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XXII CICLO

Targeting glycosyl hydrolases inhibition: synthesis of polyhydroxylated alkaloids and their iminosugar analogs

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A Marco

"...vincere la materia è comprenderla, e comprendere la materia è necessario per comprendere l'universo e noi stessi..."

Primo Levi – Il sistema periodico

Part of this thesis has been the object of publications and communications at meetings.

PUBBLICATIONS

P. Merino, I. Delso, T. Tejero, F. Cardona, M. Marradi, E. Faggi, C. Parmeggiani, A. Goti "Nucleophilic additions to cyclic nitrones en route to iminocyclitols. Straightforward Total Syntheses of DMDP, 6-deoxy-DMDP, DAB-1, CYB-3, Nectrisine and Radicamine B" *Eur J. Org. Chem.,* **2008***,* 2929-2947*.*

F. Cardona, C. Parmeggiani, E. Faggi, C. Bonaccini, P. Gratteri, L. Sim, T. M. Gloster, S. Roberts, G. J. Davies, D. R. Rose, A. Goti "Total Syntheses of Casuarine and its 6-*O*-α-Glucoside: Complementary Inhibition towards Glycoside Hydrolases of Families GH31 and GH37" *Chem. Eur. J.,* **2009**, *15*, 1627-1636.

C. Parmeggiani, D. Martella, F. Cardona, A. Goti "Total Synthesis of (−) -Uniforine A" *J. Nat. Prod.*, **2009**, *72*, 2058-2060.

M. Forcella, F. Cardona, A. Goti, C. Parmeggiani, L. Cipolla, M. Gregori, R. Schirone, P. Fusi, P. Parenti "A membrane-bound trehalase from *Chironomus riparius* larvae: purification and sensitivity to inhibition" *Glycobiology*, submitted.

C. Bonaccini, M. Chioccioli, C. Parmeggiani, F. Cardona, D. Lo Re, G. Soldaini, P. Vogel, C. Bello, A. Goti, P. Gratteri "Synthesis, biological evaluation and modelling studies of casuarine analogs: effects of structural modifications at ring B on inhibitory properties towards glucoamylases" in preparation.

F. Cardona, A. Goti, C. Parmeggiani, P. Parenti, P. Fusi, M. Forcella, L. Cipolla, T. Gloster, G. J. Davies "Casuarine-6-*O*-α-D-glucoside and its analogues strongly inhibit an insect trehalase" in preparation.

CONFERENCE PRESENTATIONS AND SEMINARS

C. Parmeggiani, F. Cardona, A. Goti, "Sintesi della Casuarina e di suoi derivati e glicoconiugati", *XXII Congresso Nazionale della Società Chimica Italiana*, Firenze, 10-15 settembre **2006**, ORG-P114.

F. Cardona, C. Parmeggiani, A. Goti, T. Pagliaccia, R. Saladino, "Total Syntheses of Pyrrolidine and Pyrrolizidine Alkaloids and their Glycoconjugates", *XXII European Colloquium on Heterocyclic Chemistry*, Bari, 2-6 settembre **2006**, SO16.

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D. Lo Re, C. Parmeggiani, F. Cradona, A. Goti, "Alcaloidi pirrolizidinici poliossidrilati (PHPAs): Sintesi di Casuarina, Glicoconiugati e Analoghi non naturali", *Riunione Scientifica Annuale della Società Chimica Italiana Sez. Toscana*, Firenze, 18 dicembre **2006**, Abstract P22.

F. Cardona, C. Parmeggiani, D. Lo Re, **A, Goti,** "Synthesis of iminosugars and related nitrogenated glycomimetics inhibitors of glycosidic enzymes", *Eight tetrahedron symposium*, Berlino (Germania), 26-29 giugno **2007**, P2.132.

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C.Parmeggiani, F. Cardona, A. Goti, "Glycosyl hydrolasesbelonging to different famiglie are selectively inhibited by casuarine and its 6-O-α-glucoside", *BIOTECH.ORG Organic Chemistry and Biotechnology: Challenges and Opportunities*, Forte dei Marmi (LU), 20-23 maggio **2009**, P-23 pag 75.

A. Goti, F. Cardona, H.-U. Reiβig, **C. Parmeggiani**, "Total synthesis of casuarine and its analogues: comparison of different methodologies", *NAT8 Giornate di Chimica delle Sostanze Naturali*, Forte dei Marmi (LU), 22-25 maggio **2009**, P26.

C.Parmeggiani, F. Cardona, A. Goti, "Pirrolizidine poliossidrilate naturali: glicomimetici potenti inibitori di glicosidasi", *XIV Corso Estivo "A. Corbella" Seminari di Sintesi Organica*, Palazzo Feltrinelli Gargnano del Garda (BS), 22-26 giugno **2009**, O17.

C.Parmeggiani, F. Cardona, A. Goti, "Controllo della regio selettività per l'ottenimento di 4-idrossi isossazolidine in cicloaddizioni di nitroni tramite la reazione di Tamao-Fleming: sintesi totale della (+)-casuarina e della (-)-uniflorina A", *XIII Convegno Nazionale sulle Reazioni Pericicliche e Sintesi di Etero e Carbocicli*, Pavia, 17-18 settembre **2009**, Abstract P28-29.

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F. Cardona, C. Parmeggiani, **A. Goti**, "A journey over the realm of highly hydroxylated pyrroline and pyrrolizidine alkaloids", *NAT8 Giornate di Chimica delle Sostanze Naturali*, Forte dei Marmi (LU), 22-25 maggio **2009**, PL5.

Summary

Chapter 1

Glycosidases inhibitors

1.1 Glycosidases

1.1.1 Introduction

Glycosidases, or glycosyl hydrolases (GH; EC 3.2.1.-), are a widespread group of enzymes that hydrolyse the glycosidic bond between two carbohydrates in a disaccharide or polysaccharide moiety or between a carbohydrate and an aglycon.[1] To date, the GHs have been classified into 108 families on the basis of amino acid sequence similarities (see the CAZYs database at www.cazy.org).

Due to the large number of proteins included in this family, a broad structural diversity is covered, but generally these enzymes have a size of approximately 500 residues and a general structure based on a barrel of β sheets surrounded by α helices (Figure 1.1). In addition to this minimum unit, glycosidases can form oligomers or have other domains that provide additional features such as molecular recognition or anchoring to membranes.

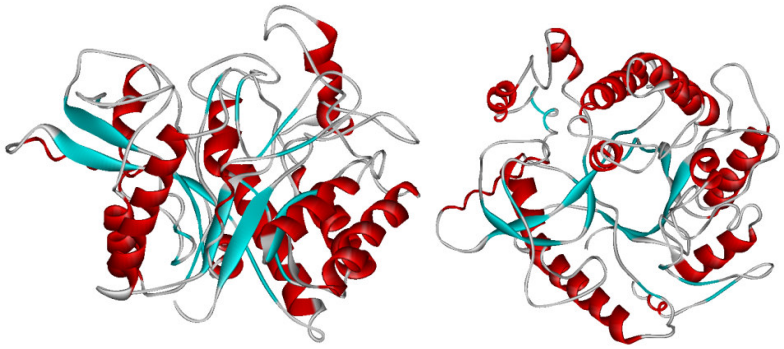


Figure 1.1. Structure of Human Cytosolic β-glucosidase[1]

Due to the function and the large number of metabolic processes in which they are involved, these enzymes are found in bacteria and prokaryotes and in higher organisms and have many different roles. In bacteria and

[1] Tribolo, S.; Berrin, J. G.; Kroon, P. A.; Czjzek, M.; Juge, N. *J. Mol. Biol.* **2007**, *370*, 964.

prokariotes glycosidases are involved in the acquisition of nutrients, in both intra- and extracellular medium; in higher organisms they are present in Golgi apparatus and in the endoplasmic reticulum, in which they are involved in processes of maturation of glycoproteins, and in the lysosomes where they carry-out the degradation of carbohydrate-based structures. Another fundamental process in which these enzymes are involved is the digestion in the digestive tract, where they degrade nutrients as lactose, sucrose, trehalose or starch, and the disappearance of these enzymes in the intestine, for example, leads to lactose intolerance in humans.

Glycosidases are involved in many metabolic pathways, from the most obvious such as the intestinal digestion of carbohydrates to complex processes of cell-cell and virus-cell recognition (Figure 1.2).

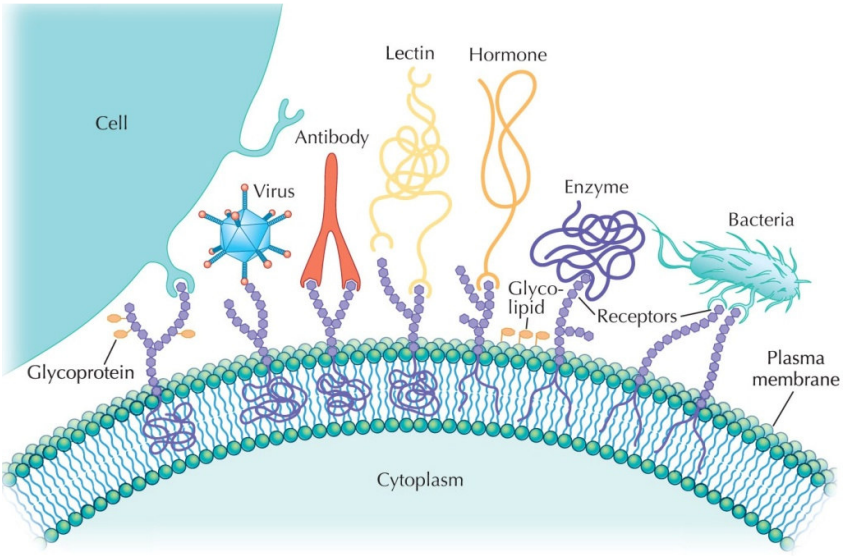
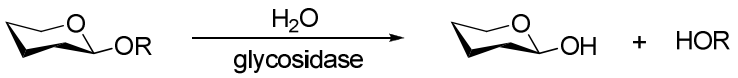


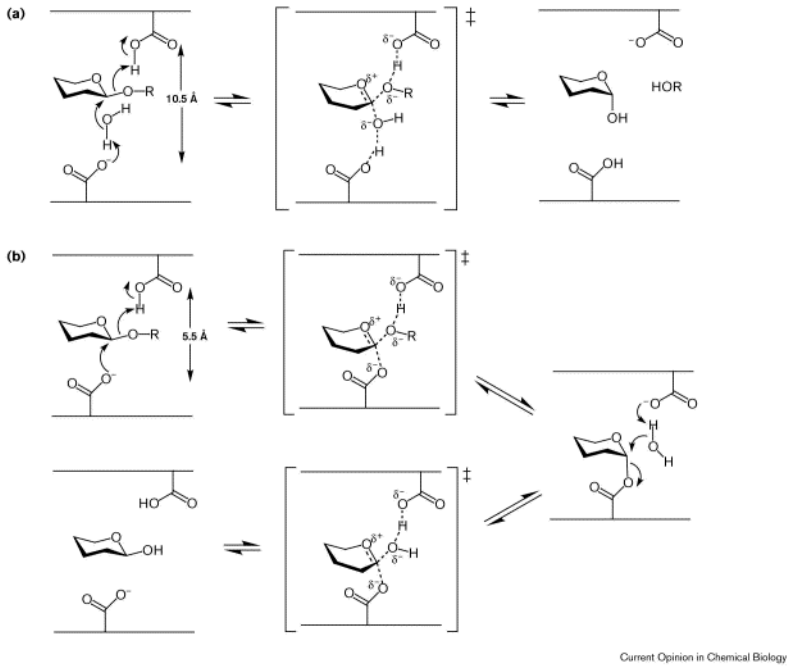
Figure 1.2. Molecular recognition

In a general scheme for the mechanism of hydrolysis (Scheme 1.1) cleavage of the glycosidic bond is achieved by attack of a water molecule catalysed by the enzyme.



Scheme 1.1. Glycosidic bond hydrolysis

The hydrolysis of the glycosidic bond is performed by two catalytic residues of the enzymes: a general acid (proton donor) and a nucleophile (base); depending on the peculiar position of these catalytic residues, hydrolysis occurs via overall retention or overall inversion of the anomeric configuration (Scheme 1.2).



Scheme 1.2. General glycosidase mechanisms for (a) an inverting β-glycosidase and (b) a retaining β-glycosidase[2]

Both mechanisms involve oxocarbenium-ion-like transition states and a pair of carboxylic acids at the active site. In inverting glycosidases, these two residues are located approximately 10 Å (+/- 2 Å) apart on average[3,4] and the

[2] Rye, C. S.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573.
[3] McCarther, J.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885.

reaction occurs via a single-displacement mechanism by a water molecule where one carboxylic acid acts as a general base and the other as a general acid (Scheme 1.2a). In retaining enzymes, the two carboxylic residues are approximately 5.5 Å apart[3,4] and the reaction proceeds via a double displacement mechanism (Scheme 1.2b).

1.1.2 How to inhibit glycosidases

The cleavage of the glycosidic bond is so general that glycosidases are involved in a large number of metabolic and catabolic processes, from the most obvious like the intestinal digestion of carbohydrates to the complex processes of cell-cell or virus-cell recognition. Here we present some important examples.

1.1.2.a Intestinal digestion: treatment of type II diabetes

The intestinal di- and oligosaccharidases are fixed components of the cell membrane of the brush border region of the small intestine wall. These enzymes digest dietary carbohydrates to monosaccharides which are absorbed through the intestinal wall. This group of enzymes include sucrase, maltase, isomaltase, lactase, trehalase and hetero-β-glucosidase. In the late 1970s, it was realized that inhibition of all or some of these enzymes could regulate the absorption of carbohydrates and such inhibitors could be used therapeutically in the oral treatment of non-insulin-dependent diabetes mellitus (NIDDM or type II diabetes).[5]

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[4] Wang, Q.; Graham, R. W.; Trimbur, D.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **1994**, *116*, 11594.
[5] Schmidt, D. D.; Frommer, W.; Müller, L.; Truscheit, E. *Naturwissenschaften* **1979**, *66*, 584.

1.1.2.b Post translational processes

The biosynthesis of the oligosaccharide chains of all the *N*-linked glycoproteins is initiated by the cotranslational transfer of a common oligosaccharide precursor (consisting of two *N*-acetylglucosamines, nine mannose and three glucose units) to the glycosylation site of newly synthesized polypeptides in the endoplasmic reticulum.[6] The *N*-glycan chain is modified by a series of reactions within the endoplasmic reticulum and the Golgi's apparatus, including trimming by the action of specific processing glycosidases. The final oligosaccharide structures depend on the polypeptides and on which processing glycosidases and glycosyltransferases are expressed in the cells. Endoplasmic reticulum glycosidases are essential for the maturation and endoplasmic reticulum quality control of glycoproteins containing *N*-glycans and for the normal development of cells, because they define the correct protein synthesis.[7]

1.1.2.c Degradation mechanisms: treatment of genetic disorders

Recent experimental data show that some human genetic diseases are due to mutations in proteins which influence their folding and lead to retaining of mutant proteins in the endoplasmic reticulum and subsequent degradation. The degradative function is carried out by more than 50 acid-dependent hydrolases contained within the lumen. Lysosomal enzymes are all *N*-glycosylated glycoproteins.[8] The glycosphingolipid (GSL) storage diseases are genetic disorders in which a mutation of one of GSL glycohydrolases blocks GSL degradation, leading to lysosomal accumulation of undegraded GSL.[9] The only

[6] a) Elbein, A. D. *Ann. Rev. Biochem.* **1987**, *56*, 497. b) Elbein, A. D. *FASEB J.* **1991**, *5*, 3055.
[7] a) Herscovics, A. *Biochim. Biophys. Acta* **1999**, *1473*, 96. b) Parodi, A. J. *Biochem. J.* **2000**, *348*, 1.
[8] Kornfeld, S.; Mellman, I. *Annu. Rev. Cell Biol.* **1989**, *5*, 483.
[9] Neufeld, E. F. *Annu. Rev. Biochem.* **1991**, *60*, 257.

successful treatment for such diseases to date is the enzyme replacement therapy for patients with type I Gaucher disease[10] and Fabry disease.[11]

1.1.2.d Molecular recognition

Molecular recognition occurs when two molecules interact in a specific, and non covalent way (such as hydrogen bridges, hydrophobic interactions, Van der Waals forces, etc.). In the biological systems there are a lot of interaction processes based on molecular recognition, as represented in Figure 1.2. The common feature for most of the recognition processes is that they are carried out by interaction of oligosaccharide chains. Therefore, the synthesis of oligosaccharides, as well as glycoproteins or glycolipids, is a fundamental step for molecular recognition and therefore for the functions of biological systems. It was shown that inhibitors of glycosidases bear interesting antiretroviral activities due to their interference with the correct glycosylation of viral envelope glycoproteins.[12]

[10] Barton, N. W.; Furbish, F. S., Murray, G. J.; Garfield, M.; Brady, R. O. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1913.
[11] Schiffmann, R.; Murray, G. J.; Treco, D.; Daniel, P.; Sellos-Moura, M.; Myers, M.; Quirk, J. M.; Zirzow, G. C.; Borowsky, M.; Loveday, K.; Anderson, T.; Gillespie, F.; Oliver, K. L.; Jeffries, N. O.; Doo, F.; Liang, T. J.; Kreps, C.; Gunter, K.; Frei, K.; Crutchfield, K.; Selden, R. F.; Brady, R. O. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 365.
[12] Greimel, P.; Spreitz, J.; Stütz, A. E.; Wrodnigg, T. M. *Curr. Top. Med. Chem.* **2003**, *3*, 513.

1.2 Iminosugars: Glycosidases Inhibitors

1.2.1 Introduction

Iminosugars are structural analogues of 'true' sugars in which the ring oxygen atom is replaced by a nitrogen atom (Figure 1.3).

sugar iminosugar

Figure 1.3. structure of iminosugars

The presence of nitrogen in the ring provides a charged site under physiological conditions which is largely responsible for their very strong interaction with glycosidases enzymes. Indeed, the protonated nitrogen atom is able to mimic the oxocarbenium ion involved in the transition state of the hydrolysis catalyzed by these enzymes. For this reason iminosugars are very potent inhibitors of glycosidases, and since these enzymes are involved in a number of essential physiological processes, they possess an immense potential as therapeutic agents and many of such applications are starting to emerge. In addition, as a result of similarities between the mechanisms of glycoside hydrolysis and glycosyl transfer, novel applications of iminosugars in the control of biological glycosylation are being uncovered which considerably extend the scope of interest of these carbohydrate mimics.

A lot of iminosugars were discovered in plants known for their therapeutic action against diarrohea, dysentery and colic, cancers, diabetes and bacterial infections and some of these have been already developed as drugs. For example, Acarbose (Figure 1.4) was introduced on the market under the name Glucobay™ in the 1990s, for the treatment of type II diabetes and *N*-butyl-DNJ, under the name Zavesca® (Figure 1.4), was released for the treatment of Gaucher's disease.

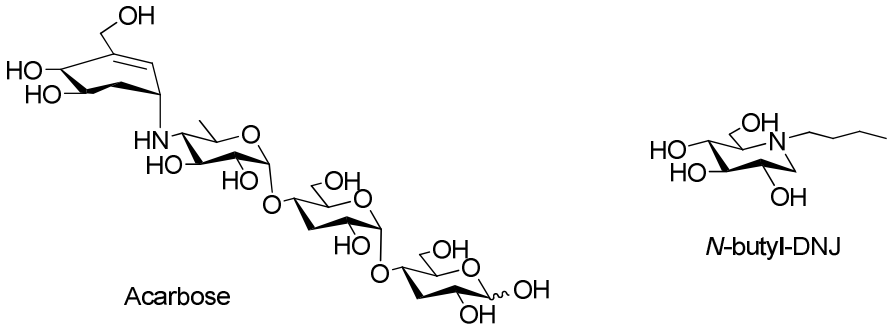

Figure 1.4. Glucobay (acarbose) and Zavesca (*N*-butyl-DNJ)

1.2.2 Classification

Iminosugars belong to the polyhydroxylated alkaloid super-family of natural products. They can be classified in several ways: according to their carbon structure, to the functional groups they contain, to the activities in which they are involved, for their therapeutic applications, or to their natural sources, etc. The systematic and intuitive classification is based on their structure.

They are present in an enormous amount of biological systems, such as plants or microorganisms. Undoubtedly the most numerous are monocyclic derivatives such as piperidines and pyrrolidine, which may also contain various substituents or functional groups to form disaccharides or oligosaccharides. Among the bicyclic iminosugars, within all the existing possibilities in nature are three basic types: pyrrolizidines, indolizidines and nortropanes. They all have at least a pyrrolidine ring.

Here we present a few examples of natural iminosugars, classified according to their structure.

1.2.2.a Piperidines

In 1966, Inouye et al.[13] discovered the first natural polyhydroxylated alkaloid, nojirimycin (NJ, Figure 1.5). Isolated from a *Streptomyces* filtrate, it was shown to actively inhibit α- and β-glucosidases and was therefore the first natural glucose mimic. Nojirimycin B (commonly called mannonojirimycin, Figure 1.5)[14] and galactostatin (galactonojirimicyn, Figure 1.5)[15] were isolated soon after.

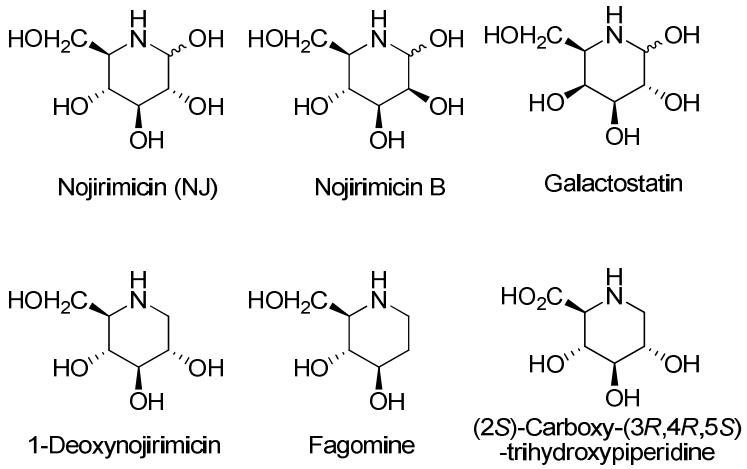

Figure 1.5. Piperidine iminosugars

Azasugars bearing a hydroxyl group at C-1 were found to be relatively difficult to isolate and handle due to their instabillity. The first report of the synthesis of an iminosugar was the synthesis of 1-deoxynojirimicin (DNJ, Figure 1.5) by Paulsen and co-workers in 1966[16] and after by Inouye et al., which was achieved through the reduction of the anomeric hydroxyl group.[17] However,

[13]a) Inouye, S.; Tsuruoka, T.; Niida, T. *J. Antibiot.* **1966**, *19*, 288. b) Inouye, S.; Tsuruoka, T.; Ito, T.;Niida, T. *Tetrahedron.* **1968**, *24*, 2125
[14] Niwa, T.; Tsouruoka, T.; Goi, H.; Kodama, Y.; Itoh, J.; Inouye, S.; Yamada, Y.; Niida, T.; Nobe, M.; Ogawa, Y.; *J. Antibiot.* **1984**, *37*, 1579.
[15] Miyake, Y.; Ebata, M. *Agric. Biol. Chem.* **1988**, *52*, 661.
[16] Paulsen, H. *Angew. Chem. Int. Ed. Engl.* **1966**, *78*, 495.
[17] Inouye, S.; Tsuruoka, T.; Ito, T.; Niida, T. *Tetrahedron* **1968**, *24*, 2125.

DNJ was soon afterwards isolated from Mulberry trees[18] as well as *Streptomyces* cultures.[19] A further DNJ analogue is the 1,2-dideoxynojirimycin, fagomine (figure 1.5). Initially found in the seeds of *Fagopyrum escuelentum* (Polygonaceae) seeds,[20] it was later discovered in the leaves and roots of Xanthocercis zambesiaca (Leguminosae) together with other fagomine analogues and derivatives.[21]

In 1984, Cenci di Bello et al. extracted from the seeds of *Baphia racemosa* the first naturally occurring azaglucuronic acid, (2*S*)-carboxy-(3*R*,4*R*,5*S*)-trihydroxypiperidine (Figure 1.5), which was found to inhibit the human liver β-D-glucuronidase and α-L-iduronidase.[22]

1.2.2.b Pyrrolidines

The five-membered pyrrolidine family of iminosugars is as important as the piperidine derivatives. As a matter of fact, five-membered ring iminosugars have been the object of extensive biological and chemical investigations.

The first natural pyrrolidine alkaloid isolated was (2*R*,3*R*,4*R*,5*R*)-2,5-dihydroxymethyl-3,4-dihydropyrrolidine (DMDP, Figure 1.6), as a mimetic of β-D-fructofuranose, from *Derris elliptica*[23] and after found in a lot of microorganisms[24] like a common metabolite.

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[18] Yagi, M.; Kouno, T.; Aoyagi, Y.; Murai, H. *Nippon Nogei Kagaku Kaishi* **1976**, *50*, 571.
[19] Murao, S. Miyata, S. *Agric. Biol. Chem.* **1988**, *52*, 661.
[20] Koyama, M.; Sakamura, S. . *Biol. Chem.* **1974**, *38*, 1111.
[21] Kato, A.; Asano, N.; Kizu, H.; Matsui, K.; Watson, A. A.; Nash, R. J. *J. Nat. Prod.* **1997**, *60*, 312.
[22] Cenci di Bello, I.; Dorling, P.; Fellows, L. E.; Winchester, B. *FEBS Lett.* **1984**, *176*, 61.
[23] Welter, A.; Jadot, J.; Dardenne, G.; Marlier, M.; Casimir, J. *Phytochem.* **1976**, *15*, 747.
[24] Watson, A. A.; Fleet, G. W. J.; Asano, N.; Molyneux, R. J.; Nash, R. J. *Phytochem.* **2001**, *56*, 265.

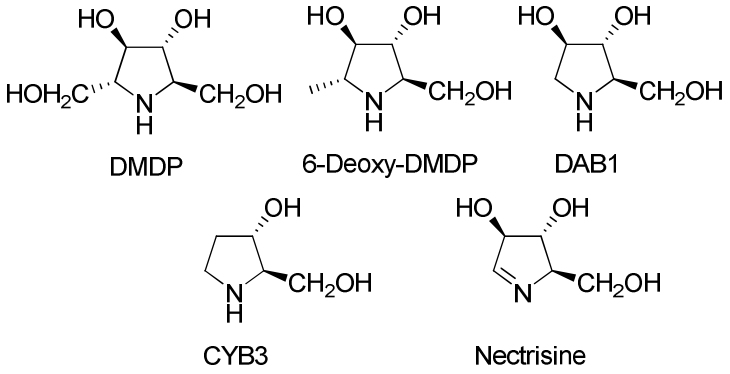

Figure 1.6. Pyrrolidine iminosugars

In fact there are many natural derivatives with a structure very similar to DMDP, in which one or more different groups or carbon atoms are removed; for instance, 6-deoxy-DMDP (Figure 1.6) or 1,4-dideoxy-1,4-imino-D-arabinitol (DAB-1, Figure 1.6)[25] or unsatured systems like nectrisine.[26]

Pyrrolidine, although structurally different from natural enzyme substrates, are generally very good inhibitors because they are also able to mimic the transition state of the enzymatic reactions.[27]

1.2.2.c Indolizidines

Indolizidines are bicyclic alkaloids where the azapyranose ring is fused to a pyrrolidine ring via *N*-bridge.

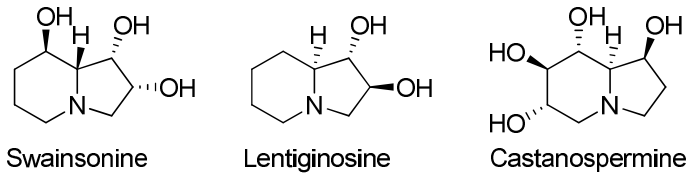

Figure 1.7. Indolizidine iminosugars

[25] Nash, R. J.; Bell, E. A.; Fleet, G. W. J.; Jones, R. H.; Williams, J. M. *J. Chem. Soc.-Chem. Commun.* **1985**, 738.
[26] Shibata, T.; Nakayama, O.; Tsurumi, Y.; Okuhara, M.; Terano, H.; Kohsaka, M. *J. Antibiot.* **1988**, *41*, 296.
[27] a)Tschamber, T.; Gessier, F.; Dubost, E.; Newsome, J.; Tarnus, C.; Kohler, J.; Neuburger, M.; Streith, J. *Bioorg. Med. Chem.* **2003**, *11*, 3559. b) Chevrier, C.; Defoin, A.; Tarnus, C. *Bioorg. Med. Chem.*, **2007**, *15*, 4125.

The first example is swainsonine (Figure 1.7), isolated in 1979 from the leaves of *Swainsona canescens* (Leguminosae).[28] Other indolizidine alkaloids are lentiginosine and castanospermine, the bicyclic equivalent of DNJ having an ethylene bridge between the hydroxymethyl group and the nitrogen atom (Figure 1.7). [29]

There have been extensive studies on the toxicity of swainsonine[28] and castanospermine[30] containing legumes *S. canescens* and *C.* a*ustrale*, respectively. In fact swainsonine was also found in locoweeds (*Astragalus* spp. and *Oxytropis* spp.), which causes the disorder 'locoism' in Western United States.[31]

1.2.2.d Pyrrolizidines

Pyrrolizidine structure is composed by two fused pyrrolidines with a bridgehead nitrogen atom.

The first natural polyhydroxylated pyrrolidine was alexine (figure 1.8) from the seeds of *Alexa leiopetala.*[32] Subsequently, australine (Figure 1.8), the 7a-epimer of alexine, was isolated from *Castanospermum australe*. These two pyrrolizidines present a pattern of substitution, on one of the two pyrrolidine rings, that is repeated in a large number of other pyrrolizidines and which reminds the structure of DMDP and castanospermine.

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[28] Colegate, S. M.; Dorling, P. R.; Huxtable, C. R. *Aust. J. Chem.* **1979**, *32*, 2257.
[29] Asano, N.; Nash, R. J.; Molyneux, R. J.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2000**, *11*, 1645.
[30] Hohenschutz, L. D.; Bell, E. A.; Jewess, P. J.; Leworthy, D. P.; Pryce, R. J.; Arnold, E.; Clardy, J. *Phytochem.* **1981**, *20*, 811.
[31] Molyneux, R. J.; James, L. F. *Science* **1982**, *216*, 190.
[32] Nash, R. J.; Fellows, L. E.; Dring, J. V.; Fleet, G. W. J.; Derome, A. E.; Hamor, T. A.; Scofield, A. M.; Watkin, D. J. *Tetrahedron Lett.* **1988**, *29*, 2487.

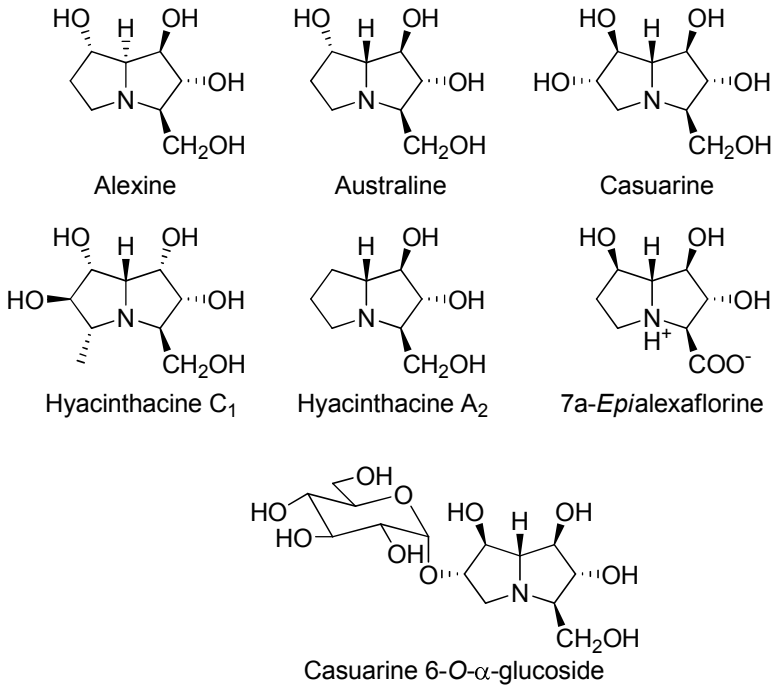


Figure 1.8. Pyrrolizidine iminosugars

Another very interesting pyrrolizidine is casuarine (Figure 1.8). This molecule was found, with its 6-*O*-α-glucoside, in the barks of *Casuarina equisetifolia*,[33] a plant used in traditional medicine in Samoa for the treatment of cancer, or in the leaves of *Eugenia jambolana*.[34]

A series of pyrrolizidines with highly diversified structures were isolated from *Hyacinthoides non-scripta* and *Scilla campanulata* (both from Hyacinthaceae family) and called hyacinthacines (for instance, hyacinthacine C_1 or hyacinthacine A_2, Figure 1.8).[35,36]

[33]Nash, R. J.; Thomas, P. I.; Waigh, R. D.; Fleet, G. W. J.; Wormald, M. R.; de Q. Lilley, P. M.; Watkin, D. J. *Tetrahedron Lett.* **1994**, *35*, 7849.
[34] Wormald, M.; Nash, R.; Watson, A. A.; Bhadoria, B. K.; Langford, R.; Sims, M.; Fleet George, W. J. *Carbohydr. Lett.* **1996**, *2*, 169.
[35] Nash, R. J.; Fellows, L. E.; Dring, J. V.; Stirton, C. H.; Carter, D.; Hegarty, M. P.; Bell, E. A. *Phytochem.* **1988**, *27*, 1403.
[36] Asano, N.; Kuroi, H.; Ikeda, K.; Kuzu, H.; Kameda, Y.; Kato, A.; Adachi, I.; Watson, A. A.; Nash, R. J.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2000**, *11*, 1.

Finally an important example is 7a-*epi*alexaflorine (Figure 1.8), extracted from *Alexa grandiflora*, in which the castanospermine hydroxymethyl group has been replaced with a carboxy group. This is the first example of a natural aminoacid in which the carboxy group is in position 3 of a pyrrolizidine.[37]

1.2.2.e Nortropanes

Nortropanes are a relatively new class of polyhydroxylated alkaloids that contain an azapyranose ring, which are isolated primarily from the *Atropa belladonna* (Solanaceae) and *Convolvulus arvenis* (Convolvunaceae) but also from Moraceae families.[38]

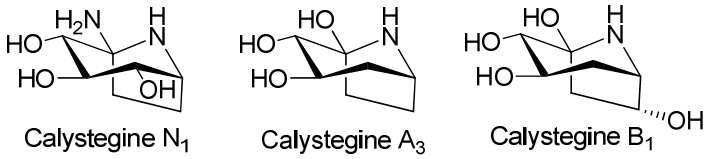
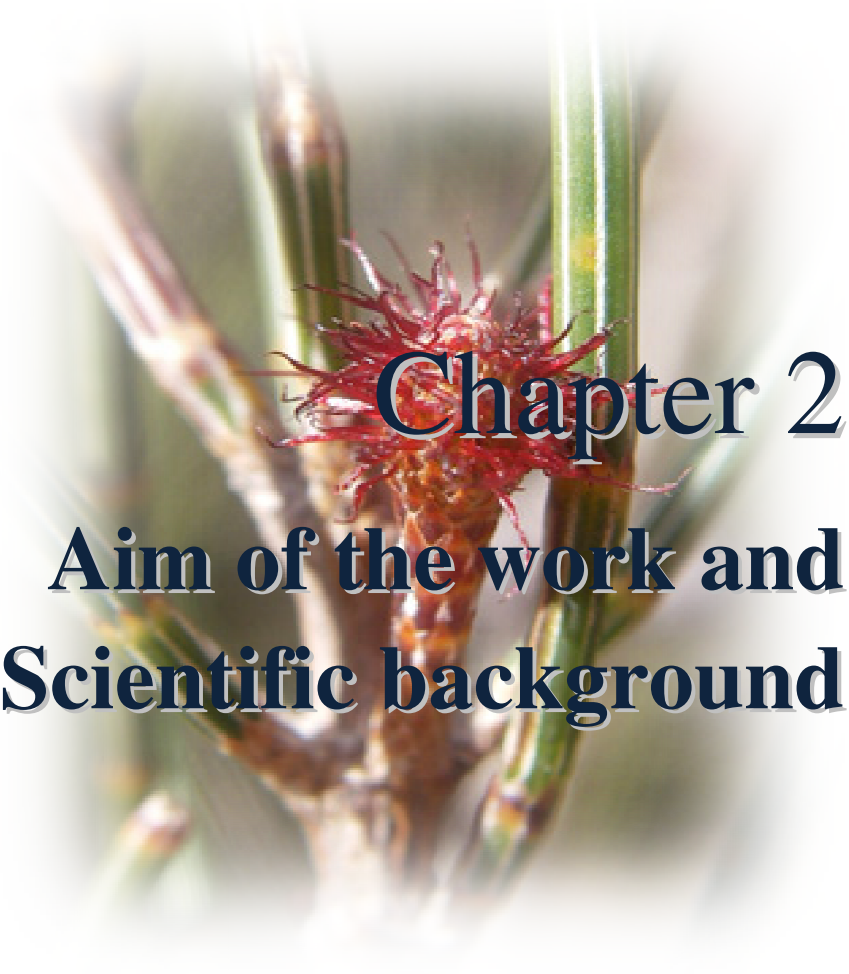


Figure 1.9. Nortropane iminosugars

These iminosugars analogues, called calystegines are interesting because they contain a tertiary hydroxyl group at the bridgehead carbon atom[29] with the exception of calystegine N_1 (Figure 1.9).[24] Based on electrophoresis separation they were divided into two groups, calystegines A and calystegines B.[39] The major component of the A group was calystegine A_3 (Figure 1.9) and of the group B was calystegine B_1 (Figure 1.9).

[37] Pereira, A. C. D.; Kaplan, M. A. C.; Maia, J. G. S.; Gottlieb, O. R.; Nash, R. J.; Fleet, G.; Pearce, L.; Watkin, D. J.; Scofield, A. M. *Tetrahedron* **1991**, *47*, 5637.
[38] Asano, N.; Oseki, K.; Tomioka, E.; Kizu, H.; Matsui, K. *Carbohydr. Res.* **1994**, *259*, 243.
[39] Tepfer, D.; Goldmann, A.; Pamboukdjian, N.; Maille, M.; Lepingle, A.; Chevalier, D.; Denarie, J.; Rosenberg, C. *J. Bacteriol.* **1988**, *170,* 1153.



Chapter 2

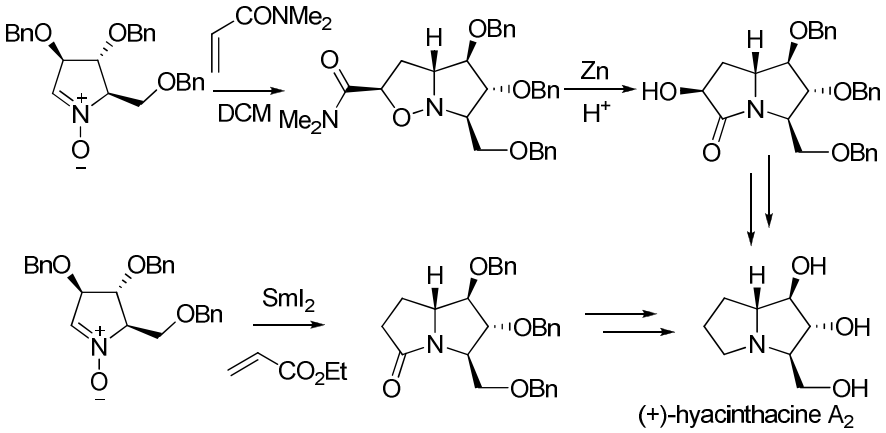
Aim of the work and Scientific background

2.1 The PhD Thesis

Due to the therapeutic properties of natural iminosugars, this work is aimed to the total synthesis of some natural pyrrolizidine and pyrrolidine alkaloids and their unnatural analogues. The natural iminosugars synthesized were chosen on the basis of their biological activity, and the unnatural ones were targeted for the purpose of molecular modelling studies.

The most important family studied is based on the casuarine structure: the total synthesis of casuarine and the first total synthesis of its 6-*O*-α-glucoside and of a series of unnatural derivatives, were accomplished.

Two different synthetic strategies were used, in order to afford the same or different iminosugars. In all cases the common building block was a carbohydrate derived nitrone (see chapter 2.2.2) previously used in our research group for the synthesis of hyacinthacine A_2 (Scheme 2.1), a related pyrrolizidine alkaloid (See Chapter 3.2.2).

