Unusual Amino Acids: Synthesis and Introduction into Naturally Occurring Peptides and Biologically Active Analogues

G. Cardillo*, L. Gentilucci and A. Tolomelli

Dip. Chimica "G. Ciamician", Università degli Studi di Bologna, Via Selmi 2, 40126 Bologna, Italy

Abstract: This review covers our recent advances in the synthesis of unusual amino acids in optically pure form, and their introduction into naturally occurring peptides with specific biological properties, or into modified bioactive peptides, aiming to obtain analogues displaying enhanced performances in term of activity, bioavailability and resistance to enzymatic hydrolysis.

Keywords: Unusual amino acids, peptides, peptidomimetics, aziridines, α-substituted-β-amino acids, β-substituted-α-amino acids, endomorphin, lysobactin.

INTRODUCTION

The design of receptor selective peptides and peptidomimetics with high potency and specificity has become one of the most important areas in bioorganic chemistry, medicinal chemistry, molecular biology and other related research areas [1]. At the present time there is a rapid growth in the number of endogenous and exogenous biologically active peptides under investigation. Most of these peptides are short lived molecules, easily degraded by enzymes with little or no therapeutic use. Therefore, it is often necessary to transform these compounds into more resistant molecules [2]. Incorporation of unusual α,α-disubstituted-α-amino acids [3], α-substituted-β-amino acids [4] or amino acid mimetics [5] into peptides results in conformational restrictions and increased rigidity, leading to enhanced resistance towards protease enzymes and to enhanced receptor selectivity. For this reason, there is considerable interest in the synthesis of unnatural amino acids in order to introduce them into a peptide sequence. Beside the introduction of constrained amino acids, cyclization is known to reduce conformational freedom. Small cyclic peptides show indeed increased resistance to enzymatic degradation and constrained flexibility as compared to their linear analogues. Consequently, they frequently exhibit higher biological selectivity and activity.

Cyclic and non-cyclic peptides with important pharmacological properties have been isolated from marine organism or from plants [6]. These compounds contain, in addition to unusual acid moieties, such as D-amino acids and hydroxy acids, non proteinogenic α- and β-amino acids, α-alkyl or α-hydroxy-β-amino acids and γ-amino acids, or nitrogen containing heterocycles.

Among them, a large number of oxazole and/or thiazole containing natural products have been isolated from marine organisms, mainly sponges and ascidians, over the last two decades [7].

In the last few years, our research group has been interested in the synthesis of biologically active peptides and peptidomimetics, using β-amino acids, aziridines and oxazolines, both as precursors of substituted amino acids and as building blocks in the synthesis of biologically active compounds.

In this mini review, the synthesis of α- and β-amino acids, substituted in α or β position with alkyl or hydroxyl group that we have developed in the last few years, is reported. In many cases, the starting step of these syntheses is the 1,4-addition of nitrogen nucleophiles to α,β-unsaturated carboxyl derivatives, carried out under high facial stereocontrol (Fig. **1**).

OPTICALLY PURE β–AMINO ACIDS AND THEIR INTRODUCTION INTO SMALL PEPTIDES

β-Amino acids[8] are an interesting class of compounds for the medicinal chemist. They are present as components in a variety of peptidic natural compounds [9], produced in animals, plants and microorganisms, which display antibiotic, antifungal, cytotoxic and other pharmacological properties. The discovery of new therapeutic agents based on non-proteinogenic amino acids stimulated the synthesis of conformationally constrained β-amino acids [10] and the importance of these compounds arises from the potential biological properties related to their structural features. In fact, the use of rigid amino acids has been the most successful approach to investigate the bioactive structure of peptides, and non proteinogenic, constrained β-amino acids are a valuable tool for probing activity changes in a peptide.

The preparation of various β-amino acids is an interesting topic that has drawn a great deal of attention, because unusual amino acids such as α-substituted- or β-substituted-β-amino acids, allow to create novel types of peptides that are catalysts or carriers of biologically active residues with pharmacologically interesting properties.

A) Synthesis of α-Substituted-β-Amino Acids Via Enzymatic Resolution of Racemates

The synthesis of enantiomerically pure α-alkyl β-amino acids has been accomplished by a variety of methods such as, for instance, the addition of chiral secondary amines to α,β-unsaturated esters [11], the condensation of disubstituted

*Address correspondence to this author at the Dip. Chimica "G. Ciamician", Università degli Studi di Bologna, Via Selmi 2, 40126 Bologna, Italy; E-mail: cardillo@ciam.unibo.it

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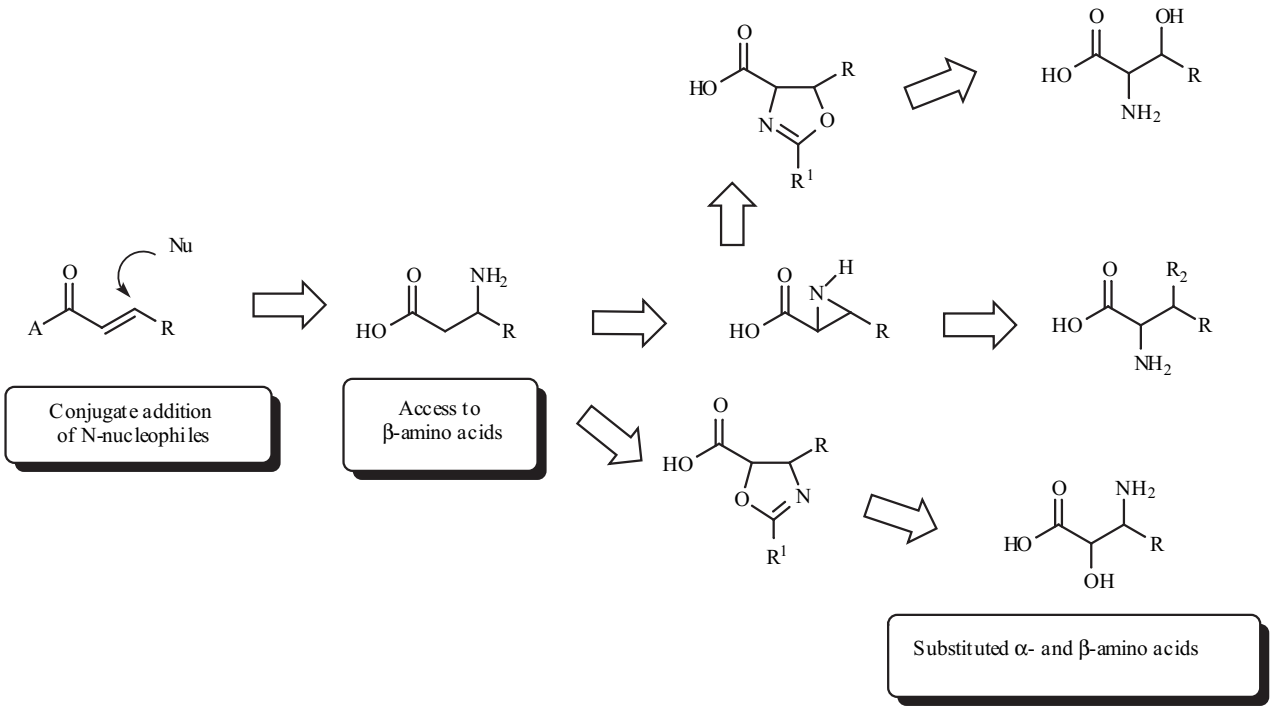


