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# Synthesis and biological evaluation of bicyclic iminosugar derivatives as inhibitors of glycosidases

Tesi di laurea sperimentale

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# Abbreviations and symbols

| | |
|---|---|
| δ | Chemical shift |
| Boc | Butoxycarbonyl |
| CuAAC | Copper(I)-catalyzed Azide-Alkyne Cycloaddition |
| Cyhex | Cyclohexane |
| DCM | Dichloromethane |
| DIPEA | *N*,*N*-Diisopropylethylamine |
| DMF | Dimethylformamide |
| DMP | Dimethoxypropane |
| DMSO | Dimethyl sulfoxide |
| EDCI | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide |
| ESI-HRMS | High resolution electrospray ionization mass spectrometry |
| $Et_2O$ | Diethyl ether |
| EtOAc | Ethyl Acetate |
| EtOH | Ethanol |
| HOBt | Hydroxybenzotriazole |
| HSQC | Heteronuclear Single-Quantum Correlation |
| *J* | Coupling constant |
| MeOH | Methanol |
| NAD | Nicotinamide adenine dinucleotide |
| NMR | Nuclear Magnetic Resonance |
| PTSA | *p*-Toluenesulfonic acid |
| r.t. | Room temperature |
| TBAF | Tetra-*n*-butylammonium fluoride |
| TBDPS | *tert*-Butyldiphenylsilyl |
| TFA | Trifluoroacetic acid |
| THF | Tetrahydrofuran |
| TLC | Thin Layer Chromatography |

*This project was realized at prof. Inmaculada Robina's laboratory, Department of Organic Chemistry, Faculty of Chemistry, University of Seville.*

## Abstract

During this work, five pyrrolizidine derivatives and one isoxazolidine derivative have been synthetized in order to evaluate their biological activities towards glycosidases, related to their configurations and type of bridge functionalities between the bicyclic iminosugar moiety and the aromatic part of the molecules. The final pyrrolizidine derivatives have been synthetized through click reactions (urea forming reaction, thiourea forming reaction and CuAAC reaction) performed on a common amino-pyrrolizidine precursor. The final isoxazolidine derivative has been synthetized through a CuAAC reaction. In addition, an indolizidine scaffold was obtained through a ring-closing metathesis on a dialkenyl pyrrolidine. This bicyclic compound could be of interest as intermediate for the synthesis of indolizidine derivatives with potential as glycosidase inhibitors. Biological evaluation towards glycosidases of the final six compounds synthetized in this work revealed that all of these compounds show inhibition towards almonds' β-glucosidase and/or jack beans' α-mannosidase.

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# 1. Introduction

## 1.1 Enzymatic inhibition: generalities[1]

An enzyme inhibitor is a molecule that can bind to the enzyme and that decreases its activity. Since controlling enzymes' activities can regulate a metabolic imbalance or kill pathogens, many drugs and pesticides are enzyme inhibitors.
The activity of an enzyme can be expressed by the velocity of the reaction that it catalyses, and this velocity can be affected by many factors such as temperature, pH, concentration of the substrate and presence of enzyme inhibitor.

In a simple enzymatic reaction, e.g. the formation of product P from substrate S, the initial velocity is proportional to the concentration of the free enzyme. In most cases this velocity reaches a maximum value, $V_{max}$, when all enzyme's molecules present in the medium are considered saturated from molecules of substrate. This kinetic model is known as classical Michaelis-Menten kinetics, whose equation for the initial velocity *v*, usually expressed in μM/min, is expressed as follows:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

The value of $K_m$, the Michaelis-Menten constant, corresponds to the concentration of substrate when the velocity is half the value of $V_{max}$. The Michaelis-Menten constant $K_m$ can be used to express the affinity of the enzyme for the substrate so it's an important parameter used to characterize enzymes. The higher the value of $K_m$ the lower the affinity of an enzyme towards the substrate, because a high concentration of the latter is needed to bind half of the molecules of enzyme in the medium.

Enzymatic inhibition can be classified as reversible or irreversible. Irreversible inhibitors bind, for example through covalent bonds, the enzyme in its active site and change the enzyme's conformation. Reversible inhibitors, on the contrary, bind the enzyme in a non-covalent way and, under certain conditions, the enzyme can recover its biological function.

Reversible inhibitors can be classified into three further classes, depending on whether they bind to the active site of the enzyme (competitives), to the enzyme-substrate complex (uncompetitives) or both (non-competitives).

In the competitive inhibition the inhibitors bind directly to the enzyme in the active site, preventing the substrate itself from binding and from reacting to form the products. The following equilibriums are formed (Fig. 1):

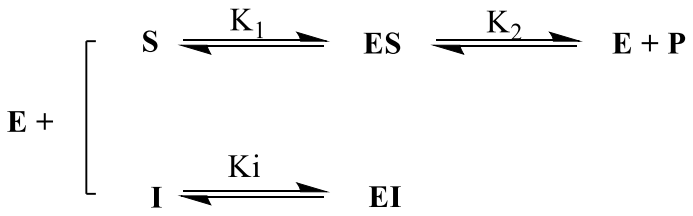



**Fig. 1** Equilibriums in competitive inhibition. E = free enzyme, S = substrate, I = inhibitor, ES = enzyme-substrate complex, EI = enzyme-inhibitor complex, P = product.

In this type of inhibition the value of $V_{max}$ remains constant while an apparent increase of the value of $K_m$ is observed. (Fig. 2)

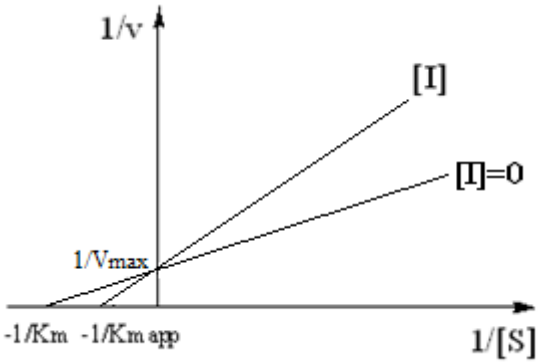



**Fig. 2** Lineweaver-Burk representation for competitive inhibition

In uncompetitive inhibition the molecule of inhibitor binds to the enzyme-substrate complex, avoiding the formation of the final products. This type of inhibition is regulated by the following equilibriums (Fig. 3):

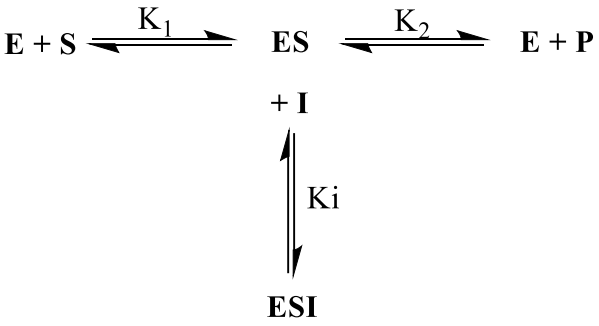



**Fig. 3** Equilibriums in uncompetitive inhibition. E = free enzyme, S = substrate, I = inhibitor, ES = enzyme-substrate complex, ESI = enzyme-substrate-inhibitor complex, P = product.

An uncompetitive inhibitor modifies both $K_m$ and $V_{max}$ values, both resulting lower than in the enzyme without inhibition. (Fig. 4)

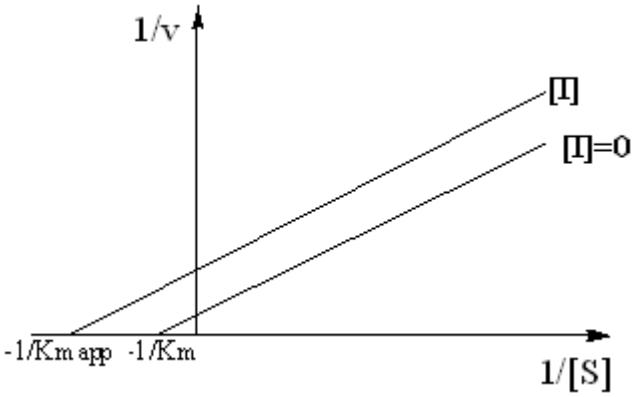



**Fig. 4** Lineweaver-Burk representation for uncompetitive inhibition

Non-competitive inhibitors bind reversely to the enzyme, usually not in its active site but in other regions, so that this binding can occur both with the free enzyme and with the enzyme-substrate complex (Fig. 5).

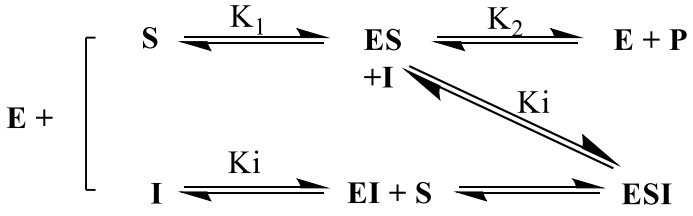



**Fig. 5** Equilibriums in non-competitive inhibition. E = free enzyme, S = substrate, I = inhibitor, ES = enzyme-substrate complex, EI = enzyme-inhibitor complex, ESI = enzyme-substrate-inhibitor complex, P = product

The non-competitive inhibition does not change the value of $K_m$, but a decreasing of the value of $V_{max}$ is observed. (Fig. 6)

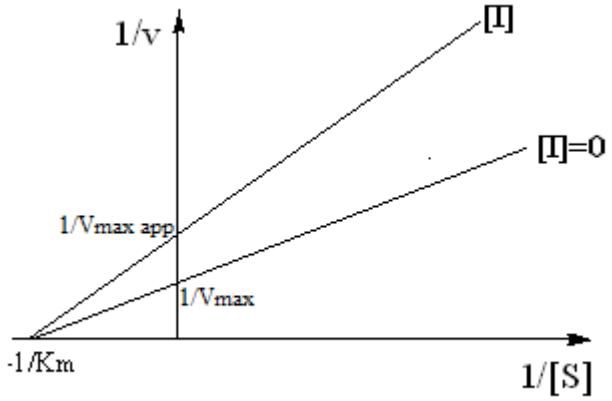



**Fig. 6** Lineweaver-Burk representation for noncompetitive inhibition

## 1.2 Glycosidases

Glycosidases, or glycoside hydrolases, are enzymes that catalyse the hydrolysis and the formation of glycosidic bonds. Glycosidases can catalyse the hydrolysis of O-, N- and S- glycosides, leading to the formation of a sugar hemiacetal or hemiketal and their corresponding free aglycone. The hydrolysis of carbohydrates is a very common and widespread biological phenomenon in living systems[2], and it can be found essentially in every domain of life, thus the control of this activity can play a key role in treating many diseases.

For example, some glycoside hydrolases are involved in the processing and biosynthesis of N-link glycoproteins in the Golgi apparatus and in the endoplasmic reticulum.[3] Cancerous tumours can express unusual carbohydrate structures resulting from an abnormal function of this biosynthesis, and the inhibition of some of these glycosidases, such as Golgi α-mannosidase II, has shown a certain potential in cancer treatment.[4]

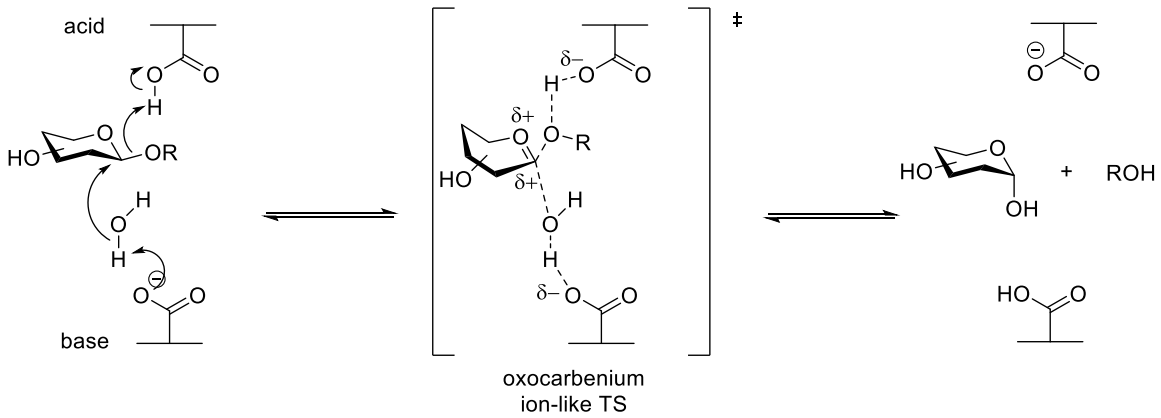
It is also known that α-L-fucosidase activity and fucose-residue levels are related to many disorders, such as fucosidosis, inflammation, cancer and cystic fibrosis.[5]

Type II diabetes can also be controlled using glycosidase inhibitors, which can prevent the glycolysis of polysaccharides and thus can regulate blood sugar levels.[6]

### 1.2.1 Mechanisms of glycosidase catalysed hydrolysis

The majority of the glycosidase enzymes acts through an acid/base catalysed mechanism via an oxocarbenium ion-like transition state. In particular, these mechanisms can be divided in two main categories: hydrolysis of the glycosidic bond with inversion of configuration, and hydrolysis of the glycosidic bond with retention of configuration; so that mechanistically speaking glycosidases can be divided into inverting enzymes and retaining enzymes. Two likely mechanisms for this type of enzymes were first proposed by Koshland[7] and, despite some refinements, they are still consider valid nowadays.

It is known that inverting glycosidases act through a single-step mechanism in which a water molecule is involved in a direct displacement at the anomeric centre (Scheme 1).