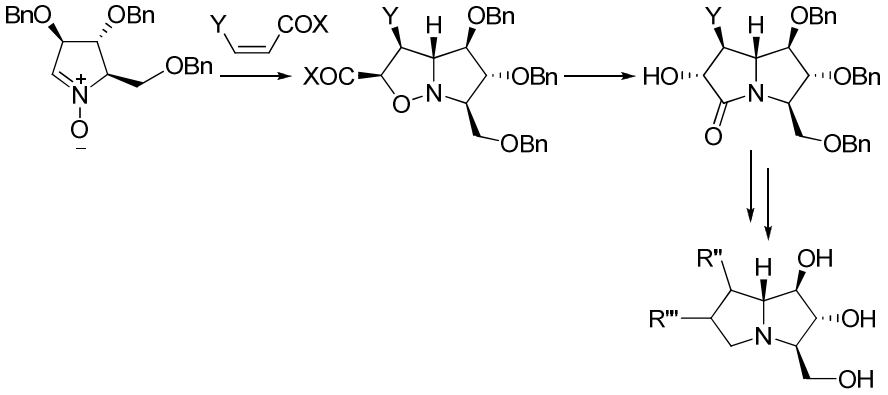


Scheme 2.1. Sintheses of Hyacinthacine A_2 by Goti and co-workers and by Py and co-workers

The same nitrone, with a different synthetic approach based on a pinacol-type coupling, was used shortly after by Py and co-workers[40] to obtain the same target.

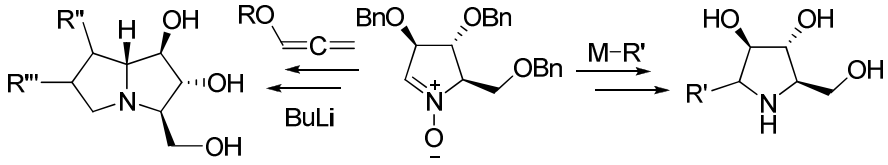
While the synthesis developed in our group was based on a 1,3-dipolar cycloaddition approach, the Py's synthetic strategy used the inversion of the C-N bond polarity in nitrones which represent the donor partner in presence of SmI_2.[40] The key step was stereoselective reductive coupling of the nitrone with ethyl acrylate, which allowed the assembly of the bicyclic core of the target molecule (Scheme 2.1).

Starting from this nitrone, our first synthetic strategy to casuarine type targets was based on the 1,3 dipolar cycloaddition reaction between a chiral nitrone and suitably substituted *cis*-alkenes (Scheme 2.2).



Scheme 2.2. 1,3-dipolar cycloaddition reaction based synthetic strategy

The alternative strategy was based on the addition of organometal derivatives to the chiral nitrone to give pyrrolidine or pyrrolizidine alkaloids (Scheme 2.3).



Scheme 2.3. Organometallic addition synthetic strategy

[40] Desvergnes, S.; Py, S.; Vallée, Y. *J. Org. Chem.* **2005**, *70*, 1459.

2.2 Nitrones

As previously mentioned, the key intermediate for both the envisaged synthetic strategies was a nitrone. Here are briefly outlined the features of nitrones with particular emphasis on their reactivity.

2.2.1 Nitrones and their reactivity

Nitrones are dipolar compounds, i.e. "electrically neutral molecules carrying a positive and a negative charge in one of their major canonical descriptions".[41] In particular, they are best represented by the two limit structures **A** and **B** (Figure 2.1), with the negative charge mostly located on the oxygen atom and the positive charge delocalized at the nitrogen atom (structure **A**) and at the C-1 carbon atom (structure **B**).

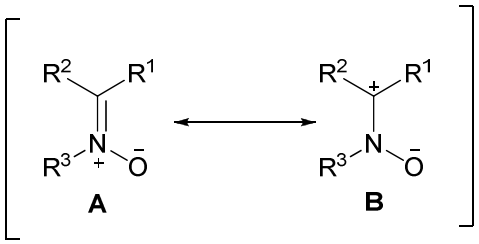
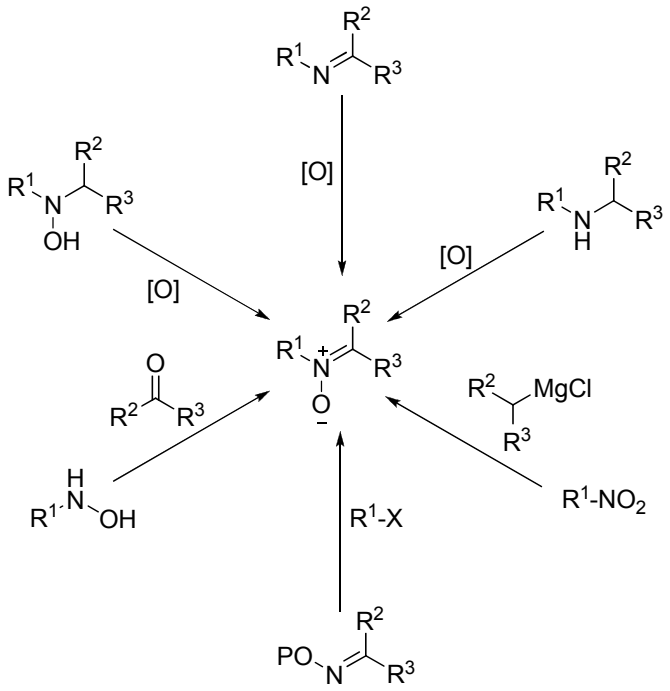


Figure 2.1. Nitrone charge delocalization

Nitrones (or azomethine oxides) were first prepared by Beckmann in 1890[42] and named after a shortening of "nitrogen-ketones" by Pfeiffer in 1916 to emphasize their similarity to ketones.[43]

To date various methods are known for the synthesis of nitrones, starting from different substrates, as described in Scheme 2.4:

[41] McNaught, A. D.; Wilkinson, A. In *IUPAC Compendium of Chemical Terminology* 2nd Edition, 1997.
[42] a) Beckmann, E. *Ber. Dtsch. Chem. Ges.* **1890**, *23*, 3331. b) Beckmann, E. *Ber. Dtsch. Chem. Ges.* **1894**, *27*, 1957.
[43] Pfeiffer, P. *Annalen* **1916**, 72.



Scheme 2.4. Synthetic strategies for the obtainment of nitrones

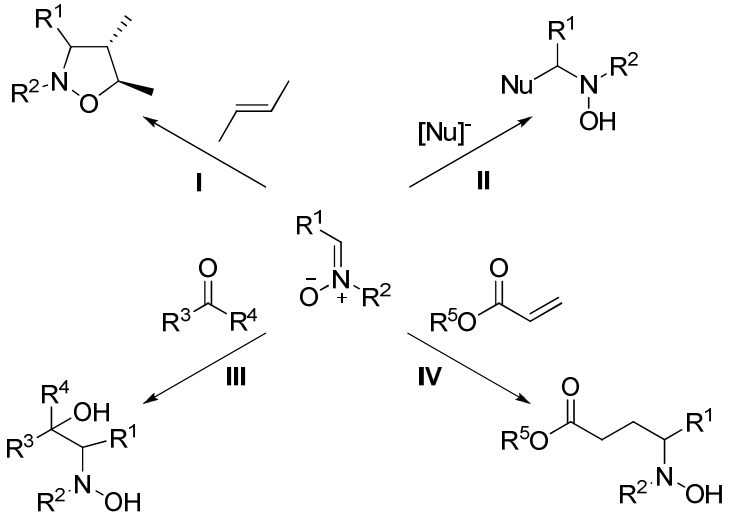
The main methods for nitrone synthesis are: oxidation of hydroxylamines, amines and imines; *N*-alkylation of oximes on nitrocompounds; condensation of carbonyl compounds with hydroxylamines and are widely described in the literature.[44]

As mentioned before, nitrones are extremely versatile compounds and they have found wide application in organic synthesis; in particular they are very useful tools for the construction of structurally complex molecules and particularly nitrogen-containing biologically active compounds.

Their chemistry is hugely varied and frequently reviewed, but is dominated by their use as 1,3-dipoles (Scheme 2.5, route **I**) for cycloaddition

[44] Revuelta, J.; Cicchi, S.; Goti, A.; Brandi, A. *Synthesis* **2007**, *4*, 485.

reactions[45] and in reaction with nucleophiles (Scheme 2.5, route **II**)[46] because of their highly polarized C=N bond.



Scheme 2.5. Nitrones reactivity

Furthermore, the synthetic applications of nitrones have recently been extended to a novel reductive cross-coupling process (pinacol-type coupling) between nitrones and carbonyl compounds (Scheme 2.5, routes **III** and **IV**),

[45] a) Tufariello, J. J. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A. Ed; John Wiley & Sons: New York **1984**. b) Confalone, P. N.; Huie, E. M. *Org. React.* **1988**, *36*, 1. c) Torssell, K. B. G. *Nitrile Oxides, Nitrones, and Nitronates in Organic Synthesis*; Feuer, H., Ed.; VCH Publishers: New York **1988**. d) Frederickson, M. *Tetrahedron* **1997**, *53*, 403. e) Gothelf, K. V.; Jørgensen, K. A. *Chem. Rev.* **1998**, *98*, 863. f) Jones, R. C. F.; Martin, J. N. In *Synthetic Applications of 1,3-Dipolar Cycloaddition Chemistry Toward Heterocycles and Natural Products*; Padwa, A.; Pearson, W. H., Eds.; John Wiley & Sons: New York **2002**. g) Osborn, H. M. I.; Gemmell, N.; Harwood, L. M. *J. Chem. Soc. Perkin Trans. 1* **2002**, 2419. h) Desimoni, G.; Tacconi, G.; Barco, A.; Pollini, G. P. *Natural Products Synthesis Through Pericyclic Reactions*; ACS Monograph n. 180; Caserio, M. C., Ed.; American Chemical Society: Washington **1983**. i) Annunziata, R.; Cinquini, M.; Cozzi, F.; Raimondi, L. *Gazz. Chim. Ital.* **1989**, *119*, 253.

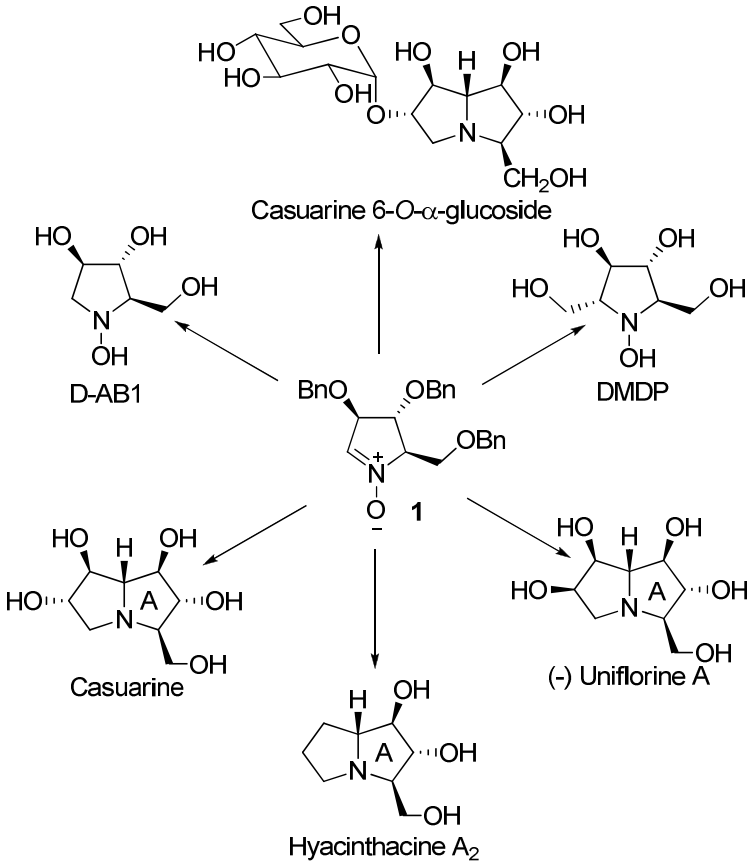
[46] a) Bloch, R. *Chem. Rev.* **1998**, *98*, 1407. b) Enders, D.; Reinhold, U. *Tetrahedron: Asymmetry* **1997**, *8*, 1895. c) Lombardo, M.; Trombini, C. *Synthesis* **2000**, 759. d) Merino, P. In *Science of Synthesis*; Padwa, A., Ed.; Thieme: Stuttgart **2004**. e) Merino, P.; Franco, S.; Merchan, F. L.; Tejero, T. *Synlett.* **2000**, 442. f) Lombardo, M.; Trombini, C. *Curr. Org. Chem.* **2002**, *6*, 695.

promoted by samarium diiodide, and other metal-mediated reactions.[47] The reactions produce vicinal amino alcohols[48] or β-amino acids,[49] thereby enhancing the synthetic versatility of these reagents. Likewise, the use of nitrones as free-radical traps (spin traps) is well established, as they have been designed to react with oxygen radicals to lengthen their half-lives and allow their detection in EPR studies.[50] Nitrones have also been used in vivo as protective agents against radicals generated by oxidative stress.[51]

[47] Cardona, F.; Goti, A. *Angew. Chem. Int. Ed.* **2005**, *44*, 7832.
[48] Masson, G.; Py, S.; Vallée, Y. *Angew. Chem. Int. Ed.* **2002**, *41*, 1772.
[49] a) Masson, G.; Cividino, P.; Py, S.; Vallée, Y. *Angew. Chem. Int. Ed.* **2003**, *42*, 2265. b) Riber, D.; Skrydstrup, T. *Org. Lett.* **2003**, *5*, 229.
[50] Janzen, E. G.; Haire, D. L.; In *Advances in Free-Radical Chemistry*; Tanner, D. D., Ed.; JAI Press: Greenwich **1990**.
[51] a) Floyd, R. A.; Hensley, K. *Ann. N. Y. Acad. Sci*. **2000**, *899*, 222. b) Floyd, R. A. *Proc. Soc. Exp. Biol. Med.* **1999**, *222*, 236.

2.2.2 The key intermediate

As already mentioned all the products synthesized derive from a common nitrone key intermediate (scheme 2.6):

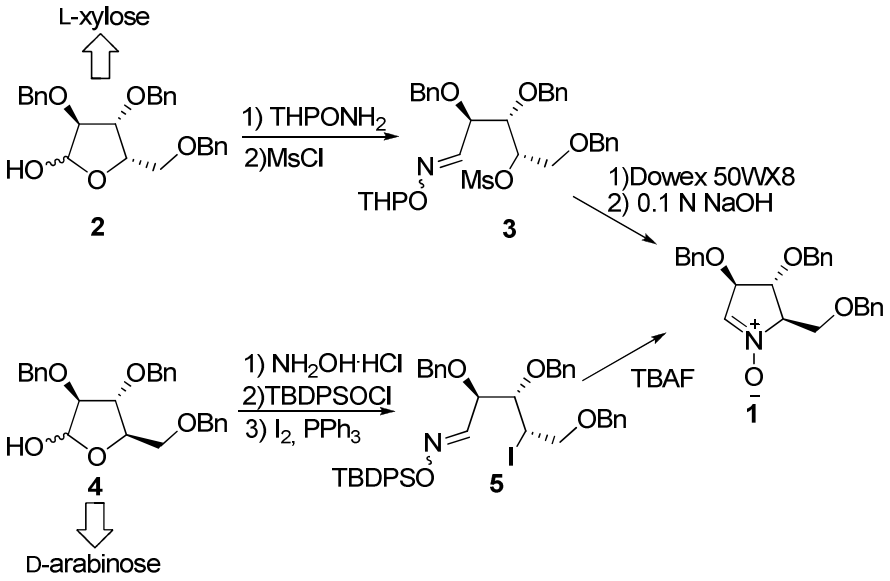


Scheme 2.6. The key intermediate

Nitrone **1** has the correct stereochemistry of the pyrrolidine alkaloids and of ring A of the pyrrolizidine alkaloids depicted in Scheme 2.6.

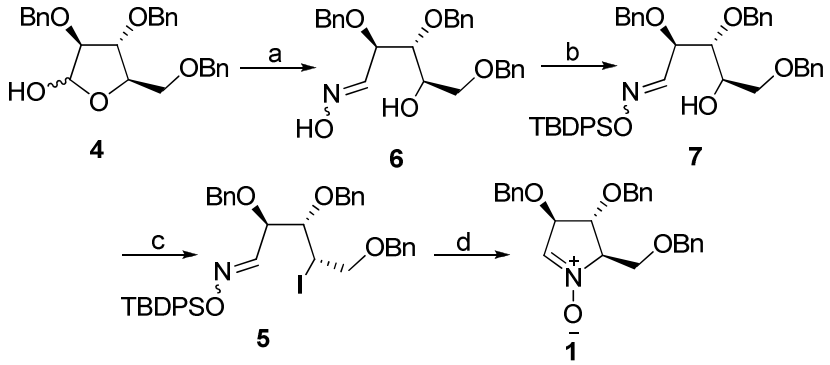
Nitrone **1** can be obtained from products of the chiral pool by a "one-pot" strategy starting from tribenzyl L-xylose,[52] or stepwise from tribenzyl D-arabinose (Scheme 2.7).[53]

[52] Cicchi, S.; Marradi, M.; Vogel, P.; Goti, A. *J. Org. Chem.* **2006**, *71*, 1614.



Scheme 2.7 Synthesis of nitrone 1

The best synthetic strategy, thanks to the greater accessibility of the starting material and the best overall yield, is that from tribenzyl D-arabinose (**4**, Scheme 2.8).



Scheme 2.8. Synthesis of nitrone 1 from D-arabinofuranose
Reaction conditions: a) $NH_2OH^{\cdot}HCl$, py, rt, 24 h, 100%; b) TBDPSCl, py, rt, 18 h, 100%; c) I_2, PPh_3, Im, toluene, 60 °C, 2 h, 85%; d) TBAF, THF, rt, 18 h, 96%

The key feature of this synthetic strategy is the double inversion of configuration at C-4 of tribenzyl D-arabinose (**4**), obtained through iodination

[53] a) Cardona, F.; Faggi, E.; Liguori, M.; Cacciarini, M.; Goti, A. *Tetrahedron Lett.* **2003**, *44*, 2315. b) Cramona, A. T.; Wightman, R. H.; Robina, I.; Vogel, P. *Helv. Chim. Acta* **2003**, *86*, 3066. c) Ref. 40

of alcohol **7** followed by cyclization of **5** to give **1** (Scheme 2.8).[53a] The two-fold inversion of configuration at C-4 of the carbohydrate that occurs in this sequence ensures the access to nitrones in wich the absolute configuration, with respect to the starting carbohydrate derivative, is overall retained at all stereocenters. Nitrone **1** has the correct relative and absolute stereochemistry at C-1, C-2 and C-3 of all the pyrrolizidine and at C-2, C-3 and C-4 of all the pyrrolidine target molecules.

2.3 Experimental Section

2.3.1 General

Commercial reagents were used as received. All reactions were magnetically stirred and monitored by TLC on 0.25 mm silica gel plates (Merck F_{254}) and column chromatography was carried out on Silica Gel 60 (32-63 μm). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. NMR spectra were recorded on Varian Mercury-400, Varian INOVA-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz), or Varian Gemini-200 (^{13}C, 50 MHz) spectrometers. Infrared spectra were recorded with a Perkin-Elmer Spectrum BX FT-IR System spectrophotometer. Mass spectra were recorded on a QMD 1000 Carlo Erba instrument by direct inlet; relative percentages are shown in brackets. ESI mass spectra were recorded by direct inlet on a ThermoScientific LCQ Fleet Ion Trap spectrometer with Surveyor Plus LC System; relative percentages are shown in brackets. ESI full MS were recorded on a Thermo LTQ instrument by direct inlet; relative percentages are shown in brackets. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter.

(1*E*)- and (1*Z*)-2,3,5,-Tri-O-benzyl-D-arabinose-*O*-oximes (**6**).[54] 2,3,5-Tri-*O*-benzyl-D-arabinose (**4**, 12.5 g, 29.7 mmol) and hydroxylamine hydrochloride (8.29 g, 119 mmol) were dissolved in dry pyridine (60 mL), and 30 g of 3Å pellets molecular sieves were added under nitrogen atmosphere. The reaction mixture was left stirring at rt, under nitrogen atmosphere for 24 h. Then, the molecular sieves were filtrated and washed several times with toluene. Evaporation under reduced pressure afforded an oil that was subsequently partially dissolved in Et_2O. After filtration, evaporation under reduced pressure of the filtrate afforded oxime **6** as a yellow oil, enough pure to be used for the

[54] Julina, R.; Vasella, A.. *Helv. Chim. Acta* **1985**, *68*, 819-830.

next step. Both diastereoisomers (*E* and *Z*) are formed (12.9 mg, quantitative yield). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.60 (d, J=7.8 Hz, 1 H, H-1 (*E*)) 7.40-7.24 (m, 15 H (*E*) + 15 H (*Z*)), 7.20 (d, J=7.0 Hz, 1 H, H-1 (*Z*)), 4.71-4.37 (m, 7 H (*Z*) + 7 H (*E*)), 4.13-4.08 (m, 1H (*E*) + 1 H (*Z*)), 3.78-3.68 (m, 3 H (*E*) + 3 H (*Z*)).

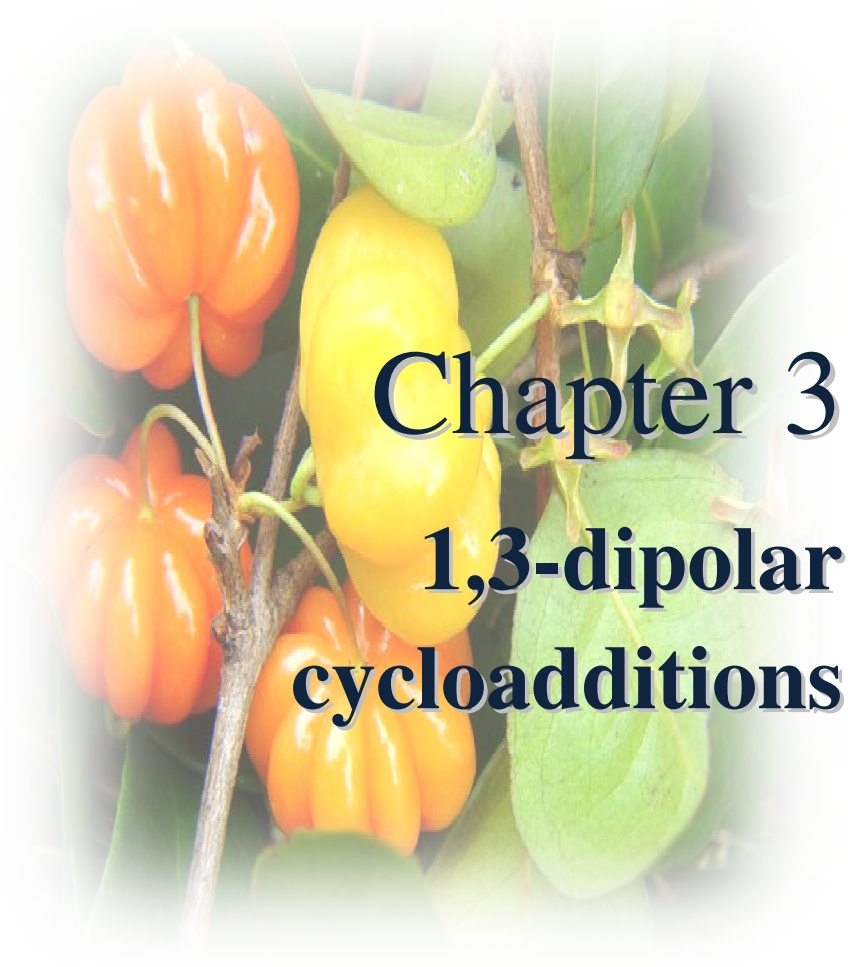
(1*E*)-and(1*Z*)-2,3,5,-tri-*O*-benzyl-D-arabinose-*O*-[(*tert*-butyl)diphenylsilyl] oximes (**7**). To a cooled (0 °C) solution of **6** (12.9 g, 29.7 mmol) in 70 mL of dry pyridine, TBDPSCl (7.73 mL, 29.7 mmol) was added dropwise under nitrogen atmosphere. The mixture was left stirring at rt under nitrogen atmosphere for 18 h. After filtration over Celite® and washing with toluene, the mixture was evaporated under reduced pressure to afford **7** as a yellow oil (20 g, quantitative yield), enough pure to be used for the next step. An analytically pure sample was obtained by filtration over a short pad of silica gel (eluent petroleum ether/ Et_2O 3:2, R_f = 0.26). $[\alpha]_D^{24}$ = – 24.4 (c 1.32, $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.92-7.76 (m, 10 H, Ar), 7.52-7.26 (m, 16 H, Ar, H-1), 4.77-4.56 (m, 6 H, Bn), 4.38-4.32 (m, 1 H), 4.15-4.06 (m, 1 H), 3.76-3.69 (m, 3 H), 1.26 (s, 9 H, *t*Bu). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 153.7 (d, C=N), 137.3, 136.6 (s, Ar), 135.0-134.3 (d, 10 C, Ar), 132.8-132.5 (s, 3 C, Ar), 129.3-124.8 (d, 15 C, Ar), 79.2 (d, 1C), 77.3 (d, 1 C), 76.6 (t, 1 C), 73.3 (t, 1 C), 72.8 (t, 1 C), 70.4 (t, 1 C), 69.5 (d, 1 C), 26.6 (q, 3 C, *t*Bu), 18.8 (s, *t*Bu). IR ($CDCl_3$): 3571, 3071, 2931, 2859 cm^{-1}. MS (EI): *m/z* 616 (M^+-*t*Bu, 5), 508 (5), 402 (5), 199 (100), 181 (45), 91 (100). Anal. Calcd. For $C_{42}H_{47}NO_5Si$ (673.90): C, 74.84; H, 7.04; N, 2.08. Found: C, 74.45; H, 7.06; N, 2.26.

(1*E*)- and (1*Z*)-2,3,5-Tri-*O*-benzyl-4-deoxy-4-iodo-L-xylose-*O*-[(*tert*-Butyl) diphenylsilyl]oximes (**5**). To a stirred solution of **7** (9.8 g, 14.5 mmol) in dry toluene (100 mL), I_2 (5.53 g, 21.8 mmol), PPh_3 (4.95 g, 18.9 mmol) and imidazole (3.05 g, 44.9 mmol) were added under nitrogen atmosphere. The brown mixture was heated at 60-65 °C for 2 h. A saturated aqueous solution of $NaHCO_3$ and $Na_2S_2O_3$ (60 ml) was added and the mixture was left stirring at rt for 15 minutes. Then, it was extracted with CH_2Cl_2 (3×80 mL). The combined

organic phases were washed with a saturated aqueous solution of NaCl, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **5** as a colourless oil, that was quickly filtered over a short pad of silica gel (eluent petroleum ether/EtOAc 10:1, R_f = 0.9) to afford pure **5** (9.6 g, 85 % yield). $[\alpha]_D^{24}$ = + 16.2 (c 1.39,$CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.78-7.62 (m, 10 H, Ar), 7.55 (d, J=7.6 Hz, 1 H, H-1), 7.40-7.10 (m, 15 H, Ar), 4.86 (d, J=11.7 Hz, 1 H), 4.66 (d, J=11.3 Hz, 1 H), 4.45 (d, J=11.3 Hz, 1 H), 4.30-4.20 (m, 5 H), 4.15-4.07 (m, 1 H), 3.74-3.69 (m, 1 H), 3.60-3.48 (m, 1 H), 1.09 (s, 9 H, *t*Bu). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 152.8 (d, C=N), 138.3-133.1 (s, 5 C, Ar), 129.7-127.5 (d, 25 C, Ar), 80.9 (d, 1 C), 77.4 (d, 1 C), 74.7 (t, 1 C), 72.8 (t, 1 C), 72.6 (t, 1 C), 71.3 (t, 1 C), 31.3 (d, CH-I), 27.1 (q, 3 C, *t*Bu), 19.3 (s, *t*Bu); IR ($CDCl_3$): 3069, 2930, 2858, 1710, 1589, 1453, 1117 cm^{-1}. MS (EI): *m/z* 657 (M^+-I, 0.3), 508 (4), 309 (20), 199 (44), 91 (100). Anal. Calcd. For $C_{42}H_{46}NO_4ISi$ (783.86): C, 64.35; H, 5.93; N, 1.79. Found: C, 64.16; H, 6.04; N, 2.10.

(3*R*,4*R*,5*R*)-3,4-dibenzyloxy-5-benzyloxymethyl-1-pyrroline-*N*-oxide (**1**). To a stirred solution of **5** (7.7 g, 9.82 mmol) in dry THF (50 mL) a 1 M solution of TBAF in THF (10.2 mL, 10.2 mmol) was added under nitrogen atmosphere. The mixture was left stirring at rt under nitrogen atmosphere for 18 h. The solvent was evaporate under reduced pressure afforded a yellow solid. Purification by flash column chromatography on silica gel (EtOAc /petroleum ether 5:1), afforded pure nitrone **1** as a yellow solid (3.94 g, 96% yield), mp 80-82 °C. $[\alpha]_d^{19}$ = – 41.7 (*c* 1.00, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41-7.30 (m, 15 H, Ar), 6.92 (br s, 1 H, H-2), 4.70 (dd, J=2.1, 1.8 Hz, 1 H, H-3), 4.65 (d, J=12.0 Hz, 1 H, Bn), 4.61-4.58 (m, 4 H, Bn), 4.55 (d, J=12.0 Hz, 1 H, Bn), 4.41 (dd, J=3.4, 2.2 Hz, 1H, H-4), 4.10-4.02 (m, 2 H, Ha-6, H-5), 3.81 (dd, J=9.9, 2.6 Hz, 1 H, Hb-6). ^{13}C NMR (50 MHz, $CDCl_3$) δ 137.7, 137.2, 137.1 (s, Ar), 132.9 (d, C-2), 128.6, 128.5-127.7 (d, 15 C, Ar), 82.7 (d, C-3), 80.3 (d, C-4), 77.4 (d, C-5), 73.9 (t, Bn), 71.9 (t, Bn), 71.6 (t, Bn), 66.1 (t, C-6). IR ($CDCl_3$): 3090, 3067, 3033, 2933, 2868, 2247, 1584, 1455, 1095 cm^{-1}. MS (EI): *m/z* 285 (6), 234 (3), 176 (6), 132 (19), 91

(100). Anal. Calcd. for $C_{26}H_{27}NO_4$ (417.50): C, 74.80; H, 6.52; N, 3.35. Found: C, 74.51; H, 6.78; N, 3.01.



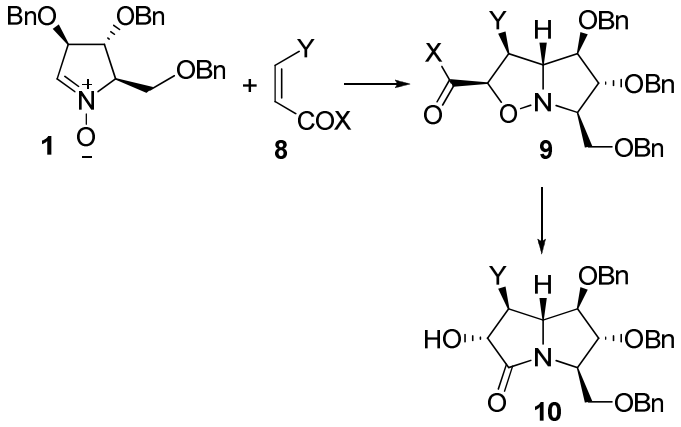
Chapter 3

1,3-dipolar cycloadditions

3.1 The synthetic strategy

Nitrones can be conveniently used in 1,3-dipolar cycloaddition reactions. The 1,3-dipolar cycloaddition reaction of nitrones to alkenes as dipolarophiles forms an isoxazolidine adduct. The synthetic utility of this reaction is demonstrated by the variety of attractive compounds which are available from isoxazolidines.

The general synthetic strategy followed for the synthesis of the pyrrolizidine alkaloids is outlined in Scheme 3.1.



Scheme 3.1. Synthetic strategy for the synthesis of pyrrolizidines

We took advantage of a stereocontrolled cyclic nitrone cycloaddition strategy[55] employing polyfunctionalyzed nitrone **1** and suitable (Z) – configurated dipolarophiles **8**, which latter were then converted into pyrrolizidinone derivatives **10** by reductive ring opening/cyclization. Intermediates **10** bear a free hydroxyl group on C-6 of the pyrrolizidine ring, thus allowing selective glucosylation in this position. A good choice of the dipolarophile is crucial for the success of the strategy.

[55] Brandi, A.; Cardona, F.; Cicchi, S.; Cordero, F. M.; Goti, A. *Chem. Eur. J.* **2009**, *15*, 7808.

The most favoured approach of alkenes to nitrone **1** occurs through *exo-anti* transition states:

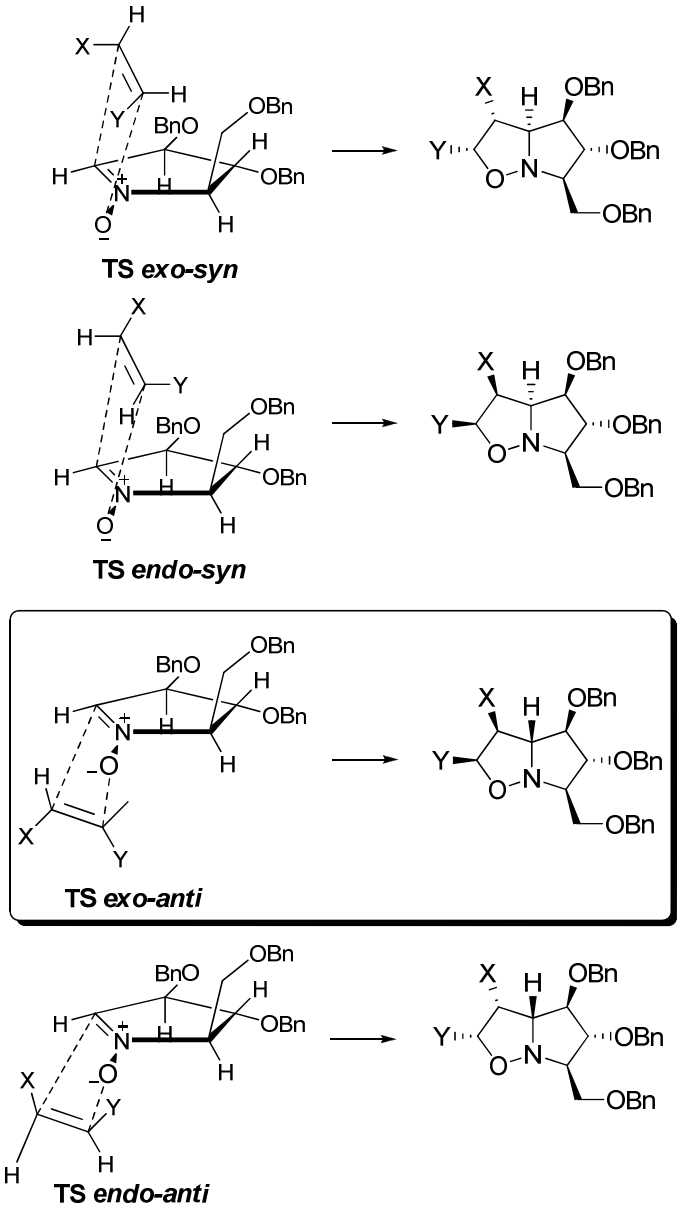


Figure 3.1. Favourite transition state of cycloaddition

The substituents at C-3 and C-5 allow to obtain exclusively *anti* cycloadducts and the steric bulk of substituent at C-4 ensures an excellent *exo* selectivity.

The complete stereoselectivity of the 1,3-dipolar cycloaddition, which allowed the selective installation of the three new stereocenters (corresponding to C-6, C-7 and C-7a in the target molecules) in one step with the required configuration, may be ascribed to the peculiar all-*trans* disposition of the benzyloxy groups in nitrone **1**, which hampered any *endo* or *syn* approaches.[44,53a]

3.2 Casuarine and its unnatural derivatives

3.2.1 Introduction

Casuarine, (1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-3-(hydroxymethyl)-1,2,6,7-tetrahydroxy pyrrolizidine (**11**, Figure 3.2), has been isolated from the bark of *Casuarina equisetifolia* L. (Casuarinaceae) and from the leaves of *Eugenia jambolana* Lam. (Myrtaceae),[33,34] two plants well known for their therapeutic action against diarrhoea, dysentery and colic,[56] breast cancer,[1] diabetes and bacterial infections.[2,57] Compound **11** is a strong inhibitor of the fungal glucoamylase from *Aspergillus niger*, with very high IC_{50} values (IC_{50} = 0.7 μM).[58]

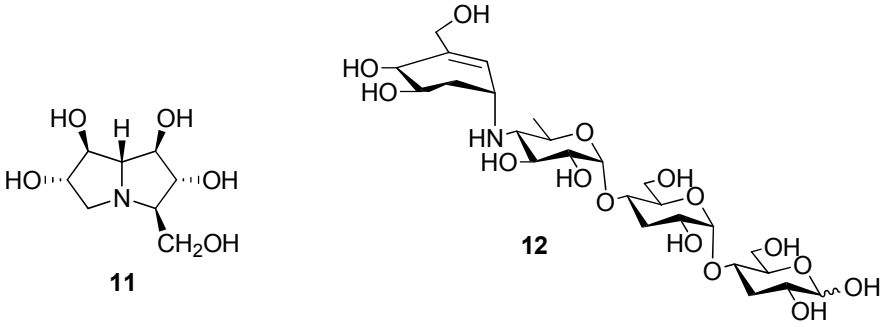


Figure 3.2. Casuarine and acarbose

Isolation and purification of **11** from natural sources are very difficult and expensive and afford only minute amounts of compound. Furthermore, very few total syntheses of casuarine (**11**) have been reported to date.[59,60] The

[56] Chopra, R. N.; Nayar, S. L.; Chopra, I. C. *Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research (India), New Dehli, **1956**, p.55; b) Nair, R. B.; Santhakumari, G. *Ancient Science of Life* **1986**, *6*, 80.

[57] a) Grover, J. K.; Yadav, S.; Vats, V. *J. Ethnopharmacol.* **2002**, *81*, 81; b) Mentreddy, S. R. *J. Sci. Food. Agric.* **2007**, *87*, 743.

[58] Kato, A.; Kano, E.; Adachi, I.; Molyneux, R. J.; Watson, A. A.; Nash, R. J.; Fleet, G. W. J.; Wormald, M. R.; Kizu, H.; Ikeda, K.; Asano, N. *Tetrahedron: Asymmetry* **2003**, *14*, 325.

[59] a) Denmark, S. E.; Hurd, A. R. *Org. Lett.* **1999**, *1*, 1311; b) Denmark, S.; Hurd, A. R. *J. Org. Chem.* **2000**, *65*, 2875; c) Izquierdo, I.; Plaza, M. T.; Tamayo, J. A. *Tetrahedron* **2005**, *61*, 6527; d) Nash, R. J.; Fleet, G. W. J.; Van Ameijde, J.; Horne, G. PCT Int. Appl. (**2006**), WO2006008493.

highly complex and challenging structure of casuarine, as it is the most highly hydroxylated pyrrolizidine alkaloid and possesses six contiguous carbon stereocenters, is presumably responsible for the limited number of synthetic approaches described.

We decided to approach the synthesis of casuarine (**11**), hyacinthacine A_2 (**13**), (−)-uniflorine A (**18**) (another natural product with inhibition properties) and some unnatural derivatives like 7-deoxycasuarine (**14**) and its lactam **15**, 7-*homo*casuarine (**16**), and 7-*epi*australine (**17**) (Figure 3.3) by the 1,3-dipolar cycloaddition strategy. Then, with these molecules in hand, we performed molecular modelling and inhibition studies.

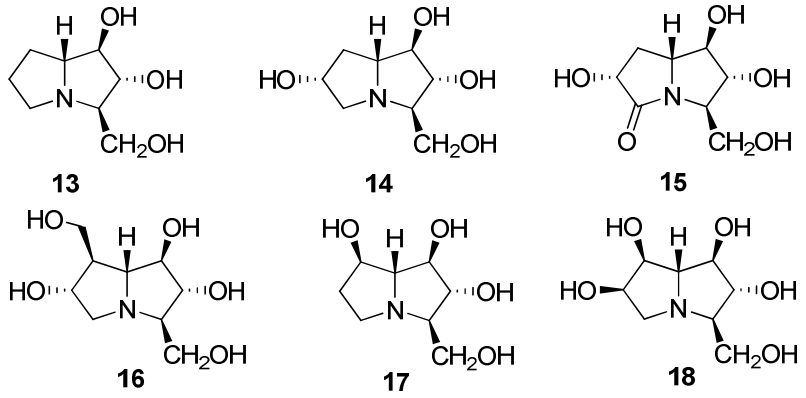


Figure 3.3. Natural and unnatural pyrrolizidine alkaloids

3.2.2 Synthesis of hyacinthacine A_2 and 7-deoxycasuarine

The 1,3-dipolar cycloaddition of nitrone **1** to dimethylacryl amide (**19**) is the first key-step for the synthesis of hyacintacine A_2 (**13**), 7-deoxycasuarine (**14**) and its lactam derivate **15** (Figure 3.3).