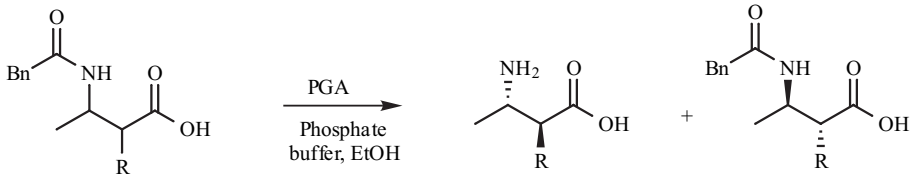
Fig. (1).

imines [12], the homologation of the corresponding α-analogues, or the functionalization at C-5 of chiral 6-alkylperhydropyrimidin-4-ones. This last method allowed us to obtain (S,S)- or (R,R)-α-alkyl β-amino acids with high stereocontrol and in high yield [13].

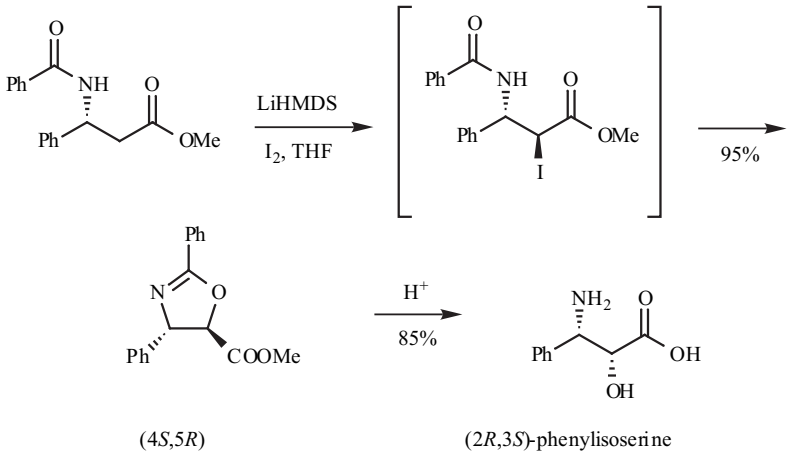
A very simple alternative procedure for the preparation of these compounds is the enzymatic hydrolysis of racemic N-phenylacetyl α-alkyl β-amino acids with penicillin G acylase (PGA) [14]. This enzyme is widely distributed among microorganisms and is used on an industrial scale for the production of 6-aminopenicillanic acids [15]. With respect to other hydrolytic enzymes, it exhibits a high affinity for the phenylacetyl moiety but a large tolerance towards the functions present in the molecule backbone. For this reason, the hydrolysis of different phenylacetic derivatives has been reported and, among them, the resolution of racemic β-amino acids catalyzed by PGA, preferentially leading to the hydrolysis of the isomer with L configuration, has been extensively studied [16]. Recently, we have reported the alkylation of *N*-phenylacetamides derived from racemic 3-aminobutanoic acid, that occurs in exclusive anti relationship [17], followed by enzymatic resolution with PGA (Scheme **1**).

This biocatalytic kinetic resolution represents a useful method for the preparation of enantiomerically pure α-alkyl β-amino acids. Furthermore, these compounds are particularly interesting since they can be easily transformed into substituted β-lactams.

Hydroxy-amino acids are important components in a variety of biologically interesting compounds. The best known molecule containing a α-hydroxy-β-amino acid is Taxol [18], a complex diterpene isolated from the bark of Taxus brevifolia, which is considered one of the most active agents in cancer chemotherapy. Though the role of the (2*R*,3*S*)-phenylisoserine side chain has not yet been fully determined, this part of the molecule seems to be fundamental in the explication of its antitumor activity. Modifications in the side chain rarely produce advantages; often leading to either a considerable reduction or a total loss of activity. Recently, taxoids bearing a difluoromethyl group in the C3' position, have been reported by Oijima *et al*. as potent anticancer agents. These analogues differ from the original diterpene for the fluorurated chain and for the substituent on the baccatin polycyclic backbone, but retain the stereochemistry of the α-hydroxy-β-amino acid [19]. Taking advantage from the use of PGA, we performed the synthesis of (2*R*,3*S*)-phenylisoserine starting from the racemic β-amino acid (Scheme **2**). [20] Enantiomerically pure (-)-(*R*)-3-amino-3-phenylpropanoic acid was obtained indeed by enzymatic kinetic resolution of the corresponding *N*-phenylacetyl derivative with penicillin G acylase (PGA)



Scheme 1.



Scheme 2.

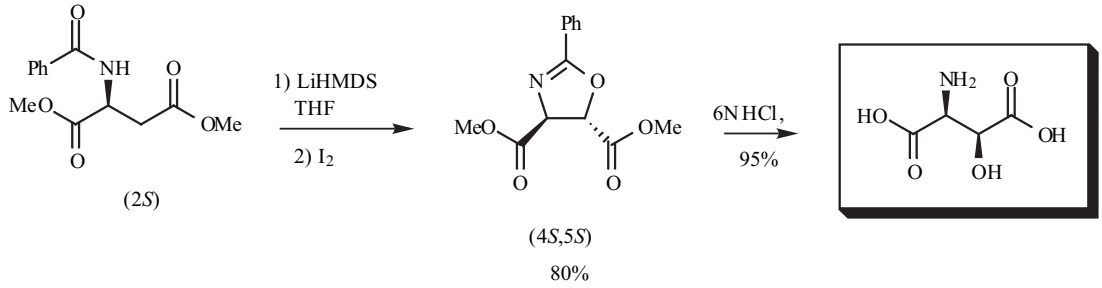
from *Escherichia coli*, immobilized on Eupergit. The key step for the introduction of hydroxyl function in the α position, was the iodination reaction of the lithium dianion of methyl (3*R*)-*N*-benzoyl-3-amino-3-phenylpropanoate, which directly afforded the corresponding trans oxazoline with high yield and diastereoselectivity. The hydrolysis under acid conditions gave (2*R*,3*S*)-phenylisoserine in excellent yield.

