UNIVERSITY OF PAVIA

Ph.D. School in Chemical and Pharmaceutical Sciences (XXXII Cycle)

Stereochemical Control in the Synthesis of AOSA: key intermediate for the preparation of Carbapenem Antibiotics

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„ I terribili semplificatori sono quelli che lavorano poi sulla paura, sono i fondamentalisti, sono i terroristi ma sono anche quelli che non vogliono faticare a capire, faticare a incontrare. Stiamo attenti ai terribili semplificatori, perché seminano nella loro irresponsabilità. E qui c'è una grande responsabilità . . . di spiegare che il mondo è complesso, che non si può essere ignoranti in un mondo come questo. “

A. Riccardi

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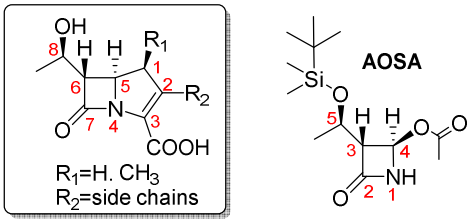
1 Abstract

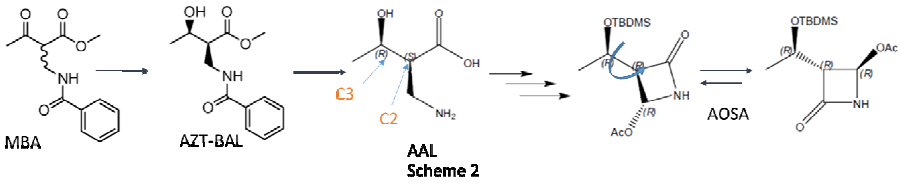
Nowadays, pharmaceutical companies are continually requested by regulatory authorities all over the world, to ensure highest quality of their products. One of the main problems concerns the control of the stereochemistry, which for chiral drugs and intermediates requires asymmetric synthesis. In this context, (*3R,4R*)-4-Acetoxy-3-[(*R*)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone, known with the acronym **AOSA** is a key intermediate, and regulatory starting material, for the preparation of carbapenem antibiotics. They are an important class of drugs that feature a wide spectrum of activity and good resistance to in vivo degradation. Aim of this project has been to build up a robust stereochemical control strategy for this key intermediate, through the preparation and characterization of a small library of selected stereoisomers to be used as reference standards in routine analytical tests.



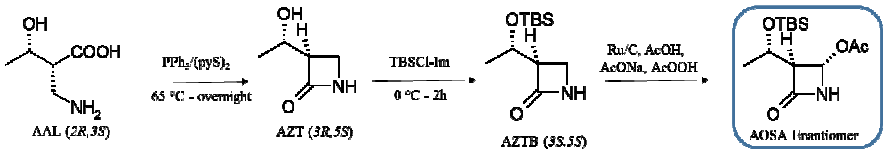
Scheme 1

Background The emergence of multi-drug resistant bacteria is a phenomenon of concern to the clinical and pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases. Carbapenems are β-lactam antimicrobial agents, with an exceptionally broad spectrum of activity, stable to most beta-lactamases. Carbapenems core structure is reported in Scheme 1. One of the main problem in managing the manufacturing of these compounds concerns the control of the stereochemistry of the sterocenters 1, 5, 6 and 8 (Scheme 1 for numbering), as its correct configuration is mandatory for the biological activity. Therefore, regulatory authorities impose severe control strategies on stereochemistry of brand new or generic drugs and often the non-active stereoisomers are required as controls.

Results and discussion: AOSA exhibits 3 sterocenters at C3, C4 and C5 (Scheme 1). Therefore, beside the right stereoisomer, there are additional 7 stereoisomers. Since absolute configuration of stereocenter in C4 is lost in subsequent reactions the main focus has been put on C3 and C5. AAL (2S,3R) (scheme 2) is the key intermediate in the synthesis of AOSA and C2 and C3 in its structure define the final AOSA absolute configuration. Therefore different protocols have been investigated to control reduction

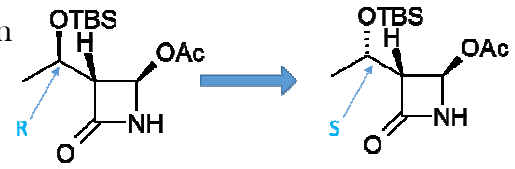


Scheme 2

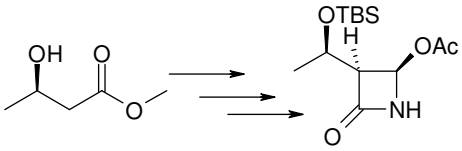


Scheme 3

reaction from MBA precursor in order to obtain different AAL isomers. The use of Biocatalyst was successful in obtaining all different AZT-BAL precursor but shown low performance in conversion. Asymmetrical Hydrogenation with chiral catalysts was then applied obtaining the *syn* couple of AAL precursor and in particular the fundamental enantiomer AAL (2R,3S). This important intermediate has been successfully carried over to AOSA enantiomer, through a route of synthesis developed specifically for this purpose (scheme 3). Since the *anti* couple of AAL precursor were not possible to be achieved with the same technology, a different approach was tested. Inversion of stereocenter in C5 was performed starting form AOSA (3R,4R,5R) following rational outlined in scheme 4. AOSA was treated with $NaBH_4$ to perform a reductive de-acetoxylation. Intermediate was then de-silylated and inversion of Hydroxyl in C5 performed through a Mitsunobu reaction. Subsequent silylation and Ruthenium mediated acetoxylation afforded AOSA(3R,4R,5S). Regarding missing forth stereoisomer AOSA (3S,4R,5R) a different protocol has been developed since performing inversion on AZT (3S,5S) would have required too much starting material considering the low overall process yield. Starting from Methyl-(R)-3-hydorxybutyrrate (scheme 5) a new synthetic pathway was developed taking advantage of its stereochemical configuration at C3. A 7 step synthesis was developed affording the forth missing stereoisomer AOSA (3S,4R,5S). The successful synthesis of these selected stereoisomers of AOSA has allowed the development of robust HPLC methods to assure the control of potential related stereoisomer in this regulatory starting material. In particular, a chiral method was developed to separate each single stereoisomer. In



Scheme 4



Scheme 5

addition, an optimized protocol of reductive de-acetoxylation has allowed us to simplify the scenario by limiting the analytical control to sterocenters at C3 and C5. Taking advantage of this achievement, a traditional and effective HPLC method was developed on de-acetoxylated derivative of AOSA (AZTB). These analytical protocols will be implemented on routine test, assuring a robust control on AOSA stereochemistry.

2 Introduction

2.1 Antimicrobial Resistance

Antimicrobial resistance (AMR or AR) is the ability of a microbe to resist the effects of medication that once could successfully treat the microbe. The term antibiotic resistance (AR or ABR) is a subset of AMR, as it applies only to bacteria becoming resistant to antibiotics. AMR is rising to dangerously high levels in all parts of the world. Bacterial resistance is not a new phenomenon. We now recognize that resistance is the inevitable consequence of organisms competing for the same ecological niche. Yet it is only during the past 80 years that resistance has been transformed by man (as the driving evolutionary force) from what might be reasonably described as stasis bacteria competing against bacteria to that of a disequilibrium of chemical warfare, where bacteria additionally compete directly with us. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. A growing list of infections – such as pneumonia, tuberculosis, blood poisoning, gonorrhea, and foodborne diseases are becoming harder, and sometimes impossible, to treat as antibiotics become less effective. Globally, 480.000 people develop multi-drug resistance each year and the cost of health care for patients with resistant infections is higher than care for patients with non-resistant infections due to longer duration of illness, additional tests and use of more expensive drugs[1]. The economic impact related to antimicrobial resistance was expected to cost over $105 billion annually worldwide and the largest relative economic impact was projected to be suffered by Africa with a drop in GDP of $2895 billion in 2050, representing 20% of the region's total economic output[2]. Without urgent actions and effective antibiotics, the success of major surgery and cancer chemotherapy would be compromised, heading for a post-antibiotic era, in which common infections and minor injuries can once again kill. In this threatening scenario pharmaceutical industry is called, now more than ever, to invest and innovate by developing new and more effective antibiotics[3].

2.2 Beta-Lactams antibiotics

β -lactams are a class of broad spectrum antibiotics, consisting of all antibiotic agents containing a β –lactam ring in their molecular structure and they work by inhibiting cell wall biosynthesis in the bacterial organism. The discovery and development of the β-lactam antibiotics are among the most powerful and successful achievements of modern science and technology. Since Fleming's accidental discovery of the penicillin-producing mold, ninety years of steady progress has followed, and today the β-lactam group of compounds are the most successful example of natural product application and chemotherapy[4]. β-lactams antibiotics are indeed an important component of the antimicrobial armamentarium of the infectious disease specialist, used in the treatment of a variety of Gram-negative and Gram-positive infections[5]. These agents represent >65% of the world antibiotic market with >50 marketed drugs of this class[6]. This class of compounds are categorized by regulatory agencies in five classes (Figure. 1):

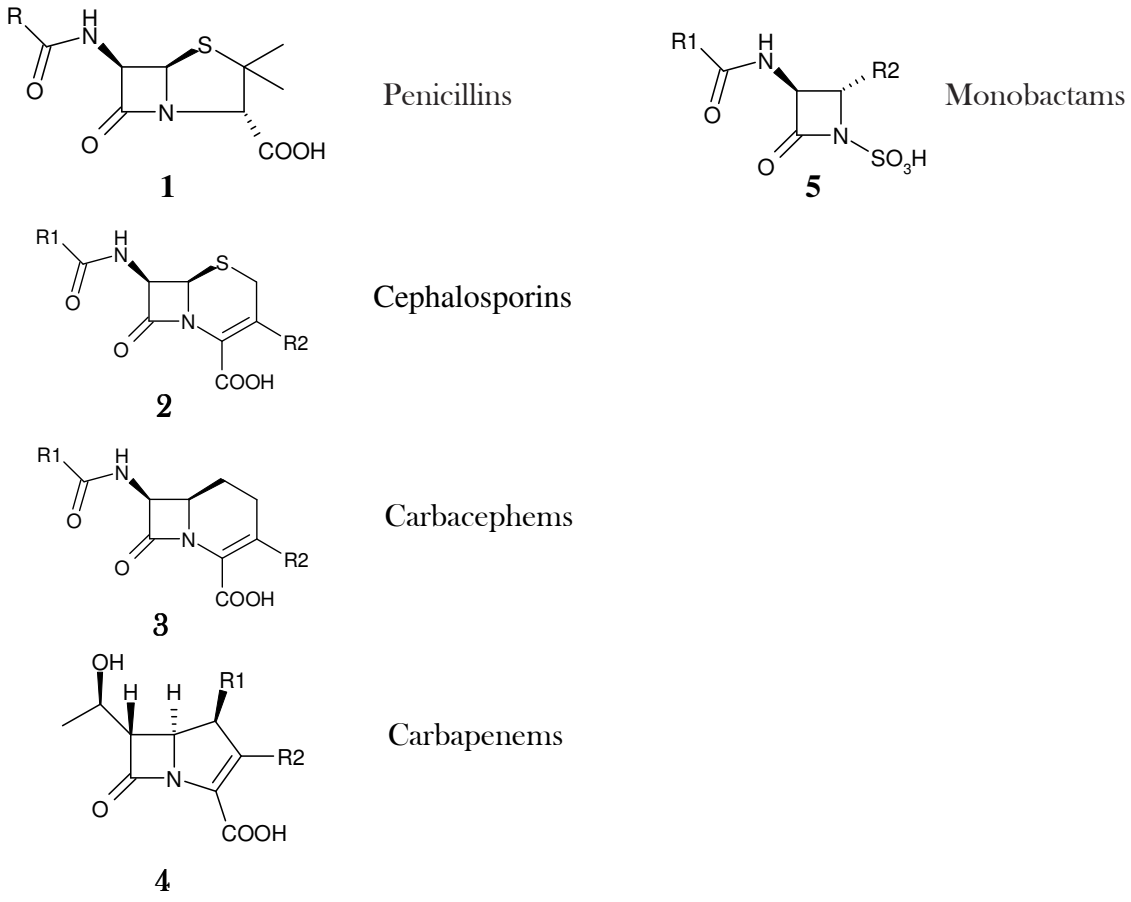


Fig. 1: classes of β –lactam antibiotics

2.3 Beta-Lactams discovery and development for clinical use

After Alexander Fleming's serendipitous discovery of penicillin and its efficacy in fighting against bacteria, the focus on this new chemical entities has been actually enormous in the following years. Lot of efforts were put to study and develop an effective synthesis on large scale to allow clinical study of penicillin but, while the discovery was made in 1928, the use of penicillin as a therapeutic agent to treat infection did not happen until 1940s with the launch of penicillin G5 in the US market[7]. Despite all efforts, the first signs of resistance to penicillin G were already evident in 1944, against *Staphylococcus aureus*. The subsequent isolation of β-lactam nucleus, 6-aminopenicillanic acid (6-APA **6**; Figure 2), proved to be the key in penicillin synthesis and modification. This important milestone opened the floodgate where novel β-lactam agents could be mass-produced by adding unusual side chains to 6-APA.

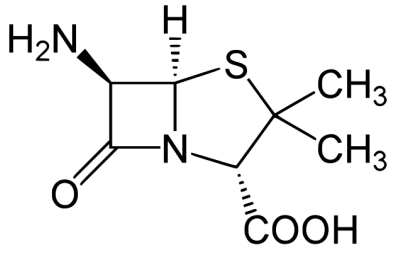


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Fig. 2: (1*S*,7*R*)-7-amino-3,3-dimethyl-6-oxo-2-thiabicyclo[3.2.0]heptane-4-carboxylic acid.

Though the generation of semi-synthetic compounds presented a great opportunity, natural sources continued to be explored. Abraham and Newton isolated cephalosporin C from a strain of *Cephalosporium acremonium*[8]. This compound generated an entire new family of β-lactam antibiotics because, instead of 6-APA, it possess a nucleus of 7-amiunocephalosporanic acid (7-ACA, Figure 3).

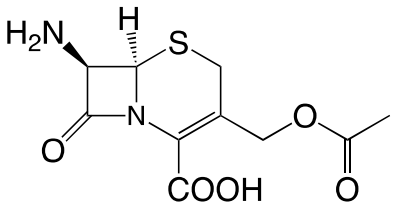

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Fig. 3: (1*S*,8*R*)-4-(acetoxymethyl)-8-amino-7-oxo-2-thiabicyclo[4.2.0]oct-4-ene-5-carboxylic acid (7-ACA)

Using 7-ACA as precursors, several generation of cephalosporin with potent broad-spectrum activity have been synthetized. Many related naturally occurring compounds with important antibiotic activity were isolated form bacteria. These include Carbapenem from *Streptomices rugosa*[9], and monobactams from *Pseudomonas acidophila*. Penem and monbactam contained different β-lactam nuclei from 6-APA and 7-ACA. Derivatives of these compounds, natural either occurring or semi-synthetic, further expanded the spectrum of β-lactam agents. In particular, Carbapenem have demonstrated a formidable efficacy and broad spectrum activity in fighting bacteria. Due to their importance, this last-resort class of β-lactams will be deeply discussed in this PhD work.

2.4 Mechanism of Action

Mechanism of action of β-lactam antibiotics consist in the inhibition of bacteria cell wall synthesis by interfering with the enzymatic transpeptidation process, which promotes polymeric peptidoglycan reticulations. This mechanism of action has been attributed to the possibility that β-lactam nucleus acts as structural analogous of D-alanine (Fig 4)

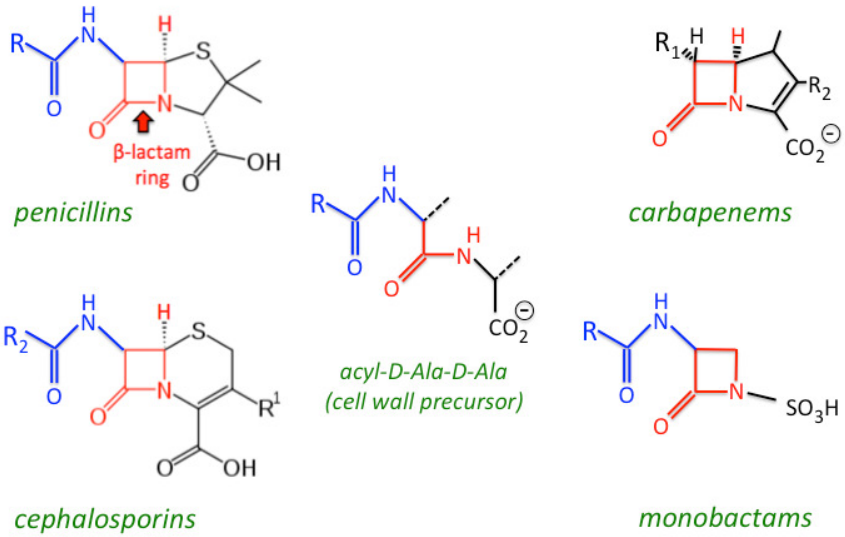
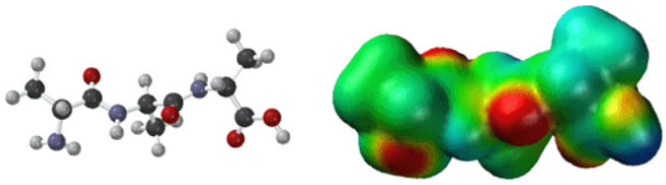


Fig 4: Core structure of beta-lactam antibiotics. All beta-lactam antibiotics contain the same core 4-member "beta-lactam" ring (highlighted in red).

The 4-member ring of beta-lactam antibiotics gives these compounds a three-dimensional shape that mimics the D-Ala-D-Ala (Fig 4) peptide terminus that serves as the natural substrate for transpeptidase activity during cell wall synthesis.

D alanine-alanine



Pennicillin

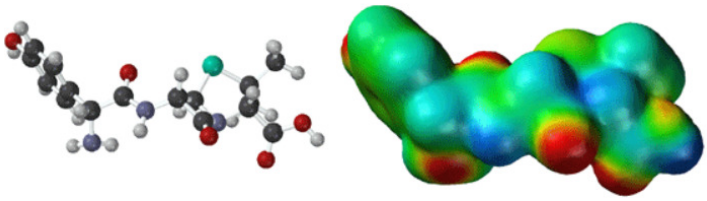


Fig *5*: Resemblance between pennicillin and D-Alanine-Alanine molecular structure[10]

As illustrated in Figure 6[11], tight binding of these drugs to the transpeptidase active site (also known as Penicillin Binding Proteins, **PBPs**)[12] inhibits cell wall synthesis, resulting in a weakened cell wall that is susceptible to lysis during periods of cell growth. One of the major driving forces of cell lysis is the very high internal osmotic pressure present in bacteria, which is caused by the presence of a high concentration of proteins and other molecules that growing bacteria need to survive.

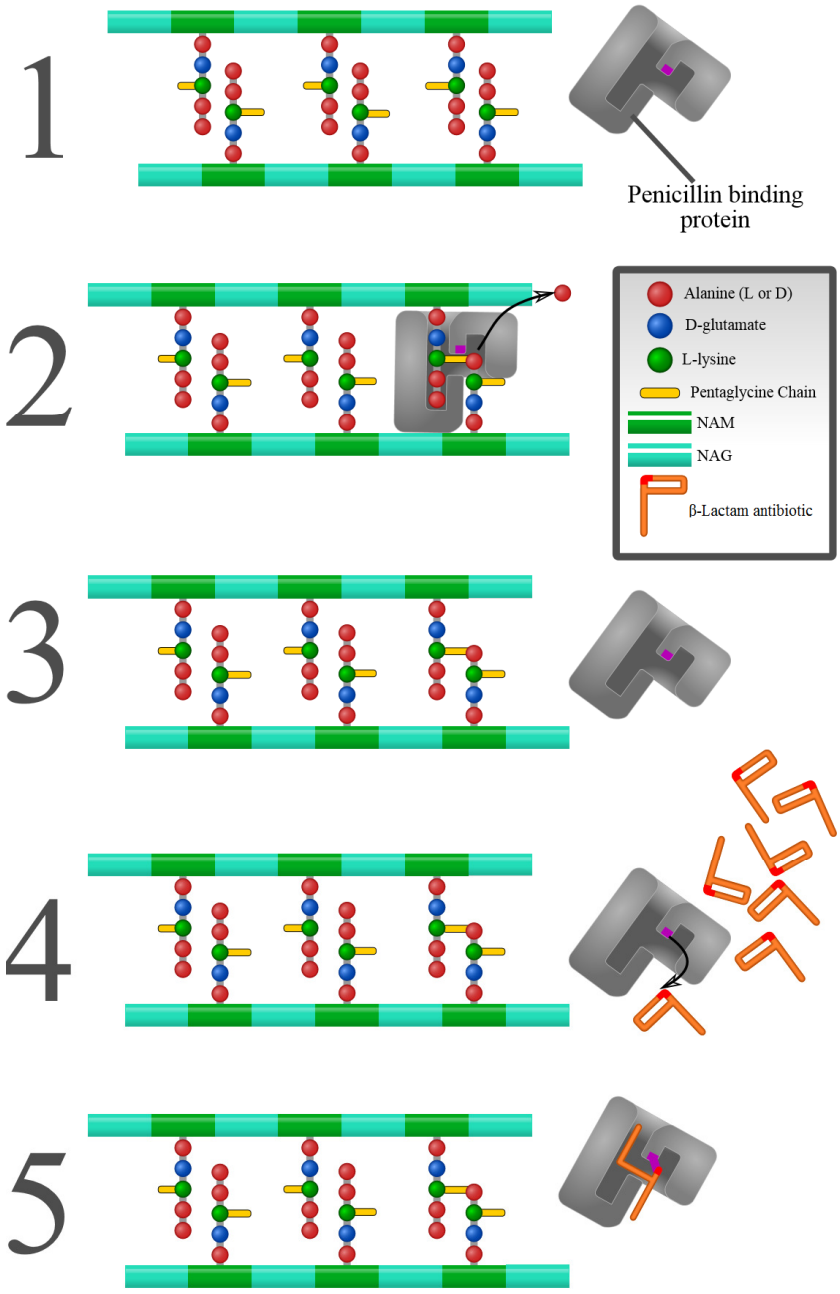


Fig 6: Mechanism of action of β-lactam antibiotics.

In the absence of drug, transpeptidase enzymes (also known as Penicillin Binding Proteins; PBP) in the cell wall catalyze cross-links between adjacent glycan chains, which involve the removal of a terminal D-alanine residue from one of the peptidoglycan precursors (highlighted by the broken oval). Glycosyltransferases (GT), which exist as either separate subunits, or tightly associated with transpeptidases (e.g. as is the case for PBP-2) create covalent bonds between adjacent sugar molecules NAM (N-acetylmuramic acid) & NAG (N-acetylglucosamine). The net result of covalent bonds

between both the peptide and sugar chains creates a rigid cell wall that protects the bacterial cell from osmotic forces that would otherwise result in cell rupture. Beta-lactam antibiotics, which include penicillins (Pen), cephalosporins (Ceph), monobactams (Mono) and carbapenems (carb) bear a structural resemblance to the natural D-Ala-D-Ala substrate for the transpeptidase, and exert their inhibitory effects on cell wall synthesis by tightly binding to the active site of the transpeptidase (PBP). In β-lactam antibiotics heterocyclic moiety is still covalently bound and cannot be removed as per D-Alanine in normal process. The process is irreversible and the result is a defected wall with inactivation of the enzyme.

Molecular target, such as PBPs, can be different and with a different distribution considering different bacteria. For instance *Staphilococcus aureus* has 4 PBPs, *E.Coli* 7 different PBPs with different affinity for β-lactams; a covalent bond of *E. Coli* PBP 1a and 1b brings to inhibition of cell wall synthesis. Inibhition of other PBPs can instead lead to retarded lysis (PBP2) or formation of long chains forms of bacteria.

β-lactam structures penetrate in bacterial cells through aqueous hydrophilic channels of external membrane, such as porins, and show different selectivity of one or more PBPs. Once covalently bonded by β-lactam synthesis of peptidoglycan is inhibited (Figure 7).

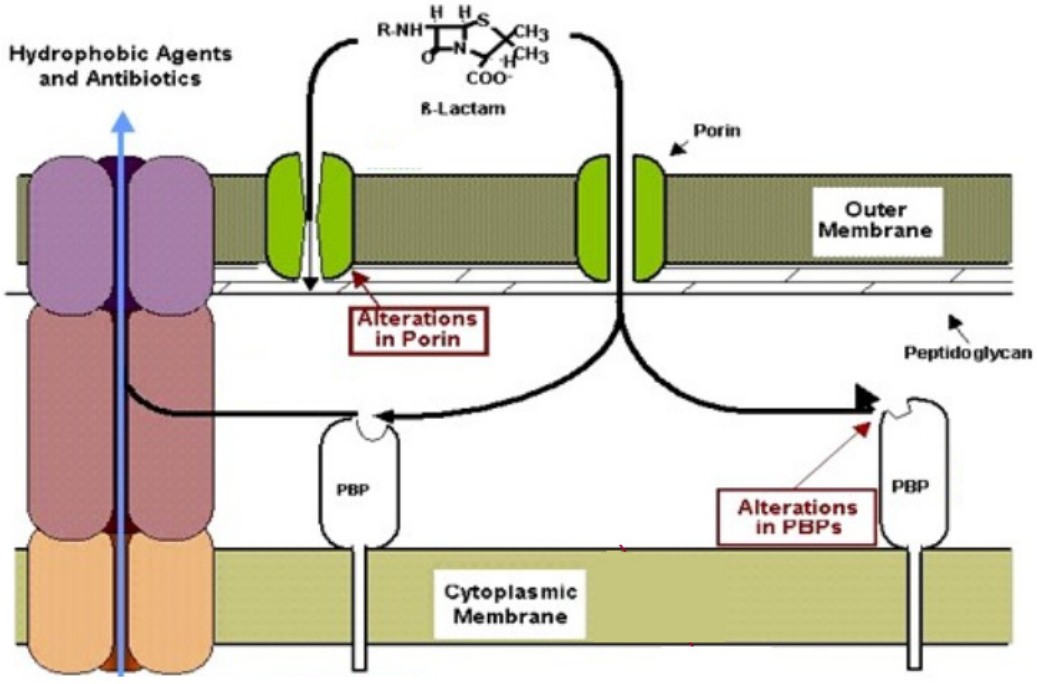


Fig 7: Mechanism of penetration of β-lactam inside a bacterial cell of Neisseria Gonorrhoeae with subsequent binding to PBP. Alteration in penetration mechanism, suc as alteration in porins, or chromosomally mediate alteration of binding site (PBPs) can lead to antibiotic resistance.

2.5 β-lactams Resistance

Antibiotics have been hailed as one of the greatest medical advance in modern age. However, Alexander Felming's less celebrate (but very important) observation was that non-lethal concentrations of penicillin could rapidly generate penicillin-resistant microbes[13]. Once an antibiotic is widely used resistance is an inevitable process (Figure 8)

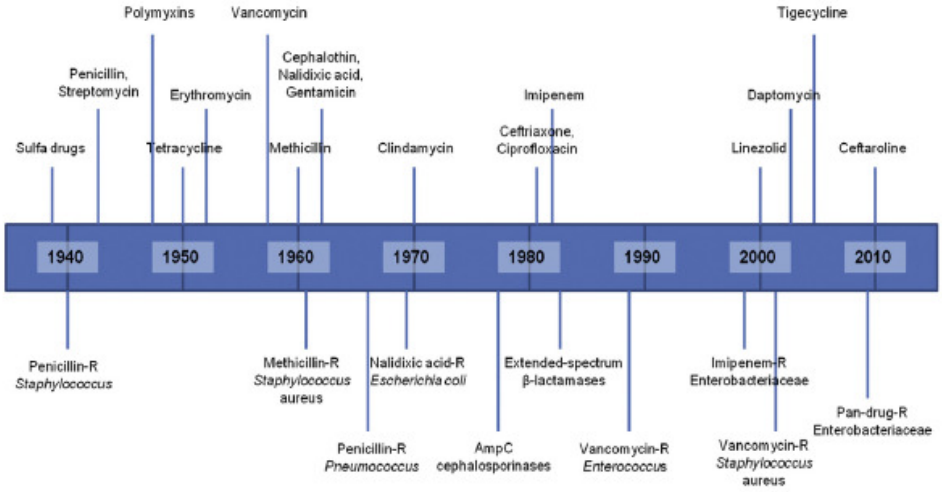


Fig 8: timeline of antimicrobial drug discovery against the development of antimicrobial drug resistance

Bacteria can acquire antibiotic resistance either through *de novo* gene mutation or by horizontal transfer of mobile genetic material. Consequently, drug targets can be altered, enzymes able to inactivate antibiotics can be expressed or efflux systems can be up-regulated[14]. This route of resistance development is often less important as cumulative point mutations are usually required for this kind of phenotypic resistance. Although it is possible for the stain harboring the mutated allele to become the predominant organism under selective pressure such as antimicrobial drug use. In contrast, the rapid and broad propagation of drug resistance is the ability for bacteria to undergo horizontal gene transfer. Antibiotic resistance genes are carried on mobile genetic elements (transposons, integrons and plasmids) which act as vectors for transfer between the same or different species. Basic mechanism of AMR can be generally summarized using the bullet-target concept[15]. Site of drug activity (target) can be altered by enzymatic modifications, transformed by genomic mutations or, bypassed metabolically. Our precious bullet (antibiotic) can undergo enzymatic inactivation and degradation, reduced accessibility in bacteria cell and increased extrusion from the cell, figure 9.

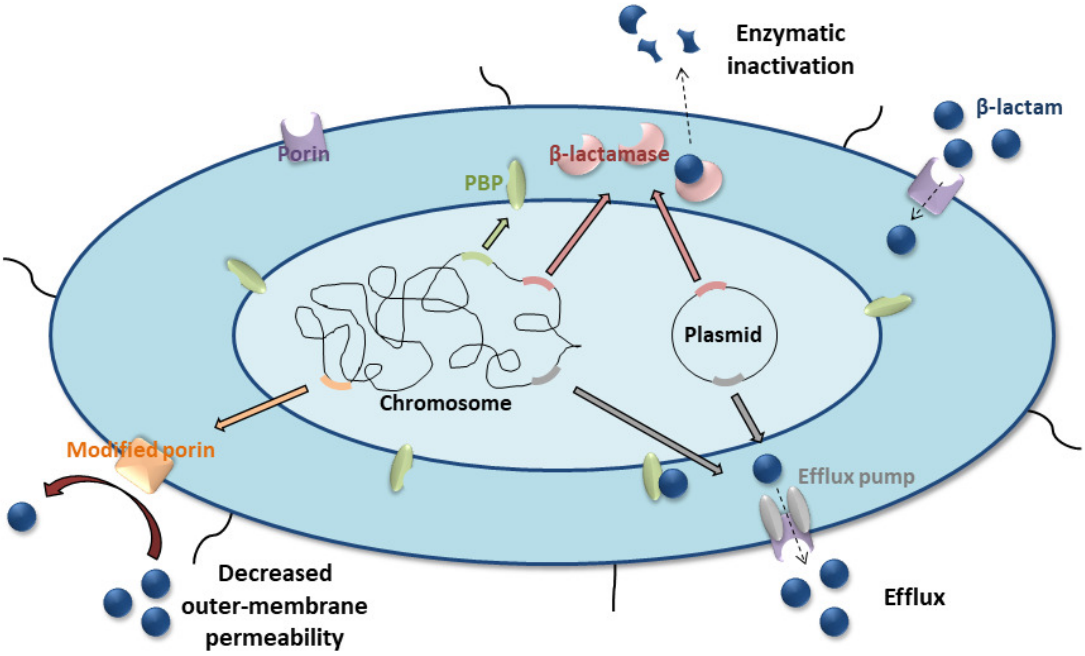


Fig 9: Primary mechanisms of b-lactam resistance in Enterobacteriaceae include the following: enzymatic inactivation of the antibiotic by chromosome-and/or plasmid-encoded enzymes possessing hydrolytic activity against b-lactam molecules; decreased outer membrane permeability through production of modified porins, loss of porins expression, or a shift in the types of porins found in the outer membrane; and efflux of the antibiotic to the outside of the bacterium through production of an efflux pump.