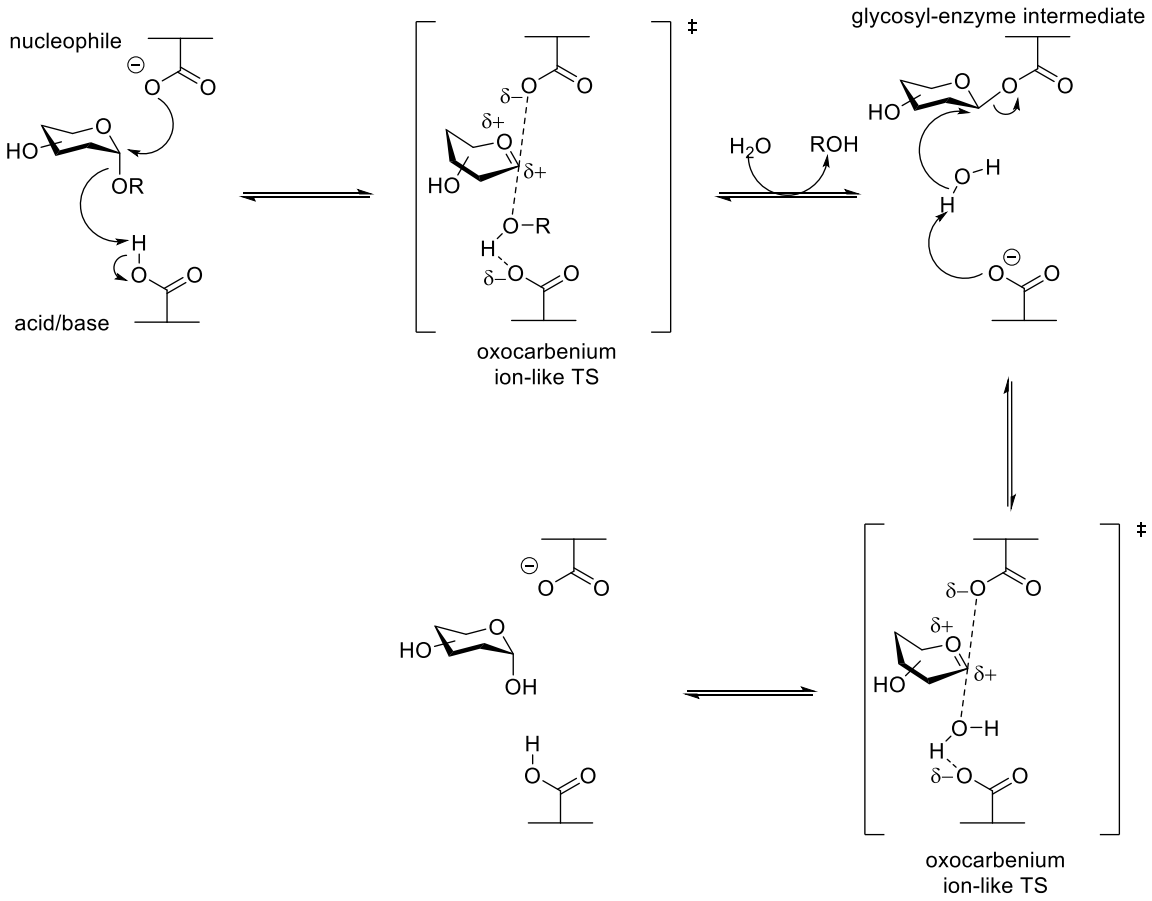



**Scheme 1.** Inverting mechanism for a generic β-glycosidase

An active site's aminoacid acts as a general base helping to deprotonate a water molecule while another amino acid, that behaves as a general acid, protonate the oxygen activating the leaving OR group of the substrate; the process then pass through an oxocarbenium-like transition state and finally the glycolysis products are released.[8] In most of the known cases, these acid and base groups are the side acid chains of glutamic or aspartic acid residues in the active site. The

oxocarbenium-ion character of the transition state is supported by several kinetics studies.

On the other hand, the mechanism with retention of configuration it is hypothesized to be a double displacement mechanism, via a covalent glycosyl-enzyme intermediate (scheme 2).




**Scheme 2.** Retaining mechanism for a generic α-glycosidase

Each of the two steps of this process passes through an oxocarbenium ion-like transition state, as in the inverting mechanism. This process is catalysed mainly by two amino acids, one acting as a nucleophile and the other acting as acid/base, depending on the step. In this mechanism, as in the previous one, the two catalytic groups are commonly the side acid chains of aspartic and glutamic residues in the active site.

In the first step, the amino acid that act like a nucleophile attacks the anomeric centre of the substrate while the other residue acts as an acid catalyst protonating the glycosidic oxygen, so that the glycosyl-ester intermediate is formed as the

glycosidic bond cleaves. At this point, the ROH molecule, that formed the aglycon part, leaves the active site and it is replaced by a water molecule. In the second step, the conjugated base of the residue, which previously acted as an acid, deprotonates the water molecule that attacks the anomeric centre and cleaves the glycosyl ester bond, giving the hydrolysis product and re-establishing the previous active site's condition.[9]

Even though the classical inverting and retaining mechanisms are the most common in this class of enzymes, several different variation of these mechanisms and a substantially different one, that catalyses the reaction with a NAD cofactor, are also known.

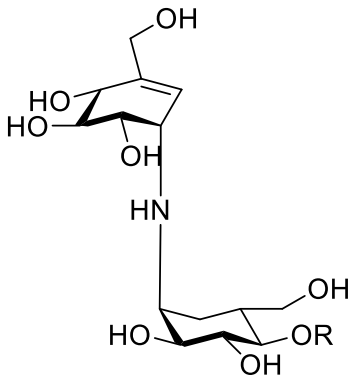
For instance, some glycosidase substrates that contain an *N*-glycolyl or *N*-acetyl group at position 2 act through a variation of the retaining mechanism, with neighbouring group participation. These enzymes do not have a catalytic nucleophile in the active site, rather the 2-acetamido group acts as an intramolecular nucleophile in the process, leading to the formation of an oxazolinium ion intermediate.[10]

In some other retaining glycosidases, it was found that the hydrolysis of the glycosidic bond requires a NAD cofactor that remains bonded to the enzyme throughout the catalysis. The mechanism starts with an initial oxidation of the hydroxyl at position 3 of the substrate by the NAD-enzyme complex, then an elimination reaction and a water addition at the anomeric centre, followed by a final reduction of the ketone previously formed, generate the final products.[11] Even though this mechanism acts through an elimination and redox reactions the overall reaction is a hydrolysis.

## 1.3 Glycosidase inhibitors

Due to the widespread biological roles that glycosidases have in living organisms, glycosidase inhibitors can have many applications in the agrochemical and pharmaceutical fields.

For example, Validoxylamine A (Fig.7) is a powerful inhibitor of trehalases in *R. solani* and other organisms, and is a product of the metabolism of Validamycin A (Fig. 7), a substance commercialized from Takeda Chemical Industries for the control of pathogens in rice and other plants.[4]




Validoxylamine A (**1**) R = H
Validamycin A (**2**) R = β-D-glucopyranosyl

**Fig.7** Chemical structures of Validoxylamine A (1) and Validamycin A (2)

In the therapeutical field glycosidase inhibitors have been found to have many applications as drugs in a broad spectrum of diseases.

For instance, in the ‘70s it was realized that inhibition of pancreatic α-amylase and other intestinal disaccharidases could regulate the absorption of carbohydrates, and thus began the search of inhibitors that could be used as a treatment in non-insulin-dependent diabetes mellitus (Type II).[4] This is the case, for instance, of Acarbose (**3**), Salacinol (**4**) and Kotalanol (**5**) (Fig. 8).

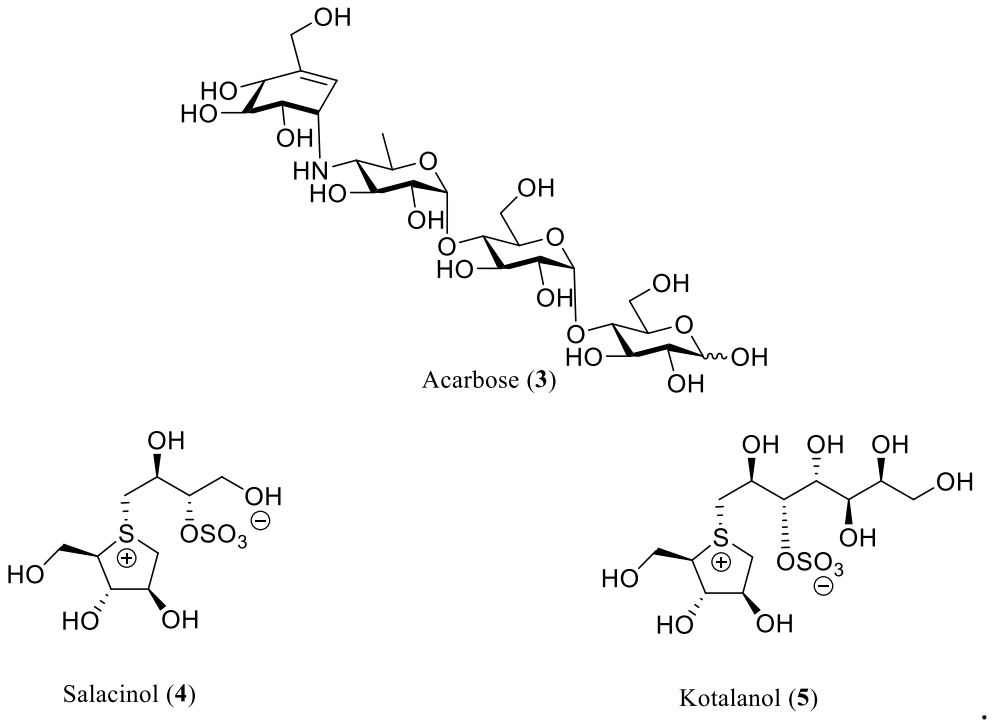



**Fig. 8** Chemical structure of Acarbose (**3**), Salacinol (**4**) and Kotalanol (**5**), inhibitors of α-glucosidases.

Acarbose is a potent sucrose inhibitor that was found to be effective in rats and healthy human volonteers.[12] In 1990, after clinical development, Arcarbose (Glucobay™) was introduced onto the market in Germany for the treatment of diabetes and was successively commercialized in Europe, Latin America and USA.[4]

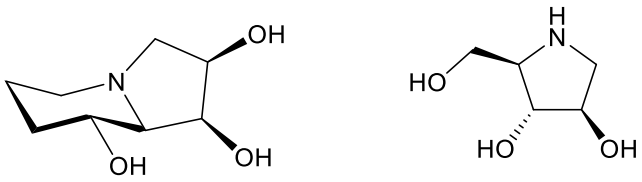
On the other hand, salacinol and kotalanol were found to be the α-glucosidase-inhibiting components of the ayurverdic traditional medicine used to treat diabetes.[13] The inhibitory activity of salacinol is almost equal to that of acarbose toward maltase and sucrase but is higher toward isomaltase.[14] Kotalanol shows a more potent inhibitory activity than acarbose and salacinol toward sucrose.[13] The efficacy of these compounds as inhibitors of glycosidases is probably due to the permanent positive charge of the sulfonium, that can mimic the positive charge of the transition state in the glycosidase-mediated hydrolysis reaction.[15]

Chemically speaking, one of the most important family of glycosidase inhibitors are without doubt the iminosugars.

### 1.3.1 Iminosugars as glycosidase inhibitors

Iminosugars, or azasugars, are structural analogues of carbohydrates where the oxygen in the ring is replaced with a nitrogen atom and many of them inhibit glycosidases in a reversible and competitive manner.[4]

Many examples of naturally occurring mono- and bicyclic iminosugars are known to be glycosidase inhibitors, such as swainsonine (**6**) and 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) (**7**) (Fig. 9)



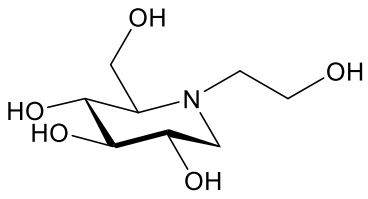

swainsonine (**6**) D-AB1 (**7**)

**Fig. 9** Chemical structure of the naturally occurring glycosidase inhibitors swainsonine (**6**) and D-AB1 (**7**).

Swainsonine (**6**) is an indolizidine alkaloid that is also a Golgi α-mannosidase II inhibitor; it has shown potential in being a drug for patients with tumours, since it leads to significant reduction in tumour masses in human patients.[16]

D-AB1 (**7**), originally isolated from a fruit, is a potent inhibitor of glycogen phosphorylase and is a possible drug candidate for the treatment of type II diabetes.[17]

Due to their biological activity towards glycosidases, iminosugars were broadly investigated in order to discover pharmacological applications. For example, miglitol (Glyset™) (Fig. 10) is an oral anti-diabetic drug, used in treatment of type II diabetes, that works inhibiting α-glucosidases.[4]




miglitol (**8**)

**Fig.10** Chemical structure of miglitol (**8**)

Iminosugars are believed to act as inhibitors towards glycosidases mimicking the oxocarbenium ion-like transition state of the enzyme-catalyzed *O*-glycoside hydrolysis; the electrostatic interaction between the protonated N at physiological pH and the active site of the enzyme is a possible mechanism to explain these inhibitions.[18] For example, nojirimycin (**9**) (Fig. 11) is very similar to glucose. The protonated inhibitor (**10**) mimics the charge development that occurs in the transition state of the cleavage mechanism of glycosidases, but has a chair conformation and not the half-chair conformation that is believed to be the conformation of the transition state (**11**).[18] (Fig. 11)

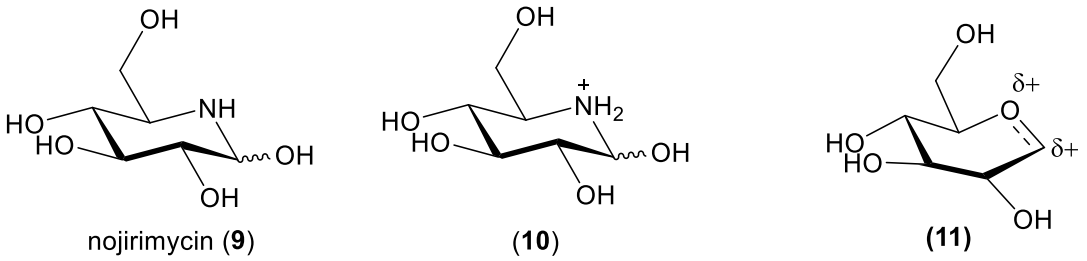



**Fig. 11** Structures of nojirimycin (**9**), protonated nojirimycin (**10**) and representative transition state of a glucosidase-catalyzed reaction (**11**)

However, many iminosugars lack selectivity toward a single glycosidase and normally have poor membrane permeability. Those are drawbacks that can limit their pharmacological application.

It is also known that while the glycone part of the inhibitor occupies the active site, the aglycone part makes additional interactions with amino acids of the allosteric site of the enzyme that can increase the affinity of the enzyme for the inhibitor or induce a fit of the glycone in the active site.[19]

For these reasons many studies focus on structural modification of known glycosidase inhibitors in order to discover more potent and selective alternatives.

For example, many natural occurring polyhydroxylated pyrrolizidine are known to be glycosidase inhibitors, such as casuarine (**12**) and australine (**13**) (Fig. 12).


**Fig.12** Chemical structures of casuarine (**12**) and australine (**13**)

Casuarine (**12**) is a potent inhibitor of amiloglucosidase of *Aspergillus niger* ($IC_{50}$=0.7 μM) but is not specific because it shows also inhibition activity towards other α- and β-glucosidases.[20] Australine (**13**) is also an inhibitor of amiloglucosidase but also shows potent inhibition towards α-glucosidase of *Aspergillus niger* ($IC_{50}$=6 μM).[21]

Many research groups[22] have developed synthetic strategies to obtain polyhydroxylated pyrrolizidines, modifying number and configuration of stereogenic centres in the pyrrolizidine moiety. However, fewer studies focus on the incorporation of substituents of different nature in the pyrrolizidine moiety.