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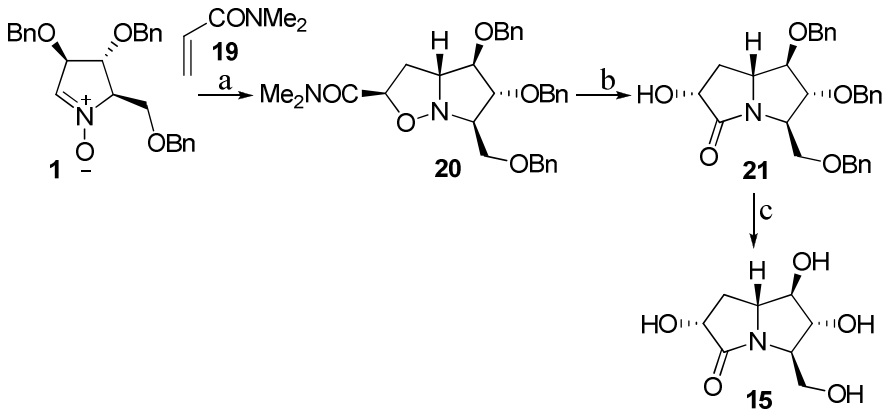
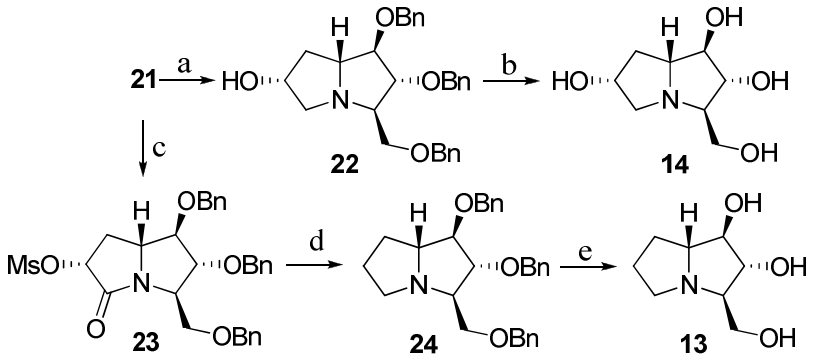
[60] Casuarine has also been obtained as a side-product during the synthesis of targeted 3-*epi*-casuarine: a) Van Ameijde, J.; Horne, G.; Wormald, M. R.; Dwek, R. A.; Nash, R. J.; Jones, P. W.; Evinson, E. L.; Fleet, G. W. J. *Tetrahedron:Asymmetry* **2006**, *17*, 2702. For related syntheses of casuarine diastereoisomers, see also: b) ref. 59d; c) Izquierdo, I.; Plaza, M. T.; Tamayo, J. A. *J. Carbohydr. Chem.* **2006**, *25*, 281; d) Ritthiwigrom, T.; Pyne, S. G. *Org. Lett.* **2008**, *10*, 2769.



Scheme 3.2. Synthesis of 7-deoxycasuarine lactam

Reaction conditions: a) DCM, rt, 3 d, 85%; b) Zn, AcOH/H_2O, 50 °C, 4 h, 80%; c) H_2, Pd/C, EtOH, 3 d, 88%.

The cycloaddition reaction gave regio- and stereo-selectively isoxazolidine **20** with 85% yield. N-O bond cleavage of **20** was then achieved by treatment with Zn in acetic acid, affording lactam **21** with 80% yield. Compound **21** was the key intermediate for the total syntheses of all the three target molecules. Hydrogenation in EtOH catalyzed by Pd/C afforded lactam **15** with 88% yield (Scheme 3.2).



Scheme 3.3. Synthesis of hyacinthacine A_2 and 7-deoxycasuarine

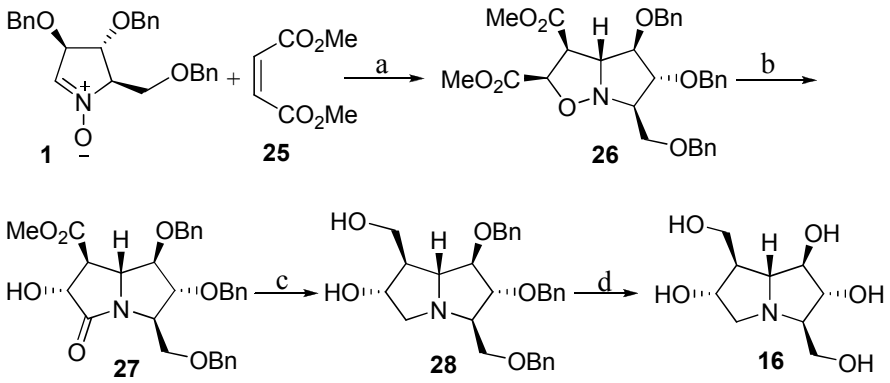
Reaction conditions: a) $LiAlH_4$, THF, reflux, 1.5 h, 75%; b) H_2, Pd/C, HCl, EtOH, 3 d, 88%; c) MsCl, NEt_3, DCM, rt, 2 h, 100%; d) $LiAlH_4$, THF, reflux, 1.5 h, 80%; e) H_2, Pd/C, HCl, MeOH, 3 d, 72%.

Reduction of the C=O bond with $LiAlH_4$ in refluxing THF gave compound **22** with 75% yield which, after catalytic hydrogenation in EtOH, afforded 7-

deoxycasuarine (**14**) in 88% yield (Scheme 3.3). Deoxygenation at C-6 was obtained through mesylation of **21** followed by treatment of mesylate **23** with $LiAlH_4$ in refluxing THF, which gave **24** with 80% yield over 2 steps. Finally catalytic hydrogenation provided hyacinthacine A_2 (**13**) with 72% yield (Scheme 3.3).

3.2.3 Synthesis of 7-*homo*casuarine

For the synthesis of unnatural 7-*homo*casuarine (**16**), dimethyl maleate (**25**) was chosen as the suitable dipolarophile. Cycloaddition reaction of nitrone **1** to **25** in dichloromethane at room temperature afforded stereoselectively adduct **26** in 78% yield (Scheme 3.4).



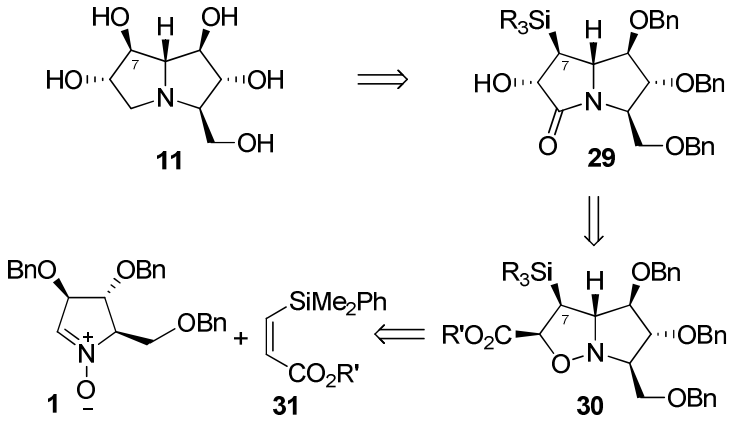
Scheme 3.4. Synthesis of 7-*homo*casuarine

Reaction conditions: a) DCM, rt, 4 d, 78%; b) Zn, AcOH/H_2O, 50 °C, 3 h, 90%; c) $LiAlH_4$, THF, reflux, 2 h, 100%; d) H_2, Pd/C, HCl, EtOH, rt, 4 d, 89%.

Treatment with Zn in acetic acid at 50°C for 3 h gave lactam **27** in 90% yield. Then, reduction of both C=O bonds, the ester and lactam moiety, with excess of $LiAlH_4$ in refluxing THF provided diol **28** quantitatively. Finally, catalytic hydrogenation with Pd/C in the presence of HCl gave unnatural 7-*homo*casuarine (**16**) in 89% yield.

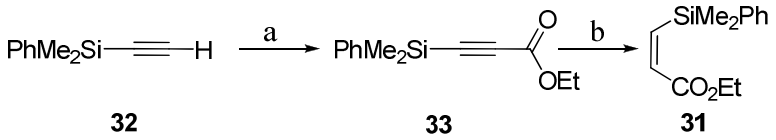
3.2.4 Syntheses of Casuarine and 7-*epi*australine

The major and most challenging problem in the synthesis of casuarine (**11**) is to install an OH group at the position 7 of the pyrrolizidine. A plausible retrosynthetic analysis suggested lactam **29** such direct precursor of casuarine (**11**) by the synthetic equivalence R_3Si = OH using a Tamao-Fleming reaction, commonly occurring with complete retention of configuration. The lactam with the silylated group at position 7 can derive from the silylated isoxazolidine **30**, inturn accessible by a cycloaddition reaction of nitrone **1** with an appropriated silylated alkene (Scheme 3.5):



Scheme 3.5. Retrosynthetic analysis for casuarine synthesis

For this reason the chosen alkene for the cycloaddition reaction was the silylated alkene **31** (Scheme 3.6).

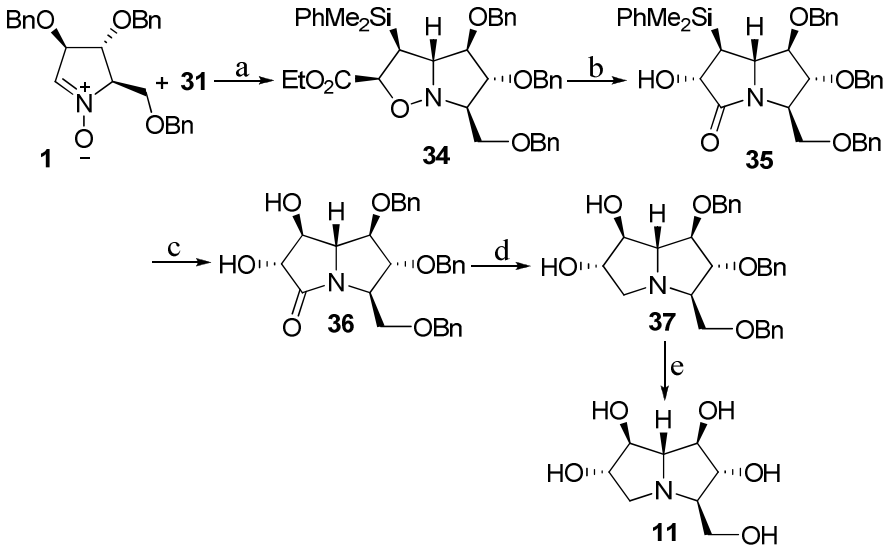


Scheme 3.6. Alkene 31 synthesis

Reaction conditions: a) 1) BuLi, THF, -78°C, 15 min.; 2) ClC=OCO_2Et, THF, -78°C, 3 h, 88% over two steps; b) H_2, Lindlar cat., 4 h, rt, 94%.

Alkyne **33** was obtained from (dimethylphenylsilyl)-acetylene (**32**) by reaction with BuLi and subsequent addition of ethyl chloroformate in 88% yield and then was hydrogenated with Lindlar catalyst and H_2 in toluene. The reaction was controlled every 30 minutes via ^{1}HNMR, and after 4 h, alkene **31** was obtained in 94% yield (Scheme 3.6).

The obtained *Z*-alkene **31** was used in the 1,3-dipolar cycloaddition reaction with nitrone **1**. Although no close example was reported, we were confident that *Z*-disubstituted alkene **31** could provide the correct regioselectivity in the cycloaddition step on the basis of related nitrone cycloaddition reactions with vinyl silanes.[61]



Scheme 3.7. Casuarine synthesis

Reaction conditions: a) DCM, rt, 36 h, 79%; b) Zn, AcOH/H_2O, 60-65°C, 5 h, 93%; c) $Hg(CF_3CO_2)_2$, TFA, AcOH, AcOOH, $CHCl_3$, rt, 76%; c) $LiAlH_4$, THF, reflux, 78%; e) H_2, Pd/C, MeOH, HCl, rt, 100%.

Indeed, the desired adduct **34** was obtained with high regio- and stereoselectivity in 79% yield (Scheme 3.7). The regiochemistry and relative stereochemistry of **34** were firmly established on the basis of COSY and NOESY experiments, which confirm the preference for an *exo-anti* transition state.

[61] DeShong, P.; Leginus, J. M.; Lander Jr, S. W. *J. Org. Chem.* **1986**, *51*, 574.

Reductive cleavage of the N-O bond[53a,62,63] with Zn in acetic acid followed by spontaneous *N*-cyclization afforded lactam **35** in 93% yield. Then, the Tamao-Fleming reaction[64] allowed oxidation of the C-Si bond with retention of configuration and 76% yield of diol **36**. Oxidation of the C-Si bond to C-OH with retention of configuration was confirmed by NOESY experiments performed on the diacetyl derivative of **36**. Finally, reduction of **36** with $LiAlH_4$ in refluxing THF (78% yield), followed by catalytic hydrogenation of pyrrolizidine **37**, occurred quantitatively which provided casuarine (**11**) as a white solid, mp 180-182 °C (Scheme 3.7). The spectroscopic data and physico-chemical properties of the synthesized compound were identical to those of natural casuarine.[33] Synthetic **11** showed a specific optical rotation ($[\alpha]^{20}_D$ = +14.4 (c 0.52, H_2O)) in good agreement with those reported for the natural and for the previously synthesized compound ($[\alpha]^{24}_D$ = +16.9 (c 0.80, H_2O);[19] $[\alpha]^{27}_D$ = +10.8 (c 1.02, H_2O),[59a] respectively). In conclusion, this total synthesis furnished casuarine in 5 steps and 44% overall yield from nitrone **1**, which compares well with the previously reported syntheses.

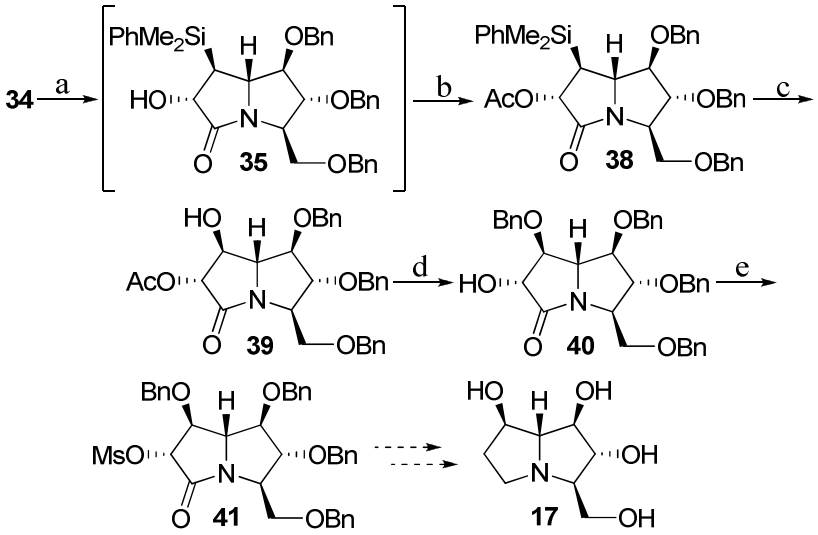
Starting from the same isoxazolidine **34**, through a protection/deprotection sequence on lactam **35**, we can also obtain *7-epi*australine (**17**) (scheme 3.8). Treatment with zinc in acetic acid/water of product **34** and in situ protection with acetic anhydride in presence of pyridine afforded **38** with 93% yield overall 2 steps, then Tamao-Fleming reaction gave product **39** in 88% yield. Lactam **39** was protected at C-7 as benzyloxy

[62] Goti, A.; Cicchi, S.; Cacciarini, M.; Cardona, F.; Fedi, V.; Brandi, A. *Eur. J. Org. Chem.* **2000**, 3633.

[63] For our related work on the synthesis of necine bases, see: a) Goti, A.; Fedi, V.; Nannelli, L.; De Sarlo, F.; Brandi, A. *Synlett* **1997**, 577; b) Goti, A.; Cacciarini, M.; Cardona, F.; Cordero, F. M.; Brandi, A. *Org. Lett.* **2001**, *3*, 1367.

[64] a) Tamao, K.; Ishida, N.; Tanaka, T.; Kumada, M. *Organometallics* **1983**, *2*, 1694; b) Tamao, K.; Ishida, N. *J. Organomet. Chem.* **1984**, *269*, C37; c) Fleming, I.; Henning, R.; Plaut, H. E. *J. Chem. Soc. Chem. Commun.* **1984**, 29; d) Fleming, I.; Henning, R.; Parker, D. C.; Plaut, H. E.; Sanderson, P. E. J. *J. Chem. Soc. Perkin Trans 1* **1995**, 317.

derivative and then OH in position 6 was deprotected to give compound **40** in which only the OH in position 6 is deprotected (75% yield over 2 steps). Mesylation of the free OH at C-6, using mesylchloride in DCM and TEA, gave product **41** in 84% yield, which can be opportunately manipulated to obtain 7-*epi*australine (**17**) (Scheme 3.8).



Scheme 3.8. Synthesis of 7-*epi*australine

Reaction conditions: a) Zn, AcOH/H_2O, 60-65°C, 2 h; b) Ac_2O, py, rt, 18 h, 93% over 2 steps; c) $Hg(CF_3CO_2)_2$, TFA, AcOH, AcOOH, $CHCl_3$, rt, 82%; d) $BnOC(=NH)CCl_3$, CF_3SO_3H, Et_2O, rt, 3 h, Ambersep 900 OH, MeOH, 75% over two steps; e)MsCl, DCM, TEA, rt, 4 h, 84%.

7-*epi*australine (**17**) remains an important goal to achieve in our research group together with australine (**42**), 6-7-di*epi*casuarine (**43**), 7-*epi*casuarine (**44**) and 5-methylcasuarine (**45**)(Figure 3.4).

All these products might be achieved starting from the same cycloaddition reaction described for the synthesis of casuarine (**11**), and the same protection/deprotection procedure illustrated before and their synthesis is studying in our laboratories.

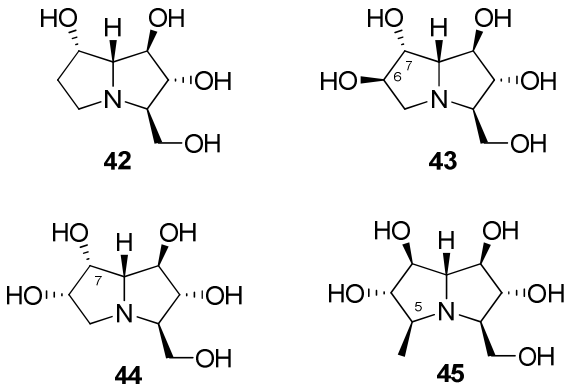


Figure 3.4. Australine (42), 6-7-di*epi*casuarine (43), 7-*epi*casuarine (44) and 5-methylcasuarine (45)

3.2.5 (–)-Uniflorine A: synthesis and structural determination

The leaves of *Eugenia uniflora* L. (Myrtaceae), an evergreen tree widely distributed in Paraguay, Uruguay, Argentina, and Brazil, furnish a natural Paraguayan medicine named Nangapiry. Infusions prepared from Nangapiry are used in folk medicines as antidiarrhoeic, diuretic, antirheumatic, antifebrile, and antidiabetic preparations.[65] Water-soluble extracts obtained from the leaves of *E. uniflora* were found to inhibit the α-glucosidases maltase and sucrase, thus accounting for the antidiabetic effect induced by Nangapiry preparations.[65] Two iminosugar alkaloids have been isolated from the water-soluble, namely uniflorine A and B, for which the structures of pentahydroxyindolizidines **46** and **47**, respectively, were proposed on the basis of NMR analyses (Figure 3.5).

Uniflorine A and B were found to inhibit rat intestinal maltase (IC_{50} values of 12 and 4.0 μM, respectively) and sucrase (IC_{50} values of 3.1 and 1.8 μM, respectively).[65]

[65] Matsumura, T.; Kasai, M.; Hayashi, T.; Arisawa, M.; Momose, Y.; Arai, I.; Amagaya, S.; Komatsu, Y. *Pharm. Biol.* **2000**, *38*, 302.

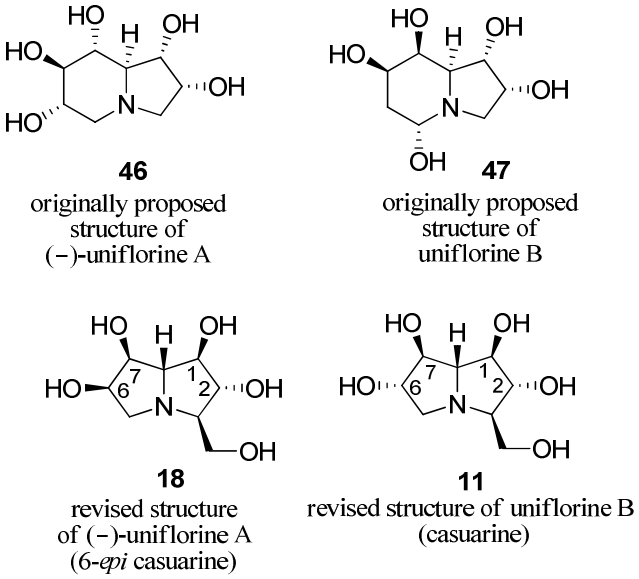


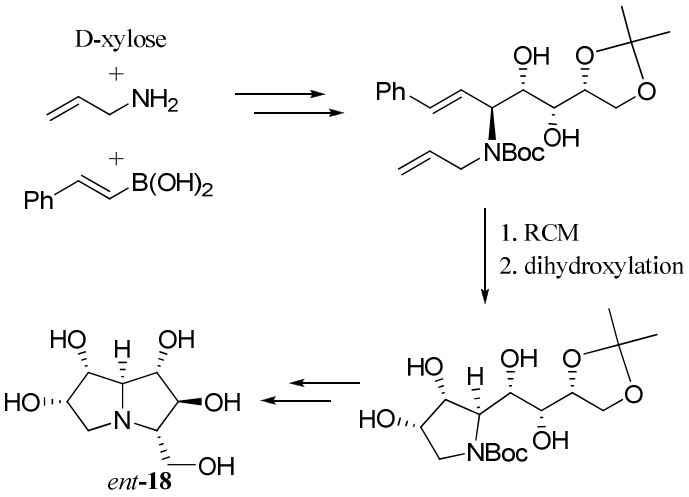
Figure 3.5. Originally proposed and revised structure of (–)-uniflorine A and uniflorine B

In 2004, Pyne and coworkers reported the total synthesis of putative uniflorine A (**46**) from L-xylose.[66] However, the NMR spectroscopic data for synthetic **46** did not match those reported for natural uniflorine A, thus proving that the original structural assignment to uniflorine A was incorrect. Other total syntheses of **46** from the group of Dhavale[67] and of diastereoisomers of **46**[68,69,70,71] showed that the NMR data of 1,2,6,7,8-pentahydroxyindolizidines differ significantly from those of the natural product named uniflorine A. Pyne and coworkers also recognized that the NMR data reported for uniflorine B and its optical rotation were in good agreement with those of the known alkaloid casuarine **11**,[70] a known inhibitor of α-

[66] Davis, A. S.; Pyne, S. G.; Skelton, B. W.; White, A. H. *J. Org. Chem.* **2004**, *69*, 3139.
[67] Karanjule, N. S.; Markad, S. D.; Dhavale, D. D. *J. Org. Chem.* **2006**, *71*, 6273.
[68] 1-*Epi*-**42** and 1,2-di-*epi*-**42**: Zhao, Z.; Song, L.; Mariano, P. S. *Tetrahedron* **2005**, *61*, 8888.
[69] 1,2-Di-*epi*-**42**: Bell, A. W.; Pickering, L.; Watson, A. A.; Nash, R. J.; Griffiths, R. C.; Jones, M. G.; Fleet, G. W. J. *Tetrahedron Lett.* **1996**, *37*, 8561.
[70] 1,2-Di-*epi*-**42** and 2-*epi*-**42**: Davis, A. S.; Ritthiwigrom, T.; Pyne, S. G. *Tetrahedron* **2008**, *64*, 4868.
[71] 8a-*Epi*-**42** and 1,2,8a-tri-*epi*-**42**: see ref. 67.

glucosidases.[38, 72] Thus, they suggested that uniflorine B was actually the known alkaloid casuarine and that uniflorine A could possess a similar 1,2,6,7-tetrahydroxy-3-hydroxymethylpyrrolizidine structure, most likely being 6-*epi*casuarine **18**, on the basis of the greatest difference of the chemical shifts at C-6 in the NMR spectra of uniflorine A and B.[70]

For these reasons we decided to embark in the total synthesis of the compound proposed to be (−)-uniflorine A (**18**) by Pyne, namely 6-*epi*casuarine, by exploiting a strategy that allowed the recent total synthesis of casuarine (**11**).[72] While our work was in progress, Pyne and Ritthiwigrom reported a total synthesis of the unnatural enantiomer of **18**, (+)-uniflorine A, which supported their structural hypothesis.[73] Their synthesis started from D-xylose and involved a boronic acid-Mannich reaction (Petasis reaction), a ring-closing metathesis (RCM) followed by osmium catalyzed syn-dihydroxylation and cyclization of the intermediate dihydroxy pyrrolidine derivative as key steps (Scheme 3.9).[73]



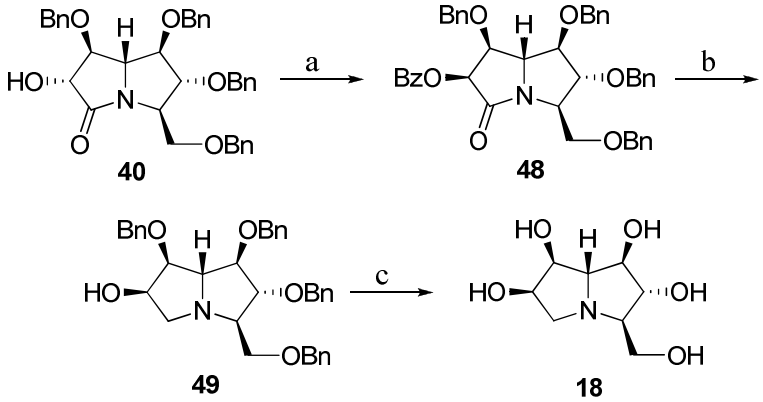
Scheme 3.9. Pyne's synthetic strategy

[72] Cardona, F.; Parmeggiani, C.; Faggi, E.; Bonaccini, C.; Gratteri, P.; Sim, L.; Gloster, T. M.; Roberts, S.; Davies, G. J.; Rose, D. R.; Goti, A. *Chem. Eur. J.* **2009**, *15*, 1627.
[73] Ritthiwigrom, T.; Pyne, S. G. *Org. Lett.* **2008**, *10*, 2769.

The pyrrolizidine obtained (*ent*-**18** ($[\alpha]^{22}_{D}$ +6.6 (*c* 0.35, H_2O) (lit.[65] for (−)-uniflorine A, $[\alpha]_D$ -4.4 (*c* 1.2, H_2O)) displayed spectroscopic data in good agreement with those of natural uniflorine A, thus confirming the postulated structural assignment as 6-*epi*casuarine **18**.

Our first total synthesis of (−)-uniflorine A (**18**) proceeded via inversion of configuration of the OH group at C-6 achieved through a Mitsunobu reaction with benzoic acid on the key intermediate **40** (Scheme 3.10) previously synthesized in the synthesis of 7-*epi*australine (**17**).[73, 74]

Indeed, the intermediate **40** bears a free OH group at C-6 which can be further manipulated independently from the other OH groups. In order to access (−)-uniflorine A, a Mitsunobu reaction was performed on **40** using THF as solvent, benzoic acid (BzOH) as the nucleophile, triphenylphosphine and, alternatively, diisopropylazodicarboxylate (DIAD) or diethylazodicarboxylate (DEAD).



Scheme 3.10. Synthesis of (–) uniflorine A

Reaction conditions: a) BzOH, PPh_3, DIAD, THF, rt, 75%; b) $LiAlH_4$, THF, reflux, 45%, H_2, Pd/C, MeOH, HCl, rt then dowex 50WX8, 6% NH_4OH, 71%.

[74] For other syntheses of casuarine, see: a) Ref. 59a; b) Ref. 59b; c) Ref. 59c; d) Ref. 60a; e) Bell, A. A.; Pickering, L.; Watson, A. A.; Robert, J. N.; Pan, Y. T.; Elbein, A. D.; Fleet, G. W. J. *Tetrahedron Lett.* **1997**, *38*, 5869.

Reaction of **40** with 1.2 equiv of BzOH, 1.2 equiv of PPh_3 and 1.2 equiv of DIAD at room temperature for 2 h allowed no conversion of starting material. When the mixture was heated at 50 °C for 4 h a complex mixture was obtained, but isolation of the desired product was unsuccessful. The use of DEAD rather than DIAD and the same reaction conditions for 18 h gave compound **48** in 63% yield. However, the best yield (75% of isolated product after purification on silica gel) was achieved performing the reaction with DIAD at room temperature for 18 h (Scheme 3.10). Inversion of configuration at C-6 was confirmed by 1D NOESY experiments performed on compound **48**. Indeed, irradiation of H-6 gave NOE enhancement at H-7 and irradiation of H-7 gave NOE at H-1 (Figure 3.6).

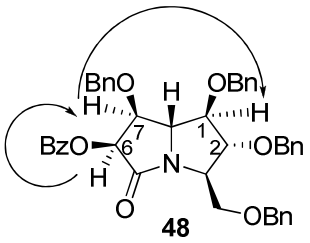


Figure 3.6. Compound 48; irradiation of H-6 gave NOE enhancement at H-7 and irradiation of H-7 gave NOE at H-1

Concomitant reduction of C=O bond and deprotection of the OH group at C-6 with $LiAlH_4$ in refluxing THF gave compound **49** in 45% yield. Finally, catalytic hydrogenation of **49** using 10% Pd on activated carbon in methanol in the presence of 0.1 ml of conc. HCl (37%), followed by absorption on an acidic resin and elution of the free base with 6% aqueous ammonium hydroxide, afforded (−)-uniflorine A (**18**) in 71% yield (Scheme 3.10). The observed NOEs are consistent with the configuration assigned to **18**, as shown in Figure 3.7. Indeed, irradiation of H-6 gave nOe at H-7 and H-1 and irradiation of H-7 gave nOe at H-1.

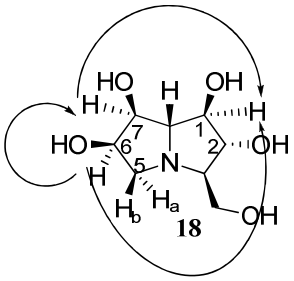


Figure 3.7. Compound 18; irradiation of H-6 gave NOE at H-7 and H-1 and irradiation of H-7 gave NOE at H-1

The ^{1}H NMR spectra (D_2O) of **18** and those of natural uniflorine A were essentially identical ($\Delta\delta$ = 0.01 - 0.13 ppm). All of the ^{13}C NMR signals of **18** (in D_2O with MeCN as an internal reference at δ 1.47) were 1.6 - 3.0 ppm upfield compared to those reported for the natural product; however, a similar difference has been previously noted also for synthetic (+)-uniflorine by Pyne and coworkers, who attributed this systematic error to a different reference (not reported) in the spectrum of the isolated natural product. Indeed, the ^{13}C NMR chemical shifts obtained for the compound synthesized by us were in good agreement with those reported for *ent*-**18** synthesized by Pyne ($\Delta\delta$ = ± 1 ppm).[73] The specific optical rotation of synthetic **18** ($[\alpha]_D^{21}$ - 6.9 (H_2O, *c* 0.42)) was essentially identical in absolute value to that reported for synthetic *ent*-**18** ($[\alpha]_D^{21}$ + 6.6 (H_2O, *c* 0.35)) and matched in sign that of the natural product ($[\alpha]_D^{21}$ - 4.4 (H_2O, *c* 1.2)). Further confirmation that natural (−)-uniflorine A is actually 6-*epi*casuarine **18**, was furnished by the melting point of synthetic **18** (177-180 °C) which was a near match to that reported for natural (−)-uniflorine A (174-178 °C).[65]

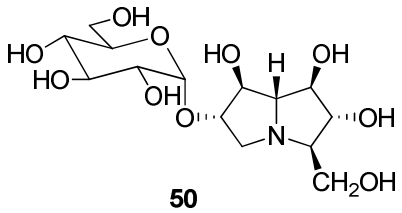
In conclusion, we reported the first total synthesis of (−)-uniflorine A in 9 steps and 11% overall yield, and we confirmed that (−)-uniflorine A is 6-*epi*casuarine.

3.3 Casuarine 6-*O*-α-D-glucoside and its unnatural analogues

3.3.1 Introduction

Trehalose is well known as a storage carbohydrate, and trehalases play the essential role in the transport of glucose in insects and fungi.[75] Trehalases inhibitors are potentially nontoxic to human, and the mode of action of these enzymes gives them a favourable biological selectivity because vertebrates do not depend on the hydrolysis of trehalose.

All powerful trehalase inhibitors reported to date are the pseudodisaccharide type of inhibitors, such as casuarine-6-*O*-α-D-glucopyranoside (**50**, Figure3.8).[58]



50

Figure 3.8. Casuarine-6-*O*-α-D-glucopyranoside

These compounds are powerful competitive inhibitors of porcine kidney trehalase. The extremely high affinity of pseudodisaccharide inhibitors derives from the synergistic interactions of an alkaloid unit and a sugar unit with two enzyme's subsites.[76] Trehalose is a blood sugar in insects and a major storage sugar in fungi and yeast. Therefore trehalases inhibitors are considered to have high potential as insecticides or fungicides.

Isolation and purification of **50** from natural sources are very difficult and expensive and afford only minute amounts of compound. As a matter of

[75] a) Elbein, A. D. *Adv.Carbohydr. Chem. Biochem.* **1974**, *30*, 227. b) Elbein, A. D.; Pan, Y. T.; Pastuszak, I.; Carroll, D. *Glycobiology* **2003***, 13,* 17R.
[76] Asano, N.; Kato, A.; Matsui, K. *Eur. J. Biochem.* **1996**, *240*, 692.

fact, **50** has never been obtained in a chemically pure form and no total synthesis of **50** has been described before.

A problematic issue in the synthesis of casuarine-6-*O*-α-glucoside (**50**) lies in the selectivity of glucosylation of the hydroxy group at C-6. Carefully planned extraction procedures have discovered recently more examples of glycosyl iminosugars isolated from natural sources, thus indicating that their occurrence in Nature is not uncommon.[77] In contrast, few syntheses of glycosyl iminosugars are reported in the literature,[78,79] and they all reflect some difficulties in targeting the final compounds with good yields and selectivities, particularly in the glucosylation step. Interestingly, their inhibitory properties differ substantially from those of the parent iminosugar, with the linked carbohydrate moiety usually generating higher selectivity. This can be due, in principle, to a different arrangement within the catalytic site or to some interactions with different subsites.

3.3.2 Syntheses of 7-deoxycasuarine and 7-*homo*casuarine glucosides and their derivatives

Before targeting the synthesis of casuarine-6-*O*-α-glucoside (**50**), we proceeded to synthesize the two unnatural analogues 7-deoxycasuarine and 7-*homo*casuarine glucosides (**51**, **52**, Figure 3.9).

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[77] Asano, N.; Yamauchi, T.; Kagamifuchi, K.; Shimizu, N.;. Takahashi, A.; Takatsuka, H.; Ikeda, K.; Kizu, H.; Chuakul, W.; Kettawan, A.; Okamoto, T. *J. Nat. Prod.* **2005**, *68*, 1238.

[78] Chemical syntheses of glycosyl iminosugars: a) Anzeveno, P. B.; Creemer, L. J.; Daniel, J. K.; King, C.-H.R.; Liu, P. S. *J. Org. Chem.* **1989**, *54*, 2539; b) Liu, P. S.; Daniel, J. K.; Rhinehart, B. L. Eur. Pat. Appl. EP297534, CA 111:7659r, **1989**; c) Ardron, H.; Butters, T. D.; Platt, F. M.; Wormald, M. R.; Dwek, R. A.; Fleet, G. W. J.; Jacob, G. S. *Tetrahedron: Asymmetry* **1993**, *4*, 2011; d) Felpin, F.-X.; Boubekeur, K.; Lebreton, J. *J. Org. Chem.* **2004**, *69*, 1497.

[79] Enzymatic syntheses of glycosyl iminosugars: a) Ref. 38; b) Asano, N.; Kato, A.; Kizu, H.; Matsui, K.; Griffiths, R. C.; Jones, M. G.; Watson, A. A.; Nash, R. J. *Carbohydr. Res.* **1997**, *304*, 173.

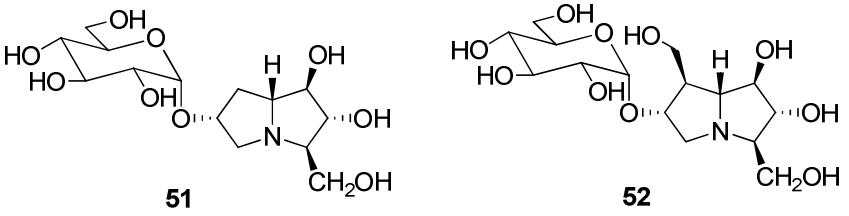
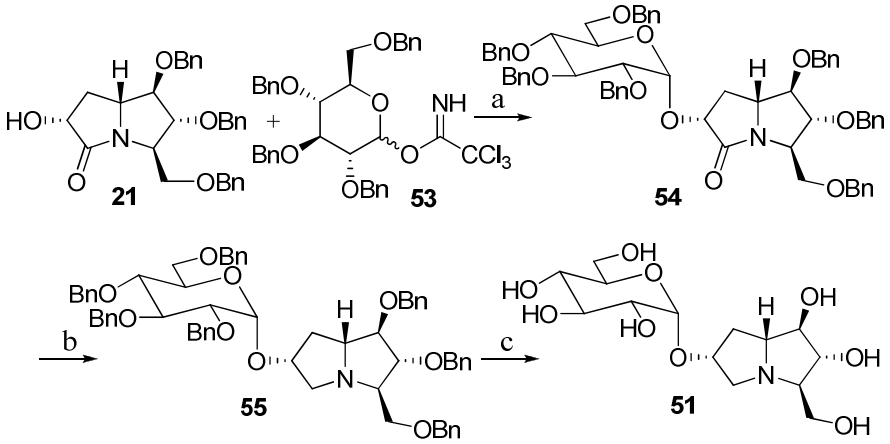


Figure 3.9. 7-Deoxycasuarine glucoside and 7-homocasuarine glucoside

For the synthesis of 7-deoxycasuarine glucoside (**51**) the glucosylation step was performed on lactam **21** with the glucosyl donor (**53**) in diethyl ether in the presence of TMSOTf, which gave **54** with 88% yield (Scheme 3.11).

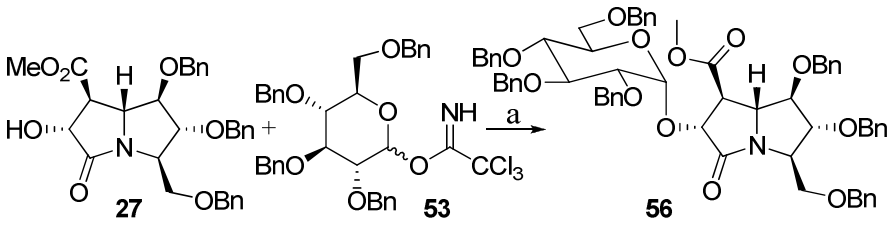


Scheme 3.11. Synthesis of 7-deoxycasuarine glucoside

Reaction conditions: a) TMSOTf, Et_2O, rt, 1 h, 88%; b) $LiAlH_4$, THF, rt, 1 h, 58%; c) H_2, Pd/C, HCl, MeOH, rt, 18 h, 77%.

Reduction of the C=O bond with $LiAlH_4$ afforded **55** with 58% yield. Finally, catalytic hydrogenation of **55** gave **51** in 77% yield. Catalytic hydrogenation was performed in the presence of some drops of HCl, as usual, in order to increase the reaction rate, since no reaction occurred under neutral conditions. Despite our concerns on carrying out the hydrogenation in acidic media, due to the presence of the hydrolytically cleavable glucosidic bond, this linkage was not affected under the standard conditions. The free amine **51** was obtained after elution trough a ionic exchange resin (Scheme 3.11).

For the synthesis of unnatural 7-*homo*casuarine glucoside (**52**) and of its lactam derivatives **57** and **60** the procedure was similar: glucosylation with the glucosyl donor **53** on lactam **27** in presence of TMSOTf afforded compound **56** in 75% yield (Scheme3.12).