This new cyclofunctionalization reaction represents a general method for the *syn* introduction of vicinal amino and hydroxyl function. Following the same reaction sequence, starting from L-aspartic acid, the enantiomerically pure β-hydroxy-aspartic acid, a component of Lysobactin antibiotic, was obtained in good yield and complete stereoselectivity (Scheme **3**) [21].

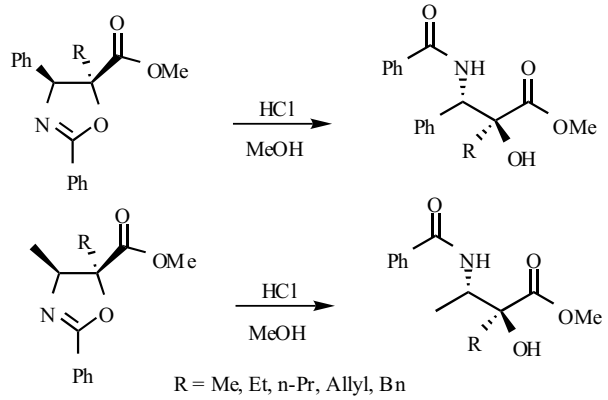
Moreover, oxazoline-carboxylates represent a protected form of hydroxy-amino acids and can be used as intermediates for the introduction of a second substituent in the α position of the amino acids [3a].

On these bases, starting from the intermediate oxazoline, a general strategy allowing the introduction of an alkyl group in the α position was developed (Scheme **4**).

The deprotonation of the activated oxazoline C4 position, followed by alkylation, afforded an easily separable mixture of α-alkylated heterocycles, whose hydrolysis leaded to the formation of α-alkyl-α-hydroxy-β-amino acids in enantiomerically pure form [22].



Scheme 3.

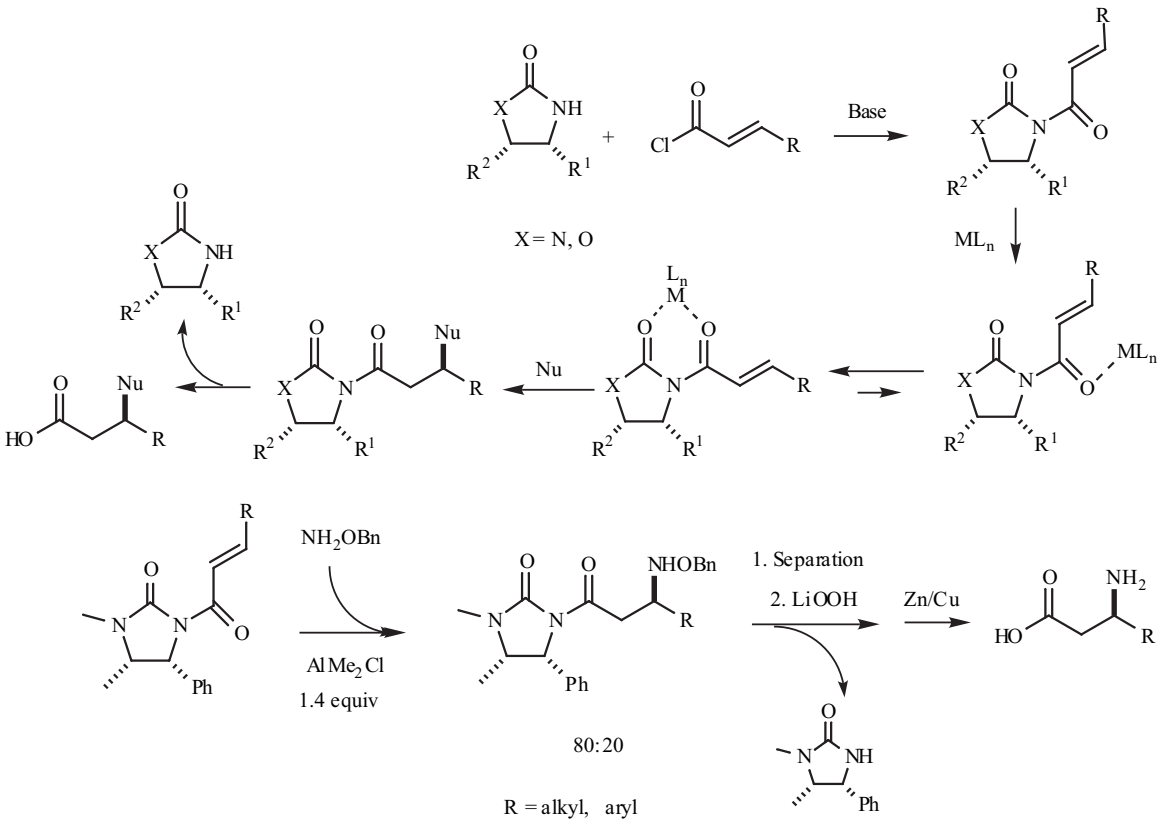


Scheme 4.

B) Synthesis of Enantiomerically Pure β-Amino Acids with the Use of Chiral Auxiliaries

In the conjugate addition of *N*-nucleophiles there are different strategies to carry out the reaction in a stereoselective fashion. Stereocontrol can be exerted by the substrate [23], by a chiral nucleophile [24], by a chiral auxiliary [25], or by a chiral Lewis acid [26].

We studied the conjugate addition of *N*-nucleophiles to α,β-unsaturated compounds carrying an imidazolidinone chiral auxiliary [27]. The addition was performed in the presence of a number of Lewis acids [28], introduced to coordinate the unsaturated system and to favour the addition by lowering the energy of LUMO. The role of the Lewis acids was not limited to increase reactivity and they also manifested a strong influence on the stereochemical outcome. Indeed, depending on the nature and on the



Scheme 5.

amount of the Lewis acid, the complex adopted different structures, monocoordinated or bicoordinated. This last complex is generally the more stable one and drives to a preferential addition to the less hindered face of the complex. When the conjugate addition was performed by choosing NH_2OBn as nucleophile, optically active β-amino acids were obtained in excellent yields, after reduction of the N-O bond in the presence of Zn/Cu and removal of the chiral auxiliary (Scheme **5**).