The most widely diffuse resistance mechanism is β-lactamases expression which use the same mechanism of PBPs. PBPs use an amino acidic serine residual moiety to form a covalent bond with the peptidoglycan chain. This bond is released when a crosslinked bond is formed with another portion of peptidoglycan. β-lactam compounds bind the same serinic moiety but in a non- reversible way, inhibiting permanently the active site. β-lactamases show a similar serinic moiety in their active site. β-lactams bind to the serine of the enzyme and are then released in a non-active form (β-lactam ring opened) Figure 10.

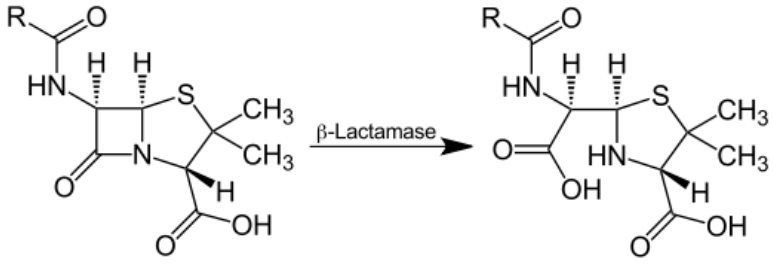


Fig. 10: penicillin ring opened inactivation by β-lactamases

Other β-lactamases act in a similar way but using a Zinc ion instead of serine to inactivate β-lactam. Antibiotic penetrates in the bacterial cell through the cell wall. At this point they undergo to the β-lactamases action which attach these molecule with a nucleophilic addition to the carbonyl of β-lactam ring leading to the formation of an inactive structure. In this way, bacteria can enzymatically destroy β-lactam antibiotics, by opening the β-lactam ring, switching off their antimicrobial activity.

2.6 Gram Positive and Gram Negative bacteria

In Gram+ bacteria, peptidoglycan polymer is very close to bacteria surface; surface's macromolecules only are external to peptidoglycan. Small molecules as β-lactams can easily penetrate the external portion of the cytoplasmic membrane to interact with PBPs. Different scenario regards Gram- bacteria. Gram- bacteria show a more complex external structure since the internal membrane (similar to Gram+) is protected from a lipopolysaccharide external membrane. External membrane acts as an impenetrable barrier for some antibiotics. Nevertheless, small hydrophilic molecules can diffuse through hydrophilic channels of the external membrane constituted by proteins called porins. Broad spectrum penicillin, as ampicillin and amoxicillin, and a good number of cephalosporins and Carbapenem diffuse through porins very rapidly, showing efficacy also against Gram- bacteria. On the other side, bacteria as *Pseudomonas aeruginosa*, are extremely resistant to a wide range of antibiotics since high permeability porins on its membrane are absent.

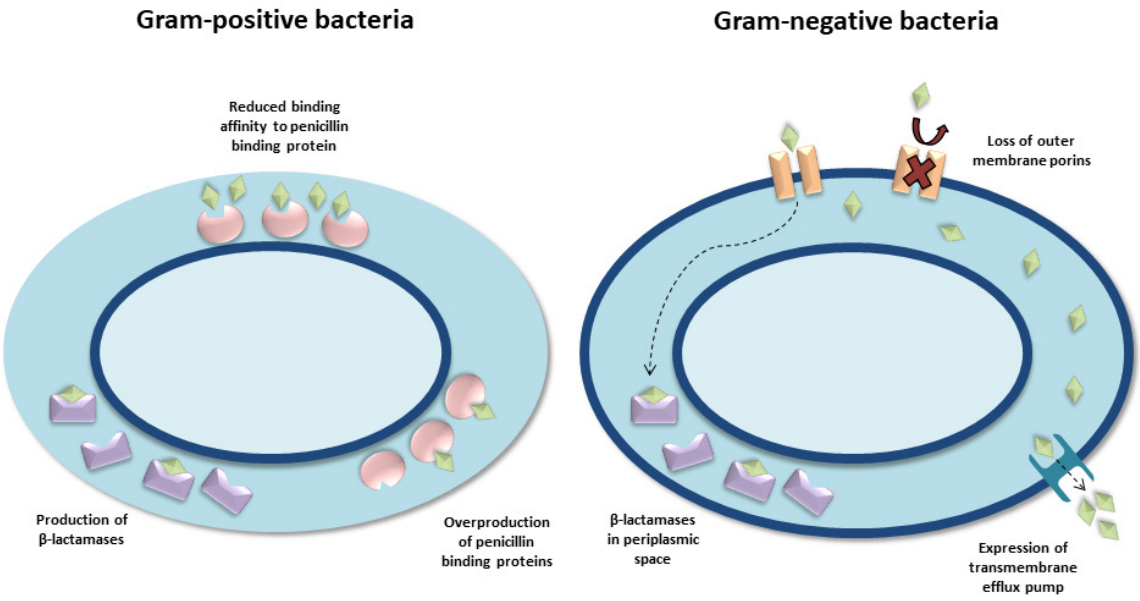


Fig 11: major mechanism of βlactam resistance in both Gram+ and Gram- bacteria.

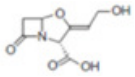
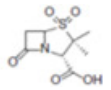
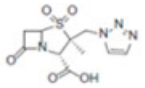
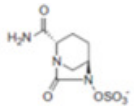
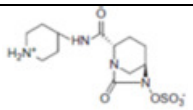
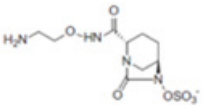
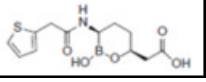
In Gram+ bacteria, βlactam resistance primarily occurs as a result of alteration of PBPs, with enzymatic degradation as minor pathway. In Gram- bacteria resistance can arise through all three processes already described in figure 11: alteration of PBPs, production of β-lactamases, and limited access to target PBPs. Due to their nature, any modulation that restrict entry (porin loss) or result in extrusion (efflux pumps) of β lactam antibiotic will confer resistance. In addition, while changes in PBPs are a fundamental base for resistance in Gram+ bacteria, they occur less frequently in Gram- bacteria, in which production of β-lactamases is the most prevalent resistance mechanism[16].

2.7 β-lactamase Inhibitors

Since the discover of the penicillin, in parallel with the research for new and more effective antibiotics, various strategies have been employed to overcome and combat resistance mechanisms. The most important strategy to "cheat" resistance has been the inhibition of β-lactamases[17] (structure reported in Table 1). The road to β-lactamases inhibitors begun in 70s when, as result of natural product screening, clavulanic acid with a novel clavam structure (they are similar to penams, but with an oxygen substituted for the

sulfur), or analogues of Clavulanic acid; see Table1) was identified as a broad spectrum inhibitor of the staphylococcal penicillinases and most of the recognized plasmid-encoded penicillinases found in enteric bacteria[18]. Clavulanic acid acts as a suicide inhibitor acylating initially the active site of serine with transient inhibition that includes hydrolysis of the inhibitor before complete enzyme inactivation. Spectrum of activity includes a good number of β-lactamases. Clavulanic acid works synergistically with penicillins and cephalosporins against β-lactamases producing bacteria through inhibition of sensitive lactamases, thus allowing the companion active antibiotic to kill bacteria. After the discovery of clavulanic acid, medicinal chemists synthetized a number of penicicllianic acid sulfones with inhibition activity. Among these Sulbactam (1978) and Tazobactam (1984) were successfully launched on market. Each followed the same inhibition/inactivation mechanism of clavulanic acid but they showed activity relevant to different class of β-lactamases increasing the spectrum of action of these new classes of compounds. After more or less two decades, a unique class of non-β-lactam β-lactamases inhibitors emerged, based on a novel bridge diazabicyclooctane (DBO) structure (table1)[19]. The first of these inhibitors, Avibactam, has a broader spectrum of activity than clavulanic acid and the sulfone inhibitors. Avibactam has been approved for clinical use in 2015 in combination with Ceftazidime, since has shown tight-binding, covalent and reversible inhibiting properties. Others DBOs Inhibitors have followed Avibactam, as Relebactam and RG6080. This last showing an additional benefit of some intrinsic antibacterial activity[20]. More recently the boronic acid inhibitor *Vaborbactam ®* represents another novel class of synthetic non β-lactam inhibitors active against serine β-lactamases. *Vaborbactam®* has been developed in combination with Meropenem to target pathogens producing serine carbapeneases[21].

Table 1: β-lactamases inhibitors of current interest

Name	Structure	Partner β-lactam	Approval Date	Inventor
Clavulanic Acid		Amoxicillin	1984	GSK
Sulbactam		Ampicillin	1986	Pfizer
Tazobactam		Piperacillin	1993	Pfizer
		Ceftolozane	2014	Cubist
Avibactam		Ceftazidime	2015	Pfizer
Relebactam		Imipenem	2019	Merck
Nacubactam		Meropenem	Not approved Phase 1	Aptuit
Vaborbactam		Meropenem	2017	Melinta

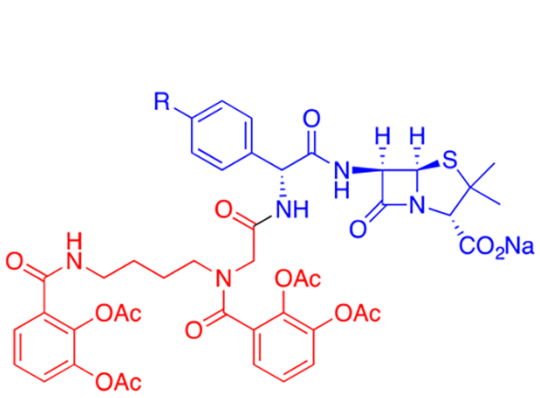
Since β-lactamases still represent one of the most relevant threat to antibiotic efficacy, perhaps the most encouraging prospect in fighting against resistance is the emerging of new classes of β-lactamases inhibitors that will provide protection for some of the most precious and vulnerable antibiotics in clinical practice, at least for the present time.

2.8 Siderophores: Trojan Horse approach to Gram negative bacteria

Microbial drug resistance in Gram- bacteria is partly due to hindered diffusion through the membrane of microbial cells and active transport mechanisms. A brand new approach to counter such resistance uses the bacterial iron transport system. Extracellular free iron is scarce in vertebrates, yet essential for microbial growth[22]. A mechanism displayed by microbial pathogens to cope with iron scarcity involves the production of Siderophores[23]. These low molecular weight molecules bear an affinity to iron that exceeds by several orders of magnitude that of transferrin, the main protein in

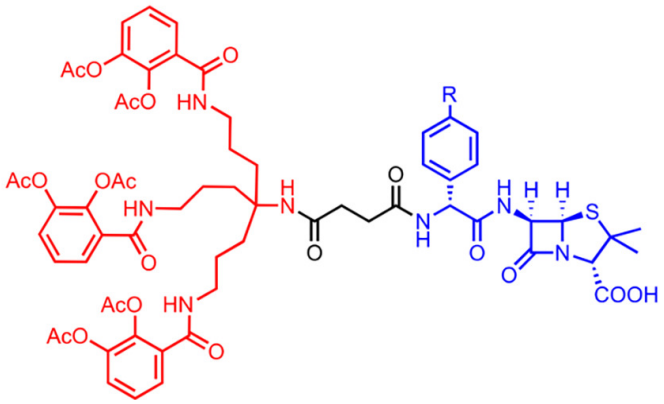
blood for iron transport[24]. Under iron starvation, siderophores are excreted, scavenge ferric ions and the complex is shuttled inside the cell. The Trojan horse approach (THA) relies on the iron-siderophore uptake system to deliver an antibiotic payload (Figure 12), a mechanism displayed by several bacteria, through the production of e.g. albomycins, ferrymicins and salmycine.

These sideromycins consist of naturally occurring hydroxamate types of siderophores, covalently linked to an antibiotic moiety[25]. Aiming to improve antibiotic uptake by pathogenic bacteria, efforts have been made in the design of siderophore-antibiotic conjugates. Typically, this involves a catechol/hydroxamate siderophore analogue and a β-lactam drug. A substantial number of different siderophore aminopenicillin conjugates have been synthesized[26]. A correlation between siderophore structure and in vitro antibacterial efficacy was found, and lead structures were optimized with respect to optimum spacer length and number of catechol moieties, figure 12.



8, R=H (ampicillin conjugate)

9, R=OH (amoxicillin conjugate)



10 R=H (ampicillin conjugate)

11 R=H (amoxicillin conjugate)

Fig. 12: examples of Siderophores (red) conjugates to penicillins (blue). Compounds **8** and **9** have shown activity vs various strains of Pseudomonas Aeruginosa. Compounds **10** and **11** have shown activity vs *E.Coli*[27]

The efficacy of traditional antibacterials, in particular reduced permeation of aminopenicillins through the outer membrane of Gram-negative pathogens, can be increased by conjugation of the antibiotic to a siderophore moiety allowing facilitated transport via essential, species specific Fe^{3+} siderophore transport pathways. In addition to the active uptake by the bacteria itself, the siderophore aminopenicillin conjugates are not substrates for efflux pumps in *P. aeruginosa*. The preclinical data on the described

conjugates indicate good in vitro activity against Gram-negative pathogens and good in vivo activity against *P. Aeruginosa*, no mutagenic potential, and good tolerance in mice and rats[28]. The activity profile and the specific active uptake in bacteria under iron depletion could be beneficial for the treatment of lung infections patients. Sideromycins represent indeed a brand new opportunity of success in fighting against resistant bacteria.

3 Carbapenems

The carbapenems are β-lactam antimicrobial agents that differ from penicillins (penams) in having a carbon atom replacing the sulphur at position 1 and an unsaturated bond between C2 and C3 in the five-member ring structure (figure 13).

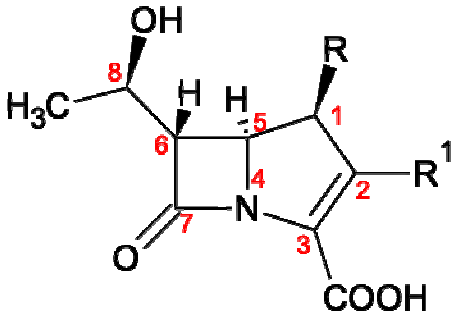
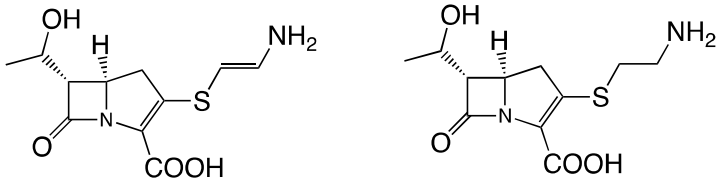


Fig. 13: Carbapenem structure. R= H, -CH_3; R^1= Side chain.

Carbapenems play key important role in our antibiotic armamentarium. Among the many hundreds of β-lactams, carbapenems show the broadest spectrum of activity and greatest potency against gram-positive and gram-negative bacteria. The broad spectrum of activity is associated with their resistance to nearly all β-lactamases. This particular stability is due to the α-1-hydroxyethyl substituent at the 6 position of the backbone. This characteristic is unique when compared with the side chains of other βlactams, which have cis configuration. As a result, they are often used as "last line agents" or "antibiotics of last resort" when patient with infections become gravely ill or are suspected of harboring resistant bacteria. Due to these mentioned characteristics Carbapenems, and in particular their stereochemical configuration, will be the main focus of this PhD work.

3.1 History

In 1960s the use of penicillins was threatened by the emerging of bacteria β-lactamases. The search for lactamases inhibitors begun right after. In 1976 the first class of β-lactamases inhibitors were discovered as olivanic acids **12** (figure 14), natural products coming from Gram-positive bacterium *Streptomyces clavoligerus*. Olivanic acids show a Carbapenem structure and act as broad spectrum β-lactam[29]. Due to their chemical instability (dimer formation, given by reactivity of free amine moiety) and the low affinity in penetrating into bacterial cell, olivanic acid has been abandoned as antimicrobial agents [30]. Shortly thereafter Thienamycin **13** (Figure 14, natural product from Streptomyces cattleya[31] was the first "carbapenem" discovered to be the parent or model compound for all carbapenems.



Olivanic acid Thienamycin

Fig 14: structure of olivanic acid and thienamycin

The hydroxyethyl side chain of Thienamycin is a radical departure from typical structure of penicillin and cephalosporins, all of which have an acylamino substituent on the β-lactam ring. The stereochemistry of this hydroxyethyl moiety is mandatary for the activity[32]. Thienamycin demonstrated potent broad-spectrum antibacterial and β-lactamase inhibitory acitivity[33] against *Pseudomonas aeruginosa* and *Bacteroids fragilis* and *Staphylococcus aureus*. Unfortunately, Thienamycin was found to be quite unstable in aqueous solution, sensitive to mild condition base hydrolysis and highly reactive to nucleophiles, hydroxylamine, cysteine and its own primary amine[34]. This found chemical instability led to the search for derivatives showing increased stability. The first developed was N-formimidoyl derivative, Imipenem **14** (Figure 15). Imipenem and Panipenem **15** (isolated right after), were more stable and less sensitive to basic hydrolysis than Thienamycin. Imipenem became, in 1985, the first commercially available carbapenem, demonstrating high affinity with PBPs and resistance to β-

lactamases[35]. However, both imipenem and Panipenem were subject to deactivation by dehydropepidase I (DHP-1) present in human renal brush border. Co-administration with an inhibitor as Cilastatine or Betamipron[36] was then necessary. Along the journey to the discover of more stable but effective carbapenems, the other currently available compounds, Meropenem **14**, biapenem **15**, ertapenem **16** and doripenem **17** (Fig16), were developed and several novel Carbapenem identified. A major advance was the introduction of a methyl group to the 1-β position (Fig. 14). This modification allowed antibiotic to be resistant against DHP-I hydrolysis[37]. Many carbapenems with this striking modification were identified in the subsequent decades (Figure 15)

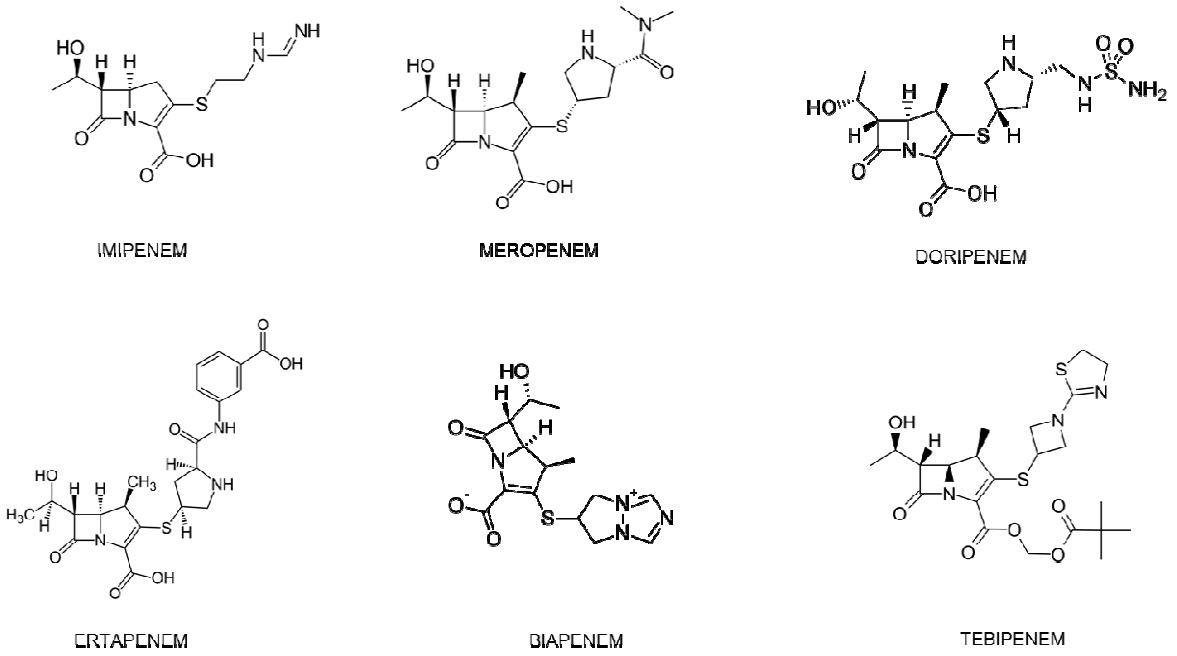


Fig 15: Carbapenem approved for regulated market

3.2 *Chemistry and Biology*

From the studies conducted on the early carbapenems, the carbon atom at the C-1 position was found to play a major role in the potency and spectrum of carbapenems and in their stability against β- lactamases. Further studies suggested that a strategically positioned hydroxyethyl R_2 (Figure 16A) side chain enhances the resistance to hydrolysis by β-lactamases[38]. In addition, carbapenems with an *R* configuration at C-8 are also very potent (Figure 16E). The *trans* configuration of the β-lactam ring at C-5 and C-6 results in stability against β-lactamases (Figure 16F)[39].

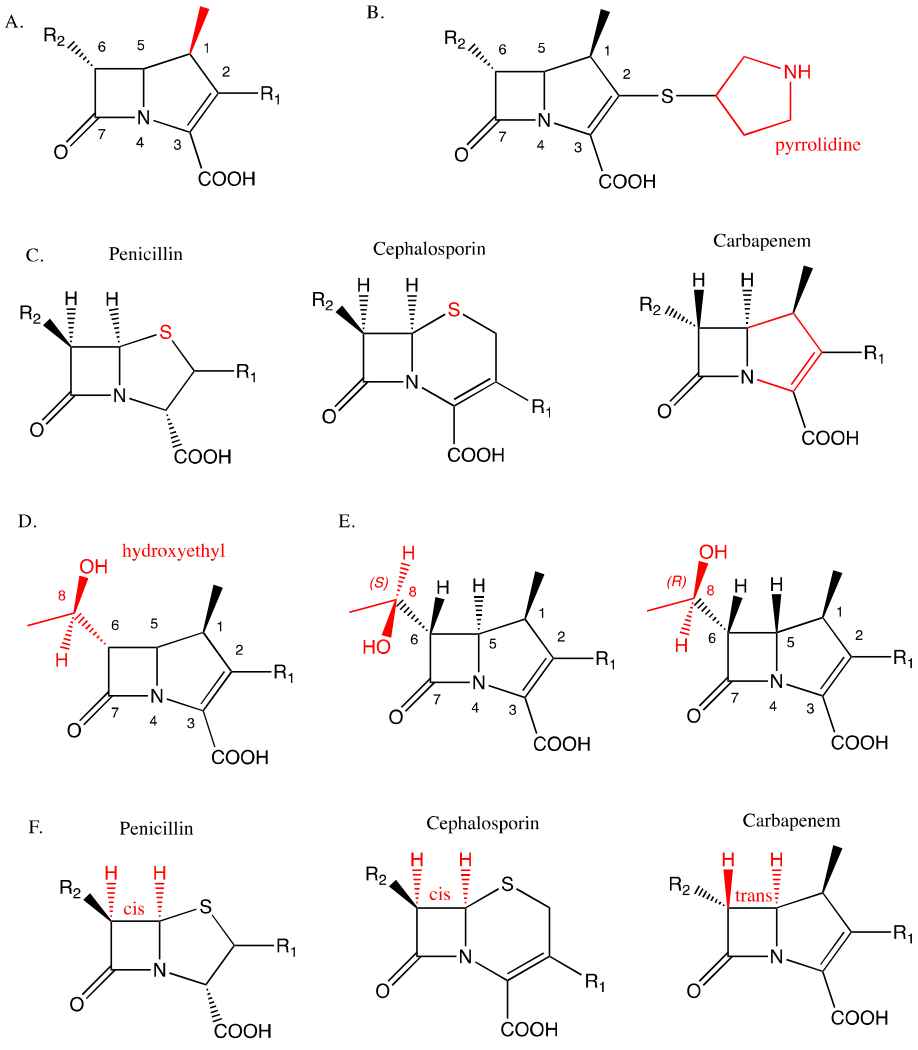


Fig 16: (A) 1-β-methyl (red) increases resistance to DHP-I. (B) The pyrrolidine ring (red) increases stability and spectrum. (C) Penicillin, cephalosporin, and carbapenem backbones. Carbapenem has a five-member ring, as does penicillin, but it has a carbon at C-1 instead of sulfur. (D) Most carbapenems have a hydroxyethyl off C-7. (E) The *R* configuration of the hydroxyethyl increases the β-lactam's potency. (F) The *trans* configuration of carbapenems at the C-5--C-6 bond increases their potency compared to penicillins and cephalosporins.

Like Thienamycin, the clinically available carbapenems exhibit the absolute configuration *R* at C-8 and relative configuration *trans* of the C-5--C-6 bond. Carbapenems with a pyrrolidine moiety (panipenem, meropenem, ertapenem, and doripenem) among various cyclic amines as a side chain have a broader antimicrobial spectrum[40].

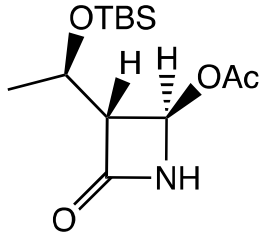
3.3 Microbiological activity

Carbapenems demonstrate an overall broader antimicrobial spectrum *in vitro* than the available penicillins, cephalosporins, and β-lactam/β-lactamase inhibitor combinations. In general, imipenem, panipenem, and doripenem are potent antibiotics against Gram-positive bacteria. Meropenem, biapenem, ertapenem, and doripenem are slightly more effective against Gram-negative organisms[41]. Ertapenem has a more limited spectrum, because it is not as active as imipenem or meropenem against *P.* aeruginosa[42] Meropenem is not as potent as imipenem or doripenem against *Acinetobacter baumannii*. Doripenem has lower MICs than do imipenem and Meropenem versus *P. aeruginosa* and *A.* baumannii[43]. In addition, doripenem is the carbapenem least susceptible to hydrolysis by carbapenemases; hydrolysis of doripenem is 2- to 150-fold slower than that of imipenem[44]. A unique application of meropenem is that when combined with clavulanic acid, it is potent at killing MDR *Mycobacterium tuberculosis*, a bacterium that typically is not susceptible to β-lactams due to a chromosomally expressed β-lactamase[45]. This ability to inhibit or kill *M. tuberculosis* is likely to be a property of other carbapenems as research in this area grows. Carbapenems can also be combined with other antimicrobials to treat serious infections. Combination therapy is a subject of intense interest, since the emergence of MDR pathogens often requires us to treat patients with more than one antibiotic[46]. Some combinations demonstrate positive effects, such as extending the spectrum or working additively or synergistically. Adverse effects include increased resistance to one of the drugs used in the combination, as well as a lack of synergy or additivity and strain dependence[47].

3.4 Synthesis

As mentioned above, several chemical approaches were developed for the synthesis of carbapenems since fermentation was not an efficient method for production[48]. Natural products (L-Cys, L-Val, L-β-amino adipic acid, and *S*-adenosyl-Met) were often used as starting material for production of carbapenems, and the synthetic approach was largely influenced by the desired stereochemistry of the final compound. In addition, once a carbapenem is developed which has an *R* configuration at C-8, is *trans* about the C-5--C-6 bond, and has a methyl at C-1 and a hydroxyethyl at C-6, most modifications are at the R1 side chain (at position C-2). Thus, carbapenems are unique compared to other β-lactams, which show differences in both R1 and R2 side chains. For these reasons, chemists have made great efforts in order to find total synthesis routes for this unique class of compounds.

From a synthetic point of view the key intermediate for carbapenem is undoubtedly compound (3R,4R)-4-Acetoxy-3-[(R)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone (AOSA) (**18**, Figure. 17)[49].

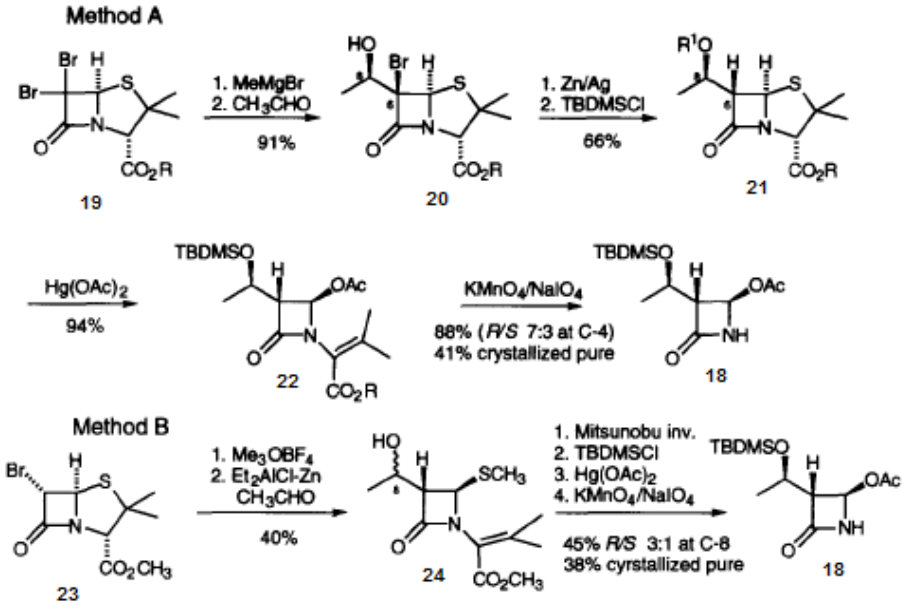


18

Fig. 17: (3R,4R)-4-Acetoxy-3-[(R)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone AOSA

AOSA is a β-lactamic compound showing three sterocenters carrying the correct absolute configuration needed for the synthesis of all carbapenems. Acetoxy group in position 4 on β-lactam ring is very important since it allows substitution reaction to pursuit in reaction needed to build up carbapenem backbone first, and then, after insertion of side chains, of the final structure of Carbapenem. Given the importance of this intermediate several synthetic routes have been developed. One of the most recent

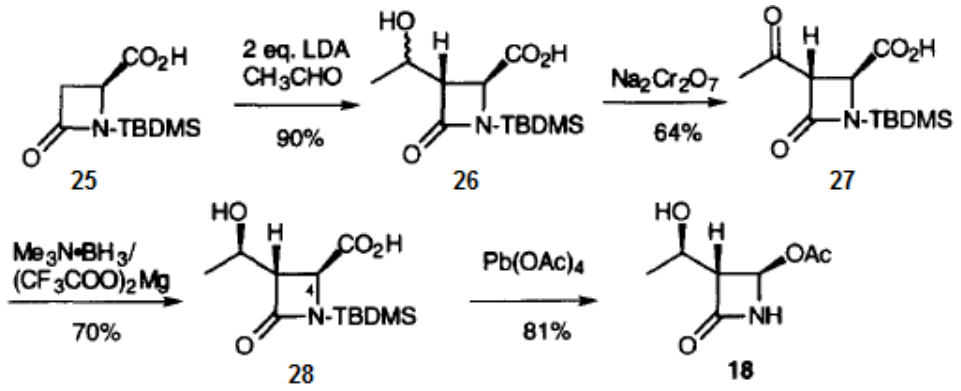
has been developed by Sankyo Co. Ltd[50], using penicillamic ester **19** as starting material. Scheme 1



Scheme 1

Two approaches were reported by this group. In method A, dibromopenicillanate **19**, obtainable from the fermentation product 6-amminopenicillanic acid, is transmetallated with methyl Grignard reagent and treated with acetaldehyde, followed by zinc-silver couple, which reduceds the second bromine, to give **21** as a 7:3 diastereomeric mixture (desired diastereoisomer shown in scheme1). The mixture is then silylated to give penicillin **21**, which is simultaneously cleaved and acetoxylated with mercuric acetate to give **22**. Final dealkylation of nitrogen on β-lactam is obtained with permanganate and periodate to give AOSA **18** (pure after crystallization). Overall process yield form **19** is 23%. The same group described also Method B outlined in Scheme 1. Starting from 6β-bromopenicillinate **23**, also obtained from 6-aminopenicillanic acid, which is cleaved with trimethyloxonium tetrafluoroborate and subjected to an aluminium mediated condensation with acetaldehyde to afford **24**, as a 3:1 diasteromeric mixture, with the wrong diasteroisomer predominating. Even in this case the mixture is silylated, treated with mercuric acetate and nitrogen deprotected in the same way to afford pure crystalline AOSA. Overall yield 15% in 5 steps. Method B, despite of lower yield is preferred since starting material is cheaper and troublesome zinc reaction is avoided.