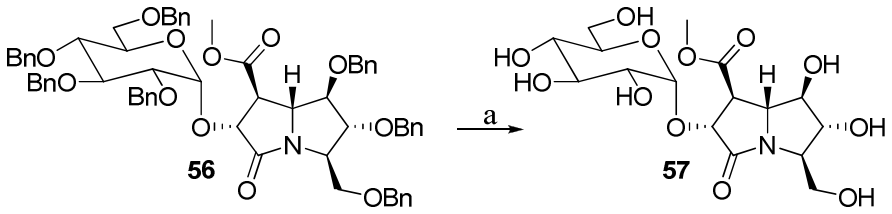


Scheme 3.12. Synthesis of glucoside 56

Reaction conditions: a) TMSOTf, Et_2O/DCM, rt, 1 h, 75%.

Intermediate **56** could be differently manipulated in order to obtain all the glucosyl derivatives **52**, **57** and **60**.

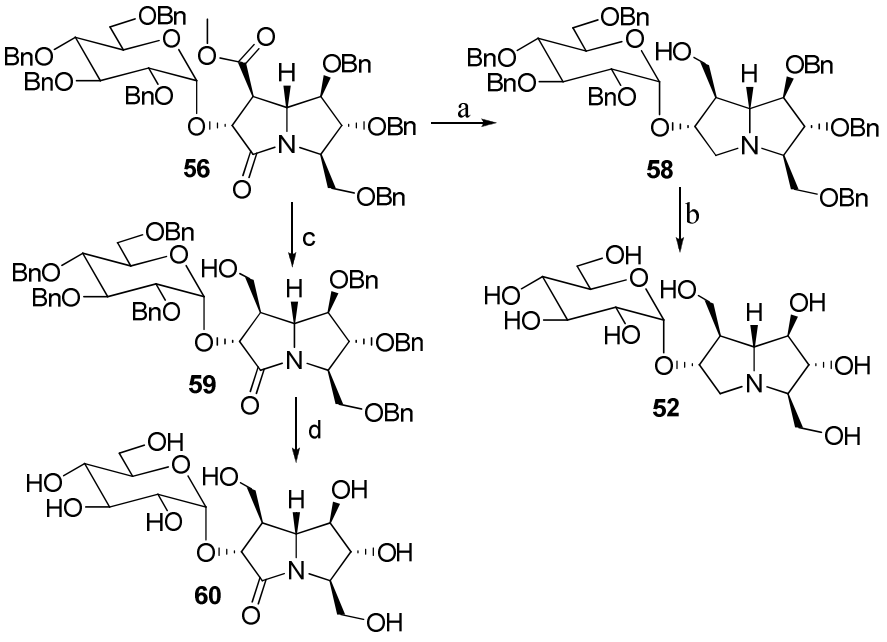
Catalytic hydrogenation of **56** afforded **57** in 96% yield. Compound **57** maintained the ester moiety at C-7 and the C=O bond at C-5 (Scheme 3.13).



Scheme 3.13. Syntheses of 7-*homo*casuarine glucoside unnatural analogue 57

Reaction conditions: a) H_2, Pd/C, MeOH/EtOAc, rt, 4 d, 96%.

Treatment of **56** with an excess of $LiBH_4$ and BH_3·THF (see below) afforded complete reduction at both the C=O bonds affording **58** in 98% yield; then catalytic hydrogenation, performed as usual, gave glucosyl derivative of 7-*homo*cauarine **52** in 79% yield. Selective reduction of the ester moiety was achieved by treatment of **56** with $LiBH_4$ in THF at room temperature for 1 h, which gave **59** in 62% yield. Finally, catalytic hydrogenation provided glucosyl derivative **60** in 72% yield (Scheme 3.14).

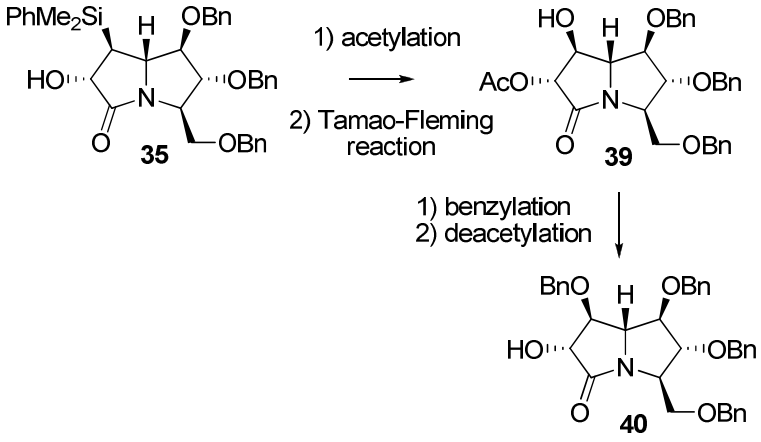


Scheme 3.14. Synthesis of 7-*homo*casuarine glucoside and its unnatural analogue 60

Reaction conditions: a) $LiBH_4$, BH_3·THF, THF, rt, 11 d, 98%; b) H_2, Pd/C, HCl, MeOH, rt, 12 h, 79%; c) $LiBH_4$, THF, rt, 18 h, 62%; d) H_2, Pd/C, MeOH, 24 h, 72%.

3.3.3 Casuarine-6-*O*-α-D-glucopyranoside and its unnatural lactam

The main problem in the synthesis of casuarine-6-*O*-α-D-glucopyranoside (**50**) was the selective glucosylation in position 6. We needed to carry out the Tamao-Fleming oxidation prior to glycosylation as the glycosidic bond did not tolerate the harsh reaction conditions of Tamao-Fleming oxidation. The problem was solved by a protection/deprotection sequence (Scheme 3.15) carried out on compound **35**, also used in the 7-*epi*australine and (−)-uniflorine A synthetic strategy, that afforded **40**, which bears the free hydroxy group at C-6 suitable to be linked to the glucose moiety.

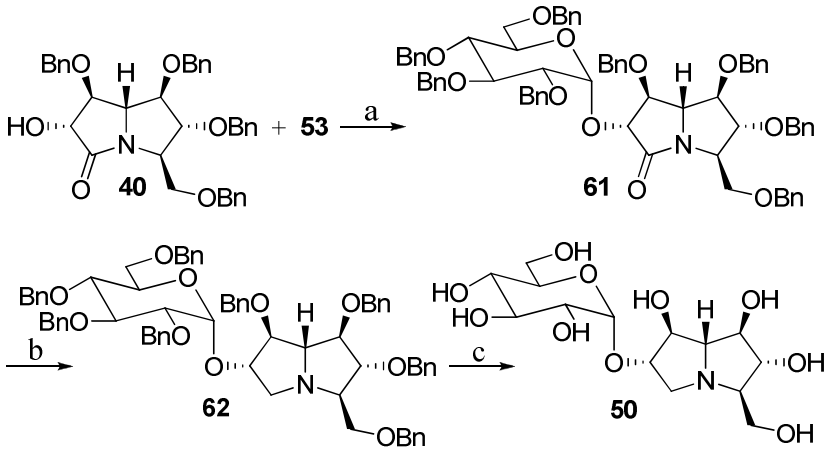


Scheme 3.15. Protection/deprotection sequence

The glucosylation reaction on **40** was carried out with the trichloroacetimidate **53** and catalytic TMSOTf in diethyl ether and afforded selectively the α-anomer **61** in 72% yield. Only traces of the β-anomer, which could not be isolated, were detected in the crude reaction mixture. Reduction of C=O double bond of **61** was not a trivial task. $LiAlH_4$, Red-Al, BH_3-SMe_2 or $LiBH_4$ in refluxing THF afforded complex mixtures of products, while at room temperature the starting material was converted only marginally. Eventually, treatment of **61** with a high excess of $LiBH_4$ in combination with BH_3-THF at room temperature for 3 days[80] afforded glucoside **62** in 68% yield. Catalytic hydrogenation finally provided casuarine-6-*O*- α -glucoside (**50**) in 77% yield as a white foam (Scheme 3.16).

^{1}H- and ^{13} C-NMR data for synthetic **50** are in good agreement with those of natural casuarine 6-*O*-α-glucoside.[34]

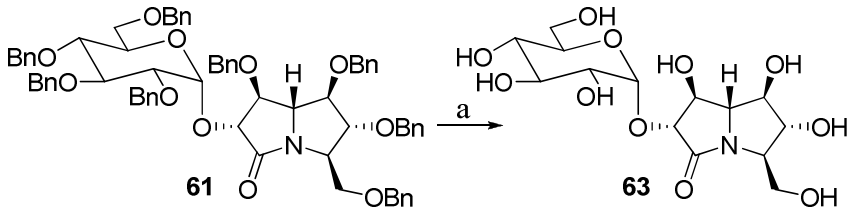
[80] Nukui, S.; Sodeoka, M.; Sasai, H.; Shibasaki, M. *J. Org. Chem.* **1995**, *60*, 398.



Scheme 3.16. Casuarine-6-*O*-α-D-glucopyranoside synthesis

Reaction conditions: a) Ac_2O, Py, rt, 15 h, 100%; b) $Hg(CF_3CO_2)_2$, TFA, AcOH, AcOOH, $CHCl_3$, 82%; c) $BnOC(=NH)CCl_3$, CF_3SO_3H, Et_2O, rt, 3 h; d) Ambersep 900 OH, MeOH, rt, 15 h, 75% (2 steps); e) TMSOTf, Et_2O, -20 °C, 40 min, 72%; f) $LiBH_4$, BH_3·THF, THF, 23 °C, 3 d, 68%; g) H_2, Pd/C, MeOH, HCl, 77%.

The hydrogenation of the full protected lactam **61** which provided glucosyl derivative **63** in 77% yield (Scheme 3.17).



Scheme 3.17. Synthesis of the lactam derivate 72

Reaction condition: a) H_2, Pd/C, MeOH/EtOAc, rt, 24 h, 77%.

The synthesis of the lactam derivatives **57**, **60** and **63** was undertaken in order to investigate the importance of the basic nitrogen atom for inhibition. With all these compounds in hand, we investigated the inhibition properties towards a wide range of commercial glycosidases (See Chapter 5).

3.4 Conclusion

In summary, we developed a novel and efficient synthetic strategy which allowed us to prepare natural iminosugars, such as casuarine, (−)-uniflorine A and hyacinthacine A_2, as well as non natural casuarine-like pyrrolizidines, such as 7-deoxycasuarine and 7-*homo*casuarine, together with natural and unnatural 6-*O*-α-glucoside derivatives.

Our methodology is based on a highly regio- and totally stereoselective 1,3 dipolar cycloaddition of suitable substituted alkenes to a carbohydrate-derived nitrone. After N-O bond cleavage of the obtained cycloadducts, the lactam intermediates were used in the synthesis of all the target compounds, including the glucosyl derivatives that were obtained by selective α-glucosylation.

All the target molecules were tested towards a wide range of glycosidases (see Chapter 5) like commercially available glycosidases, trehalases and human maltase-glucoamylase.

3.5 Experimental Section

General experimental aspect are mentioned in Chapter 2.3.1.

3.5.1 Synthesis of hyacinthacine A_2, 7-deoxycasuarine and its lactam

(2*R*,3a*R*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-[(benzyloxy)methyl]-*N*,*N*-dimethylhexahydropyrrolo [1,2-b]isoxazole-2-carboxiamide (**20**). A solution of nitrone **1** (800 mg, 1.96 mmol) and dimethylacrylamide (**19**, 242 μL, 2.35 mmol) in CH_2Cl_2 (3 mL) was stirred for 3 days at rt, then the solvent was removed under reduced pressure. Purification by flash column chromatography on silica gel (eluent petroleum ether/EtOAc 1:2) afforded pure **20** (R_f=0.35) as a white solid (858 mg, 85%), mp 72-74 °C. $[\alpha]_D^{20} = -52.5$ (c 0.86, $CHCl_3$). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.40-7.27 (m, 15 H, Ar), 4.85 (dd, *J*=7.4, 6.6 Hz, 1H, H-2), 4.64-4.50 (m, 6 H, Bn), 4.05 (dd, *J*=6.0, 3.5 Hz, 1 H, H-5), 4.01 (t, *J*=3.3 Hz, 1 H, H-4), 3.90 (ddd, *J*=9.0, 6.0, 3.1 Hz, 1 H, H-3a), 3.76 (dd, *J*=9.9, 5.0 Hz, 1 H, Ha-8), 3.65 (dd, *J*=9.9, 6.3 Hz, 1 H, Hb-8), 3.40 (m, 1 H, H-6), 3.06 (s, 3 H, NMe), 3.01 (ddd, *J*=12.5, 9.0, 6.4 Hz, 1 H, Ha-3), 2.95 (s, 3 H, NMe), 2.26 (ddd, *J*=12.6, 7.6, 6.1, 1H, Hb-3). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 168.0 (s, C=O), 138.2, 137.8, 137.5 (s, Ar), 128.4-127.5 (d, 15 C, Ar), 86.6 (d, C-5), 84.0 (d, C-4), 74.7 (d, C-2), 73.3, 72.3, 71.6 (t, Bn), 70.1 (t, C-8), 70.0 (d, C-6), 68.5 (d, C-3a), 37.0 (q, NMe), 36.1 (t, C-3), 35.8 (q, NMe). IR ($CDCl_3$): 3066, 2930, 2867, 1649, 1497, 1454, 1216, 1100 cm^{-1}. MS (EI): *m/z* 516 (M^+, 0.2), 444 (5), 197 (13), 107 (62), 90 (100), 77 (63), 56 (33). Anal. Calcd for $C_{31}H_{36}N_2O_5$ (516.63): C, 72.07; H, 7.02; N, 5.42. Found: C, 72.17; H, 7.20; N, 5.70.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-1,2-bis(benzyloxy)-3-[(benzyloxy)methyl]-6-hydroxyhexahydro-5*H*-pyrrolizin-5-one (**21**). A mixture of **20** (816 mg, 1.58 mmol) and Zn dust (407 mg) in CH_3COOH/H_2O 9:1 (12.5 mL) was heated to 50 °C for 4 h and then filtered through cotton. The solution was cooled to 0 °C

and, under vigorously stirring, saturated aqueous solution of $NaHCO_3$ (100 mL) was added until basic pH was reached. The aqueous phase was extracted with EtOAc (3 x 20 mL) and the combined organic phases were dried over Na_2SO_4. After filtration and evaporation under reduced pressure, was obtained **21** as an yellow oil, enough pure to be used for the next step (598 mg, 80%). An analytically pure sample was obtained through purification by flash column chromatography on silica gel (eluent petroleum ether/EtOAc 1:2, R_f=0.4). $[\alpha]_D^{20}$= + 3.05 (c 0.9, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38-7.26 (m, 15 H, Ar), 4.60-4.45 (m, 7 H, Bn, H-6), 4.34 (t, *J*=4.3 Hz, 1 H, H-2), 4.11 (q, *J*=4.5 Hz, 1 H, H-3), 3.87 (bs, 1 H, OH), 3.79 (dd, *J*=6.9, 5.0 Hz 1 H, H-1), 3.73 (dt, *J*=8.6, 6.5 Hz, 1 H, H-7a), 3.63 (dd, *J*=9.8, 5.5 Hz, 1 H, Ha-8), 3.52 (dd, *J*=9.8, 4.1 Hz, 1 H, Hb-8), 2.70 (ddd, *J*=12.3, 7.8, 6.1 Hz, 1 H, Ha-7), 1.80 (ddd, *J*=12.3, 10.4, 8.6 Hz, 1 H, Hb-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 174.5 (s, C=O), 137.8, 137.7, 137.4 (s, Ar), 128.5-127.6 (d, 15 C, Ar), 89.1 (d, C-1), 85.7 (d, C-2), 73.3, 72.6, 72.4 (t, Bn), 72.0 (d, C-6), 68.8 (t, C-8), 59.9 (d, C-7a), 58.6 (d, C-3), 37.2 (t, C-7). IR ($CDCl_3$): 3671, 3373, 3012, 2867, 1697, 1454, 1100 cm^{-1}. MS (EI): *m/z* 381 (M^+- Bn, 12), 336 (20), 275 (32), 180 (98), 153 (100), 88 (100). Anal. Calcd for $C_{29}H_{31}NO_5$ (473.56): C, 73.55; H, 6.60; N, 2.96. Found: C, 73.33; H, 6.80; N, 2.87.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-1,2,6-Trihydroxy-3-(hydroxymethyl)hexahydro-5*H*-pyrrolizin-5-one (**15**). To a stirred solution of **21** (150 mg, 0.32 mmol) in 15 mL of EtOH, 300 mg of Pd (10% on C) was added under nitrogen atmosphere. The suspension was stirred at rt for 3 days under hydrogen atmosphere, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a viscous oil that was purified by flash column chromatography on silica gel (eluent MeOH/EtOAc 1:3, R_f=0.14) affording pure **15** as a transparent oil (57 mg, 88 % yield). $[\alpha]_D^{20}$ = − 1.2 (c 0.25, MeOH). ^{1}H-NMR (400 MHz, CD_3OD) δ 4.54 (dd, *J*= 10.5, 7.8 Hz, 1H, H-6), 4.16 (t, *J*= 6.3 Hz, 1H, H-2), 3.84 (dd, *J*= 3.0, 8.0 Hz, 1H, Ha-8), 3.67-3.53 (m, 4H, Hb-8, H-1, H-3, H-7a), 2.78 (ddd, *J*= 12.2, 7.8, 5.9 Hz, 1H, Ha-7), 1.74 (ddd, *J*= 12.0, 10.7, 8.3 Hz,

1H, Hb-7). ^{13}C-NMR (50 MHz, CD_3OD) δ 175.29 (s, C-5), 81.76 (d, C-1), 78.30 (d, C-2), 71.66 (d, C-6), 61.99 (d, C-3), 60.17 (t, C-8), 59.82 (d, C-7a), 36.83 (t, C-7). MS: *m/z* 204 (5, M^++H), 203 (3, M^+), 185 (27, M^+-O), 172 (52), 144 (74), 126 (51), 100 (87), 86 (100), 72 (59), 57 (38). Anal. Calcd for $C_8H_{13}NO_5$ (203.19): C, 47.29; H, 6.45; N, 6.89. Found: C, 47.54; H, 6.36; N, 6.95.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-1,2-bis(benzyloxy)-3-[(benzyloxy)methyl] hexahydro-1*H*-pyrrolizin-6-ol (**22**). To a cooled (0°C) solution of **21** (255 mg, 0.54 mmol) in dry THF (6 mL), a 1 M solution of $LiAlH_4$ in THF (1.6 mL, 1.61 mmol) was added under nitrogen atmosphere. The mixture was then refluxed for 1.5 hours. Then, after cooling at 0°C, 560 µL of an aqueous saturated solution of Na_2SO_4 were added dropwise. The suspension was then filtered through Celite® and washed with EtOAc. Evaporation under reduced pressure afforded **22** as a yellow oil enough pure for the next step (185 mg, 75 % yield). An analytically pure sample was obtained through purification by flash column chromatography on silica gel (eluent petroleum ether/EtOAc 1:4, R_f=0.3). $[\alpha]_D^{20}$ = + 9.1 (c 0.83, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.36-7.24 (m, 15 H, Ar), 4.67-4.46 (m, 6 H, Bn), 4.35-4.31 (m, 1 H, H-6), 4.11 (t, *J*=4.6 Hz, 1 H, H-1), 4.07 (t, *J*=4.7 Hz, 1 H, H-2), 3.60-3.44 (m, 4 H, H-7a, H-3, Ha-8, Hb-8), 3.21 (dd, *J*=12.3, 4.5 Hz 1 H, Ha-5), 2.98 (dm, *J*=12.3 Hz, 1 H, Hb-5), 2.21 (ddd, *J*=13.8, 9.0, 5.7 Hz, 1H, Ha-7), 1.84 (dm, *J*=13.8 Hz, 1H, Hb-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.1, 137.8, 137.4 (s, Ar), 128.1-127.2 (d, 15 C, Ar), 88.9 (d, C-1), 85.2 (d, C-2), 73.6 (d, C-6), 72.9, 72.1, 71.8, 71.6 (t, Bn, C-8), 69.9 (d, C-3), 67.4 (d, C-7a), 63.3 (t, C-5), 40.0 (t, C-7). IR ($CDCl_3$): 3392, 3010, 2927, 2858, 1748, 1710, 1454, 1262 cm^{-1}. MS (EI): *m/z* 366 (6), 336 (100), 216 (61), 160 (100), 90 (100). Anal. Calcd for $C_{29}H_{33}NO_4$ (459.58): C, 75.79; H, 7.24; N, 3.05. Found: C, 75.99; H, 7.16; N, 3.02.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-3-(hydroxymethyl)hexahydro-1*H*-pyrrolizine-1,2,6-triol (7-deoxycasuarine, **14**).[81] To a stirred solution of **22** (120 mg, 0.26 mmol) in EtOH (10.5 mL), 4-5 drops of concentrated HCl and 250 mg of Pd (10% on C) were added under nitrogen atmosphere. The suspension was stirred at rt for 3 days under hydrogen atmosphere, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a viscous yellow oil (66 mg) that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (10 mL), H_2O (10 mL) to remove non amine containing products, and then with 6% NH_4OH (15 mL) to elute 7-deoxycasuarine **14** as a white solid (43 mg, 88% yield), mp 205-208°C. $[\alpha]_D^{20}$ = + 19.8 (c 0.4, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 4.44-4.38 (m, 1H, H-6), 4.05 (t, *J*=8.2 Hz, 1 H, H-1), 3.74-3.69 (m, 2 H), 3.56 (dd, *J*=11.9, 6.5 Hz, 1 H), 3.24 (ddd, *J*=12.6, 8.2, 4.2 Hz, 1 H), 3.09-3.02 (m, 2 H), 2.86 (m, 2 H, Ha-5, Hb-5), 2.12 (ddd, *J*=13.6, 8.5, 3.4 Hz, 1 H, Ha-7), 1.89 (dtd, *J*=13.6, 3.8, 1.4 Hz, 1 H, Hb-7). ^{13}C-NMR (50 MHz, D_2O) δ 82.6 (d, C-1), 79.0 (d, C-2), 75.1 (d, C-6), 72.5 (d, C-7a), 67.9 (d, C-3), 64.6 (t, C-8), 63.3 (t, C-5), 39.3 (t, C-7). MS (EI): *m/z* 190 (M^++H, 3), 189 (M^+, 1), 176 (66), 158 (M^+–CH_2OH, 65), 132 (64), 112 (23), 85 (62), 58 (100). Anal. Calcd for $C_8H_{15}NO_4$ (189.21): C, 50.78; H, 7.99; N, 7.40. Found: C, 50.37; H, 7.63; N, 7.74.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-1,2-Bis-benzyloxy-3-benzyloxymethyl-6-metansulfonyloxy-hexahydro-1*H*-pyrrolizine (**23**). To a stirred solution of **21** (95 mg, 0.20 mmol) in dry CH_2Cl_2 (0.45 mL), NEt_3 (75 µL, 0.54 mmol) was added under nitrogen atmosphere, and, at 0 °C, MsCl (20 µL, 0.26 mmol) was added dropwise. The solution was left stirring at 0 °C for 30 minutes and for 2 h at rt. The mixture was filtered through Celite® and washed with EtOAc. Evaporation under reduce pressure afforded **23,** as a white oil, (quantitative yield), enough pure to be used for the next step (110 mg, quantitative yield). $[\alpha]_D^{27}$= + 12.3 (c 1.06, CH_2Cl_2). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38-7.26 (m, 15 H, Ar), 5.33 (dd, *J* = 9.9, 8.2 Hz, 1 H, H-6), 4.61-4.43 (m, 6 H, Bn), 4.32 (t, *J* = 4.6 Hz, 1 H, H-2), 4.09

[81] Behr, J.-B.; Erard, A.; Guillerm, G. *Eur. J. Org. Chem.* **2002**, 1256.

(q, *J* = 4.3 Hz, 1 H, H-3), 3.77 (dd, *J* = 6.8, 4.9 Hz, 1 H, H-1), 3.72 (dd, *J* = 8.1, 6.5 Hz, 1 H, H-7a), 3.60 (dd, *J* = 9.9, 5.2 Hz, 1 H, Ha-8), 3.49 (dd, *J* = 9.9, 4.1 Hz, 1 H, Hb-8), 3.27 (s, 3 H, Ms), 2.76 (ddd, *J* = 12.8, 8.2, 6.2 Hz, 1 H, Ha-7), 2.00 (ddd, *J* = 12.8, 10.0, 8.2 Hz, 1 H, Hb-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 167.5 (s, C-5), 137.3-137.1 (s, 3 C, Ar), 128.3-127.4 (d, 15 C, Ar), 88.5 (d, C-1), 85.2 (d, C-2), 78.2 (d, C-6), 73.0, 72.4, 72.2 (t, Bn), 68.1 (t, C-8), 59.2 (d, C-7a), 58.6 (d, C-3), 39.7 (q, Ms), 34.6 (t, C-7). IR (CH_2Cl_2): 3065, 3033, 2868, 1717, 1454, 1364, 1267, 1179, 1104, 1028 cm^{-1}. Anal. Calcd for $C_{30}H_{33}NO_7S$ (551.20): C, 67.02; H, 6.56; N, 2.61. Found: C, 66.90; H, 6.70; N, 2.74.

(1*R*,2*R*,3*R*,7a*R*)-1,2-bis-benzyloxy-3-benzyloxymethyl-hexahydro-1*H*-pyrrolizine (**24**).[40] To a stirred solution of **23** (110 mg, 0.2 mmol) in dry THF (2.5 mL) a 1M solution of $LiAlH_4$ in THF (0.8 mL, 0.8 mmol) was added dropwise at 0 °C under nitrogen atmosphere. The mixture was heated at reflux temperature for 3 hours. An aqueous saturated solution of Na_2SO_4 (280 µL) was added dropwise and the mixture was left stirring at rt for other 10 min. After filtration through Celite®, 109 mg of a crude residue were obtained, that were purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate 1:2, R_f = 0.28) affording pure **24** (71 mg, 80 % yield) as an oil. $[\alpha]_D^{24}$ = − 5.1 (c 0.6, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37-7.25 (m, 15 H, Ar), 4.73-4.46 (m, 6 H, Bn), 4.08 (dd, *J* = 7.4, 5.9 Hz, 1H, H-2), 3.81 (t, *J* = 5.9 Hz, 1H, H-1), 3.60 (dd, J = 9.6, 4.7 Hz, 1 H, Ha-8), 3.54 (dd, *J* = 9.6, 5.7 Hz, 1 H, H-7a), 3.51-3.46 (m, 1 H, Hb-8), 3.07 (dt, *J* = 10.5, 6.1 Hz, 1 H, Ha-5), 2.98-2.94 (m, 1 H, H-3), 2.78 (dt, *J* = 10.5, 6.6 Hz, 1 H, Hb-5), 2.03-1.95 (m, 1 H, Ha-7), 1.91-1.82 (m, 1 H, Ha-6), 1.81-1.74 (m, 1 H, Hb-6), 1.72-1.62 (m, 1 H, Hb-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.2, 138.1, 137.9 (s, Ar), 128.0- 127.1 (d, 15 C, Ar), 88.6 (d, C-1), 85.5 (d, C-2), 73.0, 72.3, 71.8, 71.6 (t, C-8, Bn), 68.0 (d, C-3), 67.2 (d, C-7a), 54.8 (t, C-5), 31.4 (t, C-6), 25.5 (t, C-7).

(1*R*,2*R*,3*R*,7a*R*)-1,2-Dihydroxy-3-hydroxymethyl-hexahydro-1*H*-pyrrolizine(hyacinthacine A_2, **13**).[40, 36] To a stirred solution of **24** (25 mg, 0.056

mmol) in MeOH (2.5 mL) 3 drops of concentrated HCl and 45 mg of Pd (10% on C) were added. The mixture was left stirring at rt for 3 days under hydrogen atmosphere. The mixture was then filtered through Celite® and washed with MeOH. The solvent was evaporated under reduced pressure affording a viscous white oil, that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (10 mL), H_2O (10 mL) to remove non amine containing products and then with 6% NH_4OH (15 mL) to elute hyacinthacine A_2 **13** as a white solid (7 mg, 72% yield). $[\alpha]_D^{24}$ = + 12.4 (c 0.2, H_2O); ^{1}H-NMR (200 MHz, D_2O) δ 3.72-3.61 (m, 3 H), 3.53 (dd, *J*=11.7, 6.2 Hz, 1 H), 3.10-3.00 (m, 1 H), 2.86-2.75 (m, 1 H), 2.70-2.56 (m, 2 H), 1.90-1.58 (m, 4 H); ^{13}C-NMR (50 MHz, D_2O) δ 82.6 (d, C-1), 79.6 (d, C-2), 71.7 (d, C-3), 68.6 (d, C-7a), 65.3 (t, C-8), 57.4 (t, C-5), 32.2 (t, C-7), 27.0 (t, C-6). Anal. Calcd for $C_8H_{15}NO_3$ (173.21): C, 55.47; H, 8.73; N, 8.09. Found: C, 55.49; H, 8.61; N, 8.10.

3.5.2 Synthesis of 7-*homo*casuarine

Dimethyl(2*R*,3*S*,3a*R*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6[(-benzyloxy) methyl]hexahydropyrrolo[1,2-b]isoxazole-2,3-dicarboxilate (**26**). A solution of nitrone **1** (1.47 g, 3.52 mmol) and dimethyl maleate (**25**, 440 µL, 3.52 mmol) in CH_2Cl_2 (23 mL) was stirred at rt for 4 days, then the solvent was removed under reduced pressure. Purification of the residue by flash column chromatography on silica gel (petroleum ether/EtOAc 2:1) afforded pure **26** (R_f=0.35) as a white solid (1.54 g, 78% yield), mp 59-62 °C. $[\alpha]_D^{20}$ = – 86.0 (c 0.95, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.40-7.25 (m, 15H, Ar), 4.94 (d, *J*=8.2 Hz, 1H, H-2), 4.66-4.49 (m, 6H, Bn), 4.28 (d, *J*=7.6 Hz, 1H, H-3a), 4.12 (m, 1H, H-4), 4.09-4.08 (m, 1 H, H-5), 3.80 (s, 3 H, Me), 3.75 (s, 3H, Me), 3.79-3.74 (m, 2H, H-3, Ha-8), 3.60 (dd, *J*=9.7, 7.7 Hz, 1H, Hb-8), 3.48-3.45 (m, 1H, H-6); ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 169.4 (s, C=O), 168.9 (s, C=O), 138.1, 137.5, 137.4 (s, Ar), 128.4-127.5 (d, 15 C, Ar), 85.0, 84.3 (d, C-4, C-5) 77.8 (d, C-2), 73.4, 72.1 (t, Bn), 71.9 (d, C-6), 71.6 (t, Bn), 71.4 (d, C-3a), 70.0 (t, C-8), 54.1 (d, C-3), 52.6 (q, 2 C,

Me). IR ($CDCl_3$): 3160, 3080, 3030, 3000, 2960, 2920, 2880, 1730, 1625, 1570, 1460, 1380, 1250, 1088 cm^{-1}. MS (EI): *m/z* 561 (M^+, 0.2), 531 (0.6), 502 (M^+, 0.3), 113 (8), 91 (100), 65 (3), 77 (3). Anal. Calcd for $C_{32}H_{35}NO_8$ (561.6): C, 68.43; H, 6.28; N, 2.49. Found: C, 68.00; H, 6.42; N, 2.34.

Methyl(1*S*,2*R*,6*R*,7*R*,7a*R*)-6,7-bis(benzyloxy)-5-[(benzyloxy)methyl]-2-hydroxy-3-oxohexahydro-1*H*-pyrrolizine-1-carboxylate (**27**). A mixture of **26** (870 mg, 1.55 mmol) and Zn dust (400 mg, 6.2 mmol) in CH_3COOH/H_2O 9:1 (12.5 mL) was heated to 50 °C for 3 h and then filtered through cotton. The solution was cooled to 0°C and, under vigorously stirring, saturated aqueous solution of $NaHCO_3$ (100 mL) was added until basic pH was reached. The aqueous phase was extracted with EtOAc (3 x 60 mL) and the combined organic phases were dried over Na_2SO_4. After filtration and evaporation under reduced pressure, was obtained pure **27** as a white solid (743 mg, 90% yield), mp 111-113 °C. $[\alpha]_D^{20} = -28.7$ (c 0.635, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37-7.23 (m, 15 H, Ar), 4.76 (dd, *J*=9.8, 2.9 Hz, 1 H, H-2), 4.57-4.44 (m, 6 H, Bn), 4.31-4.28 (m, 1 H, H-5), 4.23-4.22 (m, 1 H, H-6), 3.98-3.95 (m, 1 H, H-7a), 3.92-3.90 (m, 1 H, H-7), 3.79 (s, 3H, Me), 3.58-3.50 (m, 2 H, H-8), 3.44 (d, *J*=3.1 Hz, 1 H, OH), 3.03 (t, *J*=9.3 Hz, 1 H, H-1). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 173.2, 171.1 (s, C=O), 137.7-137.2 (s, 3 C, Ar), 128.4-127.5 (d, 15 C, Ar), 87.3 (d, C-7), 84.6 (d, C-6), 74.2 (d, C-2), 73.1, 72.1, 71.8 (t, Bn), 68.1 (t, C-8), 62.7 (d, C-7a), 59.3 (d, C-5), 54.8 (d, C-1), 52.5 (q, Me). IR ($CHCl_3$): 3690, 3600-3500 (broad), 3027, 2920, 1708, 1601, 1155, 1070 cm^{-1}. MS (EI): *m/z* 513 (M^+-H_2O, 0.6), 212 (4), 91 (100), 69 (14). Anal. Calcd for $C_{31}H_{33}NO_7$ (531.6): C, 70.04; H, 2.63; N, 6.26. Found: C, 70.02; H, 2.56; N, 6.28.

(1*R*,2*R*,3*R*,6*R*,7*R*,7a*R*)-1,2-bis(benzyloxy)-3[(benzyloxy)methyl]-7-(hydroxymethyl)hexahydro-1*H*-pyrrolizin-6-ol (**28**). To a cooled (0 °C) solution of **27** (115 mg, 0.22 mmol) in dry THF (3 mL) a 1 M solution of $LiAlH_4$ in THF (1.1 mL, 1.1 mmol) was added under nitrogen atmosphere. The mixture was then refluxed for 2 hours. Then, after cooling at 0°C, 700 µL of an aqueous saturated

solution of Na_2SO_4 were added dropwise. The suspension was then filtered through Celite® and washed with EtOAc. Evaporation under reduced pressure afforded the solid **28** enough pure to be used for the next step (104 mg, quantitative yield). An analytically pure sample was obtained by filtration over a short pad of silica gel (eluent EtOAc then EtOAc /MeOH 5:1), mp 85-87 °C. $[\alpha]_D^{20}$ = + 3.58 (c 1.18, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.40-7.25 (m, 15 H, Ar), 4.64-4.56 (m, 6 H, Bn), 4.22 (q, *J*=5.6 Hz, H-6), 4.13-4.09 (m, 2 H, H-1, H-2), 3.68 (d, *J*=6.8 Hz, 2H, Ha-8, Hb-8), 3.53 (d, *J*=6.4 Hz, 2 H, Ha-9, Hb-9) 3.43-3.37 (m, 2 H, H-3, Ha-5) 3.27 (dd, *J*=7.0, 3.9 Hz, 1 H, H-7a), 2.95 (dd, *J*=10.8, 5.6 Hz, 1 H, Hb-5), 2.24 (quint., *J*=6.6 Hz, 1 H, H-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.2-137.7 (s, 3 C, Ar), 128.5-127.5 (d, 15 C, Ar), 88.0 (d, 1 C), 86.1 (d, 1 C), 76.6 (d, 1 C), 73.3 (t, 1 C), 72.4 (t, 1 C), 72.0 (t, 1 C), 71.5 (t, 1 C), 70.4 (d, 1 C), 70.4 (d, 1 C), 64.2 (t, 1 C), 62.4 (t, 1 C), 54.3 (d, 1 C). IR ($CDCl_3$): 3412, 3031, 3010, 2866, 1496, 1454, 1363, 1216, 1212, 1211, 1097 cm^{-1}. MS (EI): *m/z* 398 (M^+-C_7H_7, 11), 368 (85), 248 (11), 186 (27), 160 (30), 142 (25), 116 (22), 91 (100), 64 (21). Anal. Calcd for $C_{30}H_{31}NO_5$ (489.3): C, 73.59; H, 7.21; N, 2.86. Found: C, 73.58; H, 7.09; N, 3.18.

(1*R*,2*R*,3*R*,6*R*,7*R*,7a*R*)-3,7-bis(hydroxymethyl)hexahydro-1*H*-pyrrolizine-1,2,6-triol (7-*homo*casuarine, **16**). To a stirred solution of **28** (106 mg, 0.22 mmol), in EtOH (14 mL), 4-5 drops of concentrated HCl and 230 mg of Pd (10% on C) were added. The suspension was stirred at rt under hydrogen atmosphere for 4 days, then filtered through Celite® and washed with EtOH. Evaporation under reduced pressure afforded a viscous oil that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (10 mL), H_2O (10 mL) to remove non amine containing products and then with 6% NH_4OH (15 mL) to elute 7-*homo*casuarine (**16**). Evaporation of the solvent afforded 7-*homo*casuarine as a yellow viscous oil (38.5 mg, 89%). $[\alpha]_D^{20}$ = + 30.8 (c 0.7, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 4.39 (q, *J*=10.0 Hz, 1 H, H-6), 4.04 (t, *J*=7.8 Hz, 1 H, H-1), 3.71 (t, *J*=8.5 Hz, 1 H, H-2), 3.67 (dd, *J*=11.7, 3.5 Hz,

1 H, Ha-8), 3.55-3.44 (m, 3 H, Hb-8, Ha-9, Hb-9), 3.19 (dd, *J*=10.0, 5.2 Hz, 1 H, Ha-5), 3.02 -2.95 (m, 2 H, H-3, H-7a), 2.80 (dd, *J*=10.0, 5.2 Hz, 1 H, Hb-5), 2.21 (q, *J*=5.9 Hz, 1H, H-7). ^{13}C-NMR (50 MHz, D_2O) δ 80.2 (d, C-1), 77.5 (d, C-2), 74.5 (d, C-6), 70.6, 68.5 (d, C-3, C-7), 62.7 (t, C-8), 61.3 (t, C-9), 60.4 (t, C-5), 52.9 (d, C-7a). MS (EI): *m/z* 188 (100), 170 (10), 159 (13), 142 (14), 128 (83), 116 (26), 68 (38), 55 (24). Anal. Calcd for $C_9H_{17}NO_5$ (219.23): C, 49.31; H, 7.82; N, 6.39. Found: C, 49.06; H, 7.43; N, 6.54.

3.5.3 Syntheses of Casuarine and 7-*epi*australine

Ethyl 3-[dimethyl(phenyl)silyl]-2-propynoate (**33**). A solution of (dimethylphenylsilyl)-acetylene (**32**, 4.0 g, 25.0 mmol) in dry THF was cooled to –78 °C and *n*-butyllithium (1.6 M in hexanes, 23.5 mL, 37.4 mmol) was added dropwise. Ethyl chloroformate (7.0 g, 64.9 mmol) was added after 15 minutes and the reaction mixture was stirred at –78 °C for 3 h; then, the mixture was allowed to heat to –10 °C, H_2O (80 mL) was added dropwise and the product was extracted with petroleum ether (3 x 80 mL). The collected organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated to give the crude product, that was further purified by flash chromatography on silica gel (petroleum ether-ethyl acetate 30:1) to give the pure alkyne **33** (R_f=0.61, 5.11 g, 22.0 mmol, 88%) as a colourless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62-7.60 (m, 2 H, Ar), 7.42-7.38 (m, 3 H, Ar), 4.24 (q, *J* = 7.0 Hz, 2 H, C*H*$_2$CH$_3$), 1.32 (t, *J* = 7.0 Hz, 3 H, CH$_2$C*H*$_3$), 0.50 (s, 6 H, $SiMe_2$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 152.6 (s, C=O), 134.2 (s, Ar), 133.4 (d, 2 C, Ar), 129.7 (d, Ar), 127.8 (d, 2 C, Ar), 95.7 (s, C≡C), 91.3 (s, C≡C), 61.8 (t, *C*H$_2$CH$_3$), 13.8 (q, CH$_2$*C*H$_3$), -1.9 (q, 2 C, $SiMe_2$). IR ($CDCl_3$): 3383, 3072, 2964, 2257, 2180, 1690, 1429, 1225, 1115, 1026 cm^{-1}; MS (EI): *m/z* 217 ($M^+ -$ CH_3, 5), 189 (84), 159 (95), 145 (100), 137 (49). Anal. Calcd. for $C_{13}H_{16}O_2Si$ (232.35): C, 67.20; H, 6.94. Found: C, 67.17; H, 6.83.