C) Introduction of β-Amino Acids into Biologically Active Peptides

The design and synthesis of modified endomorphins, short peptides discovered in bovine brain in 1997 by Zadina [29], was recently developed in our laboratory. These peptides, together with enkephalins, endorphins, dynorphins, belong to the family of the endogenous opioid neuropeptides, molecules appointed by nature to pain relief in the mammalians [30]. Opioid peptides switch opioid receptors by interaction with the binding site [31]. These receptors are members of the class of G-protein coupled receptors, and are widely distributed in the central nervous system. The receptor is made of a single protein that crosses seven times the membrane, giving rise to three extracellular, and three intracellular loops, and is coupled to a G protein [32]. They can be divided into three types (μ, δ, and κ); dynorphins, endorphins, and enkephalins show a certain but not complete selectivity towards κ, μ, and δ receptors, respectively. On the contrary, endomorphins are extremely selective towards μ receptors, that are, among the others, the receptors with the most potent antinociceptive activity (Fig. **2**).

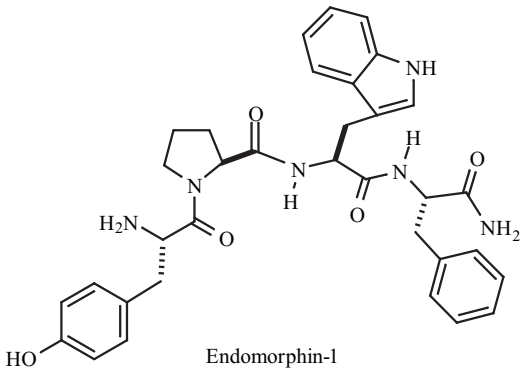
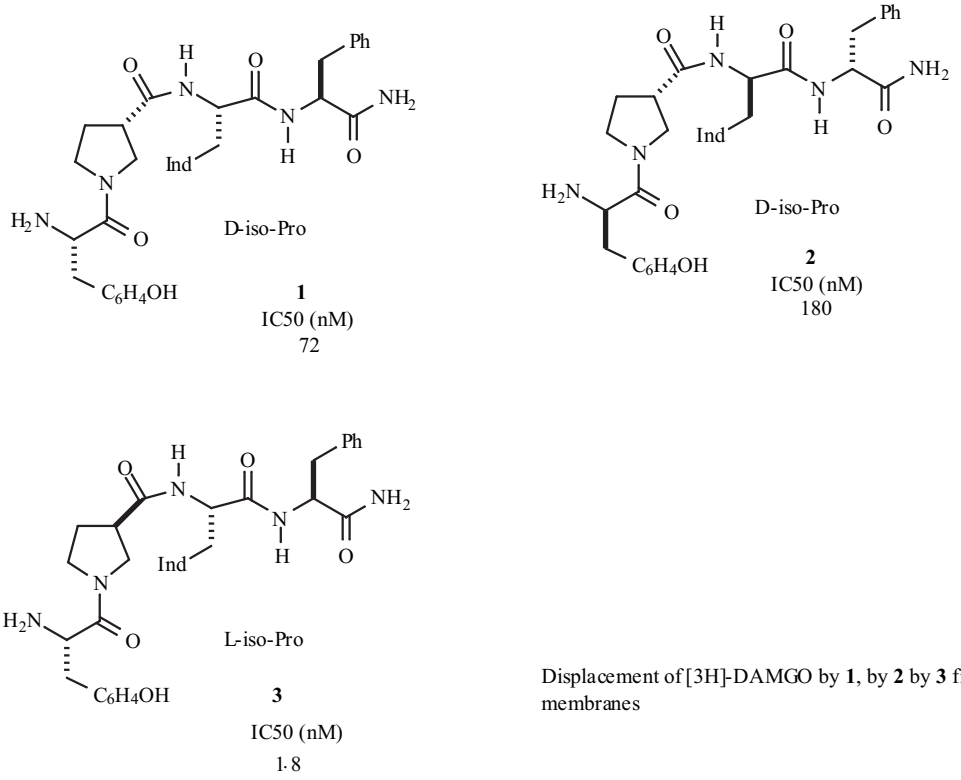


Fig. (2).

The analgesic efficacy of opioid peptides relies in their ability to inhibit the production of neuropeptides. Pain stimula are transmitted from neuron to neuron by means of the substance P. Opioid peptides are secreted by the enkephalinergic neurons and inhibit the secretion of substance P upon activation of the opioid receptors.

The role of opioid peptides as endogenous analgesics suggests a possible use as pharmacological tools for pain relief. Indeed, they appear to be deprived of the undesired secondary effects observed for morphine, mainly tolerance and dependence [33]. However, at least two major drawbacks to their use as analgesics have been proved: their rapid enzymatic degradation [34], and their scarce ability to penetrate biological barriers. To bypass these drawbacks it is possible to modify their structures by introducing unusual amino acids. A second very important point is that the investigation of the pharmacological effects displayed by the modified peptides can shed light to fundamental but still



Displacement of [3H]-DAMGO by **1**, by **2** by **3** from rat brain membranes

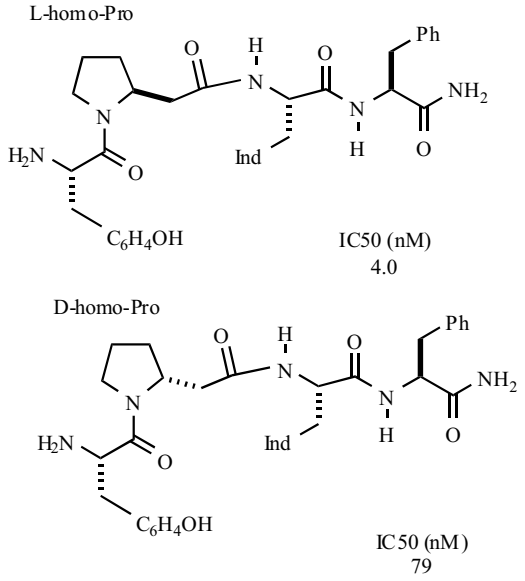
Scheme 6.

missing details of their action mechanism, in particular the biologically active conformation of the peptides at the receptors [35].

On the basis of these considerations, we synthesized a series of isomeric β-amino acids, having the amino group shifted to the β position, in order to obtain endomorphin-1 analogues [36]. Some of the β-amino acids, as β-phenylalanine and β-tryptophane, could be obtained by way of stereoselective conjugate addition of *N*-nucleophiles. [27] β-Tyrosine was prepared by means of an enzymatic resolution of racemic *N*-phenylacetamide with PGA [14]. β-proline was synthesized in both L and D configuration starting from the same hydroxy pyrrolidine upon tosylation, treatment with KCN, and acidic hydrolysis, or alternatively upon Mitsunobu displacement of the OH group with Zn tosylate, followed by the same steps as before [37].