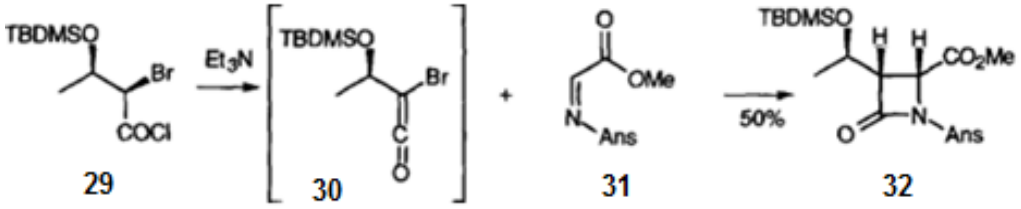
Synthetic variation has been developed by Merck[51], starting from azetidinone without the C-3 hydorxyethyl side chain and relied on the C-4 configuration to control the stereochemistry of the assembly of the side chain (scheme 2)



Scheme 2

The N-protected azetidinone **25**, derived from L-aspartic acid, was treated with two equivalents of lithium diisopropylamide and then acetaldehyde to afford an epimeric mixture of alcohols **26**. Oxidation and stereospecific reduction furnished **28**, which was oxidized with lead tetracetate under conditions that simultaneously caused decarboxylation, installed the acetoxy group and desilylated the nitrogen to give AOSA **18**.

Another interesting strategy to AOSA is the one proposed by Farmitalia S.p.a., based on the generation a chiral ketene and reacting with an achiral Schiff base through a 2+2 cycloaddition (Scheme 3)[52].



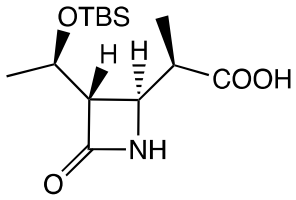
Scheme 3

Acid chloride **29**, derived from D-*allo*-threonine, is treated with base to in-situ generate Ketene **30**, and subsequent alkylation with the achiral imine **31** affording azetidinone **32**. Compound **32** can then be carried over to AOSA, involving dehalogenation, nitrogen

deprotection and transformation of the ester to acetoxy with the correct C-4 configuration.

In addition to those just discussed, there are many synthetic routes for AOSA described in literature[53]. Very important are also synthesis developed for the production of this important intermediate on large industrial scale. These latter will be described in depth in "results and discussion" section of this thesis work.

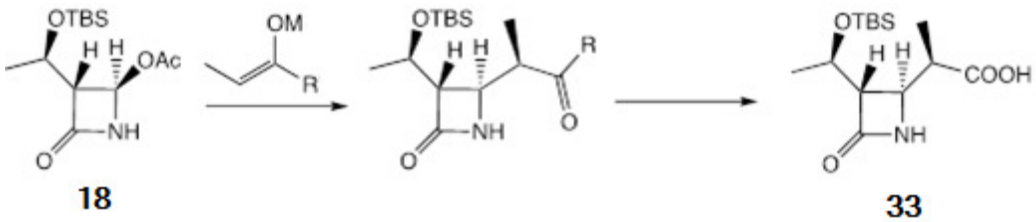
The second key intermediate for β-methyl carbapenems synthesis is compound shown in figure 18.



33

Fig. 18: β-methylazetidenone (4-BMA)

Its importance is due to the presence of β-methyl moiety on its structure. Correct absolute configuration of this methyl group is mandatary for the biological activity of the final carbapenem. Due to the importance of this advanced intermediate many elegant synthesis methods have been developed in years. The majority of routes to **33** involve an enolate addition to AOSA, schematically described in scheme 4.

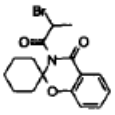
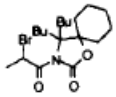
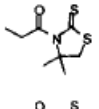
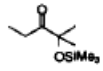


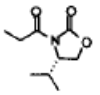
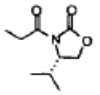
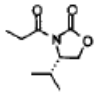
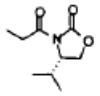
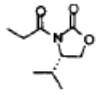
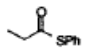
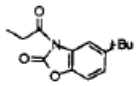
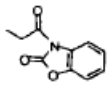
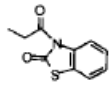
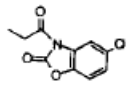
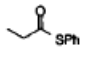
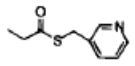
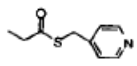
Scheme 4: enolate is added to AOSA to afford 4-BMA with de desired stereochemistry.
R = Diasteromeric auxiliary

Alternative and more proficient synthesis, applicable on large industrial scale, have been developed basing on Reformatsky type reaction. Many diasteromeric auxiliary have been investigated. The results of this approach are summarized in table 2 based on the nature

of R1, the metal or Lewis acid used in the enolization, and the stereochemical outcome in terms of the β:a ratio of the methyl group[53].

Table 2: stereospecific enolate addition to AOSA

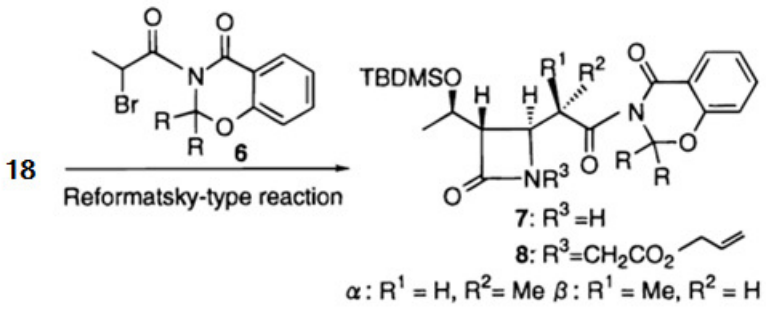
	Enolate or Precursor	Conditions	β:α ratio	Yield
A		Zn powder	92:8	75%
B		Zn dust	79:21	75%
C		Zn dust	45:55	97%
D		Zn dust	79:21	94%
E		Zn dust	91:9	99%
F		Zn dust	23:1	95%
G		$Sn(OTf)_2$, 	4:1	73%
H		$Sn(OTf)_2$, 	6.7:1	80%
I		$Sn(OTf)_2$, 	9:1	80%
J		$Sn(OTf)_2$, 	11:1	74%
K		$Sn(OTf)_2$, 	24:1	79%
L		$Sn(OTf)_2$, 	9:1	75%
M		$Sn(OTf)_2$, 	20:1	90%

	Enolate or Precursor	Conditions	β:α ratio	Yield
N		TMSCl/LDA cat. ZnI_2	4:1	93%
O		$Sn(OTf)_2$, 	92:8	80%
P		Et_2BOTf, iPr_2NEt, $ZnBr_2$	≥99:1	68%
Q		Et_2BOTf, iPr_2NEt, $BF_3{\cdot}OEt_2$	84:16	95%
R		Cp_2BOTf, iPr_2NEt, $ZnBr_2$	15:1	68%
S		Et_2BOTf, iPr_2NEt, $ZnBr_2$	1.4:1	18%
T		Et_2BOTf, iPr_2NEt, $ZnBr_2$	10:1	81%
U		Et_2BOTf, iPr_2NEt, $ZnBr_2$	15:1	56%
V		Et_2BOTf, iPr_2NEt, $ZnBr_2$	20:1	72%
W		Et_2BOTf, iPr_2NEt, $ZnBr_2$	60:1	75%
X		Et_3N, TBDMSOTf, $ZnCl_2$	10:90	91%
Y		Et_3N, TBDMSOTf, $ZnCl_2$	8:92	37%
Z		Et_3N, TBDMSOTf, $ZnCl_2$	40:60	58%

Common characteristic of all diasteromeric auxiliaries shown in table 2 is the presence of a propionilic moiety, which is mandatory for the introduction of the needed number of carbon to build up the five-member backbone of carbapenem, including the β-methyl

portion with the desired configuration. Focusing on first rows of table 2 (A – F) it is clear how the process to functionalize AOSA is a Reformatsky type reaction with metallic Zinc e 2-acylbromine carrying the diasteromeric auxiliary. Among many examples reported just few of them have been chosen to be scaled up in industrial scale to produce 4-BMA. Yield, diasteromeric ratio, cost of the diasteromeric auxiliary and its possible recovery in the waste stream, are all important parameters to be evaluated to approach an industrial scale up. In particular, diasteromeric auxiliaries A and D are the most widely for this purpose.

How outlined in table 2 and described in Professor Kondo's JOC[54] (see scheme 5) diastereoselectivity of β isomer increases at the increase of the steric hindrance of the substituent R e R_1. Yield instead decreases with the steric hindrance, Scheme 5.



entry	7	R	yield, %[a]	β:α[b]
1	**a**	Me	94	85:15
2	**b**	Bu	87	98:2
3	**c**	$C_{15}H_{31}$	64	98:2[c]
4	**d**	$-(CH_2)_4-$	77	85:15
5	**e**	$-(CH_2)_5-$	96	92:8
6	**f**	$-(CH_2)_6-$	38	89:11
7	**g**	Bn	76	99.6:0.4

Scheme 5: Steric hindrance of substituents and its effect on yield and diastereoselectivity

Prevalent formation of β isomer is easily explainable considering the formation of a six-members transition state between the imine moiety deriving from AOSA, after acetoxy elimination, and Z-isomer of Zinc enolate (Figure 19)

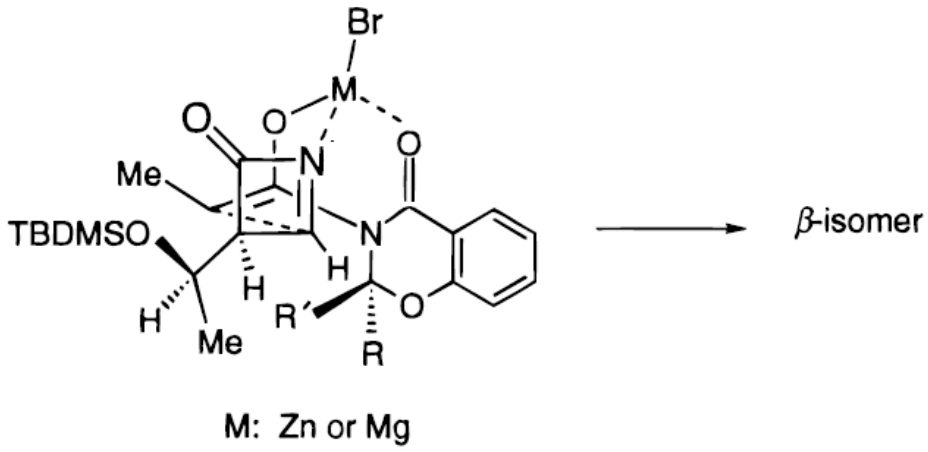
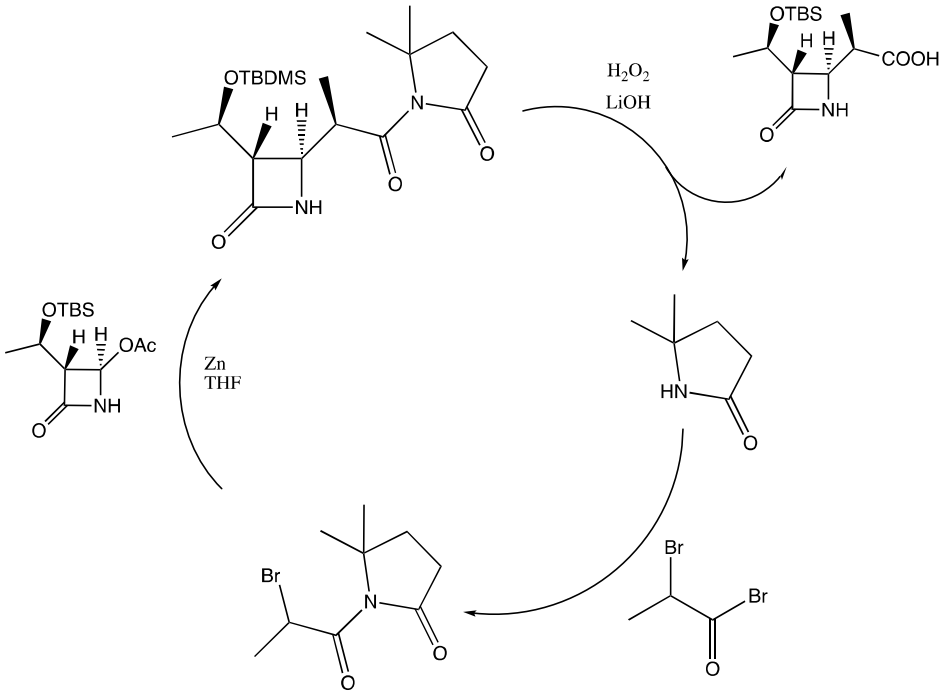


Fig 19: Zinc enolate and transition state in addition with azetidinone.

It is clear how the presence of hindered substituents on diasteromeric auxiliary does not affect diastereoselectivity, but the formation of Z-enolate and, consequently, formation of desired β isomer.

As already mentioned, mandatory characteristic for an auxiliary to be scaled up on industrial scale is the possibility to be recovered and re-used in different manufacturing campaigns. Diasteromeric compound D is often the auxiliary of choice for industrial scale manufacturing due to high yield the characteristic to be re-used in subsequent campaigns, improving cost efficiency of the process (Scheme 6).



Scheme 6: Diasteromeric auxiliary recovery process

4 Objective and purpose

Nowadays pharmaceutical companies are continually requested, by regulatory authorities all over the world, to ensure highest quality of their products. ACSDobfar is a privately held Italian company with subsidiares in 7 countries in 5 continents (Europe, North America, Asia, South America and North Africa). ACSDobfar hold in its pipeline several cutting edge anti-infective products and, in particular, it is leader manufacturer for Carbapenem in regulated market (Figure 20)

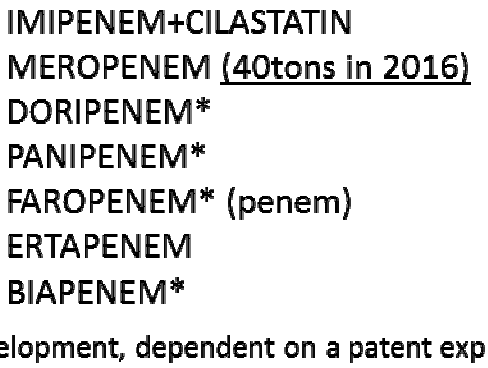


Fig 20: ACSDobfar Carbapenems pipeline

One of the main problem in managing the manufacturing of these compounds concerns the control of the stereochemistry of the sterocenters 1, 5, 6 and 8 (Fig 14 for numbering), as their correct configurations are mandatory for the biological activity. Therefore, regulatory authorities impose severe control strategies on stereochemistry of brand new or generic drugs and often the non-active stereoisomers are required as controls. As already discussed in section 2.4 azetidinone compound **18** (AOSA) is the key intermediate for the preparation of carbapenem antibiotics. AOSA shows 3 sterocenters therefore, beside the right stereoisomer (Figure 21), there are 7 possible additional stereoisomers. Considering the complexity of the possible isomerism, and the lack of structural characterization of additional stereoisomers to be used as reference standards, it is almost impossible to apply routine test to AOSA that can unambiguously assure control of all potential isomers.

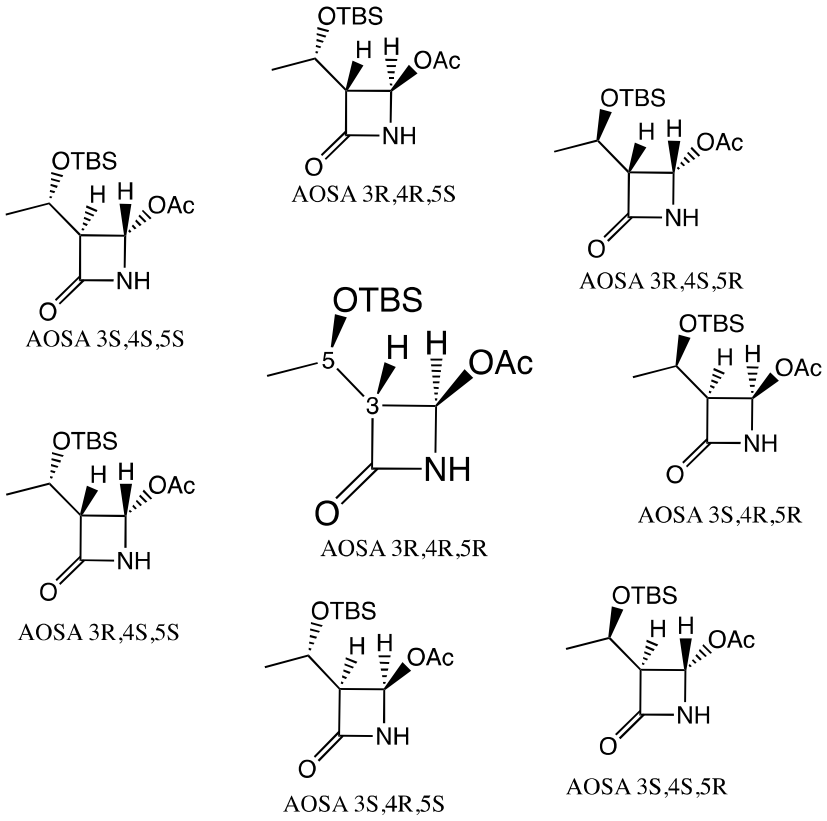


Fig 21. Possible additional AOSA stereoisomers.

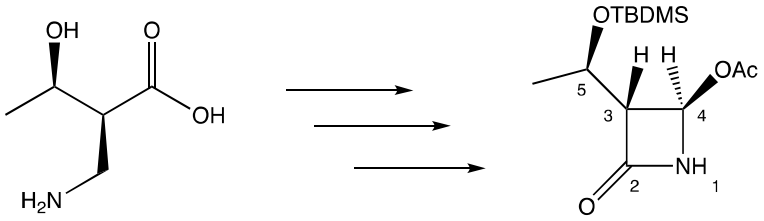
Therefore, the final aim of this PhD work is to build up a strong stereochemical control strategy for AOSA, through the preparation of selected stereoisomers which will be considered more important basing on the route of synthesis. In particular, the efforts will be focused on sterocenters 3 and 5 since their absolute configuration is retained through all subsequent synthetic steps required to achieve final carbapenem structure. Regarding stereocenter in position 4, it configuration importance is negligible, as it is lost in the subsequent step to 4-BMA **33**, through the formation of the imine derivative in Reformatsky reaction as outlined in Figure 19.

The project is expected to produce a selected reference standards library of AOSA (in particular: AOSA 3S,4S,5S; AOSA 3R,4R,5S; AOSA 3S,4R,5R), its precursors and to unambiguously define their structural characterization. This will allow us to have a more robust and fully stereocontrolled AOSA-based chemistry.

5 Results and discussion

5.1 General aspects

Several synthetic processes for AOSA are described in literature, as deeply discussed in section 2.4. Nevertheless, the most flexible for our aim is the one based on starting material (*2S,3R*)-2-(aminomethyl)-3-hydroxybutanoic acid **(AAL) 34** Scheme 6.



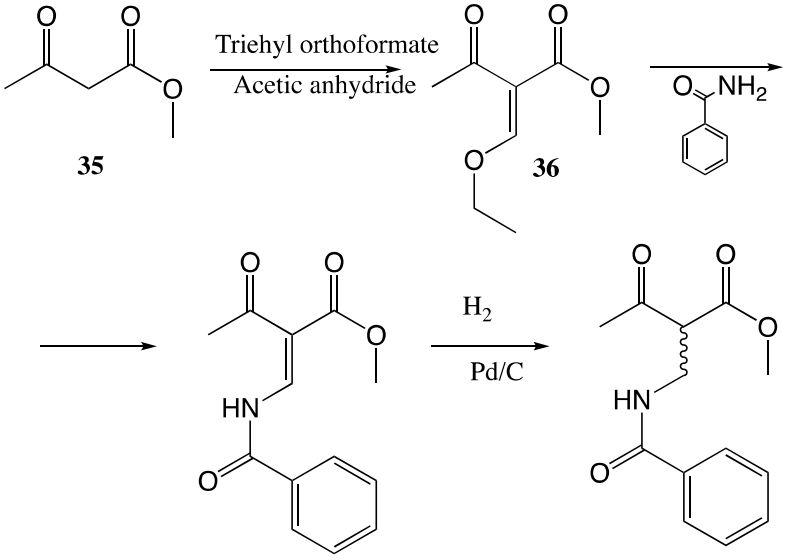
AAL (2S,3R)
34

AOSA
18

Scheme 6: Form AAL to AOSA, configuration for C3 and C5 is retained

AAL is indeed a key intermediate for AOSA's synthesis since it shows sterocenters 2 and 3 with the proper absolute configuration, which will be retained in all subsequent steps to AOSA. In other words, AAL stereochemistry controls AOSA's final absolute configuration regarding sterocenters 3 and 5. Starting from different and appropriate isomers of AAL would be possible to obtain selected AOSA stereoisomers.

Racemic AAL is widely commercially available and it is manufactured through a 5 steps synthesis[55].

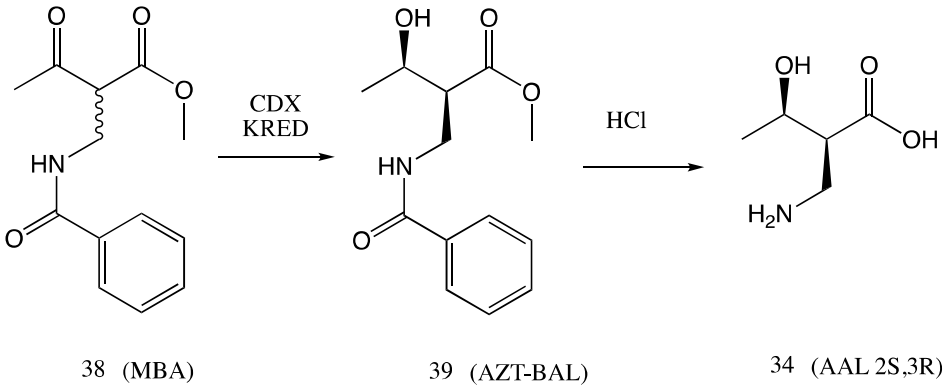


35 **36**

37 **38**
(MBA)

Scheme 7: Synthesis of MBA

Typical method, scheme 7, involves refluxing a mixture of methyl acetoacetate **35**, triethyl orthoformate, and acetic anhydride to give methyl 2-(ethoxymethylene)-3-oxobutanoate **36**, which when heated with benzamide gives methyl 2-(benzamidomethylene)-3-oxobutanoate **37**; methyl 2-benzamidomethyl-3-oxobutanoate (**38, MBA**) is finally obtained by hydrogenation in the presence of Pd/C catalysts. MBA is the precursor of AAL. The core step of the asymmetric synthesis yielding (2S,3R) AAL synthesis is undoubtedly the dynamic kinetic resolution of the MBA racemate to afford (2S,3R) AZT-BAL, which can be achieved enzymatically by using chiral catalysts. Chiral (2S,3R) AAL can be achieved by acid hydrolysis with retention of the configuration. Scheme 8

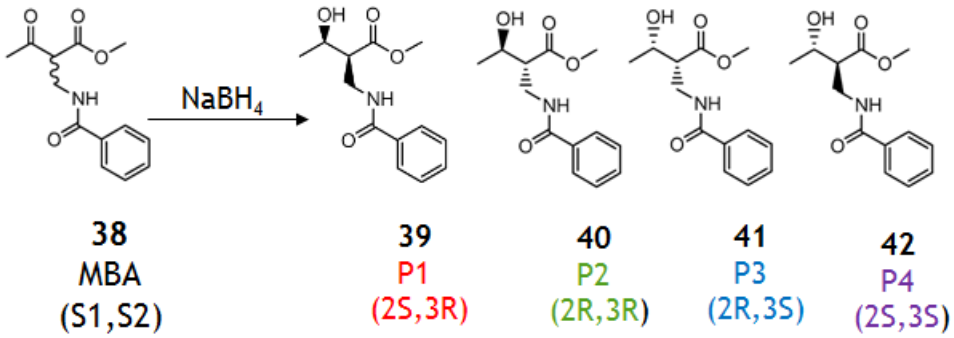


38 (MBA) 39 (AZT-BAL) 34 (AAL 2S,3R)

Scheme 8: reduction carbonyl of MBA a dynamic kinetic resolution of racemate.

Among the enzyme we used CDX KRED® is particularly effective. It is a keto-reductase belonging class enzyme (intellectual property of *Codexis ltd*) able to reduce carbonyl in position 3 to alcohol with ***R*** configuration and, in the meanwhile, to resolve recemate on sterocenter 2, giving desired configuration ***S***. This process is very effective since 100mg of KRED enzyme (using NADP as cofactor) can convert up to 100 grams of MBA **38** to AZT-BAL **39**, with an almost quantitative yield (close to 99%). Subsequent treatment of **38** with 10% HCl aqueous solution affords final AAL with the desired absolute configuration (2S,3R).

The strategy of choice to obtain different AAL isomers has initially been to focus on this reduction step from **38** to **39**. Treating racemic MBA compound **38** with $NaBH_4$ a 4 AZT-BAL isomers (P1-P4) mixture was obtained (Scheme 9).

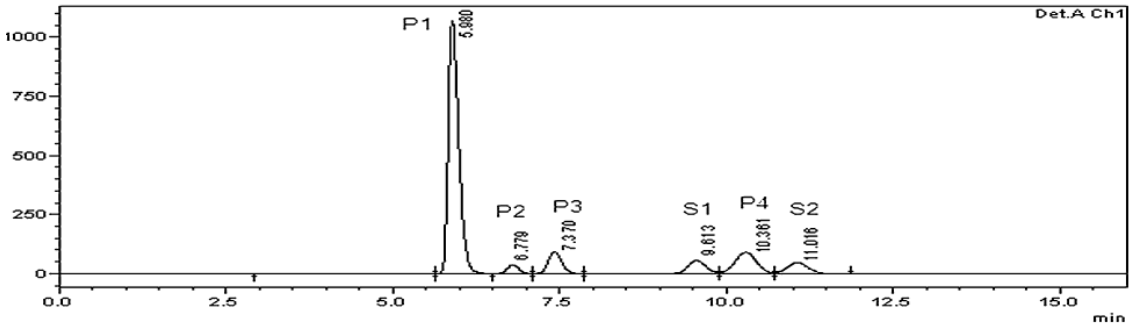


Scheme 9: Carbonyl reduction with $NaBH_4$ to afford racemic mixture

5.2 Analytical protocol

The development of a robust chiral analytical protocol was mandatory in order to effectively recognize and isolate the four stereoisomers P1-P4. This aspect was preliminary for the structure assignment step to each isomer. Surprisingly, literature survey resulted in only a very limited number of investigations dealing with reliable results on the separation of these isomers. Only a single paper by Chen, X. et al[56], reported the separation and peak attribution. Using a different chiral column, we succeeded in obtaining a quite similar separation chromatogram, Figure 22

a)



b)

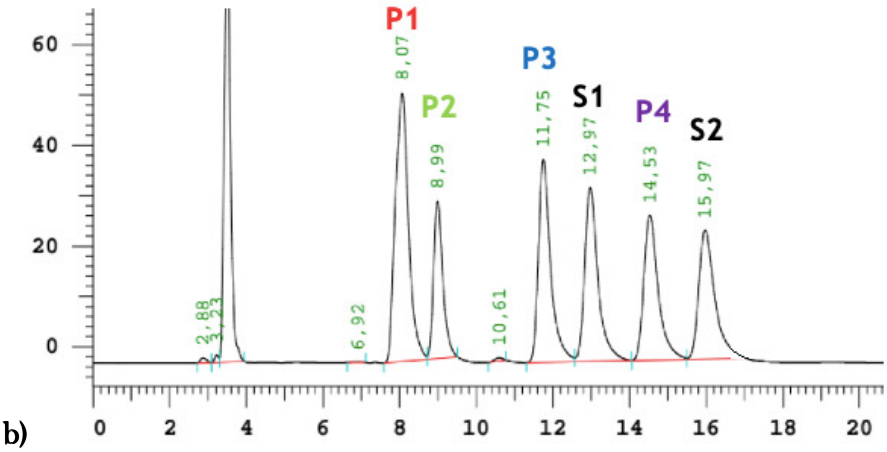


Fig 22. **a)** Chromatogram and peak elution order reported in literature by Chen, X. et al. Operative conditions: Chiralpak AY-H 250 x 4,6 mm, n-hexane/ethanol (76:24, v/v); 1.0 mL/min; 35◦C; and λ= 254 nm). **b)** Chromatogram of reduction products reproduced using different operative condition: Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/2-propanol (90:10, v/v); 1.0 mL/min; 25◦C; and λ = 254 nm)

Peak attribution, according to figure 22 as suggested by Chen et al. was not convincing since P1 (widely available in our laboratories) was injected in the HPLC showing a retention time of 11.75 minutes and MBA racemic mixture (S1 and S2) corresponded to peaks 16.7 and 18.3 minutes. We than decided to define peak elution ordered and attribution by ourselves. Crossing traditional chromatography and chiral protocols the two couple of diasteroisomers were separated and characterized by ^{1}H-NMR as *ANTI* couple and *SYN* couple. Figure 23

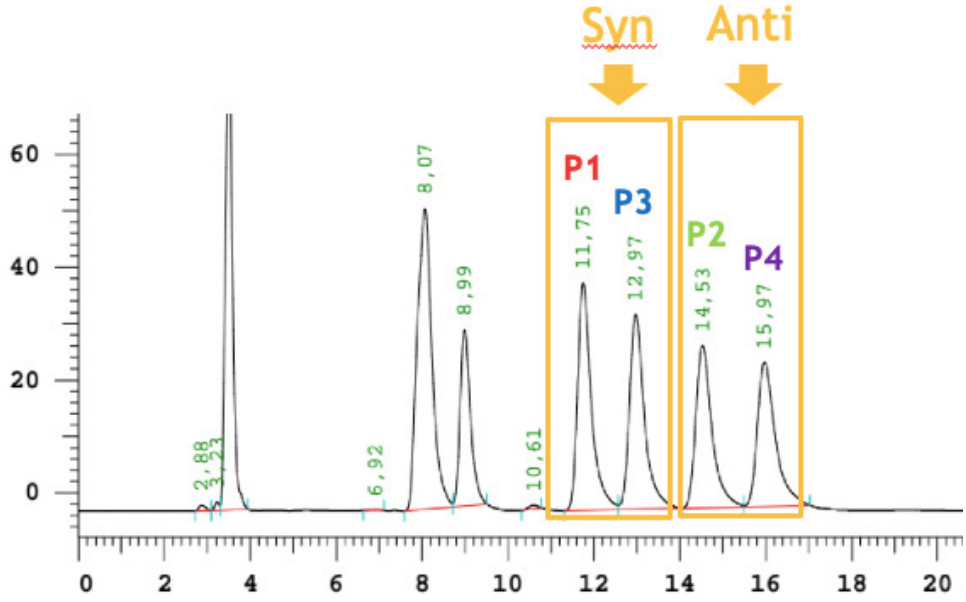


Fig 23. Correct peak attribution after diasteroisomers separation and ^{1}H-NMR acquisition.

Matching traditional and chiral analytical protocol peak elution order was then re-assigned correctly: P1 already available and its enantiomer P3 as *SYN* couple; *ANTI* couple, P2 and P4, elution order tentatively assigned based on literature data. Regarding peaks eluting at 8.07 and 8.99, chromatographic separation and isolation was attempted unsuccessfully since degradation occurred during purification process.

A robust chiral analytical protocol, was then achieved as it was suitable for the separation of each single stereoisomer. This was indeed a mandatory milestone for the purpose to obtain different AAL isomers to pursuit with the synthesis of AOSA's isomers.

But, how to reach stereoselectivity to obtain a suitable amount of every single stereoisomer?

Based on the existing industrial process and considering its good performance in terms of conversion and stereoselectivity, the enzymatic approach was the first to be investigated for the stereoselective reduction of MBA **38**.

5.3 Biocatalyst approach

Several alcohol dehydrogenase (ADH) theoretically suitable for our purpose are commercially available. ADH are a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between aldehydes or ketones and alcohols with the oxidation of nicotinamide adenine dinucleotide (NADH) to NAD^+ used as co-factors, figure 24. In biotransformation, alcohol dehydrogenases are often used for the synthesis of enantiomerically pure stereoisomers of chiral alcohols. Often, high chemo and enantioselectivity can be achieved[57].