Recently, Toyooka and cols. described the preparation of many pyrrolizidines derivatives with different aliphatic chains at position C-3[23], finding a selective inhibitor of α-fucosidase in compound **14** ($IC_{50}$= 34 μM) (Fig. 13).

At the same time, Fleet and cols.[24] described the synthesis of fluorinated pyrrolizidine, derived from australine. Compound **15** (Fig. 13), among others, showed more potent inhibition ($IC_{50}$=0.63μM) towards α-glucosidase of *A. niger* than australine and also moderate inhibition towards trehalase.

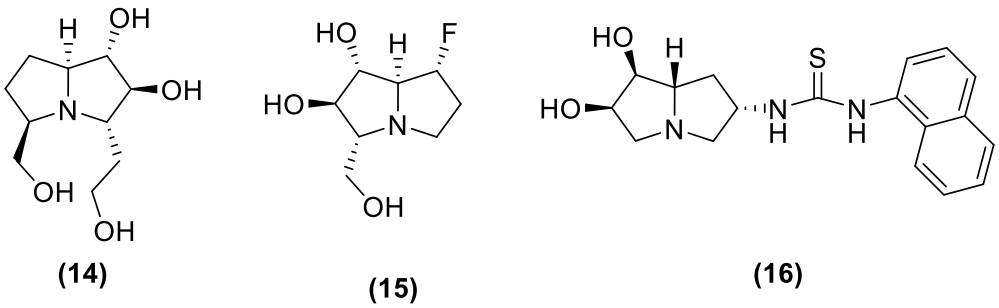
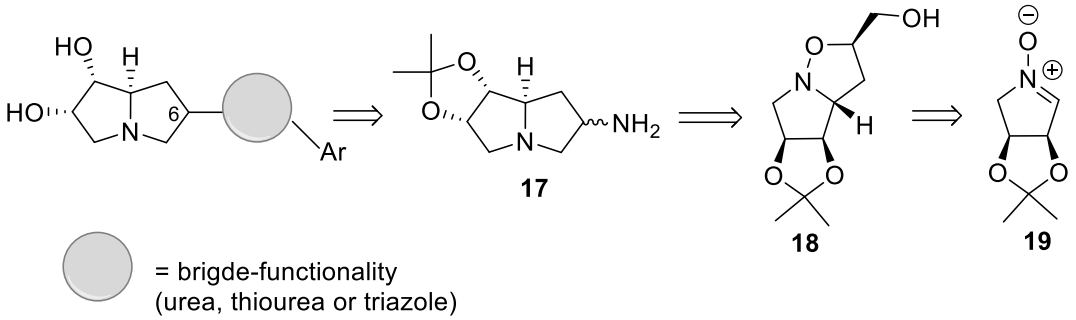



**Fig. 13** Chemical structures of compound **14**, **15** and **16**, pyrrolizidine-derivatives glycosidase inhibitors

The discovery of a potent coffee bean α-galactosidase inhibitor ($IC_{50}$= 0.37 μM), compound **16** (Fig.13), that has also shown inhibition for human lysosomal α-galactosidase ($IC_{50}$= 5.3 μM)[25], was also recently published in the research group.

## 2. Aim of the thesis

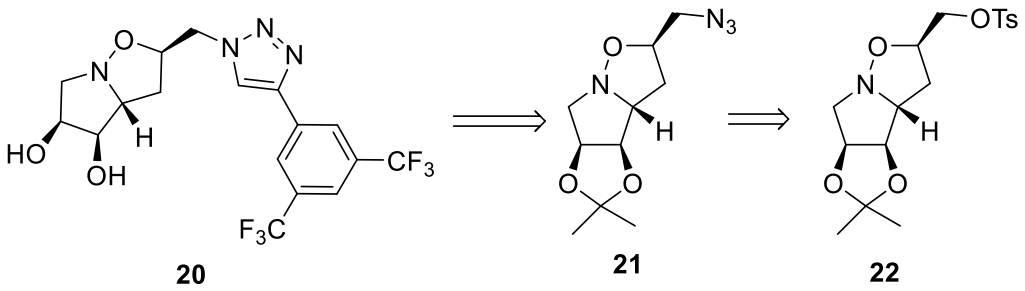
Given the well-known properties of several bicyclic iminosugar derivatives as glycosidase inhibitors, the aim of this thesis was to synthesize some molecules of this class in order to evaluate their biological activity relate to their structure.
The main part of this thesis consisted in the synthesis of different hydroxylated pyrrolizidine derivatives, starting from L-Arabinose, and in the evaluation of their biological properties as glycosidase inhibitors (Scheme 3). This project was realized at the Department of Organic Chemistry, Faculty of Chemistry, University of Seville under the supervision of prof. Inmaculada Robina.




**Scheme 3**

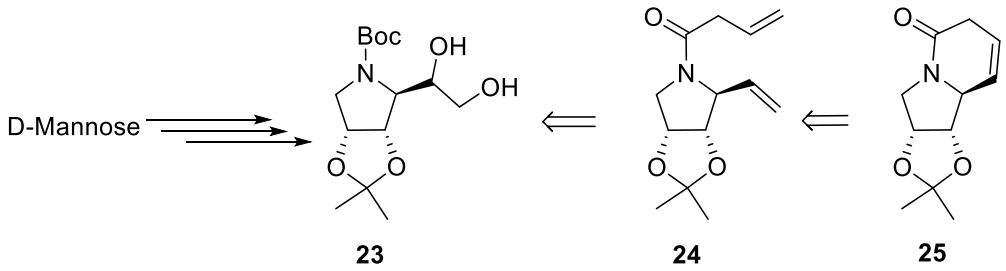
It has been observed how differences in molecular structures, such as C-6 configuration and the type of bridge-functionalities between the pyrrolizidine scaffold and the aromatic group, modify the values of enzymatic inhibition. This evaluation will be useful to choose the best leads to further carry out parallel synthesis of urea/thiourea or triazole derivatives trough reaction of **17** with a small library of isocyanates/isothiocyanates or alkynes. The *in situ* biological evaluation of this library[26] will allow the rapid identification of the best inhibitor on which conduct more accurate inhibition studies.

In addition, an isoaxozoline derivative, with a triazole bridge-functionality, was synthesized in order to evaluate its biological properties as glycosidase inhibitor and compare them with structurally similar molecules. (Scheme 4)




**Scheme 4**

Lastly, starting with an intermediate that had already been synthetized from D-mannose, the focus was to obtain the indolizidine scaffold through a ring-closing metathesis on a dialkene, in order to get to molecules further derivatized and finally tested as glycosidase inhibitors. (Scheme 5)




**Scheme 5**

The reaction of ring closing metathesis was performed using a Ruthenium Grubbs catalyst of second generation. This indolizidine-derivative compound could be used as a good intermediate in obtaining a bicyclic triazole-derivative to be biologically evaluated.

## 3. Results and discussion

### 3.1 Synthesis of the pyrrolizidine derivatives.

In the first part of this project different hydroxylated pyrrolizidine derivatives (**26a**,**b**, **27**, **28a**,**b**, Fig. 14) have been synthesized following a procedure optimized by the research group for the synthesis of the corresponding enantiomeric compounds.[25]

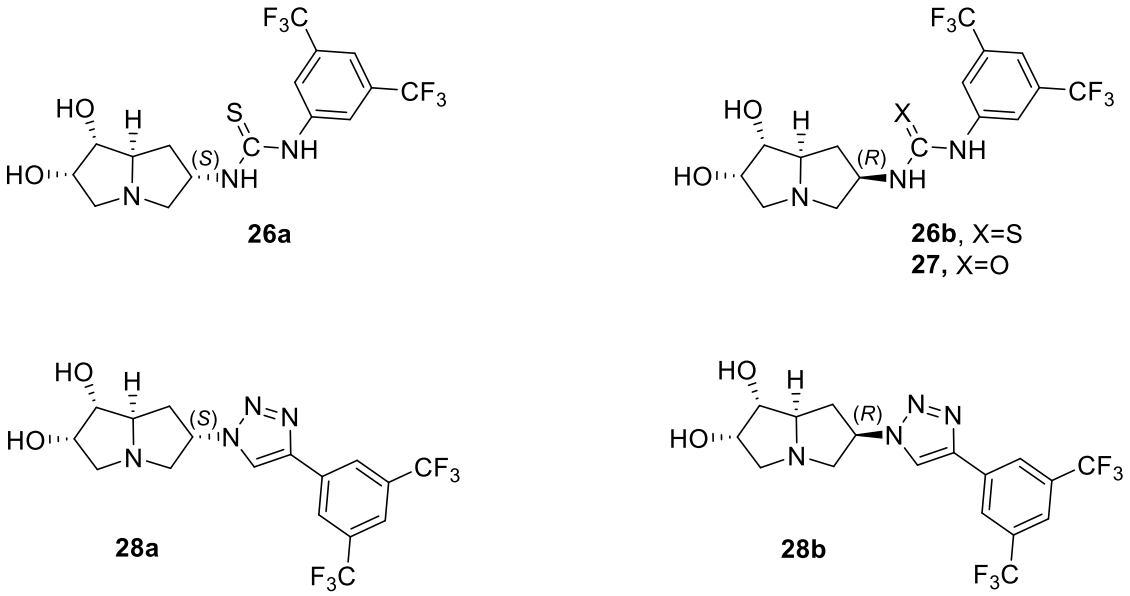
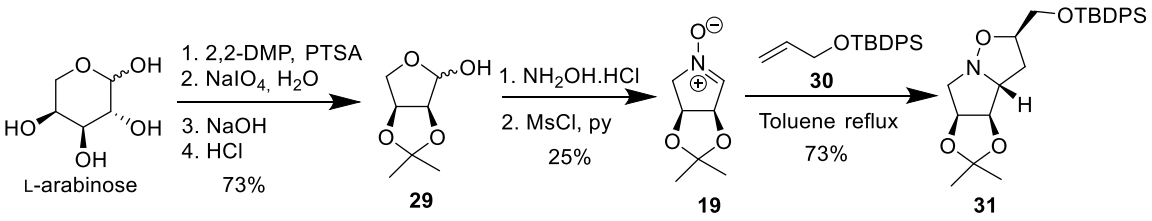



**Fig. 14** Structure of the final compounds **26a**,**b**, **27** and **28a**,**b**

These compounds have different bridge-functionalities (urea, thiourea and triazole) between the iminosugar and the aromatic group that are formed through "click reaction" on amino-pyrrolizidines **17**, prepared from L-arabinose as starting material.

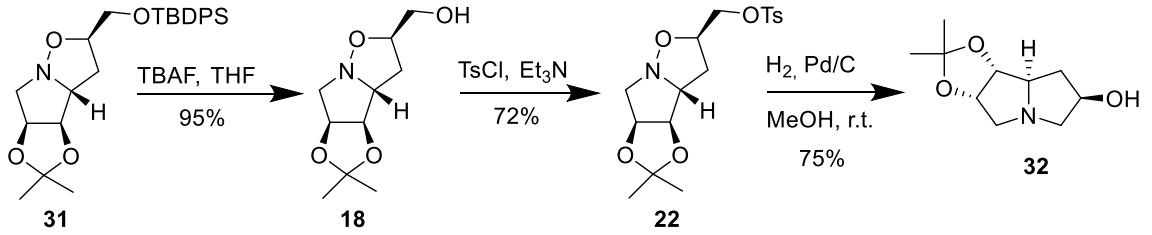
The synthetic pathway starts with the synthesis of protected eritrose **29** from L-arabinose.[27] First, L-arabinose was protected at position 3 and 4 using 2,2-DMP; then, compound **29** was obtained through periodate cleavage in 73% yield. Then, nitrone **19** was synthesized, following the procedure previously described by Goti,[28] passing through an oxime-intermediate and it was obtained in 25% yield.

The 1,3-dipolar cycloaddition between the nitrone **19** and the protected allylic alcohol **30** led to compound **31**, as a single diastereomer with a 73% yield[29] (Scheme 6).



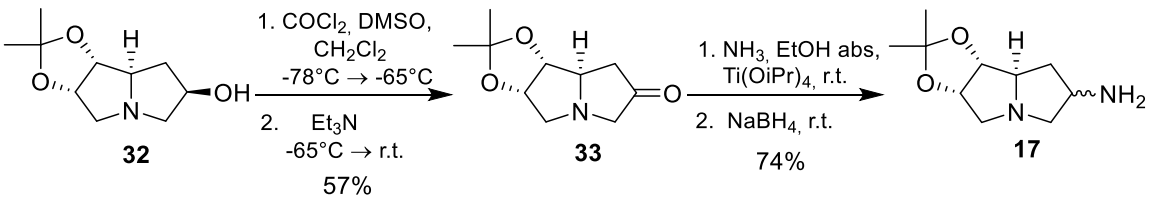

**Scheme 6**

Treatment of isoxazolidine **31** with TBAF in THF formed compound **18**, with its alcoholic function deprotected, with a yield of 95%, the latter was subsequently tosylated to achieve compound **22**. This compound was then submitted to a hydrogenation with Pd/C, obtaining pyrrolizidine **32** through the reductive cleavage of the N-O bond and the subsequent cyclization.[29] (Scheme 7)




**Scheme 7**

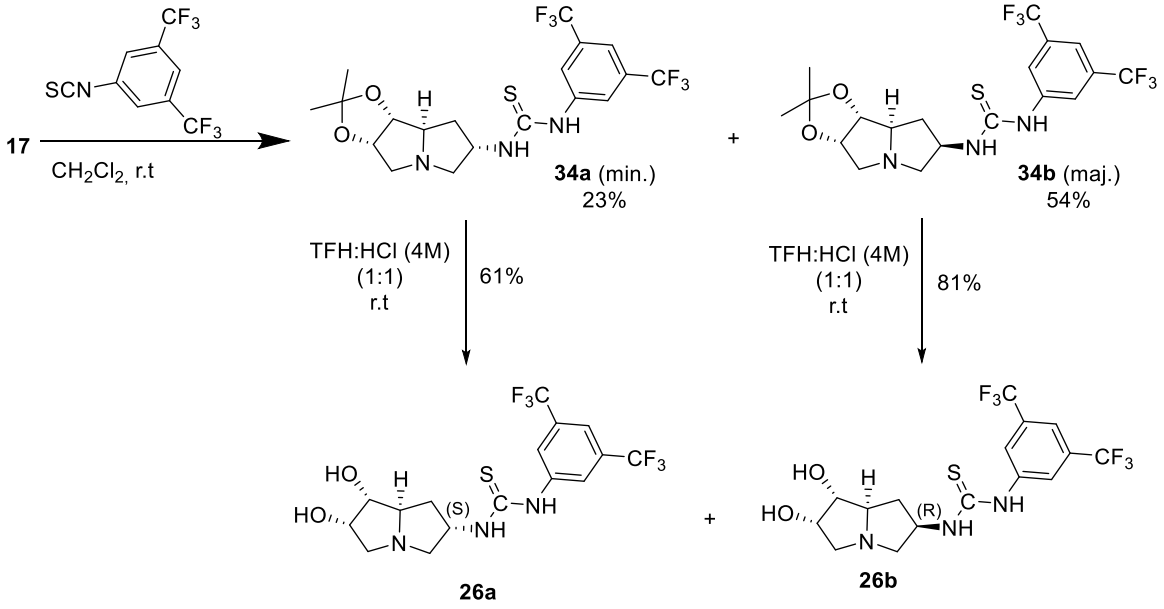
The formation of amines **17**, precursors of the final compounds, was performed in two steps: first, alcohol **32** was submitted to a Swern oxidation in order to obtain ketone **33**, that gave pyrrolizidines **17** via reductive amination, following a procedure similar to that described by Miriyala and cols.[30] (Scheme 8)