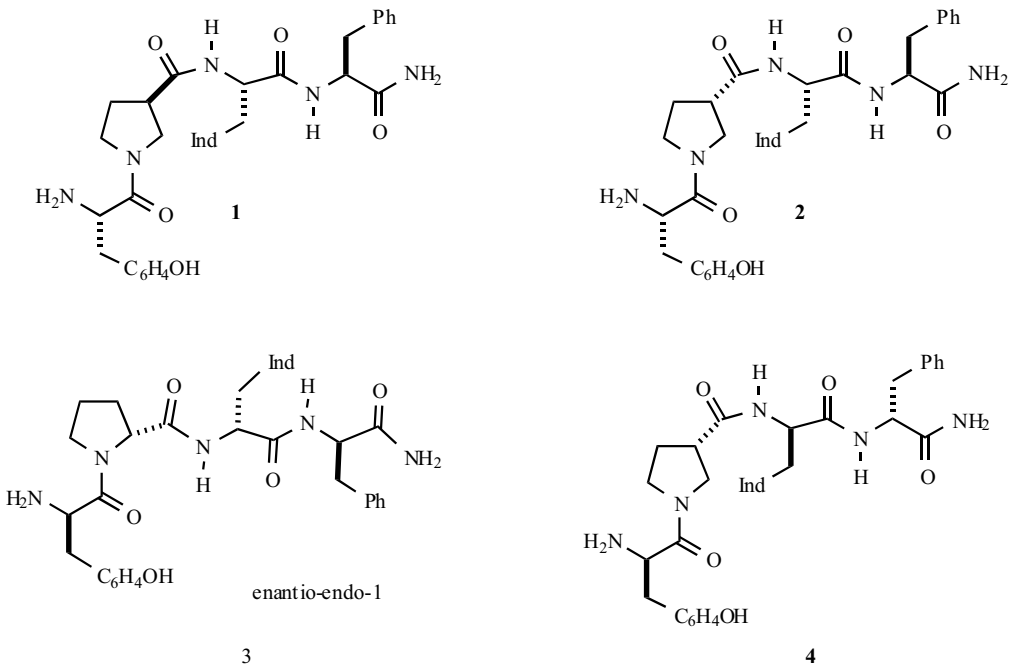
The β-isomeric amino acids were introduced in the sequence of endomorphin-1 in place of the corresponding α-residues. To test the affinity of these peptides for the μ-receptors, the ability to displace [^{3}H]-DAMGO [38], a strong radiolabelled agonist, from rat brain membranes containing the receptors, was assayed. From the inspection of the results, it turned out that the peptides containing β-phenylalanine, β-tyrosine and β-tryptophane showed very poor receptor affinities. On the contrary peptides containing L-isoproline, D-isoproline, or the one containing D-isoproline and all the other residues in D configuration, were the most effective in displacing DAMGO. Affinities are expressed in terms of IC_{50} values. The calculated values were in the nanomolar range, lower but still comparable with that of endomorphin-1, which is subnanomolar (Scheme **6**).

Further, we designed a second set of analogues containing homologated β-amino acids (Scheme **7**) [39]. The homo amino acids are currently commercially available, or can be prepared from α-amino acids by Arndt-Eistert homologation [40]. Also in this second set, the peptides with β-proline resulted to be the only ones to show a certain affinity, and in particular the IC_{50} of peptide L-homoproline was nanomolar.



Scheme 7.

To test if the modifications introduced gave to peptides an increased stability towards enzymatic degradation, the active peptides were incubated at 37°C with proteolytic enzymes [41]. The peptides containing β-isoproline and β-homoproline showed good stability in the presence of aminopeptidase-M, one of the most important enzymes



Scheme 8.

involved in the metabolism of endogenous neuropeptides, which was almost ineffective. Peptide amounts remained practically constant for hours, while, under the same conditions, endomorphin-1 was degraded in a short time (Scheme **8**).

A general effect exerted by μ-opioid receptor agonists, including endomorphin-1 and DAMGO, is the inhibition of cyclic AMP accumulation in cells. Therefore, it is possible to make an *in vitro* test of this effect to discover if a certain compound is an agonist or an antagonist [42]. The two compounds containing homoproline, the most effective in binding the receptors, suppressed forskolin-stimulated cyclic AMP production in intact SH-SY5Y cells in a concentration-dependent manner, indicating that they are real agonists.

The analgesic efficacy was also measured *in vivo* [43]. The peripheral inoculation in mice of the two analogues with homoproline gave analgesia in the tail-flick test, while under the same conditions endomorphin-1 resulted completely un-effective.

A second aspect we are dealing with, is the investigation of the bioactive conformation of endomorphin at the receptor. The X-ray structure of the receptors is not available and it can be just suggested on the basis of the data available for bovine rodopsin [44]. Therefore it is necessary to infer clues from the conformational analysis of the different ligands. However, in the case of endomorphins there is not yet a definitive and convincing model. During the last few years, several groups proposed bioactive conformations of endomorphin-1 or its analogues [45], showing tyrosine-proline bond in cis or trans configuration depending on the environmental conditions selected for the 2D NMR and molecular dynamics analysis.

Recently we made the hypothesis that endomorphin-1 could adopt at the receptor a folded structure [46]. We prepared an analogue having a carbamate group in place of tyrosine in order to increase solubility in low polarity solvents, such as $CDCl_3$, and to facilitate the formation of intramolecular H-bonds. In our opinion, this non-competitive solvent could be considered a suitable environment to simulate the hydrophobic core of a receptor. The Cbz-peptide analogue showed nanomolar affinity and adopted a folded conformation stabilized by the formation of a H-bond, a γ-turn and a ten membered ring β-turn in equilibrium.

In the same period Eguchi [47] described the agonist behaviour of a compound based on a 6,6-bicyclic scaffold as a β-turn mimetic. Both these evidences strengthen the theory that endomorphin-1 could adopt at the receptor a folded structure (Fig . **3**).