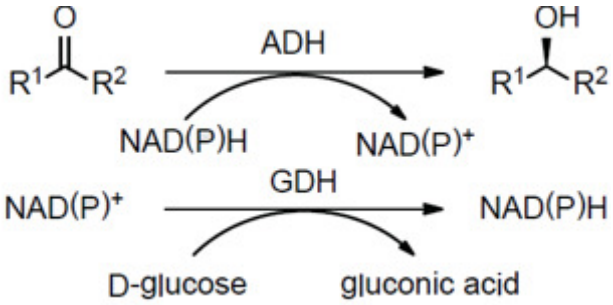


Fig 24. General ADH mechanism of action: ketone undergoes to enantioselective redaction ADH mediated, NADH co-factor is oxidized to NAD+. Glucose dehydrogenase (GDH) and D-glucose can be used to restore cofactor allowing biocatalyst to perform several cycles.

Among the large variety of ADHs available on market, we have chosen *Johnson Matthey* (*JM*) as supplier partner for our initial investigation, since this company is a long-established stakeholder of ACSDobfar for catalysis and biocatalyst supply.

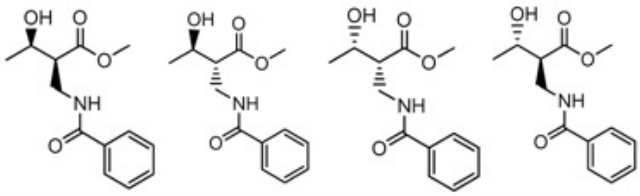
Then, we have been provided of a small library of eight different ADHs originated by recombinant expression in *E.Coli*. They have been judged to be suitable for our purpose. Their acronyms, key features and working conditions have been summarized in table 3.

Table 3: ADHs screened on reduction of MBA. All biocatalysts belong to the short chain hydrogenase/reductase family, originated by expressed recombinantly in *E.Coli*.

Enzyme	Cofactor Dependency	Optimum pH
ADH-101	$NADP^+$	7.0
ADH-104	NAD^+	6.0
ADH-105	NAD^+	6.0
ADH-110	$NADP^+$	7.0
ADH-112	NAD^+	6.5
ADH-152	$NADP^+$	6.0 - 9.5
ADH-153	$NADP^+$	6.0 - 9.5
ADH-150	$NADP^+$	7.0

Each single biocatalyst has been screened on a 5 mg MBA input scale (see experimental section for further details). After worked-up, each test has been evaluated in terms of stereoselectivity, and overall yield, evaluated by chiral chromatography. Results are summarized in table 4.

Table 4. Biocatalyst screening results: obtained isomers distribution in Area %



Screening Isomers distribution (Area%)					
Enzyme		P1(2S,3R)	P2(2R,3R)	P3(2R,3S)	P4(2S,3S)
1	*ADH-101*	80	20	n.d.	n.d.
2	*ADH-104*	-*	-*	-*	-*
3	*ADH-105*	n.d.	n.d.	60	40
4	*ADH-110*	80	20	n.d.	n.d.
5	*ADH-112*	n.d.	n.d.	80	20
6	*ADH-152*	n.d.	n.d.	30	70
7	*ADH-153*	n.d.	n.d.	90	10
8	*ADH-150*	-*	-*	-*	-*

*no conversion occurred

Data in table 4 surprisingly suggest that all the enzymes used gave us only a couple of diasteroisomers: P1 and P2 by ADH-101 and ADH-110 and P3 and P4 by ADH-105, ADH-112, ADH-152 and ADH-153, with the latter with the highest diastereoselectivity. Process led to the enantioselective reduction of ketone in 3 position but dynamic kinetic resolution of sterocenter 2 does not occur, affording one couple of diasteroisomers. The high enantioselectivity accompanied by a low diastereoselectivity was a good news for our purpose as we could generate in a limited number of experiments all four desired AZT-BAL stereoisomers to be easily separated and then carry over to AOSA. In particular, ADH-105 and ADH-110 shown the best performances for our purpose in terms of high enantioselectivity and low diastereoselectivity, Figure 25, Figure 26.

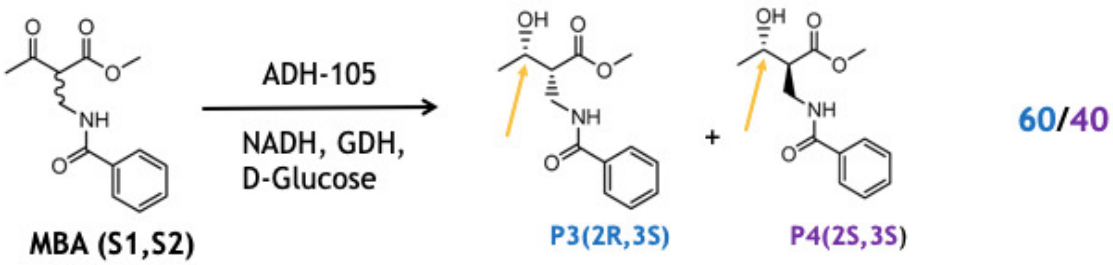


Fig 25: ADH-105 Stereoselective Carbonyl reduction occurs giving alcohol with **S** configuration

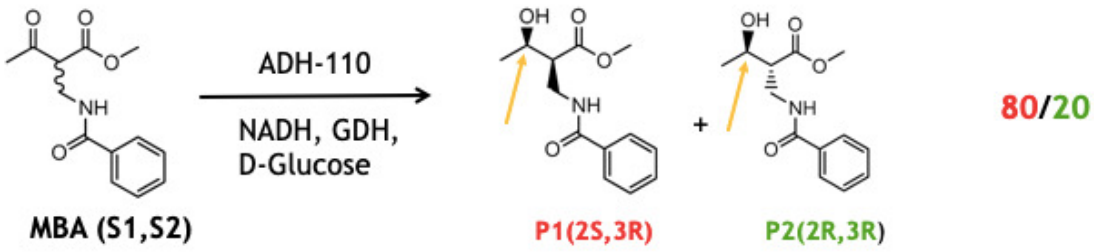


Fig 26: ADH-105 Stereoselective Carbonyl reduction occurs giving alcohol with **R** configuration

The selected enzymes (ADH-105 and ADH-110) allowed us to scaled up the asymmetric reduction on a 100 mg MBA scale in order to have enough material for the structural characterization and to pursuit the subsequent step of synthesis to AOSA. In spite of several attempts the scale up results were not so encouraging. In fact, the conversion was never higher than 40 % for both biocatalysts with a lot of starting material unreacted left and presence of minor amount of several side-products (see figure 27).

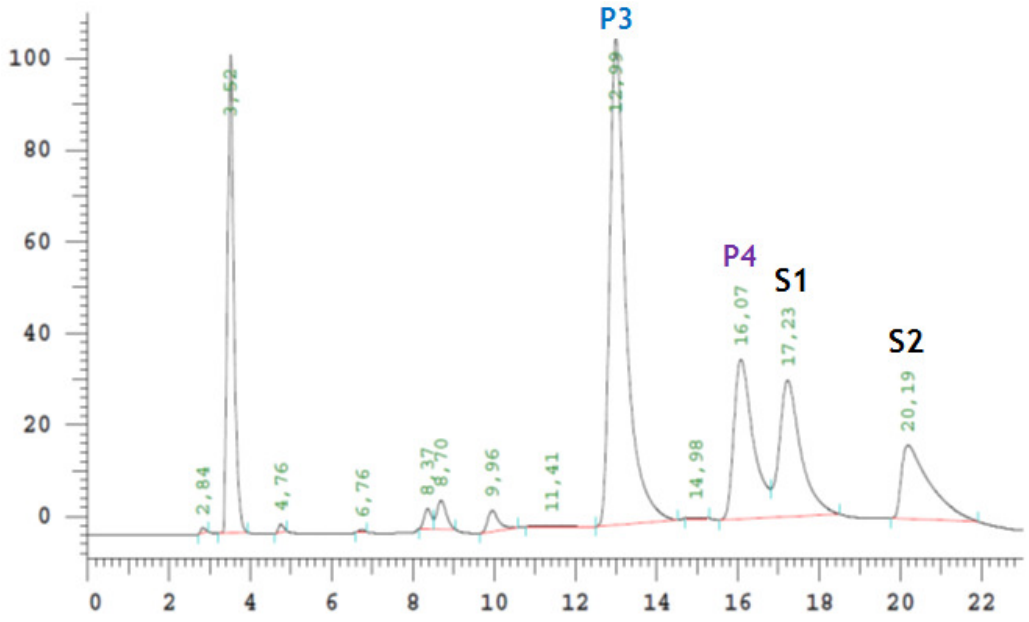


Fig 27: example chromatogram of MBA reduction with biocatalyst ADH-105 scaled up at 100mg: low conversion due to starting material left and side products formation was observed. Nevertheless, enantioselectivity was excellent.

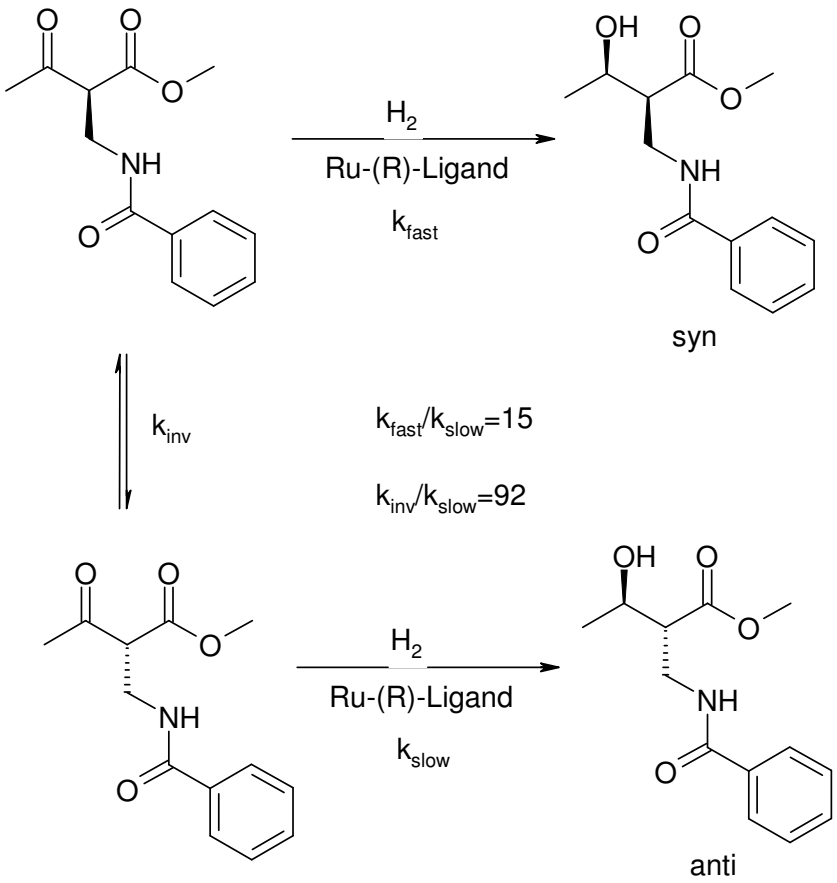
Enantioselectivity was in any case excellent (as the enantiomer was never detected by HPLC) and we succeed, after chromatographic separation, to purify enough material for

the characterization of each missing stereoisomer P2 (10mg), P3 (15mg) and P4 (10mg). This was in any case a great achievement since we succeeded in obtaining reference standards for each of our AZT-BAL target compounds, supporting and undoubtedly confirming our initial peak attribution.

Biocatalyst approach has then been fundamental to provide the necessary starting point to pursuit our primary aim. Nevertheless, this approach has been judged not suitable to move forward, since enzymatic reduction demonstrated to be not enough effective in conversion. To move forward in subsequent reactions, we had to move in a more productive reduction approach as asymmetric hydrogenation with chiral catalysts.

5.4 Asymmetric hydrogenation with chiral catalysts

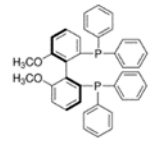
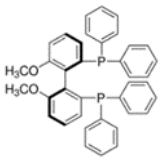
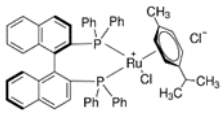
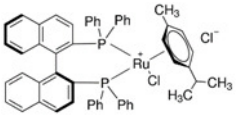
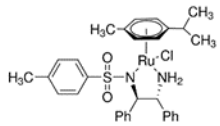
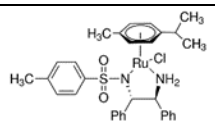
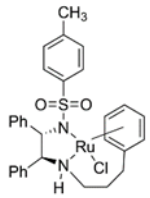
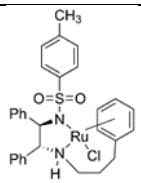
Asymmetric hydrogenation with chiral catalyst was investigated in order to obtain desired AZT-BAL isomers in a more effective and productive way, in an attempt to solve the issues on reactions yields and work-up. Even in this case, literature supporting this synthesis was very limited. Hydrogenation of this kind of substrates requires harsh conditions, including high pressure ($P \geq 50$ atm), high temperature ($T \geq 100°C$) and long reaction time (time: days). Interesting results regarding asymmetric hydrogenation of β'-keto-β amino esters to corresponding β'-hydorxy-β-amino esters are reported in an interesting paper form Xiaming et al.[58]. Good enantiomeric and diasteromeric excess have been achieved using Ru(I) as catalyst in the presence of several phosphine-based chiral ligands. These catalysts show a very interesting reactivity since they succeed in leading to enantioselective reduction of racemic 2-substituted β-ketoesters, following a dynamic kinetic resolution strategy (DKR, Scheme 10)[59].



Scheme 10: Asymmetric hydrogenation of MBA in DCM via DKR process

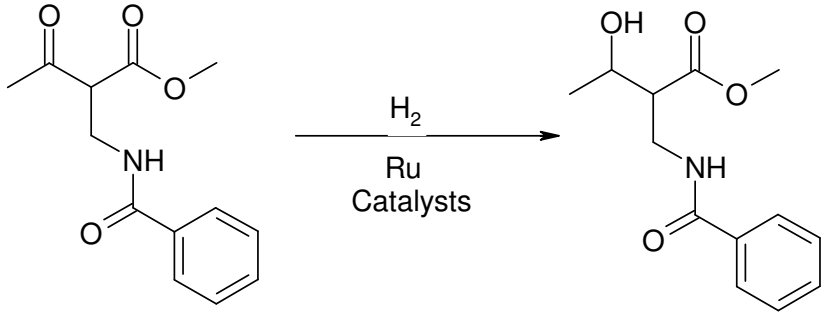
Then, we decided to test the commercial ligands described in above mentioned work[59] and to implement the study introducing a new screening, taking advantage of a brand new family of Ru(I) chiral catalysts provided by Johnson Mattey, table 5. Differently by the *in situ* catalyst **43** and **44** previously described by Xiaming[58] JM's catalyst consist in a small library of pre-formed complex of ruthenium catalyst and different chiral ligands (compounds **45, 46, 47, 48, 49, 50**).

Table 5 Ruthenium based chiral catalyst investigated in the screening

	In situ generated Catalysts		
	Metal	Ligand	
43	Ru	(S)-Segphos	
44	Ru	(R)-Segphos	
	JM Catalysts		
45	(R)-Binap RuCl p-cymene		
46	(S)-Binap RuCl p-cymene		
47	(R,R)-TsDpen-Ru(p-cymene)Cl		
48	(S,S)-TsDpen-Ru(p-cymene)Cl		
49	(S,S)-Teth-TsDpen RuCl		
50	(R,R)-Teth-TsDpen RuCl		

The listed catalysts have been tested in an screening on a 125 mg MBA input scale, according to the published conditions. Results are summarized in the following table 6.

Table 6: Results from the Ruthenium catalysis screening, referred to the reaction:



	In situ generated Catalysts					
	Metal	Ligand	Results			
			Yield %	ee%	de%	Main Product
43	Ru	(S)-Segphos	80	97.9	90.0	P3 (**41**) 2R,3S
44	Ru	(R)-Segphos	81	98.1	91.0	P1 (**39**) 2S,3R
	JM Catalysts					
45	(R)-Binap RuCl p-cymene		97	98.0	92.0	P1(**39**) 2S,3R
46	(S)-Binap RuCl p-cymene		98	97.8	89.0	P3(**41**) 2R,3S
47	(R,R)-TsDpen-Ru(p-cymene)Cl		35	80.0	91.0	P3(**41**) 2R,3S
48	(S,S)-TsDpen-Ru(p-cymene)Cl		30	60.0	89.0	P1(**39**) 2S,3R
49	(S,S)-Teth-TsDpen RuCl		-**	-	-	-
50	(R,R)-Teth-TsDpen RuCl		-**	-	-	-

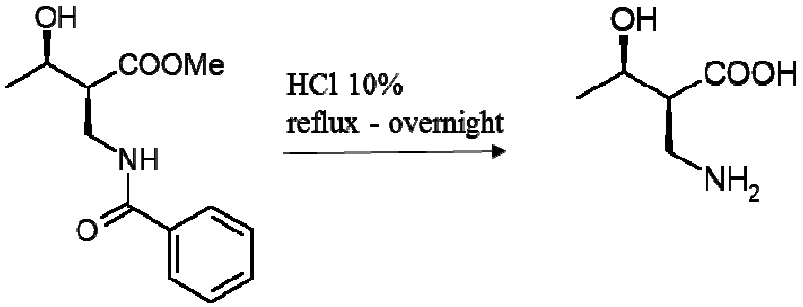
* Screening conditions: Catalyst 0.04 mol%, c=0.25M, solvent: DCM/TFE=3/1, P=50 bar, T=70°C, t=22h. *ee* and *de* were determined by chiral HPLC. Yield determined by weight after isomer purification.

** No conversion occurred

Concerning *ee* and *de*, the *in situ* pre-formed catalysts **43** and **44** have shown good performances, superimposable to what have been reported in literature. Conversion was instead found to be lower then what reported. JM's catalyst have shown good

performances. In particular, compounds **45** and **46** outlined very interesting catalytic reactivity. Specifically, very good enantiomeric excess was determined, comparable to **43** and **44**, but combined with a higher conversion percentage. on the contrary other catalysts screened (**47**, **48**, **49**, *50*) were not found to have interesting reactivity for our purpose of MBA asymmetrical hydrogenation. Looking at Table 6 it is clear how, unfortunately, all catalysts screened lead to *syn* products only, **39** and **41**, with different efficiency. No conversion to *anti* compound, **40** and **42**, occurred in any case. Nevertheless, screening was partially successful for our purpose since catalyst **46** led to the formation of AZT-BAL compound **41**, which is the enantiomer of the natural precursor of AOSA, with very good yield and selectivity. This result was fundamental to pursuit in our objective. In fact, having in our hand this precious enantiomer the achievement of a very important AOSA isomer was indeed possible. Catalyst **46** was then tested at an increased scale of 2g input MBA, affording a relevant quantity of compound **41** necessary to pursuit subsequent reaction to AOSA's enantiomer synthesis.

The subsequent deprotection step was than tested first on compound **39**, in order to evaluate performances and feasibility prior to move on more precious isomer **41**, scheme 11.

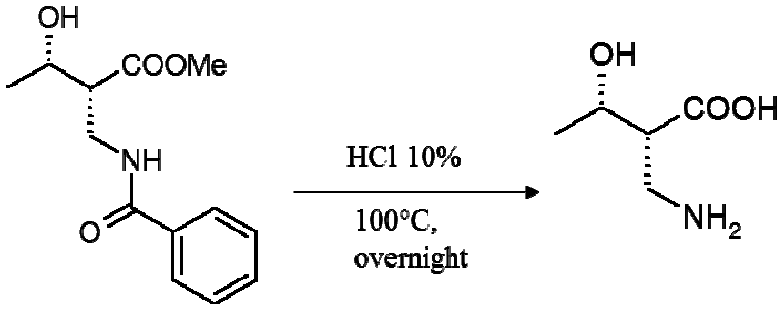


AZT-BAL (2S,3R) AAL (*2S,3R*)

Scheme 11: deprotection of AZT-BAL (**39**) to afford AAL (**34**)

Deprotection involves acid hydrolysis in 10% aqueous HCl refluxing overnight. This apparently easy step has shown unexpected complications. AAL is formally an amino acid presenting tricky characteristics since it is highly soluble in water and presents three different functional groups. In addition to these properties the absence of

chromophores makes workup, isolation and purification quite challenging. To overcome these issues after reaction completion, water has been completely removed through azeotrope distillation with toluene. Hydrochloride salt obtained as sticky oil is then dissolved in methanol and 1 equivalent of triethylamine is added. Crystallization of AAL occurs at room temperature overnight, while triethylammonium salts remain in mother liquor, with an overall yield is 55%. Optimized deprotection was then applied on enantiomer **41**, showing the same performances, affording compound **34b**, scheme 12.



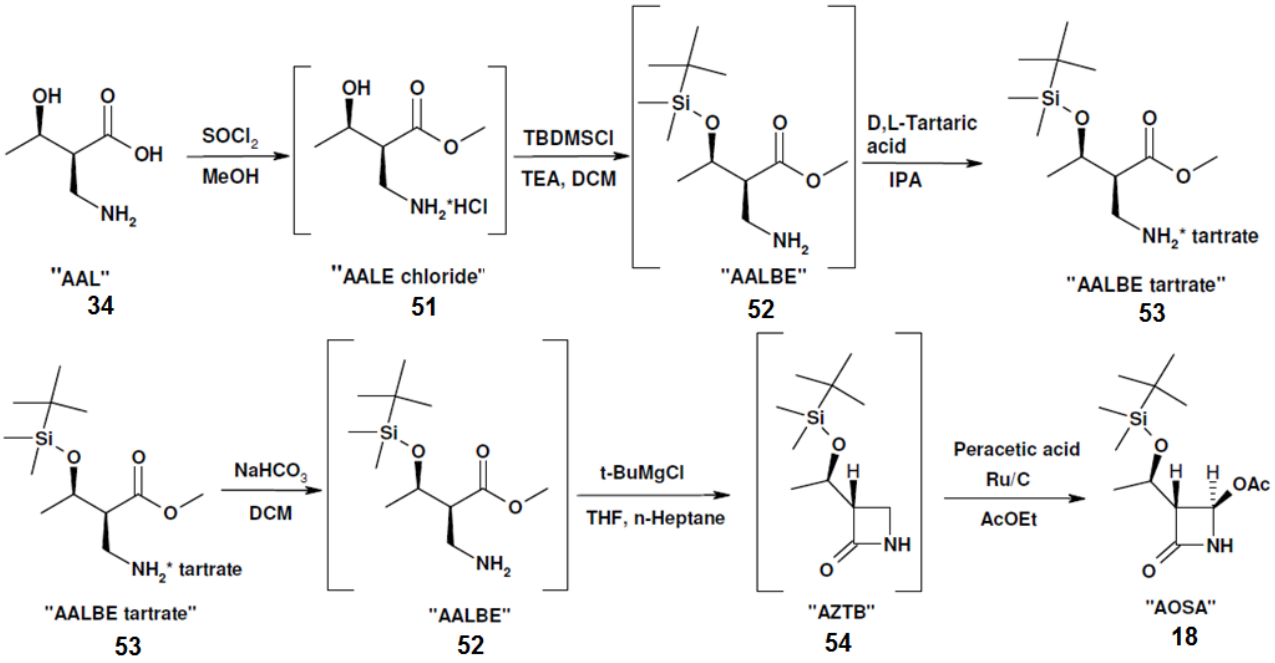
AZT-BAL (2R,3S)
41

AAL (*2R,3S*)
34b

Scheme 12: deprotection of AZT-BAL (**41**) to afford AAL (**34b**)

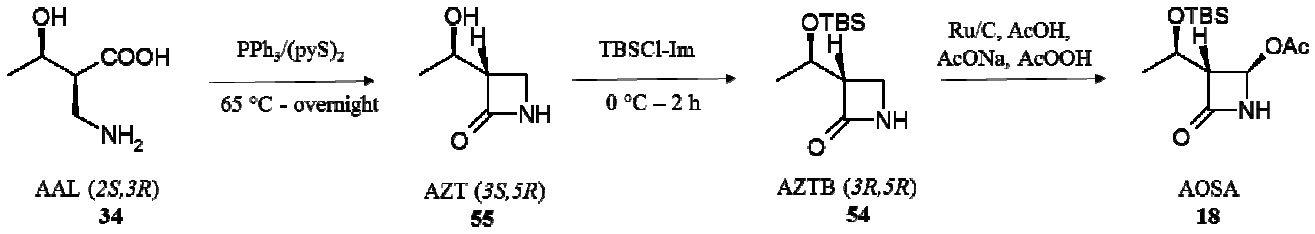
5.5 Choice of AOSA's enantiomer route of synthesis

Several AOSA's routes of synthesis have been already described in section 2.4. The choice of the most suitable route for our purpose was made evaluating all various mentioned and patented routes. ACSDobfar has developed a method for AOSA's manufacturing, owning the intellectual property, Scheme 13. This synthesis involves six steps and two isolations. ALL compound **34** is treated with $SOCl_2$ and methanol to afford the corresponding methyl ester hydrochloride *51*. Hydroxyl function is then silylated with *tert*-butyldimethylsilyl chloride (TBSCl) to afford compound *52* which is then isolated as tartrate *53*, after treatment with tartaric acid in isopropanol. Compound *53* is then treated with triethylamine to allow cyclization with tert-butyl magnesium chloride affording β-lactamic compound *54*, AZTB. Finally, treatment with peracetic acid and Ru/C leads to the formation of final intermediate AOSA (Scheme 13).



Scheme 13: ACSDobfar AOSA's manufacturing procedure.

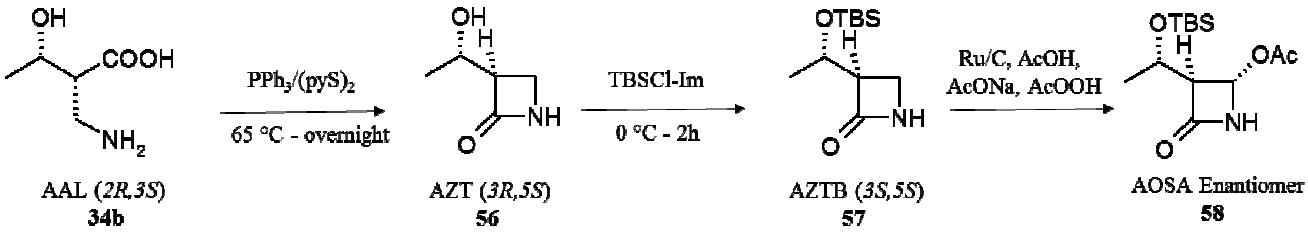
ACSDobfar's synthesis is very productive route since it does not involve chromatographic purification and uses widely available and cheap reagents in order to be cost efficient and to be easily applied on large-scale manufacturing. For our purpose, a different and more effective strategy was developed, optimized and applied, basing on existing literature and involving just three synthetic steps, scheme 14. For familiarization and optimization of various condition widely available AAL (2S, 3R) was used.



Scheme 14: Optimized route for AOSA starting from AAL involving three steps of synthesis

AAL is directly cyclized through activation of the carboxylic function with triphenylphosphine and 4,4′-dithiodipyridine in acetonitrile[60], to afford the β-lactam ***55*** AZT with 70% yield after chromatographic purification. Compound ***55*** is then silylated

following standard conditions by TMSCl and imidazole to obtain AZTB compound **54** with 80% yield after purification. Final stereospecific acetoxylation was carried over with peracetic acid and Ru/C to afford AOSA, 95% yield[61]. The optimized new synthesis has shown good overall performances in terms of yield and ease of purification of intermediates. This route was then applied on enantiomer AAL compound **34b** on 2g scale, (Scheme 15).



AAL (*2R,3S*) **34b** — AZT (*3R,5S*) **56** — AZTB (*3S,5S*) **57** — AOSA Enantiomer **58**

Scheme 15: synthesis of AOSA enantiomer form AAL 34b.

350 mg AOSA enantiomer were then obtained successfully following this route of synthesis. Having this important isomer as reference standard was really a fundamental milestone for our control strategy. ^{1}H-NMR spectra of both compounds **18** and **58** have been compared, as shown in figure 28, to confirm structure and peak overlapping for the two enantiomers.

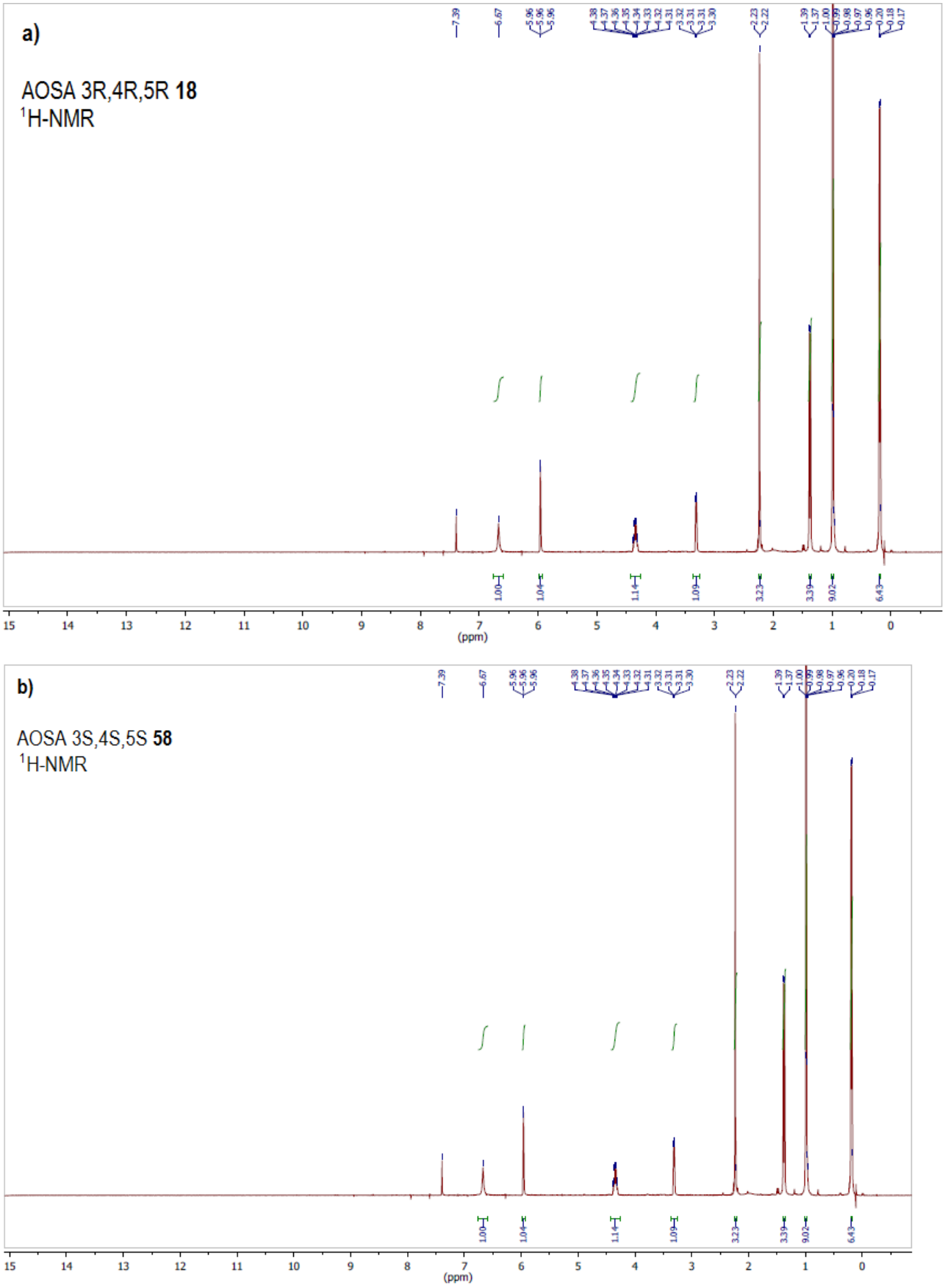


Fig. 28: a) ^{1}H-NMR (300MHz $CDCl_3$) AOSA 3R,4R,5R **18. b)** ^{1}H-NMR (300MHz CDCl3) AOSA 3S,4S,5S **58.**

5.6 AZT-BAL missing stereoisomers

Asymmetrical hydrogenation with chiral catalysts has demonstrate to be successful for the achievement of AOSA enantiomer compound **58**. Nevertheless, as outlined in table 6, this kind of approach does not lead to the formation of *anti*-couple of enantiomers, compounds **40** and **42**. Biocatalysts approach showed the possibility of formation of *anti*-couple but, as already discussed, productivity of the process was not suitable for producing enough material to move forward. To pursuit in our aim to reach at least all four AZTB compound (see figure 29) a different approach had to be developed.