**Scheme 8**

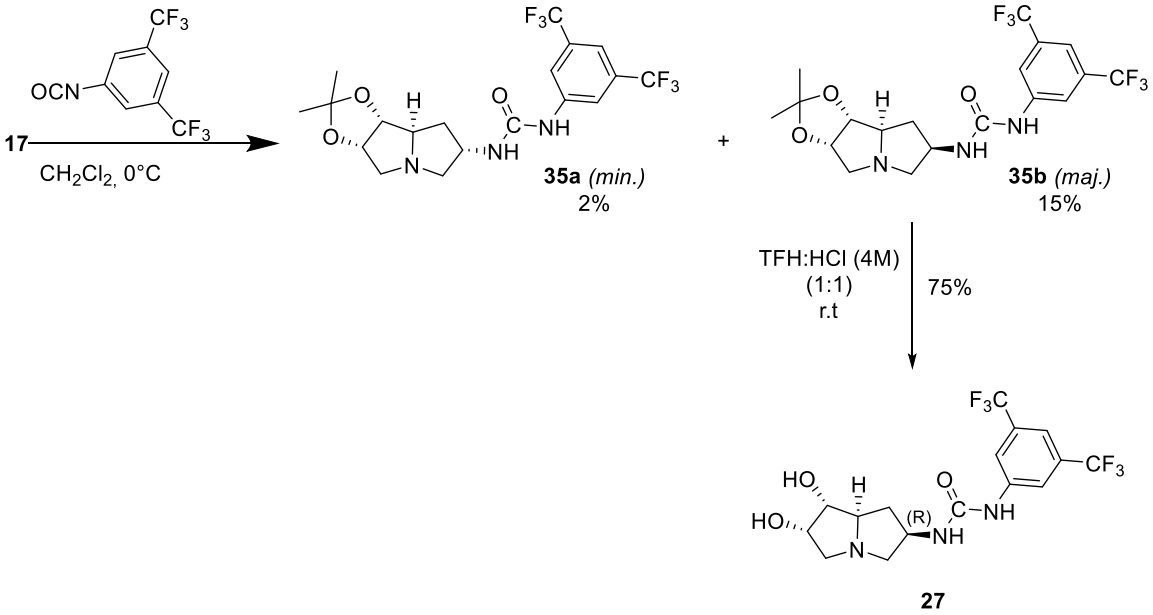
Pyrrolizidine **17** was obtained as a 2:1 mixture of diastereomers, by reductive amination. This reaction consisted in treating the ketone with $NH_3$ in the presence of $Ti(O^iPr)_4$, and then reducing the imine-intermediate *in situ* using $NaBH_4$.

Amino-thioureas **34a** and **34b** were formed treating pyrrolizidines **17** with 1-isothiocyanato-3,5-bis(trifluoromethyl)benzene and were separated using a chromatographic column, obtaining **34a** with a yield of 23% and **34b** with a yield of 54%. Finally, the deprotection of the acetal function of **34a** and **34b** carried out in acidic medium led to the final compounds **26a** and **26b** respectively with 61% and 81% yields. (Scheme 9).



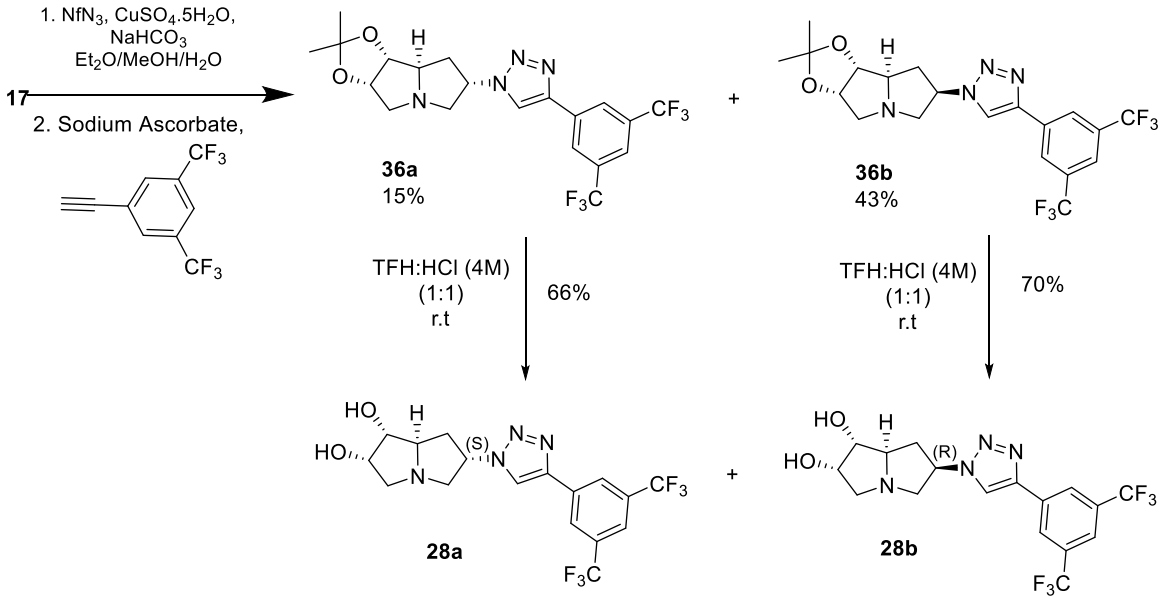

**Scheme 9**

Similarly, amino-ureas **35a** and **35b** were formed treating pyrrolizidines **17** with 1-isocyanato-3,5-bis(trifluoromethyl)benzene at 0°C, but the yield of this reaction was rather low. Several by-products were formed, probably due to the more reactive nature of isocyanates compared to isothiocyanates, even if the reaction was performed at 0°C and conducted for a shorter period of time. Compound **35a** and **35b** were formed in 2% and 15% yield respectively; finally, by the treatment of **35b** in acidic medium, the deprotected amino-urea **27** was obtained with a yield of 75%. (Scheme 10)




**Scheme 10**

On the other hand, the synthesis of compounds **28a** and **28b** was carried out following a one-pot procedure described by Chiara and cols.[31] Initially, pyrrolizidines **17** were converted into azides by reaction with $NfN_3$. Then a copper(I)-catalyzed azide alkyne cycloaddition reaction (CuAAC) with 1-ethynyl-3,5-bis(trifluoromethyl)benzene, where $Cu^{2+}$ is reduced to $Cu^{+}$ *in situ* using sodium ascorbate, followed by chromatographic purification, afforded triazoles derivatives **36a** and **36b** in 15% and 43% yields, respectively. Finally, deprotection in acidic medium of compounds **36a** and **36b** gave the dihydroxylated triazole derivatives **28a** and **28b** in 66% and 70% yields respectively. (Scheme 11)



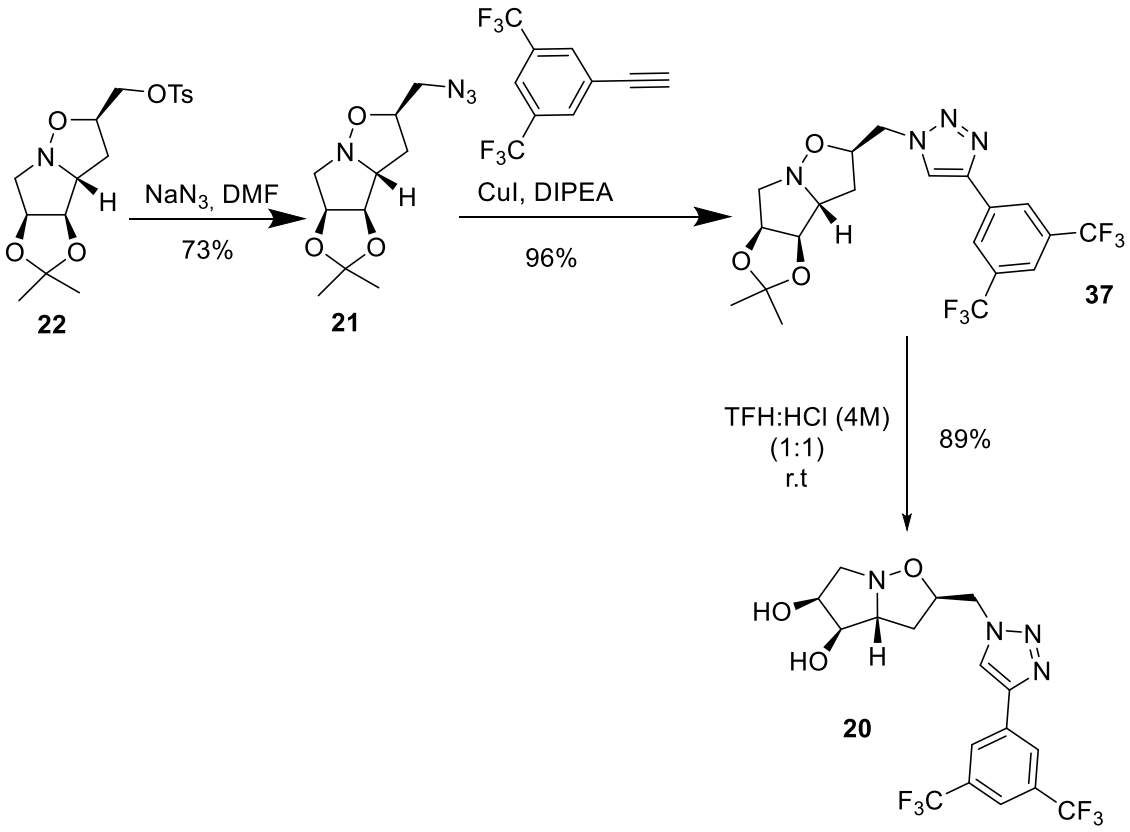

**Scheme 11**

Configurations of all the diastereomers were assigned and confirmed comparing experimental $^{1}$H-NMR and optical rotation data with those of each enantiomer.

### 3.2 Synthesis of the isoxazolidine derivative.

After obtaining pyrrolizidine derivatives **26a,b, 27**, and **28a,b**, the triazole-isoxazolidine derivative **20** was synthesized in order to evaluate its inhibition towards glycosidases.

This molecule has been synthesized through a CuAAC reaction on azide **21**, formed from intermediate **22** of the previous synthetic pathway (Scheme 12).



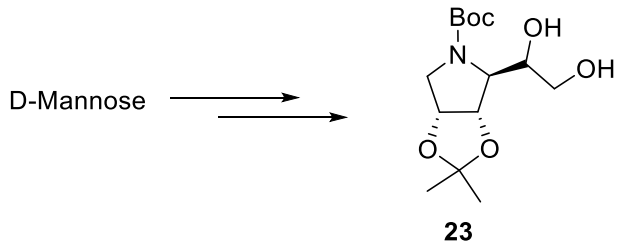

**Scheme 12**

First, tosylate derivative **22** was treated with $NaN_3$ in DMF obtaining compound **21** in 73% yield. Secondly, from this compound, through a CuAAC reaction with 1-ethynyl-3,5-bis(trifluoromethyl)benzene in presence of CuI and DIPEA, isoxazolidine derivative **37** was obtained with an excellent 96% yield.

Finally, acidic deprotection of compound **37** led to the formation of the dihydroxylated isoxazolidine derivative **20** in 89% yield.

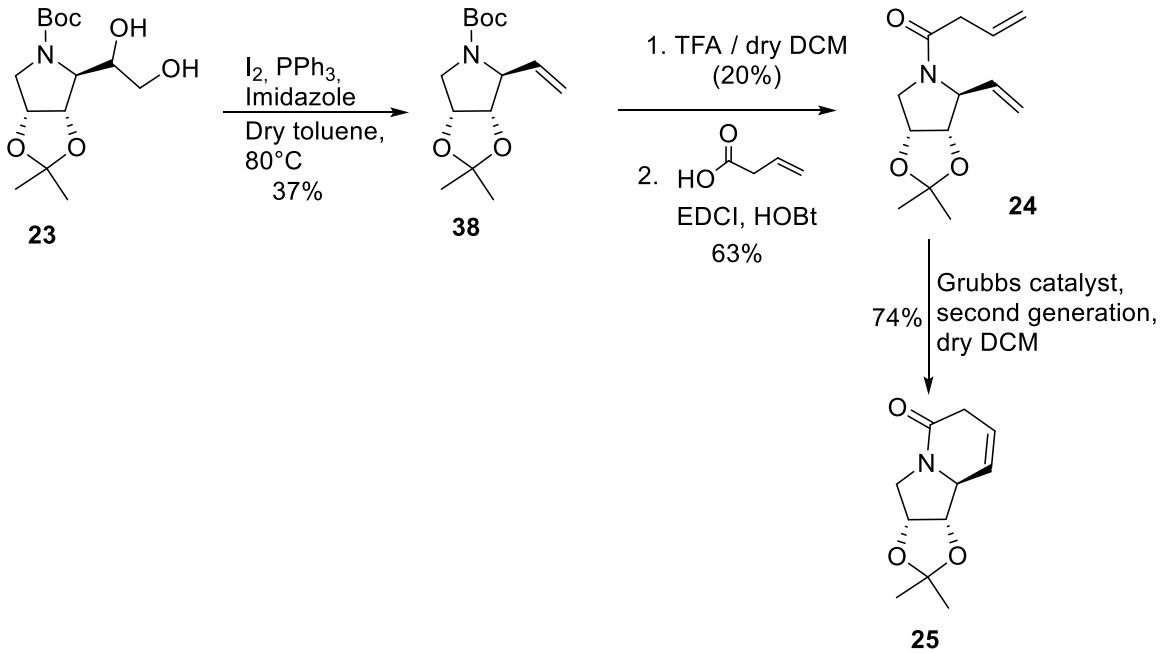
### 3.3 Ring-closing metathesis to indolizidine scaffold.

The aim of the last part of the project was to obtain an indolizidine scaffold through the reaction of ring closing metathesis of alkenes. This synthetic pathway starts from the diol **23** already synthesized from D-mannose.[32]




**Scheme 13**

The synthetic pathway to the bicyclic compound **25** is reported in scheme 14.