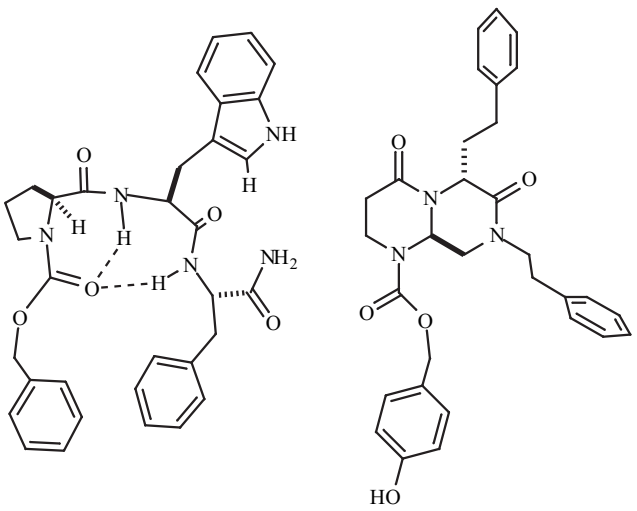
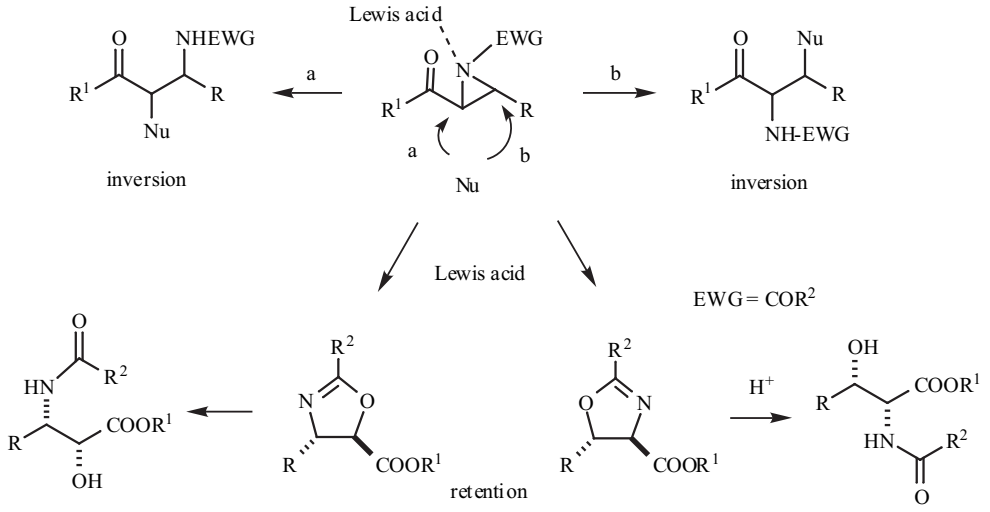


Fig. (3).



Scheme 9.

Synthesis of Enantiomerically Pure Aziridines as Intermediates in the Preparation of Hydroxy-Amino Acids

Aziridines can be regarded as substituted amino acid precursors [46]. It is well known that *N*-activated aziridines can be ring opened with a nucleophile, with inversion of the configuration, giving rise to α-substituted-β-amino acids or β-substituted-α-amino acids, depending on the nature of the nucleophile and the Lewis acid. Another reaction of *N*-acyl aziridines is the Lewis acid-promoted ring expansion to oxazolines, with retention of the configuration (Scheme **9**) [49]. This reaction occurs in regioselective fashion, depending on the groups linked to the starting aziridine, and gives rise after hydrolysis to α-hydroxy-β-amino acids or β-hydroxy-α-amino acids. This expansion was already described in literature, and its mechanism discussed in details. However, no practical purposes in a synthetic sense were reported in the literature.

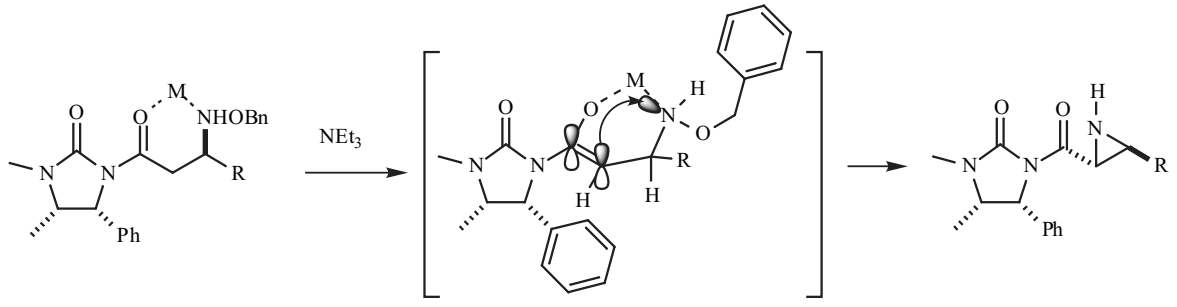
A) Synthesis of α-Amino-β-Hydroxy Acids

Beside the synthesis of β-amino acids, even the preparation of small heterocycles as precursors of hydroxy-amino acids, was developed through the conjugate addition of O-benzyl-hydroxylamine to unsaturated imides [50]. The cyclization of the 1,4-adduct by treatment with titanium or aluminum salts and triethylamine, gave indeed access to aziridine-carboxylates in optically pure form and exclusive trans configuration (Scheme **10**). The reaction mechanism was investigated, and it was found to proceed through a cyclic Evans-type enolate, undergoing cyclization after intramolecular displacement of the benzyloxy group.

The reaction is believed to adopt a SN1' mechanism. The aziridine C3-N bond breaks giving an ion pair intermediate, or an ion pair-like transition state, which closes to oxazoline with retention of the configuration. We found that, in the presence of Lewis acids, *N*-acyl aziridines carrying the imidazolidinone chiral auxiliary undergo ring expansion in an extremely fast, regioselective and stereoselective way, making it possible to use such a reaction for the preparation of hydroxy amino acids [51]. We investigated the reaction by mean of computational methods. Computations revealed a remarkably stable intermediate showing the oxygen of imidazolidinone pointing towards the incipient carbocation emerging from the C3-N bond rupture, allowing a charge delocalization. This intermediate seems to be responsible for the accelerated reaction rate and for the noteworthy regio- and stereoselectivity [52].

The ring expansion reaction of *trans* aziridines to the corresponding *trans* oxazolines easily occurs, under complete regio and stereocontrol, by treatment with Lewis acids in CH_2Cl_2 or under microwave catalyzed conditions [53]. This well known behavior has been successfully applied in our laboratory for the synthesis of several β-hydroxy-α-amino acids, such as threonine, hydroxyleucine, phenylserine, etc [54].

Once the unusual amino acid has been synthesized, the following step is its connection with a peptide sequence. With the aim to perform the direct introduction of a β-



Scheme 10.