Ethyl (2*Z*)-3-[dimethyl(phenyl)silyl]-2-propenoate (**31**) A solution of the pure alkyne **33** 5.11 g, 22.0 mmol) in toluene (55 mL) was stirred at rt under H_2

atmosphere in the presence of Lindlar catalyst (510 mg) and monitored every 30 minutes via ^{1}H NMR control. After 4 h, filtration through Celite® and evaporation of the solvent under reduced pressure afforded **31** (4.84 g, 20.7 mmol, 94%) as a pale yellow liquid, pure enough to be used for the next step. An analytically pure sample was obtained by purification on silica gel (petroleum ether-diethyl ether 20:1, R_f=0.35). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59-7.55 (m, 2 H, Ar), 7.37-7.26 (m, 3 H, Ar), 6.67 (A part of an AB system, *J* = 14.4 Hz, 1H, H-2 or H-3), 6.60 (B part of an AB system, *J* = 14.4 Hz, 1H, H-2 or H-3), 4.12 (q, *J* = 7.2 Hz, 2 H, C*H*$_2$CH$_3$), 1.21 (t, *J* = 7.2 Hz, 3 H, CH$_2$C*H*$_3$), 0.49 (s, 6 H, $SiMe_2$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.3 (s, C=O), 149.2 (d, 2 C, Ar), 139.2 (s, Ar), 136.6 (d, C=C), 133.5 (d, Ar), 128.8 (d, C=C), 127.7 (d, 2 C, Ar), 60.5 (t, *C*H$_2$CH$_3$), 14.3 (q, CH$_2$*C*H$_3$), -1.7 (q, 2 C, $SiMe_2$). IR ($CDCl_3$): 3069, 2983, 2247, 1726, 1588, 1427, 1370 cm^{-1}; MS (EI): *m/z* 235 (M^++ 1, 0.5), 234 (M^+, 0.7), 219 ($M^+ - CH_3$, 71), 191 (100), 175 (14), 157 (M^+-Ph, 68), 129 (85). Anal. Calcd. for $C_{13}H_{18}O_2Si$ (234.37): C, 66.62; H, 7.74. Found: C, 66.91; H, 8.17.

Ethyl(2*S*,3*S*,3a*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxy)methyl-3-[dimethyl(phenyl)silyl]-hexahydropyrrolo[1,2-b]isoxazole-2-carboxylate (**34**): A solution of nitrone **1** (1.23 g, 2.95 mmol) and **31** (1.17 g, 6.16 mmol) in CH_2Cl_2 (3 mL) was stirred at rt for 36 h, then the solvent was removed under reduced pressure. Purification of the residue by chromatography on silica gel (petroleum ether-ethyl acetate 7:1) afforded pure **34** (1.52 g, 2.33 mmol, 79%) as a colourless oil. R_f = 0.22 (petroleum ether-ethyl acetate 7:1). $[\alpha]_D^{22} = -76.6$ (*c* 0.56, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, 25°C) δ 7.47-7.44 (m, 2 H, Ar), 7.38-7.24 (m, 15 H, Ar), 7.21-7.17 (m, 3 H, Ar), 4.85 (d, *J* = 8.8 Hz, 1 H, H-2), 4.58 (A part of an AB system, *J* = 12.2 Hz, 1 H, Bn), 4.55 (B part of an AB system, *J* = 12.2 Hz, 1 H, Bn), 4.39 (A part of an AB system, *J* = 11.9 Hz, 1 H, Bn), 4.33 (B part of an AB system, *J* = 11.9 Hz, 1 H, Bn), 4.24 (A part of an AB system, *J* = 12.0 Hz, 1 H, Bn), 4.16 (B part of an AB system, *J* = 12.0 Hz, 1 H, Bn), 4.05-3.90 (m, 4 H, H-5, H-3a, C*H*$_2$CH$_3$), 3.78 (dd, *J* = 9.8, 4.7 Hz, 1 H, Ha-8), 3.65 (m, 1 H, H-

4), 3.57 (dd, *J* = 9.8, 8.2 Hz, 1 H, Hb-8), 3.40 (dt, *J* = 8.6, 4.7 Hz, 1 H, H-6), 2.40 (dd, *J* = 11.1, 8.8 Hz, 1 H, H-3), 1.17 (t, *J* = 7.2 Hz, 3 H, $CH_2C\mathit{H_3}$), 0.39 (s, 3 H, SiMe), 0.38 (s, 3 H, SiMe). ^{13}C-NMR (50 MHz, $CDCl_3$, 25°C) δ 170.8 (s; C=O), 138.4-137.1 (s, 4 C; Ar), 134.1-127.5 (d, 20 C; Ar), 85.3 (d; C-5), 83.9 (d; C-4), 80.7 (d; C-2), 73.4 (t; Bn), 72.7 (d; C-6), 71.8 (d; C-3a), 71.6, 71.1 (t, 2 C; Bn), 70.8 (t; C-8), 61.1 (t; $\mathit{C}H_2CH_3$), 36.9 (d; C-3), 14.0 (q; $CH_2\mathit{C}H_3$), -2.7, -4.2 (q, 2 C; $SiMe_2$). IR ($CDCl_3$): 3688, 3029, 3012, 2930, 1739, 1496, 1454, 1253 cm^{-1}; MS (70 eV): *m/z* 560 (2), 400 (53), 296 (65), 188 (40), 181 (60), 105 (48), 91 (100). Anal. Calcd for $C_{39}H_{45}NO_6Si$ (651.3): C, 71.86; H, 6.96; N, 2.15. Found: C, 71.58; H, 7.13; N, 2.08.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*S*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl-7-[dimethyl(phenyl)silyl]-6-hydroxyhexahydro-5*H*-pyrrolizin-5-one (**35**). A mixture of **34** (484 mg, 0.74 mmol) and Zn dust (244 mg) in CH_3COOH/H_2O 9:1 (8 mL) was heated to 60-65 °C for 5 h and then filtered through cotton. The solution was cooled to 0 °C and, under vigorous stirring, a saturated aqueous solution of $NaHCO_3$ (60 mL) was added until alkaline pH was reached. The aqueous phase was extracted with AcOEt (3 x 60 mL) and the combined organic phases were dried over Na_2SO_4. After filtration and evaporation under reduced pressure, a viscous oil was obtained. Purification on silica gel (petroleum ether/ethyl acetate 3:2) afforded pure **35** (420 mg, 0.69 mmol, 93%) as a colourless viscous oil. R_f = 0.4 (petroleum ether/ethyl acetate 3:2). $[\alpha]_D^{22}$ − 47.0 (*c* 1.87, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, 25°C) δ 7.55-7.53 (m, 2 H, Ar), 7.38-7.23 (m, 16 H, Ar), 7.14-7.12 (m, 2 H, Ar), 4.55 (d, *J* = 11.7 Hz, 1 H, Bn), 4.46 (s, 2 H, Bn), 4.38-4.34 (m, 2 H, Bn, H-3), 4.30 (d, *J* = 11.1 Hz, 1 H, H-6), 4.22 (d, *J* = 11.3 Hz, 1 H, Bn), 4.12-4.09 (m, 2 H, H-2, Bn), 3.62 (dd, *J* = 9.8, 4.1 Hz, 1 H, H-7a), 3.58-3.56 (m, 1 H, H-1), 3.47 (dd, *J* = 9.6, 5.8 Hz, 1 H, Ha-8), 3.43 (dd, *J* = 9.6, 8.0 Hz, 1 H, Hb-8), 2.75 (br s, 1 H, OH), 1.63 (dd, *J* = 11.1, 9.8 Hz, 1 H, H-7), 0.44 (s, 3 H, SiMe), 0.39 (s, 3 H, SiMe). ^{13}C-NMR (50 MHz, $CDCl_3$, 25°C) δ 176.6 (s, C-5), 138.0-136.3 (s, 4 C, Ar), 134.3-127.7 (d, 20 C, Ar), 89.0 (d, C-1),

84.4 (d, C-2), 73.9 (d, C-6), 73.0, 72.0, 71.5 (t, 3 C, Bn), 68.5 (t, C-8), 63.2 (d, C-7a), 59.1 (d, C-3), 38.9 (d, C-7), -3.6, -4.8 (q, 2 C, $SiMe_2$). IR ($CDCl_3$): 3400, 3000, 2850, 1700 cm^{-1}; MS (70 eV): *m/z* 516 (4), 289 (26), 181 (13), 135 (89), 91 (100). Anal. Calcd for $C_{37}H_{41}NO_5Si$ (607.81): C 73.11; H 6.80; N 2.30. Found: C 72.78; H 6.81; N 2.15.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl]-6,7-dihydroxyhexahydro-1*H*-pyrrolizine (**36**). Mercuric trifluoroacetate (64 mg, 0.15 mmol) was added to a stirred solution of **35** (47 mg, 0.079 mmol) in $CHCl_3$ (0.47 mL), acetic acid (0.12 mL) and trifluoroacetic acid (0.23 mL). The solution was stirred at rt for 1 h; then, peracetic acid (1.35 mL) was added dropwise to the mixture with ice cooling. After 1 h stirring at room temperature the solution was cooled to -10 °C and a saturated aqueous solution of $Na_2S_2O_3$ (10 mL) was added dropwise. The mixture was extracted with AcOEt (3 x 30 mL) and the combined organic layers were washed with a saturated aqueous solution of Na_2CO_3 (30 mL), dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (petroleum ether/ethyl acetate 2:3) afforded pure **36** (29 mg, 0.06 mmol, 76%) as a colourless oil. R_f = 0.2 (petroleum ether/ethyl acetate 2:3). $[\alpha]_D^{20}$ − 41.5 (*c* 0.68, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, 25°C) δ 7.36-7.20 (m, 15 H, Ar), 4.71 (d, *J* = 11.7 Hz, 1 H, Bn), 4.56-4.42 (m, 6 H, Bn, H-6), 4.26 (t, *J* = 3.6 Hz, 1 H, H-2), 4.15-4.12 (m, 2 H, H-7, H-3), 3.91 (t, *J* = 4.7 Hz, 1H, H-1), 3.66 (t, *J* = 6.3 Hz, 1 H, H-7a), 3.58 (dd, *J* = 9.7, 5.8 Hz, 1 H, Ha-8), 3.50 (dd, *J* = 9.7, 4.3 Hz, 1 H, Hb-8). ^{13}C-NMR (50 MHz, $CDCl_3$, 25°C): δ = 172.6 (s, C-5), 137.9-137.7 (s, 3 C, Ar), 128.5-127.8 (d, 15 C, Ar), 86.9 (d, C-1), 85.1 (d, C-2), 80.5 (d, C-7), 78.3 (d, C-6), 73.3 (t, Bn), 72.1 (t, 2 C, Bn), 68.3 (t, C-8), 66.8 (d, C-7a), 59.0 (d, C-3). IR ($CDCl_3$): 3372, 3011, 2866, 1703, 1454, 1214, 1102 cm^{-1}. MS (70 eV): *m/z* 428 (11), 398 (4), 368 (M^+-CH_2OBn, 3), 292 (20), 262 (12), 171 (90), 169 (57), 90 (100). Anal. Calcd for $C_{29}H_{31}NO_6$ (489.56): C 71.15; H 6.38; N, 2.86. Found: C 70.90; H 6.31; N 2.80.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl]-6,7-dihydroxyhexahydro-1*H*-pyrrolizine (**37**). To a cooled (0 °C) solution of **36** (173 mg, 0.35 mmol) in dry THF (4.2 mL) a 1 M solution of $LiAlH_4$ in THF (1.3 mL, 1.3 mmol) was added under nitrogen atmosphere. The mixture was then refluxed for 2 h. After cooling to 0 °C, a sat. Na_2SO_4 solution (600 µL) was added dropwise. The suspension was then filtered through Celite® and washed with AcOEt. Evaporation under reduced pressure afforded a viscous oil, that was quickly filtered over a short pad of silica gel (eluent: ethyl acetate) obtaining pure **37** as a viscous oil (130 mg, 0.27 mmol, 78%). R_f = 0.6 (eluent: ethyl acetate). $[\alpha]_D^{20}$ − 2.9 (*c* 0.53, $CDCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, 25°C) δ 7.32-7.23 (m, 15 H, Ar), 4.66-4.61 (m, 2 H, Bn), 4.56-4.49 (m, 4 H, Bn), 4.13-4.10 (m, 2 H, H-1, H-6), 4.04 (t, *J* = 5.6 Hz, 1 H, H-2), 3.96 (t, *J* = 5.1 Hz, 1 H, H-7), 3.51 (dd, *J* = 9.6, 5.3 Hz, 1 H, Ha-8), 3.46 (dd, *J* = 9.6, 6.3 Hz, 1 H, Hb-8), 3.40 (dd, *J* = 11.0, 5.6 Hz, 1H, Ha-5), 3.36 (t, *J* = 5.3 Hz, 1H, H-7a), 3.24 (dt, *J* = 5.6, 5.5 Hz, 1H, H-3), 2.81 (dd, *J* = 11.0, 5.3 Hz, 1H, Hb-5). ^{13}C-NMR (50 MHz, $CDCl_3$, 25°C) δ 138.2-137.9 (s, 3 C, Ar), 128.4-127.5 (d, 15 C, Ar), 86.8 (d, C-1), 85.2 (d, C-2), 81.1 (d, C-7), 78.7 (d, C-6), 73.6 (d, C-7a), 73.3, 72.5, 72.0 (t, Bn), 71. 6 (t, C-8), 69.8 (d, C-3), 59.9 (t, C-5). IR ($CDCl_3$): 3393, 2870, 2246, 1711, 1596, 1495, 1453, 1362, 1101 cm^{-1}, MS (70 eV): *m/z* 354 (M^+−CH_2OBn, 38), 234 (3), 172 (18), 160 (12), 91 (100). Anal. Calcd for $C_{29}H_{33}NO_5$ (475.58): C 73.24; H 6.99, N 2.95. Found: C 72.98; H 7.23; N 2.83.

(1*R*,*2R*,3*R*,6*S*,7*S*,7a*R*)-3-(Hydroxymethyl)hexahydro-1*H*-pyrrolizine-1,2,6,7-tetrol (casuarine, **11**). To a stirred solution of **37** (89 mg, 0.19 mmol) in MeOH (12.5 mL), 4-5 drops of conc. HCl and 100 mg of Pd (10% on C) were added. The suspension was stirred under hydrogen atmosphere for 4 d, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute casuarine

(**11**). Evaporation of the solvent afforded Casuarine as a white solid (39 mg, 0.19 mmol, 100%), m.p. 180-182 °C. $[\alpha]_D^{20}$ + 14.4 (*c* 0.52, H_2O). ^{1}H-NMR (400 MHz, D_2O, 25°C) δ 4.19-4.13 (m, 2 H, H-6, H-7), 4.15 (t, *J* = 8.2 Hz, 1 H, H-1), 3.80-3-75 (m, 2 H, H-2, Ha-8) 3.60 (dd, *J* = 11.7, 6.6 Hz, 1 H, Hb-8), 3.27 (dd, *J* = 11.8, 3.7 Hz, 1H, Ha-5), 3.10 (dd, *J* = 8.5, 2.5 Hz, 1H, H-7a), 3.07-3.04 (m, 1 H, H-3), 2.94 (dd, *J* = 11.8, 3.0 Hz, 1 H, Hb-5). ^{13}C-NMR (50 MHz, D_2O, 25°C) δ 78.9 (d, C-7), 77.8 (d, C-1), 77.6 (d, C-6), 76.6 (d, C-2), 72.7 (d, C-7a), 70.3 (d, C-3), 62.1 (t, C-8), 58.3 (t, C-5). IR (KBr): 3329, 2917, 1650, 1416 cm^{-1}. MS (70 eV): *m/z* 205 (M^+, 0.9), 188 ($M^+ - H_2O$, 0.2), 174 ($M^+ - CH_2OH$, 100), 128 (15), 102 (17), 70 (40). Anal. Calcd for $C_8H_{15}NO_5$ (205.21): C 46.82; H 7.37; N 6.83. Found: C 46.51; H 7.54; N 6.73.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*S*)-6-Acetyloxy-1,2-bis(benzyloxy)-3-[(benzyloxy) methyl]-7-[dimethyl(phenyl)silyl] -hexahydro-5H-pyrrolizin-5-one (**38**). A mixture of **34** (484 mg, 0.74 mmol) and Zn dust (244 mg) in CH_3COOH/H_2O 9:1 (8 mL) was heated to 60-65 °C for 5 h and then filtered through cotton. The solution was cooled to 0 °C and, under vigorous stirring, a saturated aqueous solution of $NaHCO_3$ (60 mL) was added until alkaline pH was reached. The aqueous phase was extracted with AcOEt (3 x 60 mL) and the combined organic phases were dried over Na_2SO_4. After filtration and evaporation under reduced pressure, a viscous oil was obtained. The crude (418 mg, 0.69 mmol) was dissolved in pyridine (1 mL) and acetic anhydride (0.5 mL) was added. The mixture was stirred at rt overnight. The mixture was concentrated under vacuum to afford an orange oil. Purification on silica gel (petroleum ether/ethyl acetate 3:1) afforded pure acetate derivative **38** (R_f=0.5, 448 mg, 0.69 mmol, 93% yield over 2 steps) as a colourless oil. $[\alpha]_D^{26} = -15.4$ (*c* 1.05, $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50-7.48 (m, 2 H, Ar), 7.39-7.24 (m, 16 H, Ar), 7.18-7.15 (m, 2 H, Ar), 5.64 (d, *J* = 11.3 Hz, 1 H, H-6), 4.59 (d, *J* = 11.7 Hz, 1 H, Bn), 4.48 (s, 2 H, Bn), 4.37 (d, *J* = 11.7 Hz, 1 H, Bn), 4.37-4.34 (m, 1 H, H-3), 4.33 (d, *J* = 11.7 Hz, 1 H, Bn), 4.21 (d, *J* = 11.7 Hz, 1 H, Bn), 4.19 (m, 1 H, H-1),

3.68-3.65 (m, 2 H, H-2, H-7a), 3.50 (dd, *J* = 9.4, 5.5 Hz, 1 H, Ha-8), 3.45 (dd, *J* = 9.4, 7.8 Hz, 1 H, Hb-8), 1.92 (s, 3 H, CH_3), 1.81 (dd, *J* = 11.3, 9.0 Hz, 1 H, H-7), 0.37 (s, 3 H, SiMe), 0.35 (s, 3 H, SiMe). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.3, 169.6 (s, 2 C, CH_3-*C*=O, C-5), 137.8-135.3 (s, 4 C, Ar), 134.0-127.5 (d, 20 C, Ar), 89.2 (d, C-2), 84.6 (d, C-1), 73.2 (d, C-6), 73.0-71.5 (t, 3 C, Bn), 68.6 (t, C-8), 62.7 (d, C-7a), 59.1 (d, C-3), 35.5 (d, C-7), 20.5 (q, CH_3), -4.6, -4.9 (q, $SiMe_2$). IR ($CDCl_3$): 3089, 3068, 2956, 2866, 1745, 1709, 1428, 1234, 1114 cm^{-1}. MS (EI): *m/z* 649 (1), 590 (3), 528 (1), 392 (2), 286 (4), 243 (5), 135 (30), 91 (100). Anal. Calcd for $C_{39}H_{43}NO_6Si$ (649.85): C, 72.08; H, 6.67; N, 2.16. Found: C, 72.08; H, 6.17; N, 2.06.

(1*R*,2*R*,3*R*,6*R*,7*S*,7a*R*)-6-Acetyloxy-1,2-bis(benzyloxy)-3-[(benzyloxy) methyl]-7-hydroxy-hexahydro-5H-pyrrolizin-5-one (**39**). Mercuric trifluoro-acetate (1.31 g, 3.08 mmol) was added to a stirred solution of **38** (1.00 g, 1.54 mmol) in $CHCl_3$ (9.1 mL), acetic acid (2.3 mL) and trifluoroacetic acid (4.6 mL). The solution was stirred at rt for 1 h, then, peracetic acid (26.4 mL) was added dropwise to the mixture with ice cooling. After 1 h stirring at rt the solution was cooled to 0 °C and a sat. $Na_2S_2O_3$ solution (120 mL) was added dropwise. The mixture was extracted with AcOEt (3 x 100 mL) and the combined organic layers were washed with a sat. Na_2CO_3 solution (4 x 60 mL), dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (petroleum ether/ethyl acetate 3:2) afforded pure **39** (R_f=0.38, 671 mg, 1.26 mmol, 82%) as a colourless oil. $[\alpha]_D^{26}$ = – 65.0 (c 0.83, $CHCl_3$), ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38-7.23 (m, 15 H, Ar), 5.29 (d, *J* = 7.6 Hz, 1 H, H-6), 4.71 (d, *J* = 11.9 Hz, 1 H, Bn), 4.58-4.45 (m, 5 H, Bn), 4.30 (dd, *J* = 4.1, 3.9 Hz, 1 H, H-2), 4.20-4.18 (m, 1 H, H-3), 4.17 (dd, *J* = 7.6, 6.4 Hz, 1 H, H-7), 3.90 (dd, *J* = 6.1, 4.4 Hz, 1 H, H-1), 3.80 (br s, 1 H, OH), 3.73 (t, *J* = 6.2 Hz, 1 H, H-7a), 3.62 (dd, *J* = 9.8, 5.9 Hz, 1 H, Ha-8), 3.53 (dd, *J* = 9.8, 4.5 Hz, 1 H, Hb-8), 2.21 (s, 3 H, CH_3). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 172.3, 166.9 (s, 2 C, C=O), 137.7-137.4 (s, 3 C, Ar),

128.4-127.6 (d, 15 C, Ar), 86.9 (d, C-1), 85.1 (d, C-2), 80.8 (d, C-6), 78.7 (d, C-7), 73.2-71.9 (t, 3 C, Bn), 68.2 (t, C-8), 67.2 (d, C-7a), 59.1 (d, C-3), 20.7 (q, CH_3). IR ($CDCl_3$): 3508, 3088, 3067, 3032, 2868, 1720, 1454, 1362, 1246, 1100 cm^{-1}. MS (EI): *m/z* 531 (6), 516 (1), 279 (1), 219 (2), 167 (7), 149 (18), 91 (100), 83 (57). Anal. Calcd for $C_{31}H_{33}NO_7$ (531.60): C, 70.04; H, 6.26; N, 2.63. Found: C, 70.01; H, 6.19; N, 2.72.

(1*R*,2*R*,3*R*,6*R*,7*S*,7a*R*)-1,2,7-Tris(benzyloxy)-3-[(benzyloxy)methyl]-6-hydroxyhexahydro-5H-pyrrolizin-5-one (**40**). To a mixture of **39** (209 mg, 0.40 mmol), 3 Å molecular sieves (300 mg) and benzyl trichloroacetimidate (202 mg, 0.80 mmol) in Et_2O (4 mL), triflic acid (0.04 mL, 0.48 mmoL) was added under nitrogen atmosphere. The mixture was stirred for 3 h at rt, filtered and washed with a sat. Na_2CO_3 solution. The organic layer was dried and evaporated under reduced pressure to afford a yellow oil, that was dissolved in MeOH (30 mL). Ambersep 900 OH (350 mg) was added and the mixture was stirred at rt for 15 h, filtered and evaporated under reduced pressure. Purification of the residue by chromatography on silica gel (petroleum ether/ethyl acetate 5:2) afforded pure **40** (R_f=0.27, 173 mg, 0.30 mmol, 75% yield over two steps) as a colourless oil. $[\alpha]_D^{25} = -32.0$ (c 0.63, $CHCl_3$), ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37-7.21 (m, 20 H, Ar), 4.81 (d, *J* = 11.8 Hz, 1 H, Bn), 4.67 (d, *J* = 11.8 Hz, 1 H, Bn), 4.59 (d, *J* = 7.8 Hz, 1 H, H-6), 4.57 (d, *J* = 11.8 Hz, 1 H, Bn), 4.54-4.42 (m, 5 H, Bn), 4.24 (dd, *J* = 3.7, 3.4 Hz, 1 H, H-2), 4.22 (m, 1 H, H-3), 4.00 (dd, *J* = 7.8, 6.9 Hz, 1 H, H-7), 3.84 (dd, *J* = 5.5, 3.7 Hz, 1 H, H-1), 3.75 (dd, *J* = 6.9, 5.5 Hz, 1 H, H-7a), 3.58 (dd, *J* = 9.9, 6.1 Hz, 1 H, Ha-8), 3.52 (dd, *J* = 9.9, 4.8 Hz, 1 H, Hb-8), 3.38 (br s, 1 H, OH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 172.5 (s, C-5), 137.8 (s, 2 C, Ar), 137.4-137.3 (s, 2 C, Ar), 128.9-127.6 (d, 20 C, Ar), 86.7 (d, C-1), 86.3 (d, C-7), 84.9 (d, C-2), 77.5 (d, C-6), 73.2-71.8 (t, 4 C, Bn), 68.4 (t, C-8), 65.9 (d, C-7a), 59.1 (d, C-3). IR ($CHCl_3$): 3654, 3066, 3031, 3011, 2868, 1709, 1454, 1364, 1102 cm^{-1}. MS (EI): *m/z* 579 (0.3), 488 (0.4), 382 (1), 261

(13), 181 (5), 91 (100). Anal. Calcd for $C_{36}H_{37}NO_6$ (579.68): C, 74.59; H, 6.43; N, 2.42. Found: C, 74.56; H, 6.37; N, 2.76.

(1*R*,2*R*,3*R*,6*R*,7*S*,7a*R*)-1,2,7-Tris(benzyloxy)-3-[(benzyloxy)methyl]-6-(methylsulfonyl)hexahydro-5H-pyrrolizin-5-one (**41**). To a solution of **40** (77 mg, 0.13 mmol) in dry DCM (2 mL), triethylamine (0.05 ml, 0.35 mmol) and mesyl chloride (0.01 mL, 0.17 mmoL) was added under nitrogen atmosphere. The mixture was stirred for 4 h at rt, then 3 ml of water were added. Afetr extraction with DCM, the organic layer was dried and evaporated under reduced pressure to afford a yellow oil, that purificated by chromatography on silica gel (petroleum ether/ethyl acetate 3:1) afforded pure **41** (R_f=0.23, 72 mg, 0.11 mmol, 84% yield) as a yellow oil. $[\alpha]_D^{25}$ = − 14.3 (c 0.52, $CHCl_3$), ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.26-7.12 (m, 20 H, Ar), 5.43 (d, *J* = 7.3 Hz, 1 H, H-6), 4.71 (d, *J* = 11.7 Hz, 1 H, Bn), 4.55 (d, *J* = 11.7 Hz, 1 H, Bn), 4.48-4.32 (m, 6 H, Bn), 4.14-4.03 (m, 3 H, H-2, H-7, H-3), 3.73 (dd, *J* = 5.4, 3.9 Hz, 1 H, H-1), 3.69-3.66 (m, 1 H, H-7a), 3.48 (dd, *J* = 9.8, 5.9 Hz, 1 H, Ha-8), 3.40 (dd, *J* = 9.8, 4.7 Hz, 1 H, Hb-8), 3.26 (s, 3 H, Ms). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 166.3 (s, C-5), 137.3, 136.9, 136.8, 136.2 (s, Ar), 128.2-127.4 (d, 20 C, Ar), 86.2 (d, C-1), 84.3 (d, C-2), 83.9 (d, C-6), 82.4 (d, C-7), 72.9, 71.9, (t, Bn), 71.7 (t, 2 C, Bn), 67.8 (t, C-8), 65.7 (d, C-7a), 59.4 (d, C-3), 40.1 (q, Ms). IR ($CHCl_3$): 3024, 1270, 1365, 1220, 1176 cm^{-1}. MS (ESI): *m/z* 680 (M + Na^+, 100). Anal. Calcd for $C_{37}H_{39}NO_8S$ (657.77): C, 67.56; H, 5.98; N, 2.13. Found: C, 66.70; H, 6.49; N, 2.15.

3.5.4 Synthesis of (−)-Uniflorine A

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*S*)-1,2,7-Tri(benzyloxy)-3-[(benzyloxy)methyl]-5-oxo-hexahydro-5*H*-pyrrolizin-6-yl benzoate (**48**). Triphenylphosphine (68 mg, 0.26 mmol) and benzoic acid (31.7 mg, 0.26 mmol) were added to a solution of **40** (125 mg, 0.216 mmol) in dry THF (4 mL), then DIAD (51 µL, 0.26 mmol) was added at 0 °C. The mixture was stirred at rt for 18 h, then the solvent was removed at reduced pressure and the crude product purified by FCC (eluent

petroleum ether:EtOAc 5:1, then 1:1), affording pure **48** (111 mg, 0.162 mmol, 75%). $[\alpha]_D^{28}$ -55.6 (CH_2Cl_2, c 0.25), ^{1}H NMR ($CDCl_3$) δ 8.00-7.98 (m, 2H), 7.52-7.48 (m, 1H), 7.37-7.33 (m, 2H), 7.28-7.13 (m, 20H, Ar), 5.63 (d, *J* = 5.4 Hz, H-6), 4.60 (d, *J* = 11.2 Hz, 1H, Bn), 4.53-4.29 (m, 7H, Bn), 4.23-4.15 (m, 2H, H-2, H-3), 4.01 (dd, *J* = 6.8, 5.4 Hz, 1H, H-7a), 3.90 (dd, *J* = 6.8, 5.4 Hz, 1H, H-7), 3.70 (dd, *J* = 5.4, 3.4 Hz, 1H, H-1), 3.63-3.52 (m, 2H, Ha-8, Hb-8), ^{13}C NMR ($CDCl_3$) δ 168.4 (s), 165.1 (s, C-5), 137.6-136.5 (s, 5C), 133.0 (d, 1C), 139.7-128.9 (d, 2C), 128.1-127.2 (d, 22C, Ar), 85.4 (d, 2C, C-1, C-2), 77.8 (d, C-7), 72.9-71.5 (t, Bn), 72.3 (d, C-6), 68.4 (d, C-7a), 67.9 (t, C-8), 59.2 (d, C-3). IR ($CDCl_3$): 1726 cm^{-1}. MS (ESI): *m/z* 706 ($M + Na^+$, 100). Anal. Calcd for $C_{43}H_{41}NO_7$ (683.29) C, 75.53; H, 6.04; N, 2.05; Found: C, 75.16; H, 6.37; N, 1.95.

(1*R*,2*R*,3*R*,6*S*,7*S*,7*aS*)-1,2,7-Tri(benzyloxy)-3-[(benzyloxy)methyl]-hexahydro-1*H*-pyrrolizin-6-ol (**49**). $LiAlH_4$ (25 mg, 0.66 mmol) was added to a solution of **48** (111 mg, 0.162 mmol) in dry THF (3 mL). The mixture was stirred at reflux under nitrogen atmosphere for 5 h, then 0.30 mL of a sat. solution of Na_2SO_4 was added dropwise at room temperature. The resulting mixture was filtered through Celite®, washed with EtOAc, then the solvent was evaporated. Purification of the crude product by FCC (eluent petroleum ether:EtOAc 1:1) gave pure **49** as a pale yellow oil (42 mg, 0.074 mmol, 45%). $[\alpha]_D^{22}$ – 6.0 (c 0.25, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.18 (m, 20H, Ar), 4.62-4.44 (m, 8H, Bn), 4.29-4.26 (m, 1H, H-6), 4.03 (pseudo t, *J* = 4.9 Hz, 1H, H-2), 3.89 (t, *J* = 4.2 Hz, 1H, H-1), 3.83 (dd, *J* = 6.9, 4.6 Hz, 1H, H-7), 3.62 (m, 1H, H-7a), 3.57-3.49 (m, 2H, H-8), 3.33-3.30 (m, 1H, Ha-5), 3.03-2.99 (m, 2H, H-3, Hb-5). ^{13}C NMR (50 MHz, $CDCl_3$) δ 137.6-137.1 (s, 4C, Ar), 128.3-127.4 (d, 20C, Ar), 85.4 (d, 2C, C-1 + C-2), 82.5 (d, C-7), 73.0-71.3 (5C, 4Bn + C-8), 70.5 and 70.3 (d, 2C, C-6 + C-7a), 69.8 (d, C-3), 60.2 (t, C-5). IR ($CDCl_3$): 3689, 3605, 2956, 2926, 2854, 1732, 161, 1457, 1378 cm^{-1}. MS (ESI) *m/z* 566 ($M + H^+$, 100). Anal. Calcd for $C_{36}H_{39}NO_5$ (565.70) C, 76.43; H, 6.95; N, 2.48; Found: C, 76,40; H, 7,37; N, 2,75.

(1*R*,2*R*,3*R*,6*S*,7*S*,7*aS*)-3-(Hydroxymethyl)hexahydro-1*H*-pyrrolizin-1,2,6,7-tetraol ((−)-uniflorine A, **18**). HCl 37% (0.1 ml) and 10% Pd on C (33 mg) were added to a solution of **49** (35 mg, 0.062 mmol) in MeOH (4 mL). The suspension was stirred under H_2 atmosphere for 4 days, then filtered through Celite® and washed with MeOH. Evaporation of the solvent under reduced pressure afforded crude **18** that was transferred to a column of Dowex 50WX8 and washed with MeOH (10 mL) and H_2O (10 mL) to remove non basic impurities, then with 6% NH_4OH (15 mL) to elute (−) - uniflorine A (**18**) as a yellowish solid (9 mg, 0.044 mmol, 71%), mp 177-180 °C. $[\alpha]_D^{21}$ − 6.9 (c 0.415, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 4.23 (q, *J* = 5.2 Hz, 1H, H-6), 4.08 (pseudo t, *J* = 4.7 Hz, 1H, H-7), 3.83 (t, *J* =7.4 Hz, 1H, H-1), 3.71 (dd, *J* = 8.8, 7.6 Hz, 1H, H-2), 3.65 (dd, *J* = 11.9, 3.7 Hz, 1H, Ha-8), 3.50 (dd, *J* = 11.9, 6.4 Hz, 1H, Hb-8), 3.08 (dd, *J* = 7.2, 5.0 Hz, 1H, H-7a), 2.97 (dd, *J* = 12.1, 5.4 Hz, 1H, Ha-5), 2.89 (dd, *J* = 12.1, 5.0 Hz, 1H, Hb-5), 2.70 (ddd, *J* = 9.1, 6.4, 3.7 Hz, 1H, H-3). ^{13}C NMR (100 MHz, D_2O) δ 78.7 (d, C-1), 77.5 (d, C-2), 75.7 (d, C-7), 72.0 (d, C-6), 71.8 (d, C-7a), 70.9 (d, C-3), 62.3 (t, C-8), 58.1 (t, C-5). MS (ESI): *m/z* 206 ($M+H^+$, 100), 158 (10), 132 (13), 118 (22), 95 (30), 79 (73), 55 (17). Anal. Calcd for $C_8H_{15}NO_5$ (205.21) C, 46.82; H, 7.37; N, 6.83; Found: C, 46.85; H, 7.68; N, 7.11.

