C and monitored by TLC (EtOAc). After 30h stirring, the reaction was quenched with saturated aqueous $NaHCO_3$ (5mL) and the solvents were evaporated under vacuum. The solid mixture was treated with a mixture of MeCN/MeOH (1/1, 10mL), filtered, and the liquid phase was concentrated under vacuum. After flash-chromatography purification (EtOAc to EtOAc/MeOH 95/5), product **7** (90mg, 35%) was obtained as a colorless oil.

$[\alpha]_D$ = 4.4° (c = 1.00, DCM)

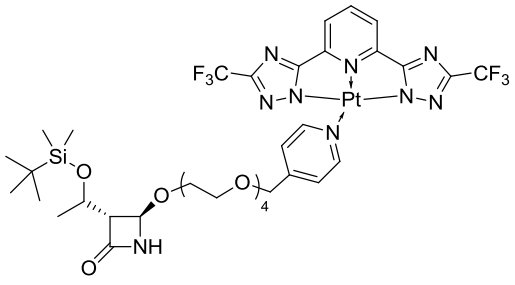
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.06 (s, 3H, MeSi), 0.07 (s, 3H, MeSi), 0.88 (s, 9H, *t*BuSi), 1.25 (d, J = 6.4Hz, 3H, Me), 2.94 (dd, J = 1.2, 4.0Hz, 1H, C*H*CONH), 3.44-3.96 (m, 16H, OC*H*$_2$C*H*$_2$O), 4.11-4.18 (m, 1H, C*H*OTBS), 4.60 (s, 2H, OC*H*$_2$Ar), 5.00 (d, J = 1.2Hz, 1H, C*H*NH), 7.29 (d, J = 5.6Hz, 2H, Ar), 7.51 (s, 1H, NH), 8.57 (d, J = 5.6Hz, 2H, Ar) ppm

^{13}C NMR (50.3MHz, $CDCl_3$): δ = -5.1, -4.2, 17.9, 22.4, 25.7, 26.5, 64.5, 65.4, 69.9, 70.2, 70.3, 70.4, 70.5, 70.7, 71.2, 71.5, 82.8, 122.2, 148.1, 149.9, 167.6 ppm

IR: ṽ = 3330, 2955, 2928, 2858, 1764, 1462, 1375, 1253, 1103, 949, 778, cm^{-1}

HPLC-MS: *R*t = 1.45min; *m*/*z* = 513 [M+H]$^+$

2,6-Bis(3-trifluoromethyl-1,2,4-triazol-5-yl)pyridin Pt(II) (3R,4R)-3-((R)-1-*t*butyldimethylsilyloxy ethyl)-4-((1-pyridin-4-yl-2,5,8,11-tetraoxatridecan-13-yl)oxy)azetidin-2-one (**8**)



In a 25mL two-neck flask with a Liebig condenser under a nitrogen atmosphere, to a stirred solution of **7** (44mg, 0.086mmol) in chloroform (7.2mL), 30mg (0.086mmol) of 2,6-bis[3-(trifluoromethyl)-1*H*-1,2,4-triazol-5-yl] pyridine, 36mg (0.086mmol) of $PtCl_2$·$(DMSO)_2$ and *N*-ethyl-*N*,*N*-diisopropylamine (36µL, 0.206mmol) were added. The mixture was heated at 50°C overnight and then analyzed by TLC (EtOAc/acetone 8/2). Direct evaporation of the reaction solvent afforded a crude product that was purified by flash-chromatography (EtOAc/acetone 95/5) to obtained **8** in discrete yield (54mg, 59%) as a yellow to orange luminescent waxy solid.

$[\alpha]_D$ = -0.4 (c = 0.91, THF)

^{1}H NMR (400MHz, $CDCl_3$): δ = -0.01 (s, 3H, MeSi), 0.00 (s, 3H, MeSi), 0.80 (s, 9H, *t*BuSi), 1.17 (d, J = 6.4Hz, 3H, Me), 2.88 (d, J = 4.4Hz, 1H, C*H*CONH), 3.46-3.85 (m, 16H, OCH_2CH_2O), 4.02-4.14 (m, 1H, C*H*OTBS), 4.68 (s, 2H, OCH_2Ar), 4.91 (s, 1H, CHNH), 7.46 (s, 1H, NH), 7.53 (d, J = 6.0Hz, 2H, Ar), 7.72 (d, J = 8.0Hz, 2H, Ar), 7.95 (t, J = 8.0Hz, 1H, Ar), 9.48 (d, J = 6.0Hz, 2H, Ar) ppm
^{13}C NMR (50.3 MHz, CDCl3): δ = -3.7, -3.6, 14.0, 22.6, 23.8, 25.6, 61.7, 66.3, 66.9, 67.4, 67.6, 70.4, 70.7, 70.8, 72.5, 82.5, 118.0, 120.8 (q, $^{1}J_{C-F}$ = 265Hz), 123.7, 142.9, 148.4, 152.6, 152.7 (q, $^{2}J_{C-F}$ = 40Hz), 152.8, 160.9, 163.5 ppm
^{19}F NMR (400MHz, $CDCl_3$): δ = -64.2 ppm
MS (ESI^+): m/z = 1077 $[M+Na]^+$
$C_{36}H_{47}F_6N_9O_7PtSi$ (1054.99): calc C 40.99, H 4.49, N 11.95; found C 40.88, H 4.20, N 12.30

7. ASYMMETRIC STRECKER REACTION

7.1 α-aminonitriles Strecker synthesis

7.1.1 α-aminonitriles Strecker synthesis using acetone-cyanohydrin

α-Aminonitrile compounds play a significant role in organic chemistry. Because of their bifunctional nature, they are versatile intermediates for the synthesis of natural and bioactive compounds, and they can be precursors to important building blocks such as naturally and non-naturally occurring α-amino acids.[141] One of the earliest procedures to obtain α-aminonitriles was the Strecker reaction, a three-component reaction between a carbonyl compound, an amine, and cyanide ion.[142] Spurred on by the ever-increasing demand for non-natural enantioenriched α-amino acids for proteomic sciences and medicinal chemistry, one of the hottest topics in recent years has been the asymmetric Strecker reaction leading to enantiopure α-aminonitriles, precursors of enantiopure α-
amino acids,[143] amines, and amine derivatives. In general, two approaches have been used to achieve successful asymmetric Strecker reactions: the addition of cyanide to chiral non-racemic imines, and the catalytic enantioselective cyanation of achiral imines.[144] As an example, we dealt some years ago with diastereoselective Strecker reactions in a study on the addition of trimethylsilyl cyanide (TMSCN) to chiral imines, where the source of chirality was the *O*protected (2*S*)-lactic aldehyde. [145] The optimal reaction conditions (Lewis acid catalysts and organic solvent)[146] varied for imines with different *N*-protecting groups. Interestingly, we observed that a preformed *N*-trimethylsilylimine was capable of reacting with TMSCN in dichloromethane without any catalyst to give chiral *N*-unprotected α-aminonitriles in good yields and with good diastereoselectivity. The first example of a diastereoselective Strecker reaction assisted by a chiral auxiliary on the amine component was reported by Harada, who used (*S*)-1-phenyl-ethylamine as the chiral auxiliary to obtain chiral (*S*)-α-amino acids after acid hydrolysis of the nitrile and cleavage and recovery of the *N*-alkyl group.[147] As a result of this approach, extensive studies involving other chiral amines, including amino acids, amino amides, amino alcohols, sulfinamides, glycosylamines, hydrazines, and others, were carried out with good results in terms of yields and diastereoselectivity. A diastereoselective Strecker reaction leading to enantiopure α-aryl glycines was recently reported by James and coworkers.[148] Recently, we reported a simple, convenient, and practical method for the synthesis of α-aminonitriles through a catalyst-free Strecker reaction of a carbonyl compound, amine, and acetone cyanohydrin in water.[149] We used acetone cyanohydrin as the cyanide source, exploiting its "in situ" dissociation into HCN and acetone. In water, the dissociation process was quite slow, but the addition of a small amount of basic compounds dramatically increased the decomposition rate. The one-pot reactions proceeded very efficiently without any catalyst at room temperature and with high chemoselectivity. α-Aminonitriles were generally obtained in good to excellent yields and, it is noteworthy that in some cases, the expected pure α-aminonitrile directly separated from

water. The next aim of the project is now to extend the protocol to the asymmetric variant. In this paper, we report the synthesis of chiral α-aminonitriles obtained by a diastereoselective Strecker reaction in water using enantiopure amines. We screened primary and secondary amines derived from L-proline, L-phenylglycine, L-phenylalanine, and L-tryptophan in a one-pot procedure with aldehydes and acetone cyanohydrin in water and obtained α-aminonitriles incorporating the chiral amine skeleton. We investigated the diastereoselectivity and the configurational stabilities of the new chiral α-aminonitriles. Furthermore, we evaluated the l-proline-derived aminonitriles as chiral precursors of unusual α-amino acids and their derivatives. As there are still only a few reports[150] dealing with the asymmetric Strecker reaction with acetone cyanohydrin as the cyanide source, a contribution to this area was highly desirable.

7.1.2 Primary chiral amines

The study of the asymmetric variant of the one-pot Strecker reaction in water using acetone cyanohydrin as the cyanide source started with some enantiopure primary amines in combination with achiral aromatic or aliphatic aldehydes (Table 7.1.1, [a]: combined yields for both isomers; [b]: amines used as hydrochloride and treated in situ with 1eq. of Et_3N). As model aldehydes, benzaldehyde (**1**), butanal (**2**), isobutyraldehyde (**3**), and pivaldehyde (**4**) were evaluated. As chiral amines, we tested the Harada's source of chirality (*S*)-1-phenylethylamine (**5**), β-aminols, β-aminoethers, such as (*S*)-2-amino-2-phenylethanol (**6**), (*S*)-2-methoxy-1-phenylethanamine (**7**), (*S*)-2-amino-3-phenylpropan-1-ol (**10**), (*S*)-2-amino-3-(1*H*-indol-2-yl)propan-1-ol (**11**), and methyl esters of L-phenylglycine (i.e., **8**), of L-phenylalanine (i.e., **9**), and of l-tryptophan (i.e., **12**). Free α-amino acids were also tested, but they did not give results. The experimental protocol consisted of pre-mixing the aldehyde and the chiral amine in a closed vial without any solvent at room temperature, followed by the addition of water and of one equivalent of acetone cyanohydrin, consecutively. The reaction mixture was subjected to orbital shaking overnight at room temperature. The reactions proceeded smoothly, and gave the products in good to excellent yields.

The best results in terms of yield were obtained with amine **5** and β-aminols **6** and **10**, which gave the aminonitriles in almost quantitative yields. Using amino acid methyl esters **8**, **9**, and **12**, the yields were lower, and we could detect in the crude mixture the presence of by-products such as aldehyde cyanohydrins; in these cases the isolation of the aminonitriles required solvent extraction followed by chromatographic purification.

As regards the diastereoisomeric outcome, amine **5** gave 2/1 mixtures of the two diastereoisomers **a** and **b** (Table 7.1.1, entries 1-4, products **13**-**16**). Aminonitriles **13**,[151] **14**,[152] **15**[153] and **16**[154] are known compounds, and the absolute configuration of the major diastereoisomers **13a**-**16a** were assigned as (*S*,*S*; i.e., 1,3-"syn"), by comparison with literature ^{1}H NMR spectroscopic data.

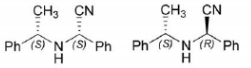
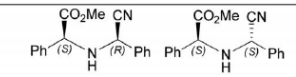
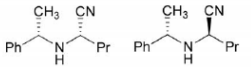
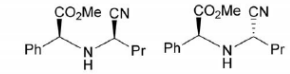
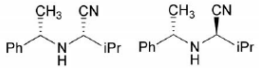
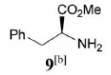
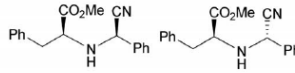
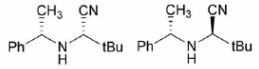
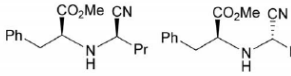
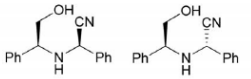
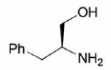
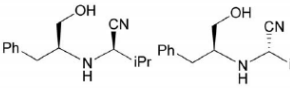
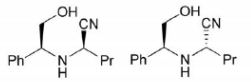
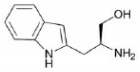
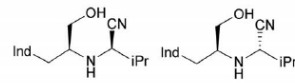
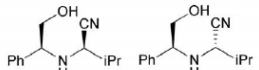
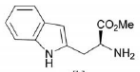
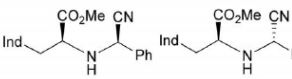
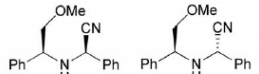
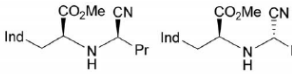
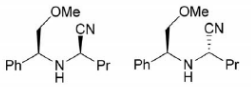
Entry	Ald., R^1	Amine	Products	Y %[a]	*dr* (**a**/**b**)	Entry	Ald., R^1	Amine	Products	Y %[a]	*dr* (**a**/**b**)
1	**1**, Ph	 **5**	 **13a** **13b**	99	69:31	10	**1**, Ph	 **8**[b]	 **22a** **22b**	73	68:32
2	**2**, Pr	**5**	 **14a** **14b**	99	74:26	11	**2**, Pr	**8**[b]	 **23a** **23b**	41	77:23
3	**3**, *i*Pr	**5**	 **15a** **15b**	99	67:33	12	**1**, Ph	 **9**[b]	 **24a** **24b**	64	67:33
4	**4**, *t*Bu	**5**	 **16a** **16b**	99	65:35	13	**2**, Pr	**9**[b]	 **25a** **25b**	25	63:37
5	**1**, Ph	 **6**	 **17a** **17b**	85	81:19	14	**3**, *i*Pr	 **10**	 **26a** **26b**	80	53:47
6	**2**, Pr	**6**	 **18a** **18b**	99	80:20	15	**3**, *i*Pr	 **11**	 **27a** **27b**	62	57:43
7	**3**, *i*Pr	**6**	 **19a** **19b**	97	83:17	16	**1**, Ph	 **12**[b]	 **28a** **28b**	31	63:37
8	**1**, Ph	 **7**	 **20a** **20b**	78	64:36	17	**2**, Pr	**12**[b]	 **29a** **29b**	40	57:43
9	**2**, Pr	**7**	 **21a** **21b**	98	71:29						

Table 7.1.1 One-pot Strecker reaction in water with 1eq. of acetone cyanohydrin

(*S*)-Phenylglycinol **6** gave a general enhancement of the diastereoisomeric ratios with all the aldehydes (Table 7.1.1, entries **5-7**, compounds **17**–**19**) up to a value of 83:17, which was obtained with compounds **19a-b**. The absolute configurations of the major diastereoisomers **17a** and **19a** were assigned as (*S*,*R*) by comparison with the ^{1}H NMR spectroscopic data for the same compounds reported in the literature.[155] For compounds **18a-b**, the configuration was assigned by comparison with 1H NMR spectra of aminonitriles **17a-b** and **19a-b**. In particular, we observed a constant and similar trend in the chemical shift of the proton on the carbon α to the CN group. The C*H*CN signal in the major diastereoisomers (i.e., **17a** and **19a**) was more shielded than the corresponding signal in the minor diastereoisomers (i.e., **17b** and **19b**). The same trend was observed for 18a and 18b, and this allowed an assignment of the configuration. Notwithstanding the difference in stereochemical descriptors of the major diastereoisomers, i.e., (*S*,*S*) for aminonitriles **13a**-**16a**, and (S,R) for **17a**-**19a**, the stereoinduction exerted by amine **6** was of the same sense as for Harada's amine **5**, giving predominantly the 1,3-"syn" stereoisomers in all cases.

The key role of the OH group in L-phenylalaninol (**6**) as an enhancer of dr was confirmed by using the corresponding *O*-methyl ether (i.e., **7**) as starting amine. In this case, lower dr values for the product aminonitriles (i.e., **20a-b** and **21a-b**) were obtained: 64:36 and 71:29, respectively. The configurations of

20a and 21a were assigned as (*S*,*R*) on the basis of the same trend of C*H*CN chemical shifts observed for compounds **17** and **19**, as discussed above. To confirm the assignment, compound **17a** was directly transformed into **20a** through simple *O*-methylation (see Supporting Information for experimental details and NMR spectroscopic data).

When methyl esters of amino acids were used as sources of chirality (Table 7.1.1, entries 10-13 and 16-17), the diastereoisomeric ratios were not outstanding, and a highest dr value of 77/23 was seen for **23a-b**, obtained from l-phenylglycine methyl ester (**8**) and butanal (**2**). The configuration of compounds **22a-b** was established to be (*S*,*R*) by chemical transformation of a 2:1 mixture of **22a**/**22b** into **17a**/**17b** by reducing the methyl ester with $NaBH_4$. The configurations of aminonitriles **23a-b**, **24a-b**, **25a-b**, **26a-b**, and **27a-b** were assigned on the basis of the ^{1}H NMR chemical shift trends of the whole series. Aminonitriles **28a-b** are known compounds, and their configurations were assigned by comparison with NMR literature data.[156] Whenever the chiral amine was more flexible on the β-carbon, as is the case for amines **10** and **11**, the diastereoselectivity dropped completely (Table 7.1.1, entries 12 and 17). α-Substituents on the aldehyde seem to have little influence on diastereoisomeric ratio, with a slight increase of dr being seen for aliphatic and α-branched aldehydes compared to benzaldehyde (Table 7.1.1, entries 2, 7, 9 and 11), with a few exceptions (Table 7.1.1, entries 2 and 13).

7.1.3 Secondary chiral amines

One major feature of our Strecker protocol is that it works well with secondary amines. Thus, we decided to test secondary chiral amines, including L-proline esters **30**– **32**, L-prolinol derivatives **33**-**34**, the (4*R*)-hydroxy-L-proline ester **35**, and (4*R*)-hydroxy-L-prolinol (**36**), with **1** and **2** as model aldehydes (Table 8.1.2, [a]: combined yields for both isomers; [b]: amines used as hydrochloride and treated in situ with 1eq. of Et_3N). The reaction of diphenylprolinol (**34**) with butanal (**2**) gave the corresponding aminonitriles (i.e., **45a-b**) in quantitative yield, and in all other cases, variable amounts of aldehyde cyanohydrins and starting materials could be detected in the crude reaction mixtures, and the products had to be purified by flash chromatography, thus lowering the yields. When L-proline esters **30**, **31**, and **32** were used as the sources of chirality, quite similar diastereoisomeric ratios were observed (average value 75:25), irrespective of the nature of the ester group (methyl, *t*butyl, or benzyl) or the aldehyde used (Table 8.1.2, entries 1-5, 10 and 11). Better diastereoselectivity were obtained with (*S*)-prolinol (**33**), which gave **42** and **43** with a *dr* of 82:18 using benzaldehyde or butanal (Table 2, entries 6 and 7). (*S*)-α,α-Diphenylprolinol **34** gave the best result, generating a single diastereoisomer with either benzaldehyde (**1**) or butanal (**2**) (Table 7.1.2, entries 8 and 9). Notably, the enantiomerically pure aminonitrile (i.e., **45a**) was obtained directly from the crude material in quantitative yield without any purification.

The absolute configuration of major or exclusive diastereoisomers of the proline-derived α-aminonitriles were assigned as (*S*,*S*) by X-ray single crystal analysis and ^{1}H NMR spectroscopy.

Entry	R^1	Amine	Products	Y %[a]	*dr* (a/b)	Entry	R^1	Amine	Products	Y %[a]	*dr* (a/b)
1	**1**, Ph	**30**[b]	**37a** + **37b**	50	75:25	8	**1**, Ph	**34**	**44a** + **44b**	50	>99:1
2	**2**, Pr	**30** [b]	**38a** + **38b**	50	75:25	9	**2**, Pr	**34**	**45a** + **45b**	97	>99:1
3	**1**, Ph	**31**	**39a** + **39b**	33	75:25	10	**1**, Ph	**35**[b]	**46a** + **46b**	58	66:34
4	**2**, Pr	**31**	**40a** + **40b**	34	75:25	11	**2**, Pr	**35**[b]	**47a** + **47b**	69	71:29
5	**1**, Ph	**32** [b]	**41a** + **41b**	53	75:25	12	**1**, Ph	**36**	**48a** + **48b**	45	86:14
6	**1**, Ph	**33**	**42a** + **42b**	35	82:18	13	**2**, Pr	**36**	**49a** + **49b**	46	86:14
7	**2**, Pr	**33**	**43a** + **43b**	31	82:18						

Table 7.1.2 One-pot Strecker reaction in water with 1eq. of acetone cyanohydrin

Products **37**–**43** and **46**–**49** showed the same trend in the ^{1}H NMR chemical shift of the C*H*CN proton, with the proton of the major diastereoisomer always more deshielded than that of the minor diastereoisomer for the whole series of compounds. Compound **45a** was analyzed by single-crystal X-ray diffraction, and the absolute structure is shown in Figure 7.1.1. To assign the absolute configuration of compound **46a**, we had to transform it into iodo derivative **50a** by a

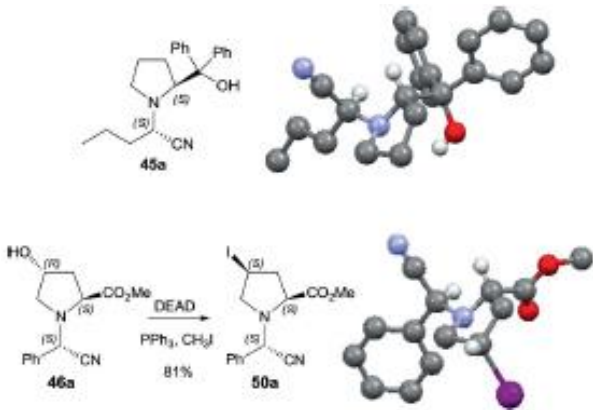


Figure 7.1.1 X-ray structures for compounds **45a** and **50a**

Mitsunobu reaction,[157] thus obtaining a crystalline product suitable for Xray diffraction analysis (Figure 7.1.1). The X-ray diffraction data of the two structures unequivocally confirmed the *S* configurations of the newly-formed stereocenters.