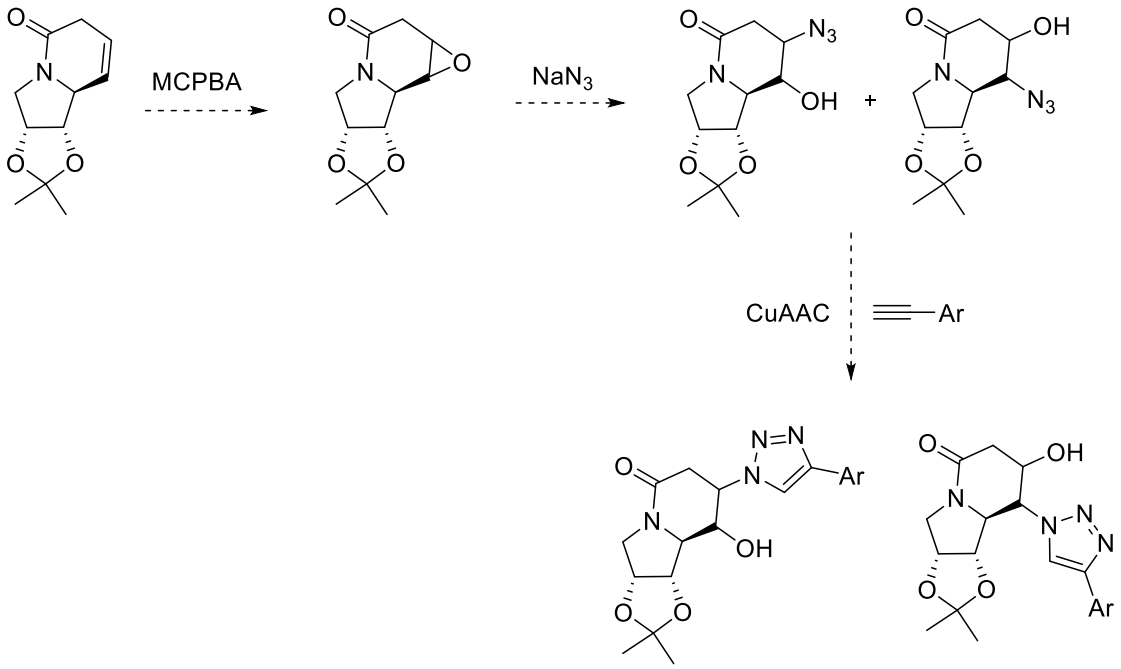

**Scheme 14**

In the first step, diol **23** was first treated with $I_2$, $PPh_3$ and imidazole in dry toluene at 80°C,[33] obtaining the alkene **38** with a yield of 37%. This compound was found to be quite volatile so the poor yield could be explained from its partial evaporation during the removal of toluene and the difficult separation of the remaining $PPh_3$ and its oxide

Secondly, compound **38** was treated with a solution of trifluoroacetic acid (TFA) in $CH_2Cl_2$ at 20% concentration, removing selectively the Boc protecting group of the amine. After the deprotection, the crude reaction was submitted, without purification, to an acylation with 3-butenoic acid, EDCI, HOBt and two equivalent of $Et_3N$ (in excess in order to neutralize the TFA left from the previous step). After the chromatographic separation, the overall yield of the two steps was 63%. Compound **24** was obtained as a mixture of two stable rotamers, as has been observed in the NMR spectra, and this is a common characteristic for this class of amide-compounds. In fact, in the $^{1}$H-NMR spectrum, acquired at room temperature, the multiplicity of the signals was too complex to be determined and in the $^{13}$C-NMR almost every signal was split for the two conformers.

Lastly, second generation Grubb's Catalyst (0.14 equiv.) was added to the dialkene **24**, diluted in dry $CH_2Cl_2$, and the mixture was heated at 60°C overnight obtaining compound **25** in 74% yield.

Starting from this bicyclic compound, with the purpose of obtaining possible glycosidase inhibitors, further steps could be the epoxidation of the double bond (stereoselectivity should be studied), the opening of the epoxide with an azide (its regio- and stereoselectivity should be studied) and a further CuAAC on the newly inserted $N_3$ group. (Scheme 15)




**Scheme 15**

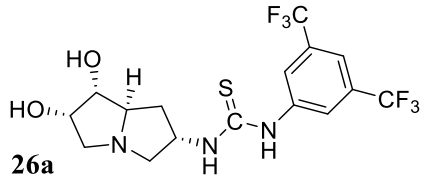
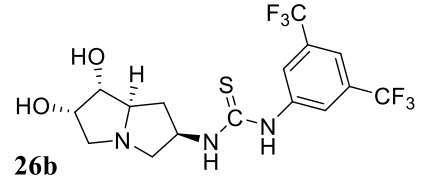
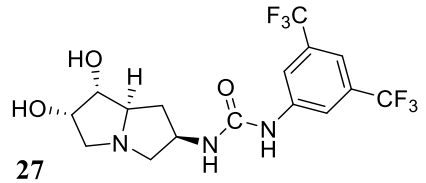
### 3.4 Biological evaluation

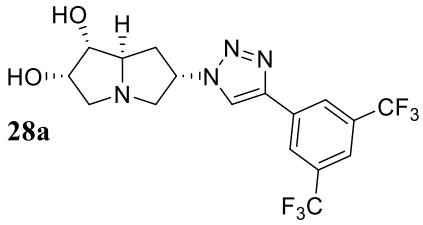
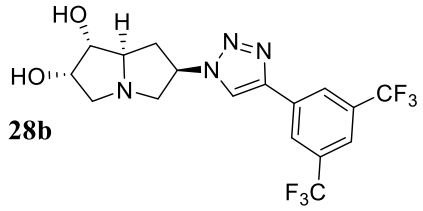
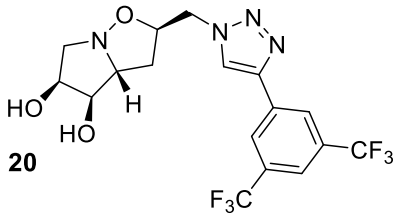
The enzymatic inhibition of the final compounds **26a,b**, **27** **28a,b** and **20** has been measured towards eleven commercially available glycosidases: β-galactosidases from *Aspergillus oryzae* and from *Escherichia coli*, α-glucosidase from rice, α-mannosidase from Jack beans, β-*N*-acetylglucosaminidase from Jack beans, α-galactosidase from coffee beans, amyloglucosidase from *Aspergillus niger*, α-glucosidase from *Saccharomyces cerevisiae*, β-glucosidase from almonds, β-mannosidase from snail and α-fucosidase from H. sapiens.

None of the compounds shows signs of inhibition with respect to nine of the eleven tested enzymes, thus they show inhibition only for the almonds' β-glucosidase and the jack beans' α-mannosidase.

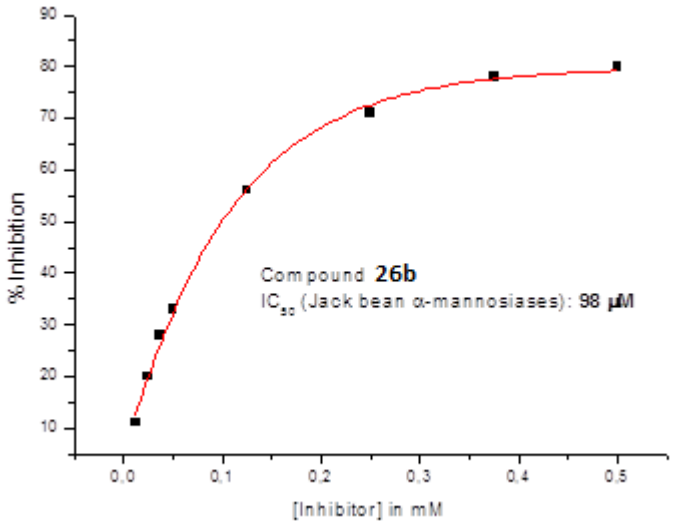
The following table (Table 1) shows the percentages of inhibition measured for each compound at 0.5 mM concentration of the inhibitor and the value of $IC_{50}$ is reported only for the more active ones.

**Table 1.** Inhibitory activities of compounds **26a**,**b**, **27**, **28a**,**b** and **20** towards glycosidases. % Inhibition at 0.5 mM of inhibitor. Optimal pH for each enzyme, 37 ºC.

| Inhibitor | β-glucosidase (almonds) | α-mannosidase (Jack beans) |
|---|---|---|
|  **26a** | 59 % | no inhibition |
|  **26b** | no inhibition | 87 %<br>($IC_{50}$ = 98 μM) |
|  **27** | 38 % | 56 % |

| | | |
|---|---|---|
|  **28a** | 27 % | 73 %<br>($IC_{50}$ = 286 μM) |
|  **28b** | no inhibition | 75 %<br>($IC_{50}$ = 142 μM) |
|  **20** | 22 % | no inhibition |

Values of $IC_{50}$ (concentration of inhibitor at which the enzymatic activity is 50% inhibited) were calculated only for **26b**, **28a** and **28b** inhibiting jack beans' α-mannosidase. For each of these compounds a curve '% of inhibition/concentration of inhibitor (mM)' was plotted and the value of $IC_{50}$ was obtained from the curve of the interpolated data, shown in figure 15.



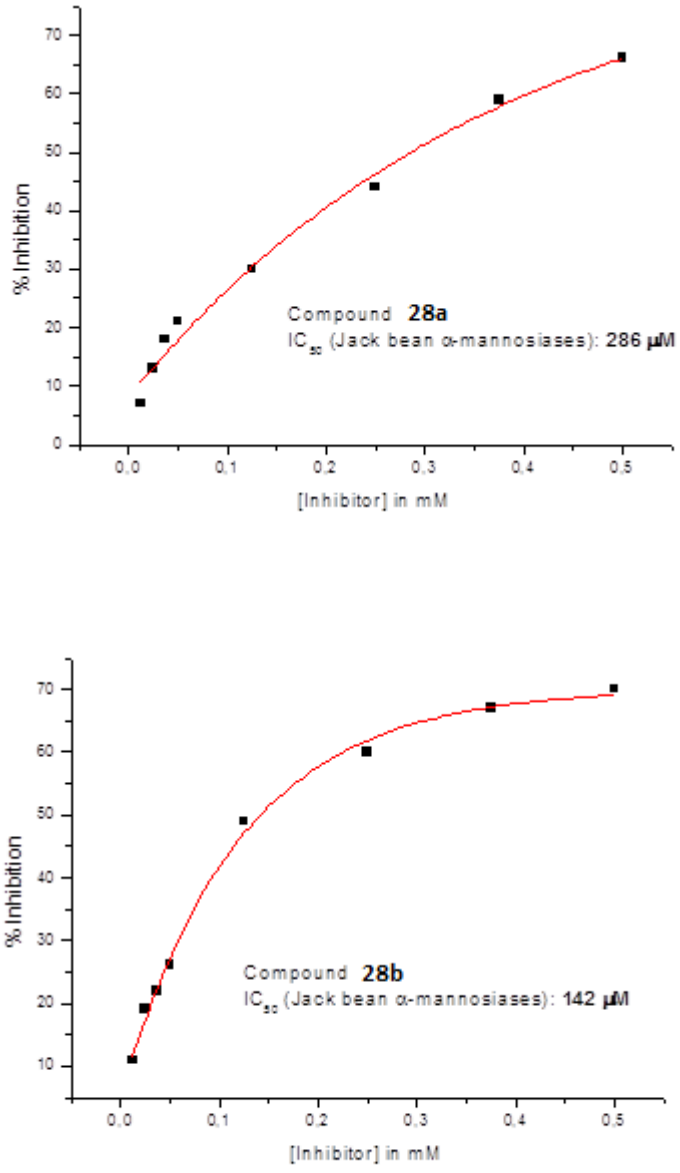



**Figure 15.** %Inhibition/[inhibitor] plots for compounds **26b**, **28a** and **28b.**

Even though the values of inhibition of these compounds are not particularly high ($IC_{50}$ ≈100-200 μM), most of them show selectivity for a single enzyme.

The most interesting results as inhibitors, in terms of potency and selectivity, are those of compounds **26b** and **28b**. A strategy to find new and better glycosidase inhibitors could be the evaluation of the biological activity of molecules with same configuration in the glycone part and same bridge functionalities as in **26b** and **28b,** but with different aromatic parts.

The thiourea-compound **26b** shows inhibition only for α-mannosidase with a percentage of 87% ($IC_{50}$ = 98 μM). When the thiourea functionality is exchanged with the urea one, as in compound **27**, a decrease in selectivity is observed, because compound **27** inhibits α-mannosidase at 56% but also the β-glucosidase enzyme at 38%.

The thiourea-compound **26a**, where the C-6 configuration is opposite to that in compound **26b**, only shows inhibition for the β-glucosidase, with a percentage of 59%, as opposed to compound **26a**, that inhibits α-mannosidase.

For what concerns the triazole-compounds, the major diastereomer **28b** only shows inhibition for α-mannosidase at 75% ($IC_{50}$ = 142 μM) whereas the minor **28a** shows a lower selectivity with 73% of inhibition ($IC_{50}$ = 286 μM) for α-mannosidase and also a weak inhibition (27%) for β-glucosidase.

Compound **20** shows only a weak inhibition of 22% for β-glucosidase; this compound has some structure similarity with compound **28a** but has an oxygen in the cycle and an additional $CH_2$ before the triazole that change the orientation of the aglycone and these changes involve the loss of inhibition for α-mannosidase.

We can also observe, knowing the inhibition values for *ent*-**26a**, *ent*-**26b**, *ent*-**27**, *ent*-**28a** and *ent*-**28b**[25] that there is a general trend of this enantiomers to be more potent but also slightly less selective than the ones synthesized in this work. Another difference is that the enantiomers show inhibition for α-galactosidase, α-mannosidase and α-fucosidase, while the compounds presented in this thesis compounds inhibit α-mannosidase and β-glucosidase.

## 4. Conclusions

During this work, five pyrrolizidine derivatives, compounds **26a**,**b**, **27** and **28a**,**b** and one isoxazolidine derivative, compound **20**, have been synthetized. The biological evaluation of these compounds was conducted in order to relate their configurations and type of bridge functionalities between the bicyclic iminosugar moiety and the aromatic part of the molecules, to their inhibition activities towards glycosidases. All the final compounds synthesized in this work show inhibition towards almonds' β-glucosidase and/or jack beans' α-mannosidase; the best results in term of potency and selectivity are those of compound **26b** (jack beans' α-mannosidase, $IC_{50}$ = 98 μM) and **28b** (jack beans' α-mannosidase, $IC_{50}$ = 142 μM). A strategy to find new and better glycosidase inhibitors could be the evaluation of the biological activity of molecules with same configuration in the glycone part and same bridge functionalities as in **26b** (thiourea) and **28b** (triazole), but with different aromatic parts.

A compound with indolizidine scaffold, compound **25**, was obtained through a ring-closing metathesis on a dialkenyl pyrrolidine. This bicyclic compound could be of interest as intermediate for the future synthesis of indolizidine derivatives with potential as glycosidase inhibitors.