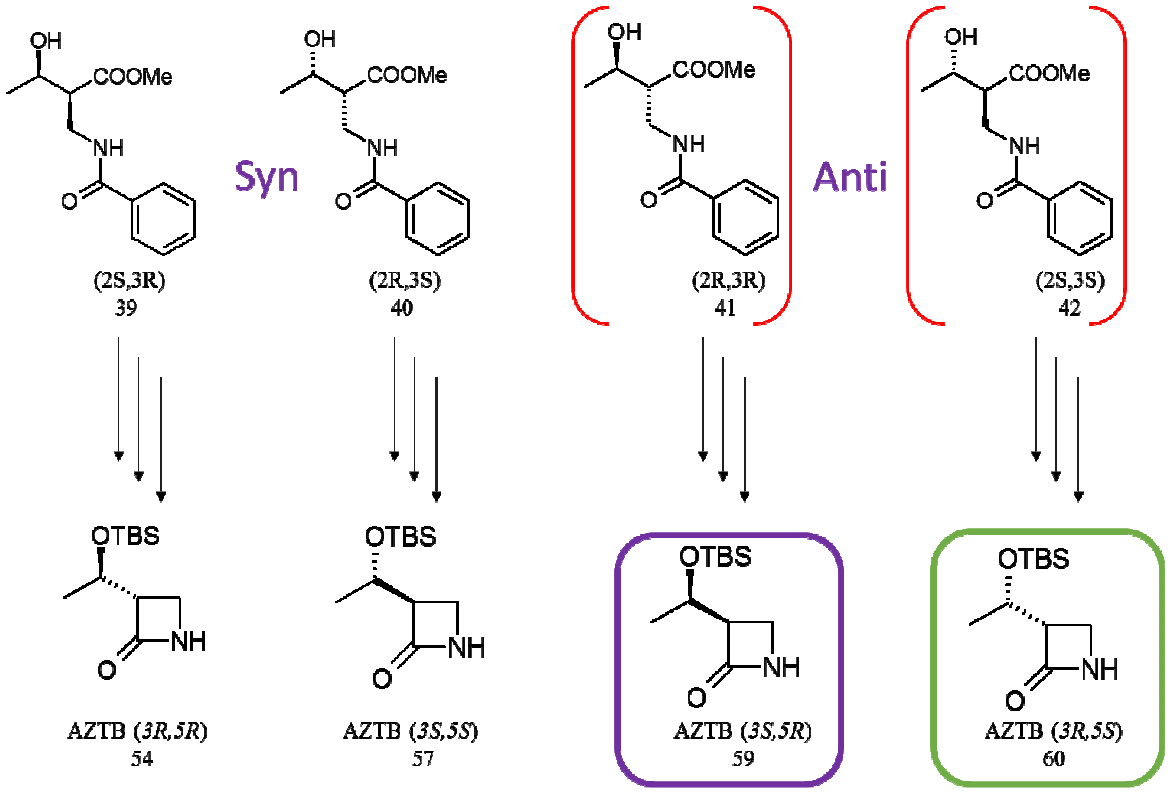


Fig 29: due to the impossibility to have anti-couple of compounds, **41** and **42**, corresponding AZTB advanced intermediates had to be achieved with a different strategy.

In fact, as already discussed, AZTB is an advanced intermediate showing sterocenters 3 and 5 with the absolute configuration that will be retained through all subsequent synthetic steps. For all these reasons, we decided to target directly these advanced intermediates **59** and **60**, putting in place a new synthetic strategy by-passing AZT-BAL precursors.

5.7 Synthesis of AZTB 3R, 5S (60)

AZTB (3R, 5S) **60** synthesis has been optimized and achieved through a different "retrosynthetic" approach. We decided to start form widely available compound **18** AOSA since, comparing with our target compound and looking at their structures, they differ just for one stereocenter in 5 position, Figure 30.

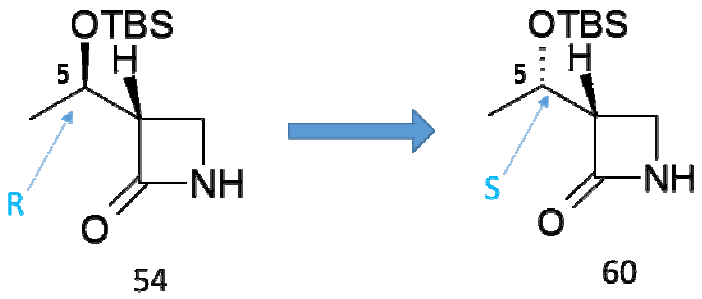
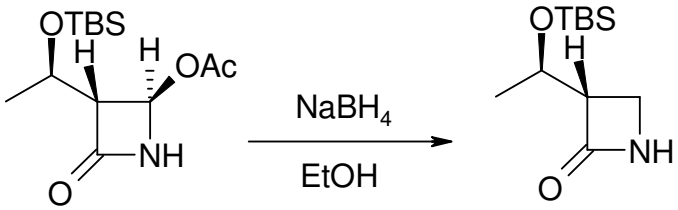


Fig 30: AZTB compound 54, natural AOSA precursor, differ from AZTB 60 just for the absolute configuration of stereocenter in 5 position.

At first, the idea was to simplify stereochemistry of AOSA by removing acetoxyl functional group in position 4, in order to obtain directly ATZB **54** and then proceed through other steps with the inversion of stereocenter 5. Threating AOSA **18** with sodium born hydride in ethanol, we had the first evidences of this possibility. Using 1 equivalent of $NaBH_4$ at room temperature as mentioned in literature, we observed formation of several side-product achieving an overall yield of 45% AZTB (Scheme 16)[62].

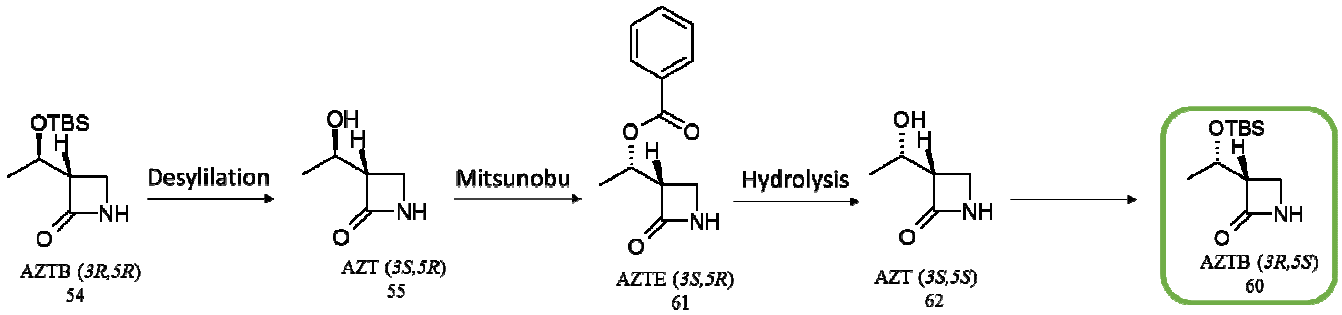


Scheme 16: reductive de acetoxylation of AOSA to afford AZTB

Adjusting condition to 3 equivalents of $NaBH_4$ added portion wise at -10°C the reaction was then optimized increasing yield up to 95% in AZTB. This trivial reaction is very important in the objective to build up an analytical control strategy. Having demonstrated the possibility to remove stereocenter at C4 with high conversion we had

in our hands the possibility to move forward. In fact, this reductive de-acetoxylation of AOSA to quantitatively yield AZTB has allowed us to simplify the defense of AOSA stereochemistry limiting the discussion to sterocenters 3 and 4 given that, as deeply discussed, stereocenter in 4 is in any case lost in subsequent steps to carbapenem. AOSA can, in this way, be easily converted to corresponding AZTB and the other 3 possible stereoisomers can be controlled directly trough a traditional HPLC protocol. The development of this efficient analytical protocol in this regard will be discussed in section 4.12

In addition, having the possibility to start form AOSA allowed us to have AZTB available in large quantity very easily. This was indeed important since the original planned synthesis to achieve the target compound **60** involved four steps, starting from AZTB, as shown in scheme 17.



Scheme 17: synthesis of AZTB **60** starting from AZTB ***54***.

Plan foresaw initial desilylation of AZTB to afford AZT, inversion of stereocenter 5 through *Mitsunobu* reaction with benzoic acid of afford the corresponding benzoate ester AZTE. Then hydrolysis and re-silylation of alcohol have been performed to give target compound **60**. Starting from AZTB desilylation was easily achieved treating compound ***54*** with tetrabutylammonium fluoride affording AZT ***55*** with 65% yield after purification. At this point inversion of stereocenter 5 was then tried in typical *Mitsunobu* reaction condition (triphenylphosphine, benzoic acid, overnight at room temperature in Toluene) using Diethyl azodicarboxylate (DEAD) as condensing agent. Reaction was unexpectedly tricky. No formation of desired compound **61** was observed in any condition. Benzoic anhydride was the main product with formation of triphenylphosphine oxide. In addition, several degradation by-products were observed.

Main degradation product isolated, hydroxyl β-elimination with double bond formation, is shown in figure 31.

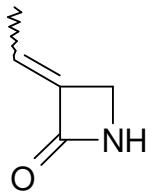


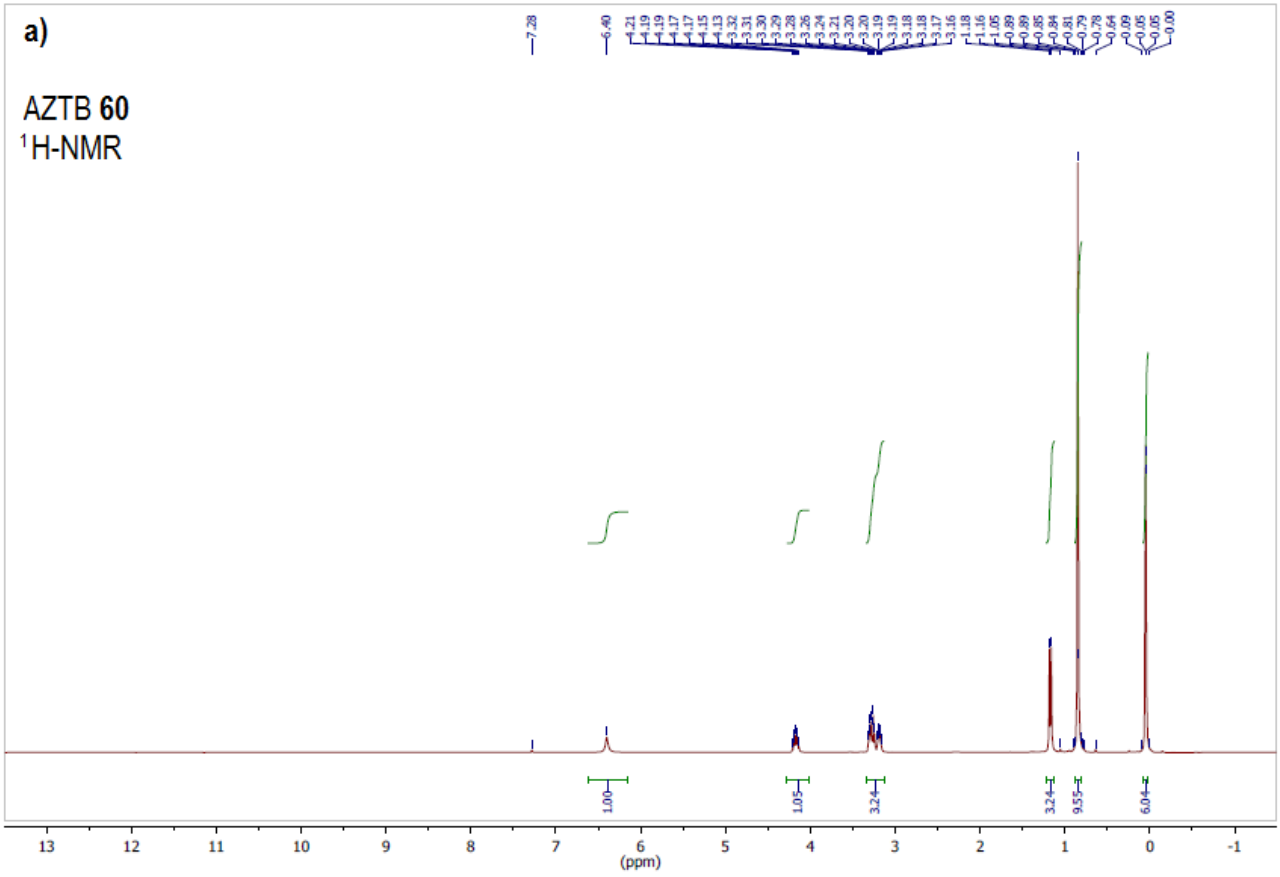
Fig 31: β-elimination product

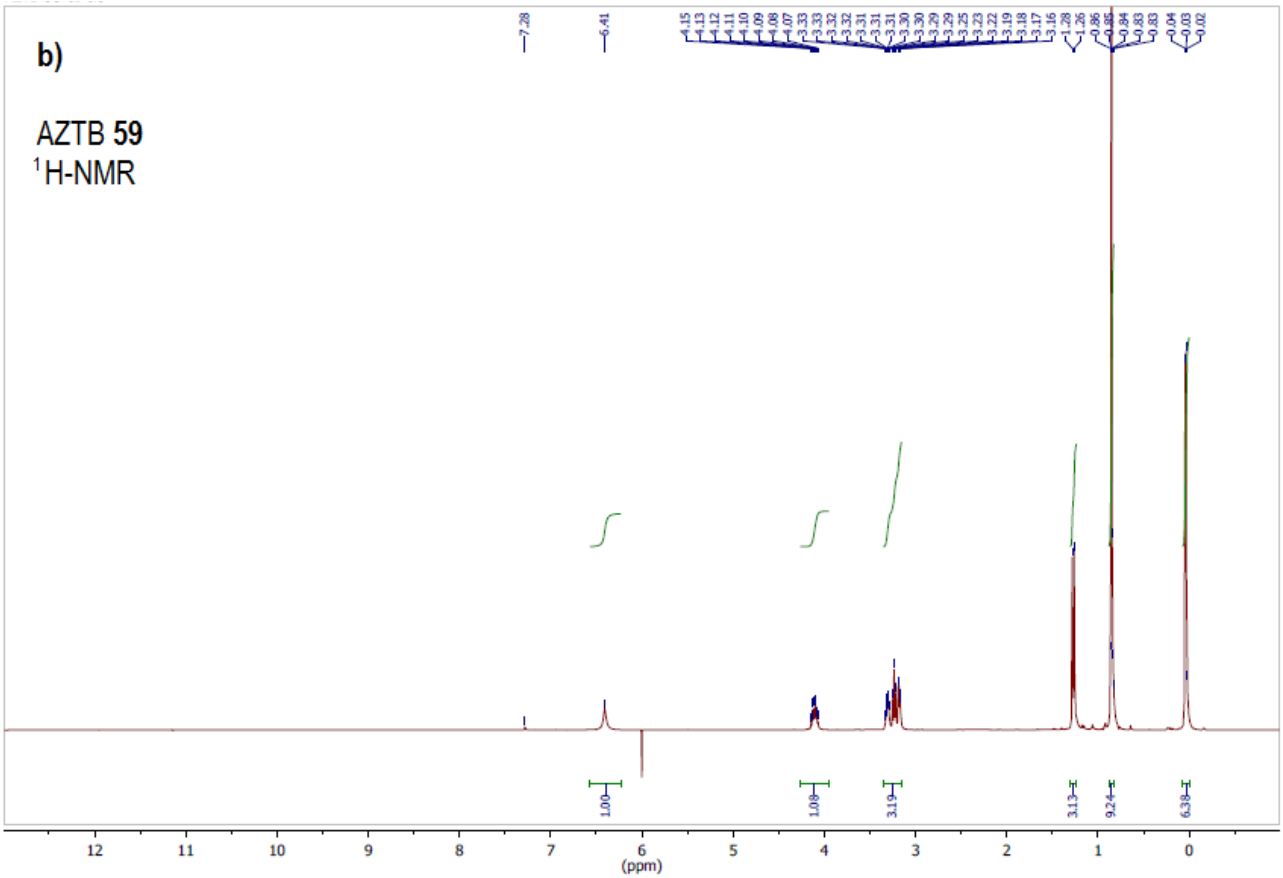
AZT is in fact quite unstable in solution. Similar reactivity has been found on AZT at room temperature in different solvent such as dichloromethane and acetone. Increased stability was instead found in ethyl acetate and toluene.

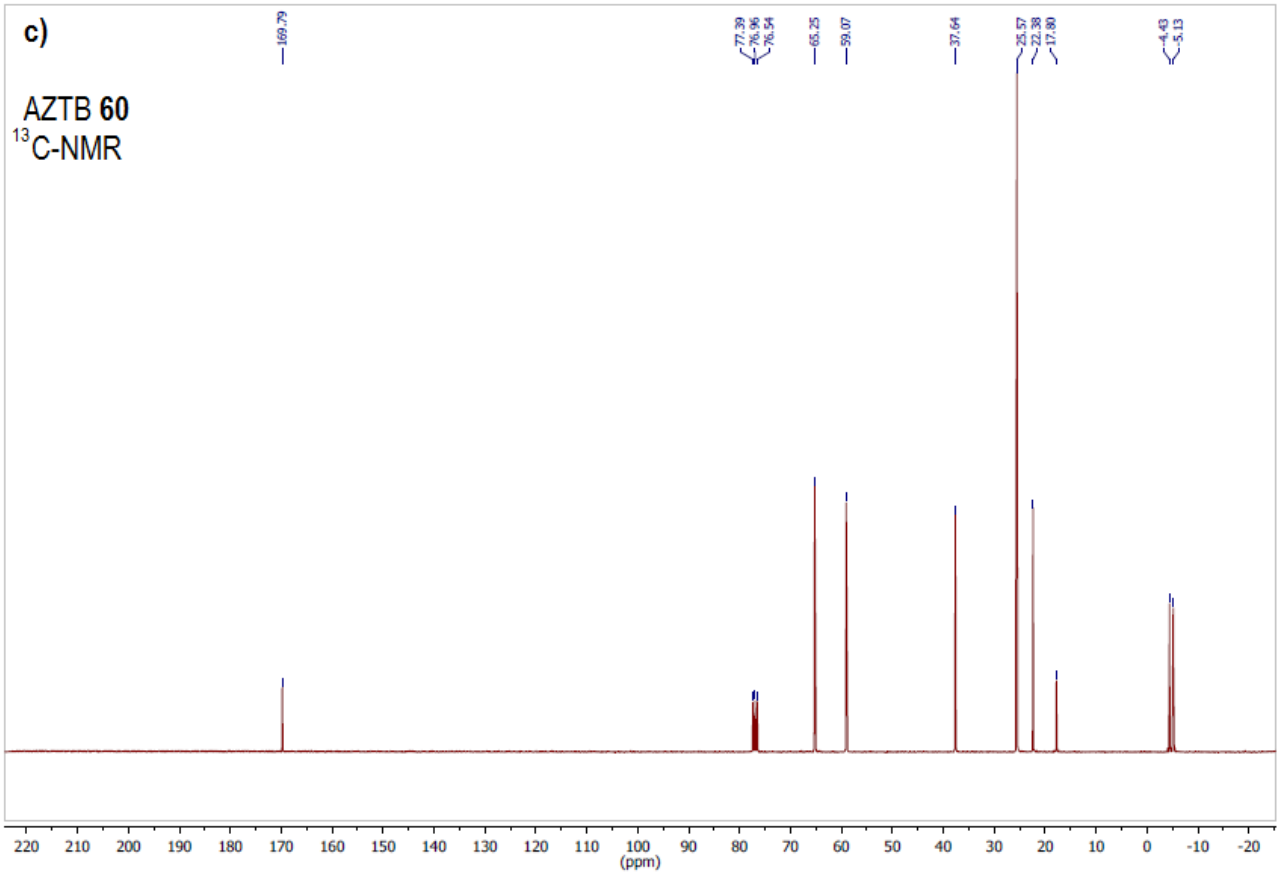
As *Mitsunobu* reaction is known to be highly sensitive to the steric hindrance of the starting alcohol, several methods were tried to overcome this drawback. We tried to perform the nucleophilic substitution by replacing benzoic acid by a stronger acid (nitrobenzoic acid). However, additional degradation reactions and the formation of side-products were observed. The activation of the hydroxy function was then tested by using a mixture of DIAD (diisopropyl azodicarboxylate)/PPh_3 with similar disappointing results.

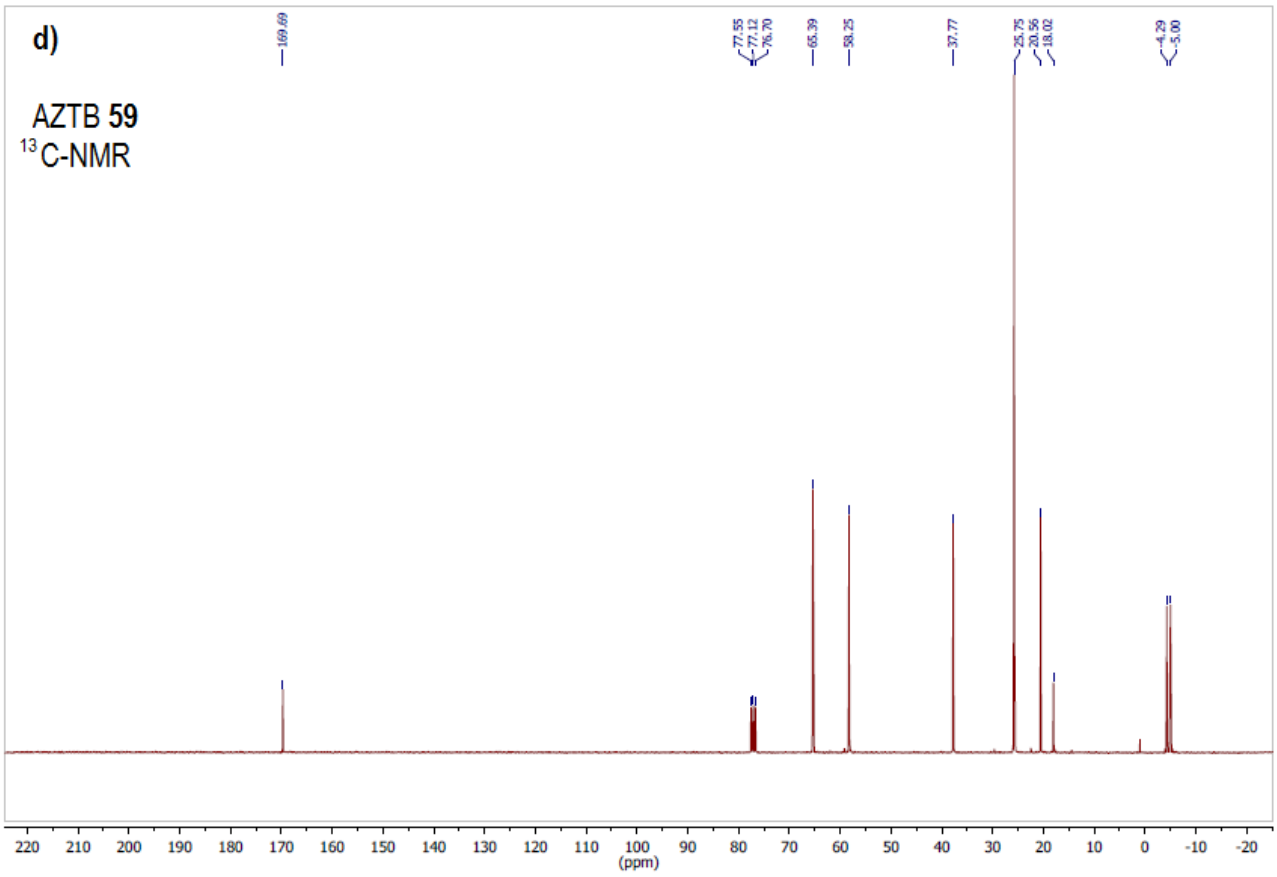
Searching in the literature on similar substrate[63], this issue was brilliantly overcome replacing DEAD with tetramethylazo dicarboxamide (TMAD), as activating agent, coupled with a more nucleophilic phosphine such as PBu_3. In these condition compound **61** was obtained with an acceptable yield of 39%. The limited formation of side products made also the purification step easier. AZTE ester **61** was the treated with methanol and sodium hydroxide at room temperature overnight to afford AZT **62** with the desired C5 absolute configuration. At last, silylation with TBSCl and imidazole was performed at r.t. overnight to afford target reference standard compound **60** with an overall yield from AZTB **54** of 25%. With this important advanced intermediate, another milestone was achieved.

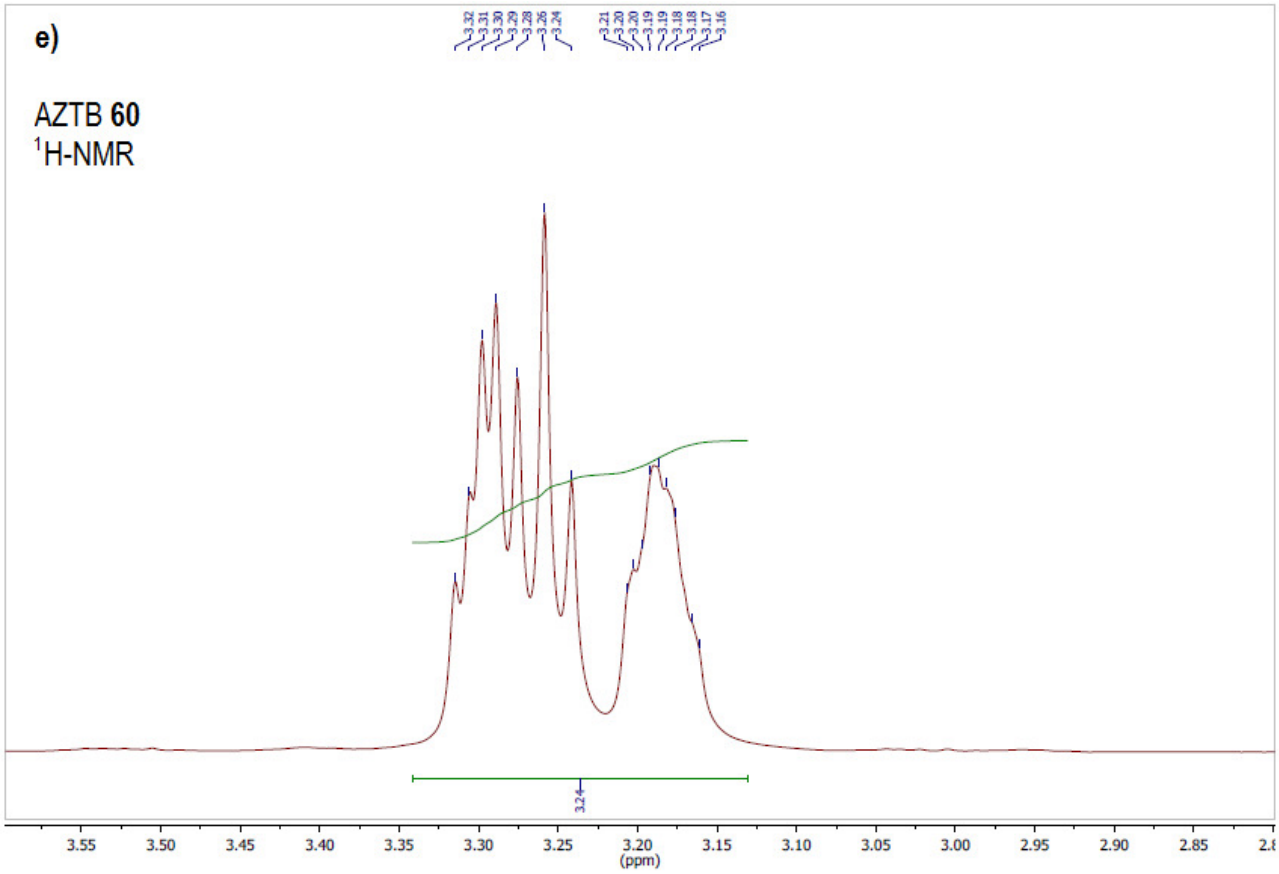
AZTB **54** was characterized by NMR and spectra compared with AZTB **60** in order to outline similarity and differences. Figure 32.


a)
AZTB 60
1H-NMR
(ppm)


b)
AZTB 59
1H-NMR
(ppm)


c)
AZTB 60
13C-NMR
169.79
77.39
76.96
76.54
65.25
59.07
37.64
25.57
22.38
17.80
-4.43
-5.13
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
(ppm)


d)
AZTB 59
13C-NMR
169.69
77.55
77.12
76.70
65.39
58.25
37.77
25.75
20.56
18.02
-4.29
-5.00
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
(ppm)


e)
AZTB 60
1H-NMR
3.32
3.31
3.30
3.29
3.28
3.26
3.24
3.21
3.20
3.20
3.19
3.19
3.18
3.17
3.16
3.24
3.55
3.50
3.45
3.40
3.35
3.30
3.25
3.20
3.15
3.10
3.05
3.00
2.95
2.90
2.85
(ppm)

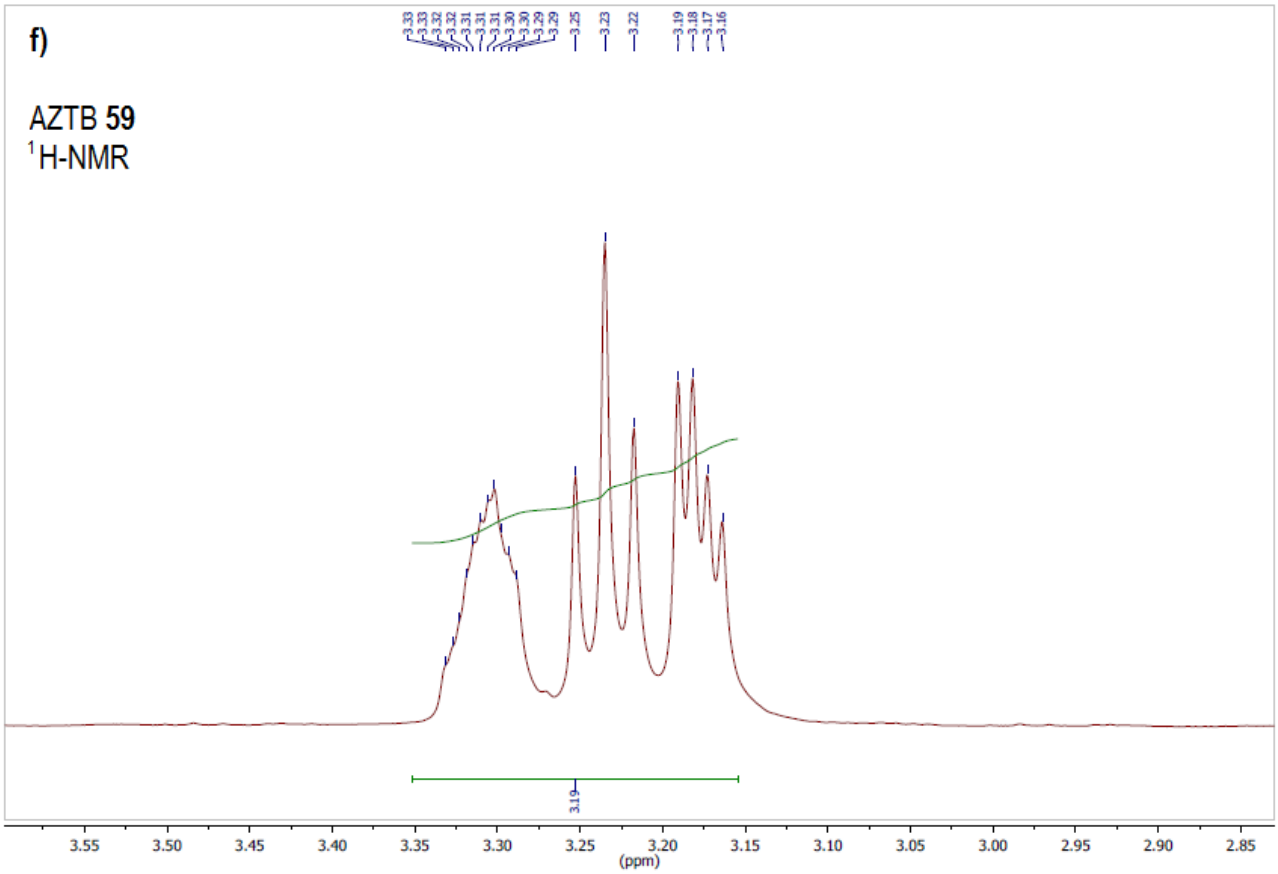

f)
AZTB 59
1H-NMR
3.33
3.33
3.32
3.32
3.31
3.31
3.31
3.30
3.30
3.29
3.29
3.25
3.23
3.22
3.19
3.18
3.17
3.16
3.19
3.55
3.50
3.45
3.40
3.35
3.30
3.25
3.20
3.15
3.10
3.05
3.00
2.95
2.90
2.85
(ppm)

Fig. 32: a) ^{1}H-NMR (300MHz $CDCl_3$) AZTB **60. b)** ^{1}H-NMR (300MHz CDCl3) AZTB ***59*. c)** ^{13}C-NMR ($CDCl_3$) AZTB **60. d)** ^{13}C-NMR ($CDCl_3$) AZTB ***59*. e)** ^{1}H-NMR (300MHz $CDCl_3$) AZTB **60** zoom 2.8-3.4 ppm**. f)** ^{1}H-NMR (300MHz $CDCl_3$) AZTB ***59*** zoom 2.8-3.4 ppm**.**

As outlined in figure 32, NMR spectra confirmed structure for AZTB ***59***. Comparison between the two diasteroisomers AZTB ***59*** and **60** spectra shows just little difference in general chemical shifts. Going more in detail and focusing on region between 2.8 and 3.4 ppm (figure 32 **e** and **f**) a substantial difference was observed.