The stereochemical assignment using 1H NMR signal trends was confirmed by several chemical transformations. Thus, compound **46a** was transformed into **48a** by methylester reduction with $NaBH_4$, and in the same way, **47a** was transformed into **49a**, and **37a** into **42a**. Finally, compound **37a** was converted into **44a** by Grignard addition.

For the one-pot Strecker reaction, two mechanisms have been proposed: one going via an imine (or iminium ion) intermediate, the other via an aminol intermediate.[158] The imine route is more plausible in those cases where the Strecker reaction is carried out in organic solvents and in the presence of a dehydrating agent that could force the elimination of water. However, Tanaka et al. demonstrated that imines could be efficiently formed also in water by mixing aromatic aldehydes and amines; the Schiff bases separated from the aqueous medium as crystalline products in structure of the amine on the formation of iminium intermediates with aldehydes in water showed that aminol and iminium species were intermediates with high barriers, especially for secondary cyclic amines such as proline methyl ester and pyrrolidine.[159]

In case of L-prolinols, because of the presence of a primary alcohol in the side chain, the formation of bicyclic 1,3-oxazolidines, as well as imine/imonium and aminol intermediates, could be possible (Figure 7.1.2).[160]

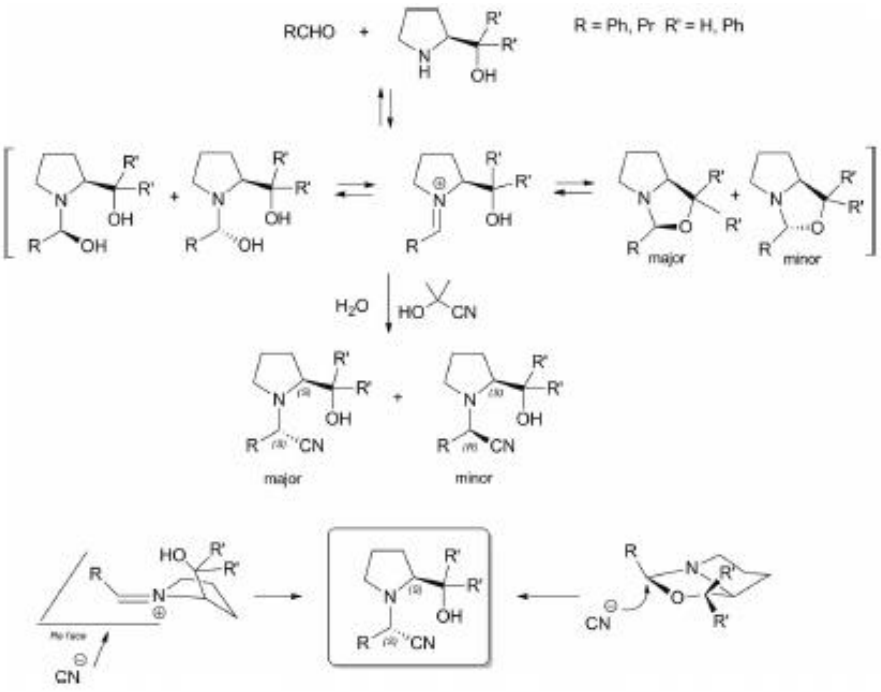
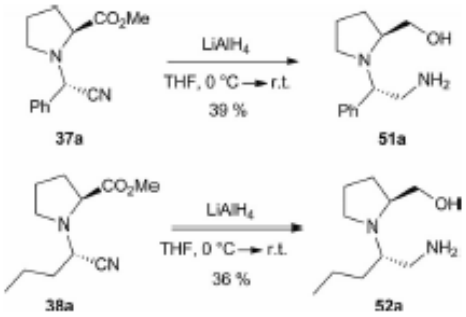


Figure 7.1.2 Reaction intermediates and models for the asymmetric induction

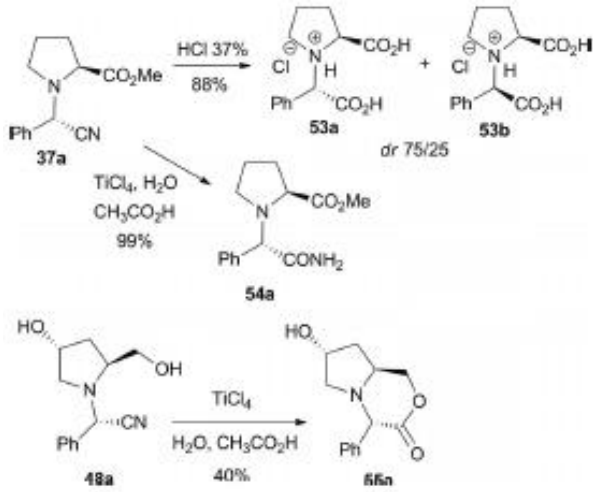
To obtain some evidence about the intermediates involved in our catalyst-free one-pot reaction in water, NMR analysis was performed with benzaldehyde (**1**), butanal (**2**), L-prolinol (**33**), and L-diphenylprolinol

(**34**). An equimolar mixture of the aldehyde and the amine was pre-mixed at room temperature, and aliquots were diluted in NMR tubes in two solvents, D_2O or $CDCl_3$, and quickly analyzed. We obtained resolvable spectra that indicated the presence of several species in solution (see Supporting Information for details and spectra). When L-prolinol (**33**) was mixed with benzaldehyde (**1**) in $CDCl_3$ and D_2O, two new distinct species with specific signals in the range 5.4-5.5ppm were observed, together with some remaining starting materials. The spectra of butanal (**2**) and prolinol (**33**) showed similar species with signals at 4.0-4.5ppm; in D_2O, traces of the aldehyde hydrate and an imonium species appeared. Upon addition of acetone cyanohydrin, the resonances of the two new species disappeared, and those of the corresponding aminonitriles appeared. The rapid consumption of the intermediate species agreed with our previously reported observation that acetone cyanohydrin decomposes quickly in H_2O in the presence of traces of amine or imine species to give acetone and HCN. Careful analysis of ^1H and ^{13}C NMR spectra and comparison with authentic spectra of 1,3-oxazolidines reported in the literature[161] allowed us to assign a bicyclic 1,3-oxazolidine structure to the two diastereoisomeric intermediates. However, the diastereoisomeric ratio of the 1,3-oxazolidines (7/3) observed in the NMR analysis did not strictly correspond to the *dr* of the final aminonitrile products (for instance **42a**/**42b** 82:18, see Table 7.1.2), because of epimerization phenomena (see below). Interestingly, the ^1H NMR analysis of the reaction of diphenylprolinol (**34**) with aldehydes **1** or **2** in $CDCl_3$ revealed, as well as large amounts of starting materials remaining, the presence of only one isomer of 1,3-oxazolidine,[162] and this corresponds to the fact that a single diastereoisomer of aminonitrile was obtained in the Strecker reaction with both aldehydes (Table 7.1.2, entries 8 and 9). The presence of bicyclic intermediates could provide indirect evidence for a high reactivity of the iminium species, which presumably have a very short lifetime in aqueous solution and undergo a rapid intramolecular cyclization. However, despite the presence of several reactive intermediates, upon the addition of acetone cyanohydrin to the aqueous solution, all of the productive equilibria shifted to form the aminonitrile products. As a tentative model for the asymmetric induction exerted by the l-prolinols, the predominant or exclusive formation of (*S*,*S*)-aminonitriles could derive from addition to the *Re* face of the iminium, or from bimolecular nucleophilic substitution from the bottom face of the 1,3-oxazolidine (Figure 7.1.2).All of the proline-derived α-aminonitriles were new compounds, and bearing in mind the importance and the utility of proline derivatives in organocatalysis and metal catalysis, the preservation of l-proline and (4*R*)-4-hydroxy-(*S*)-proline scaffolds in the target molecules allowed the formation of very interesting bifunctional compounds suitable for the design and synthesis of new chiral ligands. Thus, we investigated the reactivity of some of the proline-derived aminonitriles in reduction and hydrolysis, just to explore their transformation into new chiral aminols and amino acids. We treated aminonitriles **37a** and **38a** with $LiAlH_4$, and obtained chiral 1,5-aminols **51a** and **52a**, respectively, by concomitant reduction of the ester and nitrile groups (Scheme 7.1.1).



Scheme 7.1.1 Reduction of some chiral α-aminonitriles

According to the literature, hydrolysis of the nitrile group should be a routine transformation. We found, however, that the hydrolysis of pure aminonitrile **37a** occurred under drastic conditions with an excess of aqueous HCl (37%) for 24h at reflux, and gave a 75/25 mixture of two diastereoisomers **53a** and **53b** in 88% total yield (Scheme 7.1.2). To avoid the epimerization, we tried several milder conditions reported in the literature, such as HCl (5% in dioxane),[163] sulfuric acid in DCM at 0°C, or $NaHCO_3$ (1M aqueous).[164] None of these reactions resulted in the formation of dicarboxylic acids, only epimerization of the starting aminonitriles and retro-Strecker products were observed.



Scheme 7.1.2 Hydrolysis of some chiral α-aminonitriles

Even alcoholysis procedures with HCl in methanol designed to transform the cyanide group into a methyl ester[165] did not succeed. On the other hand, good results were obtained in the hydrolysis of the nitrile to an amide. Using a stoichiometric amount of $TiCl_4$ in acetic acid and 4eq. of water,[166] pure **37a** was converted into amide **54a** in quantitative yield without any epimerization. It is interesting that when the same procedure was used with 4-hydroxy-prolinol **48a**, bicyclic lactone **55a** was formed, due to an intramolecular cyclization. This result on the epimerization phenomena in the hydrolysis reaction, as well as the observation of unexpected spontaneous epimerization in stored samples, prompted us to face the problem of configurational stability of α-aminonitriles. For example, we observed that isolated minor diastereoisomer **37b** partially converted into the major diastereoisomer (i.e., **37a**) over time, whereas

major diastereoisomer **37a** did not epimerize. Also, in a 86/14 mixture of compounds **48a**–**b**, minor diastereoisomer **48b** completely isomerized into **48a** within a month. The epimerization of aryl-α-aminonitriles has already been reported in the literature. Sakurai, for instance, observed isomerization of diastereoisomeric α-aryl aminonitriles when the compounds were dissolved in DMSO, or after heating.[167] We so prepared DMSO-d_6 solutions of isolated pure diastereoisomers **37a** and **37b** and we heated them at 80°C for 24h. ^{1}H NMR spectra of both of the samples showed the formation of traces of benzaldehyde and L-proline methyl ester, and also of epimerization products with a final diastereoisomeric ratio of 68/32 (**37a**/**37b**) starting from **37a**, and 65/35 (**37a**/**37b**) starting from **37b**. Moreover, the two separated diastereoisomeric aminonitriles, **46a** and **46b**, after being heated without solvent for 24h at 80°C, gave identical ^{1}H NMR spectra recorded in $CDCl_3$ that showed epimerization products in both samples with a convergent diastereoisomeric ratio **46a**/**46b** of 72/28. Even in those cases, we observed traces of starting materials, benzaldehyde and *trans* 4-hydroxy-(*S*)-proline methyl ester, together with two further diastereoisomers derived from the partial racemization (about 15%) of the carbon α to the ester group. In the case of butanal derivatives **47a**-**b**, the same experiment gave a complex product mixture, and the spectra were not resolvable. The presence of starting materials, and the observation that final diastereoisomeric ratios obtained in the isomerization experiments were consistent with the diastereoisomeric ratios obtained in the Strecker protocol (Table 7.1.2, entries 1 and 10), suggest that epimerization takes place by a retro-Strecker dehydrocyanation/cyanation mechanism.

7.2 Experimental section

7.2.1 General informations

As previously reported (Paragraph 2.3.1).

7.2.2 Crystal data

Single-crystal X-ray diffraction data were collected for **45a** and **50a** at r.t. with an Oxford Diffraction Xcalibur diffractometer equipped with a Mo-K_α radiation source (λ = 0.71073Å) and a Sapphire3 CCD detector. The structure was solved using SHELXS-97 (others) or SIR-92 (iodioderiv) programs; SHELXL-97 was used for structural refinement.[168]

7.2.3 Synthesis

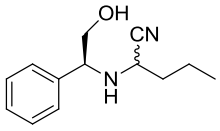
Amines **5**, **6**, **8**, **9**, **10**, **12**, **30**, **31**, **32**, **33**, **34** and **35** were purchased from Sigma-Aldrich and used without purification, Amines **7**,[169] **11**[170] and **36**[171] were synthesized following published procedures. Compounds

13, **14**, **15**, **16**, **17**, **19** and **28** were known, and after the synthesis gave spectroscopic data consistent with the published data.

General Procedure for α-aminonitriles 13-29 and 37-49

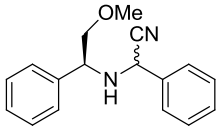
The chosen aldehyde (1mmol) and amine (1mmol) were mixed in an orbital shaker at room temp. in a 5mL vial equipped with a screw cap. After 10min, water (4mL) and acetone cyanohydrin (1mmol) were added, and the cap was closed. If the amine was used as its hydrochloride, triethylamine (1mmol) was added prior to the addition of the aldehyde. The mixture was stirred in an orbital shaker for 20h. The reaction mixture was poured into brine (5mL) and extracted with DCM (2x10mL), then the combined extracts were dried with Na_2SO_4 and concentrated. If necessary, the products were purified by flash chromatography. Diastereoisomeric ratios were determined from ^{1}H NMR spectra or HPLC data of the crude material.

2-(2-Hydroxy-1-phenylethyl)amino pentanenitrile (**18**)



The major diastereoisomer was separated by flash chromatography, the minor isomer was characterized in the crude mixture.
Data for major isomer **18a** (2*R*,2*S*): yellow syrup
$[\alpha]_D$ = +157.37° (c = 0.64, DCM)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.92 (t, J = 7.2Hz, 3H, Me), 1.48-1.58 (m, 2H, $CH_3CH_2CH_2$), 1.74-1.79 (dt, J = 7.2, 7.2Hz, 2H, $CH_3CH_2CH_2$), 2.34 (bs, 2H, OH and NH), 3.30 (t, J = 7.2 Hz, 1H, CHCN), 3.61 (dd, J = 9.6, 11.2Hz, 1H, CHOH), 3.80 (dd, J = 4.0, 11.2 Hz, 1H, CHOH), 4.13 (dd, J = 4.0, 9.6Hz, 1H, CHPh), 7.28-7.39 (m, 5H, Arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 18.9, 35.6, 47.7, 63.1, 67.2, 120.1, 127.7, 128.3, 128.8, 138.1 ppm
IR: ṽ = 3415, 3325, 2962, 2932, 2227, 1065 cm^{-1}
$C_{13}H_{18}N_2O$ (218.29): calc C 71.53, H 8.31, N 12.83; found C 71.99, H 8.45, N 12.72
HPLC-MS: Rt = 7.35min; *m*/*z* = 219 $[M+H]^+$, 241 $[M+Na]^+$.
Data for minor isomer **18b** (2*S*,2*S*): yellow syrup
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.95 (t, J = 7.2Hz, 3H, Me), 1.48-1.58 (m, 2H, $CH_3CH_2CH_2$), 1.69-1.78 (m, 2H, $CH_3CH_2CH_2$), 2.46 (bs, 2H, OH and NH), 3.55-3.88 (m, 3H, CHCN, CH_2OH), 3.99 (dd, J = 4.4, 7.6Hz, 1H, CHPh), 7.28-7.39 (m, 5H, arom) ppm
HPLC-MS: Rt = 6.51min; *m*/*z* = 219 $[M+H]^+$, 241 $[M+Na]^+$

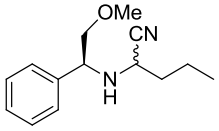
2-[(2-Methoxy-1-phenylethyl)amino]-2-phenylacetonitrile (**20**)



Characterized as a diastereoisomeric mixture, signals refer to the major diastereoisomer, only the main signals of the minor diastereoisomer are reported.
Data for major isomer **20a** (2*R*,2*S*) and minor isomer **20b** (2*S*,2*S*): orange oil.
^{1}H NMR (400MHz, $CDCl_3$): δ = 3.33 (s, 3H, OCMe minor), 3.40 (s, 3H, OMe), 3.46-3.54 (m, 2H, CH_2OMe), 3.96 (dd, J = 3.2, 8.8Hz, 1H, PhC*H*CH_2O minor), 4.40 (dd, J = 5.6, 8.0Hz, 1H, PhC*H*CH_2O), 4.49 (s, 1H, CHCN), 4.71 (s, 1H, CHCN minor), 7.27-7.43 (m, 6H, arom), 7.50-7.54 (m, 4H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 51.8, 58.6, 60.6, 76.9, 118.6, 127.2, 127.8, 128.4, 128.7, 128.8, 128.9, 135.1, 138.1 ppm
IR: ṽ = 3325, 2890, 2228, 1108 cm^{-1}

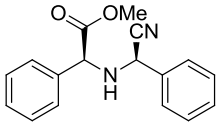
HPLC-MS: Rt = 9.68min, minor isomer, *m*/*z* = 240 $[M\text{-}CN]^+$, 267 $[M+H]^+$; Rt = 10.19min, major isomer, *m*/*z* = 240 $[M\text{-}CN]^+$, 267 $[M+H]^+$
Compound **20a** was also obtained starting from **17a**.

2-[(2-Methoxy-1-phenylethyl)amino]pentanenitrile (**21**)



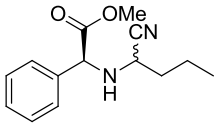
Characterized as a diastereoisomeric mixture, signals refer to the major diastereoisomer.
For the minor diastereoisomer only the main signals of are reported.
Data for major isomer **21a** (2*R*,2*S*) and minor isomer **21b** (2*S*,2*S*): orange oil.
^{1}H NMR (400MHz, $CDCl^3$): δ = 0.91 (t, J = 7.2Hz, 3H, Me), 0.93 (t, J = 7.2Hz, 3H, Me minor), 1.49-1.55 (m, 2H, $CH_3CH_2CH_2$), 1.63-1.69 (m, 2H, CH_3CH_2 CH_2, minor), 1.71-1.78 (m, 2 H, $CH_3CH_2CH_2$), 3.25 (t, J = 7.2Hz, 1H, CHCN), 3.36–3.44 (m, 2H, C*H*2OMe), 3.41 (s, 3H, OMe), 3.76 (t, J = 6.8Hz, 1H, CHCN), 4.13 (dd, J = 3.6, 7.6Hz, 1H, NHC*H*Ph), 4.25 (dd, J = 5.2, 8.0Hz, 1H, NHC*H*Ph), 7.28-7.39 (m, 5H, Ar) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.5, 18.9, 35.8, 47.5, 58.6, 60.2, 77.0, 120.3, 127.7, 128.2, 128.7, 138.4 ppm
IR: ṽ = 3326, 2962, 2930, 2225, 1689, 1455, 1118 cm^{-1}
HPLC-MS: Rt = 9.13min, minor isomer; *m*/*z* = 233 $[M+H]^+$, 438 $[2M\text{-}CN]^+$;
Rt = 9.68min, major isomer; *m*/*z* = 233 $[M+H]^+$, 438 $[2M\text{-}CN]^+$

Methyl 2-((Cyano-phenyl-methyl)amino)-2-phenylacetate (**22a**)



Characterized as a diastereoisomeric mixture, yellow oil.
Data for major isomer **22a** (2*R*,*S*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.78 (dd, J = 3.6, 10.4Hz, 1 H, NH), 3.71 (s, 3H, Me), 4.50 (d, J = 10.4Hz, 1H, PhC*H*CO_2), 4.77 (d, J = 3.6 Hz, 1 H, PhC*H*CN), 7.35-7.55 (m, 5H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 51.7, 52.7, 63.2, 118.2, 127.3, 128.1, 128.8, 128.9, 129.0, 129.2, 134.2, 136.1, 171.7 ppm
IR: ṽ = 3327, 2252, 1739, 1453 cm^{-1}
HPLC-MS: Rt = 9.00min; *m*/*z* = 254 $[M\text{-}CN]^+$, 276 $[M\text{-}HCN+Na]^+$, 281 $[M+H]^+$, 303 $[M+Na]^+$, 319 $[M+K]^+$
Data for minor isomer **22b** (2*S*,*S*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.54 (dd, J = 6.4, 6.8Hz, 1H, NH), 3.74 (s, 3H, Me), 4.62 (d, J = 6.8Hz, 1H, PhC*H*CO_2), 4.87 (d, J = 6.8Hz, 1H, C*H*CN), 7.35-7.55 (m, 5H, arom) ppm
IR: ṽ = 3327, 2252, 1453 cm^{-1}
HPLC-MS: Rt = 8.83min; *m*/*z* = 254 $[M\text{-}CN]^+$, 276 $[M\text{-}HCN+Na]^+$, 281 $[M+H]^+$, 303 $[M+Na]^+$, 319 $[M+K]^+$

Methyl 2-(1-cyanobutylamino)-2-phenylacetate (**23**)



Characterized as a diastereoisomeric mixture, yellow oil.
Data for major isomer **23a** (2*R*,*S*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.91 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$), 1.47-1.62 (m, 2H, $CH_3CH_2CH_2$), 1.74-1.80 (m, 2H, $CH_3CH_2CH_2$), 2.45 (d, J = 11.6Hz, 1H, NH), 3.25 (dt, J = 6.8, 11.6Hz, 1H, C*H*CN), 3.71 (s, 3H, CO_2CH_3), 4.64 (s, 1H, CHPh), 7.33-7.43 (m, 5H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.3, 18.8, 35.4, 47.4, 52.7, 63.2, 119.7, 128.1, 128.8, 128.9, 137.1, 171.9 ppm
IR: ṽ = 3331, 3031, 2960, 2875, 2253, 2226, 1739, 1455, 1436 cm^{-1}
HPLC-MS: Rt = 8.62min; *m*/*z* = 220 [M-CN]+, 247 $[M+1]^+$, 269 $[M+Na]^+$, 285 $[M+K]^+$.