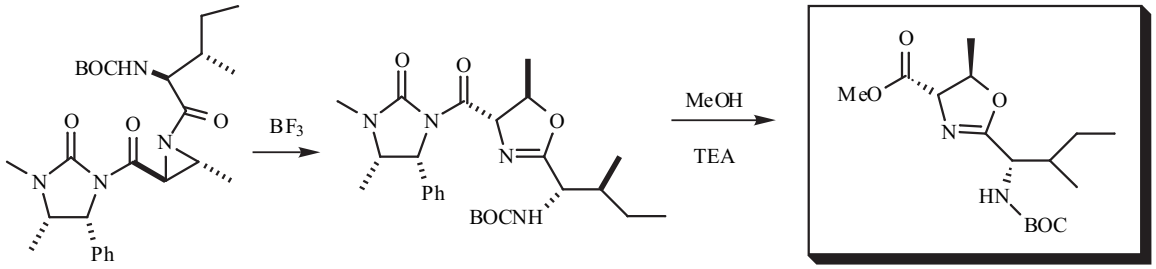
hydroxy-α-amino acid into a dipeptide, we thought that the acyl group of the *N*-activated-aziridine could originate from an amino acid. Therefore, the heterocycle was coupled with an amino acid through the standard procedure for peptide synthesis, and the acylated product was treated with a Lewis acid. The expected expansion gave the corresponding enantiomerically pure oxazoline [55].

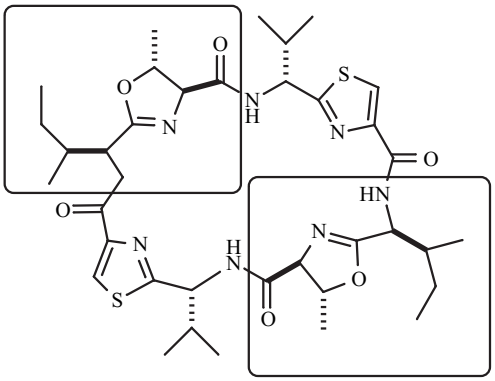
In recent years a wide variety of cyclopeptide alkaloids have been isolated from marine source. Among them, the Lissoclinum class [56], which includes lissoclinamide, westiellamide [57] and ascidiacyclamide [58], is characterized by an alternating sequence of oxazole, thiazole, oxazoline and thiazoline moieties. These compounds are secondary metabolites of algae, fungi and primitive marine organisms and possess unique pharmacologically attractive properties, including cytotoxic, immunoregolatory, antineoplastic and antibiotic activities. The particular structure, the size and the conformation of these macrocycles suggested that their activity *in vivo* could be due to ion chelation and transport [59]. Their isolation in significant quantities from marine natural sources is quite difficult and several research groups focused their attention on the synthesis and structural assignment of these metabolites and related analogues.

The activation of (2*S*,3*R*)-3-methyl-aziridine-2-carboxylate with isoleucine followed by ring expansion in the presence of boron trifluoride, furnished in excellent yield the corresponding *trans* oxazoline [54]. The non-destructive removal of the chiral auxiliary allowed a fragment of the ascidian compound Ascidiacyclamide to be obtained. On the other hand, after hydrolysis under acidic conditions and removal of the chiral auxiliary, the dipeptide isoleucine-threonine was isolated in enantiomerically pure form. Following the same reaction pathway, the *N*-leucyl-aziridine, submitted to rearrangement, gave the corresponding *trans* oxazoline, which represent the monomer of the cyclic trimer Westiellamide (Scheme **11**).

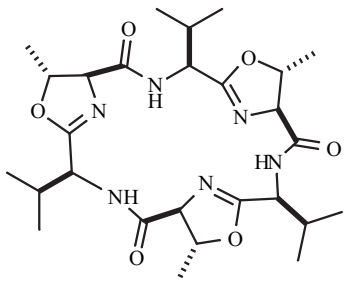
This useful method also allowed the synthesis of different small fragments of the depsipeptides Lysobactin and Katanosin [60].

These macrocyclic antibiotics were isolated in 1988 respectively from a species of Lysobacter (ATCC 53042) [61] and from the culture broth of a strain related to the genus Cytophaga [62]. They display high biological activity against methicillin and vancomycin-resistant bacteria, but their toxicity is slightly stronger [63]. They play an important role in the inhibition of the cell wall peptidoglycan formation but their mechanism of action seems to be different from that of Vancomycin. *In vitro* studies with a wall membrane of *Staphylococcus aureus*, showed that these macrocyclic lactones block transglycosilation by inhibiting the formation of lipid intermediates and the formation of nascent peptidoglycan [64]. The backbones of Lysobactin and Katanosin A and B, contain eleven amino acids, five of which are syn or anti β-hydroxy-α-amino acids. The difference between these molecules lies in the stereochemistry of the allo-threonine residue and in the side chain of the residue directly linked to this unusual amino acid (Fig. **4**).





Ascidiacyclamide



Westiellamide

Scheme 11.

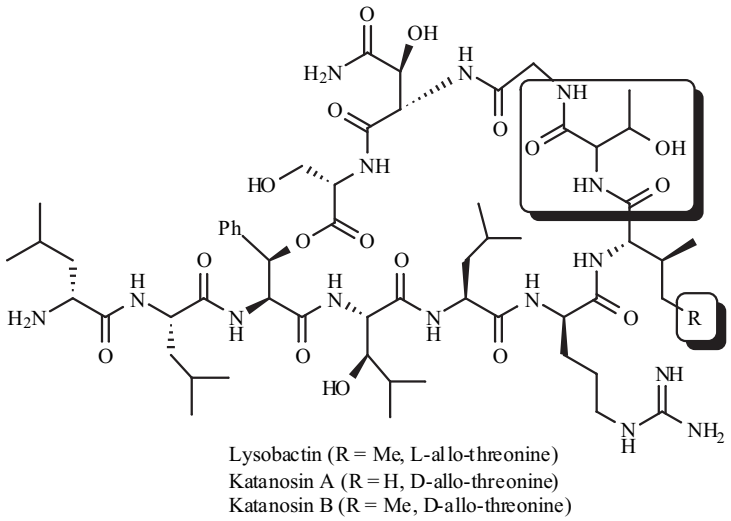


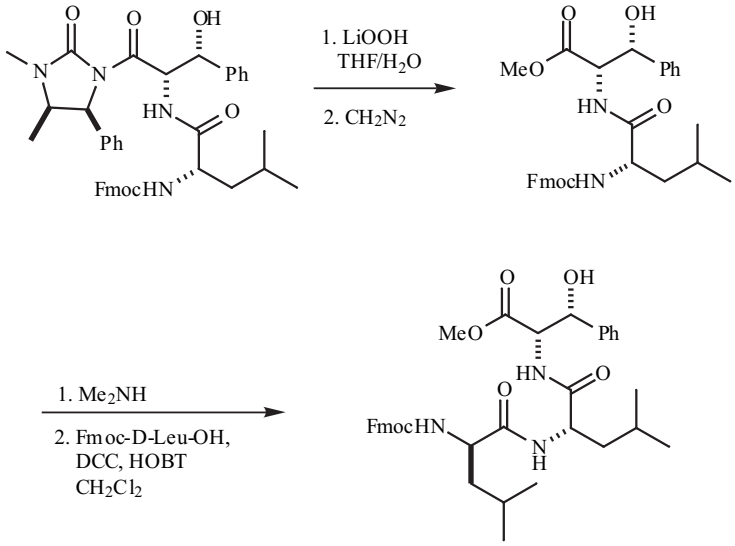
Fig. (4).