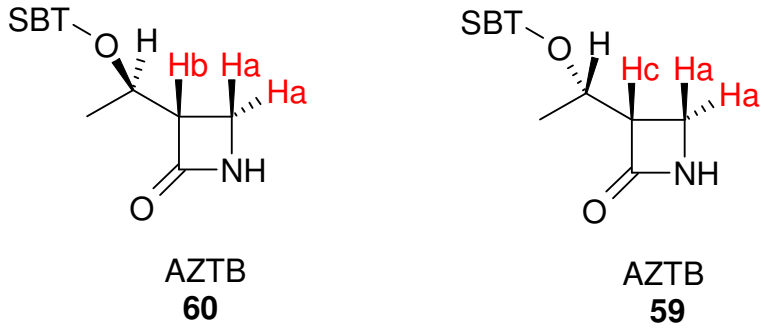


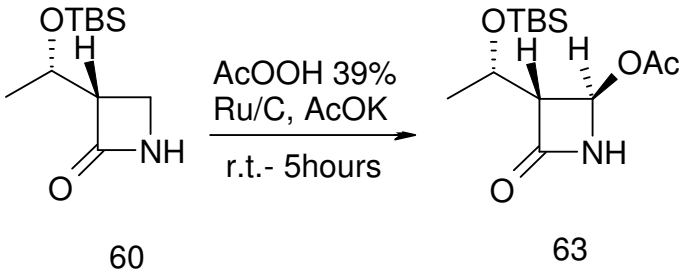
Fig 33. Protons with chemical shift change in in AZTB 60 and 59.

Multiplet relevant to Hb (figure 33) shows a shift from 3.19 to 3.31 ppm. At the same time multiplet relevant to one of the diasterotopic protons Ha shifts from 3.30 to 3.19 ppm.

To complete the study, ***59*** reactivity in Ruthenium mediated- acetoxylation condition was investigated trying to achieve the corresponding AOSA with C5-S inversion.

5.8 Synthesis of AOSA 3R, 4R, 5S (63)

AZTB **60** has been treated with peracetic acid and Ru/C to be carried over to corresponding AOSA (3R, 4R, 5S), scheme 18.



60 63

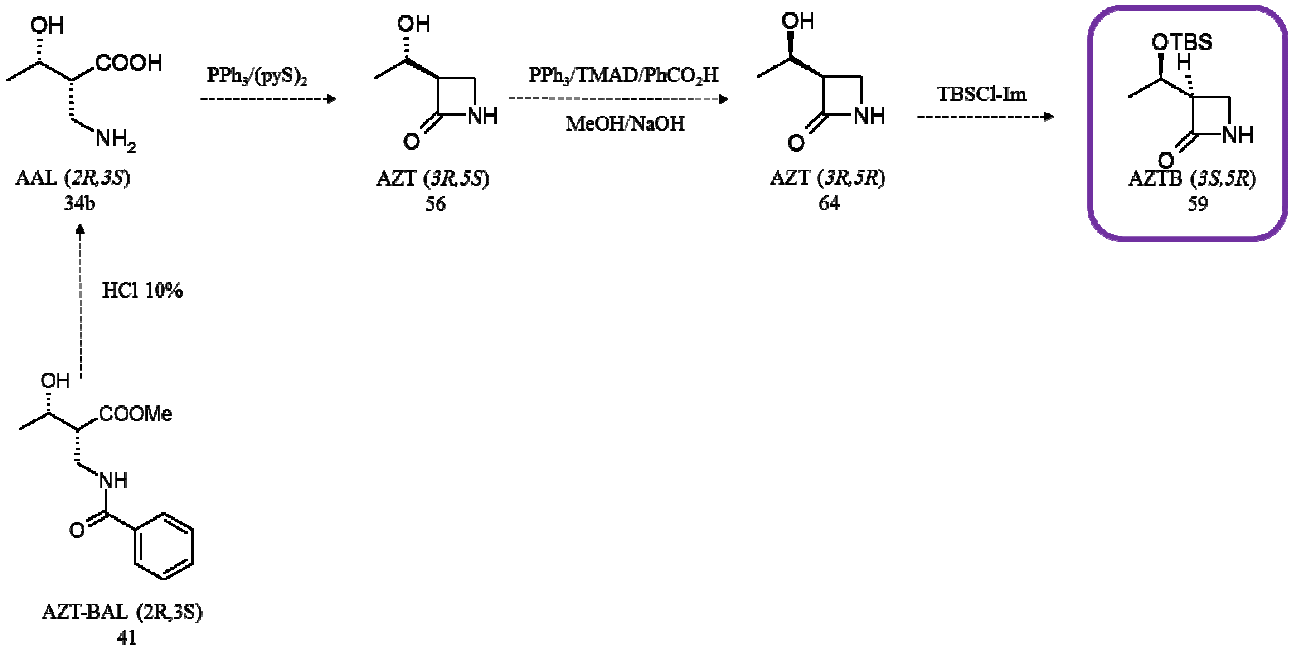
Scheme 18: acetoxylation of AZTB 3R, 5S to afford AOSA 3R, 4R, 5S

Acetoxylation method was applied as per ACSDobfar manufacturing procedure described in scheme 13. Reaction has generally very good performances on natural AZTB 3R, 5R; very high yield usually achieved, close to 95%, very good in-solution purity profile are observed with no side product and final intermediate AOSA is isolated through crystallization with no need of further purifications. Applying the same procedure to isomer **60** a different scenario was observed. After 5 hours reaction side-product formation was observed and starting material still unreacted was present. In any case, desired main product **63** was obtained isolated and characterized, with an overall yield of 55%.

Regarding seteroselectivity of reaction, unexpectedly, just the formation of 4R stereoisomer was observed. Independently by configuration of C5, acetoxylation occurs on *Re* face of AZTB, based on the steric hindrance of stereocenter C3.

5.9 Synthesis of AZTB 3S, 5R (59)

Synthesis of AZTB *59* has been, unexpectedly, the most challenging part of our control strategy. Actually, considering all synthetic work already developed, the synthesis of ***59*** was theoretically already possible following the route reported in scheme 19.



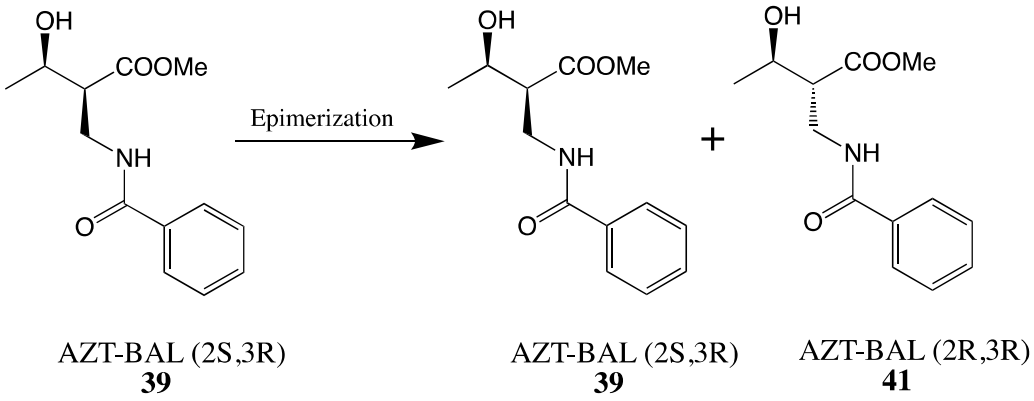
Scheme 19: synthesis of AZTB 3S, 5R starting from AZT-BAL 2R, 3S

Starting form asymmetrical hydrogenation product **41**, deprotection can be performed to afford AAL enantiomer **34b** followed by cyclization to obtain AZT *56*. At this point inversion of stereocenter in C5 can be tackled using procedure successfully developed for compound **60**, using TMAD and PBu_3. Subsequently saponification and silylation with TBSCl would afford target AZTB *59*. Nevertheless, overall yield of the whole process would be around 10%, based on the results acquired on the same synthesis for different isomers. Considering this, almost 30g of compound **41** would be necessary to obtain at least 300mg of *59*, minimum quantity needed for a reference standard. In addition, autoclave needed for high pressure hydrogenation has a maximum capacity of 70ml, with a maximum output for run of 2g AZT-BAL. Easy to understand how this would be an unlikely scenario since a reference standard, to be used in routine test, should be, if possible, easily available in a short time when needed.

For these reasons, once again, a different approach for AZTB *59* was studied in order to afford last desired stereoisomer in a more direct and easy way.

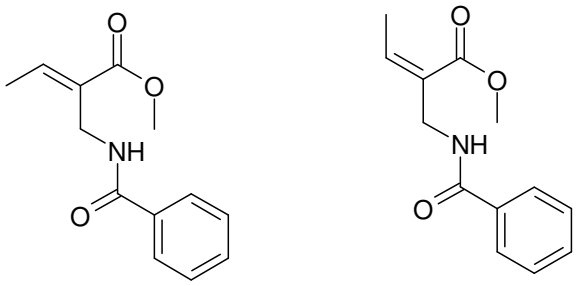
5.10 Racemization of AZT-BAL 2S, 3R (39)

First tentative to set a new route for AZTB *59*, was thought considering epimerization at C2 of optically pure compound AZT-BAL **39**, widely available through Codex® KRED enzymatic synthesis, scheme 20.



Scheme 20: Epimerization of C2 on AZT-BAL 2S, 3R

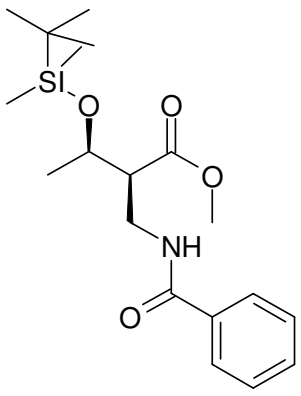
Taking advantage of the acidity of proton at the α-position (C2), the idea was to promote keto-enol equilibrium with the right base in order to induce epimerization at C2. The results would be having a mix of two diasteroisomers **39** and **41** that could be easily separated and then carried over to AZTB. Several conditions were then tested studying this possibility. First, typical thermodynamic conditions were tested, treating **39** at room temperature with tert-butanol/potassium tert-butoxide. Just after one hour, both starting material and desired product were not present, formation of two unknown compound was observed. Main products of reaction were then isolated and characterized, structures showed in figure 34.



65 **66**

Fig 34. β-elimination products

Characterized structures correspond to β-elimination products **65** and **66**. AZT-BAL undergoes to β-elimination very easily under these conditions. Different conditions have then been explored. Potassium tert-butoxide has been dissolved in THF and **39** dissolved at -78°C. Different temperature have been screened trying to achieve racemization containing β-elimination. No reaction was observed at from -78 up to 0°C. At 10°C slow reactivity was shown affording in any case β-elimination product. Different base has then been tested. Lithium diisopropylamide (LDA) 1M in THF has been test and, even in this case different temperatures have been screened. Starting from -78°C up to 0°C no reaction occurred. At +5°C reactivity is observed leading, even in this case, to the formation of **65** and **66**. β-elimination is indeed a typical reactivity of this substrate in basic condition. Protection by silylation of hydroxyl was then explored trying to give to the substrate a different reactivity allowing racemization and inhibiting β-elimination, Figure 35.



67

Fig. 35. AZT-BAL protected as TBS

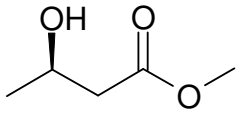
Different silylation condition were tested (TBSCl/imidazole or triethylamine) but, surprisingly, compound **67** has demonstrated impossible to be achieved with this approach.. This was quite disappointing since this kind of silylation occurs with no particular issues on similar molecules such as AZT *55* (see scheme 14) and AALE *51* (see scheme 13). Looking at various structure, protection present on the amine seems to act a steric hindrance role in this kind of reaction. To overcome this issue compound *52* was synthetize following a similar route described in scheme 14, and easily protected with benzoyl chloride and pyridine affording desired compound **67** with 90% yield.

Attemps of racemization were then also approached on **67**. Different temperature and bases were tested with not encouraging results, since β elimination was found to be the only possible reactivity even on this protected substrate as per AZT-BAL.

Racemization approach was the abandoned and a completely different synthesis was thought and developed in order to achieve last missing stereoisomer.

5.11 Synthesis of 59 from Methyl (R)-3-hydroxybutyrate

Exploring literature and various commercially available starting materials, a possible alternative synthesis for the forth missing stereoisomer *59* was thought. The synthesis involves optical pure substrate **68** as starting material, figure 36.

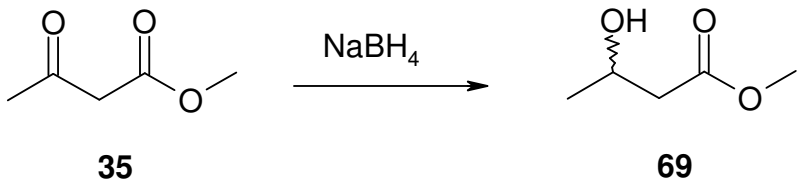


68

Fig. 36: Methyl (R)-3-hydorxybutyrate

Methyl-(R)-3-hydorxybutyrate shows desired absolute configuration of alcoholic function in C3 and it easily commercially available. Starting from this advanced starting material a new potential synthesis was planned to achieve *59* since one of the two stereocenter is

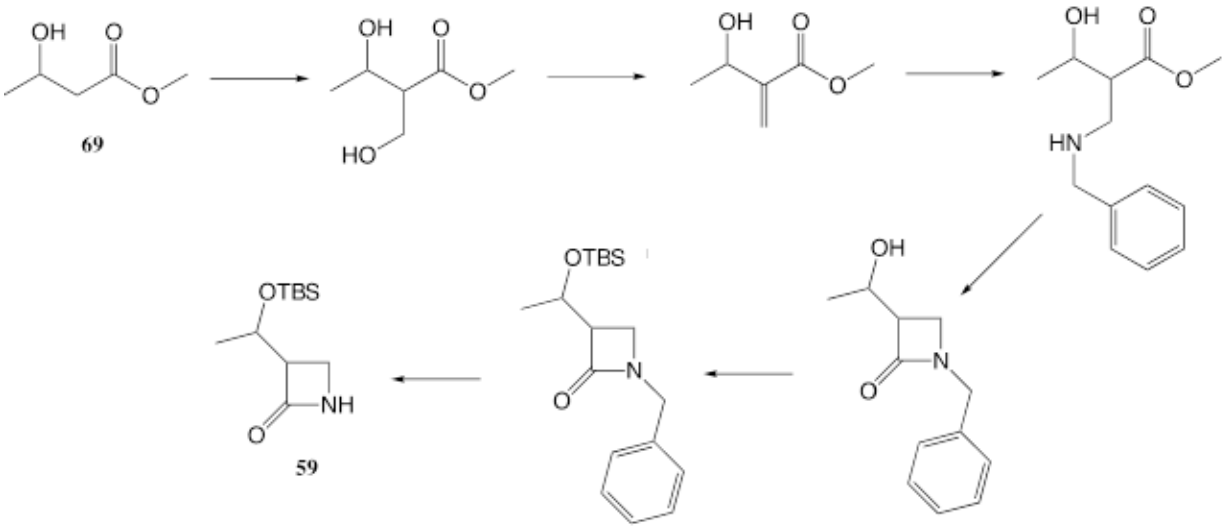
already present and forming stereocenter in C2 couple of diasteroisomer will be produced with the possibility of chromatographic separation. For the development and optimization of this brand new route of synthesis, the less precious and easily achievable racemic compound **69** was use and obtained treating methyl acetoacetate **35** with $NaBH_4$. Figure 37.



35 **69**

Fig 37: Racemic 3-hydorxybutyrate used for familiarization, obtained from methyl acetoacetate by treatment with $NaBH_4$

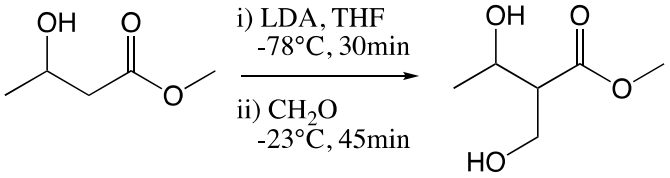
Total synthesis is shown in figure 35 and involves seven subsequent steps starting from **69**, Scheme 21. Starting from the racemic mixture **69** two couple of enantiomers will be forming. Separation of *anti* and *syn* couple can be performed at the end of the synthesis. This would be quite easy having already in our hand, reference standards for all other three AZTB isomers **54**, **57**, **60**. Various steps are described below.



Scheme 21. Potential synthesis of AZTB *59* starting from **69**

Starting form racemic compound **69** enolate on C2 is formed by treatment with LDA at -78°C in THF[64], temperature has then allowed rising and p-formaldehyde is added at -23°C. Reaction leads to the formation of desired compound **70** after workup, but with

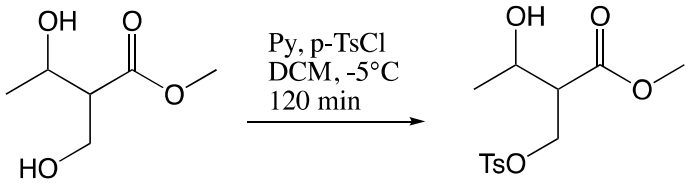
quite low conversion (around 25%). Different conditions were then investigated to improve yield of this step.



69 **70**

Scheme 22

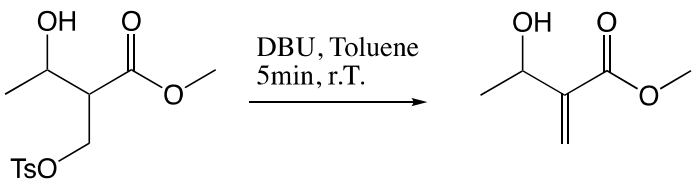
We try to improve reactivity of electrophile employing pure formaldehyde in reaction with enolate. Producing formaldehyde from p-formaldehyde through 200°C pyrolysis in a side vessel and bubbling sub surface in THF solution with a gentle nitrogen stream we observed a quicker conversion increased up to 60%. Compound **70** is known to be quite instable so, after workup and mass confirmation product has been carried over to subsequent step without any further purification. Compound **70** is then been treated with 4-toluensulfonyl chloride and pyridine in dry DCM for 5hours to afford **71**, scheme 23.



70 **71**

Scheme 23

After mass confirmation, product has not been isolated (expected and known to be unstable and prone to elimination) is carried over directly to subsequent elimination step. **71** has then been treated with 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) in toluene for few minutes at 25°C, to easily afford **72** with 50% overall yield from **69** after workup and purification, scheme 24.

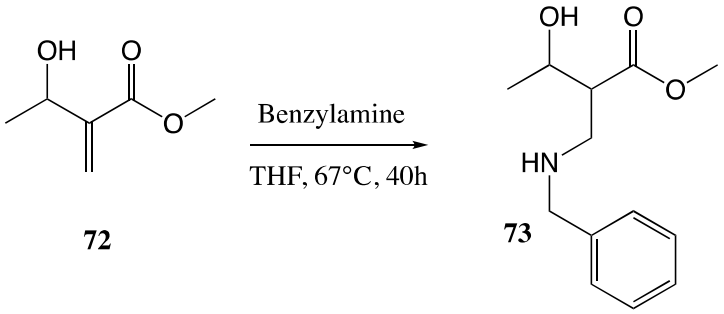


71 **72**

Scheme 24

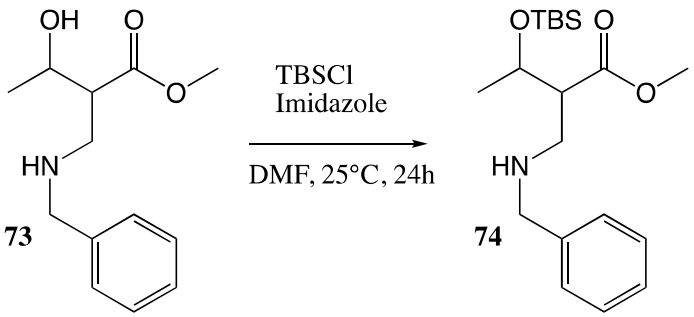
72 has been characterized and compared with commercially available reference standard confirming desired structure.

With this important intermediate achieved on racemate our efforts were focused on aza-michael reaction which forms a new stereocenter on C2. Our principal aim was to push for the formation of the *anti* couple of enantiomers. Looking in literature *syn* couple is undoubted the most favorite one in these kind of reaction. Using methanol as solvent for **72** in aza-michael reaction with benzyl amine, 95/5 *syn*/*anti* ratio is easily achieved[65]. Using aprotic solvent as THF ratio can be instead switched to a 25/75 syn/anti, a ratio more suitable for our purpose. For these reason THF was tested for aza-michael reaction on **72** with benzyl amine, scheme 25.



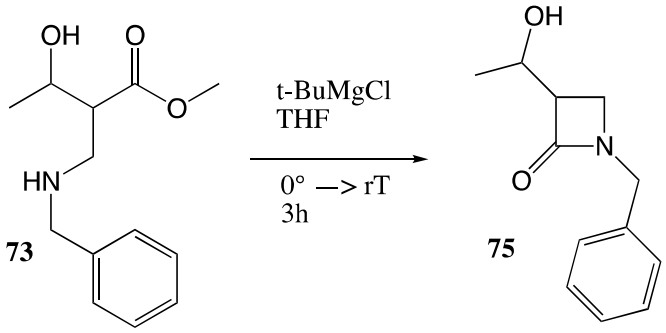
Scheme 25

Treating **72** with benzyl amine in THF for 40h at 70°C, aza-michael product **73** was achieved. Due to the free amine present, purification of crude material was quite difficult, in particular we did not succeed in separating the two diasteroisomers couple syn/anti. We then decided to move forward, after mass confirmation, with the subsequent steps, postponing isomers separation to more advanced intermediates. **73** was then treated with TBSCl to afford silylated derivative **74**, prior to proceed with cyclization, scheme 26.



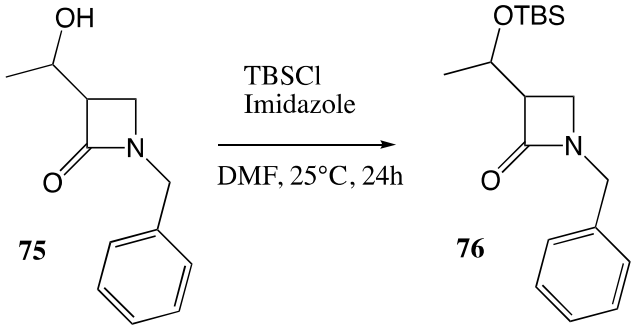
Scheme 26

Similar on what previously observed on similar "amine protected" compound AZT-BAL reaction presented some issues. Conversion was low, 20%, with several side products forming including β-elimination product. Strategy was then changed approaching cyclization directly on **73**, scheme 27.



Scheme 27

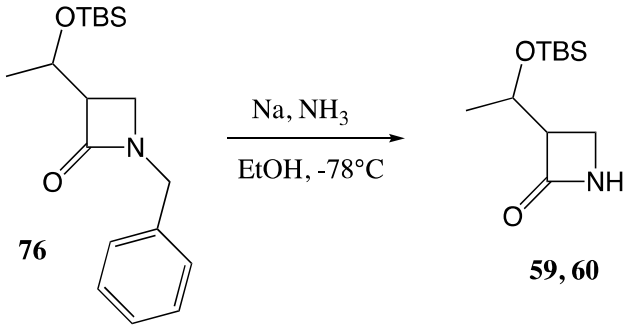
Treating **73** with tert-butylmagnesium bromide in THF a good conversion was observed in 3 hours, reaction was clean and diasteroisomers couple **75** (d.r. syn/anti 25/75, by HPLC. Isomer attribution based on *d.r* of Aza-Michael starting compound **73** described in literature[65] was isolated with a good yield of 88% after purification. Diasteroisomers couple was difficult to separate even at this stage, we then decided to carry over both isomers to AZTB analogous, performing purification at the last step. Diasteroisomers couple **75** was treated with TBSCl in DMF, scheme 28, to afford **76**.



Scheme 28

Reaction was quite slow, even after 36 hours at 25°C, conversion was not completed (20 area % reagent left). Nevertheless, work up was carried over and diasteroisomers couple **76** isolated after purification. Structural identity of the compounds was confirmed by NMR spectroscopy and mass spectrometry characterization.

HPLC analysis showed a consistent d.r. of 20/80 syn/anti. Finally, deprotection of **76** was performed using *Birch reaction* condition, scheme 29.

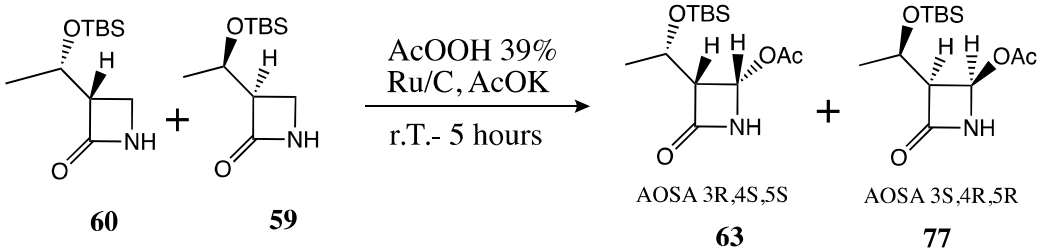


76 **59, 60**

Scheme 29

Substrate **76** was treated with metallic sodium, liquid ammonia and ethanol at -78°C. Reaction is very fast, 20 minute to afford AZTB final intermediate with 93%yield. Surprisingly, conversion was clean and formation of just one product was observed. Work up was then performed by removing toluene, deprotection by-product, and crude material purified to isolate AZTB mix. Comparing isolated product with other AZTB reference standards in our hands, just the anti couple of AZTB **59** and **60** were found. *Syn* couple, present in starting material, has been unexpectedly purged in reaction, affording just the major and desired *anti* couple of enantiomers. Enantiomer mixture was then characterized by NMR confirming structure by comparison with pure enantiomer **59**.

Enantiomers mixture of AZTB **59** and **60** was then carried over to AOSA corresponding enantiomers mixture. **59** and **60** were treated with peracetic acid following ACSDobfar manufacturing procedure, scheme 30.



60 **59** AOSA 3R,4S,5S **63** AOSA 3S,4R,5R **77**

Scheme 30

As expected, similar low performances in conversion compared with AZTB **60** were observed. Nevertheless AOSA **63** and **77** were obtained with 52% yield. Enantiomer mixture was then characterized by NMR confirming structure by comparison with pure enantiomer **63**. Racemic mixture was then analyzed with HPLC chital protocol to check retention time of the two enantiomers (figure 38).