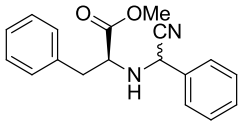
Data for minor isomer **23b** (2*R*,*S*)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.97 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$), 1.47-1.62 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.74-1.80 (m, 2H, CH$_3$CH$_2$C*H*$_2$), 2.04 (bs, 1 H, NH minor), 3.72 (s, 3H, CO_2CH_3), 3.68-3.76 (m, 1H, C*H*CN), 4.65 (s, 1H, PhCH), 7.33-7.43 (m, 5H, arom) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 18.8, 35.5, 48.8, 52.7, 63.3, 119.8, 127.6, 128.6, 128.7, 136.3, 172.3 ppm

HPLC-MS: Rt = 8.32min; *m*/*z* = 220 $[M-CN]^+$, 247 $[M+H]^+$, 269 $[M+Na]^+$, 285 $[M+K]^+$

Methyl 2-((cyano-phenylmethyl)amino)-3-phenylpropanoate (**24**)



Characterized as a diastereoisomeric mixture, pale yellow oil.

HPLC-MS: Rt = 9.40min (major+minor); *m*/*z* 268 $[M-CN]^+$, 295 $[M+H]^+$, 317 $[M+Na]^+$, 333 $[M+K]^+$.

Data for major isomer **24a** (2*R*,*S*)

^{1}H NMR (400MHz, CDCl3): δ = 2.42 (bs, 1H, NH), 2.96 (dd, J = 8.4, 13.6Hz, 1H, PhC*H*HCH), 3.12 (dd, J = 5.2, 13.6Hz, 1H, PhCH*H*CH), 3.70 (dd, J = 5.2, 8.4Hz, 1H, C*H*CH$_2$Ph), 3.75 (s, 3H, Me), 4.58 (s, 1H, C*H*CN), 7.19-7.56 (m, 10H, arom) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 39.7, 52.2, 52.9, 60.4, 118.6, 126.9, 127.4, 128.5, 128.9, 129.1, 129.3, 134.3, 136.8, 173.6 ppm

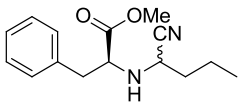
IR: ṽ = 3328, 2952, 2925, 2293, 2229, 1736 cm^{-1}.

Data for minor isomer **24b** (2*S*,*S*)

^{1}H NMR (400MHz, $CDCl_3$): δ = 2.10 (br. s, 1H, NH), 2.93-3.00 (m, 1 H, PhC*H*H), 3.14 (dd, J = 5.6, 14.0Hz, 1H, PhCH*H*), 3.79 (s, 3H, Me), 3.88 (dd, J = 5.6, 8.0Hz, 1 H, C*H*CH$_2$), 4.88 (s, 1H, C*H*CN), 7.19-7.56 (m, 10 H, arom) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 39.4, 52.1, 53.3, 59.6, 118.4, 127.1, 127.5, 128.6, 129.0, 129.1, 129.2, 134.2, 136.2, 173.6 ppm

Methyl 2-(1-cyanobutylamino)-3-phenylpropanoate (**25**)



Diastereoisomers separated by flash chromatography.

Data for major isomer **25a** (1*R*,*S*): pale yellow oil

$[\alpha]_D$ = 292.6 (*c* = 1.25, $CHCl_3$)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.89 (t, J = 6.8Hz, 1H, C*H*$_3$CH$_2$CH$_2$), 1.40-1.49 (m, 2H, C*H*$_2$), 1.57–1.69 (m, 2H, C*H*$_2$), 2.03 (dd, J = 7.7, 10.4Hz, 1H, NH), 2.86 (dd, J = 7.6, 13.6Hz, 1H, CH*H*Ph), 3.02 (dd, J = 5.6, 13.6Hz, 1H, C*H*HPh), 3.27 (dt, J = 7.2, 10.4Hz, 1H, C*H*CN), 3.62 (ddd, J = 5.6, 7.6, 7.7Hz, 1H, CH$_2$C*H*NHCO$_2$Me), 3.74 (s, 3H, Me), 7.17-7.31 (m, 5H, arom) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 13.3, 18.7, 35.7, 39.8, 49.4, 52.1, 61.2, 119.9, 126.8, 128.4, 129.3, 136.9, 173.7 ppm

IR: ṽ = 3328, 3002, 2874, 2225, 1956, 1888, 1737, 1205 cm^{-1}

$C_{15}H_{20}N_2O_2$ (260.33): calc C 69.20, H 7.74, N 10.76; found C 69.25, H 7.81, N 10.68

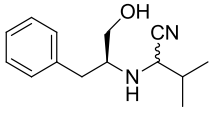
HPLC-MS: Rt = 9.16min; *m*/*z* = 261 $[M+H]^+$, 283 $[M+Na]^+$.

Data for minor isomer **25b** (1*S*,*S*)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.91 (t, J = 7.2Hz, 1H, C*H*$_3$CH$_2$CH$_2$),1.40-1.49 (m, 2H, CH$_2$), 1.57-1.69 (m, 2H, CH$_2$), 21.81 (bs, 1H, NH), 2.92 (dd, J = 8.0, 14.0Hz, 1H, CH*H*Ph), 3.12 (dd, J = 5.6, 14.0Hz, 1H, C*H*HPh), 3.70-3.76 (m, 1H, C*H*CN), 3.72 (s, 3H, Me), 3.81-3.86 (m, 1H, CH$_2$C*H*NHCO$_2$Me), 7.17-7.31 (m, 5H, arom) ppm

HPLC-MS: Rt = 8.90min; *m*/*z* = 261 $[M+H]^+$, 283 $[M+Na]^+$

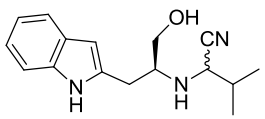
2-(1-Hydroxy-3-phenylpropan-2-yl amino)-3-methylbutanenitrile (**26**)



Characterized as a diastereoisomeric mixture enriched in the minor stereoisomer after flash chromatography, pale yellow oil.

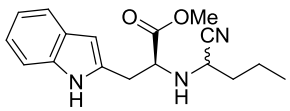
Data for major isomer **26a** (2*R*,1*S*)
^{1}H NMR (400MHz, CDCl3): δ = 0.98-1.03 [m, 6H, CH(Me)$_2$], 1.90-1.96 [m, 1H, C*H*(Me)2], 2.65 (dd, J = 7.2, 13.2Hz, 1H, CHC*H*HPh), 2.77 (dd, J = 6.0, 13.2Hz, 1H, CHCH*H*Ph), 3.09-3.16 (m, 1H, CH$_2$C*H*CH$_2$), 3.30 (d, J = 5.6 Hz, 1H, CHC*H*CN), 3.42 (dd, J = 7.2, 11.2Hz, 1H, CHC*H*HOH), 3.74 (dd, J = 4.0, 11.2Hz, 1H, CHCH*H*OH), 7.24-7.39 (m, 5H, arom) ppm
HPLC-MS: Rt = 7.22min, *m*/*z* = 206 [M-CN]$^+$, 233 [M+H]+, 255 [M+Na]$^+$.
Data for minor isomer **26b** (2*S*,1*S*)
^{1}H NMR (400MHz, CDCl$_3$): δ = 1.00 (d, J = 6.8Hz, 3 H, CH$_3$CHC*H*$_3$), 1.05 (d, J = 6.8Hz, 3H, C*H*$_3$CHCH$_3$), 1.61 (bs, 1H, OH), 1.88-1.99 [m, 1H, CHC*H*(Me)$_2$], 2.14 (bs, 1 H, NH), 2.80 (dd, J = 8.4, 13.6Hz, 1H, CHC*H*HPh), 2.94 (dd, J = 6.0, 13.6Hz, 1H, CHCH*H*Ph), 3.17-3.22 (m, 1H, CH$_2$C*H*CH$_2$), 3.43-3.47 (m, 2H, CHC*H*HOH, CHC*H*CN), 3.66 (dd, *J* = 3.2, 11.2 Hz, 1 H, CHCH*H*OH), 7.24_7.39 (m, 5 H, arom) ppm
^{13}C NMR (100MHz, CDCl$_3$): δ = 17.9, 19.1, 31.7, 38.0, 54.9, 58.1, 61.9, 119.1, 126.9, 128.8, 129.2, 137.2 ppm
IR: ṽ = 3442, 3338.7, 2963, 2929, 2227, 1120 cm^{-1}
HPLC-MS: Rt = 6.87min; *m*/*z* = 206 [M-CN]$^+$, 233 [M+H]$^+$, 255 [M+Na]$^+$

2-((1-Hydroxy-3-(1H-indol-2-yl)propan-2-yl)amino)-3-methylbutanenitrile (**27**)



Characterized as a diastereoisomeric mixture, pale yellow oil.
Data for major isomer **27a** (2*R*,1*S*)
^{1}H NMR (400MHz, CDCl$_3$): δ = 0.98-1.00 [m, 6H, CH(Me)$_2$], 1.68-2.04 (bs, 2H, OH, N*H*CHCN), 1.86-1.97 [m, 1H, CHC*H*(Me)$_2$], 3.01 (ddd, J = 0.8, 8.0, 14.4Hz, 1H, IndC*H*HCH), 3.07 (ddd, J = 0.8, 8.4, 14.4Hz, 1H, IndCH*H*CH), 3.28-3.34 (m, 1H, CH$_2$C*H*CH$_2$), 3.46-3.53 (m, 2H, CHC*H*CN, CHC*H*HOH), 3.70 (dd, J = 3.6, 10.8Hz, 1H, CHCH*H*OH), 7.06-7.25 (m, 3H, arom), 7.37-7.40 (m, 1H, arom), 7.62-7.66 (m, 1H, arom), 8.09 (bs, 1H, NHInd, minor), 8.14 (bs, 1H, NHInd) ppm
^{13}C NMR (100MHz, CDCl$_3$): δ = 17.8, 19.1, 26.8, 31.8, 55.2, 57.6, 62.4, 111.2, 111.4, 118.7, 119.5, 119.6, 122.2, 122.5, 127.4, 136.2 ppm
IR: ṽ = 3412, 3057, 2228, 1457, 1031 cm^{-1}
HPLC-MS: Rt = 7.66min; *m*/*z* = 245 [M-CN]$^+$, 294 [M+Na]$^+$
Data for minor isomer **27b** (2*S*,1*S*)
^{1}H NMR (400MHz, CDCl$_3$): δ = 0.98-1.00 [m, 6 H, CH(Me)$_2$], 1.68-2.04 (bs, 2H, OH, N*H*CHCN), 1.86-1.97 [m, 1H, CHC*H*(Me)$_2$], 2.81 (ddd, J = 0.8, 3.6, 14.4Hz, 1H, IndC*H*HCH), 2.96 (ddd, J = 0.8, 6.0, 14.4Hz, 1H, IndCH*H*CH), 3.23-3.29 (m, 1H, CH$_2$C*H*CH$_2$), 3.46-3.53 (m, 2H, CHC*H*CN, CHC*H*HOH), 3.77 (dd, J = 3.6, 10.8Hz, 1H, CHCH*H*OH), 7.06-7.25 (m, 3H, arom), 7.37-7.40 (m, 1H, arom), 7.62-7.66 (m, 1H, arom), 8.09 (bs, 1H, NHInd) ppm.
^{13}C NMR (100MHz, CDCl$_3$): δ = 17.9, 19.2, 27.4, 31.7, 55.3, 58.4, 64.3, 111.2, 111.8, 118.7, 119.5, 119.8, 122.3, 122.6, 127.6, 136.3 ppm
HPLC-MS: Rt = 7.35min; *m*/*z* = 245 [M-CN]$^+$, 294 [M+Na]$^+$

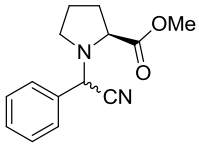
Methyl 2-(1-cyanobutyl)amino-3-(1H-indol-2-yl)propanoate (**29**)



Characterized as a diastereoisomeric mixture, pale yellow oil.
Data for major isomer **29a** (1*R*,*S*)
^{1}H NMR (400MHz, CDCl$_3$): δ = 0.87 (t, J = 7.2 Hz, 3 H, C*H*$_3$CH$_2$CH$_2$), 1.39-1.53 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.64-1.70 (m, 2H, CH$_3$CH$_2$C*H*$_2$CH), 1.74 (bs, 1H, CHN*H*CHCN), 3.10 (ddd, J = 0.8, 7.2, 14.8Hz, 1H, IndC*H*HCH), 3.21 (ddd, J = 0.8, 5.2, 14.8Hz, 1H, IndCH*H*CH), 3.41 (t, J = 8.0 Hz, 1H, CH$_2$C*H*CN), 3.71-3.76 (m, 1H, CH$_2$C*H*COMe), 3.71 (s, 3H, CO$_2$Me), 7.07-7.24 (m, 3H, arom), 7.35-7.39 (m, 1H, arom), 7.61-7.64 (m, 1H, arom), 8.08 (bs, 1H, NHInd) ppm

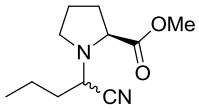
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.2, 18.6, 28.9, 35.3, 48.9, 52.1, 60.5, 110.8, 111.1, 118.6, 119.6, 119.9, 122.1, 122.8, 127.3, 136.0, 174.1 ppm
IR: ṽ = 3445, 2977, 2227, 1736, 1494, 1155 cm^{-1}
HPLC-MS: Rt = 8.78 min; m/z = 273 $[M-CN]^+$, 300 $[M+H]^+$, 322 $[M+Na]^+$
Data for minor isomer **29b** (1R,S)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.90 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$CH$_2$), 1.39-1.53 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.64-1.70 (m, 2H,CH$_3$CH$_2$C*H*$_2$CH), 2.09 (bs, 1H, CHN*H*CHCN), 3.15 (ddd, J = 0.8, 8.4, 14.4 Hz, 1 H, IndC*H*HCH), 3.31 (ddd, J = 0.8, 4.8, 14.8 Hz, 1H, IndCH*H*CH), 3.66 (t, J = 6.8 Hz, 1H, CH$_2$C*H*CN), 3.73 (s, 3H, CO_2Me) , 3 .96 (dd , J = 5. 2 , 8. 4Hz, 1H, CH$_2$C*H*COMe), 7.07-7.24 (m, 3H, arom), 7.35-7.39 (m, 1H, arom), 7.61-7.64 (m, 1H, arom), 8.12 (bs, 1H, NHInd) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.3, 18.7, 29.2, 35.7, 49.3, 52.1, 59.4, 110.3, 111.2, 118.6, 119.5, 119.9, 122.3, 122.7, 127.3, 136.2, 173.8 ppm
HPLC-MS: Rt = 8.63 min; m/z = 273 $[M-CN]^+$, 300 $[M+H]^+$, 322 $[M+Na]^+$

Methyl 1-(cyanophenylmethyl)pyrrolidine-2-carboxylate (**37**)



Diastereoisomers separated by flash chromatography.
Data for major isomer **37a** (S,1S): yellow oil.
$[\alpha]_D$ = -94.06° (c = 0.64, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.79-1.87 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 2.06-2.14 (m, 1H, C*H*HCHCO$_2$Me), 2.21-2.31 (m, 1H, CH*H*CHCO$_2$Me), 2.55 (dt, J = 8.8, 8.8Hz, 1H, CH$_2$CH*H*N), 2.72-2.76 (m, 1 H, CH$_2$C*H*HN), 3.61 (dd, J = 7.2, 9.2Hz, 1H, CHHC*H*CO$_2$Me), 3.80 (s, 3H, Me), 5.37 (s, 1H, CHCN), 7.35-7.43 (m, 3H, arom), 7.57-7.60 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 22.8, 28.8, 48.2, 52.1, 58.1, 63.0, 116.0, 127.6, 128.7, 128.8, 133.8, 173.3 ppm
IR: ṽ = 2953, 2227, 1743, 1206 cm^{-1}
$C_{14}H_{16}N_2O_2$ (244.29): calc C 68.83, H 6.60, N 11.47; found C 69.02, H 6.71, N 11.57
HPLC-MS: Rt = 8.37min; m/z = 245 $[M+H]^{1+}$, 267 $[M+Na]^+$
Data for minor isomer **37b** (S,1R): yellow oil.
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.58-1.98 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 2.00-2.09 (m, 1H, C*H*HCHCO$_2$Me), 2.12-2.22 (m, 1H, CH*H*CHCO$_2$Me), 2.91-2.99 (m, 1H, C*H*HN), 3.32 (s, 3H, Me), 3.33-3.38 (m, 1H, CH$_2$CH*H*N), 3.48 (dd, J = 4.0, 9.2Hz, 1H,CH$_2$C*H*CO$_2$Me), 5.17 (s, 1H, CHCN), 7.35-7.41 (m, 3H, arom), 7.45-7.52 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 23.9, 30.4, 51.6, 53.1, 58.7, 61.0, 116.6, 128.3, 128.6, 129.1, 132.9, 174.0 ppm
HPLC-MS: Rt = 7.27min; m/z = 245 $[M+H]^+$, 267 $[M+Na]^+$

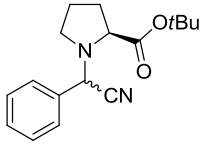
Benzyl 1-(1-cyanobutyl)methylpyrrolidine-2-carboxylate (**38**)



The major diastereoisomer was separated by flash chromatography, the minor isomer was characterized in the crude mixture.
Data for major isomer **38a** (S,1S): pale yellow oil.
$[\alpha]_D$ = -64.37° (c = 1.4, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.97 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$CH$_2$), 1.43-1.60 (m, 2H, CH$_3$C*H*$_2$), 1.70-1.82 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.87-1.94 (m, 2H, CH$_3$CH$_2$C*H*$_2$), 1.99-2.07 (m, 1H, C*H*HCHCO$_2$Me), 2.12-2.22 (m, 1H, CH*H*CHCO$_2$Me), 2.59 (dt, J = 8.8, 8.4Hz, 1 H, CH*H*N), 3.11 (ddd, J = 4.4, 6.4, 8.8Hz, 1H, C*H*HN), 3.43 (dd, J = 6.4, 8.4Hz, 1H, CHCO$_2$Me), 3.73 (s, 3H, Me), 3.97 (t, J = 7.6Hz, 1H, CHCN) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.3, 19.2, 23.0, 28.6, 34.4, 47.9, 52.0, 54.0, 63.5, 117.8, 173.3 ppm
IR: ṽ = 2960, 2876, 2223, 1747, 1175 cm^{-1}
$C_{11}H_{18}N_2O_2$ (210.14): calc C 62.83, H 8.63, N 13.32; found C 62.78, H 8.71, N 13.48
HPLC-MS: Rt = 7.49min; m/z = 130 $[M-CH_3(CH_2)_2CHCN+H]^+$, 211 $[M+1]^+$

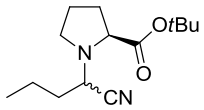
Data for minor isomer **38b** (S,1R)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.94 (t, J = 8.0 Hz, 3H, C*H*$_3$CH$_2$CH$_2$), 1.43-1.60 (m, 2H, CH$_3$C*H*$_2$), 1.70-1.82 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.87-1.94 (m, 2H, CH$_3$CH$_2$C*H*$_2$), 1.99-2.07 (m, 1H, C*H*HCHCO$_2$Me), 2.12-2.22 (m, 1H, CH*H*CHCO$_2$Me), 2.80-2.87 (m, 1H, CH*H*N), 3.19–3.24 (m, 1H, C*H*HN), 3.56 (dd, J = 4.0, 12.0Hz, 1H, C*H*CO$_2$Me), 3.76-3.81 (m, 1H, C*H*CN), 3.82 (s, 3H, Me) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 19.3, 24.1, 29.2, 34.7, 47.9, 51.9, 3.7, 63.5, 118.2, 175.0 ppm
HPLC-MS: Rt = 6.92min; *m*/*z* = 211 $[M+H]^+$

_t_Butyl 1-(cyanophenylmethyl)pyrrolidine-2-carboxylate (**39**)



Diastereoisomers separated by flash chromatography.
Data for major isomer **39a** (*S*,1*S*): pale yellow oil.
$[\alpha]_D$ = -54.53° (c = 0.27, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.52 [s, 9H, CMe_3], 1.78-1.84 (m, 2H, CH$_2$C*H*$_2$CH$_2$) , 2.02-2.10 (m, 1H, C*H*HCHCO$_2$Me), 2.16-2.25 (m, 1H, CH*H*CHCO$_2$Me), 2.54 (dt, J = 7.6, 9.2 Hz, 1H, CH$_2$CH*H*N), 2.71 (ddd, J = 4.4, 6.0, 9.2Hz, 1H, C*H*HN), 3.48 (dd, J = 6.8, 8.8Hz, 1H, C*H*CO$_2$*t*Bu), 5.39 (s, 1H, CHCN), 7.34-7.43 (m, 3H, arom), 7.58-7.61 (m, 2H, arom) ppm
^{13}C NMR (50.3MHz, $CDCl_3$): δ = 22.7, 28.1, 28.8, 48.2, 58.2, 63.6, 81.4, 116.2, 127.6, 128.7 (2 C), 134.1, 171.9 ppm
IR: $\tilde{\nu}$ = 2959, 2874, 2227, 1735, 1010 cm^{-1}
$C_{17}H_{22}N_2O_2$ (286.37): calc C 71.30, H 7.74, N 9.78; found C 71.53, H 7.86, N 9.84.
HPLC-MS: Rt = 10.88min; *m*/*z* = 172 $[M-t\text{Bu-CN+3H}]^+$, 260 $[M-CN]^+$, 287 $[M+H]^+$, 309 $[M+Na]^+$, 325 $[M+K]^+$
Data for minor isomer **39b** (*S*,1*R*): pale yellow oil.
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.57 (s, 9 H, CMe_3), 1.82-1.89 (m, 1H, CH$_2$C*H*HCH$_2$), 1.92-1.98 (m, 1 H, CH2CH*H*CH2), 1.99–2.07 (m, 1 H, C*H*HCHCO2Me), 2.09-2.18 (m, 1H, CH*H*CHCO$_2$Me), 2.54 (dt, *J* = 6.4, 9.2Hz, 1H, CH$_2$CH*H*N), 3.35-3.40 (m, 2H, C*H*HN, C*H*CO$_2$*t*Bu), 5.16 (s, 1H, CHCN), 7.35-7.39 (m, 3H, arom), 7.52–7.54 (m, 2 H, arom) ppm
HPLC-MS: Rt = 10.09min; *m*/*z* = 172 $[M-t\text{Bu-CN+3H}]^+$, 260 $[M-CN]^+$, 287 $[M+H]^+$, 309 $[M+Na]^+$, 325 $[M+K]^+$