3.5.5 Syntheses of 7-deoxycasuarine and 7-*homo*casuarine glucoside and their derivatives

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy-5-oxo casuarine (**54**). A solution of glucopyranosyl tricholoroacetimidate **53**[82] (190 mg, 0.29 mmol) and pyrrolizidine **21** (79 mg, 0.17 mmol) in dry Et_2O (3 mL) was stirred for 10 minutes at rt under nitrogen atmosphere in the presence of 3 Å molecoular sieves (150 mg). After cooling to – 20°C and addition of TMSOTf (20 µL, 0.09 mmol), stirring was continued for 40 minutes;

[82]Rathore, T.; Hashimoto, K.; Igarashi, H.; Nukaya, D.; Fullerton, S. *Tetrahedron* **1985**, *41*, 5427.

during this period the temperature was raised to rt. The mixture was washed with a saturated aqueous solution of Na_2CO_3 (2 mL), the combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by flash column chromatography on silica gel (pentane/EtOAc 5:1, increasing polarity) to afford pure **54** as a colourless oil (140 mg, 88 % yield). $[\alpha]_D^{22}$ = + 44.0 (c 1.23, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.48-7.46 (m, 2 H, Ar), 7.38-7.25 (m, 31 H, Ar), 7.17-7.14 (m, 2 H, Ar), 5.68 (d, *J* = 3.5 Hz, 1 H, H-1), 5.05 (d, *J* = 11.3 Hz, 1 H, Bn), 5.02 (d, *J* = 12.1 Hz, 1 H, Bn), 4.86 (d, *J* = 10.5 Hz, 1 H, Bn), 4.79 (d, *J* = 10.9 Hz, 1 H, Bn), 4.75 (d, *J* = 11.7 Hz, 1 H, Bn), 4.65 (dd, *J* = 10.1, 8.6 Hz, 1 H, H-6'), 4.60-4.46 (m, 9 H, Bn), 4.37 (dd, *J* = 5.5, 5.1 Hz, 1 H, H-2'), 4.05 (m, 1 H, H-3'), 4.01 (t, *J* = 9.4 Hz, 1 H, H-4), 3.83 (dd, *J* = 7.4, 5.5 Hz, 1 H, H-1'), 3.76-3.58 (m, 7 H, H-2, H-3, H-5, Ha-6, Hb-6, H-7a', Ha-8'), 3.54 (dd, *J* = 9.8, 3.5 Hz, 1 H, Hb-8'), 2.60 (m, 1 H, Ha-7'), 2.01 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 171.3 (s, C-5'), 138.8-137.4 (s, 7 C, Ar), 128.5-127.4 (d, 35 C, Ar), 95.6 (d, C-1), 89.0 (d, C-1'), 86.1 (d, C-2'), 81.5 (d, C-4), 78.8, 77.4 (d, 2 C), 75.7, 75.2 (t, Bn), 73.6 (d, C-6'), 73.5, 73.3, 72.5, 72.3, 71.6 (t, Bn), 70.8 (d, 1 C), 69.0 (t, C-8'), 68.6 (t, C-6), 59.2 (d, 1 C), 58.3 (d, C-3'), 34.9 (t, C-7'). IR ($CDCl_3$): 3090, 3067, 3033, 2868, 1702, 1454, 1363, 1099, 1071 cm^{-1}. MS (EI): *m/z* 1018 (100, $[M+Na]^+$), 996 (35, M^+). Anal. Calcd for $C_{63}H_{65}NO_{10}$ (996.19): C, 75.96; H, 6.58; N, 1.41. Found: C, 75.75; H, 6.82; N, 1.44.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy casuarine (**55**). To a cooled (0°C) solution of **54** (73 mg, 0.053 mmol) in dry THF (0.75 mL) a 1 M solution of $LiAlH_4$ in THF (0.250 mL) was added under nitrogen atmosphere. The mixture was stirred at rt for 1 h, then, after cooling at 0 °C, a saturated aqueous solution of Na_2SO_4 (0.3 mL) was added dropwise. The suspension was then filtered through Celite®, washed with EtOAc and concentrated under reduced pressure to afford the crude product, that was purified by flash column chromatography on silica gel (EtOAc /petroleum ether 7:3, R_f=0.35) to give pure **55** as a colourless oil (30 mg, 58 %). $[\alpha]_D^{29}$ = + 36.7 (c

1.20, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.17 (m, 35 H, Ar), 4.95 (d, *J* = 10.9 Hz, 1 H, Bn), 4.87 (d, *J* = 10.7 Hz, 1 H, Bn), 4.84 (d, *J* = 3.5 Hz, 1 H, H-1), 4.78 (d, *J* = 10.7 Hz, 1 H, Bn), 4.73 (d, *J* = 11.7 Hz, 1 H, Bn), 4.72 (d, *J* = 12.1 Hz, 1 H, Bn), 4.65 (d, *J* = 12.1 Hz, 1 H, Bn), 4.63 (d, *J* = 12.5 Hz, 1 H, Bn), 4.59-4.48 (m, 7 H, Bn), 4.33 (m, 1 H, H-6'), 4.13 (dd, *J* = 6.6 Hz, 1 H, H-1'), 4.07 (dd, *J* = 7.4, 7.0 Hz, 1 H, H-2'), 3.95 (t, *J* = 9.4 Hz, 1 H, H-3), 3.79 (m, 1 H, H-5), 3.75 (dd, *J* = 10.5, 3.5 Hz, 1 H, Ha-6), 3.67 (dd, *J* = 9.4 Hz, 1 H, H-4), 3.65 (dd, *J* = 10.5, 1.7 Hz, 1 H, Hb-6), 3.58-3.46 (m, 5 H, H-2, H-3', H-7a', Ha-8', Hb-8'), 3.19 (dd, *J* = 11.3, 4.3 Hz, 1 H, Ha-5'), 3.05 (m, 1 H, Hb-5'), 2.21 (m, 1 H, Ha-7'), 1.93 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.7, 138.6, 138.5, 138.3, 138.2, 138.1, 137.9 (s, Ar.), 128.6-127.4 (d, 35 C, Ar), 96.3 (d, C-1), 88.1 (d, C-1'), 84.6 (d, C-2'), 81.8 (d, C-3), 79.9 (d, 1 C), 78.8 (d, C-6'), 77.6 (d, C-4), 75.6-72.2 (t, 7 C, Bn), 72.1 (t, C-8'), 70.7 (d, C-5), 68.5 (t, C-6), 67.6 (d, 1 C), 66.0 (d, 1 C), 58.7 (t, C-5'), 37.8 (t, C-7'). IR ($CDCl_3$): 3088, 3066, 3031, 2926, 2866, 1496, 1453, 1363, 1207, 1070 cm^{-1}. MS (EI): *m/z* 1004 ($M+Na^+$, 32), 982 (M^+, 100). Anal. Calcd for $C_{63}H_{67}NO_9$ (982.21): C, 77.04; H, 6.88; N, 1.43. Found: C, 77.38; H, 6.74; N, 1.73.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-deoxycasuarine (**51**). To a stirred solution of **55** (46 mg, 0.05 mmol) in MeOH (5 mL), 20 mg of Pd (10% on C) and 2 drops of HCl were added. The suspension was stirred at rt under hydrogen atmosphere for 12 h when filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute the product **51**. Evaporation of the solvent afforded the product as a colourless highly hygroscopic solid (13 mg, 77% yield). $[\alpha]_D^{29}$ = + 97.8 (c 0.88, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 4.89 (d, *J* = 3.9 Hz, 1 H, H-1), 4.38 (m, 1 H, H-6'), 4.03 (t, *J* = 8.2 Hz, 1 H, H-1'), 3.77-3.55 (m, 7 H, H-2', Ha,b-8'), 3.46 (dd, *J* = 9.9, 3.9 Hz, 1 H, H-2), 3.36-3.28 (m, 2 H, H7a'), 3.22 (ddd, *J* = 9.6, 6.0, 3.5 Hz, 1 H, H-3'), 3.19-3.16 (m, 1 H, Ha-5'), 3.01 (dd, *J* = 13.1, 3.7 Hz, 1

H, Hb-5'), 2.20-2.13 (m, 1 H, Ha-7'), 2.09-2.06 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, D_2O) δ 99.6 (d, C-1), 81.8 (d, C-1'), 81.0 (d, C-6'), 77.9 (d, C-2'), 74.8, 74.4, 73.2, 71.8, 71.4, 68.3 (d, C-2, C-3, C-4, C-5, C-3', C-7a'), 63.5, 62.6 (t, C-6, C-8'), 60.2 (t, C-5'), 37.7 (t, C-7'). ^{1}H-NMR (400 MHz, CD_3OD) δ 4.92 (d, *J* = 3.7 Hz, 1 H, H-1), 4.51 (m, 1 H, H-6'), 4.13 (t, *J* = 8.0 Hz, 1 H, H-1'), 3.89-3.63 (m, 7 H, H-2', Ha,b-6, Ha,b-8'), 3.47 (dd, *J* = 9.8, 3.8 Hz, 2 H), 3.45-3.43 (m, 1 H, H7a'), 3.39-3.31 (m, 3 H, H-3', Ha-5'), 3.12 (dd, *J* = 12.7, 3.6 Hz, 1 H, Hb-5'), 2.30-2.19 (m, 2 H, Ha,b-7'). ^{13}C-NMR (50 MHz, CD_3OD) δ 96.9 (d, C-1), 79.3 (d, C-1'), 77.5 (d, C-6'), 75.4 (d, C-2'), 72.3, 72.0, 70.9, 69.6, 69.2 (d, C-2, C-3, C-4, C-5, C-3'), 66.4 (d, C-7a') 60.4, 60.3 (t, C-6, C-8'), 57.1 (t, C-5'), 35.1 (t, C-7'). MS (EI): *m/z* 350 ($M^{\cdot+}-1$, 0.4), 320 (100), 172 (9), 158 (42), 68 (21). Anal. Calcd for $C_{14}H_{25}NO_9$ (351.35): C, 47.86; H, 7.17; N, 3.99. Found: C, 47.26; H, 7.90; N, 3.80.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-methoxycarbonyl-5-oxo-casuarine (**56**). A solution of glucopyranosyl tricholoroacetimidate **53**[82] (890 mg, 1.30 mmol) and pyrrolizidine **27** (429 mg, 0.81 mmol) in Et_2O/DCM 3:1 was stirred for 10 minutes at rt under nitrogen atmosphere in the presence of 3 Å molecular sieves (200 mg). After cooling to −20°C and addition of TMSOTf (73 μL, 0.40 mmol), stirring was continued for 1 hour; during this period the temperature was raised to rt. The mixture was washed with 8 mL of a saturated aqueous solution of Na_2CO_3 and the combined organic layer was dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc 7:1) to afford pure **56** (R_f=0.26 petroleum ether/EtOAc 3:1) as a colourless oil (598 mg, 75%). $[\alpha]_D^{21}$ = + 32.7 (c 0.2, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45-7.11 (m, 35 H, Ar), 5.80 (d, *J* = 3.5 Hz, 1 H, H-1), 5.01 (d, *J* = 10.6 Hz, 2 H, Bn), 5.00 (d, *J* = 9.6 Hz, 1 H, H-6'), 4.82 (d, *J* = 10.5 Hz, 1 H, Bn), 4.75 (d, *J* = 10.8 Hz, 1 H, Bn), 4.71 (d, *J* = 12 Hz, 1 H, Bn), 4.64 (d, *J* = 12 Hz, 1 H, Bn), 4.57-4.46 (m, 8 H, Bn), 4.27 (t, *J* = 4 Hz, 1 H, H-2), 4.17-4.14 (m, 1 H, H-3'), 3.96 (dd, *J* = 6.4, 4.4 Hz, 1 H, H-1'), 3.94 (dd, *J* = 10.4 Hz, 1 H, H-3), 3.86 (dd, *J* = 6.4, 1

H, H-7a'), 3.77 (dd, *J* = 10 Hz, 1 H, H-5), 3.71-3.62 (m, 7 H, OCH_3, H-6a, H-6b, H-4, H-2), 3.57 (dd, *J* = 9.6 Hz, 1 H, H-8a'), 3.51 (dd, *J* = 9.6, 4.4 Hz, 1 H, H-8b'), 3.26 (dd, *J* = 10, 2 Hz, 1 H, H-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 170.7, 170.0 (s, C=O). 138.6-137.1 (s, 7 C, Ar.), 128.3-127.2 (d, 35 C, Ar), 95.2 (d, C-1), 87.8 (d, C-1'), 85.2 (d, C-2'), 81.1 (d, C-3), 78.5 (d, C-2), 76.7 (d, C-6'), 75.5 (t, Bn), 75.2 (d, C-5), 74.9-71.8 (t, 6 C, Bn), 70.5 (d, C-4), 68.3 (t, C-8'), 67.8 (t, C-6), 61.8 (d, C-7a'), 58.5 (d, C-3'), 52.3 (q, Me), 51.84 (d, C-7'). IR ($CDCl_3$): 2925, 2867, 1737 (C=O), 1711 (C=O), 1454, 1071 cm^{-1}. HRMS (ESI) for $C_{65}H_{67}NO_{12}Na$ $[M+Na]^+$ calculated: 1076.4556; found: 1076.4553. Anal. Calcd for $C_{65}H_{67}NO_{12}$ (1054.23): C, 74.05; H, 6.41; N, 1.33. Found: C, 73.63; H, 6.43; N, 0.99.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-methoxycarbonyl-5-oxo-casuarine (**57**). To a stirred solution of **56** (125 mg, 0.118 mmol) in MeOH/AcOEt 3:1 (12 mL), 150 mg of Pd (10% on C) were added. The suspension was stirred at rt under hydrogen atmosphere for 4 days, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afford pure **57** as a waxy solid (48 mg, 96%). $[\alpha]_D^{21}$ = + 75.3 (c 0.15, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 5.18 (d, *J* = 3.6 Hz, 1 H, H-1), 4.97 (d, *J* = 9.6 Hz, 1 H, H-6'), 4.08 (dd, *J* = 6.8, 6.4 Hz, 1 H, H-2'), 3.88 (dd, *J* = 7.6, 7.2 Hz ,1 H, H-1'), 3.80-3.55 (m, 11H, H-5, OCH_3, H-4, H-3, Ha-6, Hb-6, Ha-8', H-7a', H-3'), 3.41 (dd, *J* = 10, 4 Hz, 1 H, H-2), 3.37 (dd, *J* = 9.6 Hz, 1 H, Hb-8'), 3.28 (dd, *J* = 9.2 Hz, 1 H, H-7'). ^{13}C-NMR (50 MHz, D_2O) δ 171.9, 171.5 (s, C=O), 98.8 (d, C-1), 79.2 (d, C-6'), 78.3, 77.3, 72.2, 72.1, 70.9, 68.6, 61.4, 60.9 (d, 1 C), 59.5, 52.2 (t, 1 C), 52.8 (q, OMe), 52.2 (d, 1 C). IR (KBr): 3420 (OH), 1710 (C=O), 1684 (C=O), 1205, 1143, 1024 cm^{-1}. HRMS (ESI) for $C_{16}H_{25}NO_{12}Na$ $[M+Na]^+$ calculated: 446.1269; found: 446.1266. Anal. Calcd for $C_{16}H_{25}NO_{12}$ (423.37): C, 45.39; H, 5.95; N, 3.31. Found: C, 44,73; H, 6,27; N, 3,37.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-[(hydroxy)methyl]-casuarine (**58**). To a cooled (0°C) solution of **56** (100 mg, 0.09 mmol) in dry THF (1.5 mL) a 2 M solution of $LiBH_4$ in THF (0.9 mL) and a

1M solution of BH_3 in THF (1.8 mL) were added dropwise. The reaction mixture was stirred at rt for 5 days and then, after cooling to -20°C, H_2O was added dropwise. The mixture was filtered through Celite® and washed with $CHCl_3$, then concentrated under reduced pressure afford a viscous oil. Purification by flash column chromatography on silica gel (EtOAc/hexane 1:3) afforded pure **58** (R_f=0.28) as a colourless oil (89 mg, 98%). $[\alpha]_D^{20}$ = + 25.3 (c 0.6, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.14 (m, 35 H, Ar), 4.88-4.82 (m, 2H, Bn), 4.79 (d, *J* = 3.6 Hz, 1 H, H-1), 4.74-4.45 (m, 10 H, Bn), 4.37-4.28 (m, 3H, H-1', Bn), 4.17-4.02 (m, 3 H), 3.89-3.84 (m, 2 H), 3.77-3.73 (m, 1 H), 3.66-3.42 (m, 9 H), 3.34 (dd, *J* = 13.5, 5.3 Hz, 1 H), 2.36 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.2-137.8 (s, 7 C, Ar), 128.6-127.5 (d, 35 C, Ar), 95.5 (d, C-1), 86.9, 83.2, 81.8, 79.7, 79.6, 78.9, 77.8 (d, 1C), 75.7, 75.3, 73.7, 73.3, 72.9, 72.7, 72.2 (t, 1C), 71.2, 70.3 (d, 1 C), 69.0, 68.1, 66.9, 61.7 (t, 1 C), 53.9 (d, C-7'). IR ($CDCl_3$): 3480 (OH), 3032, 2925, 2870, 2386, 1454, 1078 cm^{-1}. HRMS (ESI) for $C_{64}H_{69}NO_{10}$ [M^+] calculated: 1012.4994; found: 1012.4993. Anal. Calcd for $C_{64}H_{69}NO_{10}$ (1012.23): C, 75.94; H,6.87; N,1.38; Found: C, 75.46; H, 7.05; N, 1.45.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-hydroxymethil-casuarine (**52**). To a stirred solution of **58** (89 mg, 0.09 mmol) in MeOH (8 mL), 20 mg of Pd (10% on C) and 2 drops of HCl were added. The suspension was stirred at rt under hydrogen atmosphere for 2 days when filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute the product **52**. Evaporation of the solvent afforded the product as a colourless highly hygroscopic solid (27 mg, 79%). $[\alpha]_D^{26}$ = + 102.5 (c 0.105, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 4.89 (d, *J* = 3.7 Hz, 1 H, H-1), 4.26-4.25 (m, 1 H, H-6'), 4.08 (t, *J* = 7.7 Hz, 1 H, H-1'), 3.77-3.42 (m, 10 H), 3.32 (dd, *J* = 9.8, 9.0 Hz, 1 H, H-3'), 3.24-3.15 (br m ,3 H, Ha-5'), 3.04 (br m, 1 H, H-7a'), 2.50-2.48 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, D_2O) δ 96.9 (d,C-1),

79.8 (d, C-1'), 79.1 (d, C-2'), 76.0 (d, C-6'), 72.3, 71.9, 70.6, 69.2, 68.6 (d, 6 C, C-2, C-3, C-4, C-5, C-3', C-7a'), 61.2, 60.9, 60.0 (t, C-6, C-8', C-9'), 51.3 (t, C-5'). IR (KBr):3399, 1419, 1023 cm^{-1}. MS (EI): *m/z* 351 (26); 350 (85); 207 (22), 188 (37); 110 (19), 91 (22), 73 (44), 60 (100), 55 (64). Anal. Calcd for $C_{15}H_{27}NO_{10}$ (381.38): C, 47.24; H, 7.14; N, 3.67; Found: C, 46.50; H, 7.65; N, 3.29.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-[(hydroxy)methyl]-5-oxo-casuarine (**59**). To a cooled (0°C) solution of **56** (221 mg, 0.21 mmol) in dry THF (1.5 mL) a 2 M solution of $LiBH_4$ in THF (0.42 mL) was added dropwise. The reaction mixture was stirred at rt overnight and then, after cooling to 0°C, H_2O was added dropwise. The mixture was then filtered through Celite®, washed with $CHCl_3$ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (EtOAc/hexane 1:4) to afford pure **59** (R_f=0.33 EtOAc/petroleum ether 1:3) as a colourless oil (134 mg, 62%). $[\alpha]_D^{23}$ = + 44.4 (c 0.4, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.47-7.08 (m, 35 H, Ar), 5.68 (d, *J* = 3.6 Hz, 1 H, H-1), 5.06 (dd, *J* = 2 Hz, 2 H, Bn), 4.81-4.75 (m, 3 H, Bn), 4.72 (d, *J* = 7.6 Hz, 1 H, H-6'), 4.60-4.40 (m, 9 H, Bn), 4.26 (dd, *J* = 3.6, 4 Hz, 1 H, H-2'), 4.21 (m, 1 H, H-3'), 3.94 (t, *J* = 9.2 Hz, 1 H, H-3) 3.87-3.66 (m, 6 H, H-5, Ha-6, H-7a', Ha,b-8', H-1',), 3.60 (dd, *J* = 9.2, 3.6 Hz, 1 H, H-2), 3.45 (d, *J* = 5.2 Hz, 2 H, Ha,b-9'), 3.37 (dd, *J* = 7.6, 1 H, Hb-6), 3.26 (dd, *J* = 10.8, 9.2 Hz, 1 H, H-4), 3.13 (t, 1 H, OH), 2.44 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 171.4 (s, C=O), 138.4-136.7 (s, 7 C, Ar), 128.7-127.3 (d, 35 C, Ar), 94.9 (d, C-1), 87.7 (d, C-1'), 86.1 (d, C-2'), 81.4 (d, C-3), 78.2 (d, C-2), 77.4 (d, C-4), 75.6 (d, C-6'), 75.6, 74.9, 73.3, 73.0, 71.9, 71.7, 71.2 (t, Bn), 70.5 (d, C-5), 68.8 (t, C-6), 68.7 (t, C-9'), 60.5 (d, C-7a'), 58.4 (t, C-8'), 58.4 (d, C-3'), 50.4 (d, C-7'). IR ($CDCl_3$): 3463 (OH), 3032, 2925, 2870, 1703 (C=O), 1454, 1078 cm^{-1}. HRMS (ESI) for $C_{64}H_{67}NO_{11}Na$ $[M+Na]^+$ calculated: 1048.4606; found: 1048.4602. Anal. Calcd for $C_{64}H_{67}NO_{11}$ (1026.22) C, 74.90; H, 6.58; N, 1.36; Found: C, 74,65 ; H, 6,69; N, 1,53.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-hydroxymethil-5-oxo-casuarine (**60**). To a stirred solution of **59** (134 mg, 0.13 mmol) in MeOH (13 mL), 230 mg of Pd (10% on C) were added. The suspension was stirred at rt under hydrogen atmosphere for 24 h when filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afford pure **60** as a waxy solid (37 mg, 72%). $[\alpha]_D^{26}$ = + 59.3 (c 0.75, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 5.18 (d, *J* = 4 Hz, 1 H, H-1), 4.57 (d, *J* = 9.6 Hz, 1 H, H-6'), 4.09 (dd, *J* = 6.8, 6.4 Hz, 1 H, H-2'), 3.82-3.58 (m, 10 H, H-1', H-3', Ha,b-8', Ha-9', H-3, H-4, H-5, Ha,b-6), 3.50-3.45 (m, 2 H, H-7a', H-2), 3.34 (dd, *J* = 9.6, 9.2 Hz, 1 H, Hb-9'), 2.50-2.44 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, D_2O) δ 173.9 (s, C=O), 98.6 (d, C-1), 79.9 (d, C-6'), 77.9, 77.3, 72.7, 72.5, 71.2, 69.3, 62.1, 61.2 (d, 1 C), 60.3, 59.8, 59.7 (t, 1 C), 50.0 (d, 1 C). IR (KBr): 3378 (OH), 1689 (C) cm^{-1}. HRMS (ESI) for $C_{65}H_{67}NO_{12}Na$ $[M+Na]^+$ calculated: 418.1320; found: 418.1312. Anal. Calcd for $C_{15}H_{25}NO_{11}$ (395.36): C, 45.57; H, 6.37; N, 3.54; Found: C, 44.80; H, 6.28; N, 3.04.

3.5.6 Casuarine-6-*O*-α-D-glucopyranoside and its unnatural lactam

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tetra(benzyloxy)-5-oxo-casuarine (**61**). A solution of glucopyranosyl tricholoroacetimidate (**53**, 230 mg, 0.33 mmol) and pyrrolizidine **40** (115 mg, 0.20 mmol) in dry diethyl ether (4 mL) was stirred for 10 min at room temperature under nitrogen atmosphere in the presence of 3 Å molecoular sieves (150 mg). After cooling to –20 °C and addition of trimethylsilyl trifluoromethanesulfonate (18 μL, 0.10 mmol), stirring was continued for 40 min; during this period the temperature was raised to rt. The mixture was washed with a sat. Na_2CO_3 solution (2 mL), dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate 5:1) to afford pure **61** (159 mg, 0.144 mmol, 72%) as a colourless oil. R_f = 0.32 (petroleum ether/ethyl acetate 5:1). $[\alpha]_D^{24}$ = + 51.5 (*c* 0.87, $CHCl_3$). ^{1}H-

NMR (400 MHz, $CDCl_3$, 25°C) δ 7.47-7.12 (m, 40 H, Ar), 5.69 (d, *J* = 3.5 Hz, 1 H, H-1), 5.01 (d, *J* = 10.8 Hz, 1 H, Bn), 5.00 (d, *J* = 11.9 Hz, 1 H, Bn), 4.84 (d, *J* = 10.9 Hz, 1 H, Bn), 4.77 (d, *J* = 10.8 Hz, 1 H, Bn), 4.75 (d, *J* = 10.5 Hz, 1 H, Bn), 4.73 (d, *J* = 8.2 Hz, 1 H, H-6'), 4.64-4.32 (m, 11 H, Bn), 4.31 (dd, *J* = 4.1, 3.7 Hz, 1 H, H-2'), 4.21-4.17 (m, 2 H, H-3', H-7'), 3.99 (dd, *J* = 9.4, 9.2 Hz, 1 H, H-4), 3.99-3.94 (m, 2 H, H-5, H-1'), 3.74 (dd, *J* = 6.6, 6.4 Hz, 1 H, H-7a'), 3.68 (dd, *J* = 9.6, 9.2 Hz, 1 H, H-3), 3.64 (dd, *J* = 9.6, 3.5 Hz, 1 H, H-2), 3.61-3.53 (m, 3 H, Ha-6, Ha-8', Hb-8'), 3.45 (dd, *J* = 10.7, 1.7 Hz, 1 H, Hb-6). ^{13}C-NMR (50 MHz, $CDCl_3$, 25°C) δ 169.4 (s, C-5'), 138.9-137.2 (s, 8 C, Ar), 128.5-127.5 (d, 40 C, Ar), 95.2 (d, C-1), 87.2 (d, C-1'), 86.1 (d, C-2'), 84.4 (d, C-7'), 81.6 (d, C-4), 78.6 (d, C-2), 77.9 (d, C-6'), 77.3 (d, C-3), 75.7, 74.9 (t, 2 C, Bn), 73.3 (t, 2 C, Bn), 72.3 (t, Bn), 72.0 (t, 2 C, Bn), 71.7 (t, Bn), 70.4 (d, C-5), 69.2 (t, C-8'), 68.1 (t, C-6), 65.8 (d, C-7a'), 58.9 (d, C-3'). IR ($CHCl_3$): 3066, 3032, 3010, 2867, 1711, 1454, 1098 cm^{-1}. HRMS (ESI) calcd for $C_{70}H_{71}NO_{11}Na$ $[M+Na]^+$: 1124.4919; found: 1124.4918. Anal. Calcd for $C_{70}H_{71}NO_{11}$ (1102.31): C, 76.27; H, 6.49; N, 1.27: Found: C, 76.53; H, 6.76; N, 1.38.

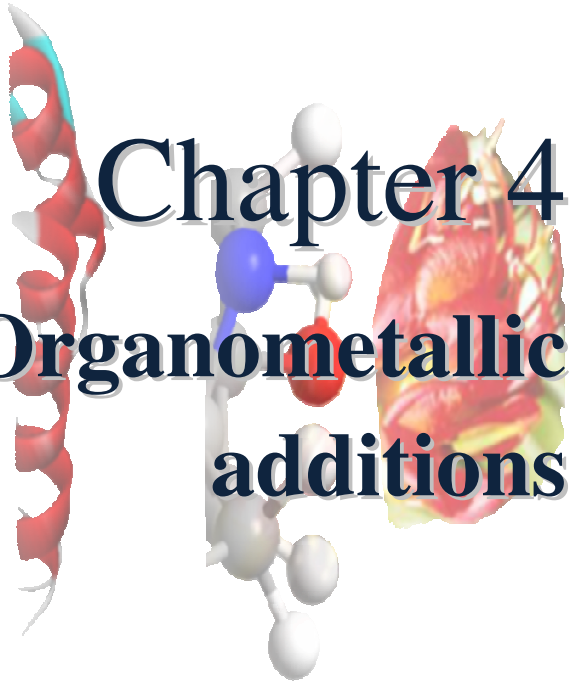
(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tetra(benzyloxy)-casuarine (**62**). To a stirred solution of **61** (134 mg, 0.13 mmol) in MeOH (13 mL), 230 mg of Pd (10% on C) were added. The suspension was stirred at rt under hydrogen atmosphere for 24 h when filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afford pure **62** as a waxy solid (37 mg, 72%). $[\alpha]_D^{26}$ = + 59.3 (c 0.75, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 5.18 (d, *J* = 4 Hz, 1 H, H-1), 4.57 (d, *J* = 9.6 Hz, 1 H, H-6'), 4.09 (dd, *J* = 6.8, 6.4 Hz, 1 H, H-2'), 3.82-3.58 (m, 10 H, H-1', H-3', Ha,b-8', Ha-9', H-3, H-4, H-5, Ha,b-6), 3.50-3.45 (m, 2 H, H-7a', H-2), 3.34 (dd, *J* = 9.6, 9.2 Hz, 1 H, Hb-9'), 2.50-2.44 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, D_2O) δ 173.9 (s, C=O), 98.6 (d, C-1), 79.9 (d, C-6'), 77.9, 77.3, 72.7, 72.5, 71.2, 69.3, 62.1, 61.2 (d, 1 C), 60.3, 59.8, 59.7 (t, 1 C), 50.0 (d, 1 C). IR (KBr): 3378 (OH), 1689 (C) cm^{-1}. HRMS (ESI) for $C_{65}H_{67}NO_{12}Na$ $[M+Na]^+$ calculated: 418.1320; found: 418.1312. Anal. Calcd for

$C_{15}H_{25}NO_{11}$ (395.36): C, 45.57; H, 6.37; N, 3.54; Found: C, 44.80; H, 6.28; N, 3.04.

6-α-D-Glucopyranosyl-*O*-casuarine (**50**). A solution of **62** (196 mg, 0.18 mmol) in MeOH (25 mL) was stirred at room temperature under H_2 atmosphere for 24 h in the presence of 10% Pd/C (350 mg) and 4-5 drops of conc. HCl. Filtration through Celite® afforded a waxy solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute pure **50** (50 mg, 0.14 mmol, 77%) as a white foam. An analytically pure sample was obtained by filtration through DOWEX 50WX8-200. $[\alpha]_D^{23}$ = + 91.9 (*c* 0.35, H_2O). ^{1}H-NMR (400 MHz, D_2O, 25°C) δ 4.89 (d, *J* = 3.7 Hz, 1 H, H-1), 4.24 (t, *J* = 3.0 Hz, 1 H, H-7'), 4.09 (dt, *J* = 4.3, 3.0 Hz, 1 H, H-6'), 4.00 (t, *J* = 8.2 Hz, 1 H, H-1'), 3.79-3.75 (m, 1 H, Ha-6), 3.68-3.56 (m, 5 H, H-3, H-5, Hb-6, H-2', Ha-8'), 3.49 (dd, *J* = 11.8, 6.3 Hz, 1 H, Hb-8'), 3.47 (dd, *J* = 10.0, 3.7 Hz, 1 H, H-2), 3.31 (t, *J* = 9.4 Hz, 1 H, H-4), 3.13 (dd, *J* = 12.9, 4.3 Hz, 1 H, Ha-5'), 3.01-2.96 (m, 3 H, H-3', Hb-5', H7a'). ^{13}C-NMR (50 MHz, D_2O, 25°C) δ 96.7 (d, C-1), 83.0 (d, C-6'), 77.4 (d, C-7'), 76.9 (d, C-1'), 76.1 (d, C-2'), 72.1 (d, C-7a'), 71.9 (d, C-3), 71.5 (d, C-5), 70.3 (d, C-2), 68.9 (d, C-4), 68.6 (d, C-3'), 62.0 (t, C-8'), 59.8 (t, C-6), 55.1 (t, C-5'). HRMS (ESI) calcd for $C_{14}H_{26}NO_{10}$ $[M+H]^+$: 368.1551; found: 368.1544. Anal. Calcd for $C_{14}H_{25}NO_{10}$ (367.35): C, 45.77; H, 6.86; N, 3.81. Found: C, 45.39; H, 6.57; N, 3.46.

6-α-D-Glucopyranosyl-*O*-5'-oxo-casuarine (**63**). To a stirred solution of **61** (159 mg, 0.144 mmol) in MeOH/EtOAc 7:1 (12 mL), 180 mg of Pd (10% on C) were added. The suspension was stirred at rt under hydrogen atmosphere for 24, then filtered through Celite® and then washed with MeOH. Evaporation under reduced pressure afforded pure **63** (42 mg, 0.110 mmol, 77 % yield) as an hygroscopic pale yellow oil. $[\alpha]_D^{24}$ = + 37.6 (c 0.28, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 5.14 (d, *J* = 3.7 Hz, 1 H, H-1), 4.53 (d, *J* = 8.4 Hz, 1 H, H-6'), 4.27 (dd, *J* = 8.4, 7.0 Hz, 1 H, H-7'), 4.08 (t, *J* = 6.1 Hz, 1 H, H-2'), 3.90 (t, *J* = 6.8 Hz, 1

H, H-1'), 3.78-3.58 (m, 7 H, H-3, H-3',H-5, Ha,b-6, Ha,b-8'), 3.50-3.44 (m, 2 H, H-2, H-7a'), 3.34 (t, *J* = 9.5 Hz, 1 H, H-4). ^{13}C-NMR (50 MHz, D_2O) δ 170.8 (s, C-5'), 98.0 (d, C-1), 81.6 (d, C-6'), 78.2 (d, 1 C), 77.3 (d, C-7'), 76.9 (d, C-2'), 71.9 (d, 1 C), 71.5 (d, 1 C), 70.5 (d, 1 C), 68.6 (d, C-4), 64.7 (d, 1 C), 60.7 (d, 1 C), 59.6 (t, 1 C), 58.8 (t, 1 C). MS (ESI): *m/z* 404 (100, M^++Na), 405 (16), 406 (4); Anal. Calcd for $C_{14}H_{23}NO_{11}$ (381.33): C, 44.10; H, 6.08; N, 3.67 Found: C, 43.96; H, 6.19; N, 3.42.



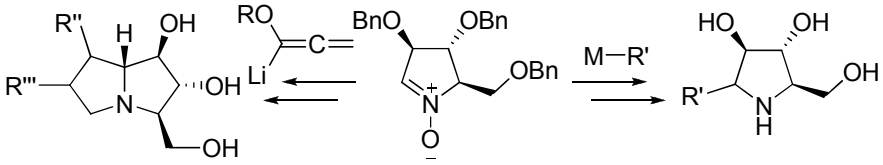
Chapter 4

Organometallic additions

4.1 Introduction

In this chapter, organometallic additions to the key nitrone for obtaining two different classes of polyhydroxylated alkaloids will be discussed.

As mentioned (Chapter 2.1), organometallic addition on a chiral pyrroline nitrone can be used to obtain pyrrolidine and pyrrolizidine alkaloids:



Scheme 4.1. General organometallic addition synthetic strategy

The different procedures utilized for obtaining both the classes of products are discussed herein.

Part of the work described in section 4.2 was developed in the Laboratory of Asymmetric synthesis of Prof. Pedro Merino, Department of Organic Chemistry, University of Zaragoza (Aragona, Spain); and some of the work described in section 4.3 was developed in the research group of Prof. Hans-Ulrich Reiβig, Freie Universität Berlin, Institut für Chemie und Biochemie Organische Chemie (Berlin, Germany).

4.2 Synthesis of pyrrolidine alkaloids

4.2.1 Introduction

A great variety of alkaloids possessing the structure of polyhydroxylated pyrrolidines, otherwise known as iminocyclitols, has been isolated from natural sources, including plants and microorganisms,[83] and many of them are powerful inhibitors of biologically important enzymes such as glycosidases and those closely related with the metabolism of *N*-linked glycoproteins.[84] Due to that biological activity, iminocyclitols might constitute powerful tools against cancer,[85] viral infections[86] or diabetes.[87] Naturally occurring DMDP (**64**), a fairly widespread secondary metabolite firstly isolated from *Derris elliptica* (Leguminosae)[23] and since then found in microorganisms and plant species of quite unrelated families,[88] and its unnatural synthetic analogues are known to

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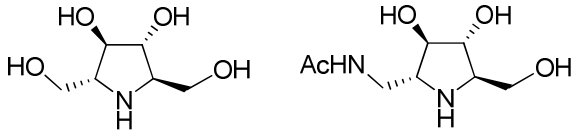
[88] a) Juettner, F.; Wessel, H. P. *J. Phycol.* **2003**, *39*, 26. b) Satoshi, W.; Hideki, K.; Kouzo, N.; Hiroshi, A. *Bioscience, Biotech. Biochem.* **1995**, *59*, 936. c) Ref. 77. d) Molyneux, R. J. *Phytochem. Anal.* **1993**, *4*, 193. e) Fellows, L. E. *Pesticide Sci.* **1986**, *17*, 602.

be selective inhibitors of glycosidases and showed to have potentialities to be used as antiviral and anticancer therapeutic drugs as well as immunomodulators.[89]



64 **65**

Figure 4.1. DMDP (64) and *N*-acetyl-2-(aminomethyl) iminocyclitol (65)

The biological activity of iminocyclitols is due to the structural similarity of the conjugates ammonium acids, formed by protonation under physiological conditions, with the flattened half-chair conformation found for the transition structures of substrates involved in the mechanism of glycosidases.[90] In the particular case of *N*-acetyl-β-hexosaminidases, important enzymes whose inefficiency results in osteoarthritis, it has been suggested the participation of the neighboring C-2 acetamido group of the substrate.[91] Following this hypothesis, Wong and co-wokers[92] found that *N*-acetyl-2-(aminomethyl) iminocyclitol **65** and structural analogues are potent inhibitors of *N*-acetyl-β-hexosaminidases, and hence are expected to have potential as chondrotherapeutic agents.[93] Closely related derivatives of **65** bearing lipophilic aliphatic or aromatic amides attached to C-1 have been found to inhibit β-glucosidase in the nanomolar range.[94] Simpler synthetic analogues of

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65 are also inhibitors of several glycosidases as reported by Vogel.[95] Other syntheses of polyhydroxylated 2-(aminomethyl)pyrrolidines have been reported by Jäger[96] and Correia.[97] Several of these compounds have also been used as ligands forming chelates with a variety of metals including vanadium,[98] gold,[99] zinc,[100] palladium,[101] nickel,[102] ruthenium,[103] platinum,[104] rhodium,[105] titanium and zirconium,[106] that served as catalysts in a variety of enantioselective reactions such as homocoupling of boronic acids[107] and

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Bioorg. Med. Chem. Lett. **2001**, *11*, 1339. c) Wrodnigg, T. M.; Diness, F.; Gruber, C.; Häusler, H.; Lundt, I.; Rupitz, K.; Steiner, A. J.; Stütz, A. E.; Tarling, C. A.; Withers, S. G.; Wölfler, W. *Bioorg. Med. Chem. Lett.* **2004**, *12*, 3485.

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[103] Jones, M. D.; Paz, A. A. F.; Davies, J. E.; Raja, R.; Klinowski, J.; Johnson, B. F. G. *Inorg. Chim. Acta* **2004**, *357*, 1247.

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[107] Gonzalez-Arellano, C.; Corma, A.; Iglesias, M.; Sanchez, F. *J. Chem. Soc. Chem. Commun.* **2005**, 1990.

hydrogenation.[108] Platinum-complexes of 2-(aminomethyl) pyrrolidines have also been evaluated against several human cancer lines[104b] and their DNA-binding properties have been studied.[104c]

4.2.2 The synthetic strategy

We decided to take advantage of the availability of nitrone **1**, which can be easily prepared from carbohydrates (See chapter 2.2.2), thus providing the configuration of three stereogenic centers of the pyrrolidine ring as derived from the chiral pool precursor. To obtain the target compounds we addressed the synthetic plan illustrated in Scheme 4.2, where nitrone **A** serves as electrophile in nucleophilic addition reactions.

The additions were expected to be highly stereoselective, according to previous experience,[109] by virtue of the presence of vicinal stereogenic centers that would discriminate between the two faces of the nitrone.

In order to introduce suitable groups we have chosen as nucleophiles hydroxymethyl anion synthetic equivalents,[110] cyanide (as an aminomethyl synthon equivalent)[111] and Grignard reagents.[112] To gain access to other

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[108] a) Jones, M. D.; Raja, R.; Meurig; T. J.; Johnson, B. F. G. *Topics in Catalysis* **2003**, *25*, 71. b) Raja, R.; Thomas, J. M.; Jones, M. D.; Johnson, B. F. G.; Vaughan, D. E. W. *J. Am. Chem. Soc.* **2003**, *125*, 14982.

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[110] For a previous study with acyclic nitrones, see: Merino, P.; Franco, S.; Merchan, F. L.; Revuelta, J.; Tejero, T. *Tetrahedron Lett.* **2002**, *43*, 459.

[111] For previous studies on the reactivity of cyanide nucleophiles with nitrones see: a) Merino, P.; Tejero, T.; Revuelta, J.; Romero, P.; Cicchi, S.; Mannucci, V.; Brandi, A.; Goti, A. *Tetrahedron: Asymmetry* **2003**, *14*, 367. b) Merino, P.; Lanaspa, A.; Merchan, F. L.; Tejero, T. *J. Org. Chem.* **1996**, *61*, 9028. (c) Merchan, F. L.; Merino, P.; Tejero, T. *Tetrahedron Lett.* **1995**, *36*, 6949.

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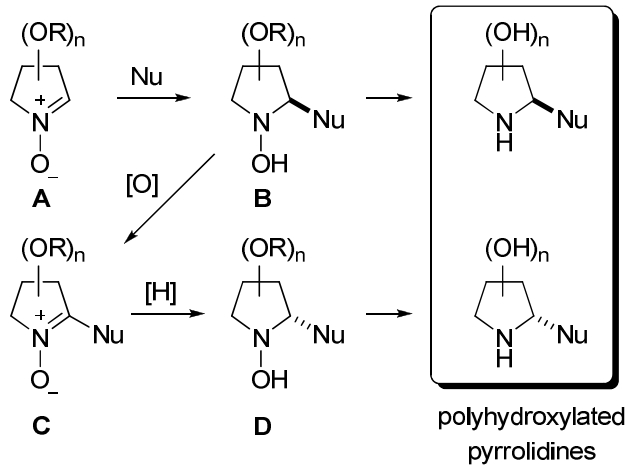
isomers, an oxidation-reduction protocol was envisaged on the hydroxylamines **B** immediately obtained after the nucleophilic addition step. The route involved oxidation of compounds **B** to nitrones **C**, followed by stereoselective reduction to epimeric hydroxylamines **D** (Scheme 4.2).



Scheme 4.2. Synthetic strategy

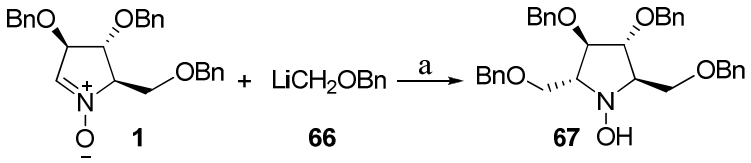
Finally, further elaboration of intermediates **B** and **D** would allow the obtention of the target polyhydroxylated pyrrolidines. It should be pointed out that the oxidation of **B** to furnish **C** may account with regioselectivity since two possible isomers can be formed, being possible to predict to some extent the major one. This feature, combined with a suitable study on the reduction scheme, gives a great malleability to our approach, which will allow one to play around with a wide panel of substrates leading to an ample diversity of configurational combinations.

Synlett **2003**, 35. g) Cardona, F.; Moreno, G.; Guarna, F.; Vogel, P.; Schuetz, C.; Merino, P.; Goti, A. *J. Org. Chem.* **2005**, *70*, 6552. For a review, see: h) Ref. 46 c.

4.2.3 Synthesis of DMDP and structural analogues

The synthetic strategy required the addition of an organometallic reagent to nitrone **1**. The synthetic equivalent chosen for the hydroxymethyl anion was the benzyl-protected (hydroxymethyl)-lithium derivatives **66**, generated in situ from the corresponding tributyltin derivative as described.[113] The choice of **66** as organometal derivative for the synthesis of DMDP (**64**) is due to the possibility of removing all the benzyl groups in one step at a later stage of the synthesis.

The nucleophilic addition of this reagent to nitrone **1** allowed formation of only one diastereoisomer, namely hydroxylamine **67** with 71% yield. As expected, the Grignard reagent reacted with the cyclic nitrone at the less hindered face opposite to the OBn substituent at C-3 (Scheme 4.3).



Scheme 4.3. Organometallic addition on nitrone 1

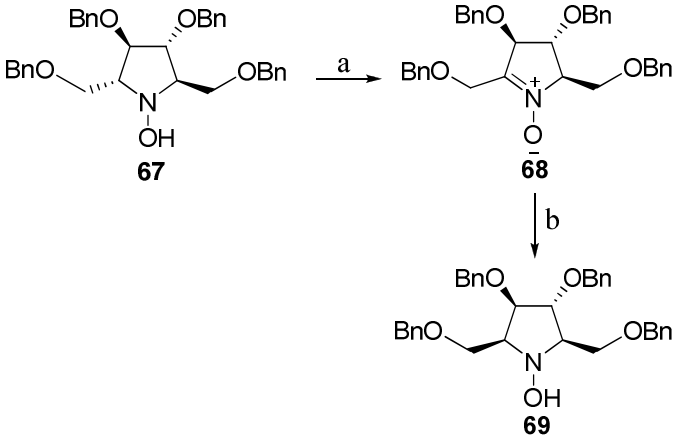
Reaction condition: a) THF, -80°C, 71%.