# 5. Experimental section

## 5.1 Materials

All the reactants used, if not differently indicated in the synthesis section, were commercially available.

## 5.2 Experimental methods and Instrumentation

For the reactions conducted under inert atmosphere were used $N_2$ "U" commercial quality and Ar "N-48" commercial quality.

Solvent evaporation was performed under reduced atmosphere at temperatures below 50°C.

For chromatography the following stationary phases were utilized:

- Silica gel *60 (Merck Kiesekger 60 $F_{254}$* or *Kieselgel 60 $F_{254}$*, 0.2 mm thick) for TLC
- Silica gel 60 (*E. Merck*, particle size 0.043-0.063 and 0.063-0.200 mm) for chromatography under gravity or compressed air.

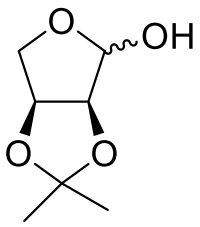
The optical rotations **($[\alpha]_D$)** were measured with a spectropolarimeter *Jasco P-2000*, using the sodium emission line ($\lambda$ = 589 nm) in a 1 cm-long cell, and at the temperature and with the solvent indicated for each case.

$^1$H-NMR and $^{13}$C-NMR spectra were acquired with instruments Bruker AMX 300 of the General Service of the University of Seville (CITIUS). The solvents used were $CDCl_3$ and $CD_3OD$ at room temperature. The values of chemical shift ($\delta$) are given in ppm and calibrated with the solvent signals; the values of coupling constants (*J*) are given in Hz. The assignation of each signal was confirmed and completed by 2D homonuclear correlation experiments and 2D HSQC experiments.

The ESI (ElectroSpray Ionization) mass spectra were performed with Micromass AutoSpeQ instrument of the general service of the University of Seville. For each experiment, the value of m/z registered is compared, up to the fourth decimal, with that of the molecular ion, which is calculated with the more abundant isotopes.

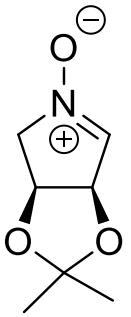
## 5.3 Synthesis

### (2*R*,3S)-2,3-*O*-isopropylidendioxy-1-tetrahydrofuro-1-ol (29)[27]




To a solution of L-Arabinose (10 g; 67 mmol) in DMF (140 mL) 2,2-DMP (34 mL, 266.4 mmol) and PTSA (0.259 g, 1.33 mmol) are added, and the obtained solution is then stirred at room temperature. After 4 hours $Na_2CO_3$ is added until the pH was neutral, the solution is filtered and concentrated. The crude is then dissolved in $H_2O$ and washed several times with cyclohexane. After that, $NaIO_4$ (17g, 80 mmol) is added to the aqueous phase and the solution is stirred at room temperature for 2.5 hours. Later, NaOH 1 M is added to the solution until the pH is higher than 7 and the solution is stirred during 1 hour at room temperature. Finally, HCl 1 M is added to neutralize the solution and the aqueous phase is extracted several times with EtOAc. The organic phases are reunited, dried over $Na_2SO_4$, filtered and concentrated. The resulting crude is purified by column chromatography on silica gel ($Et_2O$/Cyhex 2/1 → 1/1→ 2/1) obtaining compound **29** (7.78 g, 48.6 mmol, 73%) as a mixture of diastereoisomers.

**(2*R*,3S)-2,3-*O*-isopropylidendioxy-1-pyrroline 1-oxide (19)**



To a solution of **29** (6.42 g, 40.1 mmol) 0.5 M in dry pyridine (70 mL), molecular sieves of 3 Å (35 g) and $NH_2OH \cdot HCl$ (3.34 g, 48.1 mmol) are added. The resulting solution is stirred at room temperature under inert atmosphere for 17 hours. After that time, the solution is cooled at 0°C and a solution of MsCl (4.2 mL, 43 mmol) in dry pyridine (70 mL) is added rapidly. The solution is stirred at room temperature under inert atmosphere for 24 hours. Finally, the reaction mixture is diluted with $CH_2Cl_2$, filtered on celite and washed several times with $CH_2Cl_2$ and EtOAc. The resulting crude is then purified by a column chromatography on silica gel (EtOAc/MeOH 15/1 → 10/1) and compound **19** (1.60 g, 10.2 mmol, 25%) is obtained as a pale yellow solid.

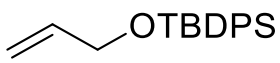
Data for compound **19**:

$[\alpha]_D^{29}$ 28.26 (c 0.500, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 158.0809, calculated for $C_7H_{11}NO_3$ $[M+H]^+$: 158.0812

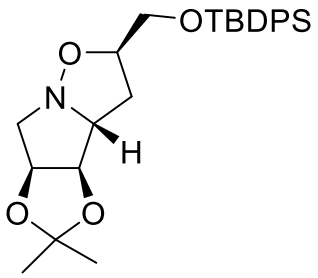
The NMR data of product **19** are consistent with those of its enantiomer.[28]

**(Allyloxy)(*tert*-butyl)diphenylsilane (30)**[34]




To a solution of allylic alcohol (3.0 g, 52.5 mmol) and imidazole (4.2 g, 63 mmol) in dry DMF (65 mL) cooled to 0°C, TBDPSCl (15.1 mL, 56.85 mmol) is added. The reaction is stirred at r.t. under inert atmosphere for one day. After that time, the mixture is cooled to 0°C and 75 mL of water are added. The phases are separated and the aqueous phase is extracted with EtOAc. The combined organic phases are washed with water and with brine, dried over $Na_2SO_4$, filtered and concentrated. The resulting crude is purified by a column chromatography on silica gel (EtOAc/Cyhex 1/50) obtaining compound **30** (14.01 g, 47.2 mmol, 90%) as a colourless oil.

**(2*R*,3a*R*,4*R*,5*S*)-Hexahydro-2-*tert*-butyldiphenylsilyloxymethyl-4,5-*O*-isopropylidendioxy-pyrrolo[1,2-b] isoxazole (31)**




A solution of **19** (2.83 g, 18.0 mmol) and **30** (10.65 g, 36.0 mmol) in toluene (217 mL) is stirred at reflux for 5 hours. After that time, the reaction mixture is cooled until room temperature is reached and the solution is concentrated. The resulting crude is then purified by a column chromatography on silica gel (EtOAc/Cyhex 1/5) and compound **31** (5.95 g, 13.1 mmol, 73%) is obtained as a colourless oil.

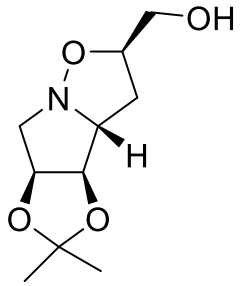
Data for compound **31**:

$[\alpha]_D^{29}$ -21.25 (c 0.845, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 454.2402, calculated for $C_{26}H_{35}NO_4Si$ $[M+H]^+$: 454.2408

The NMR data of product **31** are consistent with those of its enantiomer.[29]

**(2*R*,3a*R*,4*R*,5*S*)-Hexahydro-2-hydroxymethyl-4,5-*O*-isopropylidendioxy-pyrrolo[1,2-b] isoxazole (18)**




To a solution of **31** (2.709 mmol) in THF (22.7 mL) a solution of TBAF 1M in THF (3.0 mL, 3.0 mmol) is added; the reaction mixture is stirred at room temperature for 2 hours, and then, mixture is concentrated. The resulting crude is purified by a column chromatography on silica gel ($Et_2O$ → $Et_2O$/MeOH 9/1) obtaining compound **18** (554 mg, 2.57 mmol, 95%) as a white solid.

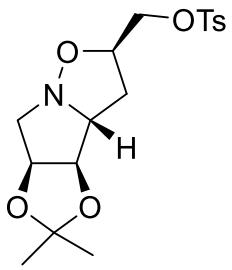
Data for compound **18**:

$[\alpha]_D^{27}$ -42.0 (c 0.330, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 216.1226, calculated for $C_{10}H_{17}NO_4$ $[M+H]^+$: 216.1230

The NMR data of product **18** are consistent with those of its enantiomer.[29]

**(2*R*,3a*R*,4*R*,5*S*)-Hexahydro-4,5-*O*-isopropylidendioxy-2-tosyloxymethyl-pyrrol[1,2-b] isoxazole (22)**




Compound **18** (2.44g, 11.3 mmol) is dissolved in 90 mL of dry $CH_2Cl_2$ under inert atmosphere and are cooled to 0°C. After that, $Et_3N$ (7.9 mL 56.5 mmol) is added dropwise and then TsCl (6.46 g, 33.9 mmol) is added. The reaction mixture is stirred at r.t. for 4 h. After that time, the reaction mixture is cooled to 0°C and 25 mL of water are added dropwise, the resulting mixture is left stirring for 15 minutes. The organic phase is then washed with water and with brine, dried over $Na_2SO_4$, filtered and concentrated. The crude is purified by a column chromatography on silica gel (EtOAc/Cyhex 2/1 → EtOAc/Cyhex 1/2) and compound **22** (2.99 g, 8.09 mmol, 72%) is obtained as a white solid.

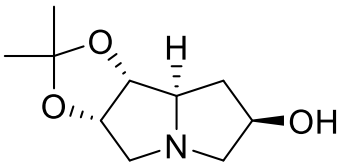
Data for compound **22**:

$[\alpha]_D^{26}$ -43.75 (c 0.240, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 370.1311, calculated for $C_{17}H_{24}NO_6S$ $[M+H]^+$: 370.1319

The NMR data of product **22** are consistent with those of its enantiomer.[25]

**(1*R*,2*S*,6*R*,7a*R*)-1,2,6-Trihydroxypyrrolizidine (32)**




Compound **22** (2.99 g, 8.12 mmol) is dissolved in 160 mL of MeOH, and a catalytic quantity of Pd/C is added to the reaction mixture. The flask is then saturated with $H_2$ at 1 atmosphere and the reaction is stirred during 4 hours at r.t. The reaction mixture is then concentrated and the crude is purified with a chromatographic silica column (EtOAc/MeOH 3/1 → EtOAc/MeOH 1/2) obtaining compound **32** (1.21 g, 6.09 mmol, 75%) as a white solid.

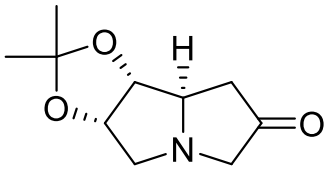
Data for compound **32**:

$[\alpha]_D^{30}$ 23.12 (c 0.950, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 200.1277, calculated for $C_{17}H_{24}NO_6S$ $[M+H]^+$: 200.1281

The NMR data of product **32** are consistent with those of its enantiomer.[29]

**(1*R*,2*S*,7a*R*)-1,2-*O*-Isopropylidendioxy-pyrrolizidin-6-one (33)**



A solution of oxalyl chloride (1.0 mL, 12.2 mmol) in dry $CH_2Cl_2$ (15 mL) is cooled to -78 °C and then dry DMSO is added (1.7 mL, 24.4 mmol). The mixture is left reaching -65°C, then a solution of compound **32** (1.21 g, 6.09 mmol) in dry $CH_2Cl_2$ (19 mL) is added and the reaction mixture is stirred for 5.5 h at -65°C under inert atmosphere. After that time, $Et_3N$ (4.2 mL, 30.4 mmol) is added dropwise, the reaction is left reaching r.t. and then is concentrated. The resulting crude is purified by a column chromatography on silica gel ($Et_2O$/Acetone 3/1) and compound **33** (0.68 g, 3.47 mmol, 57%) is obtained as an orange solid.

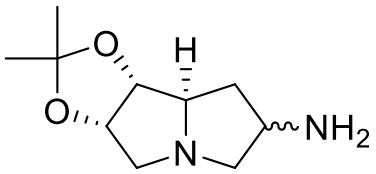
Data for compound **33**:

$[\alpha]_D^{30}$ 146.44 (c 0.550, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 198.1123, calculated for $C_{10}H_{15}NO_5$ $[M+H]^+$: 198.1125

The NMR data of product **33** are consistent with those of its enantiomer.[25]

**6-Amino-1,2-*O*-isopropylidendioxy-pyrrolizidine (17)**



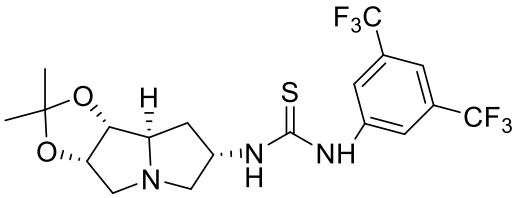

A solution of compound **33** (684 mg, 3.47 mmol) and $Ti(O^iPr)_4$ (2.0 mL, 6.9 mmol) in 25 mL of EtOH is saturated with gaseous $NH_3$ at one atmosphere and the reaction mixture is stirred for 4.75 h at r.t. After that time, $NaBH_4$ (197 mg, 5.20 mmol) is added and the reaction is stirred for 2 h. Then, 18 mL of $NH_4OH$ 2M are added and the mixture is filtered using celite, washed many times with EtOH and AcOEt and concentrated. The resulting crude is purified by column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 7/1/0.05 → 5/1/0.05) obtaining, as a mixture of diastereoisomers (2:1), compound **17** (510 mg, 2.57 mmol, 74%) as a pale yellow oil.

Data for compound **17**:

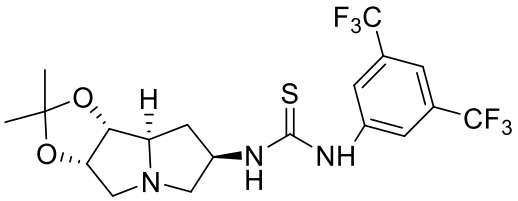
ESI-HRMS *m/z* obsd. 199.1439, calculated for $C_{10}H_{12}N_2O_2$ $[M+H]^+$: 199.1441

The NMR data are of product **17** consistent with those of their enantiomer.[25]

***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*S*,7a*R*)-1,2-*O*-isopropylidendioxy-pyrrolizidin-6-yl]thiourea (34a)** and ***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*R*,7a*R*)-1,2-*O*-isopropylidendioxy-pyrrolizidin-6-yl]thiourea (34b)**




**34a**




**34b**

To a solution of **17** (158 mg, 0.795 mmol) in 8 mL of dry $CH_2Cl_2$, under inert atmosphere, 1-isothiocyanate-3,5-bis(trifluoromethyl)benzene (370 μL, 1.99 mmol) is added. The reaction mixture is stirred at r.t. for 3.75 h and then is concentrated. The resulting crude is then purified by column chromatography on silica gel (DCM/MeOH 40/1 → 20/1) obtaining compound **34a** (86 mg, 0.180 mmol, 23%) and compound **34b** (201 mg, 0.428 mmol, 54%) as white solids.