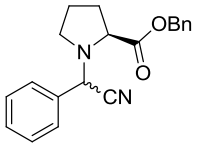
_t_Butyl 1-(1-cyanobutyl)pyrrolidine-2-carboxylate (**40**)



Characterized as a diastereoisomeric mixture, pale yellow oil.
IR: ν˜ = 2964, 2933, 2223, 1739, 1150, 1100 cm^{-1}
Data for major isomer **40a** (*S*,1*S*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.98 (t, J = 7.6Hz, 3H, C*H*$_3$CH$_2$CH), 1.47 [s, 9H, CMe_3] , 1.49-1.59 (m, 4H, C*H*$_2$C*H*$_2$CH$_3$), 1.74-1.81 (m, 1H, C*H*HCH$_2$N), 1.82-1.91 (m, 1H, CH*H*CH$_2$N), 1.94-2.02 (m, 1H, C*H*HCHCOOCH$_3$), 2.07-2.15 (m, 1H, CH*H*CHCO$_2$Me), 2.57 (dt, *J* = 8.0, 8.8Hz, 1H, CH*H*N), 3.07-3.12 (m, 1H, CH*H*N), 3.29 (dd, J = 6.8, 8.8Hz, 1H, C*H*CO$_2$CH$_3$), 3.99 (t, J = 7.6Hz, 1H, C*H*CN) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 19.3, 22.9, 28.1, 28.6, 34.5, 47.9, 54.1,
64.2, 81.2, 118.1, 171.9 ppm
HPLC-MS: Rt = 10.26min; *m*/*z* = 275 $[M+Na]^+$
Data for minor isomer **40b** (*S*,1*R*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.94 (t, J = 7.2Hz, 3H, C*H*$_3$CH$_2$CH), 1.46 (s, 9H, CMe_3), 1.49-1.59 (m, 4H, C*H*$_2$C*H*$_2$CH$_3$), 1.74-1.81 (m, 1H, C*H*HCH$_2$N), 1.82-1.91 (m, 1H, CH*H*CH$_2$N), 1.94-2.02 (m, 1H, C*H*HCHCOOCH$_3$), 2.07-2.15 (m, 1H, CH*H*CHCO$_2$CH$_3$), 2.77-2.83 (m, J = 8.8Hz, 1H, CH*H*N), 3.18-3.22 (m, 1H, CH*H*N), 3.39 (dd, J = 3.2, 9.6Hz, 1H, C*H*CO$_2$CH$_3$), 3.78 (dd, J = 6.4, 9.6Hz, 1H, C*H*CN) ppm

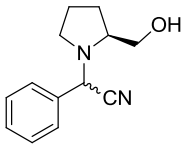
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 19.4, 24.3, 27.9, 29.7, 35.0, 47.9, 55.7, 64.2, 80.8, 118.5, 174.0 ppm
HPLC-MS: Rt = 9.68min; *m*/*z* = 275 $[M+Na]^+$

Benzyl 1-(cyanophenylmethyl)pyrrolidine-2-carboxylate (**41**)



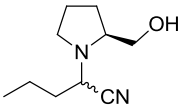
Diastereoisomers separated by flash chromatography.
Data for major isomer **41a** (*S*,1*S*): pale yellow oil.
$[\alpha]_D$ = -65.20° (c = 1.19, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$) : δ = 1.58-1.87 (m, 2H, $CH_2CH_2CH_2$), 2.09-2.18 (m, 1H, C*H*HCHCO$_2$Bn), 2.21-2.31 (m, 1H, CH*H*CHCO$_2$Me), 2.59 (dt, J = 8.0, 8.4Hz, 1H, CH_2CHHN), 2.70-2.75 (m, 1H, CH_2CHHN), 3.65 (dd, J = 6.8, 8.8Hz, 1H, CHHC*H*CO$_2$Bn), 5.21 (d, J_{AB} = 12.4Hz, 1H, C*H*HPh), 5.26 (d, J_{AB} = 12.4Hz, 1H, CH*H*Ph), 5.35 (s, 1H, CHCN), 7.35-7.41 (m, 8H, arom), 7.50-7.52 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 22.8, 28.6, 48.1, 58.0, 62.9, 66.6, 115.9, 127.4, 128.2, 128.3, 128.5, 128.6, 128.7, 133.7, 135.5, 172.4 ppm
IR: ṽ = 3064, 3033, 2926, 2850, 2227, 1956, 1888, 1743, 1175 cm^{-1}
$C_{20}H_{20}N_2O_2$ (320.38): calc C 74.98, H 6.29, N 8.74; found C 75.07, H 6.34, N 8.69
HPLC-MS: Rt = 10.86 min; *m*/*z* = 321 $[M+H]^+$, 343 $[M+Na]^+$
Data for minor isomer **41b** (*S*,1*R*): pale yellow oil.
$[\alpha]_D$ = -17.93° (c = 0.99, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.88-1.99 (m, 2H, $CH_2CH_2CH_2$), 2.01-2.08 (m, 1H, C*H*HCHCO$_2$Bn), 2.10-2.22 (m, 1H, CH*H*CHCO$_2$Bn), 2.98 (dt, J = 6.8, 8.8Hz, 1H, CH_2CHHN), 3.37 (dt, J = 2.8, 8.8Hz, 1H, C*H*HN), 3.52 (dd, J = 4.0, 9.6Hz, 1H, CHHC*H*CO$_2$Bn), 4.71 (d, J_{AB} = 12.0Hz, 1H, C*H*HPh), 4.80 (d, J_{AB} = 12.4Hz, 1H, CH*H*Ph), 5.17 (s, 1H, CHCN), 7.18-7.20 (m, 2H, arom), 7.31-7.39 (m, 6H, arom), 7.46-7.51 (m, 2H, arom) ppm
^{13}C NMR (50 MHz, CDCl3): δ = 23.8, 30.4, 52.8, 58.4, 60.9, 66.1, 116.7, 128.0, 128.1, 128.2, 128.3, 128.5, 128.9, 132.9, 135.4, 173.3 ppm
IR: ṽ = 3064, 3033, 2952, 2828, 2226, 1743, 1166 cm^{-1}
$C_{20}H_{20}N_2O_2$ (320.38): calc C 74.98, H 6.29, N 8.74; found C 75.21, H 6.44, N 8.61.
HPLC-MS: Rt = 9.98min; *m*/*z* = 321 $[M+H]^+$, 343 $[M+Na]^+$, 359 $[M+K]^+$

2-(2-(Hydroxymethyl)pyrrolidin-1-yl)-2-phenylacetonitrile (**42**)



Characterized as a diastereoisomeric mixture.
Signals refer to the major diastereoisomer, only the main signals of the minor diastereoisomer are reported, pale yellow oil.
Data for major isomer **42a** (2*S*,2*S*) and minor isomer **42b** (2*R*,2*S*)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.67-1.74 (m, 1H, NCH_2CH_2CHHCH), 1.76-1.87 (m, 2H, CH_2CHHCH_2, NCH_2CH_2CHHCH), 1.99-2.04 (m, 1H, CH_2CHHCH_2), 2.15 (bs, 1H, OH), 2.62 (dt, *J* = 8.8, 9.6Hz, 1H, CH_2CHHN), 2.68-2.73 (m, 1H, CH*H*N), 2.88-2.95 (m, 1H, minor) , 3.00-3.04 (m, 1H, minor), 3.11-3.16 (m, 1H, C*H*CH_2OH), 3.61 (dd, *J* = 3.6, 11.2Hz, 1H, CHC*H*HOH), 3.82 (dd, *J* = 3.2, 11.2Hz, 1H, CHCH*H*OH), 5.11, (s, 1H, CHCN minor), 5.27 (s, 1H, CHCN), 7.37-7.42 (m, 3H, arom), 7.43-7.52 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 23.1, 27.5, 49.2, 57.9, 62.6, 63.2, 116.3, 127.5, 128.7, 128.8, 134.1 ppm
IR: ṽ = 3424, 3063, 3033, 2924, 2875, 2227, 1602, 1452, 1077 cm^{-1}
HPLC-MS: Rt = 5.36min, minor isomer; *m*/*z* = 190 $[M-CN]^+$; Rt = 6.38min, major isomer; *m*/*z* = 217 $[M+H]^+$, 190 $[2M-CN]^+$
Compound **42a** was also obtained starting from **37a** by reduction of the ester functionality with $NaBH_4$.

2-(2-Hydroxymethyl-pyrrolidin-1-yl) pentanenitrile (**43**)



Characterized as a diastereoisomeric mixture.
Signals refer to the major diastereosiomer, only the main signals of the minor diastereoisomer are reported, pale yellow oil.

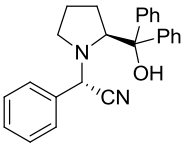
Data for major isomer **43a** (2*S*,2*S*) and minor isomer **43b** (2*R*,2*S*)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.98 (t, J = 7.2 Hz, 3H, C*H*$_3$CH$_2$CH$_2$), 1.46-1.56 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.71-1.87 (m, 4H, CH$_3$CH$_2$C*H*$_2$, CH$_2$C*H*$_2$CH$_2$), 1.92-1.98 (m, 2H, CH$_2$CH$_2$C*H*$_2$CH), 2.63 (dt, J = 6.8, 8.8Hz, 1H, CH$_2$C*H*HN), 2.7-2.83 (m, 1 H, minor), 2.92-2.97 (m, 1H, C*H*CH$_2$OH), 3.03-3.08 (m, 1H, CH$_2$CH*H*N), 3.11-3.17 (m, 1H, minor), 3.46 (ddd, J = 3.6, 8.8, 12.0Hz, 1H, CHC*H*HOH), 3.63 (ddd, J = 2.8, 2.8, 12.0Hz, 1H, CHCH*H*OH), 3.72 (dd, J = 6.0, 9.2Hz, 1H, CHCN minor), 3.83 (dd, *J* = 7.6, 8.0Hz, 1H, CHCN) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 13.4, 19.3, 23.4, 27.2, 34.7, 48.7, 53.5, 62.5, 62.8, 118.0 ppm

IR: ν̃ = 3423, 2962, 2960, 2875, 2224, 1640, 1043 cm^{-1}

(S)-2-[(S)-2-Hydroxydiphenylmethylpyrrolidinyl]-2-phenylacetonitrile (**44a**)



White solid, M.p. 105-112°C

$[\alpha]_D$ = 21.39° (c = 0.38, $CHCl_3$)

^{1}H NMR (400MHz, $CDCl_3$): δ = 1.55-1.79 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.84-1.91 [m, 1H, CH$_2$C*H*HCHC(Ph)$_2$OH], 2.04-2.14 [m, 1H, CH$_2$CH*H*CHC(Ph)$_2$OH], 2.67-2.76 (m, 2H, CH$_2$CH$_2$N), 4.03 (s, 1H, C*H*CN), 4.15 (bs, 1H, OH), 4.33 [dd, J = 4.4, 9.2Hz, 1H, CHHC*H*C(Ph)$_2$OH], 7.17-7.23 (m, 3H, arom), 7.29-7.38 (m, 8H, arom), 7.60-7.62 (m, 2H, arom), 7.78-7.80 (m, 2H, arom) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 23.8, 29.4, 50.7, 59.1, 68.9, 78.2, 116.6, 125.2, 125.4, 126.7, 127.1, 127.4, 128.3, 128.7, 128.8, 128.9, 133.9, 145.5, 147.1 ppm

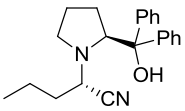
IR: ν̃ = 3500, 2970, 1682, 1596 cm^{-1}

$C_{25}H_{24}N_2O$ (368.47): calc C 81.49, H 6.57, N 7.60; found C 81.62, H 6.68, N 7.51

HPLC-MS: Rt = 12.07min; *m*/*z* = 342 [M-CN]$^+$, 369 [M+H]$^+$, 391 [M+Na]$^+$

Compound **44a** was also obtained in 41% yield starting from **37a** by Grignard addition.

(S)-2-[(S)-2-Hydroxydiphenylmethyl-pyrrolidin-1-yl]pentanenitrile (**45a**)



White solid, M.p. 107-114°C

$[\alpha]_D$ = -36.21° (c = 0.62, $CHCl_3$)

^{1}H NMR (400MHz, $CDCl_3$): δ = 0.66 (t, J = 6.8 Hz, 1H, C*H*$_3$CH$_2$), 1.06-1.16 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.37-1.46 (m, 1H, CH$_3$CH$_2$C*H*H), 1.48-1.58 (m, 1H, CH$_3$CH$_2$C*H*H), 1.73-1.84 [m, 3H, C*HH*C*H*HCHC(CPh$_2$OH)] , 1.93-2.06 [m, 1H, CH$_2$CH*H*CH(CPh2OH)], 2.71-2.79 (m, 2H, CHCN, CH$_2$C*H*HN), 3.09-3.14 (m, 1H, CH$_2$CH*H*N), 3.96 (bs, 1H, OH), 4.10 [dd, *J* = 3.6, 9.2Hz, C*H*(CPh$_2$OH)], 7.16-7.21 (m, 2H, arom), 7.27-7.34 (m, 4H, arom), 7.53-7.56 (m, 2 H, arom), 7.61-7.63 (m, 2H, arom) ppm

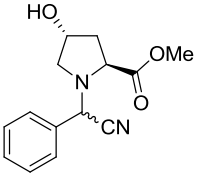
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.0, 18.9, 24.0, 28.9, 34.5, 50.1, 54.7, 69.6, 77.9, 118.4, 125.1, 125.4, 126.5, 126.8, 128.1, 128.5, 145.4, 147.1 ppm

IR: ν̃ = 3411, 3066, 3023, 2923, 2224, 1141 cm^{-1}

$C_{22}H_{26}N_2O$ (334.45): calc C 79.00, H 7.84, N 8.38; found C 79.28, H 7.92, N 8.44

HPLC-MS: Rt = 11.78min; *m*/*z* = 308 [M-CN]+, 335 [M+H]$^+$

Methyl 1-(cyanophenylmethyl)-4-hydroxypyrrolidine-2-carboxylate (**46**)



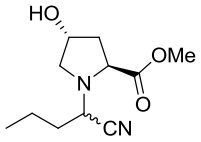
Diastereoisomers separated by flash chromatography.

Data for major isomer **46a** (2*S*,4*R*,1*S*): white solid, M.p. 88-92°C

$[\alpha]_D$ = -74.20 (c = 1.5, $CHCl_3$)

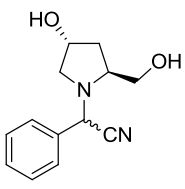
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.77 (bs, 1H, OH), 2.23-2.27 (m, 2H, C*H*$_2$CHCO$_2$Me), 2.57 (dd, J = 4.0, 10.4Hz, 1H, CHC*H*HN), 3.08 (dd, J = 5.2, 10.4Hz, 1H, CHCH*H*N), 3.80 (s, 3H, Me), 3.92 (dd, J = 7.6, 8.8Hz, 1H, CHHC*H*CO$_2$Me), 4.37-4.42 (m, 1H, C*H*OH), 5.38 (s, 1H, C*H*CN), 7.37-7.43 (m, 3H, arom), 7.57-7.59 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 38.7, 52.2, 56.7, 57.9, 61.6, 69.2, 116.2, 127.5, 128.8, 128.9, 133.5, 172.8 ppm
IR: ṽ = 3178, 2227, 1740, 1207 cm^{-1}
$C_{14}H_{16}N_2O_3$ (260.29): calc C 64.60, H 6.20, N 10.76; found C 65.02, H 6.18, N 10.68
HPLC-MS: Rt = 5.41min; *m*/*z* = 234 [M-CN]$^+$, 261 [M+H]$^+$, 283 [M+Na]$^+$
Data for minor isomer **46b** (2*S*,4*R*,1*R*): white solid, M.p. 103-104°C.
$[\alpha]_D$ = -13.10° (c = 1.5, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.99 (bs, 1H, OH), 2.11 (dddd, *J* = 0.8, 4.8, 8.4, 9.2Hz, 1H, CHC*H*HCHCO$_2$Me), 2.18-2.25 (m, 1H, CHCH*H*CHCO$_2$Me), 2.89 (ddd, J = 0.8, 4.0, 9.6Hz, 1H, CHC*H*HN), 3.38 (s, 3H, Me), 3.55 (dd, J = 4.8, 9.6 Hz, 1H, CHCH*H*N), 3.75 (dd, J = 6.4, 8.4Hz, 1H, CHHC*H*CO$_2$Me), 4.55-4.60 (m, 1H, C*H*OH), 5.16 (s, 1H, C*H*CN), 7.37-7.41 (m, 3H, arom), 7.47-7.50 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): *δ* = 39.4, 51.7, 57.9, 59.6, 60.6, 69.8, 116.6, 128.3, 128.7, 129.2, 132.4, 173.2 ppm
IR: ṽ = 3377, 2943, 2230, 1739, 1207 cm^{-1}
$C_{14}H_{16}N_2O_3$ (260.29): calc C 64.60, H 6.20, N 10.76; found C 64.81, H 6.29, N 10.64
HPLC-MS: Rt = 3.99min; *m*/*z* = 234 [M-CN]$^+$, 261 [M+H]$^+$, 283 [M+Na]$^+$, 543 [2M+Na]$^+$

Methyl 1-(1-cyanobutyl)-4-hydroxypyrrolidine-2-carboxylate (**47**)



Diastereoisomers separated by flash chromatography.
Data for major isomer **47a** (2*S*,4*R*,1*S*): pale yellow oil.
$[\alpha]_D$ = -103.90° (c = 0.6, $CHCl_3$)
^{1}H NMR (400MHz, CDCl3): δ = 0.97 (t, J = 7.6 Hz, 3H, C*H*$_3$CH$_2$CH$_2$), 1.48-1.60 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.73-1.79 (m, 3H, CH$_3$CH$_2$C*H*$_2$, OH), 2.12-2.24 (m, 2H, C*H*$_2$CHCO$_2$Me), 2.63 (dd, J = 3.6, 10.4Hz, 1H, CHHC*H*HN), 3.43 (dd, J = 5.2, 10.4Hz, 1H, CHHCH*H*N), 3.73-3.78 (m, 1H, CHHC*H*CO$_2$Me), 3.75 (s, 3H, C*H*$_3$), 3.99 (t, J = 7.6Hz, 1H, CHHC*H*CN), 4.45-4.51 (m, 1H, C*H*OH) ppm
^{13}C NMR (50.3MHz, $CDCl_3$): δ = 12.9, 18.7, 34.0, 38.1, 51.8, 53.5, 56.0, 61.7, 68.6, 117.5, 172.7 ppm
IR: ṽ = 3450, 2959, 2874, 2226, 1743, 1438, 1090 cm^{-1}
$C_{11}H_{18}N_2O_3$ (226.27): calc C 58.39, H 8.02, N 12.38; found C 58.48, H 8.11, N 12.21
HPLC-MS: Rt = 3.99min; *m*/*z* = 200 [M-CN]$^+$, 227 [M+H]$^+$, 249 [M+Na]$^+$
Data for minor isomer **47b** (2*S*,4*R*,1*R*)
^{1}H NMR (400MHz, $CDCl_3$) : *δ* = 0.94 (t, J = 7.2 Hz, 1H, C*H*$_3$CH$_2$CH$_2$), 1.42-1.57 (m, 2H, CH$_3$C*H*$_2$CH$_2$), 1.59-1.72 (m, 2H, CH$_3$CH$_2$C*H*$_2$), 1.87 (bs, 1H, OH), 2.14 (dddd, J = 0.8, 5.6, 8.4, 14.0Hz, 1H, CHC*H*HCHCO$_2$Me), 2.22-2.28 (m, 1H, CHCH*H*CHCO$_2$Me), 2.82 (ddd, J = 0.8, 4.4, 10.0Hz, 1H, CHC*H*HN), 3.42 (dd, J = 5.6, 10.0Hz, 1H, CHCH*H*N), 3.75 (s, 3H, Me), 3.76-3.82 (m, 2H, C*H*CO$_2$Me, C*H*CN), 4.52-4.58 (m, 1H, CH$_2$C*H*OH)
HPLC-MS: Rt = 3.31min; *m*/*z* = 200 [M-CN]$^+$, 227 [M+H]$^+$, 249 [M+Na]$^+$

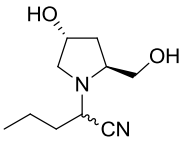
2-(4-Hydroxy-2-hydroxymethyl-pyrrolidin-1-yl)-2-phenylacetonitrile (**48**)



Compound **48a** (2*S*, 4*R*, 1*S*), the major isomer, was obtained pure and characterized after spontaneous isomerization of a **48a**/**48b** mixture, pale yellow solid, M.p. 105-107°C.
$[\alpha]_D$ = -110.91° (c = 0.66, MeOH)
^{1}H NMR (400MHz, $CDCl_3$): δ = 1.95 (ddd, J = 4.4, 8.0, 13.0Hz, 1H, CHC*H*HCH), 1.92-2.11 (bs, 2H, OH), 2.06 (ddd, J = 6.8, 8.4, 13.0 Hz, 1H, CHCH*H*CH), 2.59 (dd, J = 4.8, 10.0Hz, 1H,

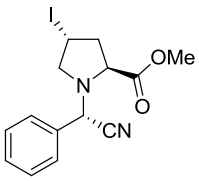
CHC*H*HN), 3.01 (dd, J = 6.0, 10.0Hz, 1H, CHCH*H*N), 3.39-3.45 (m, 1H, CHHC*H*CH_2OH), 3.62 (dd, J = 4.0, 12.0Hz, 1H, CHC*H*HOH), 3.85 (dd, J = 3.2, 12.0Hz, 1H, CHCH*H*OH), 4.31-4.36 (m, 1H, C*H*OH), 5.32 (s, 1H, CHCN), 7.37-7.42 (m, 3H, arom), 7.48-7.51 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 37.1, 57.4, 57.7, 61.5, 62.7, 69.4, 116.4, 127.4, 128.9 (2C), 133.7 ppm
IR (nujol): $\tilde{\nu}$ = 3334, 2229 cm^{-1}
$C_{13}H_{16}N_2O_2$ (232.28): calc C 67.22, H 6.94, N 12.06; found C 66.89, H 6.98, N 11.99
HPLC-MS: Rt = 2.49min (major + minor); *m*/*z* = 206 $[M-CN]^+$, 233 $[M+H]^+$, 255 $[M+Na]^+$, 487 $[2M+Na]^+$
Data for minor isomer **48b** (2*S*,4*R*,1*R*): obtained only as a diastereoisomeric mixture, only the main signals are reported.
^{1}H NMR (400MHz, $CDCl_3$): δ = 3.85 (dd, J = 5.2, 11.6Hz, 1H, CHCH*H*OH), 4.40-4.50 (m, 1H, C*H*OH), 5.16 (s, 1H, CHCN) ppm
Compound **48a** was also obtained starting from **46a** by reduction of the ester functionality with $NaBH_4$.