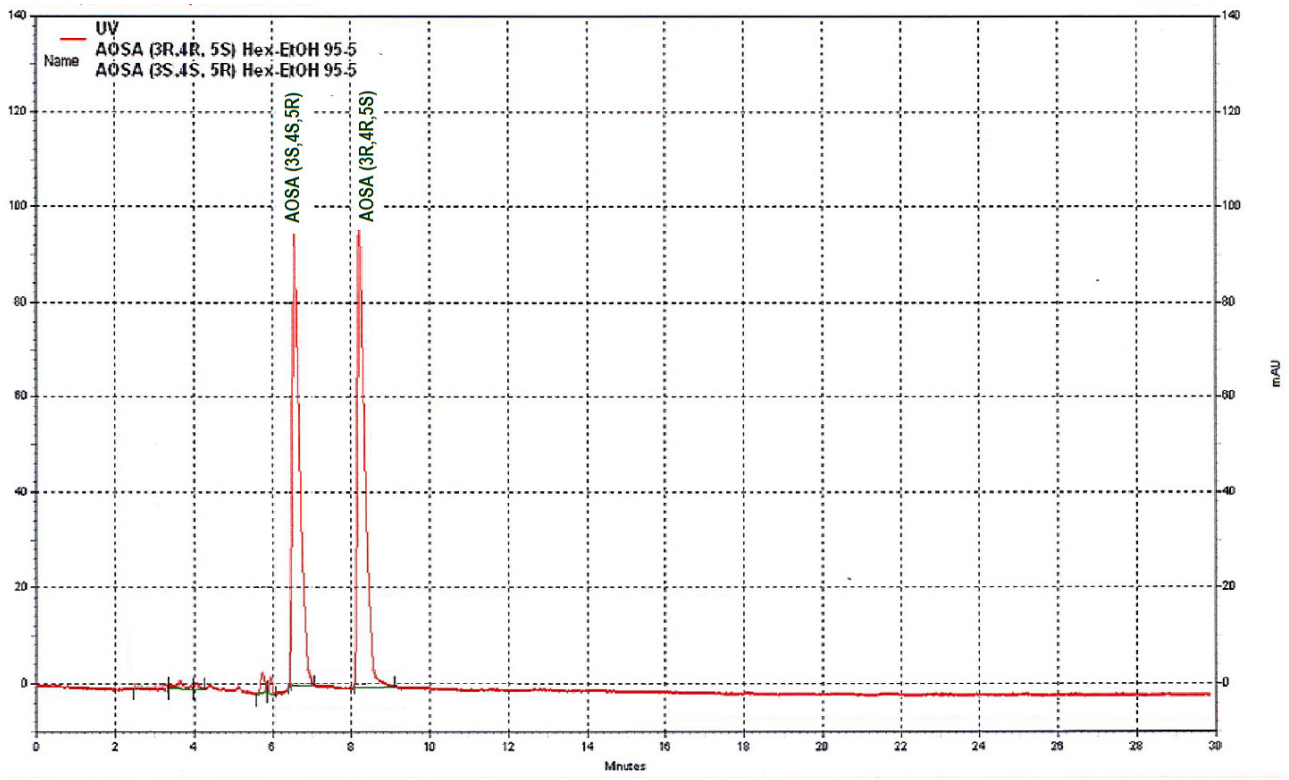
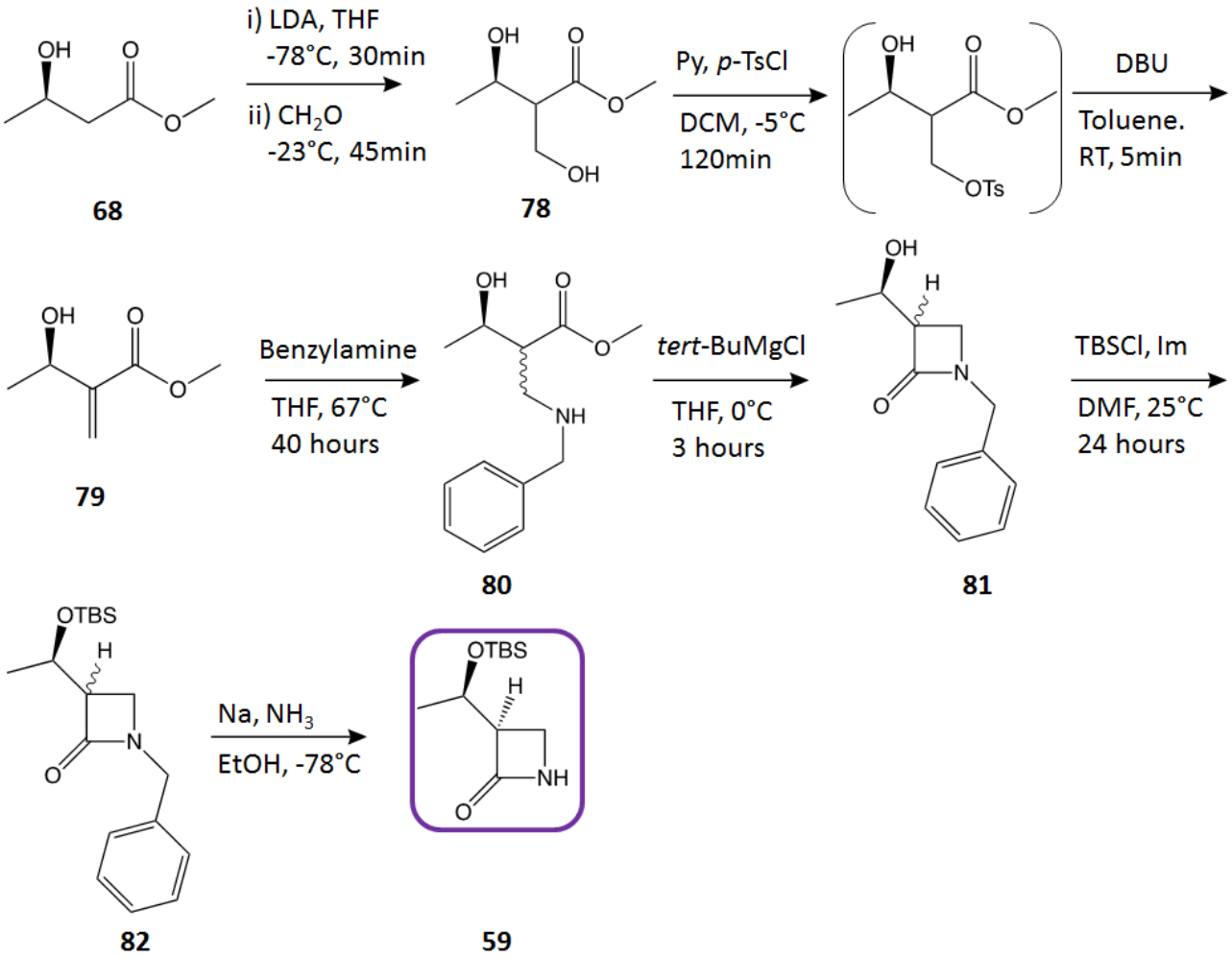


Fig. 38: Chromatograms showing retention time comparing AOSA (3R,4S,5S) **63**, AOSA (3S,4R,5R) **77** . Analytical method: Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/EtOH (95/5, v/v); 1.0 mL/min; 25◦C; λ= 210 nm)

Route of synthesis developed reveled to be successful for anti AZTB. The Same successful strategy was then applied on optical pure compound **68** in order to obtain pure AZTB *59*, scheme 31.



Scheme 31

Compound **68** shown the same performances thought all synthetic steps outlining just little variation in terms of yield and purity but behaving in line with the results observed with non-optically pure compound. Compound **68** was treated with LDA in THF to promote enolate formation at -78°C. Subsequent reaction with in-situ generated formaldehyde afforded crude compound **78.** This time crude material was purified through a silica gel chromatography allowing characterization of pure diol **78.** Treatment with p-TsCl followed by elimination with DBU afforded one pot **79** with 70% yield after purification. Aza-michael was then performed treating with benzyl amine in THF. Even on optically pure compound, *d.r.* observed for product **80** was similar to the non-optically pure substrate (75/25, anti/syn), results aligned with literature[65] also in this case. Cyclization was then performed treating with *tert*-butyl magnesium chloride and affording compound **81** with a 77% overall yield from **79.** Silylation with TBSCl was

performed obtaining **82** with a 78% yield and, subsequently, birch reaction finally affording target compound ***59***. Differently from what already observed on reaction performed on compound **76**, *syn* diasteroisomer ***54*** was found to be present in crude material in a 10 area % ratio with ***59***. Pure compound ***59*** was then obtained after chromatographic purification on silica gel. Characterization confirmed structure by comparison with reference standard **60**.

To complete our library, even this last achieved AZTB stereoisomer ***59*** was then carried over to AOSA corresponding stereoisomer **77**, following the ACSDobfar protocol already described in scheme 30. As expected, similar low performances in conversion compared with AZTB **60** were observed. Nevertheless AOSA **77** was obtained with 40% yield, finally affording our last missing AOSA stereoisomer.

5.12 *Analytical protocols development*

As already discussed, principal aim of this work was to obtain reference standards for AOSA's isomers in order to put in place robust analytical protocols applicable as routine test on incoming raw material. All incoming starting materials are checked and released by quality control prior to be used in manufacturing chain of API. Every material is defined by an analytical monograph including all routine test to be applied with all specification needed for a product to be released and used. AOSA has a complete analytical monograph including related substances HPLC analysis. Nevertheless, different isomers are not mentioned and relative retention time neither. In more detail, a chiral HPLC method is present in the monograph to control enantiomer but having, since today, no reference standard was impossible to assure that the method in place is able to separate and control enantiomeric purity. This has always been a serious lack for the control of this very important regulatory starting material. So, having finally AOSA enantiomer compound **58**, it has been injected in the analytical chiral system, figure 39.

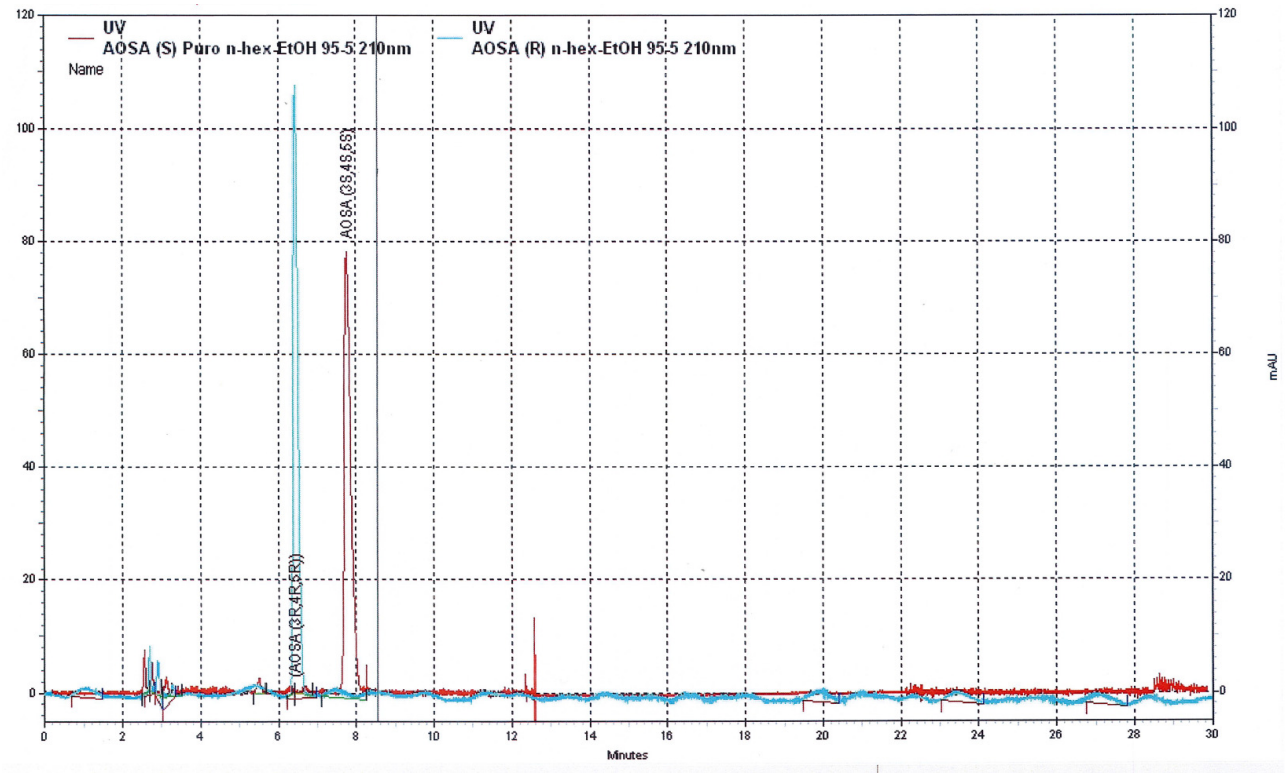


Fig 39: Chromatograms showing retention time comparing AOSA (3R,4R,5R) **18** in blue and AOSA (3S,4S,5S) **58** in red. Analytical method: Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/EtOH (95/5, v/v); 1.0 mL/min; 25◦C; λ= 210 nm

As shown in chromatogram AOSA's enantiomer **58** elutes with a different retention time. The method is indeed able to separate the two enantiomer and can be consider suitable for the control of AOSA's optical purity.

For completion, also other AOSA isomers were also injected in the system as shown in figure 40.

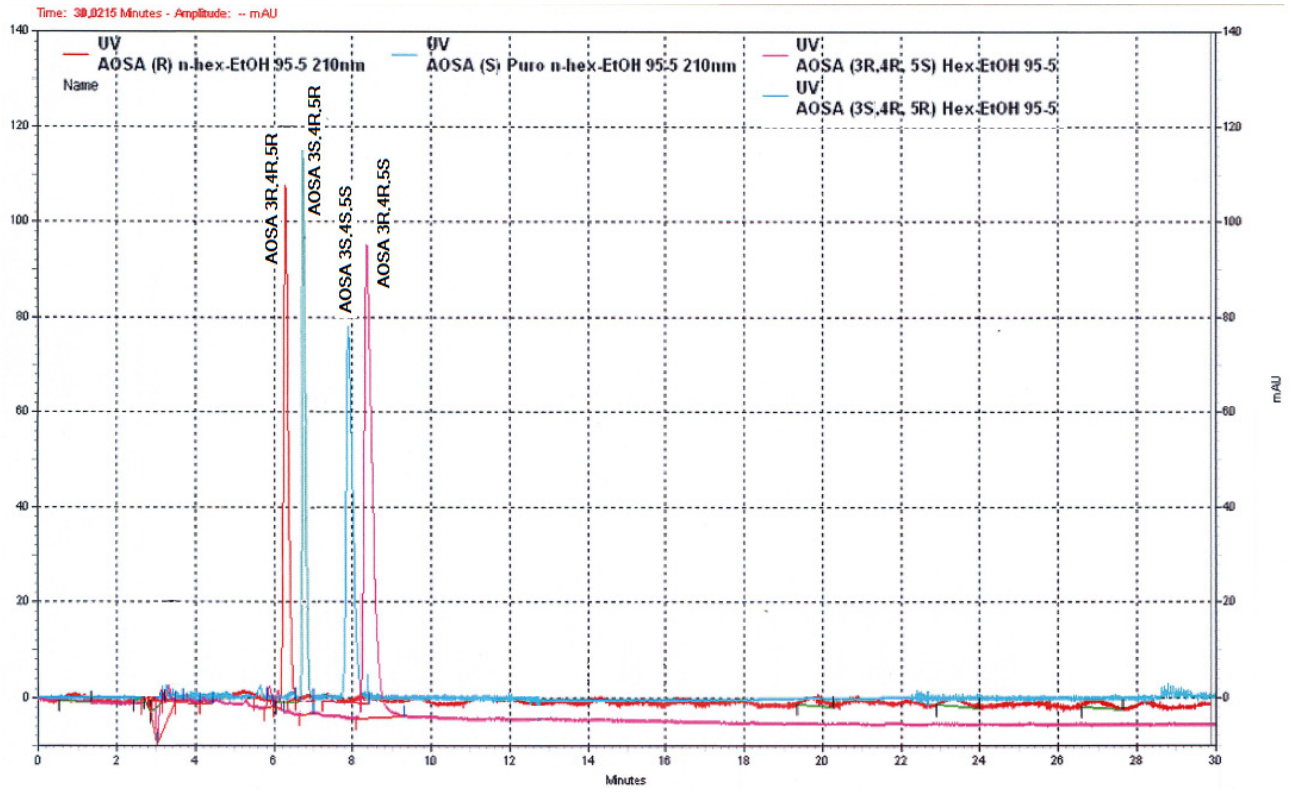


Fig. 40: Chromatograms showing retention time comparing AOSA (3R,4R,5R) **18** in red, AOSA (3S,4S,5S) **58** in blue, AOSA (3R,4R,5S) **63** in purple and AOSA (3S,4R,5R) **77** in green. Analytical method: Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/EtOH (95/5, v/v); 1.0 mL/min; 25◦C; λ= 210 nm)

The chiral analytical system demonstrated to be suitable for the separation of all four AOSA isomers.

Excluding AOSA's enantiomer, to control other possible stereoisomers without recurring to chiral method, a traditional HPLC method on AOSA derivative AZTB was also put in place. As already shown in section 4.5 AOSA can be easily converted to its advanced precursor AZTB, with a trivial treatment with $NaBH_4$

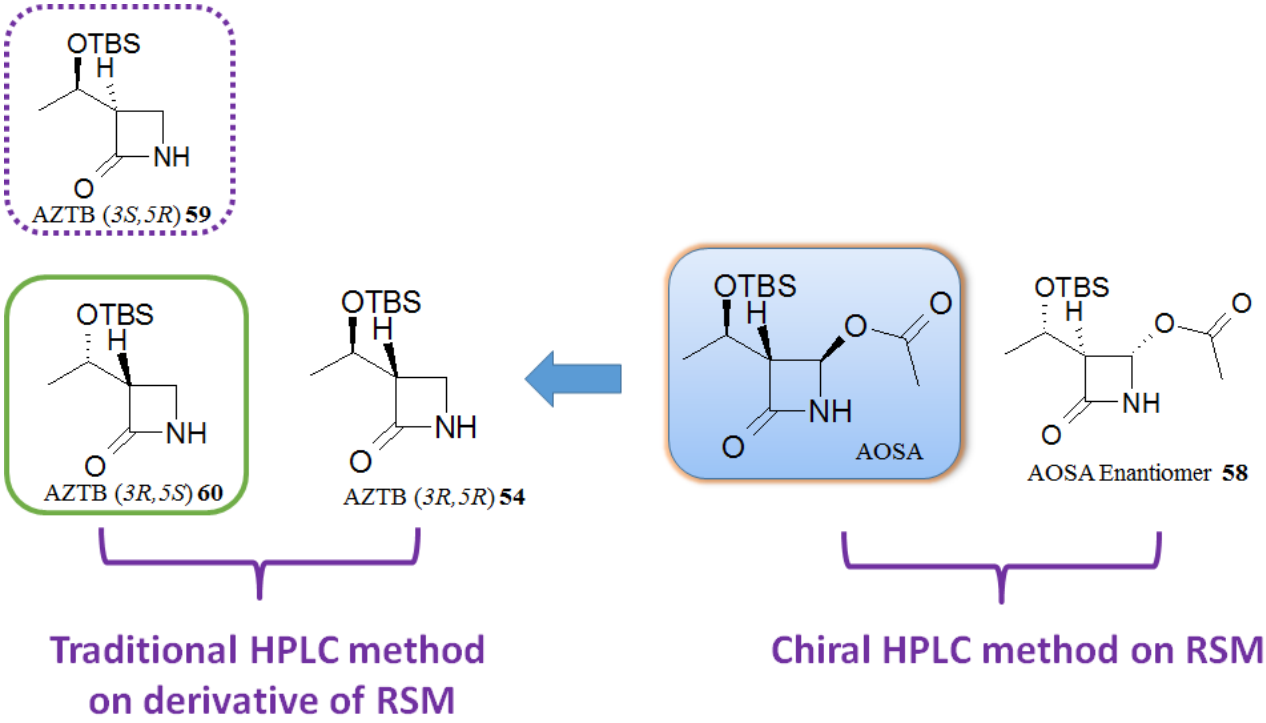


Fig 41: converting AOSA in its precursor AZTB a traditional HPLC method can be applied.

As shown in figure 41, converting AOSA in AZTB analogue, a traditional HPLC method can be applied to control presence of stereoisomers **60** and *59*, respectively precursors of AOSA **63** and **77**. In addition, it is enough to have just one of two isomers as reference standard, to check to easily control both compounds with a simple traditional HPLC injection. Analytical method has been then developed in this regard and isomers injected to define retention time, figure 42.

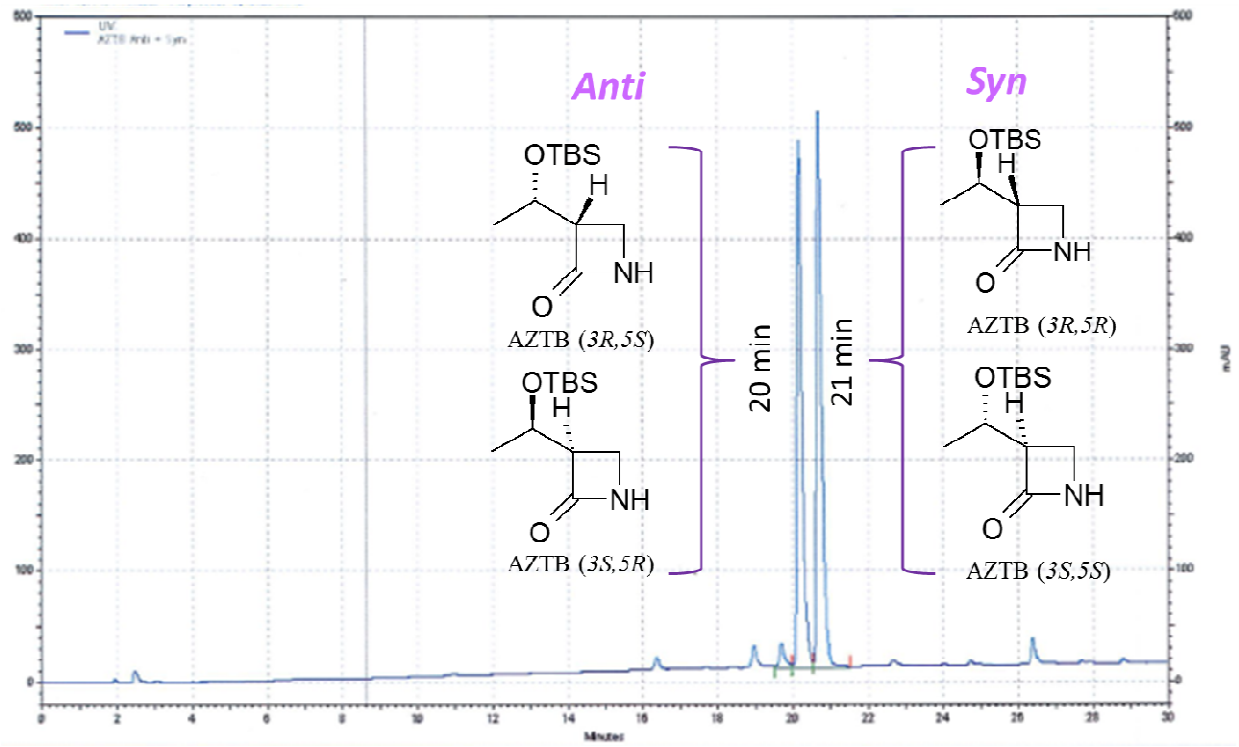


Fig 42: traditional HPLC method can effectively separate syn and anti couple of AZTB isomers. Operative conditions: Lichrospher® 10 column, RP-18e, 5µm, 250mm. l = 210nm, column temperature 25°C. Mobile phase A: Water 1% phosphoric acid; Mobile phase B: Acetonitrile. Gradient: B% →**t** 0=10; **t** 30min=100.

The development of these analytical protocols, with the availability of all needed reference standard, represent indeed a great achievement. The initial aim, to build up a robust control strategy for this fundamental regulatory starting material, has been successfully accomplished. Routine test will be implemented and applied, from now on, to incoming AOSA in our manufacturing chain.

6 Experimental section

Preparation of Methyl 2-Benzamidomethyl-3-oxobutanoate MBA 38. To a mixture of methyl acetoacetate **35** (1.16 g, 10 mmol) and *N*-(hydroxymethyl)benzamide (1.51g, 10 mmol) in 20ml of diethylethere cooled to 0 °C is slowly added BF_3 etherate solution (2.5 ml, 20 mmol) with stirring. The rate of addition of BF_3 etherate solution is maintained in such a way that the reaction temperature does not exceed 5 °C and the addition time is about 1 h. Upon complete addition, the reaction temperature is raised to 25°C and reacted for 2.5 h with stirring. The reaction mixture is added to a solution of sodium acetate in water (3.0g sodium acetate in 6 mL water), mixed well, and then allowed to separate. The aqueous layer is extracted twice with methylene chloride, each time with 30 mL. The organic layers are combined and dried with anhydrous magnesium sulfate. After removal of solvents under vacuum, the residue is recrystallized in ethyl acetate and *n*-hexane (1:1) and dried to yield **38** (1.69g, 68%).

^{1}H NMR (300 MHz - $CDCl_3$): 2.32 (s, 3H), 3.76 (s, 3H), 3.92 (m, 1H), 3.93 and 3.95 (m, 2H), 6.82 (s, 1H), 7.41 (dd, 2H), 7.49 (m, 1H), 7.72 (d, 2H). ^{13}C NMR ($CDCl_3$): 30.1. 37.8, 52.8, 58.3, 127.1, 128.7, 131.8, 134.1, 167.7, 169.2, 202.6.

Preparation of (2S,3R)-Methyl 2-(benzamidomethyl)-3-hydroxybutanoate 39 (enzymatic): In In round bottom flask are charged 300ml of isopropanol, 330ml of buffer solution (triethanolamine/magnesium sulphate heptahydrate pH=7) at room temperature and 77g of MBA **38**. Temperature is adjusted at +40°C. 150mg KRED Enzyme CDX are then charged and 15mg NADP dissolved in 7.5ml of buffer solution. The reaction mass is maintained at +40°C for 8hrs. 150mg KRED are then added and 15mg NADP in 7.5ml of buffer solution. The reaction mass is mantained at +40°C for 8hrs. After reaction completion checked by TLC (Hexane/acetate 8/2), add 225ml of Brine solution and 300ml of ethyl acetate. After phases separation, organic layer is washed with 260ml of water. Organic phase is then dried with sodium sulfate an concentrated to oil yielding **39** (75g 90%)

^{1}H NMR (300 MHz - $CDCl_3$): 1.27 (d, 3H), 2.64 (m, 1H), 3.53e3.43(m, 2H), 3.74 (s, 3H), 4.41(m, 1H), 7.05 (bs, 1H), 7.43 (m, 3H), 7.57 (m, 1H), 7.81 (m, 2H).

^{13}C NMR ($CDCl_3$): 20.69, 37.76, 51.84, 52.41, 65.36, 126.97, 128.52, 131.74, 133.57, 168.64, 174.16.

Preparation of Methyl 2-(benzamidomethyl)-3-hydroxybutanoate racemate 39, 40, 41, 42: in a round bottomed flask 0.2 g of $NaBH_4$ are added with 14 ml of Ethanol. An etanolic solution of **MBA** 38 (2g in 14ml of Ethanol) is the added in 30 minutes at 0°C. Reaction is kept at 0°C for 2 hours. After reaction completion aqueous HCl 3.7% is added drop wise at the same temperature till pH=7. 50 ml of ethyl acetate and 50 ml of purified water are then added, stirred for 30 minutes and stop to allow phases separation. After phase separation water is back-extracted with 20 ml of ethyl acetate and then organic phases collected, dried over magnesium sulphate and solvent evaporated to afford a dense oil containing the racemic mixture. HPLC: Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/2-propanol (90/10, v/v); 1.0 mL/min; 25◦C; wavelength = 254 nm). Retention times: **39**=11.7min, **40**=12.9min, **41**=14.5min, **42**=15.9min. *Syn* couple (**39**, **41**) and *anti* couple (**40**, **42**) are then separated on silica chromatographic column (hexane/acetate 8/2).

Syn couple (**39**, **41**) ^{1}H NMR (300 MHz - $CDCl_3$) 1.28 (d, 3H), 2.63 (m, 1H), 3.59e3.41(m, 2H), 3.74 (s, 3H), 4.41(m, 1H), 7.01 (bs, 1H), 7.44 (m, 3H), 7.56 (m, 1H), 7.81 (m, 2H).

^{13}C NMR ($CDCl_3$): 20.70, 37.78, 51.84, 52.43, 65.38, 126.99, 128.56, 131.74, 133.60, 168.65, 174.17.

Anti couple (**40**, **42**) ^{1}H NMR ($CDCl_3$): 1.26 (d, 3H), 2.64e2.60 (m, 1H), 3.60e3.55 (m, 1H), 3.73 (s, 3H), 4.01e3.98 (m, 1H), 4.18e4.11 (m, 2H), 6.96 (s, 1H), 7.46e7.44 (m, 2H), 7.54e7.49 (m, 1H), 7.80e7.75 (m, 2H). ^{13}C NMR (CDCl3): 20.9, 38.0, 52.1, 52.7, 65.6, 127.2, 128.8, 132.0, 168.9.

General procedure for preparation of Methyl 2-(benzamidomethyl)-3-hydroxybutanoate 39, 40, 41, 42 by enzymatic screening: total reaction volume 500µl. Enzyme 5mg. **MBA 38** 50mM in DMSO. Reaction buffer: potassium phosphate 250mM containing $NAD(P)^+$ (1mM) at required pH (see table 3). Cofactor system: GDH (1mg/ml) D-glucose (1.1eq compared to **38**). Procedure: weigh 5 mg of ADH in to a 1.5ml reaction tube. Add 500µl of the reaction buffer to the ADH and mix until the enzyme is

dissolved. Start reaction by adding 38 and incubate the reaction in a shaking incubator at 30°C. after 24hours extract the reaction with ethyl acetate (2x0.6ml). Organic phases are collected and checked by Chiral HPLC. Lux® 5µm Amylose-1 LC column 250 x 4,6 mm. (n-hexane/2-propanol (90/10, v/v); 1.0 mL/min; 25◦C; wavelength = 254 nm). Single diasteroisomers couple have been separated on silica chromatographic column (hexane/acetate 8/2).

General procedure for preparation of Methyl 2-(benzamidomethyl)-3-hydroxybutanoate 39 and 41 through asymmetrical hydrogenation: to an autoclave for high-pressure hydrogenation 0.02mmol of catalyst (**43**, **44**, **45**, **46**, **47**, **48**, **49**, **50**) are added to a mixture of dichloromethane and trifluoroethanol (12ml/4ml). 0.5mmol of **38** are then added and solution degassed with argon for 10 minutes. Autoclave is then closed and purged three times with nitrogen/Hydrogen cycles. Reaction is then stirred at 50 bar of Hydrogen for 24 hours at 70°C. After 24h solvent are evaporated, oil dissolved in ethyl acetate and checked by HPLC. Crude material is purified on silica chromatographic column (hexane/acetate 8/2).

39: ^{1}H NMR (300 MHz - $CDCl_3$): 1.27 (d, 3H), 2.64 (m, 1H), 3.53e3.43(m, 2H), 3.74 (s, 3H), 4.41(m, 1H), 7.05 (bs, 1H), 7.43 (m, 3H), 7.57 (m, 1H), 7.81 (m, 2H).

^{13}C NMR ($CDCl_3$): 20.69, 37.76, 51.84, 52.41, 65.36, 126.97, 128.52, 131.74, 133.57, 168.64, 174.16.

41: ^{1}H NMR (300 MHz - $CDCl_3$): 1.18 (d, 3H), 2.63 (m, 1H), 3.60e3.64(m, 2H), 3.70 (s, 3H), 4.01(m, 1H), 7.24 (bs, 1H), 7.45 (m, 3H), 7.58 (m, 1H), 7.85 (m, 2H).

^{13}C NMR ($CDCl_3$): 20.81, 37.91, 51.81, 52.40, 65.60, 126.90, 128.47, 131.64, 133.61, 168.65, 174.04.

Preparation of (2S,3R)-2-(aminomethyl)-3-hydroxybutanoic acid 34: 2g of **39** are suspended in 20 ml of aqueous 10% HCl. Mixture is hated at reflux for 15 hours. Solution is then cooled to 50°C and washed twice with 50ml of toluene. Water is the evaporated at rotavapor maintain temperature below 70°C. 100 ml of toluene are then added and distill under vacuum to remove residual water azeotrpically. 10 ml of methanol are then added at 0°C and 0.3ml (1eq) of triethylamine is added dropwise.

Solution is stirred overnight at the same temperature, crystallization occurs. Solid is filtered and washed with 3ml of cold methanol to afford **34** (1.1g, 55%yield).

34: ^{1}H NMR (300 MHz - D_2O): 1.32 (d, 3H), 2.56 (m, 1H), 3.33 (d, 2H) and 4.10 (dq, 1H). ^{13}C NMR (D_2O): 20.68, 38.73, 52.37, 67.80, 178.26

Preparation of (3S)-3-[(1R)-1-hydroxyethyl]azetidin-2-one AZT *55* from AAL 34: 100mg of **34** are suspended in 20ml of acetonitrile. 262mg (1.2eq) of triphenylphosphine and 198mg of 2,2'-dithiopyridine (1.2eq) are added to the suspension and mixture refluxed overnight. Solvent is then distilled to oil and crude material purified on a silica gel column (hexane/Ethyl acetate 1/1) to separate triphenylphosphine oxide and thione to yield ***55*** (90mg, 70%).

^{1}H NMR (300 MHz - D_2O): 1.28 (d, 3H), 3.33 (m, 1H), 3.35 (m, 2H) 4.20 (m, 1H) 4.80 (s, 1H) ^{13}C NMR (D_2O): 20.05, 38.16, 56.63, 64.69, 172.33.

Preparation of (R)-3-((R)-1-((tert-butyldimethylsilyl)oxy)ethyl)azetidin-2-one AZTB *54* from AZT *55*: 300mg of ***55*** are added to 10 ml of dimethylformammide cooled at 0°C. 270 mg of imidazole and 470mf of TBSCl are then added maintaining temperature at 0°C. Reaction is let stirred overnight at room temperature. After reaction completion 20 ml of ethyl acetate and 30ml of water are added to the mixture. After phases separation aqueous phase is back-extracted with 20ml of ethyl acetate. Organic phases are then collected and dried over magnesium sulfate. Solvent is then distilled and crude material purified over silica gel chromatography (Cyclohexane/Ethyl acetate 6/4) affording **54** with 80%yield.