The method reported above, that allows the direct introduction of β-hydroxy-α-amino acids into dipeptide fragments, has been successfully applied in our laboratory for the synthesis of leucine-phenylserine dipeptide (Scheme **12**) and isoleucine-threonine dipeptide. By coupling other amino acids, under the standard conditions for the synthesis of peptides in solution, we could obtain significant portions of the antibiotic backbone [59].

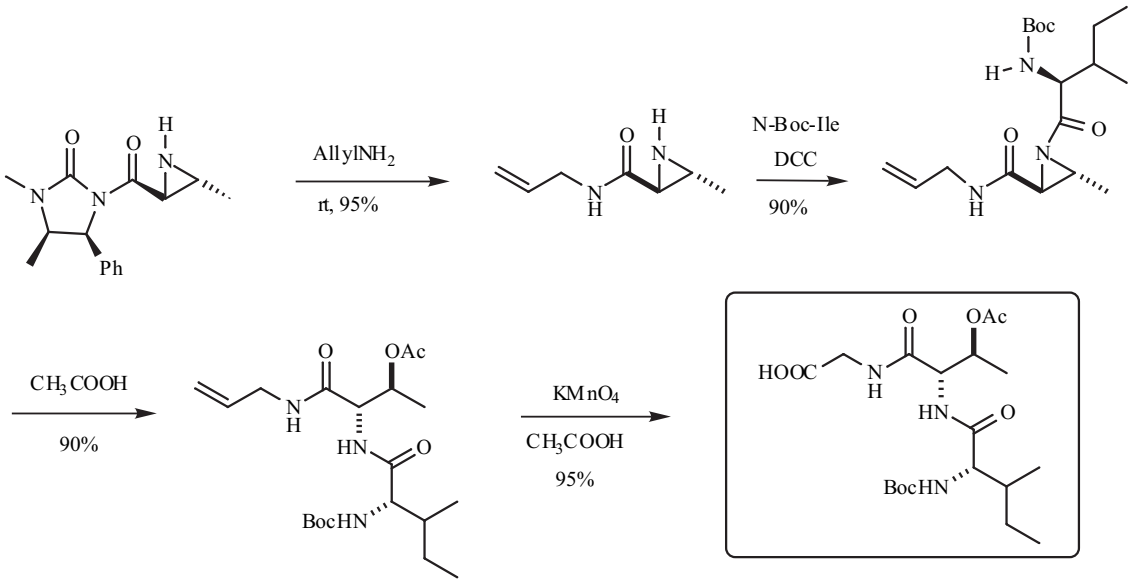
Anyway the ring expansion reaction of *trans* aziridines to the corresponding *trans* oxazolines allowed us to obtain, under complete regio and stereocontrol, exclusively *syn* β-hydroxy-α-amino acids containing peptides. Since the ring opening of activated aziridines occurs with inversion of configuration, we explored this last approach for the preparation of *allo*-threonine containing sequences (Scheme **13**) [65]. The removal of the chiral imidazolidinone auxiliary with neat allylamine, inhibited the ring expansion reaction and allowed the *N*-isoleucyl-heterocycle to be opened with acetic acid. Treatment of the allylamide moiety, a masked glycine residue, with $KMnO_4$ gave Ile-*allo*-Thr-Gly tripeptide fragment. By changing the stereochemistry of the starting aziridine, it is possible to synthesize with this procedure important fragments of both Lysobactin or Katanosin.

B) Synthesis of α-Hydroxy-β-Amino Acids

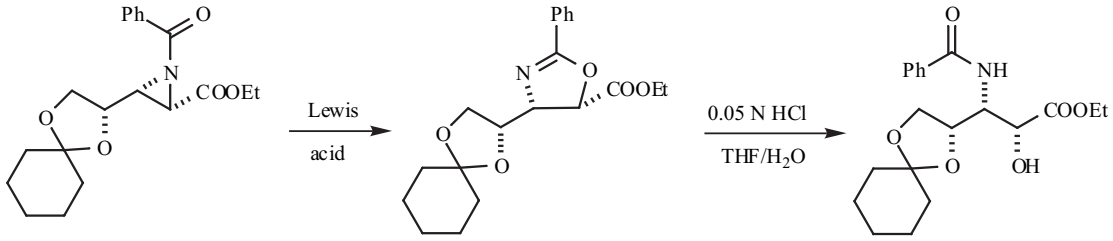
Polyhydroxylated amino acids are compounds of particular interest, for their utility as alkaloid and azasugar precursors [66]. Actually, polyhydroxylated β-amino acids are much less known, and in the five carbon series, only the 3-amino-3-deoxy D-arabinonic acid has been synthesized [67]. For these reasons, we envisaged the utility to define new ways to the synthesis of polyhydroxylated amino acids using *N*-acyl-aziridine-2-esters derived from D-glyceraldehyde as polyhydroxylated amino pentanoic acid precursors [68]. Following the synthetic protocol reported above, we submitted trans-aziridines to rearrangement in the presence of Lewis acids and we observed the fast and regioselective formation of trans-oxazoline-5-ester with retention of the configuration (Scheme **14**). It is important to



Scheme 12.



Scheme 13.



Scheme 14.

underline that the regiochemistry of the product in this case is opposite to that of the examples reported before, this different behaviour being to ascribe to the presence of the ester moiety in place of the chiral imidazolidinone and to the oxygenated group linked to the C3. The mild hydrolysis of this heterocycle gave the α-hydroxy β-amino acid, which is a protected form of the unnatural 3-amino 3-deoxy D-xylonic acid in optically pure form.

CONCLUSION

The synthesis and the study on the structure/activity relationship of receptor selective peptides and peptidomimetics, have recently shown that changing few bonds and angles in a ligand can have a major effect on the binding properties and on the resistance to enzymatic degradation. The synthesis of unusual amino acids and heterocyclic scaffolds as building blocks of conformationally constrained peptides and peptidomimetics, has become one of the main challenges in synthetic chemistry, since it can give access to new classes of ligands and provide critical insight into biologically active conformations. Moreover, the presence of unusual amino acids in the backbone of macrocyclic peptides with interesting pharmacological properties, isolated from natural sources, prompted us to focus our attention on the development of original and straightforward methods for the preparation of these building blocks.

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