2-(4-Hydroxy-2-hydroxymethyl-pyrrolidin-1-yl) pentanenitrile (**49**)



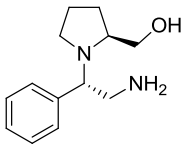
Characterized as a diastereoisomeric mixture, signals refer to the major diastereoisomer, only the main signals of the minor diastereoisomer are reported.
Data for major isomer **49a** (2*S*,4*R*,2*S*) and minor isomer **49b** (2*S*,4*R*,2*S*): pale yellow oil.
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.97 (t, J = 7.2 Hz, 3H, C*H*$_3$$CH_2CH_2$), 0.98 (t, J = 7.6 Hz, 3H, C*H*$_3$$CH_2CH_2$, minor), 1.45-1.68 (m, 2H, CH_3C*H*$_2$$CH_2$), 1.66-1.79 (m, 2H, CH_3CH_2C*H*$_2$), 1.89 (ddd, J = 4.0, 7.6, 13.0Hz, 1H, CHC*H*HCH), 1.96-2.07 (m, 1H, CHCH*H*CH), 2.63 (dd, J = 4.0, 9.6Hz, 1H, CHC*H*HN), 2.77 (dd, J = 5.6, 9.6Hz, 1H, CHC*H*HN, minor), 3.20-3.26 (m, 1H, CHC*H*CH2OH), 3.34 (dd, J = 5.6, 10.0Hz, 1H, CHCH*H*N), 3.48 (dd, *J* = 3.2, 12.0Hz, 1H, CHC*H*HOH), 3.55-3.59 (m, 1H, CHC*H*HOH minor), 3.68 (dd, J = 3.2, 12.0Hz, 1H, CHCH*H*OH), 3.77 (dd, J = 6.4, 10.0Hz, 1H, CHCN, minor), 3.87 (t, J = 8.0Hz, 1H, CHCN), 4.40 (m, 1H, C*H*OH) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 13.3, 19.2, 34.6, 36.9, 53.2, 57.0, 61.5, 61.7, 69.7, 118.1 ppm
IR: $\tilde{\nu}$ = 3406, 2961, 2874, 2226, 1097 cm^{-1}
HPLC-MS: Rt = 1.79min (major + minor); *m*/*z* = 172 $[M-CN]^+$, 199 $[M+H]^+$, 221 $[M+Na]^+$
Product **49a** was also obtained starting from **42a** by reduction of the ester functionality with $NaBH_4$.

Methyl (2S,4S)-1-[(S)-cyanophenylmethyl]-4-iodopyrrolidine-2-carboxylate (**50a**)



DEAD (40% solution in toluene, 107mg, 0.61mmol) was added dropwise to a stirred solution of **46a** (134mg, 0.51mmol) and triphenylphosphine (161mg, 0.61mmol) in THF (1.5mL) at 0°C, and then methyl iodide (38μL, 0.61mmol) was added. After 20min, the solution was warmed to room temperature and stirred for 5h until TLC showed that the reaction was complete. The reaction mixture was concentrated to dryness under reduced pressure, and the residue was purified by flash chromatography to give **50a** (153mg, 0.41mmol, 81%) as a white solid, M.p. 80-85°C.
$[\alpha]_D$ =-28.55° (c = 0.20, $CHCl_3$)
^{1}H NMR (400MHz, $CDCl_3$): δ = 2.66 (ddd, J = 6.0, 6.4, 14.4Hz, 1H, CHIC*H*HCHCO_2Me), 2.91 (ddd, J = 6.8, 9.2, 14.4Hz, 1H, H*H*CHCO_2Me), 2.99 (dd, *J* = 4. 0, 11.2Hz , 1H, NC*H*HCHI), 3.05 (dd, J = 6.0, 11.2Hz, 1H, NCH*H*CHI), 3.76 (dd, J = 6.8, 8.8Hz, CH_2C*H*CO_2Me), 3.84 (s, 3H, Me), 4.32 (m, 1H, C*H*I), 5.49 (s, 1H, C*H*CN), 7.37-7.46 (m, 3H, arom), 7.64-7.66 (m, 2H, arom) ppm
^{13}C NMR (100MHz, $CDCl_3$): δ = 15.3, 41.8, 52.5, 57.8, 59.1, 62.2, 116.4, 127.3, 128.9, 129.0, 133.2, 171.8 ppm
IR: $\tilde{\nu}$ = 3028, 2950, 2230, 1746, 1493, 1130 cm^{-1}
$C_{14}H_{15}IN_2O_2$ (370.19): calc C 45.52, H 4.08, N 7.57; found C 45.62, H 4.11, N 7.42
HPLC-MS, Rt = 9.93min; *m*/*z* 371 [M+H]+, 393 $[M+Na]^+$

[(S)-1-((S)-2-amino-1-phenylethyl)pyrrolidin-2-yl]methanol (**51a**)



A solution of $LiAlH_4$ (2M in Et_2O, 500µL) was added dropwise to a stirred solution of cyanoester **37a** (0.2mmol) in THF (1.7mL) at 0°C under an inert atmosphere. After 15min, the solution was warmed to room temperature. The progress of the reaction was monitored by TLC, and at completion after 4h tartrate Na-K salt (saturated aqueous) was added with vigorous stirring to favor phase separation. The separated organic phase was dried (Na_2SO_4) and concentrated, and the residue was purified by flash chromatography (eluent $CHCl_3$/MeOH/NH_4OH, 60/30/1) to yield 17mg of product **51a** (39%).

$[\alpha]_D$ = 1.68° (c = 0.31, DCM)

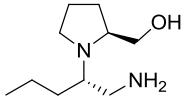
^{1}H NMR (400MHz, D_2O): δ = 1.62-1.71 (m, 2 H, $NCH_2CH_2CH_2$), 1.78-1.87 (m, 2H, $NCH_2CH_2CH_2$), 2.66 (m, 1H, $NCHHCH_2CH_2$), 2.91 (m, 1H, $NCHHCH_2$), 3.02 (m, 2H, CH_2NH_2), 3.15 (m, 2H, CHN, CH_2OH), 3.35 (dd, J = 4.4, 12.4Hz, 1H, CH_2OH), 3.74 (dd, J = 4.8, 10.8Hz, 1H, PhCHN), 7.40-7.53 (m, 5H, Ph) ppm

^{13}C NMR (50.3MHz, D_2O): δ = 24.4, 28.7, 43.6, 52.9, 60.6, 64.9, 68.0, 127.7, 127.9, 128.3, 128.6, 128.8, 139.2 ppm

IR (neat): $\tilde{\nu}$ = 3358, 2924, 1599, 1493, 1453, 1379, 1074, 1040, 766, 735, 704 cm^{-1}

HPLC-MS: Rt = 1.26min; *m*/*z* = 221 $[M+H]^+$

[(S)-1-((S)-1-aminopentan-2-yl)pyrrolidin-2-yl] methanol (**52a**)



Following the same procedure as above, starting from **38a**. Yield 36%, orange oil.

$[\alpha]_D$ = +68.0° (*c* = 1.50, $CHCl_3$)

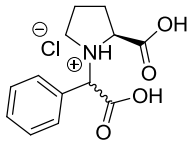
^{1}H NMR (400MHz, $CDCl_3$): δ = 0.93 (t, J = 6.4Hz, 3H, $CH_3CH_2CH_2$), 1.31-1.46 (m, 4H, $CH_3CH_2CH_2$), 1.63-1.82 (m, 4H, $NCH_2CH_2CH_2$), 2.61-2.82 (m, 4H,$CHCH_2NH_2$, $NCH_2CH_2CH_2$), 2.97-3.13 (m, 5H, OH, NH_2, $NCHCH_2NH_2$, CH_2CHCH_2OH), 3.30 (dd, J = 4.8, 10.8Hz, 1H, CHHOH), 3.48 (dd, J = 3.6, 10.8Hz, 1H, CHHOH) ppm

^{13}C NMR (100MHz, $CDCl_3$): δ = 14.3, 20.4, 24.5, 29.0, 31.7, 42.4, 50.0, 58.8, 60.9, 64.4 ppm

IR: $\tilde{\nu}$ = 3280, 2957, 2927, 2870, 1660, 1106 cm^{-1}

$C_{10}H_{22}N_2O$ (186.29): calc C 64.47, H 11.90, N 15.04; found C 64.58, H 11.99, N 14.78

2-Carboxy-1-(carboxyphenylmethyl)pyrrolidin-1-ium chloride (**53**)



A solution of **37a** (2.75mmol, 670mg) in HCl (37%, 12mL) was heated at reflux for 24h, and after completion of the reaction, the water was removed. The crude material was washed with DCM to obtain 692mg of **53** (88%) as a mixture of diastereoisomers (*de* 78:22). Characterized as a diastereoisomeric mixture. Signals refer to the major diastereoisomer, only the main signals of the minor diastereoisomer (white solid) are reported.

Data for major isomer **53a** (2*S*,*S*) and minor isomer **53b** (2*S*,*R*)

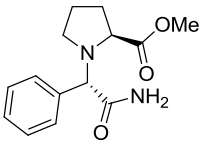
^{1}H NMR (400MHz, CD_3OD) : δ = 2.02-2.31 (m, 3H, CH_2CH_2, CH_2CHHCH), 2.39-2.46 (m, 1 H, $CH_2CHHCHCO_2H$), 2.54-2.64 (m, 1H, $CH_2CHHCHCO_2H$, minor), 3.36-3.46 (m, 1H, $NCHHCH_2$, minor), 3.56-3.62 (m, 1H, $NCHHCH_2$), 3.59-3.67 (m, 1H, CH_2CHHN, minor), 3.95-4.01 (m, 1H, $NCHHCH_2$), 4.39-4.43 (m, 1H, CH_2CHCO_2H, minor), 4.49 (dd, *J* = 6.4, 9.2 Hz, 1H, CH_2CHCO_2H), 5.45 (s, 1H, PhCH), 5.55 (s, 1H, PhCH, minor), 7.51-7.62 (m, 5 H, Ph) ppm

Data for major diastereoisomer **53a**

^{13}C NMR (100MHz, CD_3OD): δ = 25.2, 31.4, 57.9, 67.7, 2.3, 131.3, 131.5, 132.2, 132.9, 170.7, 171.8 ppm

HPLC-MS: Rt = 1.48min; *m*/*z* = 250

Methyl (2S)-1-((S)-2-amino-2-oxo-1-phenylethyl)pyrrolidine-2-carboxylate (**54a**)



Methyl ester **37a** (1.64mmol, 400mg), acetic acid (9.84mmol, 637μL), water (6.56mmol, 118μL), $TiCl_4$ (3.28mmol, 360μL), and DCM (1mL) were added in that order to a 5mL vial equipped with a screw cap. The solution was stirred overnight on an orbital shaker, and the reaction was monitored by TLC. When the reaction was complete, DCM was added, and the organic phase was separated and discarded. The aqueous solution was treated with saturated $NaHCO_3$ until basic pH was reached, and then it was extracted with DCM. The combined organic extracts were dried with Na_2SO_4 and concentrated in vacuum to give 429mg of **54a** in quantitative yield, white solid, M.p. 149-154°C.

$[\alpha]_D$ = +2.83° (c = 1.20, $CHCl_3$)

^{1}H NMR (400MHz, $CDCl_3$): δ = 1.81-1.96 (m, 3H, $CH_2CH_2CH_2$, C*H*HCHCO$_2$), 2.00-2.08 (m, 1H,CH_2CH*H*CHCO$_2$), 2.71-2.77 (m, 1H, CH_2CH*H*N), 3.27-3.32 (m, 1H, CH_2C*H*HN), 3.36 (dd, J = 4.0, 9.6Hz, 1H, CH_2C*H*CO$_2$), 3.50 (s, 3H, Me), 4.24 (s, 1H, PhC*H*CONH$_2$), 5.73 (bs, 1H, CON*H*H), 7.29-7.33 (m, 5H, arom), 7.66 (bs, 1H, CONH*H*) ppm

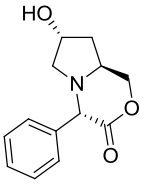
^{13}C NMR (100MHz, $CDCl_3$): δ = 24.2, 30.6, 51.7, 54.4, 61.7, 73.2, 128.5, 128.6, 129.2, 136.5, 174.8, 175.9 ppm

IR: $\tilde{\nu}$ = 3397, 2919, 1725, 1667, 1129 cm^{-1}

$C_{14}H_{18}N_2O_3$ (262.30): calc C 64.10, H 6.92, N 10.68; found C 63.99, H 6.94, N 10.72

HPLC-MS: Rt = 3.41min; *m*/*z* = 263 [M+H]$^+$, 285 [M+Na]$^+$, 547 [2M+Na]$^+$

(4S,7R,8aS)-7-Hydroxy-4-phenyltetrahydro-1H-pyrrolo[2,1-c][1,4]-oxazin-3(4H)-one (**55a**)



The product was obtained in 40% yield after flash chromatography following the same procedure reported for the synthesis of amide **54a** from aminol **48a**.

Pale yellow oil.

$[\alpha]_D$ = –2.47° (c = 0.15, $CHCl_3$)

^{1}H NMR (400MHz, CDCl3): δ = 1.92 (ddd, J = 4.8, 9.6, 14.0Hz, 1H, CHOHC*H*HCH), 2.09-2.17 (m, 1H, CHOHC*H*HCH), 2.91 (dd, J = 3.6, 10.0Hz, 1H, NC*H*HCHOH), 3.12-1.15 (d, J = 10.0Hz, 1H, NC*H*HCHOH), 3.84-3.92 (m, 1H, CH_2C*H*CH_2OCO), 4.14 (dd, J = 4.8, 11.6Hz, CHCH*H*OCO), 4.17 (dd, J = 4.4, 11.6Hz, CHCH*H*OCO), 4.49-4.51 (m, 1H, CH_2C*H*OHCH$_2$), 4.60 (s, 1H, C*H*Ph), 7.37-7.40 (m, 3H, arom), 7.64-7.66 (m, 2H, arom) ppm

^{13}C NMR (100MHz, CDCl3): δ = 37.6, 52.9, 62.2, 65.8, 68.4, 71.3, 127.1, 128.2, 128.7, 135.5, 170.9 ppm

IR: $\tilde{\nu}$ = 3362, 2924, 2853, 1737, 1042 cm^{-1}

$C_{13}H_{15}NO_3$ (233.26): calc C 66.94, H 6.48, N 6.00; found C 66.88, H 6.53, N 6.09

HPLC-MS, Rt = 2.44min; *m*/*z* = 234 [M+H]$^+$, 256 [M+Na]$^+$, 272 [M+K]$^+$, 489 [2M+Na]$^+$

8. LACCASE MEDIATED OXIDATIONS

8.1 Laccase-Mediator Oxidations Systems (LMS) for alcohols

8.1.1 Introduction

Oxidations are pivotal reactions in organic synthesis and are widely used in industrial processes for the synthesis of bulk, fine and specialty chemicals.[172] At the same time, oxidations are among the most polluting and hazardous processes, delivering toxic waste, as in the case of traditional stoichiometric oxidants based on Cr^{VI} or Mn^{VII} salts. To develop cleaner and eco-efficient catalytic oxidation processes, a number of greener catalytic methodologies have been developed by using less toxic metal catalysts and molecular oxygen or air as the oxidant.[173] However, many of these systems still require harsh reaction conditions, give metal-containing wastes, and selectivity can be difficult to control. Hence, to develop more benign and selective redox processes, biocatalysis is emerging as a valuable tool.[174] Bio-oxidations have the added value of high levels of selectivity (regio-, chemo-, and stereo-) that are reliable even for fine chemicals with complex structures and possessing oxidation-sensitive functional groups.[175]

Considering the oxidation of alcohols, an important transformation in the synthesis of fine chemicals, more frequently biocatalysis made use of oxidoreductases (dehydrogenases and oxidases), whereas peroxidases and monooxygenases were employed to a lesser extent.[176] Some biocatalytic methods that used whole cells were also reported.[177] As representative examples, the enantioselective oxidation of 2-phenylpropanol by *Acetobacter aceti*[178] and the oxidation of primary alcohols to aldehyde by *Gluconobacter oxydans*[179] have been reported. In these cases, intact cells were used as the oxidizing agent. Laccases (EC 1.10.3.2) belong to the multi-copper family of oxidases. These enzymes contain four copper centers per protein molecule and catalyze the oxidation of electron-rich aromatic substrates, usually phenols or aromatic amines, by using oxygen as the electron acceptor.[180] Because water is the only byproduct formed, in principle, they are ideal catalysts for sustainable chemical and technological processes. Laccases are widely distributed in nature and perform a multiplicity of functions linked to either synthetic or degradation processes.[181] Fungal laccases, for instance, play a critical role in lignin and humus degradation,[182] and they are of particular interest because such enzymes are secreted extracellularly in response to simple inducers; this makes their production and purification relatively simple. Laccases have broad industrial applications, for instance, in pulp and paper industry, biosensor technology, in the organic synthesis of useful compounds, and offer great interest in environmental biotechnology.[183]

Although the natural substrates of laccases are the phenolic residues of lignin, the inclusion of appropriate mediators in the laccase-mediator system (LMS) makes the oxidation of nonphenolic substrates accessible.[184] The application of the LMS in the oxidation of alcohols is well documented in the literature.[185] Commonly, laccases oxidize secondary alcohols to ketones and primary alcohols to the

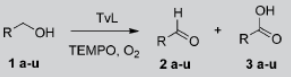
corresponding aldehydes, whereas the overextended oxidation of primary alcohols to carboxylic acid was less reported.[186]

Herein, we report developments in chemoenzymatic oxidation by commercial laccase from *Trametes versicolor* (TvL) of some primary alcohols to the carboxylic acids or aldehydes and of selected secondary alcohols to ketones with a particular emphasis on stereoselectivity. Moreover, within an interdisciplinary project dedicated to improving the sustainable production of active pharmaceutical compounds by the use of chemoenzymatic processes,[187] we report herein an important application of laccase TvL in the bio-oxidation of some (S)-profenols to (S)-profens, which are important benchmark drugs in the class of non-steroidal anti-inflammatory drugs (NSAIDs). To the best of our knowledge, laccase-mediated oxidation of this important class of substrate has not yet been reported; the only example reported is the bio-oxidation of 2-flurbiprofenal by using an engineered alcohol dehydrogenase (ADH).

8.1.2 Laccase alcohol oxydation to carbonyls or carboxylic acids

The use of isolated laccases for alcohol oxidation to obtain aldehydes was already reported. As a first attempt, we used the commercially available fungal laccase from TvL (Sigma-Aldrich) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; free radical) as a mediator. Reactions were conducted on benzyl alcohol (**1a**; 0.5 mmol) as a model compound, in NaOAc buffer (pH 4.5, 0.1m), and TEMPO (20 mol%) in open vials to ambient air. As expected, the reaction gave benzaldehyde (**2a**) and better yields were obtained in the absence of acetate buffer (Table 8.1.1, entries 1 and 2). However, for prolonged reaction times with closed vials under an oxygen atmosphere, a considerable amount of benzoic acid was obtained (Table 9.1.1, entries 3 and 4). Thus, to increase the efficiency of the process leading to carboxylic acids, further experiments were conducted and the most effective reaction conditions in terms of enzyme amount, solvent and cosolvent, pH, and oxygen source were found (Table 8.1.1). The best experimental conditions were found to be as follows: 1) the use of TEMPO in 20% molar ratio, other mediators, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole (HOBT), and *p*-OH- and *p*-amino- TEMPO, were used without positive results; 2) several cosolvents and additives were tested, such as THF, acetone, DMSO, MeCN, *t*BuOMe, DCM, *t*BuOH, Triton X, two ionic liquids, NaCl, and NBu_4HSO_4, but better results were obtained in pure water or with little amounts of acetone; 3) different pH conditions were explored and better results were obtained with buffers at pH 4.5 or no buffer (the pH becomes acidic during the reaction course); and 4) better results were obtained by bubbling oxygen into the reaction balloon or vial, then closing it with a cap. As far as the reaction time is concerned, reactions were monitored by TLC, HPLC, or NMR spectroscopy generally every 24h and stopped at complete alcohol conversion or when substrate conversion did not proceed further. Representative results on several primary alcohols are reported in Table 8.1.1 ([a] Procedure A: substrate (0.5mmol), enzyme (5mg, 68U), mediator TEMPO (20mol%), solvent (6mL), O_2 bubbled through a closed vial. [b] Ratio between starting alcohol, aldehyde and acid was evaluated by ^{1}H NMR

spectroscopy, or ^{19}F for entries 24 and 25. [c] Formation of acetal between aldehyde and alcohol was observed. [d] Formation of acetophenone, a known by-product of 2-phenylpropanal (**2u**), was observed. [e] Procedure B was used).