Data for compound **34a**:

$[\alpha]_D^{23}$ 33.14 (c 0.6, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 470.1324, calculated for $C_{19}H_{22}F_6N_3O_2S$ $[M+H]^+$: 470.1331

The NMR data of compound **34a** are the same of the data of its enantiomer.[25]

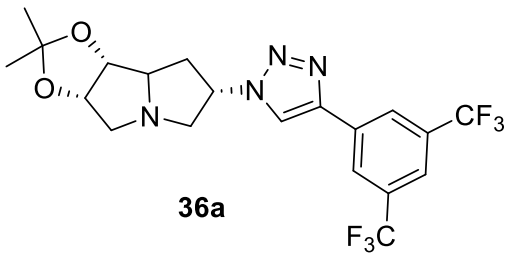
Data for compound **34b**:

$[\alpha]_D^{23}$ 31.00 (c 0.73, $CH_2Cl_2$)

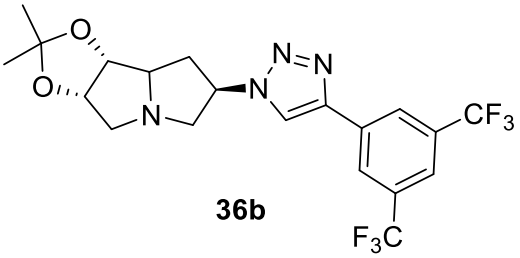
ESI-HRMS *m/z* obsd. 470.1323, calculated for $C_{19}H_{22}F_6N_3O_2S$ $[M+H]^+$: 470.1331

The NMR data of compound **34b** are consistent with those of its enantiomer.[25]

**(1*R*,2*S*,6*S*,7a*R*)-1,2-*O*-Isopropylidendioxy-6-[(4-(3,5-bis(trifluoromethyl)phenyl))-1*H*-1,2,3-triazol-1-yl)]pyrrolizidine (36a)** and **(1*R*,2*S*,6*R*,7a*R*)-1,2-*O*-isopropylidendioxy-6-[(4-(3,5-bis(trifluoromethyl)phenyl))-1*H*-1,2,3-triazol-1-yl)]pyrrolizidine (36b)**




**36a**




**36b**

Compound **17** (0.160 g, 0.81 mmol) is dissolved in $H_2O$ (1.1 mL) and MeOH (2.9 mL), and $NaHCO_3$ (0.271 g, 3.23 mmol), a solution of $NfN_3$ (0.471 g, 1.45 mmol) in $Et_2O$ (2.2 mL) and $CuSO_4 \cdot 5H_2O$ (20 mg, 0.081 mmol) are added. The reaction is stirred at r.t. during 6 h, then 1-ethynyl-3,5-bis(trifluoromethyl)benzene (0.211 g, 0.89 mmol,) and sodium ascorbate (0.240 g, 1.21 mmol) are added. The reaction mixture is stirred overnight at r.t. After that time, the reaction mixture is concentrated, redissolved in $CH_2Cl_2$ and then washed with a saturated solution of $NaHCO_3$ in water. The organic phase is dried over $Na_2SO_4$, filtered and concentrated. The resulting crude is purified by a column chromatography on silica gel ($Et_2O$/Acetone 15/1 → 4/1) obtaining compound **36a** (56 mg, 0.12 mmol, 15%) and compound **36b** (158 mg, 0.34 mmol, 43%) as pale yellow oils.

Data for compound **36a**:

$[\alpha]_D^{23}$ 7.20 (c 0.53, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 463.1556, calculated for $C_{20}H_{21}F_6N_4O_2$ $[M+H]^+$: 463.1563

The NMR data of product **36a** are consistent with those of its enantiomer.[25]

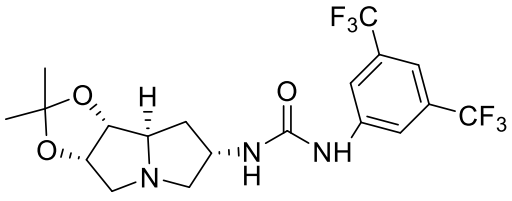
Data for compound **36b**:

$[\alpha]_D^{29}$ 37.07 (c 0.97, $CH_2Cl_2$)

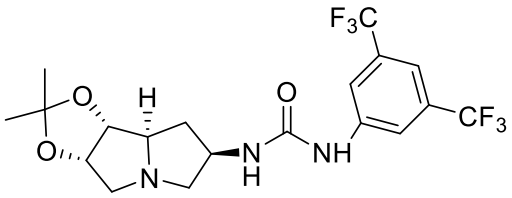
ESI-HRMS *m/z* obsd. 463.1556, calculated for $C_{20}H_{21}F_6N_4O_2$ $[M+H]^+$: 463.1563

The NMR data of product **36b** are consistent with those of its enantiomer.[25]

***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*S*,7a*R*)-1,2-*O*-isopropylidendioxy-pyrrolizidin-6-yl]urea (35a)** and ***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*R*,7a*R*)-1,2-*O*-isopropylidendioxy-pyrrolizidin-6-yl]urea (35b)**




**35a**




**35b**

To a solution of compound **17** (0.153 g, 0.77 mmol) in dry $CH_2Cl_2$ (8.3 mL) at 0 °C 1-isocyanate-3,5-bis(trifluoromethyl)benzene (0.217g, 0.85 mmol) is added dropwise. The reaction is stirred for 1 h at 0°C under inert atmosphere. After that time the mixture is concentrated and then purified by a column chromatography on silica gel (DCM/MeOH 20/1 → 10/1 ) obtaining compound **35a** (8 mg, 0.02 mmol, 2.2%) and compound **35b** (0.52 mg, 0.11 mmol, 15%) as white solids.

Data for compound **35a**:

$[\alpha]_D^{30}$ 32.79 (c 0.77, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 454.1552, calculated for $C_{19}H_{22}F_6N_3O_3$ $[M+H]^+$: 454.1560

The NMR data are consistent with those of its enantiomer.[25]

Data for compound **35b**:

$[\alpha]_D^{30}$ 25.24 (c 0.765, $CH_2Cl_2$)

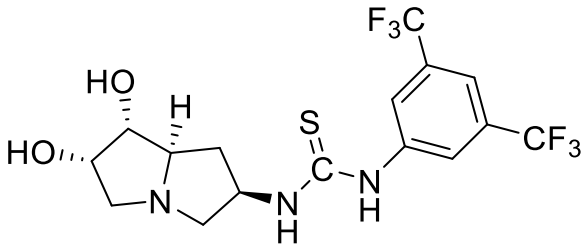
ESI-HRMS *m/z* obsd. 454.1553, calculated for $C_{19}H_{22}F_6N_3O_3$ $[M+H]^+$: 454.1560

The NMR data are consistent with those of its enantiomer. [25]

**General procedure for acidic deprotection:**

A solution of the protected compound (0.5 mmol) in 12 mL of a mixture of HCl 4M/ THF (1/1) is stirred at r.t. for 3.5 h. After that time, the reaction mixture is concentrated and the residue is dissolved in 9 mL of THF, neutralized with 7 mL of $NH_4OH$ (25%) and concentrated. The crude is then purified by column chromatography on silica gel.

***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*R*,7a*R*)-1,2-dihydroxy-pyrrolizidin-6-yl]thiourea (26b)**




Following the general procedure for acidic deprotection on **34b** (0.122 g, 0.26 mmol) and purification through column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 5/1/0.01) compound **26b** (91 mg, 0.212 mmol, 81%) is obtained as a white solid.

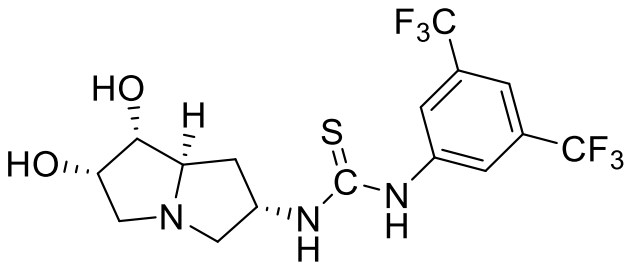
Data for compound **26b**:

$[\alpha]_D^{29}$ 14.52 (c 0.65, MeOH)

ESI-HRMS *m/z* obsd. 430.1012, calculated for $C_{16}H_{18}F_6N_3O_2S$ $[M+H]^+$: 430.1018

The NMR data are consistent with those of its enantiomer. [25]

***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*S*,7a*R*)-1,2-dihydroxy-pyrrolizidin-6-yl]thiourea (26a)**




Following the general procedure for acidic deprotection on **34a** (0.073g, 0.15 mmol) and purification through column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 5/1/0.01) compound **26a** (41 mg, 0.94 mmol, 61%) is obtained as a white solid.

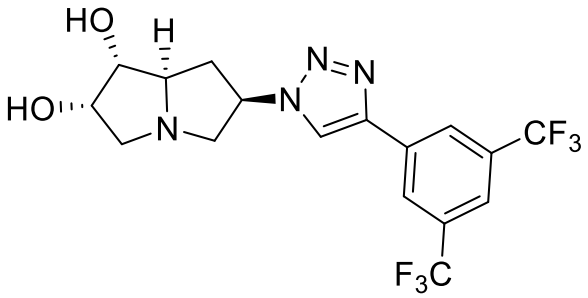
Data for compound **26a**:

$[\alpha]_D^{29}$ -10.98 (c 0.65, MeOH)

ESI-HRMS *m/z* obsd. 430.1012, calculated for $C_{16}H_{18}F_6N_3O_2S$ $[M+H]^+$: 430.1018

The NMR data are consistent with those of its enantiomer.[25]

**(1*R*,2*S*,6*R*,7a*R*)-6-(4-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl)hexahydro-1H-pyrrolizine-1,2-diol (28b)**




Following the general procedure for acidic deprotection on **36b** (0.148 g, 0.32 mmol) and purification through column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 5/1/0.02) compound **28b** (95 mg, 0.22 mmol, 70%) is obtained as a white solid.

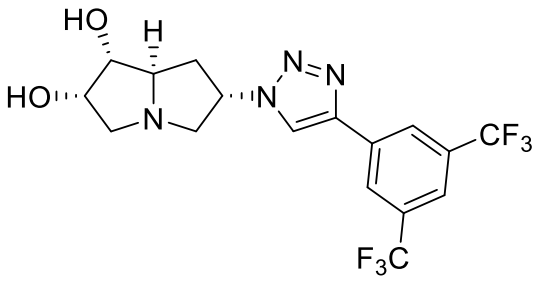
Data for compound **28b**:

$[\alpha]_D^{29}$ 20.03 (c 0.61, MeOH)

ESI-HRMS *m/z* obsd. 423.1240, calculated for $C_{17}H_{17}F_6N_4O_2$ $[M+H]^+$: 423.1250

The NMR data are consistent with those of its enantiomer.[25]

**(1*R*,2*S*,6*S*,7a*R*)-6-(4-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl)hexahydro-1H-pyrrolizine-1,2-diol (28a)**




Following the general procedure for acidic deprotection on **36a** (0.046 g, 0.10 mmol) and purification through column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 8/1/0.01) compound **28a** (28 mg, 0.06 mmol, 66%) is obtained as a white solid.

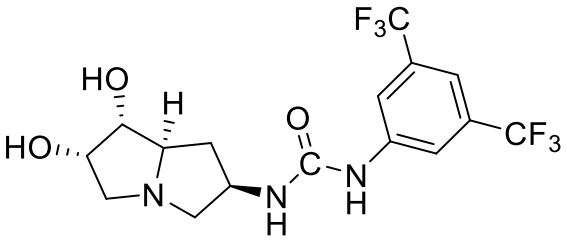
Data for compound **28a**:

$[\alpha]_D^{29}$ -6.73 (c 0.695, MeOH)

ESI-HRMS *m/z* obsd. 423.1242, calculated for $C_{17}H_{17}F_6N_4O_2$ $[M+H]^+$: 423.1250

The NMR data are consistent with those of its enantiomer.[25]

***N*-[(3,5-Bis(trifluoromethyl)phenyl)]-*N'*-[(1*R*,2*S*,6*R*,7a*R*)-1,2-dihydroxy-pyrrolizidin-6-yl]urea (27)**




Following the general procedure for acidic deprotection on **35b** (0.035 g, 0.07 mmol) and purification through column chromatography on silica gel (DCM/MeOH/$NH_4OH$ 5/1/0.2) compound **27** (22 mg, 0.05 mmol, 75%) is obtained as a white solid.

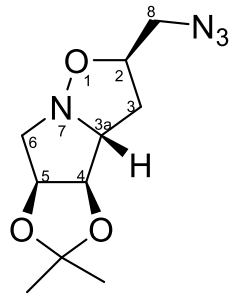
Data for compound **27**:

$[\alpha]_D^{29}$ 14.63 (c 0.82, MeOH)

ESI-HRMS *m/z* obsd. 414.1239, calculated for $C_{16}H_{18}F_6N_3O_3$ $[M+H]^+$: 414.1247

The NMR data are consistent with those of its enantiomer.[25]

**(2*R*,3a*R*,4*R*,5*S*)-Hexahydro-4,5-*O*-isopropylidendioxy-2-azidomethyl-pyrrol[1,2-b] isoxazole (21)**




To a solution of compound **22** (0.150 g, 0.41 mmol) in DMF (4 mL) $NaN_3$ (0.066g, 1.01 mmol) is added. The reaction mixture is stirred at 70°C for 3 h. After that time, the solvent is removed and the crude is dissolved in $CH_2Cl_2$, washed with water and brine. The organic phase is dried over $Na_2SO_4$, filtered and evaporated. The crude is then purified by a chromatography column on silica gel ($Et_2O$/Cyhex 1/1) obtaining compound **21** (71 mg, 0.29 mmol, 73%) as a pale yellow oil.

Data for compound **21**:

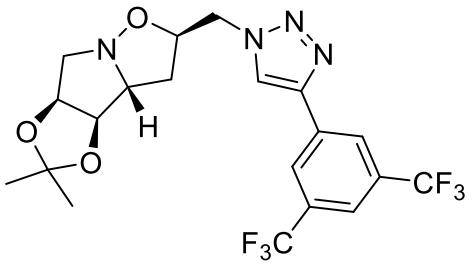
$[\alpha]_D^{30}$ -64.25 (c 0.93, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 241.1294, calculated for $C_{10}H_{16}N_4O_3$ $[M+H]^+$: 241.1295

$^1$H-NMR (300 MHz, $CDCl_3$, δ ppm, *J* Hz) 4.93 (dt, 1H, $J_{5\text{-}6}$=4.6, $J_{5\text{-}4}$=6.5, H-5), 4.57 (dd, 1H, $J_{4\text{-}3a}$= 2.5 , $J_{5\text{-}4}$= 6.5 H-4), 4.33 (ddd, 1H, *J* =4.27, $J_{3\text{-}2}$=6.9, *J* = 11.03, H-2), 3.76 (dt, 1H $J_{4\text{-}3a}$=2.5, $J_{3\text{-}3a}$ =6.9, H-3a), 3.40 (dd, 1H, $J_{8.1\text{-}2}$=6.53, $J_{8.1\text{-}8.2}$=12.7, H-$8_1$), 3.34 (d, 2H, $J_{5\text{-}6}$=4.6, H-6), 3.19 (dd, 1H $J_{8.1\text{-}8.2}$= 12.7, $J_{8.2\text{-}2}$=4.1, H-$8_2$), 2,26 (t, 2H, $J_{3\text{-}3a}$ = $J_{3\text{-}2}$=6.9, H-3), 1.51 (s, 3H, $C(CH_3)_2$), 1.31 (s, 3H, $C(CH_3)_2$).