^{1}H NMR (300 MHz – $CDCl_3$): 0.05 (s, 6H), 0.79 (s, 9H), 3.17 (m, 1H) 3.22 (m, 2H) 4.17 (m, 1H) 6.40 (bs, 1H) ^{13}C NMR ($CDCl_3$): 4.43, 5.13, 17.80, 22.38, 25.57, 37.64, 59.07, 65.25, 169.79

Preparation of (3R,4R)-4-Acetoxy-3-[(R)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone AOSA 18: 1g of 54 is added to 20ml of ethyl acetate at room temperature under nitrogen flow. 0.25mg of Ru/C, 860mg of sodium acetate and 0.8ml of acetic acid are the added at the same temperature. Peracetic acid 39%in water (5,6ml) is the charged dropwise. Reaction is let stirring for 1 h. After reaction completion 2ml of 2% aqueous

sodium metabisulfite is added at room temperature and let stir for 10 minutes. Solid precipitation occurs. Solid is filtered and washed twice with 10ml of diethyl ethere and 5ml of water. Filtrate is collected and pH adjusted to 5 with NaOH 15% in water. Phases are then separated and aqueous phase back-extracted with 10 ml of ethyl acetate. Organic layers are then collected and wash twice with a 5% sodium bicarbonate solution in water (30ml each), and once with 30 ml of purified water. Organic phases are dried over magnesium sulfate and solvent distilled to afford 1,1 g oil. Crude material is purified over chromatographic silica gel column (cyclohexane/Ethyl acetate 7/3) to afford **18** (1g, 95%yield)

^{1}H NMR (300 MHz – $CDCl_3$): 0.12 (m, 6H) 0.91 (s, 9H), 1.31 (d, 3H), 2.15 (s, 3H), 3.23 (m, 1H), 4.27 (m, 1H), 5.90 (d, 1H), 7.14 (bs, 1H).

^{13}C NMR ($CDCl_3$): 4.46, 5.33, 17.78, 20.76, 22.19, 25.56, 63.72, 64.92, 74.97, 56.65, 64.69, 166.43, 171.16.

Preparation of (2R,3S)-2-(aminomethyl)-3-hydroxybutanoic acid 34b: 2g of **41** are suspended in 20 ml of aqueous 10% HCl. Mixture is hated at reflux for 15 hours. Solution is then cooled to 50°C and washed twice with 50ml of toluene. Water is the evaporated at rotavapor maintain temperature below 70°C. 100 ml of toluene are then added and distill under vacuum to remove residual water azeotrpically. 10 ml of methanol are then added at 0°C and 0.3ml (1eq) of triethylamine is added dropwise. Solution is stirred overnight at the same temperature, crystallization occurs. Solid is filtered and washed with 3ml of cold methanol to afford **34b** (1.1g, 55%yield).

34b ^{1}H NMR (300 MHz - D_2O): 1.32 (d, 3H), 2.56 (m, 1H), 3.33 (d, 2H) and 4.10 (dq, 1H). ^{13}C NMR (D_2O): 20.70, 38.75, 52.40, 67.83, 178.29

Preparation of (3R)-3-[(1S)-1-hydroxyethyl]azetidin-2-one AZT *56* from AAL 34b: 1g of **34b** are suspended in 200ml of acetonitrile. 2.62g (1.2eq) of triphenylphosphine and 1.98g of 2,2'-dithiopyridine (1.2eq) are added to the suspension and mixture refluxed overnight. Solvent is then distilled to oil and crude material purified on a silica gel column (hexane/Ethyl acetate 1/1) to separate triphenylphosphine oxide and thione to yield ***56*** (900mg, 70%).

56: ^{1}H NMR (300 MHz - D_2O): 1.28 (d, 3H), 3.33 (m, 1H), 3.35 (m, 2H) 4.20 (m, 1H) 4.80 (s, 1H) ^{13}C NMR (D_2O): 19.62, 37.77, 56.22, 64.30, 172.03.

Preparation of (S)-3-((S)-1-((tert-butyldimethylsilyl)oxy)ethyl)azetidin-2-one AZTB *57* from AZT *56*: 600mg of ***56*** are added to 20 ml of dimethylformammide cooled at 0°C. 540 mg of imidazole and 940mg of TBSCl are then added maintaining temperature at 0°C. Reaction is let stirred overnight at room temperature. After reaction completion 40 ml of ethyl acetate and 60ml of water are added to the mixture. After phases separation aqueous phase is back-extracted with 40ml of ethyl acetate. Organic phases are then collected and dried over magnesium sulfate. Solvent is then distilled and crude material purified over silica gel chromatography (Cyclohexane/Ethyl acetate 6/4) affording ***57*** with 81%yield.

^{1}H NMR (300 MHz – $CDCl_3$): 0.08 (s, 6H), 0.88 (s, 9H), 1.20 (d, 3H) 3.20 (m, 1H) 3.35 (m, 2H) 4.22 (m, 1H) 5.97 (bs, 1H)

^{13}C NMR ($CDCl_3$): 4.40, 5.11, 17.83, 22.41, 25.59, 37.58, 59.19, 65.21, 169.50

Preparation of (3S,4S)-4-Acetoxy-3-[(S)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone *58*: 1g of ***57*** is added to 20ml of ethyl acetate at room temperature under nitrogen flow. 0.25mg of Ru/C, 860mg of sodium acetate and 0.8ml of acetic acid are the added at the same temperature. Peracetic acid 39%in water (5,6ml) is the charged dropwise. Reaction is let stirring for 1 h. After reaction completion 2ml of 2% aqueous sodium metabisulfite is added at room temperature and let stir for 10 minutes. Solid precipitation occurs. Solid is filtered and washed twice with 10ml of diethyl ethere and 5ml of water. Filtrate is collected and pH adjusted to 5 with NaOH 15% in water. Phases are then separated and aqueous phase back-extracted with 10 ml of ethyl acetate. Organic layers are then collected and wash twice with a 5% sodium bicarbonate solution in water (30ml each),

and once with 30 ml of purified water. Organic phases are dried over magnesium sulfate and solvent distilled to afford 1,1 g oil. Crude material is purified over chromatographic silica gel column (cyclohexane/Ethyl acetate 7/3) to afford **58** (1g, 95%yield)

^{1}H NMR (300 MHz – $CDCl_3$): 0.18 (s, 6H) 0.98 (s, 9H), 1.37 (d, 3H), 2.22 (s, 3H), 3.31 (m, 1H), 4.34 (m, 1H), 5.96 (m, 1H), 6.67 (bs, 1H).

^{13}C NMR ($CDCl_3$): 4.45, 5.32, 17.79, 20.75, 22.18, 25.55, 63.72, 64.90, 74.98, 56.63, 64.69, 166.40, 171.14.

Preparation (R)-3-((R)-1-((tert-butyldimethylsilyl)oxy)ethyl)azetidin-2-one AZTB *54* from AOSA 18: 20g of **18** are dissolved in 130ml of ethanol at 0°C. 2,6g of $NaBH_4$ are then added portion wise in 1 hour keeping the temperature. After complete charge mixture is stirred at 10°C for 3o minutes. After reaction completion maintaining the temperature 3ml of acetic acid and 1 ml of HCl 1 N are added carefully, precipitation occurs. Solid is filtered and solvent distilled off. Oil is dissolved in 150ml of cyclohexane at 40°C and solution filtered to eliminate undissolved material. Solvent is then distilled obtaining ***54*** as a grey solid (14.6g, 90%yield)

^{1}H NMR (300 MHz – $CDCl_3$): 0.06 (s, 6H), 0.78 (s, 9H), 3.18 (m, 1H) 3.23 (m, 2H) 4.16 (m, 1H) 6.41 (bs, 1H) ^{13}C NMR ($CDCl_3$): 4.43, 5.13, 17.80, 22.38, 25.57, 37.64, 59.07, 65.25, 169.79

Preparation of (3S)-3-[(1R)-1-hydroxyethyl]azetidin-2-one AZT *55* from AZTB *54*: 30g of 54 are dissolved in 600ml of acetonitrile at room temperature. 100 ml of aqueous HCl 1N are the added and mixture let stirring for two hours. After reaction completion pH is adjusted to 7 by adding 30 ml of NaOH 15% in water. Solvent is then concentrated under vacuum. Residual oil is dissolved in 500ml of THF and concentrated twice. 21g of crude material are obtained. Solid is then suspended in 250 ml of diethyl ether and filtered obtaining 15g of ***55*** as a white solid (95% yield)

^{1}H NMR (300 MHz - D_2O): 1.28 (d, 3H), 3.33 (m, 1H), 3.35 (m, 2H) 4.20 (m, 1H) 4.80 (s, 1H) ^{13}C NMR (D_2O): 19.65, 37.78, 56.22, 64.33, 172.13.

Preparation of (S)-1-((S)-2-oxoazetidin-3-yl)ethyl benzoate 61: 9g of ***55*** are suspended in 55 ml of toluene at room temperature. Then tributhylphosphine and benzoic acid are added. Mixture is cooled to 0°C and 20g of TMAD are charged and mixture stirred at the same temperature for 30 minutes. Solution is then heated at 60°C and stirred for 20 hours until reaction completion. Solvent in then removed under vacuum and crude material purified on a silica gel chromatographic column (Hexane/Ethyl Acetate 4/6) affording 7.8g of **61** as a yellow oil (39%yield).

^{1}H NMR (300 MHz - $CDCL_3$): 1.65 (d, 3H), 3.32 (m, 1H), 3.49 (m, 1H) 5.52 (m, 1H) and 6.51 (br, 1H) ^{13}C NMR ($CDCL_3$): 17.46, 38.15, 43.11, 67.84, 123.54, 133,03, 139.68, 165.85, 168,16.

Preparation of (3S)-3-[(1S)-1-hydroxyethyl]azetidin-2-one 62: 7.8g of 61 are dissolved in 400ml of methanol. 25ml of NaOH 0.5M are the added at room temperature and stirred overnight. After reaction completion 2ml of acetic acid are added (pH=6.8) and solvent removed under vacuum. Crude material is purified on silica gel chromatographic column (ethyl acetate/methanol 95/5) affording 2.5g of **62** as a green oil.

^{1}H NMR (300 MHz - D_2O): 1.30 (d, 3H), 3.40 (m, 1H), 3.45 (m, 2H) 4.30 (m, 1H) 4.85 (s, 1H) ^{13}C NMR (D_2O): 19.80, 37.98, 56.00, 64.46, 172.23.

Preparation of (R)-3-((S)-1-((tert-butyldimethylsilyl)oxy)ethyl)azetidin-2-one 60: 2,4g of **62** are suspended in 30ml of DMF at 0°C. 1,6g of imidazole and 3.3g of TBSCl are then charged maintaining temperature at 0°C. Reaction is let stirred overnight at room temperature. After reaction completion 120 ml of ethyl acetate and 180ml of water. After phases separation aqueous phase is back-extracted with 120ml of ethyl acetate. Organic phases are then collected and dried over magnesium sulfate. Solvent is then distilled and crude material purified over silica gel chromatography (Cyclohexane/Ethyl acetate 6/4) affording 2.2g of **60** as colorless oil (25% overall yield form ***54***).

^{1}H NMR (300 MHz – $CDCl_3$): 0.06 (s, 6H), 0.86 (s, 9H), 1.19 (d, 3H), 3.18 (m, 1H), 3.32 (m, 2H), 4.18 (m, 1H), 6.31 (bs, 1H) ^{13}C NMR ($CDCl_3$): 4.41, 5.13, 17.81, 22.39, 25.58, 37.63, 59.09, 65.24, 169.74.

Preparation of (3R,4R)-4-Acetoxy-3-[(S)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone 63: 1g of **60** is added to 20ml of ethyl acetate at room temperature under nitrogen flow. 0.25mg of Ru/C, 860mg of sodium acetate and 0.8ml of acetic acid are the added at the same temperature. Peracetic acid 39%in water (5,6ml) is the charged dropwise. Reaction is let stirring for 1 h. After reaction completion 2ml of 2% aqueous sodium metabisulfite is added at room temperature and let stir for 10 minutes. Solid precipitation occurs. Solid is filtered and washed twice with 10ml of diethyl ethere and 5ml of water. Filtrate is collected and pH adjusted to 5 with NaOH 15% in water. Phases are then separated and aqueous phase back-extracted with 10 ml of ethyl acetate. Organic layers are then collected and wash twice with a 5% sodium bicarbonate solution in water (30ml each), and once with 30 ml of purified water. Organic phases are dried over magnesium sulfate and solvent distilled to afford 1,1 g oil. Crude material is purified over chromatographic silica gel column (cyclohexane/Ethyl acetate 7/3) to afford **63** (500mg, 40%yield)
^{1}H NMR (300 MHz – $CDCl_3$): 0.17 (s, 6H), 0.96 (s, 9H), 1.40 (d, 3H), 2.20 (s, 3H), 3.32 (m, 1H), 4.35 (m, 1H), 5.78 (s, 1H), 6.80 (bs, 1H). ^{13}C NMR ($CDCl_3$): 4.26, 5.16, 17.82, 20.72, 25.57, 64.20, 64.51, 75.91, 166.09, 171.02.

Preparation of methyl (2S,3R)-2-(aminomethyl)-3-((tert-butyldimethylsilyl)oxy)butanoate *52* from 34: intellectual property of ACSDobfar spa.

Preparation of methyl (2S,3R)-2-(benzamidomethyl)-3-((tert-butyldimethylsilyl)oxy) butanoate 67: 6.2g of ***52*** are dissolved in 40ml of dichloromethane. 1eq of pyridine is the added at 0°C followed by 1.4eq of benzoyl chloride dropwise, maintaining the temperature. After reaction completion, after 2 hours, 40ml of water are added and pH adjusted to 7 with 10% aqueous sodium bicarbonate. After phase separation DCM layer is washed with 40ml of brine. Organic phase is then dried over $MgSO_4$ and concentrated under vacuum to afford 8.6g of crude material. Mixture is then purified by silica gel chromatography (Hexane/Ethyl acetate 1/1). Obtained 5g of **67**(76% yield).
^{1}H NMR (200 MHz – $CDCl_3$): 0.07 (m, 6H) 0.86 (s, 6H), 1.33 (d, 3H), 2.71 (m, 1H), 3.59e4.20 (m, 2H), 3.97 (s, 3H), 4.45 (m, 1H), 6.86 (bs, 1H), 7.37 (m, 3H). 7.73 (m, 2H).

Preparation of Methyl-3-hydroxybutyrate 69: 5g of **35** are dissolved in 12ml of ethanol at 0°C. 12 ml of ethanol containing 0.6g of $NaBH_4$ are then charged dropwise maintaining the temperature. Reaction is then stirred for 2.5 hours. After reaction completion acetic acid is added until hydrogen formation stops, pH is the adjusted to 7 with HCl 1N. Solid precipitation occurs. Solid is the filtered and solvent concentrated to oil under vacuum. 40ml of cyclohexane are then added at 40°C till complete dissolution and then solvent distilled to oil under vacuum (operation repeated three times). Pale white solid **69** is then obtained (4g, 80% yield). LC-MS confirmed structure $[M+H]^+$=103

Preparation of methyl 3-hydroxy-2-(hydroxymethyl)butanoate 70: 15ml of LDA 1M in THF are cooled to -78°C. 2,3g of **69** dissolved in 40ml of THF are the added dropwise. Solution is stirred for 30 minutes at -78°C then temperature is increased at -23°C. in a side vessel 5,5g of p-formaldehyde are heated at 200°C to promote de-polymerization and formation of formaldehyde gas which is bubbled in the THF solution in 45minutes with a gentle nitrogen stream. Reaction is stirred for additional 30 minutes and then quenched 40 ml of a 5% NH_4Cl solution and 40ml of HCl 1N. aqueous mixture is extracted three times with 200ml of ethyl acetate. Organic phases are then collected and washed with 40 ml of 10% aqueous sodium bicarbonate and with 40ml of brine. Organic rich solution is the dried over sodium sulfate and solvent distilled under vacuum to afford 2 g of crude **70** used directly in subsequent step after LC-MS confirmation ($[M+H]^+$=148).

Preparation of methyl 3-hydroxy-2-({[(4-methylphenyl)sulfonyl]oxy}methyl)butanoate 71: 1g of **70** is dissolved in 50ml of dry dichloromethane. 1.42 g of *p*-TsCl and 10ml of pyridine are then added at 5°C and reaction stirred overnight. After reaction completion checked by LC-MS 30ml of purified water are added, phases separated and aqueous one extracted three times with 50 ml of dichloromethane. Organic layers are collected and washed with 30ml of aqueous sodium bicarbonate 10% and 50ml of brine. Rich organic phase is dried over sodium sulfate and solvent distilled under vacuum to afford **71** as a dense oil 0.9g. Crude material is used directly in subsequent step after LC-MS confirmation ($[M+H]^+$=302).

Preparation of methyl 3-hydroxy-2-methylidenebutanoate 72: 0.9g of 71 is dissolved in 3ml of toluene. 1ml of DBU in 5ml of toluene is the added at room temperature for 5 minutes. After reaction completion checked by TLC, 40ml of water are then added, phases separated and aqueous layer extracted three times with 20ml of dichloromethane. Organic phases are then collected and dried over sodium sulfate. Solvent is distilled under vacuum to oil and crude material purified on a chromatographic column (Ethyl acetate/Hexane 8/2) to afford **72** with a 50% overall yield form **69**.

^{1}H NMR (300 MHz – $CDCl_3$): 1.35 (d, 3H), 3.76 (s, 3H), 4.60 (m, 1H), 5.82 (s, 1H), 6.19 (s, 1H). ^{13}C NMR ($CDCl_3$): 22.01, 51.72, 66.78, 123.95, 143.57, 166.20.

Preparation of methyl 3-hydroxy-2-[(phenylamino)methyl]butanoate 73: 0.7g of **72** are dissolved in 20ml of THF. 0.3ml of benzyl amine is then added at room temperature. Temperature is adjusted to 65°C and reaction let stir for 72hours. After reaction completion solvent is removed under vacuum. 50ml of ethyl acetate and 50ml of purified water are then added. After phases separation aqueous layer is back-extracted twice with 50ml of ethyl acetate. Organic phases are collected and washed with 50ml of brine. Organic phase is then dried over sodium sulphate and solvent removed under vacuum to afford **73** (0.75g, of crude use directly in following step). LC-MS confirmation ($[M+H]^+$=237)

Preparation of 1-benzyl-3-(1-hydroxyethyl)azetidin-2-one 75: 0.75g of **73** are dissolved in 4.2ml of dry THF. Solution is cooled at 0°C and 7.4ml of tert-butyl magnesium chloride 1M in THF are added. Reaction is stirred for 4hours at room temperature. After reaction completion, solution is cooled at 0°C and 8.4 ml of 10% aqueous solution of NH_4Cl is added. Mixture is stirred for 10 minutes and Dicalite is added. Solid is filtered off and solution concentrated under vacuum to 9ml. residue is extracted three times with 15ml of ethyl acetate. Organic layers are collected, dried over sodium sulfate and solvent removed under vacuum to afford **75** (0.5g, 77% yield). LC-MS confirmation ($[M+H]^+$=205).

Preparation of 1-benzyl-3-(1-((*tert*-butyldimethylsilyl)oxy)ethyl)azetidin-2-one 76: 0.5g of **75** are dissolved in 5ml of DMF at 0°C. 0.45 g of imidazole and 0.40 g of TBSCl are the added. Reaction is stirred overnight. After reaction completion 20 ml of diethyl ether are added and washed five times with 5 ml of water. Organic layer is dried over sodium sulfate and solvent removed under vacuum to afford **76** as a yellow oil (0.62g, 80%yield).
^{1}H NMR (300 MHz – $CDCl_3$): 0.08 (d, 6H) 0.88 (s, 9H), 1.27 (m, 1H), 1.37 (m, 3H), 3.25 (m, 3H), 4.17 (m, 1H), 7.33 (m, 5H).
^{13}C NMR ($CDCl_3$): 20.48, 25.64, 40.81, 45.75, 56.52, 57.47, 65.41, 127.47, 128.08, 128.65, 135.66, 167.96.

Preparation of 3-(1-((*tert*-butyldimethylsilyl)oxy)ethyl)azetidin-2-one 59 and 60: to 10 ml of liquid ammonia at -78°C, 0.3g of sodium metallic are added. After dissolution solution becomes blu and 0.7ml of dry ethanol are added followed by 0,2g of metallic sodium. A solution of 150mg of **76** in 5ml of THF at-78°C are then added dropwise. Reaction is stirred for 20min maintaining the temperature at -78°C. The reaction is then quenched by addition of 2g of ammonium chloride. The system is taken at room temperature. Temperature is adjusted to room and 50ml of DMC are added. Solid is filtered and solution treated with sodium sulfate. Solvent is concentrate under vacuum to afford **59** and **60** after chromatographic purification on silica column. (100mg, 93% yield)
^{1}H NMR (200 MHz – $CDCl_3$): 0.07 (m, 6H) 0.88 (m, 6H), 1.29 (d, 3H), 3.23 (m, 2H), 3.36 (m, 1H), 4.14 (m, 1H), 6.02 (bs, 1H).
^{13}C NMR ($CDCl_3$): 4.41, 5.13, 17.81, 22.39, 25.58, 37.63, 59.09, 65.24, 169.74.

Preparation of (3R,4R)-4-Acetoxy-3-[(S)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone 63 and 77 from racemic mixture AZTB 59 and 50: 100mg of **60** is added to 5ml of ethyl acetate at room temperature under nitrogen flow. 0.025mg of Ru/C, 8,6mg of sodium acetate and 0.08ml of acetic acid are the added at the same temperature. Peracetic acid 39%in water (0.56ml) is the charged dropwise. Reaction is let stirring for 1 h. After reaction completion 0.2ml of 2% aqueous sodium metabisulfite is added at room temperature and let stir for 10 minutes. Solid precipitation occurs. Solid is filtered and washed twice with 10ml of diethyl ethere and 5ml of water. Filtrate is collected and

pH adjusted to 5 with NaOH 15% in water. Phases are then separated and aqueous phase back-extracted with 10 ml of ethyl acetate. Organic layers are then collected and wash twice with a 5% sodium bicarbonate solution in water (10ml each), and once with 10 ml of purified water. Organic phases are dried over magnesium sulfate and solvent distilled to afford 100 mg oil. Crude material is purified over chromatographic silica gel column (cyclohexane/Ethyl acetate 7/3) to afford **63** and **77** (50mg, 40%yield).
^{1}H NMR (300 MHz – $CDCl_3$): 0.19 (s, 6H), 0.98 (s, 9H), 1.39 (d, 3H), 2.22 (s, 3H), 3.39 (m, 1H), 4.39 (m, 1H), 5.71 (s, 1H), 6.82 (bs, 1H). ^{13}C NMR ($CDCl_3$): 4.27, 5.20, 17.00, 20.80, 25.50, 64.22, 64.59, 75.98, 166.10, 171.09.

Preparation of methyl (R)-3-hydroxy-2-(hydroxymethyl)butanoate 78: 15ml of LDA 1M in THF are cooled to -78°C. 2,3g of **68** dissolved in 40ml of THF are the added dropwise. Solution is stirred for 30 minutes at -78°C then temperature is increased at -23°C. in a side vessel 5,5g of p-formaldehyde are heated at 200°C to promote de-polymerization and formation of formaldehyde gas which is bubbled in the THF solution in 45minutes with a gentle nitrogen stream. Reaction is stirred for additional 30 minutes and then quenched by addition of 40 ml of a 5% NH_4Cl solution and 40ml of HCl 1N. aqueous mixture is extracted three times with 200ml of ethyl acetate. Organic phases are then collected and washed with 40 ml of 10% aqueous sodium bicarbonate and with 40ml of brine. Organic rich solution is the dried over sodium sulfate and solvent distilled under vacuum to afford 2 g of crude material. Diol compound was then purified on chromatographic column 8/2 cyclohexane acetate affording 78 1.2 g of pure **78** with 55% yield. LC-MS confirmation ($[M+H]^+$=148).

Preparation of methyl (R)-3-hydroxy-2-methylidenebutanoate 79: 1g of **78** is dissolved in 50ml of dry dichloromethane. 1.42 g of *p*-TsCl and 10ml of pyridine are then added at 5°C and reaction stirred overnight. After reaction completion checked by LC-MS 30ml of purified water are added, phases separated and aqueous one extracted three times with 50 ml of dichloromethane. Organic layers are collected and washed with 30ml of aqueous sodium bicarbonate 10% and 50ml of brine. Rich organic phase is dried over sodium sulfate and solvent distilled under vacuum to afford a dense oil 0.9g. Crude material is dissolved in 3ml of toluene. 1ml of DBU in 5ml of toluene is the added at

room temperature for 5 minutes. After reaction completion checked by TLC, 40ml of water are then added, phases separated and aqueous layer extracted three times with 20ml of dichloromethane. Organic phases are then collected and dried over sodium sulfate. Solvent is distilled under vacuum to oil and crude material purified on a chromatographic column (Ethyl acetate/Hexane 8/2) to afford **79** with a 70% yield.
^{1}H NMR (300 MHz – $CDCl_3$): 1.36 (d, 3H), 3.77 (s, 3H), 4.61 (m, 1H), 5.82 (t, 1H), 6.20 (s, 1H). ^{13}C NMR ($CDCl_3$): 22.02, 51.72, 66.79, 123.90, 143.50, 166.21.

Preparation of methyl (R)-3-hydroxy-2-[(phenylamino)methyl]butanoate 80: 0.7g of **79** are dissolved in 20ml of THF. 0.3ml of benzyl amine is then added at room temperature. Temperature is adjusted to 65°C and reaction let stir for 72hours. After reaction completion solvent is removed under vacuum. 50ml of ethyl acetate and 50ml of purified water are then added. After phases separation aqueous layer is back-extracted twice with 50ml of ethyl acetate. Organic phases are collected and washed with 50ml of brine. Organic phase is then dried over sodium sulphate and solvent removed under vacuum to afford **80** (0.77g, 60%yield). Crude material was used directly in subsequent step after retention time comparison with **73** and LC-MS confirmation.

Preparation of 1-benzyl-(R)-3-(1-hydroxyethyl)azetidin-2-one 81: 0.75g of **80** are dissolved in 4.2ml of dry THF. Solution is cooled at 0°C and 7.4ml of tert-butyl magnesium chloride 1M in THF are added. Reaction is stirred for 4hours at room temperature. After reaction completion, solution is cooled at 0°C and 8.4 ml of 10% aqueous solution of NH_4Cl is added. Mixture is stirred for 10 minutes and Dicalite is added. Solid is filtered off and solution concentrated under vacuum to 9ml. residue is extracted three times with 15ml of ethyl acetate. Organic layers are collected, dried over sodium sulfate and solvent removed under vacuum to afford **81** (0.5g, 77% yield from **79**)
^{1}H NMR (300 MHz – $CDCl_3$): 1.26 (m, 6H) 2.03 (s, 3H), 3.03 (m, 1H), 3.21 (m, 2H), 4.05 (m, 2H), 4.35 (m, 2H), 7.31 (m, 5H). ^{13}C NMR ($CDCl_3$): 20.92, 41.52, 45.84, 57.06, 66.28, 127.30, 127.94. 128.60, 135.42, 170.83

Preparation of 1-benzyl-3-(1-((*tert*-butyldimethylsilyl)oxy)ethyl)azetidin-2-one 82: 0.5g of **81** are dissolved in 5ml of DMF at 0°C. 0.45 g of imidazole and 0.40 g of TBSCl are the added. Reaction is stirred overnight. After reaction completion 20 ml of diethyl ether are added and washed five times with 5 ml of water. Organic layer is dried over sodium sulfate and solvent removed under vacuum to afford **82** as a yellow oil (0.62g, 80%yield).
^{1}H NMR (300 MHz – $CDCl_3$): 0.09 (d, 6H) 0.87 (s, 9H), 1.27 (m, 1H), 1.38 (m, 3H), 3.24 (m, 3H), 4.16 (m, 1H), 7.32 (m, 5H).
^{13}C NMR ($CDCl_3$): 20.47, 25.63, 40.81, 45.74, 56.51, 57.48, 65.40, 127.46, 128.06, 128.61, 135.65, 167.93.

Preparation of 3-(1-((*tert*-butyldimethylsilyl)oxy)ethyl)azetidin-2-one *59*: to 10 ml of liquid ammonia at -78°C, 0.3g of sodium metallic are added. After dissolution solution becomes blu and 0.7ml of dry ethanol are added followed by 0,2g of metallic sodium. A solution of 150mg of **82** in 5ml of THF at-78°C are then added dropwise. Reaction is stirred for 20min maintaining the temperature at -78°C. Mixture is the quenched with 2g of ammonium chloride. The system is then taken at room temperature and 50ml of DMC are added. Solid is filtered and solution treated with sodium sulfate. Solvent is concentrate under vacuum to afford ***59*** after chromatographic purification on silica column. (100mg, 93% yield)
^{1}H NMR (300 MHz – $CDCl_3$): 0.06 (s, 6H) 0.86 (s, 9H), 1.19 (d, 3H), 3.18 (m, 1H), 3.29 (m, 2H), 4.16 (m, 1H), 6.31 (bs, 1H).
^{13}C NMR ($CDCl_3$): 4.41, 5.13, 17.81, 22.39, 25.58, 37.63, 59.09, 65.24, 169.74.

Preparation of (3R,4R)-4-Acetoxy-3-[(S)-1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone 77: 100mg of ***59*** is added to 5ml of ethyl acetate at room temperature under nitrogen flow. 0.025mg of Ru/C, 8,6mg of sodium acetate and 0.08ml of acetic acid are the added at the same temperature. Peracetic acid 39%in water (0.56ml) is the charged dropwise. Reaction is let stirring for 1 h. After reaction completion 0.2ml of 2% aqueous sodium metabisulfite is added at room temperature and let stir for 10 minutes. Solid precipitation occurs. Solid is filtered and washed twice with 10ml of diethyl ethere and 5ml of water. Filtrate is collected and pH adjusted to 5 with NaOH 15% in water. Phases are then separated and aqueous phase back-extracted with 10 ml of ethyl acetate.

Organic layers are then collected and wash twice with a 5% sodium bicarbonate solution in water (10ml each), and once with 10 ml of purified water. Organic phases are dried over magnesium sulfate and solvent distilled to afford 100 mg oil. Crude material is purified over chromatographic silica gel column (cyclohexane/Ethyl acetate 7/3) to afford **77** (50mg, 40%yield).

^{1}H NMR (300 MHz – $CDCl_3$): 0.18 (s, 6H), 0.97 (s, 9H), 1.41 (d, 3H), 2.20 (s, 3H), 3.33 (m, 1H), 4.36 (m, 1H), 5.79 (s, 1H), 6.82 (bs, 1H). ^{13}C NMR ($CDCl_3$): 4.28, 5.18, 17.80, 20.76, 25.52, 64.21, 64.51, 75.93, 166.10, 171.05.

7 Acronyms and abbreviation list

4-BMA: (3S,4S)-4-[(R)-1-carboxyethyl]-3-[(R)-1-(t-butyldimethylsilyloxy)ethyl]-2-azetidinone

6-APA: (2*S*,5*R*,6*R*)-6-Amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

7-ACA: 3-(Acetyloxymethyl)-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

AAL: 2-(aminomethyl)-3-hydroxybutanoic acid

AALE: 2-oxoazetidin-3-yl)ethyl benzoate

ADH: Alcohol dehydrogenase

AMR: Antimicrobial resistance

AOSA: 4-Acetoxy-3-[1'((t-butyldimethylsilyl)oxy)ethyl]-2-azetidinone

API: Active pharmaceutical ingredients

AZT: 3-[1-hydroxyethyl]azetidin-2-one

AZTB: 3-(1-((tert-butyldimethylsilyl)oxy)ethyl)azetidin-2-one

AZT-BAL: Methyl 2-(benzamidomethyl)-3-hydroxybutanoate

DBO: Diazabicyclooctane

DBU: Diazabicycloundecene

DCM: Dichloromethane

DEAD: Diethyl azodicarboxylate

DHP: Dehydropeptidase

DIAD: Diisopropyl azodicarboxylate

DKR: Dynamic kinetic resolution

DMF: Dimethylformammide

DMSO: Dimethylsulfoxide

GDH: Glucose dehydrogenase

GT: Glycosyltransferases

KRED: Codexis alcohol dehydrogenase

LDA: Lithium diisopropylamide

MBA: Methyl 2-Benzamidomethyl-3-oxobutanoate

NAD: Nicotinammide adenina dinucleotide

NADP: Nicotinammide adenina dinucleotide phosphate

NAG: N-acetylglucosamine

NAM: N-acetylmuramic acid

PBP: Penicillin binding protein

PBu_3: Tributhyl phosphine

PPh_3: Triphenyl phosphine

p-TsCl: p-tolunesulfonyl chloride

Py: Pyridine

TBSCl: tert-butyldimethylsilil chloride

TFE: Triflouroethanol

THA: Trojan horse approach

THF: Tetrahydorfuran

TLC: Thin layer chromatography

TMAD: Tetramethylazo dicarboxamide

TMSCl: Trimethyl silyl chloride

8 Literature

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