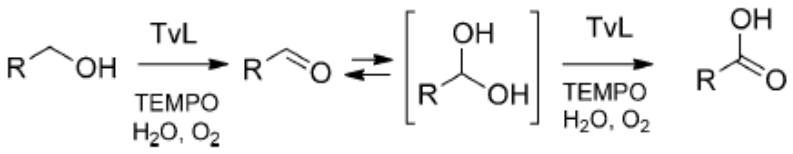


Entry	RCH_2OH (1 a–u)	Conditions[a]	*t* [d]	RCH_2OH (1 a–u)	Yield[b] [%] RCHO (2 a–u)	RCOOH (3 a–u)
1	benzyl alcohol (1 a)	H_2O, pH 4.5 0.1 M, RT	6	80	20	–
2	1 a	H_2O, RT	3	50	50	–
3	1 a	H_2O, RT	8	–	50	50
4	1 a	H_2O, 30 °C	6	–	51	49
5	1 a	H_2O, 30 °C, no enzyme	7	100	–	–
6	cinnamyl alcohol (1 b)	H_2O, RT	8	–	99	–
7	*p*-OMe benzyl alcohol (1 c)	H_2O, RT	5	65	35	–
8	1 c	H_2O, acetone 10 %, RT	6	32	68	–
9	2,4-dimethoxybenzyl alcohol (1 d)	H_2O, RT	5	74	26	–
10	1 d	H_2O, acetone 10 %, 30 °C	5	75	14	11
11	3,4-dimethoxybenzyl alcohol (1 e)	H_2O, RT	7	–	95	5
12	1 e	H_2O, acetone 10 %, RT	7	–	87	13
13	4-nitrobenzyl alcohol (1 f)	H_2O, RT	5	61	33	6
14	2,4-dinitrobenzyl alcohol (1 g)	H_2O, RT	6	25		75
15	pentafluorobenzyl alcohol (1 h)	H_2O, RT	5	–	13[c]	67
16	3,5-ditrifluoromethylbenzyl alcohol (1 i)	H_2O, RT	5	25	48[c]	21
17	2-pyridinemethanol (1 j)	H_2O, RT	0.5	–	–	>99
18	3-pyridinemethanol (1 k)	H_2O, RT	2	–	–	>99
19	4-pyridinemethanol (1 l)	H_2O, RT	4	–	–	>99
20	furfurol (1 m)	H_2O, RT	6	98	traces	–
21	2-thienylmethanol (1 n)	H_2O, RT	7	–	74	26
22	octanol (1 o)	H_2O, RT	8	66	30	traces
23	cyclohexylmethanol (1 p)	H_2O, RT	6	99	–	–
24	trifluoroethanol (1 q)	H_2O, RT	13	85	7	8
25	1 q	H_2O, pH 4.5 0.5 M, RT	43	10	–	90
26	4-phenyl-1-butanol (1 r)	H_2O, RT	7	81	19	–
27	3-phenyl-1-propanol (1 s)	H_2O, RT	6	76	12	12
28	2-phenylethanol (1 t)	H_2O, RT	6	traces	traces[d]	75
29	2-phenylpropanol (1 u)	H_2O, pH 4.5 0.1 M, RT	6	–	traces[d]	60
30	1 u	H_2O, RT	6	–	traces[d]	74
31	1 u	H_2O, acetone 10 %, RT	2	14	–	86
32	(2*S*)-2-phenylpropanol (1 u)	H_2O, RT[e]	5	–	–	>99 (>99 *ee*)
33	(2*R*)-2-phenylpropanol (1 u)	H_2O, RT[e]	5	–	–	>99 (>99 *ee*)

Table 8.1.1 Bio-oxidation of primary alcohols with Laccase from TvL

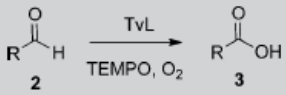
For benzyl alcohols **1a** and **1c-i**, the efficiency in the oxidation to acids strongly depends on the nature of the substituents on the aromatic ring. Donor-substituted benzyl alcohols **1c-e** provided null or modest results, whereas acceptor-substituted benzyl alcohols gave the corresponding benzoic acids in modest to good yields, as in the case of **1g** and **1h** (Table 8.1.1, entries 14 and 15). The best results were obtained with heteroaromatic primary alcohols, in particular, pyridilmethanols **1j-l** gave the corresponding pyridil–carboxylic acids quantitatively (Table 8.1.1, entries 17-19). Aliphatic alcohols **1o** and **1p** reacted poorly, but activated **1q** gave the trifluoroacetic acid, even if extended reaction times were required (Table 8.1.1, entry 25); for substrate **1q**, a buffered solution (pH 4.5) was necessary because of the incoming strong acidity of trifluoroacetic acid, as a matter of fact, in H_2O alone the oxidation to acid was poor (Table 8.1.1, entry 24).

The oxidation of primary alcohols to the corresponding carboxylic acids can be considered a two-step oxidation: first to aldehyde and then to the corresponding carboxylic acid (Scheme 8.1.1). Oxidation in water of aldehydes to acids would proceed through a germinal diol intermediate.[188]



Scheme 8.1.1 The two-step oxidation of primary alcohols

To ascertain effective catalysis on both oxidative steps, we tested some aldehydes as substrates (Table 9.1.2, [a] Procedure A: substrate (0.5mmol), enzyme (5mg), mediator TEMPO (20mol%), solvent (6mL), O2 bubbled through a closed vial. [b] Yields of products isolated after acid-base workup). As a first test, we tried **2a** as a substrate under standard conditions and benzoic acid was successfully obtained (Table 9.1.2, entry 2). With no enzyme, the oxidation was poor; thus giving evidence for a low efficiency of spontaneous aldehyde oxidation (Table 8.1.2, entry 1). Substituted pyridine-carbaldehyde **2j** gave the corresponding acid in excellent yield, whereas **2g** gave a poorer result probably because of the hydrophobicity of the substrate and poor solubility in water (Table 8.1.2, entry 3).



Entry	RCHO (2)	Conditions[a]	*t* [d]	Yield of acid (3)[b] [%]
1	Benzaldehyde (**2a**)	H_2O, 30 °C, no enzyme	6	17
2	**2a**	H_2O, 30 °C	6	77
3	2,4-dinitrobenzaldehyde (**2g**)	H_2O, RT	6	30
4	2-pyridincarboxaldehyde (**2j**)	H_2O, 30 °C	1	>99
5	**2j**	H_2O, pH 4.5k, 2 M, 30 °C	1	>99
6	**2j**	H_2O, RT	0.5	>99
7	3-pyridincarboxaldehyde (**2k**)	H_2O, 30 °C	1	>99
8	**2k**	H_2O, pH 4.5, 2 M, 30 °C	1	>99
9	2-phenylpropanal (**2u**)	H_2O, RT, no enzyme	7	–
10	**2u**	H_2O, 30 °C	7	>99

Table 8.1.2 Bio-oxidation of aldehydes with Laccase from TvL

Concerning the mechanism of the LMS oxidation, Baiocco et al. proposed two possible routes by which the mediator could oxidize the substrate: the Electron Transfer (ET) and Hydrogen Abstraction Transfer (HAT) routes.[189] Kinetic studies on TEMPO strongly supported an ionic hydrogen abstraction route[190] that has precedents in the efficient oxidation procedures of alcohols by TEMPO with chemical oxidants.[191] The effectiveness of the catalytic activity of TEMPO in alcohol oxidation by O_2 is due to the intermediate formation of the oxamonium salt, which is the actual oxidant species[192] and continuously restored by laccase. A tentative ionic route for the two-step oxidation of alcohols to carboxylic acids by TvL laccase/TEMPO is proposed in Figure 8.1.1. The efficiency of the process could depend on the facility of hydrogen abstraction[193] on the starting alcohol and the gem-diol intermediate, together with the hydration equilibrium of the aldehyde. If the hydrogen acidity were too low, oxidation would be difficult.

Moreover, if the solubility of the aldehyde in H_2O were too low and/or its hydration equilibrium unfavorable, the reaction could stop at the aldehyde level. From this perspective, the results obtained, for instance, with **1o**, **1p**, or cynnamyl alcohol (**1b**) could be interpreted.

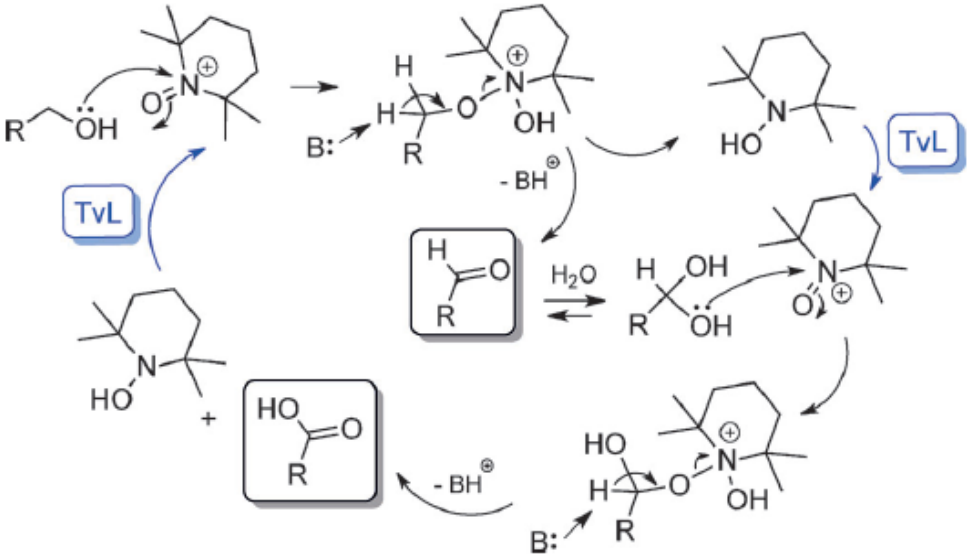
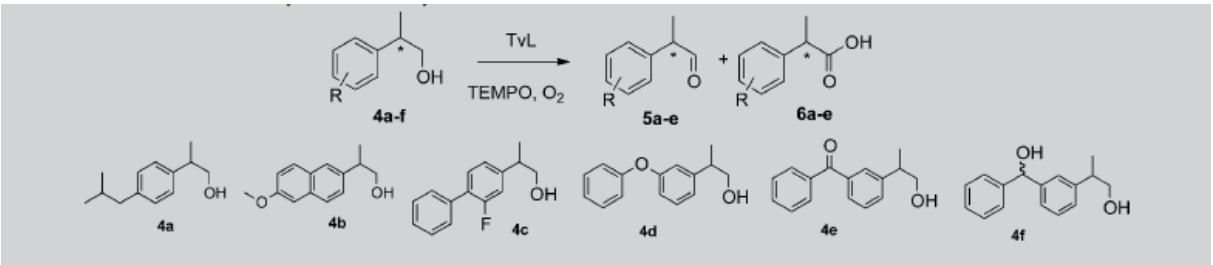


Figure 9.1.1 The ionic route for the two-step oxidation of alcohols

Bio-oxidations by laccases are usually conducted in acetate buffer at pH 4.5-4.8. However, we observed improved yields with unbuffered solutions in water. This result could derive from a salting-out effect in buffered solutions with a lowered solubility of reagents or intermediates. Moreover, it is known that the stability of the TEMPO nitroxyl radical in acidic medium, such as in acetate buffer, is low, and for longer reaction times the mediator decomposed to a greater extent.[194] A better result with unbuffered aqueous solutions could be thus consistent with the pH-dependent stability of the mediator. However, in the case of **1q** (Table 8.1.1, entries 24 and 25), acetate buffer was necessary because during the reaction progress, the incoming trifluoroacetic acid strongly decreases pH and could decompose the mediator and/or denature enzyme over long reaction times. Following our interest in the oxidation of arylpropanols,[195] we then tried the optimized protocol on **1u**, which successfully gave the 2-phenylpropanoic acid in good yields (Table 8.1.1, entries 29-33). The LMS oxidation worked well: conversion was always complete, the selectivity to the carboxylic acid was good, and only traces of the corresponding aldehyde **2u** were detected in the crude reaction mixture. The efficiency and selectivity strongly depended on the relative positions (span) of the aromatic ring with the hydroxy group: **1t** and **1u** were efficiently converted into the carboxylic acids, on the contrary **1r** and **1s** gave none or only traces of the corresponding acids (Table 8.1.1, entries 26-29). To rule out the possibility that any racemization of the stereogenic center occurred during bio-oxidation, we tested (2S)- or (2R)-phenylpropanol (Table 8.1.1, entries 32 and 33). The enantiomeric purity of the starting alcohols was completely retained in the final acids. For a five-day reaction in simple water, enantiomerically pure (2R)- or (2S)-phenylpropionic acids were thus quantitatively obtained.

The good result obtained with **1u** prompted us to explore the laccase-catalyzed oxidation of a series of 2-arylpropanols (Table 9.1.3, [a] Procedure B: substrate (1mmol), enzyme (20mg/mmol), mediator TEMPO (20mol%), solvent (20mL), O_2 bubbled in a closed vial. [b] Yields of products isolated after acid–base workup. [c] S refers to the marked stereogenic center. [d] Yield refers to ketoprofen **6e**). Our attention was addressed to some profenols, **4a-f**, as industrially relevant substrates. All profenols could be oxidized in good to excellent yields with a total retention of configuration when (S)-2-arylpropanols were used (Table 8.1.3, entries 2, 6, 9, 10, 12 and 14). The enantiomerically pure 2-arylpropanols were obtained through enzymatic reduction of the corresponding 2-arylpropanals following the Dynamic Kinetic Resolution (DKR) protocol we developed.

The use of a 10% organic cosolvent, such as acetone or DMSO, gave improved results, depending on the substrate; for instance, compound **4b** gave a better result with acetone 10%, whereas **4c** gave a better result with DMSO (Table 8.1.3, entries 6 and 9). In case of **4e** and **4f**, the use of 10% acetone was detrimental (Table 8.1.3, entries 13 and 15) and resulted in poor yields of ketoprofen; a significant amount of by-products, such as methylarylketones, and partially oxidized products were obtained.

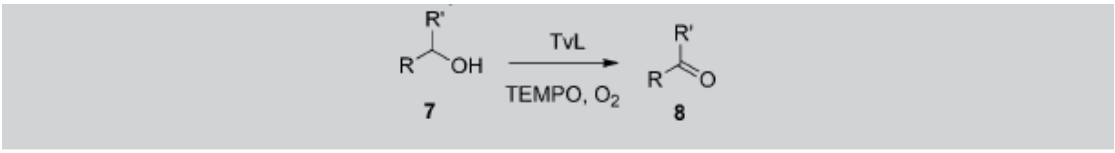


Entry	Alcohol (4a–f)	Conditions[a]	t [d]	Yield[b] [%] aldehyde	acid
1	ibuprofenol (4a)	H_2O, pH 4.5, 0.1 M, RT	6	traces	–
2	(*S*)-ibuprofenol (4a)	H_2O, RT	6	–	79 (*S*/*R* > 99:1)
3	ibuprofenol (4a)	H_2O, acetone 10%, RT	7	–	72
4	naproxenol (4b)	H_2O, pH 4.5, 0.1 M, RT	6	–	–
5	naproxenol 4b	H_2O, DMSO 10%, RT	6	traces	–
6	(*S*)-naproxenol (4b)	H_2O, acetone 10%, RT	7	–	92 (*S*/*R* > 99:1)
7	flurbiprofenol (4c)	H_2O, pH 4.5 0.1 M, RT	6	traces	40
8	flurbiprofenol (4c)	H_2O, RT	4	traces	35
9	(*S*)-flurbiprofenol (4c)	H_2O, DMSO 10%, RT	7	–	99 (*S*/*R* > 99:1)
10	(*S*)-fenoprofenol (4d)	H_2O, RT	7	–	91 (*S*/*R* > 99:1)
11	fenoprofenol (4d)	H_2O, acetone 10%, RT	7	10	13
12	(*S*)-ketoprofenol (4e)	H_2O, RT	7	–	95 (*S*/*R* > 99:1)
13	ketoprofenol (4e)	H_2O, acetone 10%, RT	7	traces	13
14	(*S*)-ketoprofendiol[c] (4f)	H_2O, RT	7	–	82 *S*/*R* > 99:1)[d]
15	ketoprofendiol (4f)	H_2O, acetone 10%, RT	7	traces	24[d]

Table 8.1.3 Bio-oxidation of profenols to profens

To expand the scope, some secondary alcohols were tested under the optimized reaction conditions (Table 8.1.4, [a] Procedure C: the same as A. [b] Yield calculated from NMR spectroscopic analysis of the crude reaction mixture). Cyclohexanol (**7a**), 1-phenylethanol (**7b**), and 1-phenylpropanol (**7c**) gave good results

in the production of the corresponding ketones (Table 8.1.4, entries 1-4). 2-Substituted cyclohexanols **7h-i** gave worse yields (Table 8.1.4, entries 10-13), but they showed stringent stereospecific behavior: only the 1,2-cis-cyclohexanols were oxidized. This is consistent with previously reported results on 2-methylcyclohexanols oxidation by cytochrome P-450; it showed higher reactivity for cis-2-methylcyclohexanol than that of the trans isomer due to steric hindrance.[196] Interesting and promising results were obtained with α-hydroxyacids or α-hydroxyesters. Mandelic acid (**7e**) gave the corresponding oxoacid in satisfactory yields (Table 8.1.4, entries 6 and 7), whereas its methyl ester (**7d**) gave a better result with quantitative yield in a very short reaction time (Table 8.1.4, entry 5).



Entry	RR′CHOH	Conditions[a]	*t* [d]	Yield of RCOR′[b] [%]
1	cyclohexanol (**7a**)	H_2O, RT	6	80
2	1-phenylethanol (**7b**)	H_2O, RT	6	>99
3	**7b**	H_2O, pH 4.5, 0.1 M, RT	7	77
4	1-phenylpropanol (**7c**)	H_2O, RT	7	>99
5	mandelic acid methyl ester (**7d**)	H_2O, RT	0.1	>99
6	mandelic acid (**7e**)	H_2O, pH 4.5, 0.1 M, RT	8	66
7	**7e**	H_2O, RT	8	60
8	diphenylmethanol (**7f**)	H_2O, acetone 10%	7	61
9	lactic acid ethyl ester (**7g**)	H_2O, RT	8	50
10	*cis*-2-methoxycyclohexanol (**7h**)	H_2O, RT	7	12
11	*trans*-2-methoxycyclohexanol (**7h**)	H_2O, RT	7	0
12	*cis*-2-methylcyclohexanol (**7i**)	H_2O, RT	7	42
13	*trans*-2-methylcyclohexanol (**7i**)	H_2O, RT	7	0
14	(−)-menthol (**7j**)	H_2O, RT	7	0

Table 8.1.4 Bio-oxidation of secondary alcohols

Conclusions

There is a great need for sustainable oxidation of fine chemicals by employing clean primary oxidants, such as oxygen and greener catalysts; thus avoiding the use of harsh organic and inorganic oxidants, even in catalytic amounts. From this point of view, the combination of two green and efficient catalysts, such as the commercially available enzyme laccase TvL and the stable free radical TEMPO, offers great opportunities. We widened the range of applicability by exploring the oxidation, in water, of some primary alcohols to the corresponding carboxylic acids or aldehydes and of selected secondary alcohols to ketones. Moreover, we succeeded in an important application: the development of the Laccase-Mediator

System (LMS) oxidation of 2-arylpropanols (profenols) to the corresponding 2-arylpropionic acids (profens), in high yields and with complete retention of configuration.

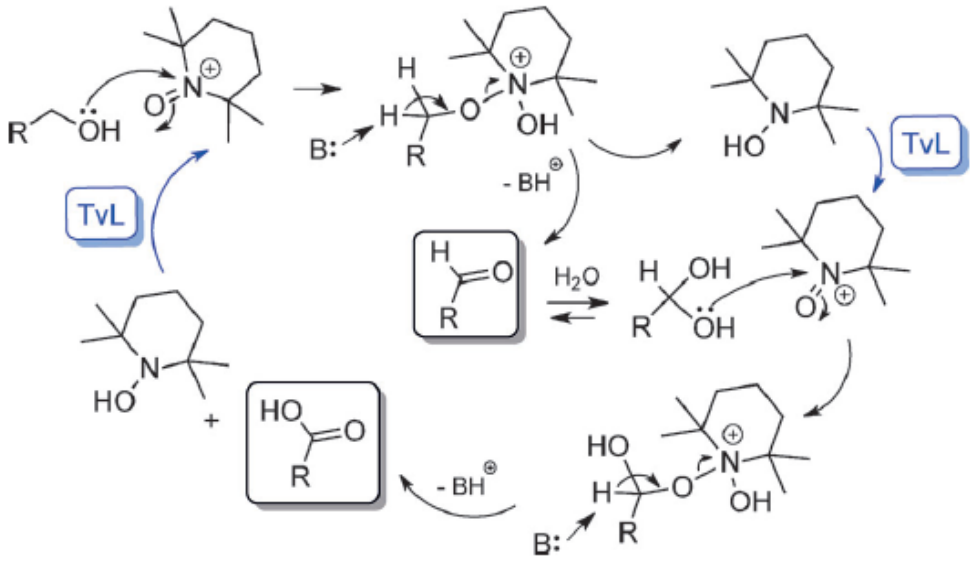


Figure 8.1.2 Chemoenzymatic route to enantiomerically pure arylpropanoic acids

Thus, the chemoenzymatic reduction of arylpropanals we already successfully developed through the DKR process, coupled with the chemoenzymatic oxidation reported herein, depict a more environmentally friendly alternative route to the synthesis of enantiomerically pure profens and contributes to improved sustainability in the synthesis of this important class of drugs (Figure 8.1.2, where HLADH = Horse Liver Alcohol DeHydrogenase, NADH/NAD^+ = Nicotinamide Adenine Dinucleotide redox couple).

8.2 Laccase-Mediator Oxidations Systems (LMS) for amines

8.2.1 Introduction

Selective oxidation of amines is an important tool to get functional-group interconversions in organic synthesis. Depending on amine, catalytic system, reaction conditions, and oxidizing agent, a panel of different products could be obtained, such as, for instance, carbonyl compounds, amides, nitriles, and so on (Figure 8.2.1).[197] Great progress has been made in recent years in developing catalytic and selective methods for amine oxidation.[198] Focusing on primary amines, some relevant transformations should be considered: the oxidation to nitriles or carbonyl compounds, the oxidative self-condensation of the starting substrates to give imines, and with a second amine, the oxidative cross-coupling to give cross imines (Figure 8.2.1).