Contrary to what found for acyclic α-alkoxy nitrones, stereocontrol of the nucleophilic additions to cyclic derivatives could not be achieved using Lewis acids.[114, 111c] In order to gain access to C-2 epimers of hydroxylamine **67** it was then necessary to apply an oxidation (to nitrone) – reduction (to

[113] Still, W. C. *J. Am. Chem. Soc*. **1978**, *100*, 1481.
[114]a) Merino, P. *Compt. Rend. Chimie* **2005,** *8*, 775. b) Merino, P. In *Targets in Heterocyclic Systems. Chemistry and Properties* (Eds.: O. A. Attanasi, D. Spinelli), Italian Society of Chemistry, Rome, **2003**, Vol. 7, pp. 140. c) Ref. 46e. d) Goti, A.; Cicchi, S.; Cordero, F. M.; Fedi, V.; Brandi, A. *Molecules* **1999**, *4*, 1. e) Brandi, A.; Cardona, F.; Cicchi, S.; Cordero, F. M.; Goti, A. *Curr. Trends in Org. Synth.* **1999**, 213. f) Ref.47.

hydroxylamine) sequence, which had been successfully used in Prof. Merino laboratories for the synthesis of substituted prolines.[115,116]

Because of its C_2 symmetry, no regioselectivity issue applied to the oxidation of hydroxylamine **67** with manganese (IV) oxide[117] and nitrone **68** was afforded quantitatively (Scheme 4.4). The oxidation was found to be clean, no purification of the obtained nitrone being necessary after usual work-up.



Scheme 4.4. Oxydation/reduction strategy

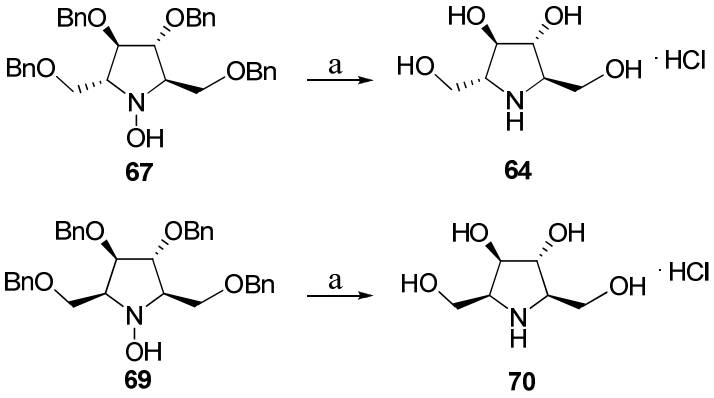
Reaction conditions: a) MnO_2, DCM, r.t., 100%. b) L-Selecride, THF, -80°C, 18 h, 100%.

The reduction of nitrone **68** with L-Selectride was found to be completely selective towards the expected isomer. The absolute configuration of the newly created stereogenic center in hydroxylamines **67** and **69** was unambiguously ascertained by 1D NOE and 2D NOESY and COSY NMR experiments, which unequivocally showed the expected interactions between the protons of the pyrrolidine ring. For compound **67** both the ^{1}H and ^{13}C

[115] a) Marradi, M.; Cicchi, S.; Delso, J. I.; Rosi, L.; Tejero, T.; Merino, P.; Goti, A. *Tetrahedron Lett.* **2005**, *46*, 1287. b) Merino, P.; Delso, I.; Tejero, T.; Cardona, F.; Goti, *Synlett* **2007**, 2651.
[116] Merino, P.; Revuelta, J.; Tejero, T.; Cicchi, S.; Goti, A. *Eur. J. Org. Chem.* **2004**, 776.
[117] Cicchi, S.; Marradi, M.; Goti, A.; Brandi, A. *Tetrahedron Lett.* **2001**, *42*, 6503-6505.

spectra showed signals only consistent with the presence of a symmetry plane in the molecule.

The target polyhydroxylated pyrrolidines were finally obtained by concomitant reduction and deprotection of the precursor hydroxylamines (Scheme 4.5).



Scheme 4.5. Hydrogenation/deprotection of protected hydroxylamines

Reaction conditions: a) H_2, 10% $Pd(OH)_2$-C, 20 atm, HCl-MeOH, r.t., 6 h, 100%.

Compounds **67** and **69** were subjected to hydrogenolysis under 20 atm of hydrogen in HCl (conc): methanol and in the presence of 10% Pearlman's catalyst to give the corresponding hydrochlorides in nearly quantitative yield. Thus, the hydrochloride of DMDP·HCl[118] (**64**) and its C-2 epimer **70**[119] were obtained from nitrone **1** in 71% overall yield (Scheme 4.5).

[118] For representative and leading references on previous syntheses of DMDP and analogues, see: a) Behr, J. B.; Guillerm, G. *Tetrahedron Lett.* **2007**, *48*, 2369. b) Zhou, X.; Liu, W.-J.; Ye, J.-L.; Huang, P.-Q. *Tetrahedron* **2007**, *63*, 6346. c) Vyavahare, V. P.; Chattopadhyay, S.; Puranik, V. G.; Dhavale, D. D. *Synlett* **2007**, 559. d) Trost, B. M.; Horne, D. B.; Woltering, M. J. *Chem. Eur. J.* **2006**, *12*, 6607. e) Garcia-Moreno, M. I.; Aguilar, M.; Mellet, C. O.; Fernandez, J. M. G. *Org. Lett.* **2006**, *8*, 297. f) Izquierdo, I.; Plaza, M. T.; Rodriguez, M.; Franco, F.; Martos, A. *Tetrahedron* **2005**, *61*, 11697. g) Badorrey, R.; Cativiela, C.; Diaz-de-Villegas, M. D.; Diez, R.; Galvez, J. A. *Synlett* **2005**, 1734. h) Donohoe, T. J.; Headley, C. E.; Cousins, R. P. C.; Cowley, A. *Org. Lett.* **2003**, *5*, 999. i) Garcia, A. L. L.; Correia, C.;R. D. *Tetrahedron Lett.* **2003**, *44*, 1553. j) Izquierdo, I.; Plaza, M. T.; Franco, F. *Tetrahedron: Asymmetry* **2002**, *13*, 1503.

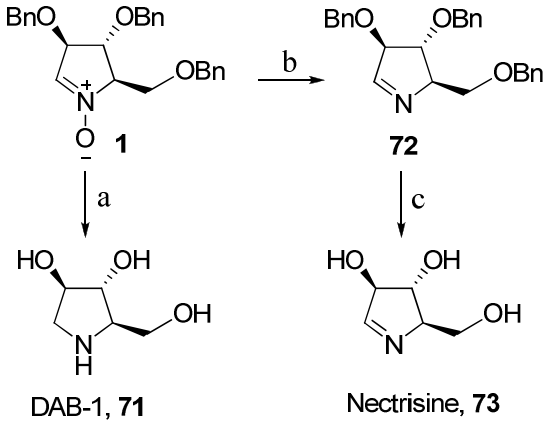
[119] Izquierdo-Cubero, I.; Plaza Lopez-Espinosa, M. T.; Robles-Diaz, R.; Franco-Montalban, F. *Carbohydr. Res.* **2001**, *330*, 401.

The complete deprotection and reduction of **67** allowed, as in the other cases, an easy manipulation of the final compound as the corresponding hydrochloride **64**·HCl, which showed to be stable at 25 °C for months without appreciable decomposition. Nevertheless, in order to compare the spectroscopic and physical properties of DMDP with those reported in the literature, the obtained pyrrolidine was also characterized as the free base after deposition onto a DOWEX 50W8-200 exchange resin and elution with 3N NH_4OH in MeOH. Since some controversy about the presence of hydrochloride salts and free bases has been reported with other isomers,[120] it should be pointed out that a trivial analysis of the ^1H NMR showed more deshielded signals for the hydrochlorides than for the free base. In addition, the typical signal corresponding to ammonium species is revealed at 7-8 ppm in the spectrum of **64**·HCl (as a consequence of the high symmetry around the nitrogen atom), thus unequivocally confirming the presence of the hydrochloride salt. Also, the elemental analyses fully agree with both hydrochloride and free salt. The analytical and spectroscopic data of **64** were in agreement, the NMR data being an exact match, with those previously reported.[121]

Nitrone **1** is also itself a direct precursor of polyhydroxylated pyrrolidines of interest such DAB-1 (**71**), a potent inhibitor of α-glucosidases, α-D-arabinosidase, β-glucosidase and α-mannosidase, isolated from *Angylocalyx* sp. (Leguminosae).[122,123] This compound was obtained in high yield

[120] Kumar, V.; Ramesh, N. G. *Tetrahedron* **2006**, *62*, 1877.
[121] For a copy of NMR of (+)-DMDP see: Fechter, M. H.; Gradnig, G.; Berger, A.; Mirtl, C.; Schmid, W.; Stutz, A. E. *Carbohydr. Res.* **1998**, *309*, 367.
[122] a) Jones, D. W. C.; Nash, R. J.; Bell, E. A.; William, J. M. *Tetrahedron Lett.* **1985**, *26*, 3125. b) Fleet, G. W. J.; Nicholas, S. J.; Smith, P. W.; Evans, S. V.; Fellows, L. E.; Nash, R. J. *Tetrahedron Lett.* **1985**, *26*, 3127.
[123] Asano, N.; Kizu, H.; Oseki, K.; Tomioka, E.; Matsui, K.; Okamoto, M.; Baba, M. *J. Med. Chem.* **1995**, *38*, 2349.

(86%) by direct hydrogenation of nitrone **1** with Pd-C in HCl-MeOH (Scheme 4.6).



Scheme 4.6. Syntheses of DAB-1 and nectrisine

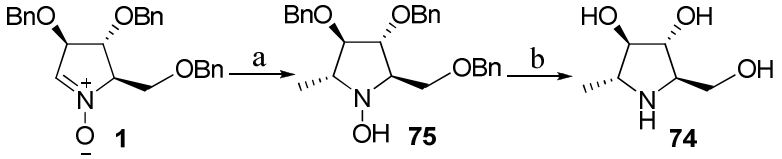
Reaction conditions: a) H_2, Pd-C, HCl-MeOH, r.t., 15 h then Dowex WX8-200, 98%. b) $(MeO)_3P$, Ph_3P, TEA, reflux, 62%. c) BCl_3, DCM, 67%.

Nectrisine **73** (also known as FR-900483), isolated from the fungus *Nectria lucida* F-4490 (Ascomycetes)[124] is an inhibitor of glucosidases and mannosidases possessing also immunostimulating properties. Deoxygenation of nitrone **1** to imine **72** was obtained using a modification of a reported procedure,[125] which employs trimethyl phosphine in refluxing TEA. The addition of 10 mol% of triphenylphosphine to trimethylphosphite in triethylamine allowed **72**[126] to be afforded in good yield. Debenzylation of **72** with BCl_3,[126] in order to preserve the imine functionality, gave nectrisine **73**, which was found to be unstable in D_2O solution,[127] affording hydrated

[124] Shibata, T.; Nakayama, O.; Tsurumi, Y.; Okuhara, M.; Terano, H.; Kohsaka, M. *J. Antibiot.* **1988**, *41*, 296.
[125] Milliet, P.; Lusinchi, X. *Tetrahedron* **1979**, *35*, 43.
[126] Bosco, M.; Bisseret, P.; Bouix-Peter, C.; Eustache, J. *Tetrahedron Lett.* **2001**, *42*, 7949.
[127] Otero, J. M.; Soengas, R. G.; Estevez, J. C.; Estevez, R. J.; Watkin, D. J.; Evinson, E. L.; Nash, R. J.; Fleet, G. W. J. *Org. Lett*. **2007**, *9*, 623.

derivatives. On the other hand, ^{13}C NMR data of the crude compound were in agreement with those reported in the literature. [128]

The synthesis of 6-deoxy-DMDP **74**, an inhibitor of β-mannosidase, β-galactosidase, and α-fucosidase, isolated from *Angylocalyx* sp. (Leguminosae)[129] was afforded through quantitative and complete stereoselective addition of methylmagnesium bromide to nitrone **1** (Scheme 4.7).



Scheme 4.7. Synthesis of 6-deoxy-DMDP

Reaction conditions: a) MeMgBr, THF, 0°C, 100%. b) H_2, Pd-C, HCl-MeOH, r.t., 16 h then Dowex WX8-200, 87%.

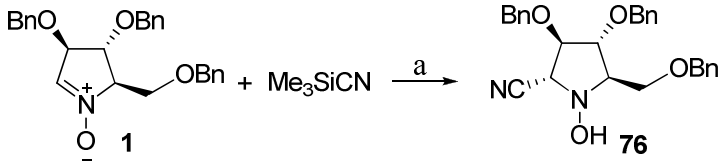
As expected, the Grignard reagent reacted with the cyclic nitrone at the less hindered face opposite to the C-3 OBn substituent, to afford hydroxylamine **75** quantitatively. Hydrogenation of **75** with 10% Pd-C in HCl-MeOH followed by elution through DOWEX WX8-200 with 6% ammonium hydroxide furnished the free amine **74** in 87% overall yield over two steps.

4.2.4 Synthesis of 2-(aminomethyl)pyrrolidines

Nitrone **1** was converted into the corresponding α-cyano hydroxylamine **76** by reaction with trimethylsilyl cyanide in methanol (Scheme 4.8). Trimethylsilyl cyanide was slowly converted *in situ* into HCN,[130] which reacted

[128] Kim, Y. J.; Takatsuki, A.; Kogoshi, N.; Kitahara, T. *Tetrahedron* **1999**, *55*, 8353.
[129] a) Molyneux, R. J.; Pan, Y. T.; Tropea, J. E.; Elbein, A. D.; Lawyer, C. H.; Hughes, D. J.; Fleet, G. W. J. *J. Nat. Prod.* **1993**, *56*, 1356. b) Yasuda, K.; Kizu, H.; Yamashita, T.; Kameda, Y.; Kato, A.; Nash, R. J.; Fleet, G. W. J.; Molyneux, R. J.; Asano, N. *J. Nat. Prod.* **2002**, *65*, 198.
[130] Singh, N.; Mohan, S. *J. Chem. Soc., Chem. Commun.* **1969**, 868.

smoothly with nitrone, no additive being necessary,[131] to provide the free hydroxylamine **76** enough pure to be used for the next step.

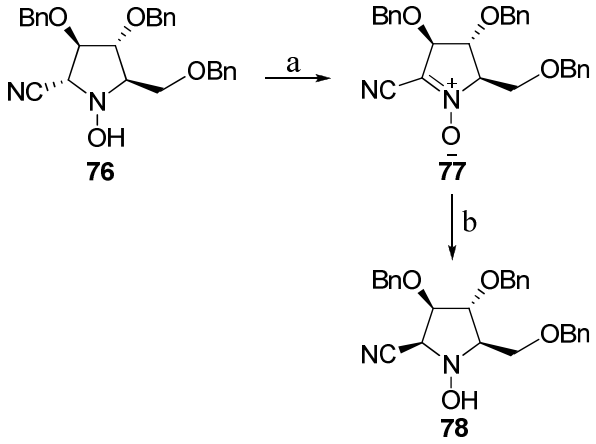


Scheme 4.8. Synthesis of the 2-cyano-*N*-hydroxypyrrolidine 76

Reaction condition: a) MeOH, r.t., 100%.

The selectivity of the reaction was complete and only the isomer showing a *trans* disposition to the C3 substituent could be detected by ^{1}H NMR.

Since all attempts to invert the stereocontrol in the hydrocyanation of nitrones had been unsuccessful,[111a] we focused our attention on the above described oxidation-reduction protocol to obtain epimeric hydroxylamine at C-2. This was accomplished by oxidation of **76** to nitrone **77** with manganese (IV) oxide[117] as described above, followed by stereoselective reduction with sodium borohydride (Scheme 4.9).



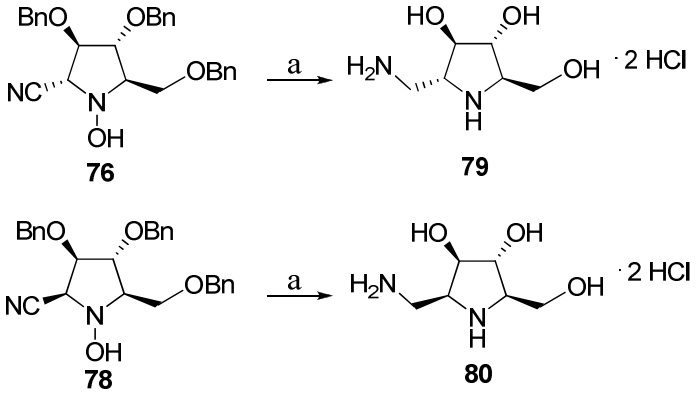
Scheme 4.9. Synthesis of cyano derivative 78

Reaction conditions: a) MnO_2, DCM, 100%. b) $NaBH_4$, MeOH, 100%.

[131] Merino, P.; Tejero, T. *Tetrahedron* **2001**, *57*, 8125.

The oxidation and reduction were found to be completely regio- and stereoselective, respectively. Presumably, the regioselectivity of the reaction was controlled by conjugation of the incipient nitrone functionality with the cyanide moiety, so that the oxidation is directed solely towards the cyanide side. The relative stereochemistry of pyrrolidine substituents in **76** and **78**, and thus the absolute configuration of the new stereogenic centers was confirmed by 2D NMR (NOESY and COSY) experiments.

The palladium-catalyzed hydrogenation-deprotection methodology used for the synthesis of **67** and **69** in Scheme 4.5 was then applied to the synthesis of 2-(aminomethyl)pyrrolidines. When compounds **76** and **78** were subjected to hydrogenolysis under 150 bar of hydrogen in 1N HCl in MeOH, the corresponding dihydrochlorides **79** and **80** were obtained in quantitative yield (Scheme 4.10).



76 **79**

78 **80**

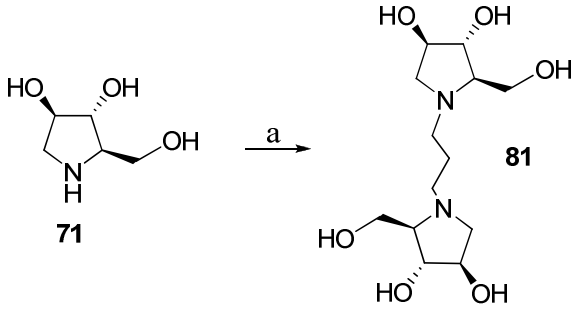
Scheme 4.10. Synthesis of 2-(aminomethyl)pyrrolidines

Reaction conditions: a) H_2, 5% $Pd(OH)_2$-C, 150 atm, HCl-MeOH, r.t., 6 h, 100%.

4.2.5 Synthesis of *N*-alkylated and dimeric derivatives of DAB-1

In order to increase the inhibition of trehalases, docking studies have shown that DAB-1 dimers fit good with the enzyme catalytic site. The synthesis is easy and can be accomplished by the addition of dibromo-alkanes to DAB-1 (**71**).

Unfortunately, the reaction took place only with dibromopropane in refluxing MeOH/dioxane affording the dimer **81** (Scheme 4.11):

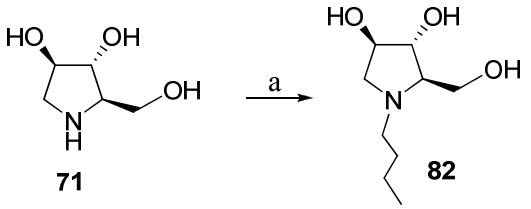


Scheme 4.11. Synthesis of DAB-1 dimer 81

Reaction conditions: a) $Br(CH_2)_3Br$, $NaHCO_3$, dioxane-MeOH, reflux., 24 h, 58%.

The reactions with di-bromoethane, di-iodoethane and di-chloroacetone, in the same conditions or changing the solvent (CH_3CN) or the base (K_2CO_3), gave complex mixtures. An attempt using microwaves as heating source gave no reaction also.

Similarly we attempt the alkylation at the nitrogen atom with bromoalkanes and we obtained the butyl derivate **82** (Scheme 4.12):



Scheme 4.12. Synthesis of alkylated derivate 82

Reaction conditions: a) *n*-BuBr, $NaHCO_3$, dioxane-MeOH, reflux., 20 h, 64%.

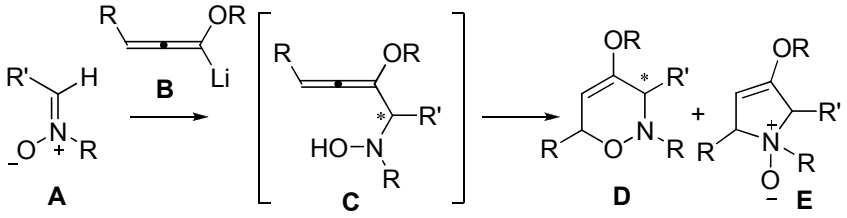
From the reaction with bromododecane in the same conditions or using microwave, only starting material was recovered.

4.3 Pyrrolizidine alkaloids

4.3.1 Introduction

Synthesis and use of allene derivatives have been rapidly expanded in preparative organic chemistry.[132] In recent years, both acceptor-substituted allenes and electon-rich allenes, in particular methoxyallene, have emerged as versatile key building blocks for the synthesis of several natural products and related compounds.

A most important aspect for applications of alkoxyallenes is the relatively high acidity of the hydrogen atom at C-1. Alkoxyallenes can smoothly be deprotonated at this position by butyllithium.[133] The resulting lithium compound can react with different electrophiles leading to C-1 substituted derivatives. In particular the addition on lithiated alkoxyallenes (**B**) to nitrones (**A**) allowed formation of the allenyl hydroxylamine (**C**). In some cases it was possible to isolate it, with subsequent cyclization to 1,2-oxazines (**D**) or amine oxide (**E**) (Scheme 4.13).[134]



Scheme 4.13. Synthetic strategy

We applied this strategy aimed to the synthesis of pyrrolizidine alkaloids such as australine (**42**) and 6,7-di-*epi*casuarine (**43**).

[132] *The Chemistry of Ketenes, Allenes and Related Compounds*; Patai, S., Ed.; Wiley: New York, **1980**.
[133] Hoff, S.; Brandsma, L; Arens, J. F. *Recl. Trav. Chim. Pays-Bas* **1968**, *87*, 916.
[134] Pulz, R.; Cicchi, S.; Brandi, A.; Reiβig, H.-U. *Eur. J. Org. Chem.* **2003**, 1153.

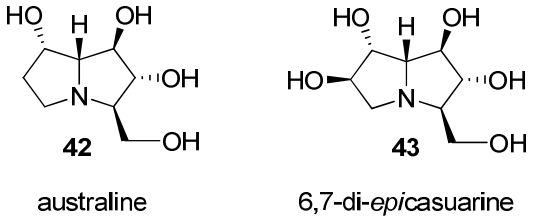
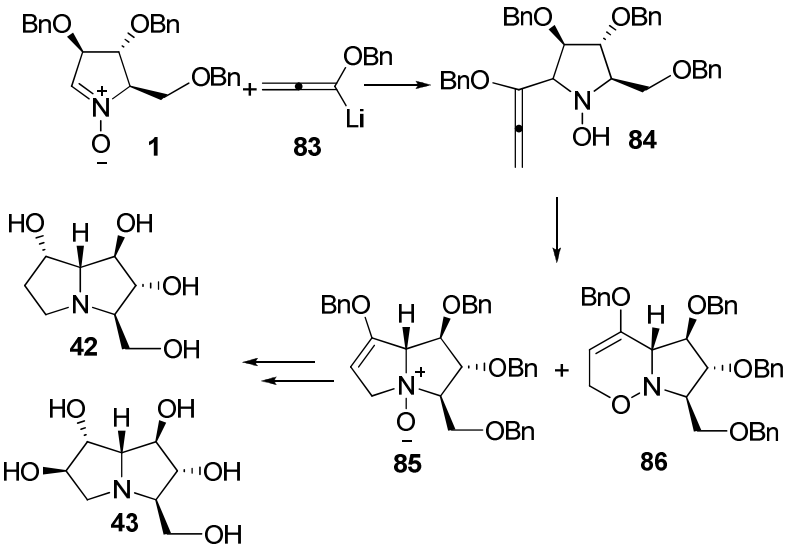


Figure 4.2. Australine and 6,7-di-*epi*casuarine

The synthetic strategy involves an organometallic addition reaction of lithiated benzyloxyallene **83** to nitrone **1** with formation of adduct **84**. Spontaneous cyclization of **84** can, in principle, furnish N-oxide derivative **85** or 1,2-oxazolidine derivative **86** (Scheme 4.14) which can be differently manipulated to obtain the target molecules.



Scheme 4.14. Synthetic strategy for the synthesis of australine and 6,7-di-*epi*casuarine

We first developed the synthetic strategy using methoxy allene, which is more easily available than benzyloxy allene. This also allowed to study the inhibitory properties of methoxy derivatives of the target molecules, 7-methoxyaustraline **87** and 7-methoxy-6,7 di-*epi*casuarine **88** (Figure 4.3).

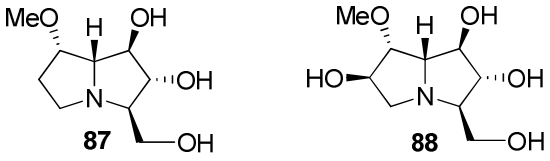
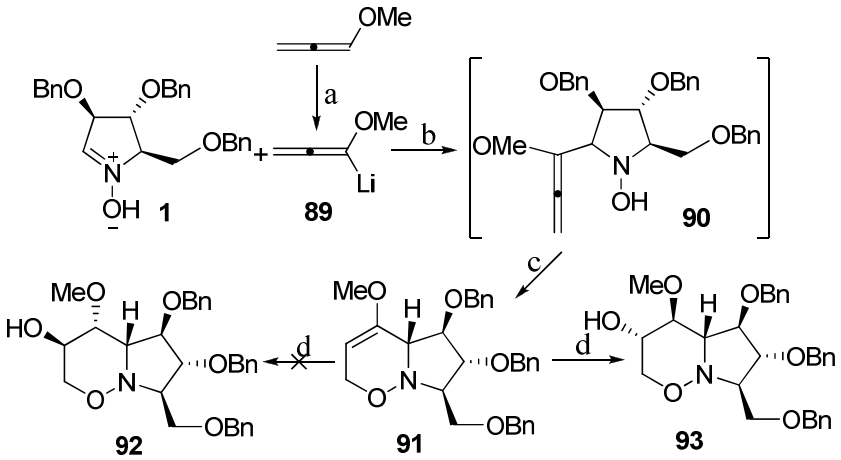


Figure 4.3. 7-Methoxyaustraline 96 and 7methoxy-6,7-di-*epi*casuarine

4.3.2 Syntheses of methoxy derivatives

Attempted syntheses of **87** and **88** started from the reaction of nitrone **1** with lithiated methoxy allene **89**, which provided adduct **90** that could not be isolated. The crude was stirred at rt in DCM affording only 1,2-oxazine derivative **91** (Scheme 4.15); no formation of the N-oxide derivative was observed.



Scheme 4.15. Synthesis of adduct 93

Reaction conditions: a) BuLi, -78°C, THF, 15 min; b) -78°C, THF, 2 h; c) DCM, rt, 48 h; 78% over three steps; d) BH_3·THF, THF, 2 h, rt, then NaOH, H_2O_2, 18 h, rt, 84% over two steps.

Contrary to our expectation, hydroboration of **91** did not afford the desired stereoisomer **92**; compound **93** was formed instead, as demonstrated by its NMR spectra (Scheme 4.15).

We expected that BH_3 would hydroborate from the apparently more accessible upper side of the molecule affording **92**. The opposite result may be rationalized hypothesing that the nitrogen atom coordinates BH_3 in the most

stable trans-fuse conformer, favouring its attack from the lower side of the molecule and affording adduct **93** (Figure 4.4).

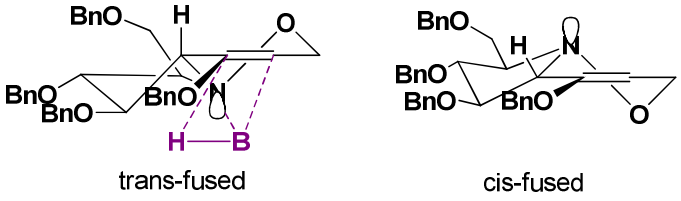


Figure 4.4. Coordination of the nitrogen atom to BH_3

With compound **93** in hand we completed the total synthesis of methoxy derivatives of casuarine **94** and 7-*epi*australine **95** (Figure 4.5), indeed, it was interesting to study their inhibition properties in order to understand the role of modification at C-7 of a very active molecule such as casuarine **11** (See chapter 5).

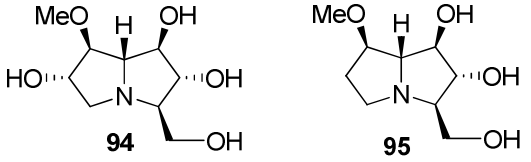
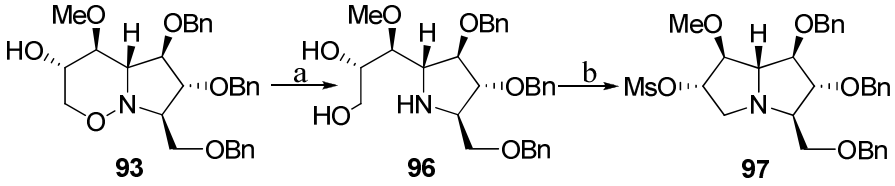


Figure 4.5. 7-Methoxycasuarine 94 and 7-*epi*-7-methoxyaustraline 95

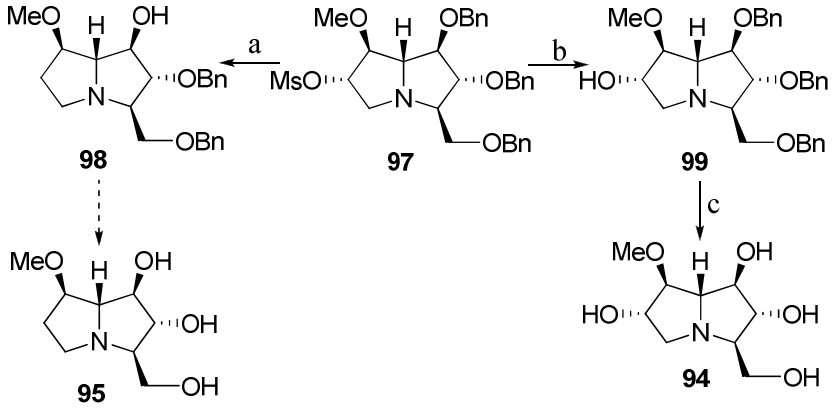
We proceeded with the opening of the N-O bond mediated by SmI_2, affording in 94% yield compound **96**, which was mesylated with mesylchloride in DCM and in the presence of triethylamine to obtain intermediate **97** after spontaneously cyclization(Scheme 4.16).



Scheme 4.16. Synthesis of mesylate derivative 97

Reaction conditions: a) SmI_2, THF, rt, 4 h, 94%; b) MsCl, DCM, TEA, rt, 1.5 h; 85%.

Mesylate derivative **97** can be differently manipulated to obtain both the desired products **94** and **95** (Scheme 4.17).



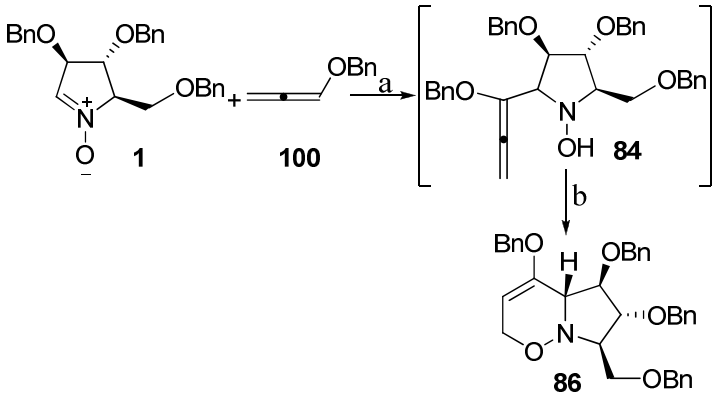
Scheme 4.17. Syntheses of 94 and 95

Reaction conditions: a) DIBAL-H, THF, rt, 2 h, 50%; b) $LiAlH_4$, THF, rt, 2 h, 85%; c) H_2, Pd/C, MeOH, HCl cat., rt, 18 h, 62%.

Treatment of **97** with DIBAL-H afforded the deoxygenated **98** which can be hydrogenated in order to obtain **95**. In contrast, reduction of **97** with $LiAlH_4$ gave demesylation to deprotected derivative **99** which afforded, after hydrogenation, 7-methoxycasuarine **94** (Scheme 4.17).

4.3.3 Syntheses of casuarine and 7-*epi*australine

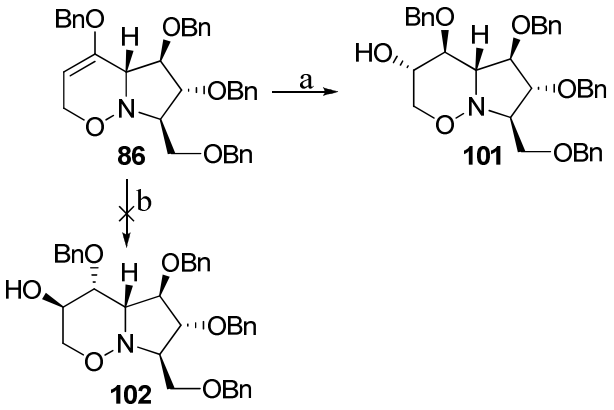
Reaction of nitrone **1** with lithiated benzyloxy allene **100** afforded adduct **84** which could not be isolated and cyclised spontaneously to give, also in this case, oxazine **86** without any formation of N-oxide **85** (scheme 4.18).



Scheme 4.18. Synthesis of compound 86

Reaction conditions: a) BuLi, -78°C, THF, 2 h; b) DCM, rt, 48 h; 81% over two steps.

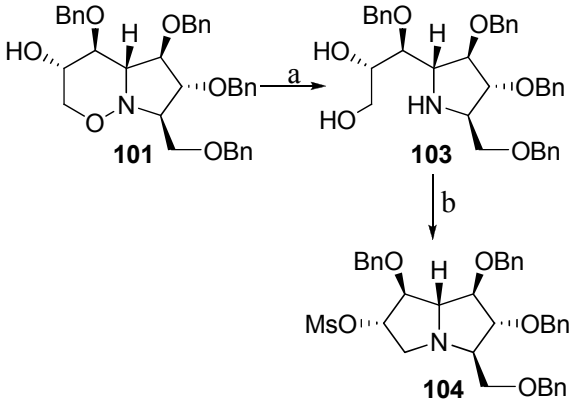
Also in this case treatment with BH_3 afforded **101** and not the desired product **102**. In order to obtain **102**, the reaction was performed with the more hindered 9-BBN but only starting material was recovered (Scheme 4.19) in this case.



Scheme 4.19. Synthesis of compound 101

Reaction conditions: a) BH_3·THF, THF, rt, 2 h, then NaOH, H_2O_2, rt, 18 h, 81% over two steps; b) 9-BBN, THF, rt, 2 h, then NaOH, H_2O_2, rt, 18 h.

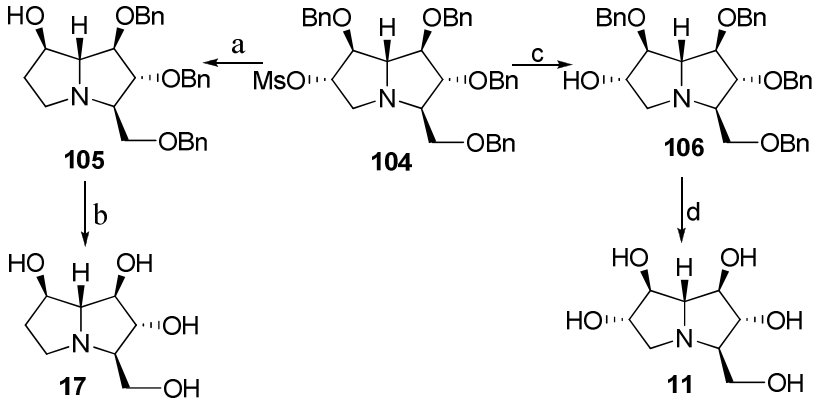
Oxazine **101** was treated with SmI_2 affording **103**, in 81% yield, which was mesylated to obtain pyrrolizidine **104** after spontaneously cyclization (scheme 4.20).



Scheme 4.20. Synthesis of compound 104

Reaction conditions: a) SmI_2, THF, rt, 4 h, 81%; b) MsCl, DCM, TEA, rt, 1.5 h; 94%.

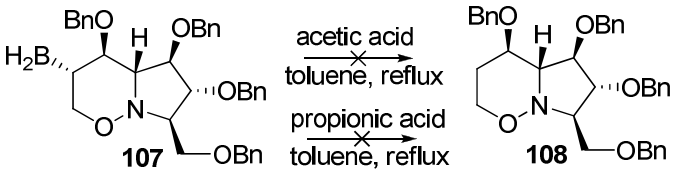
Treatment of **104** with DIBAL-H afforded the deoxygenated **105** which was hydrogenated to obtain 7-*epi*australine **17**. Treatment with $LiAlH_4$ of **104** afforded deprotected derivative **106** which gave, after hydrogenation, casuarine **11** (Scheme 4.21).



Scheme 4.21. Syntheses of 7-*epi*australine (17) and casuarine (11)

Reaction conditions: a) DIBAL-H, THF, rt, 2 h, 50%; b) H_2, Pd/C, MeOH, HCl cat., rt, 18 h, 100%; c)$LiAlH_4$, THF, rt, 2 h, 86%; d) H_2, Pd/C, MeOH, HCl cat., rt, 18 h, 100%.

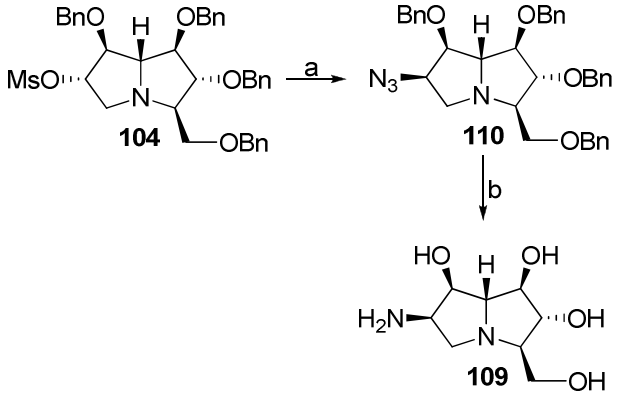
In order to shorter the total synthesis of 7-*epi*australine (**17**), after hydroboration, compound **107** was treated with acetic acid or propionic acid, but no desired compound **108**, which would have not required deoxygenation step, was obtained (Scheme 4.22).



Scheme 4.22. Synthesis of compound 108

In order to eliminate the deprotection step after mesylation in the casuarine (**11**) synthesis, we performed mesylation with only 1 eq. of mesyl chloride on compound **103**, but we obtained a complex mixture of mono-mesylate derivatives, starting material and compound **104**.

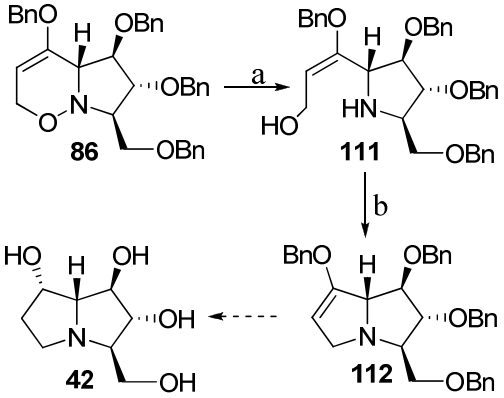
In order to obtain 7-*epi*aminocasuarine (**109**), a reaction with NaN_3 was performed on the same intermediate **104** affording compound **110**, which can be hydrogenated in the presence of Pd/C to obtain 7-*epi*aminocasuarine (**109**) (scheme 4.23).



Scheme 4.23. Synthesis of 7-*epi*-aminocasuarine

Reaction conditions: a) NaN_3, DMF, 100°C, 48 h, 62%; b) H_2, Pd/C, MeOH, HCl, rt, 48 h, 80%.

The total synthesis of australine (**42**) is still a target of our research group. For this reason we are investigating a new synthetic approach starting from oxazine **86** which, through N-O bond cleavage afforded compound **111** (Scheme 4.24).



Scheme 4.24. New route for the synthesis of australine

Reaction conditions: a) $Mo(CO)_6$, $NaBH_4$, CH_3CN/H_2O, reflux, 3 h, 45%; b) MsCl, DCM, TEA, rt, 3 h, 65%.

Compound **111** was treated with mesyl chloride affording **112** which can be hydrogenated in order to obtain australine. At the current stage, only preliminary, but promising results have been collected. Work is underway in our laboratory to target the synthesis of australine (**42**).

4.4 Conclusion

In the first part of this chapter, the diastereoselective synthetic methodology studied allowed the preparation of several families of polyhydroxylated pyrrolidines and variants thereof with a great versatility. The complete diastereoselectivity found in nucleophilic additions to the key cyclic nitrone and the possibility of configurational inversion at the newly created stereocenter, through an oxidation-reduction protocol, makes this methodology complementary to other nucleophilic additions to acyclic nitrones tunable with Lewis acids. The protocol was used to synthesize 2-(hydroxymethyl)- and 2-(aminomethyl)- polyhydroxylated pyrrolidines enantiospecifically, offering a new route to such systems competing with previously reported strategies. Finally, the work provided access to naturally occurring alkaloids DMDP, 6-deoxy-DMDP, DAB-1, and nectrisine as well as several structural analogues. The total synthesis of other natural polyhydroxylated pyrrolidines and analogues using the present methodology is under investigation in our laboratories.