$^{13}$C-NMR (75.4 MHz, $CDCl_3$, δ ppm, J Hz) δ 113.2 (Cq $C(CH_3)_2$), 84.9 (CH, C-4), 80.6 (CH, C-5), 76.2 (CH, C-2), 71.3 (CH, C-3a), 60.6 ($CH_2$, C-6), 53.7 ($CH_2$, C-8), 36.26 ($CH_2$, C-3), 27.0 ($CH_3$, $C(CH_3)_2$), 25.0 ($CH_3$, $C(CH_3)_2$).

**(2*R*,3a*R*,4*R*,5*S*) -Hexahydro–4,5 isopropylidendioxy-2- [(4-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl)methyl)] pyrrolo [1,2-b]isoxazole (37)**



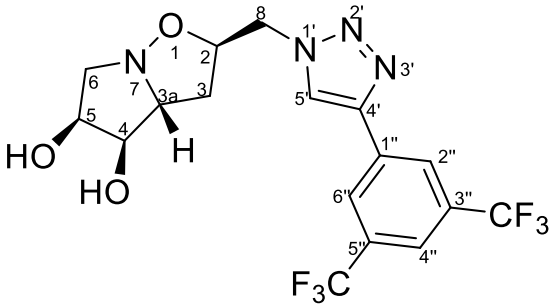
To a solution of **21** (47 mg, 0.20 mmol) in toluene (1.8 mL) 1-ethynyl-3,5-bis(trifluoromethyl)benzene (71μL, 0.392 mmol), DIPEA (127 μL, 0.745 mmol,) and CuI (12 mg, 0.06 mmol) are added. The reaction mixture is stirred at 50 °C for 5 h, then 12 mL of a aqueous saturated solution of $NaHCO_3$ is added and the mixture is extracted with EtOAc. The combined organic phase is dried over $Na_2SO_4$, filtered and concentrated. The crude is purified by column chromatography on silica gel (EtOAc/Cyhex 2/1) obtaining compound **37** (90 mg, 0.19 mmol, 96%) as a white solid.

Data for compound **37**:

$[\alpha]_D^{26}$ -50.43 (c 0.89, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 479.1505, calculated for $C_{20}H_{20}F_6N_4O_3$ $[M+H]^+$: 479.1512

**(2*R*,3a*R*,4*R*,5*S*)-Hexahydro-2-((4-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl)methyl) pyrrolo[1,2-b]isoxazole-4,5-diol (20)**




Following the general procedure for acidic deprotection on **37** (0.069 g, 0.15 mmol), compound **20** (57 mg, 0.13mmol, 89%) is obtained as a white solid after purification through column chromatographic (DCM/MeOH/$NH_4OH$ 8/1/0.01).

Data for compound **20**:

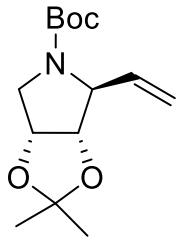
$[\alpha]_D^{30}$ -43.14 (c 0.935, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 479.1505, calculated for $C_{20}H_{20}F_6N_4O_3$ $[M+H]^+$: 479.1512

$^1$H-NMR (300 MHz, MeOD, δ ppm, *J* Hz) δ 8.59 (s, 1H, H-5'), 8.45 (s, 2H, H-2", H-6"), 7.93 (s, 1H, H-4''), 4.71-4.49 (m, 3H, H-8 and H-2), 4.30 (q, 1H, $J_{4\text{-}5}$=$J_{5\text{-}6}$=5.0, H-5), 3.95 (t, 1H, $J_{4\text{-}5}$=$J_{4\text{-}3a}$=5.0 H-4), 3.71-3.62 (m, 1H, H-3a), 3.37-3.23 (m, 2H, H-6), 2.40 (ddd, 1H, $J_1$= 3.2, $J_2$=6.3, $J_{3.1\text{-}3.2}$=12.9, H-$3_1$), 2.33-2.21 (m, 1H, H-$3_2$).

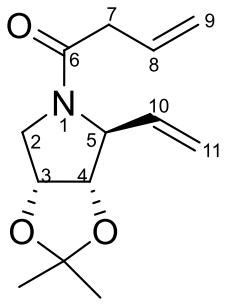
$^{13}$C-NMR (75.4 MHz, MeOD, δ ppm, *J* Hz) δ 145.9 (Cq, C-4'), 134.6 (Cq, C-1''), 133.5 (q, Cq, $J_{CF}$=37, C-3", C-5"), 126,7 (m, CH, C-2'', C-6''), 124.9 (CH, C-5'), 124.8 (q, $J_{CF}$=270, $CF_3$), 122.3 (m, CH, C-4''), 77.9 (CH, C-4), 76.8 (CH, C-2), 72.6 (CH, C-5), 71.2 (CH, C-3a), 62.3 ($CH_2$, C-6), 53.1 ($CH_2$, C-8), 37.4 ($CH_2$, C-3).

**(2*S*, 3*S*, 4*R*) *N*-*tert*-Butoxycarbonyl-2-vinyl-3,4-isopropylidendioxy-pyrrolidine (38)**[33]




A mixture of diol **23** (645 mg, 2.13 mmol), $PPh_3$ (2.250 g, 8.50 mmol) and imidazole (0.579 g, 8.50 mmol) are dissolved in dry toluene (34 mL). The solution is heated up to 80°C and $I_2$ (1.619 g, 6.38 mmol) is added slowly. The reaction mixture is stirred at 80°C under inert atmosphere for 1h. Afterwards, the reaction mixture is left reaching r.t., 40 mL of cold $Et_2O$ are added and the flask is cooled down in the fridge. The crude is then filtered over celite and concentrated. Due to the quite volatile nature of the product, attention is advised. The crude is purified by column chromatography on silica gel ($Et_2O$/CyHex 1/2) and compound **38** (214 mg, 0.79 mmol, 37%) is obtained as white solid.

**(2*S*,3*S*,4*R*) *N*-(but-3-en-1-one)-2-vinyl-3,4-isopropylidendioxy-pyrrolidine (24)**




To a solution of **38** (0.337 g, 1.25 mmol) in dry $CH_2Cl_2$ (7.2 mL), TFA (1.8 mL) is added dropwise. The solution is stirred at r.t. under inert atmosphere during 2.5 h. After that time, the mixture is concentrated and dried under vacuum. The crude product is dissolved in $CH_2Cl_2$ and $Et_3N$ (348 μL, 2.506 mmol), HOBt·$H_2O$ (0.1919 g, 1.253 mmol), EDCI·HCl (0.240 g, 1.25 mmol) and 3-butenoic acid (107 μL, 1.253 mmol) are added. The reaction is stirred overnight at r.t. and under inert atmosphere. After that time, the mixture is washed once with HCl 0.5M, once with a aqueous saturated solution of $NaHCO_3$ and once with brine. The organic phase is then dried over $Na_2SO_4$, filtered and concentrated. The crude is purified by column chromatography on silica gel (AcOEt/CyHex 1/2) obtaining compound **24** (189 mg, 0.79 mmol, 63%), as a mixture of two stable rotamers, as a white solid.

Data for compound **24**:

$[\alpha]_D^{30}$ 26.22 (c 0.54, $CH_2Cl_2$)

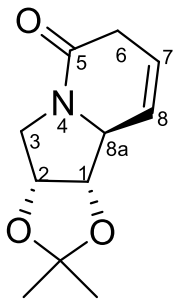
ESI-HRMS *m/z* obsd. 260.1260, calculated for $C_{13}H_{19}NO_3$ $[M+H]^+$: 260.1257

$^{1}$H-NMR (300 MHz, $CDCl_3$, δ ppm, *J* Hz) δ 6.05-5.88 (m, 1H, H-8), 5.83-5.64 (m, 1H, H-10), 5.27-5.02 (m, 4H, H-9, H-11), 5.00-4.95 and 4.63-4.49 (m, 2H, H-4, H-5), 4.78-4.69 (m, 1H, H-3), 4.24-3.29 (m, 2H, H-2), 3.20-2.94 (m, 2H, H-7), 1.43 (s, 3H, $C(CH_3)_2$), 1.30 (s, 3H, $C(CH_3)_2$).

$^{13}$C-NMR (75.4 MHz, $CDCl_3$, δ ppm, *J* Hz) δ 170.5 (C=O, C-6, maj.), 169.5 (C=O, C-6, min.), 133.6 (CH, C-8, maj.), 132.8 (CH, C-8, min.), 131.4 (CH, C-10, maj.), 131.1 (CH, C-10, min.), 118.3 (CH, C-11*, min.) 118.0 (CH, C-11*, maj.), 116.7 (CH, C-9*, maj.), 116.1 (CH, C-9*, min.), 112.0 (Cq, $C(CH_3)_2$, min. and maj.), 84.8 (CH, C-4*, maj.), 83.3 (CH, C-4*, min.), 79.4 (CH, C-3, min.), 78.1 (CH, C-3, maj.), 66.2 (CH, C-5*, maj.), 64.1 (CH, C-5*, min.), 52.7 ($CH_2$, C-2, min.), 51.2 ($CH_2$, C-2, maj.), 40.1 ($CH_2$, C-7, min.), 39.0 ($CH_2$, C-7, maj.), $26.9_2$ ($CH_3$, $C(CH_3)_2$, maj.), $26.8_8$ ($CH_3$, $C(CH_3)_2$, min.), 25.0 ($CH_3$, $C(CH_3)_2$, maj.), 24.9 ($CH_3$, $C(CH_3)_2$, min.).

*C-5 and C-4 and C-11 and C-9 signals could be exchanged.

**(1*S*,2*R*,8a*S*)-2-en-1,2-isopropylidendioxy indolizidin-5-one (25)**




To a solution of **24** (0.069 g, 0.29 mmol) in 10 mL of dry $CH_2Cl_2$ a solution of second generation Grubb's Catalyst (37 mg, 0.04 mmol) in $CH_2Cl_2$ is added. The reaction mixture is then heated at 60°C, and stirred overnight at reflux under inert atmosphere. Then, the reaction mixture is concentrated, and the crude is purified by column chromatography on silica gel (EtOAc/Cyhex 5/1). Compound **25** (45 mg, 0.22 mmol, 74%) is obtained as a white solid.

Data for compound **25**:

$[\alpha]_D^{26}$ -57.72 (c 0.605, $CH_2Cl_2$)

ESI-HRMS *m/z* obsd. 210.1124, calculated for $C_{11}H_{15}NO_3$ $[M+H]^+$: 210.1125.

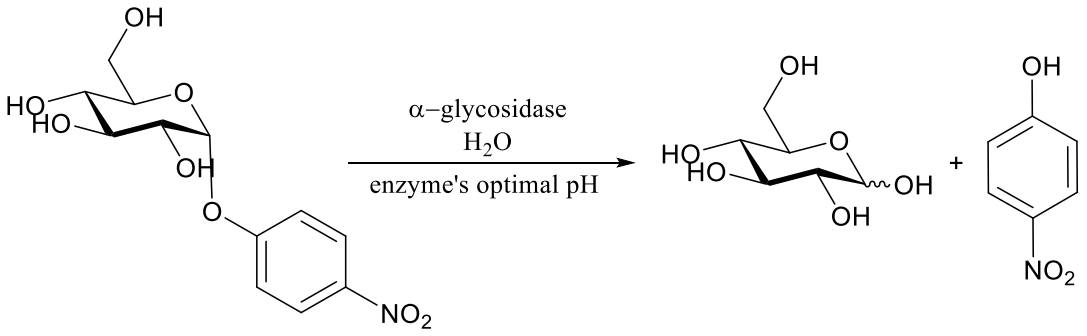
$^1$H-NMR (300 MHz, $CDCl_3$, δ ppm, *J* Hz) δ 5.97 (d, 1H *J*= 10.3, H-8), 5.86-5.78 (m, 1H, H-7), 4.72 (td, 1H, $J_{3.1\text{-}2}$=2.5, $J_{2\text{-}3.2}$=$J_{1\text{-}2}$=6.5, H-2), 4.42 (dd 1H $J_{3.2\text{-}2}$=6.5, $J_{3.1\text{-}3.2}$= 13.7, H-$3_2$), 4.23-4.30 (t, 1H, $J_{1\text{-}2}$=$J_{1\text{-}8a}$= 6.5 H-1), 4.07-3.97 (m, 1H, H-8a), 3.36 (dd, 1H, $J_{3.1\text{-}2}$= 2.5, $J_{3.1\text{-}3.2}$= 13.7, H-$3_1$), 3.01-2.79 (m, 2H, H-6), 1.54 (s, 3H, $C(CH_3)_2$), 1.36 (s, 3H, $C(CH_3)_2$).

$^{13}$C-NMR (75.4 MHz, $CDCl_3$, δ ppm, J Hz) δ 166.0 (C=O), 123.6 (CH, C-7), 122.7(CH, C-8), 114.2 (Cq, $C(CH_3)_2$), 83.4 (CH, C-1), 76.8 (CH, C-2), 63.3 (CH, C-8a), 48.4 ($CH_2$, C-3), 32.3 ($CH_2$, C-6), 27.8 ($CH_3$, $C(CH_3)_2$), 25.7 ($CH_3$, $C(CH_3)_2$).

## 5.4 Biological evaluation tests

The biological evaluation tests were performed by Dr Antonio J. Moreno Vargas, University of Seville, using the method described by Saul and cols.[35] with *p*-nitrophenylglycosides commercially available, appropriated for each enzyme.

This compound could be used in this analysis because it mimics a substrate for this class of enzymes and the following reaction (Scheme 16) can take place:




**Scheme 16.** Example of enzymatic cleavage of a *p*-nitrophenylglycoside.

At pH=9,8 (pH value reached at the end of the enzymatic tests adding a solution of sodium borate) *p*-nitrophenol is considered completely deprotonated and the quantity of *p*-nitrophenate, which is intensely yellow, formed in each case can be measured through visible absorption spectroscopy at 405 nm. In this conditions, values of absorbance of the formed *p*-nitrophenate vary linearly with time of reaction and enzyme concentration.

Percentages of inhibition can be obtained comparing absorbance values between experiments with and without the inhibitor, conducted in the same condition of temperature, time and pH. These experiments are conducted twice and the given result is the average value of the two measurements.

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