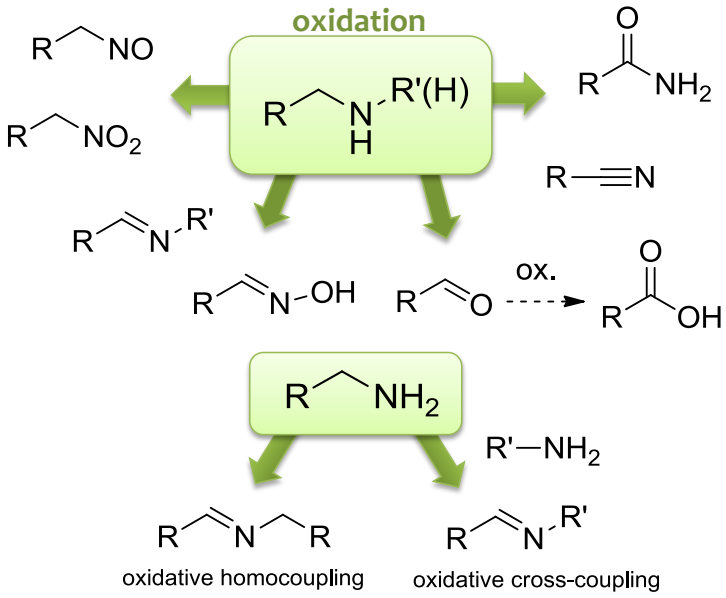


Figure 8.2.1 Functional group diversity generated by amine oxidation

These oxidative dehydrogenation of amines have often been based on transition metal complexes, as catalysts, under aerobic conditions with molecular oxygen as final oxidant[199] and in water as solvent.[200] Other approaches utilized Cu salts/*N*-oxyl radical systems,[201] metal-organic framework solids,[202] gold catalysis,[203] metal free aerobic conditions[204] and, very recently, TiO_2 photocatalytic oxidation in water.[205] However, many of these systems still require harsh reaction conditions, give metal containing wastes, and selectivity can be difficult to achieve and control.

Biocatalysis is emerging as a valuable tool to develop more benign and selective redox processes.[206] Bio-oxidations could have higher selectivity (regio- chemo- or stereo-) suitable even for fine chemicals with complex structures and oxidation sensitive functional groups.[207] In nature, copper amine oxidases (CAOs, EC 1.4.3.21-2) couple the oxidation of primary amines to aldehydes with the reduction of molecular oxygen to hydrogen peroxide using ortho-quinone cofactors.[208]

Laccases belong to the multi-copper family of oxidases (EC 1.10.3.2), they contain four copper centers per protein molecule and catalyze the oxidation of electron rich aromatic substrates, usually phenols or aromatic amines using oxygen as the electron acceptor.[209] Being water the only by-product, Laccases are ideal catalysts for sustainable chemical and technological processes. In fact, they can be used in organic synthesis, have industrial applications, and offer great applications in environmental biotechnology.[210] Although the natural substrates of laccases are phenolic residues of lignin, the use of mediators in the laccase-mediator system (LMS) makes accessible the oxidation of non-phenolic substrates.[211]

Application of LMS in bio-oxidation of alcohols is well documented in the literature.[212] Recently, we reported an application of LMS in the oxidation of some primary alcohols to the corresponding aldehydes and carboxylic acids. Moreover, we succeeded in a relevant application, developing the LMS-oxidation of 2-arylpropanols (Profenols) to the corresponding 2-arylpropionic acids (Profens), in high yields and with

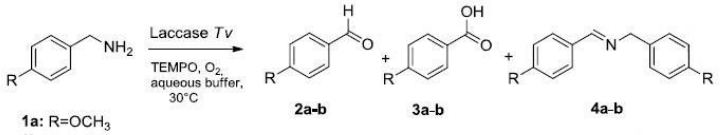
a complete retention of configuration.[213] Concerning the oxidation of amines, few applications of Laccases were reported.[214]

Herein we describe a selective oxidation of amines employing Laccase from *Trametes versicolor* (Laccase Tv) as the enzyme, TEMPO as mediator and O_2 as oxidant, in buffered water as a solvent. We found that, depending on the reaction conditions, the bio-oxidation could be selectively driven to give the corresponding aldehydes or imines in good yields.

8.2.2 Laccase amine selective oxidation to aldehydes or imines

Bio-oxidation of amines was initially investigated starting from the reaction conditions optimized for alcohol oxidation, as we previously reported: Laccase *Tv* (Sigma-Aldrich, 5mg, 50units), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; free radical) 20mol% in water (6mL) at room temperature with O_2 bubbled into the reaction vessel for 30s. In a preliminary attempt *p*OMe-benzylamine (**1a**, 0.5mmol), chosen as a model substrate, did not react (Table 8.2.1, entry 1). The use of unbuffered water as reaction medium, which gave good results with alcohols, in this case failed maybe due to inactivation of Laccase *Tv* at the basic pH generated by amine dissolution in H_2O.[215] The use of acetate buffer at pH 4.5 then resulted in a successful reaction and **1a** was quantitatively converted in the corresponding aldehyde **2a** in 24 hours (Table 8.2.1, entry 2). The product **2a** was easily isolated in quantitative yields from the reaction mixture by a simple solvent extraction. We extended the reaction time to 7 days to test the possibility of a further oxidation of the aldehyde to the corresponding *p*OMe-benzoic acid (**3a**), but only the aldehyde was recovered (Table 8.2.1, entry 3). Use of a lower amount of the enzyme was evaluated and it was observed that the efficiency of the bio-oxidation was maintained until 0.1mg (1U) of Laccase *Tv*, (Table 8.2.1, entry 6), even if after 24 hours the reaction was not complete (conversion 83%) and a considerable amount of the imine **4a** was isolated after work-up (Table 8.2.1, entry 6). On extending the reaction time to 7 days aldehyde **2a** was quantitatively recovered (Table 8.2.1, entry 7). Our attention was then focused on the aqueous reaction medium, and we observed that on lowering the concentration of acetate buffer from 0.5M to 0.2M, a complete conversion was reached in a shorter reaction time (Table 8.2.1, entry 8 versus 2). To stoichiometrically buffer the amine basicity and use the lowest amount of acetate, unbuffered H_2O with 1eq of acetic acid as additive was tested on **1a**, and an efficient oxidation to aldehyde **2a** was achieved (Table 8.2.1, entry 9). In this condition amine and acetic acid formed the corresponding ammonium salt and the amount of free amine in the aqueous solution depends on the hydrolysis constant of the salt. On consuming the free-amine by oxidation, the pH of the reaction solution lowered by the increasing release of acetic acid from the salt hydrolysis. At neutral pH, phosphate buffer pH 7, conversions of 50% and 83% were reached in 24 and 48 hours, respectively. It is interesting to note that at this pH a complete selectivity towards the imine **4a** was obtained after work-up (Table 8.2.1, entries 10-12).

TEMPO, the redox mediator in the oxidation with Laccase, is needed in sub-stoichiometric amounts because Laccases constantly restore the oxamonium ion responsible of the oxidation of the substrate.[216] We then tested the reaction on lowering the amount of TEMPO: from 20 to 10mol% at pH 4.5 the reaction proceeded well with complete conversion and total product selectivity towards the aldehyde, whereas using 5 and 2.5mol% lower conversion and selectivity were obtained (Table 8.2.1, entries 13, 14 and 17). On lowering TEMPO mol% at pH 7, the reaction was slowed down and the conversion was poor but after the work-up only the imine **4a** was recovered (Table 8.2.1, entries 15 and 16). On extending the reaction time to 4 days with TEMPO 2.5mol%, the conversion was complete and the imine **4a** was isolated in 95% yields (Table 8.2.1, entry 18). Under standard conditions in 24 hours (TEMPO 20mol% and acetate buffer, Table 8.2.1, entry 19) benzylamine **1b** gave quantitatively benzaldehyde **2b**, on extending the reaction time to 10 days, as well as on lowering the buffer concentration to 0.2M, considerable amounts of benzoic acid were detected in the reaction mixture (Table 8.2.1, entries 20 and 21). To force the obtainment of the carboxylic acid, the reaction was kept at 50°C for long times but only the aldehyde was recovered (Table 8.2.1, entry 23).



Entry	Substrate	Buffer, pH, conc.	Enzyme amount [mg]	TEMPO [%]	Time	Conversion [%][b]	Selectivity 2 / 3 / 4 [c]	Product [Y%][d]
1	**1a**	Unbuffered water, rt	5	20	8d	0	-	-
2	**1a**	acetate, pH 4.5, 0.5M	5	20	24h	>99	100 / 0 / 0	**2a** (>99)
3	**1a**	acetate, pH 4.5, 0.5M	5	20	7d	>99	100 / 0 / 0	**2a** (>99)
4	**1a**	acetate, pH 4.5, 0.5M	2.5	20	24h	>99	100 / 0 / 0	**2a** (>99)
5	**1a**	acetate, pH 4.5, 0.5M	1	20	24h	>99	100 / 0 / 0	**2a** (>99)
6	**1a**	acetate, pH 4.5, 0.5M	0.1	20	24h	83	60 / 0 / 40	-
7	**1a**	acetate, pH 4.5, 0.5M	0.1	20	7d	>99	100 / 0 / 0	**2a** (96)
8	**1a**	acetate, pH 4.5, 0.2M	5	20	3.5h	>99	100 / 0 / 0	**2a** (95)
9	**1a**	1eq acetic acid	5	20	2.5h	>99	100 / 0 / 0	**2a** (>99)
10	**1a**	phosphate, pH 7, 0.5M	5	20	24h	50	0 / 0 / 100	**4a** (32)
11	**1a**	phosphate, pH 7, 0.5M	5	20	48h	83	0 / 0 / 100	**4a** (80)
12	**1a**	phosphate, pH 7, 0.5M	0.1	20	24h	15	0 / 0 / 100	**4a** (8)
13	**1a**	acetate, pH 4.5, 0.5M	5	10	24h	>99	100 / 0 / 0	**2a** (>99)
14	**1a**	acetate, pH 4.5, 0.5M	5	5	24h	62	60 / 0 / 40	-
15	**1a**	phosphate, pH 7, 0.5M	5	5	24h	45	0 / 0 / 100	**4a** (24)
16	**1a**	phosphate, pH 7, 0.5M	5	2.5	24h	35	0 / 0 / 100	**4a** (20)
17	**1a**	acetate, pH 4.5, 0.5M	5	2.5	24h	44	80 / 0 / 20	-
18	**1a**	phosphate, pH 7, 0.5M	5	2.5	4d	>99	0 / <5 / 95	**4a** (95)
19	**1b**	acetate, pH 4.5, 0.5M	5	20	24h	>99	100 / 0 / 0	**2b** (98)
20	**1b**	acetate, pH 4.5, 0.5M	5	20	10d	>99	57 / 43 / 0	-
21	**1b**	acetate, pH 4.5, 0.2M	5	20	10d	>99	61 / 39 / 0	-
22	**1b**	acetate, pH 4.5, 0.5M	5	2.5	3d	90	75 / 0/ 25	-
23	**1b**	acetate, pH 4.5, 0.5M, 50°C	5	20	10d	>99	100 / 0 / 0	**2b** (>99)
24	**1b**	phosphate, pH 7, 0.5M	5	2.5	2d	40	0 / 0 / 100	**4b** (36)
25	**1b**	phosphate, pH 7, 0.5M	5	2.5	4d	>99	0 / <5 / 95	**4b** (92)

Table 8.2.1 Optimization of reaction conditions[a]

At pH 7 and TEMPO 2.5 mol%, conversion and selectivity towards the imine **4b** was complete, and only traces of a further oxidation to benzoic acid were observed after 4 days (entries 24 and 25 in Table 8.2.1,

always with: [a] Reaction conditions: substrate (0.5mmol), aqueous buffer (6mL), O_2 bubbled in closed vial. [b] Conversion determined on the crude reaction mixture after the work-up. [c] Ratio between acid, aldehyde and imine has been evaluated by ^{1}HNMR. [d] Yields determined on the crude by ^{1}H NMR after solvent extraction and evaporation).

From the initial screening, pH and buffer concentration emerged as important parameters on kinetic and selectivity of the bio-oxidation. Therefore, we performed time course experiments for the oxidation of amine **1a** under three different medium conditions: acetate buffer pH 4.5 0.5M and 0.2M, or H_2O with 1 equivalent of acetic acid as additive (Figure 8.2.2, with amine **1a** consumption, filled tag, and aldehyde **2a** formation, empty tag, depending on buffer concentration or additive). The reaction mixture was analysed via HPLC by sampling until complete conversions. In acetate buffer 0.5M the reaction required 24 hours to give the aldehyde **2a**, whereas on decreasing the buffer concentration (0.2M) or in the presence of acetic acid in H_2O the reaction was faster and complete in 3-4 hours. It is indeed likely that a high buffer concentration could be detrimental, at some extent, for TEMPO oxidation by LMS or for solubility of reactants in the aqueous medium and resulted in a worsening of the whole process.

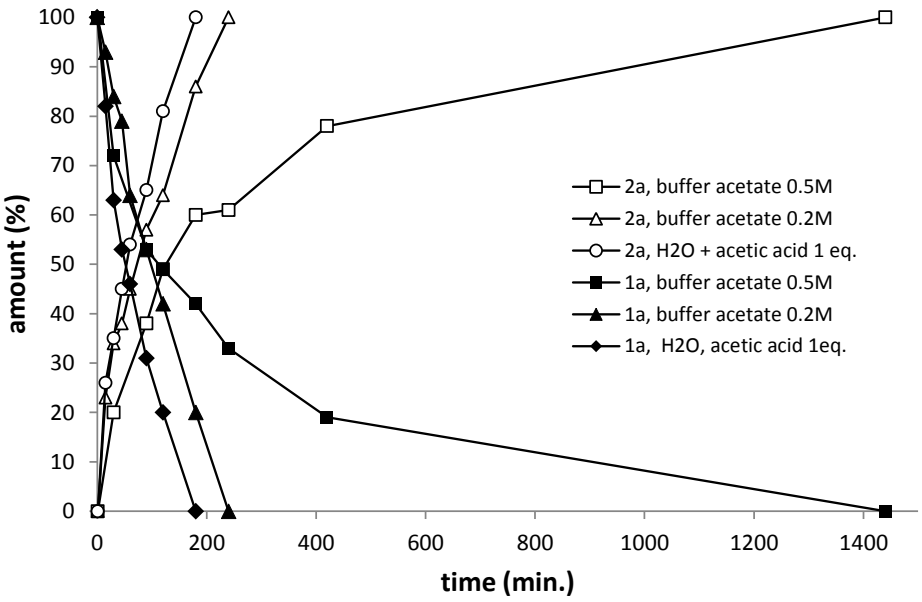


Figure 8.2.2 Time course of enzymatic oxidation

The bio-oxidation reaction is quite clean and its progress could be easily monitored by ^{1}H NMR spectroscopy: as the amine **1a** was consumed the aldehyde **2a** appeared. Figure 8.2.3 reports a time course ^{1}H NMR analysis performed in D_2O with 1 equivalent of acetic acid as additive. In detail, in a NMR tube, **1a** (0.05mmol), D_2O (0.6mL) and acetic acid (0.05mmol) were mixed, then TEMPO (0.01mmol) and Laccase *Tv* (0.5mg, 5U) were added and finally O_2 was bubbled for 30 second, the spectrum was recorded (at t = 2min, upper spectrum, Figure 8.2.3), and the NMR tube was then stirred on an orbital shaker at 150rpm at 30°C. After 60 minutes the tube was re-analyzed and a second spectrum was obtained (Figure 8.2.3).

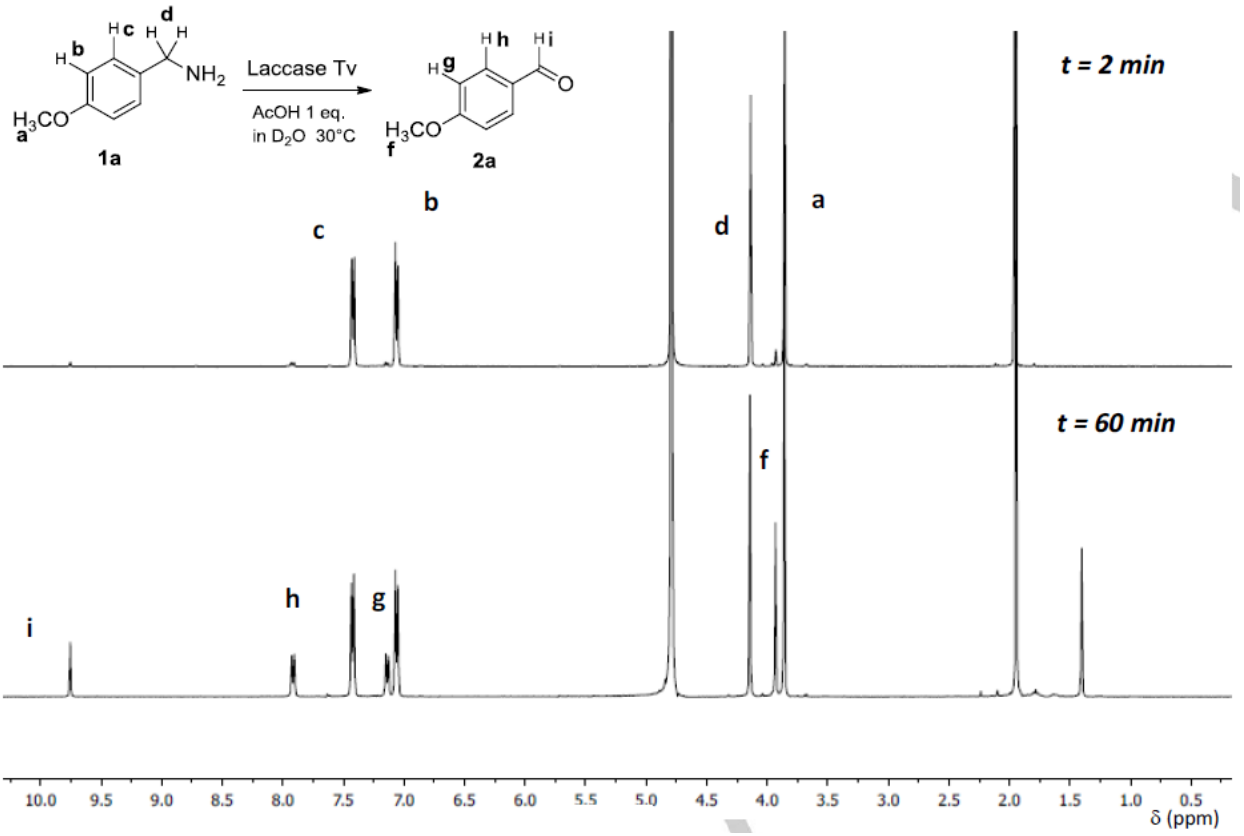


Figure 8.2.3 ^{1}H NMR analysis for Laccase Tv/Tempo oxidation of amine **1a** to **2a**

The NMR analysis showed that from the very beginning together with ^{1}H signals of the starting amine (a-d), traces of the aldehyde **2a** appeared (f-i). After 60 min the aldehyde was as expected much more abundant (about 30%) and noteworthy ^{1}H NMR analysis showed that during the reaction course no other detectable intermediates or by-products were present in the reaction mixture. With a similar NMR protocol, we analyzed the reaction under the conditions to selectively get the imine. Amine **1a** Laccase Tv/TEMPO in buffer phosphate at pH 7 in D_2O were mixed, then O_2 was bubbled for 30 second. On direct sampling and NMR analysis of the reaction mixture, after 2min, only the starting amine **1a** was detected (Figure 8.2.4), after 4h we found a 1/1 mixture of amine **1a** and aldehyde **2a** and no imine present, while after the work-up, imine **4a** was the only product detected (Figure 9.2.4). As a control experiment, equimolar amounts of the commercially available aldehyde **2a** and the amine **1a** were mixed in buffer phosphate at pH 7 in D_2O but in 24h no traces of the corresponding imine **4a** were detected.

It could be then concluded that the imine is not a reaction intermediate and its quantitative obtainment occurred during work-up when the starting amine underwent to condensation with the aldehyde just formed in the bio-oxidation. Having explored the reaction conditions for amines **1a** and **1b**, the optimal conditions to selectively obtain aldehydes (**2a-b)** or imines (**4a-b**) were then established as follows: buffer acetate pH 4.5 at shorter reaction time for obtaining aldehydes, and eventually carboxylic acids at longer reaction time, buffer phosphate pH 7 for obtaining imines.

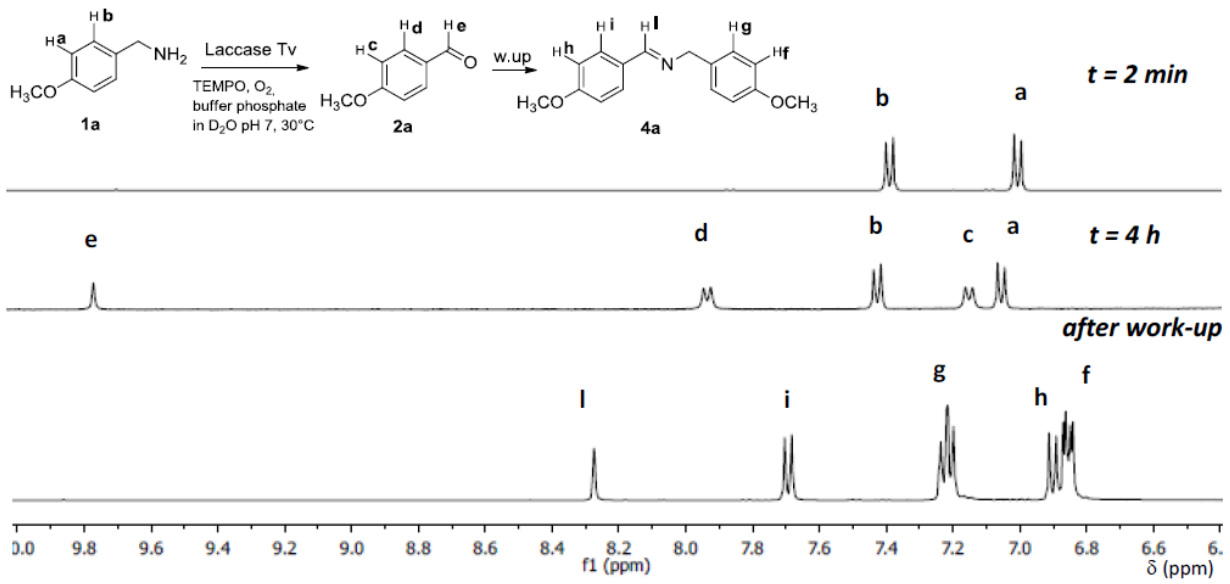


Figure 9.2.4 [1]H NMR analysis for Laccase Tv/Tempo oxidation of amine **1a** to **2a**

Concerning the mechanism of the amine oxidation by Laccase-TEMPO, a tentative hypothesis could be formulated starting from the ionic route proposed for LMS oxidation of alcohols (Figure 8.2.5). The effectiveness of the process could depend on the facility of hydrogen abstraction on the starting amine supported by observation of an easier oxidation of benzylamines than alkyl amines, together with ammonia elimination to give the aldehyde. If the medium conditions slow down the reaction rate at a 50% conversion in the work-up procedure condensation of the residual starting amine with the aldehyde gave the imine. Noteworthy, the aerobic oxidative homo-coupling of amines to imines is going to take great attention as a valuable alternative to the traditional amine–carbonyl condensation,[217] and this is the first result on such an oxidation obtained with enzymatic catalysis.

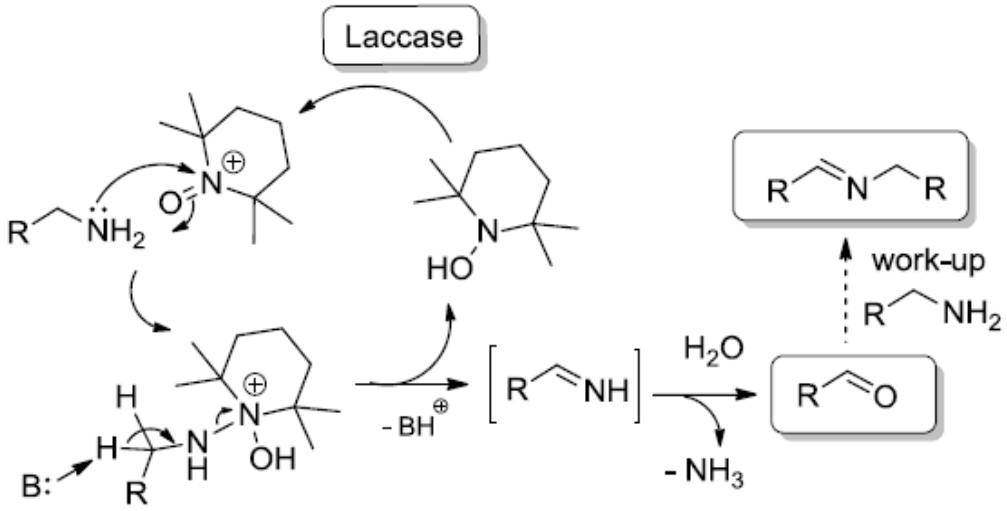
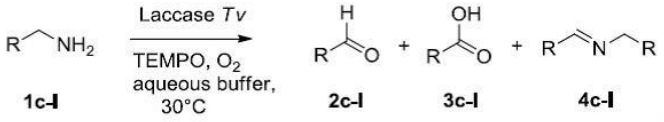


Figure 8.2.5 Proposed mechanism for the bio-oxidation of benzylamines

To study the applicability of the amine bio-oxidation, the scope of the reaction was extended to a series of primary amines (Table 8.2.2, [a] Reaction conditions: substrate (0.5mmol), aqueous buffer (6mL), enzyme (5mg), O_2 bubbled in closed vial. [b] Conversions were evaluated on crude after work-up. [c] Ratio between acid, aldehyde and imine has been evaluated by ^{1}H NMR. [d] Yields determined on isolated products. [e] Polymerization products), under the optimized conditions for a controlled product selectivity. Octylamine **1c**, as a model of aliphatic amines, did not react (Table 8.2.2, entry 1) .



1c-l **2c-l** **3c-l** **4c-l**

Entry	Substrate	Buffer, pH, conc.	TEMPO [%]	Time	Conv. [%][b]	Selectivity 2 / 3 / 4 [c]	Product [Y%][d]
1	octylamine, **1c**	acetate, pH 4.5, 0.5M	20	7d	0	-	-
2	3,4-dihydroxybenzylamine,**1d**	acetate, pH 4.5, 0.5M	20	24h	>99	-	- [e]
3	pyridine-3yl-methanamine, **1e**	acetate, pH 4.5, 0.5M	20	7d	-	-	-
4	2-methoxybenzylamine, **1f**	acetate, pH 4.5, 0.5M	20	24h	>99	100 / 0 / 0	**2f** (98)
5	"	acetate, pH 4.5, 0.5M	20	7d	>99	90 / 10 / 0	-
6	"	acetate, pH 4.5, 0.2M	20	10d	>99	61 / 39 / 0	-
7	"	phosphate, pH 7, 0.5M	5	7d	>99	<5 / 0 / 95	**4f** (95)
8	3,4-dimethoxybenzylamine, **1g**	acetate, pH 4.5, 0.5M	20	24h	>99	100 / 0 / 0	**2g** (98)
9	"	acetate, pH 4.5, 0.5M	20	7d	>99	88 / 12 / 0	-
10	"	acetate, pH 4.5, 0.2M	20	10d	>99	61 / 39 / 0	-
11	"	phosphate, pH 7, 0.5M	5	5d	>99	0 / <5 / 95	**4g** (80)
12	*p*methylbenzylamine, **1h**	acetate, pH 4.5, 0.5M	20	24h	>99	100 / 0 / 0	**2h** (95)
13	"	acetate, pH 4.5, 0.5M	20	7d	>99	77 / 23 / 0	-
14	"	acetate, pH 4.5, 0.2M	20	10d	>99	43 / 57 / 0	-
15	"	1eq acetic acid	20	7d	>99	66 / 34 / 0	-
16	"	phosphate, pH 7, 0.5M	5	7d	57	0 / 0 / 100	**4h** (44)
17	3,5-bis(trifluoromethyl) benzylamine,**1i**	acetate, pH 4.5, 0.5M	20	24h	>99	57 / 0 / 43	-
18	"	phosphate, pH 7, 0.5M	5	4d	59	0 / 0 /100	**4i** (44)
19	*o*chlorobenzylamine, **1j**	acetate, pH 4.5, 0.5M	20	24h	>99	100 / 0 / 0	**2j** (99)
20	"	acetate, pH 4.5, 0.5M	20	7d	>99	50 / 50 / 0	-
21	"	acetate, pH 4.5, 0.2M	20	10d	>99	<5 / 95 / /0	**3j** (88)
22	"	1eq acetic acid	20	7d	>99	10 / 90 / 0	-
23	"	phosphate, pH 7, 0.5M	5	5d	>99	<5 / 0 / 95	**4j** (92)
24	*p*fluorobenzylamine, **1k**	acetate, pH 4.5, 0.5M	20	24h	>99	100 / 0 / 0	**2k** (97)
25	"	acetate, pH 4.5, 0.5M	20	7d	>99	75 / 25 / 0	-
26	"	acetate, pH 4.5, 0.2M	20	10d	>99	59 / 41 / 0	-
27	"	phosphate, pH 7, 0.5M	5	7d	94	0 / 0 / 100	**4k** (92)
28	*p*nitrobenzylamine, **1l**	acetate, pH 4.5, 0.5M	20	24h	>99	50 / 0 / 50	-
29		phosphate, pH 7, 0.5M	5	4d	50	0 / 0 / 100	**4l** (36)

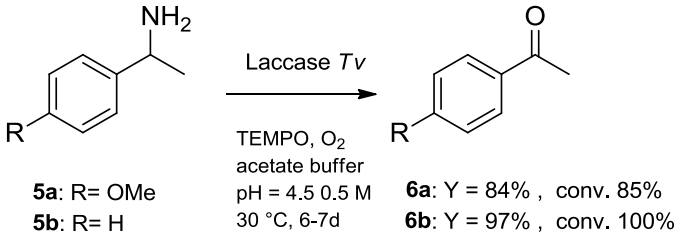
Table 8.2.2 Bio-oxidation of primary amines[a]

This result is indeed consistent with the proposed mechanism of a Laccase-Mediator System (LMS), which supported an ionic hydrogen abstraction route for oxidation with TEMPO[218] (Figure 9.2.5): in this case the α-proton of **1c** acidity was not adequate for an efficient hydrogen abstraction. On the other hand dihydroxybenzylamine **1d** and pyridine-3-yl-methanamine **1e** were completely converted but in a

complex mixture of by-products (Table 8.2.2, entries 2 and 3). Actually, Laccases are well known to oxidize phenolic compounds which are their natural substrates in lignin and to polymerize aniline.[219]

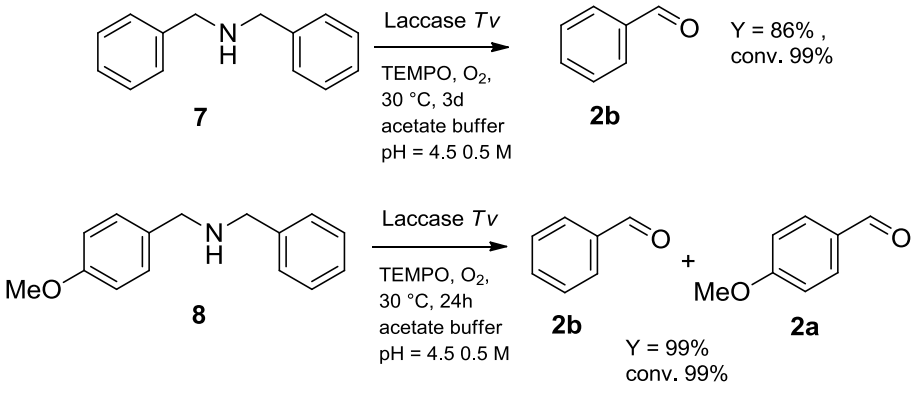
Most of the benzyl amines **1f**-**1l** were selectively converted in the corresponding aldehydes or imines in good yields depending on the selected reaction conditions. Electronic effects associated with electron donating and electron withdrawing substituents on the phenyl ring have little effect on the efficiency of the oxidation reaction. The amines 3,5-bis(trifluoromethyl)-benzylamine (**1i**) and *p*nitrobenzylamine (**1l**) at pH 4.5 gave a 1/1 mixture of the corresponding aldehydes and imines (Table 9.2.2, entries 17 and 28), whereas at pH 7 the selectivity for the imines **4i** and **4l** was successfully obtained, albeit with lower yields (Table 9.2.2, entries 18 and 29). Product selectivity towards the carboxylic acids was obtained with buffer acetate 0.2M and a significant amount of acid was obtained with most benzylamines and, remarkably, *o*chlorobenzylamine **1j** in 10 days was converted in the corresponding acid **3j** in 88% yield (Table 9.2.2, entry 21).

We also applied the Laccase/TEMPO system in the oxidation of α-substituted benzylamine such as 1-arylethylamines **5a**-**b** (Scheme 9.2.2): in both cases the corresponding acetophenones **6a**-**b** were obtained in good conversions and yields.



Scheme 8.2.2 Oxidation of α-substituted benzylamines to give ketones

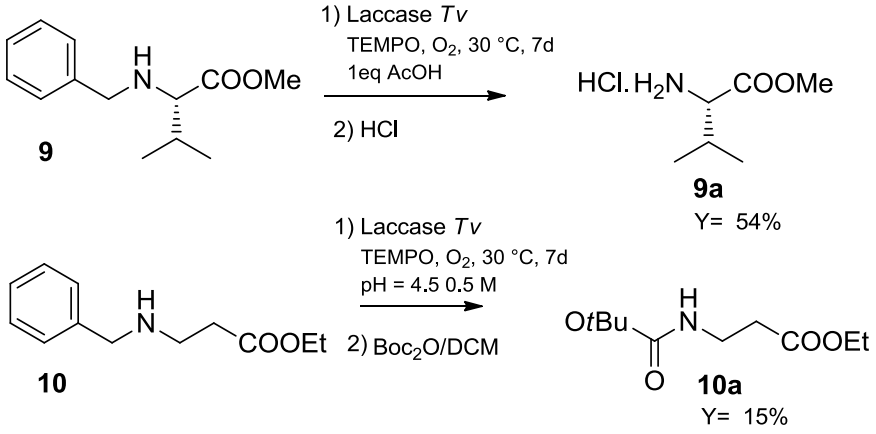
The bio-oxidation was successful also with secondary benzylamines **7** and **8**, as reported in Scheme 9.2.3. Both symmetrical or unsymmetrical substituted benzylamines were readily oxidized to the corresponding aldehydes.



Scheme 8.2.3 Oxidation of secondary dibenzylamines to give aldehydes

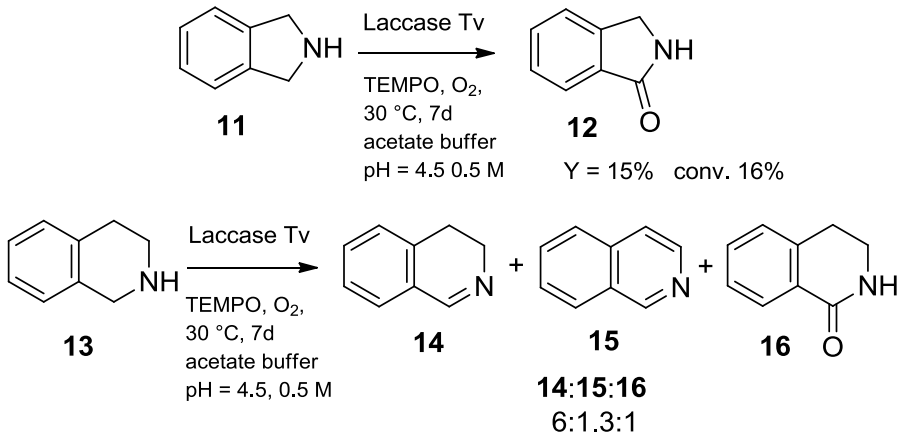
The easy bio-oxidation of benzylamines suggested a tentative exploration in selective oxidation of unsymmetrical secondary amines such as *N*-benzylaminoesters in which the oxidation resulted in the elimination of the benzyl groups as in deprotection steps (as depicted in Scheme 8.2.4). Two substrates were tested, the *N*-benzylvaline methylester **9** and the *N*-benzyl β-alanine ethylester **10**.

Notwithstanding a total conversion of the starting material, products were difficulty recovered as ammonium salt (**9a**) or after derivatization as their corresponding *t*butyloxycarbonylamino derivative (**10a**).



Scheme 8.2.4 Oxidation of *N*-benzyl-aminoesters

Finally, two heterocyclic amine 2,3-dihydro-isoindole (**11**) and tetrahydro-isoquinoline (**13**) were tested (Scheme 8.2.5).



Scheme 8.2.5 Oxidation of isoindoline and isoquinoline

Oxidation of dihydro-isoindole **11** was not efficient, giving poor conversion and yields after 7 reaction days, but selective: dihydro-isoindolone **12** was the only product obtained. In the case of tetrahydro-isoquinoline **13,** conversion was complete in 7 days, but in the reaction mixture, after the work-up, three oxidation products were detected: among them 3,4-dihydro-isoquinoline **14** was the main component, detected by NMR on crude.

8.3 Experimental section

8.3.1 General informations

As previously reported (Paragraph 2.3.1).

8.3.2 Laccase alcohol oxidation to carbonyls or carboxylic acids

Starting materials: alcohols and aldehydes used as starting materials in Tables 8.1.1, 8.1.2 and 8.1.4 were commercially available or known compounds.

Primary alcohols **1c**, **1d**, **1f**, **1j**, **1k**, **1l** and **1p** were obtained by reduction of the corresponding commercial aldehyde with $NaBH_4$ in MeOH; compound **1g** was obtained by reduction of the corresponding aldehyde with BH_3·THF (5eq) in THF; **1h** and **1p** were obtained from the corresponding carboxylic acids by reduction with borane-dimethylsulfide (BH_3·Me_2S) in Et_2O; **1s** was obtained by reduction from **1b** with H_2 on Pd/C.

Racemic 2-arylpropanols **4a-f** in Table 8.1.3 were obtained by BH_3·Me_2S reduction of racemic commercial acids, enantiomerically pure (S)-**4a-f** were obtained by enantioselective biocatalysis starting from the corresponding racemic aldehydes.

Secondary alcohols **7a**, **7b**, **7f**, **7i**, and **7j** were obtained by $LiAlH_4$ reduction from the corresponding ketone in Et_2O; **7d** was obtained by acid-catalyzed esterification of **7e** and MeOH.

Procedure A

Experimental oxidation procedure for compounds reported in Tables 8.1.1 and 8.1.2: TEMPO (0.1mmol) and the enzyme (5mg) were added to a stirred solution of the alcohol or aldehyde (0. mmol) in the appropriate solvent (6mL) in a 10mL vial with a screw cap, then O_2 was bubbled for 30s. The solution was stirred on an orbital shaker at 150rpm, retained, and the reaction was monitored by TLC. When the reaction was complete, the aqueous solution was kept at 0°C and adjusted to pH 2 by slow addition of aqueous HCl (1M). The acid aqueous phase was then extracted with DCM (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum, and analyzed by HPLC and ^1H and ^{19}F NMR for alcohols **1h** and **1i**; ^{19}F NMR for **1q**. In the case of compounds **1j**, **1k**, **1l**, **2j** and **2k** the crude aqueous phase was directly lyophilized and analyzed by ^1H NMR spectroscopy. Spectroscopic data were consistent with those reported in the literature and in the NMR spectroscopy database (Reaxys and AIST SDBS).

Procedure B

Experimental procedure for the synthesis of 2-arylpropionic acids (profens; Table 9.1.3): TEMPO (0.2mmol) and the enzyme (20mg) were added to a stirred solution of the alcohol **4a-f** (1mmol) in the appropriate solvent (20mL) in a 50mL balloon. O_2 was bubbled for 30s and then the balloon was closed

with a cap. The solution was stirred on an orbital shaker at 15rpm and kept at room temperature. The reaction course was monitored by TLC. When the starting material disappeared, an aqueous saturated solution of $NaHCO_3$ at 0°C was added to the flask followed by DCM (2mL). The organic layer was separated and discharged, then the aqueous solution was kept at 0°C and adjusted to pH 2 by slow addition of aqueous HCl (1M). The acid aqueous phase was then extracted with DCM (3x15mL). The collected organic phases were dried over Na_2SO_4, filtered, and concentrated in vacuum to afford 2-arylpropionic acids. NMR spectroscopy and HPLC data of obtained products were consistent with those previously reported. Enantiomeric excess values were obtained on HPLC chiral columns.

Procedure C

Experimental oxidation procedure for compounds reported in Table 9.1.4: TEMPO (0.1mmol) and the enzyme (5mg) were added to a stirred solution of the alcohol (0.5mmol) in the appropriate solvent (6mL) in a 10mL vial with a screw cap, and then O_2 was bubbled for 30s. The solution was stirred on an orbital shaker at 150rpm, maintained at room temperature, and the reaction was
monitored by TLC. In case of compounds **7b**, **7c**, **7d**, and **7f**, when the reaction was complete, the aqueous solution was extracted with EtOAc (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum, and analyzed by HPLC and ^1H and ^{13}C NMR spectroscopy. In case of compound **7e**, when the reaction was complete, the aqueous solution was kept
at 0°C and adjusted to pH 2 by slow addition of aqueous HCl (1M). The acid aqueous phase was then extracted with DCM (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum, and analyzed by HPLC and ^1H and ^{13}C NMR spectroscopy. In the case of compounds **7a**, **7g**, **7h**, **7i**, and **7j**, when the reaction was complete, the aqueous solution was extracted
with Et_2O (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum, and analyzed by HPLC and ^1H and ^{13}C NMR spectroscopy. Spectroscopic data were consistent with those reported in the literature and in the NMR spectroscopy database.

8.3.3 Laccase amine selective oxidation to aldehydes or imines

Starting amines **1a-l**, **5a-b**, **7**, **11**, **13**, Laccase from *Trametes versicolor* and TEMPO were purchased from Sigma-Aldrich (Sigma 51639, 10U/mg), Benzylamines **8**, **9** and **10** were prepared by alkylation with benzylbromide. All the obtained products were known and their spectroscopic data were consistent with those reported in the literature and in NMR database (Reaxys and AIST SDBS).

General procedure for aldehydes

To a stirred solution of the amine (0.5mmol) in the appropriate solvent, (acetate buffer pH 4.5 0.5M or 1eq. of acetic acid in H_2O) (6mL) in a 10mL vial with a screw cap, TEMPO (0.1mmol) and the enzyme (5mg, 50U) were added. O_2 was bubbled for 30 seconds and the vial was closed. The solution was stirred

on an orbital shaker at 150rpm and kept at 30°C in thermostat. After completion (TLC monitoring), the aqueous solution was extracted with EtOAc (3x5mL). The aqueous phase was then adjusted to pH 2 by slow addition of aqueous HCl (1M) and then extracted with EtOAc (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum and analyzed by ^{1}H NMR and ^{13}C NMR.

General procedure for imines

To a stirred solution of the amine (0.5mmol) in buffer phosphate pH 7.5 0.5M (6mL) in a 10mL vial with a screw cap TEMPO (0.025mmol) and the enzyme (5mg) were added and then O_2 was bubbled for 30 seconds. The solution was stirred on an orbital shaker at 150rpm and kept at 30°C in thermostat. When the reaction was complete or after 7 days the aqueous solution was extracted with DCM (3x5mL) and EtOAc (3x5mL). The aqueous phase was then adjusted to pH 9 by slow addition of aqueous NaOH (1M) and then extracted with DCM (3x5mL). The collected organic phases were dried over Na_2SO_4, filtered, concentrated in vacuum, and analyzed by ^{1}H NMR and ^{13}C NMR.

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