In the second part of this chapter, nucleophilic additions of allenes to the key nitrone were studied in order to obtain natural iminosugars and unnatural casuarine-like pyrrolizidines. The target molecules were studied toward amyloglucosidase from *Aspergillus Niger* (see Chapter 5) with interesting results.

4.5 Experimental Section

General experimental aspect are mentioned in Chapter 2.3.1.

4.5.1 Synthesis of DMDP and structural analogues

(2*R*,3*R*,4*R*,5*R*)-3,4-bis(benzyloxy)-2,5-bis(benzyloxymethyl)-1-hydroxy pyrrolidine (**67**) To a cooled (-80 °C) solution of **66** (0.850 g, 2 mmol) in anhydrous THF (15 mL), butyllithium (3.2 mL of a 1.6 M solution in hexanes, 2 mmol) was slowly added via syringe under an argon atmosphere. The resulting solution was stirred for 6 min at which time a cooled (-80 °C) solution of **1** (0.334 g, 0.8 mmol) in THF (5 mL) was added during a period of 5 min via cannula. After 15 min at -80 °C the reaction was quenched with sat aq NH_4Cl (1 mL) and the reaction mixture was allowed to warm to room temperature. The reaction mixture was treated with additional sat aq NH_4Cl (20 mL) and diethyl ether (30 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (2 x 30 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$), filtered and evaporated under reduced pressure afforded pure **67** (0.306 g, 71%) as an oil; $[\alpha]_D^{25}$ = + 21 (*c* 0.25, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.34-7.20 (m, 20H, Ar), 6.04 (s, 1H, OH), 4.54 (2H, d, *J* = 12.1 Hz, Bn), 4.50 (2H, d, *J* = 12.1 Hz, Bn), 4.49 (2H, d, *J* = 11.8 Hz, Bn), 4.43 (2H, d, *J* = 11.8 Hz, Bn), 4.00 (dd, *J* = 3.7, 1.5 Hz, 2H, H-3), 3.78 (dd, *J* = 9.6, 4.8 Hz, 2H, H-6), 3.63 (dd, *J* = 9.6, 6.2 Hz, 2H, H-7), 3.51 (m, 2H, H-2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 133.0 (s, 2C, Ar), 132.9 (s, 2C, Ar), 123.6-122.6(d, 8C, Ar), 122.4 (d, 4C, Ar), 78.6 (d, 2C, C-3, C-4), 68.2 (t, 2c, Bn), 66.5 (t, 2C, Bn), 64.4 (d, 2C, C-2, C-5), 62.6 (t, 2C, C-6, C-7). IR (KBr): 2441 (ν, OH), 3028 (ν, =CH), 2912 (ν, -CH), 2000-1800 (overtones, Bn) cm^{-1}. Anal Calcd. for $C_{34}H_{37}NO_5$ (539.66): C, 75.67; H, 6.91; N, 2.60. Found: C, 75.52; H, 6.83; N, 2.87.

(2*R*,3*R*,4*R*)-3,4-bis(benzyloxy)-2,5-bis(benzyloxymethyl)-3,4-dihydro-2H-pyrrole-1-oxide (**68**). To a cooled (0°C) solution of **67** (1.078 g, 2 mmol) in

CH_2Cl_2 (30 mL), activated manganese (IV) oxide (2.09 g, 2.4 mmol) was added portionwise. After 15 min of stirring at 0 °C the reaction mixture was warmed to room temperature and stirring was continued until complete disappearance of the starting material (TLC, c.a. 2 h). Then, the reaction mixture was filtered through a pad of Celite® and anhydrous $MgSO_4$ and the resulting filtrate was evaporated under reduced pressure afforded pure **68** (1.075 g, 100%) as an oil. $[\alpha]_D^{25}$ = - 38 (*c* 0.96, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.13-7.28 (m, 20H, Ar), 4.68 (s, 1H, H-3), 4.59 (d, *J* = 14.4 Hz, 1H, H-6), 4.53 (d, *J* = 11.7 Hz, 1H, Bn), 4.48 (d, *J* = 12.0 Hz, 1H, Bn), 4.47 (s, 2H, Bn), 4.47 (d, *J* = 11.7 Hz, 1H, Bn), 4.41 (d, *J* = 12.0 Hz, 1H, Bn), 4.39 (d, *J* = 11.9 Hz, 1H, Bn), 4.35 (d, *J* = 11.9 Hz, 1H, Bn), 4.25 (dt, *J* = 14.4, 1.3, Hz, 1H, H-6), 4.14 (dd, *J* = 2.9, 1.7, Hz, 1H, H-4), 3.94-3.99 (m, 1H, H-5), 3.87 (dd, *J* = 10.1, 5.8, Hz, 1H, H-9), 3.72 (dd, *J* = 3.3, 10.1 Hz, 1H, H-9). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 143.9 (s, C-2), 137.7 (s, 4C, Ar), 137.5-127.6 (d, 12C, Ar), 127.0 (d, 4C, Ar), 83.4 (d, C-3), 78.9 (d, C-4), 78.2 (d, C-5), 73.6 (t, Bn), 73.5 (t, Bn), 72.1 (t, Bn), 71.6 (t, C-6), 66.8 (t, C-9), 62.4 (*t,* Bn). Anal Calcd. for $C_{34}H_{35}NO_5$ (537.75): C, 79.95; H, 6.56; N, 2.61. Found: C, 79.88; H, 6.73; N, 2.90.

(2*R*,3*R*,4*R*,5*S*)-3,4-bis(benzyloxy)-2,5-bis(benzyloxymethyl)-1-hydroxy pyrrolidine (**69**). A cooled (-80 °C) solution of **68** (0.538 g, 1 mmol) in anhydrous THF (10 mL) was treated dropwise with L-Selectride (2 mL of a 1M solution in hexanes, 2 mmol) under an argon atmosphere. After stirring for 2 h at -80 °C the reaction misture was allowed to warm to 0 °C at which time sat aq NH_4Cl (5 mL) was added. The resulting mixture was treated with diethyl ether (15 mL); the organic layer was separated and the aqueous layer was extracted with diethyl ether (2 x 15 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$) and evaporated under reduced pressure without exceeding 20 °C to give pure **69** (0.540 g, 100%) as an oil. This compound could not be characterized since it showed to be rather unstable

being oxidized immediately to the precursor nitrone **68**. Because of this reason it was used immediately in the next step without any purification.

(2*R*,3*R*,4*R*,5*R*)-2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine hydrochloride (DMDP, **64**). A solution of hydroxylamine **67** (0.540 g, 1 mmol) in methanol (10 mL) was treated with $Pd(OH)_2$-C (15 mg) and a 1M solution of HCl in methanol. The resulting mixture was stirred under 20 atm of hydrogen for 6 h. The catalyst was eliminated by filtration through a pad of Celite®, the filtrate was treated with 3M HCl in methanol and the resulting solution was stirred at room temperature for additional 10 min. The solvent was eliminated under reduced pressure to afforded pure **64** (0.2 g, 100 %) as a white solid; mp >150 °C (dec). $[\alpha]_D^{25}$ = + 48 (*c* 0.32, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 4.01-3.96 (m, 2H, H-3), 3.84 (dd, *J* = 12.7, 3.7 Hz, 2H, H-4), 3.76 (dd, 2H, *J* = 12.7, 5.7 Hz, 2H, H-4), 3.44–3.50 (m, 2H, H-2). ^{13}C NMR (100 MHz, D_2O) δ 74.2 (d, 2C, C-3), 62.4 (d, 2C, C-2), 57.8 (t, 2C, C-4). Anal Calcd. for $C_6H_{14}ClNO_4$ (199.63): C, 36.10; H, 7.07; N, 7.02. Found: C, 36.32; H, 7.21; N, 7.17. In order to compare with the literature data an analytical sample of the free amine was obtained by passing the hydrochloride through a Dowex 50WX8 ion-exchange resin. Elution with 3M ammonia in methanol afforded after evaporation the free base of **64** as a white solid: mp 114-117 °C (Lit.[9d] mp 115-117 °C); $[\alpha]_D^{25}$ = + 53 (*c* 0.95, H_2O) (Lit. [9d] $[\alpha]_D$ = + 55.4 (*c* 1.3, H_2O)). ^{1}H-NMR (400 MHz, D_2O) δ 3.72-3.77 (m, 2H, H-3), 3.61 (dd, *J* = 11.9, 6.3 Hz, 2H, H-4), 3.53 (dd, 2H, *J* = 11.9, 4.3 Hz, 2H, H-4) 2.92-2.99 (m, 2H, H-2). ^{13}C NMR (100 MHz, D_2O) δ 77.5 (d, 2C, C-3), 61.6 (t, 2C, C-4), 61.5 (d, 2C, C-2). Anal Calcd. for $C_6H_{13}NO_4$ (163.17): C, 44.16; H, 8.03; N, 8.58. Found: C, 43.87; H, 8.05; N, 8.53.

(2*S*,3*R*,4*R*,5*R*)-2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine hydrochloride (**70**) A solution of hydroxylamine **69** (0.540 g, 1 mmol) in methanol (10 mL) was treated with $Pd(OH)_2$-C (15 mg) and a 1M solution of HCl in methanol. The resulting mixture was stirred under 20 atm of hydrogen for 6 h. The catalyst was eliminated by filtration through a pad of Celite®, the

filtrate was treated with 3M HCl in methanol and the resulting solution was stirred at room temperature for additional 10 min. The solvent was eliminated under reduced pressure afforded pure **70** (0.2 g, 100 %) as a white solid; mp >150 °C (dec). $[\alpha]_D^{25} = -5$ (*c* 0.75, MeOH) (Lit.[59] $[\alpha]_D = -3.4$ (*c* 1.0, MeOH)). ^{1}H-NMR (400 MHz, D_2O) δ 4.16 (dd, *J* = 3.6, 2.2 Hz, 1H, H-4), 3.98 (dd, *J* = 3.6, 2.2 Hz, 1H, H-3), 3.70–3.92 (m, 5H, H-5, Ha-6, Hb-6, Ha-7, Hb-7), 3.49 (ddd, *J* = 8.4, 4.6, 3.6 Hz, 1H, H-2). ^{13}C NMR (100 MHz, D_2O) δ 75.8 (d, C-3), 74.3 (d, C-4), 66.6 (d, C-2), 62.9 (d, C-5), 59.1, 56.7 (t, C-6, C-7). Anal Calcd. for $C_6H_{14}ClNO_4$ (199.59): C, 36.10; H, 7.07; N, 7.02. Found: C, 35.83; H, 7.15; N, 6.86.

(2*R*,3*R*,4*R*)-2-(hydroxymethyl)-3,4-dihydroxypyrrolidine (DAB-1, **71**). A solution of nitrone **1** (0.430 g, 1.03 mmol) in methanol (8 mL) was treated with Pd-C (420 mg) and concentrated HCl (4 drops). The resulting mixture was stirred under hydrogen atmosphere for 15 h. The catalyst was eliminated by filtration through a pad of Celite®, then the filtrate was concentrated under reduced pressure and passed through a short pad of Dowex WX8-200 eluting with water, MeOH and finally with a 6% NH_4OH solution. Concentration of the fractions eluted with NH_4OH afforded a brown oil (135 mg, 98% yield); $[\alpha]_D^{23} = +5.6$ (*c* 0.285, H_2O) (Lit.[63] $[\alpha]_D = +6.3$ (*c* 1, H_2O)). ^{1}H-NMR (400 MHz, D_2O) δ 4.03 (dt, *J* = 5.7, 3.9 Hz, 1 H, H-4), 3.73 (dd, *J* = 5.7, 3.6 Hz, 1 H, H-3), 3.63 (dd, *J* = 11.5, 4.9 Hz, 1 H, Ha-6), 3.55 (dd, *J* = 11.5, 6.4 Hz, 1 H, Hb-6), 3.02 (dd, *J* = 12.2, 5.7 Hz, 1 H, Ha-5), 2.90 (m, 1 H, H-2)2.74 (dd, *J* = 12.3, 3.9 Hz, 1 H, Hb-5). ^{13}C NMR (50 MHz, D_2O) δ 78.0 (d, C-3), 76.4 (d, C-4), 64.4 (d, C-2), 61.0 (t, C-6), 49.6 (t, C-5). Anal Calcd. for $C_5H_{11}NO_3$ (133.12): C, 45.10; H, 8.33; N, 10.52. Found: C, 44.82; H, 8.11; N, 10.23.

(2*R*,3*R*,4*R*)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2*H*-pyrrole (**72**). To a solution of nitrone **1** (285 mg, 0.68 mmol) in 20 mL of a 10:1 mixture of trimethyl phosphite and TEA, triphenylphosphine (75 mg, 0.30 mmol) was added and the mixture was heated at reflux for 4 h. After evaporation under reduced pressure, purification by flash column

chromatography (eluent petroleum ether/EtOAc, 1.5:1) afforded pure **72** (170 mg, 62%) as an oil; $[\alpha]_D^{22} = -10.4$ (*c* 0.76, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 2.34 Hz, 1 H, H-5), 7.23 – 7.40 (m, 15 H, Ar), 4.59 - 4.66 (m, 7 H, Bn, H-4), 4.18 - 4.21 (m, 1 H, H-2), 4.14 (t, *J* = 3.8 Hz, 1 H, H-3) 3.79 (dd, *J* = 9.8, 4.5 Hz, 1 H, Ha-6), 3.58 (dd, *J* = 9.8, 6.3 Hz, 1 H, Hb-6). ^{13}C NMR (50 MHz, D_2O) δ 165.9 (d, C-5), 138.1, 137.8, 137,4 (s, Ar), 128.5-127.6 (d, 15 C, Ar), 90.7 (d, C-4), 84.5 (d, C-3), 76.9 (d, C-2), 73.4, 72.4, 72.1 (t, Bn), 71.1 (t, C-6). Anal Calcd. for $C_{26}H_{27}NO_3$ (401.45): C, 77.78; H, 6.78; N, 3.49. Found: C, 77.58; H, 6.56; N, 3.31.

(2*R*,3*R*,4*R*)-2-(hydroxymethyl)-3,4-dihydro-2*H*-pyrrole-3,4-diol (Nectrisine, **73**). To a solution of **72** (0.1 g, 0.25 mmol) in dry CH_2Cl_2 (0.5 mL) a 1M solution of BCl_3 in CH_2Cl_2 (1.75 mL) was added at -78 °C under nitrogen atmosphere. The mixture was stirred for 3 h, while the temperature raised to -40 °C. Then, a saturated aqueous solution of $NaHCO_3$ was added until neutral pH was reached. The solvent was evaporated under reduced pressure and the residue was taken up with 10 mL of AcOEt and stirred for 10 minutes. After decantation, this procedure was repeated 3 times. The combined organic phases were concentrated, affording a white solid, that was purified by flash column chromatography (eluent EtOAc/MeOH, 2:1) affording pure **73** (22 mg, 67% yield) as an oil. ^{13}C NMR (50 MHz, D_2O) δ 170.6 (d), 83.4 (d), 78.3 (d), 76.8 (d), 61.3 (t). Nectrisine (**73**) was found to be unstable in D_2O solution, due to formation of hydrated derivatives.[47]

(2*R*,3*R*,4*R*,5*R*)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-1-hydroxy-5-methyl pyrrolidine (**75**). To a cooled (0 °C) solution of nitrone **1** (0.418 g, 1 mmol) in anhydrous THF (10 mL), methyl magnesium bromide (3 mL of a 3M solution in THF, 3 mmol) was added dropwise under an argon atmosphere. The resulting solution was stirred at 0 °C for 3 h, at which time the reaction was quenched with sat aq NH_4Cl (10 mL). The reaction mixture was diluted with diethyl ether (10 mL), the organic layer was separated and the aqueous layer

was extracted with diethyl ether (2 x 10 ml). The combined organic extracts were washed with brine (20 mL), dried over $MgSO_4$, filtered and the solvent was eliminated under reduced pressure. The crude product was purified by column chromatography (Hexane/EtOAc, 4:1) to give pure **75** (0.434 g, 100%) as an oil. $[\alpha]_D^{24} = -12.5$ (*c* 0.135, $CDCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.25–7.37 (m, 15H, Ar), 4.95 (bs, 1H, OH), 4.45–4.56 (m, 6H, Bn), 3.90 (dd, *J* = 4.1, 3.1 Hz, 1H, H-2), 3.72–3.76 (m, 2H, H-3, H-4), 3.62 (dd, *J* = 9.6, 6.5 Hz, 1H, Ha-6), 3.51–3.55 (m, 1H, Hb-6), 3.37–3.40 (m, 1H, H-5), 1.26 (d, *J* = 6.4 Hz, 3H, Me). ^{13}C NMR (50 MHz, $CDCl_3$) δ 135.3–135.6 (s, 3C, Ar), 125.4–126.8 (d, 15C, Ar), 85.0 (d, C-3), 81.8 (d, C-2), 71.4, 71.1, 69.7 (t, Bn), 69.4 (t, C-6), 67.2 (d, C-4), 62.3 (d, C-5), 11.7 (q, Me). Anal Calcd. for $C_{27}H_{31}NO_4$ (433.49): C, 74.80; H, 7.21; N, 3.23. Found: C, 74.78; H, 6.91; N, 3.44.

(2*R*,3*R*,4*R*,5*R*)-2-(hydroxymethyl)-3,4-dihydroxy-5-methyl pyrrolidine (6-deoxy-DMDP, **74**). A solution of hydroxylamine **75** (0.141 g, 0.32 mmol) in methanol (20 mL) was treated with 10% Pd-C (170 mg) and 6 drops of conc HCl were added. The resulting mixture was stirred at room temperature under hydrogen for 16 h. The catalyst was eliminated by filtration through a pad of Celite®, the filtrate was passed through a Dowex 50WX8 ion-exchange resin. Elution with 3M ammonia in methanol afforded after evaporation pure **74** (0.41 g, 87%) as a syrup. $[\alpha]_D^{20} = +44.4$ (*c* 0.71, MeOH) (Lit. [69a] $[\alpha]_D = +26.2$ (*c* 1.1, MeOH) for the natural compound, $[\alpha]_D = +42.9$ (*c* 0.72, MeOH) for the synthetic compound. ^{1}H-NMR(400 MHz, D_2O) δ 3.69 (t, *J* = 7.0 Hz, 1 H, H-3), 3.55 (dd, *J* = 11.4, 4.9 Hz, 1 H, Ha-6), 3.51-3.45 (m, 2 H, Hb-6, H-4), 2.92 (td, 6.6, 4.9 Hz, 1 H, H-2), 2.83 (dq, *J* = 8.2, 6.4 Hz, 1 H, H-5), 1.06 (d, *J* = 6.4 Hz, 3 H, Me). ^{13}C NMR (50 MHz, D_2O) δ, 82.7 (d, C-4), 78.2 (d, C-3), 62.5 (t, C-6), 61.5 (d, C-2), 55.7 (d, C-5), 17.4 (q, Me). Anal Calcd. for $C_6H_{13}NO_3$ (147.14): C, 48.97; H, 8.90; N, 9.52. Found: C, 48.59; H, 9.02; N, 9.78.

4.5.2 Synthesis of 2-(aminomethyl)pyrrolidines

2*R*,3*R*,4*R*,5*R*)-3,4-bis(benzyloxy)-5-(benzyloxymethyl)-1-hydroxy pyrrolidine-2-carbonitrile (**76**) To a solution of nitrone **1** (0.417 g, 1 mmol) in MeOH (10 mL) was added trimethylsilyl cyanide (0.100 g, 1 mmol) under an argon atmosphere. The resulting solution was stirred at room temperature for 10 h, at which time the solvent was rotatory evaporated, without exceeding 35 °C, afforded a crude product which was purified by column chromatography (Hexane/EtOAc, 4:1) to give pure **76** (0.444 g, 100 %) as an oil; $[\alpha]_D^{25}$ = + 9 (*c* 1.08, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.30 (m, 15H, Ar), 6.81 (s, 1H, OH), 6.59 (d, *J* = 12.2 Hz, 1H, Bn), 6.54 (d, *J* = 11.7 Hz, 1H, Bn), 6.51-6.42 (m, 4H, Bn), 4.25 (d, *J* = 1.9 Hz, 1H, H-2), 4.19 (t, *J* = 2.4 Hz, 1H, H-3), 3.97 (dd, *J* = 6.7, 2.3 Hz, 1H, H-4), 3.76 (dd, *J* = 10.6, 3.5 Hz, 1H, Ha-6), 3.59 (dd, *J* = 10.6, 3.5 Hz, 1H, Hb-6), 3.32 (dt, *J* = 3.5, 7.0 Hz, 1H, H-5). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 137.5, 137.4, 136.6 (s, Ar), 128.8-127.9 (d, 15 C, Ar), 115.8 (s, CN), 83.7 (d, C-3), 81.3 (d, C-4), 73.4, 72.3, 72.2 (t, Bn), 69.5 (d, C-5), 66.3 (t, C-6), 61.3 (d, C-2). Anal Calcd. for $C_{27}H_{28}N_2O_4$ (444.47): C, 72.95; H, 6.35; N, 6.30. Found: C, 73.05; H, 6.28; N, 6.48.

(2*R*,3*R*,4*R*)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-5-cyano-3,4-dihydro-2H-pyrrole-1-oxide (**77**). To a cooled (0°C) solution of **76** (0.888 g, 2 mmol) in CH_2Cl_2 (30 mL), activated manganese (IV) oxide (2.09 g, 2.4 mmol) was added portionwise. After 15 min of stirring at 0 °C the reaction mixture was warmed to room temperature and stirring was continued until complete disappearance of the starting material (TLC, c.a. 2 h). Then, the reaction mixture was filtered through a pad of Celite® and anhydrous $MgSO_4$ and the resulting filtrate was evaporated under reduced pressure afforded pure **77** (0.885 g, 100 %) as an oil; $[\alpha]_D^{25}$ = - 35 (*c* 2.50, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.10 (m, 15H, Ar), 4.84 (dd, *J* = 2.0, 0.6 Hz, 1 H, H-4), 4.76 (d, *J* = 11.8 Hz, 1H, Bn), 4.65 (d, *J* = 11.8 Hz, 1H, Bn), 4.59 (d, *J* = 12.0 Hz, 1H, Bn), 4.55 (d, *J* = 11.8 Hz, 1H, Bn), 4.51

(d, *J* = 12.0, 1H, Bn), 4.50 (d, *J* = 11.8 Hz, 1H, Bn), 4.40 (dd, *J* = 3.6, 2.0 Hz, 1H, H-3), 4.11-4.07 (m, 1H, H-2), 4.01 (dd, *J* = 10.4, 4.9 Hz, 1H, Ha-6), 3.71 (dd, *J* = 3.2, 10.4 Hz, 1H, Hb-6). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 137.1, 136.5, 136.4 (s, Ar), 128.7-127.8 (d, 15C, Ar), 118.0 (s, C-5), 111.1 (s, CN), 82.0 (d, C-4), 79.5 (d, C-2), 79.3 (d C-3), 73.6, 72.2, 72.1 (t, Bn), 65.7 (t, C-6). Anal Calcd. for $C_{27}H_{26}N_2O_4$ (442.45): C, 73.28; H, 5.92; N, 6.33. Found: C, 73.41; H, 5.78; N, 6.49.

(2S,3*R*,4*R*,5R)-3,4-bis(benzyloxy)-5-(benzyloxymethyl)-1-hydroxypyrrolidine-2-carbonitrile (**78**). To a cooled (0 °C) solution of nitrone **77** (0.442 g, 1 mmol) in MeOH (6 mL), sodium borohydride (76 mg, 2 mmol) was added portionwise. The reaction mixture was stirred for an additional hour and sat aq NH_4Cl (5 mL) was added. After stirring for 15 min the reaction mixture was diluted with diethyl ether (20 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (2 x 20 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$ filtered and the solvent was eliminated under reduced pressure afforded pure **78** (0.444 g, 100 %) as an oil. $[\alpha]_D^{25}$ = + 11 (*c* 0.27, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.33-7.08 (m, 15H, Ar), 4.60 (d, *J* = 12.0 Hz, 1H, Bn), 4.48 (d, *J* = 12.0 Hz, 1H, Bn), 4.47 (s, 2H, Bn), 4.34 (d, *J* = 11.8 Hz, 1H, Bn), 4.29 (d, *J* = 11.8 Hz, 1H, Bn), 3.95 (s, 2H, H-2 and H-3), 3.76 (d, *J* = 5.2 Hz, 1H, H-4), 3.59 (dd, *J* = 10.0, 5.4 Hz, 1H, Ha-6), 3.54 (dd, *J* = 10.0, 5.6 Hz, 1H, Hb-6), 3.03 (c, *J* = 5.4 Hz, 1H, H-5). ^{13}C-NMR (100MHz, $CDCl_3$) δ 137.8, 137.2, 136.6 (s, Ar), 128.6-127.7 (d, 15C, Ar), 116.3 (s, CN), 81.7 (d, C-3), 79.7 (d, C-4), 73.4, 72.6, (t, Bn), 71.9 (d, C-5), 71.8 (t, Bn), 68.7 (t, C-6), 62.1 (d, C-2). Anal Calcd. for $C_{27}H_{28}N_2O_4$ (444.47): C, 72.95; H, 6.35; N, 6.30. Found: C, 73.14; H, 6.21; N, 6.57.

(2*R*,3*R*,4*R*,5*R*)-2-(aminomethyl)-5-(hydroxymethyl)-3,4-dihydroxy-pyrrolidine dihydrochloride (**79**). A solution of hydroxylamine **76** (0.444 g, 1 mmol) in methanol (10 mL) was treated with $Pd(OH)_2$-C (15 mg) and a 1M solution of HCl in methanol. The resulting mixture was stirred under 20 atm of hydrogen for 6 h. The catalyst was eliminated by filtration through a pad of

Celite®, the filtrate was treated with 3M HCl in methanol and the resulting solution was stirred at room temperature for additional 10 min. The solvent was eliminated under reduced pressure to afford pure **79** (0.235 g, 100 %) as a white solid; mp >150 ºC (dec). $[\alpha]_D^{25}$ = + 43 (*c* 1.43, H_2O); ^{1}H-NMR (400 MHz, D_2O) δ 4.27 (dd, *J* = 3.5, 1.4 Hz, 1H, H-3), 4.05 (dd, *J* = 2.7, 1.7 Hz, 1H, H-4), 4.02 (dt, *J* = 6.6, 3.6 Hz, 1H, H-2), 3.90 (dd, *J* = 12.1, 4.8 Hz, 1H, Ha-6), 3.76 (dd, *J* = 12.1, 8.8 Hz, 1H, Hb-6), 3.62 (ddd, *J* = 8.8, 4.9, 2.4 Hz, 1H, H-5), 3.54 (dd, *J* = 13.9, 6.8 Hz, 1H, Ha-7), 3.42 (dd, *J* = 13.9, 6.2 Hz, 1H, Hb-7). ^{13}C-NMR (100MHz, D_2O) δ 75.6 (d, C-4), 74.5 (d, C-3), 68.3 (d, C-5), 59.3 (t, C-6), 58.7 (d, C-2), 35.4 (t, C-7). Anal Calcd. for $C_6H_{16}Cl_2N_2O_3$ (235.07): C, 30.65; H, 6.86; N, 11.92. Found: C, 30.50; H, 6.93; N, 11.70.

(2*S*,3*R*,4*R*,5*R*)-2-(aminomethyl)-5-(hydroxymethyl)-3,4-dihydroxy-pyrrolidine dihydrochloride (**80**). A solution of hydroxylamine **78** (0.444 g, 1 mmol) in methanol (10 mL) was treated with $Pd(OH)_2$-C (15 mg) and a 1M solution of HCl in methanol. The resulting mixture was stirred under 20 atm of hydrogen for 6 h. The catalyst was eliminated by filtration through a pad of Celite®, the filtrate was treated with 3M HCl in methanol and the resulting solution was stirred at room temperature for additional 10 min. The solvent was eliminated under reduced pressure to afford pure **80** (0.235 g, 100 %) as a white solid; mp >150 ºC (dec). $[\alpha]_D^{25}$ = + 43 (*c* 1.43, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 4.27 (dd, *J* = 3.5, 1,4 Hz, 1H, H-3), 4.05 (dd, *J* = 2.7, 1.7 Hz, 1H, H-4), 4.02 (dt, *J* = 6.6, 3.6 Hz, 1H, H-2), 3.90 (dd, *J* = 12.1, 4.8 Hz, 1H, Ha-6), 3.76 (dd, *J* = 12.1, 8.8 Hz, 1H, Hb-6), 3.62 (ddd, *J* = 8.8, 4.9, 2.4 Hz, 1H, H-5), 3.54 (dd, *J* = 13.9, 6.8 Hz, 1H, Ha-7), 3.54 (dd, *J* = 13.9, 6.2 Hz, 1H, Hb-7). ^{13}C NMR (100 MHz, D_2O) δ 75.6 (d, C-4), 74.5 (d, C-3), 68.3 (d, C-5), 59.3 (t, C-6), 58.7 (d, C-2), 35.4 (t, C-7). Anal Calcd. for $C_6H_{16}Cl_2N_2O_3$ (235.07): C, 30.65; H, 6.86; N, 11.92. Found: C, 30.89; H, 7.01; N, 11.83.

4.5.3 Synthesis of *N*-alkylated and dimeric derivatives of DAB-1

(2*R*,3*R*,4*R*,2'*R*,3'*R*,4'*R*)-1,1'-propane-1,3-diylbis[2(hydroxymethyl)-pyrrolidine-3,4-diol] (**81**) To a solution of DAB-1 **71** (68 mg, 0.51 mmol) in MeOH/dioxane 3/2 (5 mL) was added $NaHCO_3$ (128 mg, 1.53 mmol) and 1,3 di-bromopropane (0.03 ml, 0.26 mmol). The mixture was refluxing for 24 h, at which time the mixture was filtered and the solvent was rotatory evaporated afforded a crude product which was purified by column chromatography (DCM/MeOH/NH_3, 10:5:0.5) to give pure **81** (91 mg, 58 %) as an oil. $[\alpha]_D^{25} = -54$ (*c* 0.40, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 4.01 (ddd, *J* = 5.5, 2.7, 2.3 Hz, 2H,H-4), 3.81 (dd, *J* = 4.9, 2.9 Hz, 2H, H-3), 3. 61 (d, *J* = 5.5 Hz, 4H, H-6), 2.94 (dd, *J* = 11.3, 1.6 Hz, 2H,Ha-5), 2.75 (dt, *J* = 12.0, 8.2 Hz, 2H,Ha-7), 2.69 (dd, *J* = 11.3, 5.7 Hz, 2H, Hb-5), 2.48 (q, *J* = 5.2 Hz, 2H, H-2), 2.35 (dt, *J* = 12.0, 7.8 Hz, 2H, Hb-7), 1.61 (quint., *J* = 7.8 Hz, 2H, H-8) ^{13}C-NMR (50 MHz, D_2O) δ 78.0 (d, 2C, C-3), 74.5 (d, 2C, C-4), 71.2 (d, 2C, C-2), 60.2 (t, 2C, C-6), 57.4 (t, 2C, C-5), 52.3 (t, 2C, C-7), 24.0 (t, C-8). MS (EI): *m/z* 288 ($M^+ - H_2O$, 9), 174 (M^+-DAB-1, 7), 156 ($M^+ - DAB\text{-}1 - H_2O$, 12), 146 ($M^+ - DAB\text{-}1 - CH_2CH_2$, 87), 129 ($M^+ - DAB\text{-}1 - CH_2CH_2 - OH$, 42), 116 (DAB-1−OH, 100). Anal Calcd. for $C_{13}H_{26}N_2O_6$ (306.36): C, 50.97; H, 8.55; N, 9.14. Found: C, 51.58; H, 8.68; N, 8.67.

(2*R*,3*R*,4*R*)-1-butyl-2-(hydroxymethyl)pyrrolidine-3,4-diol (**82**). To a solution of DAB-1 **71** (70 mg, 0.53 mmol) in MeOH/dioxane 3/2 (5 mL) was added $NaHCO_3$ (134 mg, 1.59 mmol) and bromobutane (0.07 ml, 0.64 mmol). The mixture was refluxing for 24 h, at which time the mixture was filtered and the solvent was rotatory evaporated afforded a crude product which was purified by column chromatography (DCM/MeOH/NH_3, 10:3:0.3) to give pure **82** (64 mg, 0.34 mmol, 64 %) as an oil; $[\alpha]_D^{25} = -50$ (*c* 0.67, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 3.99 (dt, *J* = 5.8, 2.4 Hz, 1H,H-4), 3.81 (dd, *J* = 4.9, 2.7 Hz, 1H, H-3), 3. 64-3.56 (m, 2H, Ha-6 , Hb-6), 2.90 (dd, *J* = 11.3, 2.0 Hz, 1H,Ha-5), 2.71 (dddd, *J* = 11.7, 10.9, 5.5 Hz, 1H,Ha-7), 2.64 (dd, *J* = 11.3, 5.9 Hz, 2H, Hb-5), 2.44 (dt, *J*

= 5.5, 5.2 Hz, 1H, H-2), 2.27 (dddd, *J* = 11.9, 10.5, 5.3 Hz, 1H, Hb-7), 1.43-1.27 (m, 2H, Ha-8, Hb-8), 1.19 (sest., *J* = 7.4 Hz, 2H, Ha-9, Hb-9), 0.78 (t, *J* = 7.4, 3H, H-10). ^{13}C-NMR (50 MHz, D_2O) δ 79.2 (d, C-3), 75.5 (d, C-4), 72.0 (d, C-2), 61.4 (t, C-6), 58.4 (t, C-5), 55.4 (t, C-7), 29.1 (t, C-8), 20.3 (t, C-9), 13.4 (q, C-10). MS (EI): *m/z* 189 (M^+, 3), 158 (40). Anal Calcd. for $C_9H_{19}NO_3$ (189.25): C, 57.12; H, 10.12; N, 7.40. Found: C, 56.95; H, 9.96; N, 7.16.

4.5.4 Syntheses of methoxy derivatives

(4a*S*,5*R*,6*R*,7*R*)-5,6-bis(benzyloxy)-7-[(benzyloxy)methyl]-4-methoxy-4a,5,6,7-tetrahydro-2H-pyrrolo[1,2-b][1,2]oxazine (**91**) To a stirred solution of methoxyallene (0.17 mL, 2.01 mmol) in dry THF (10 mL), a solution of BuLi 2.5 M in THF (1.86 mmol, 0.74 mL) were added at -40°C under argon atmosphere. The mixture was stirring at -40°C for 10 minutes under argon atmosphere, then a solution of nitrone **1** (450 mg, 1.08 mmol) in dry THF (10 mL) were added at -78°C. The mixture was stirring at -78°C under argon atmosphere for 2 hours. A TLC control (DCM/AcOEt 8:1) showed the disappearance of the starting material (R_f = 0.11) and the appearance of a new product (R_f = 0.59). H_2O (17 mL) was added dropwise then the mixture was warm up to rt. The mixture was extracted with AcOEt (3 x 20 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 2:1) afforded pure **91** (R_f=0.32, 389 mg, 0.80 mmol, 74 % yield) as a yellow oil. $[\alpha]_D^{20}$ = + 7.8 (c 0.4, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.43-7.25 (m, 15 H, Ar), 4.74 (t, *J* = 2.9 Hz, 1 H, H-3), 4.69-4-49 (m, 6 H, Bn), 4.39 (t, *J* = 2.3 Hz, 1 H, Ha-2), 4.33-4.29 (m,2 H, H-5, Hb-2), 4.09 (t, *J* = 4.3 Hz, 1 H, H-6), 3.75 (d, *J* = 6.4 Hz, 1 H, H-4a), 3.64-3.59 (m, 2 H, H-7, Ha-8), 3.62 (s, 3 H, Me), 3.57-3.52 (m, 1 H, Hb-8). ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm) 152.1 (s, C-4), 138.4-138.3 (s, 3 C, Ar), 128.4-127.7 (d, 12 C, Ar), 91.4 (d, C-3), 87.6 (d, C-5), 86.1 (d, C-6), 73.5, 72.2, 72.0 (t, Bn), 69.4 (t, C-8), 68.2 (d, C-

7), 66.0 (d, C-4a), 63.6 (t, C-2), 54.5 (q, Me). IR (KBr): 3029, 2929, 2898, 2859, 1675, 1452, 1360, 1221, 1072, 695 cm^{-1}. HRMS (ESI) for $C_{30}H_{35}NO_5$ $[M+H]^+$ calculated: 488.2392; found: 488.2427; Anal. Calcd for $C_{30}H_{33}NO_5$ (487.59): C, 73.90; H, 6.82; N, 2.87. Found: C, 73.65; H, 6.75; N, 2.88.

(3*S*,4*S*,4a*R*,5*R*,6*R*,7*R*)-5,6-bis(benzyloxy)-7-[(benzyloxy)methyl]-4-methoxyhexahydro-2H-pyrrolo[1,2-b][1,2]oxazin-3ol (**93**) To a stirred solution of **91** (177 mg, 0.36 mmol) in dry THF (8 mL), a solution of BH_3·THF 1M in THF (1.44 mmol, 1.44 mL) was added at -30°C under argon atmosphere. The solution was stirred at -30°C for 5 minutes and then at rt for 2 h. A TLC control (Hex/AcOEt 2:1) showed the disappearance of the starting material (R_f = 0.57) and the appearance of a new product (R_f = 0.86), then a solution of NaOH 2N (2.16 mL) and H_2O_2 (0.72 mL) were added dropwise at -10°C and the mixture was stirring overnight. Then a saturated aqueous solution of $Na_2S_2O_3$ (2.5 mL) was added dropwise and the mixture was stirring for few minutes. The mixture was extracted with Et_2O (3 x 15 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (hexane/ethyl acetate 1:1) afforded pure **93** (R_f=0.27, 145 mg, 0.29 mmol, 80 % yield) as a colourless oil. $[\alpha]_D^{20}$ = – 26.1 (c 0.93, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.36-7.25 (m, 15 H, Ar), 4.63-4.44 (m, 6 H, Bn), 4.33 (dd, *J* = 7.0, 2.7 Hz, 1 H, H-5), 4.09 (t, *J* = 3.0 Hz, 1 H, H-6), 4.02 (d, *J* = 3.5 Hz, 1 H, Ha-2), 3.76-3.66 (m, 4H, Hb-2, Ha-8, H-3, H-7), 3.51 (dd, *J* = 8.9, 7.4 Hz, 1 H, Hb-8), 3.46 (s, 3 H, Me), 3.34 (t, *J* = 5.8 Hz, 1 H, H-4), 3.24 (dd, *J* = 7.0, 6.2 Hz, 1H, H-4a), 2.48 (d, *J* = 3.5 Hz, 1H, OH). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 138.2, 138.1, 137.9 (s, Ar), 128.5-127.7 (d, 12 C, Ar), 85.7 (d, C-6), 84.9 (d, C-5), 79.1 (d, C-4), 73.5, 72.1, 71.8 (t, Bn), 69.3 (t, C-2), 68.7 (t, C-8), 68.7 (d, 2 C, C-7, C-3), 68.6 (d, C-4a), 59.0 (q, Me). IR (KBr): 3430, 3029, 2913, 2864, 1093, 734, 695 cm^{-1}. HRMS (ESI) for $C_{30}H_{36}NO_6$ $[M+H]^+$ calculated: 506.2498; found: 506.2537; Anal. Calcd for $C_{30}H_{35}NO_6$ (505.60): C, 71.27; H, 6.98; N, 2.77. Found: C, 71.00; H, 7.23; N, 2.82.

(2'*S*,3'*S*)-3-{(2*R*,3*R*,4*R*,5*R*)-3,4-bis(benzyloxy)-5-[(benzyloxy)methyl] pyrrolidin-2-yl}-3'-methoxypropane-1,2-diol (**96**). To a stirred solution of 1,2-diiodoethane (586 mg, 2.08 mmol) in dry THF (12 mL), Sm (341 mg, 2.27 mmol) was added at rt under argon atmosphere. The solution was stirred at rt for 2 h, then a solution of **93** (318 mg, 0.63 mmol) in dry THF (12 mL) was added and the mixture was stirring at rt for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.31) and the appearance of a new product (R_f = 0.02), then a saturated aqueous solution $NaHCO_3$ (6.0 mL) was added and the mixture was extracted with Et_2O (3 x 20 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (dichloromethane/methanol 15:1) afforded pure **96** (R_f=0.50, 298 mg, 0.59 mmol, 94 % yield) as an orange oil. $[\alpha]_D^{20}$ = + 2.94 (c 0.34, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.35-7.23 (m, 15 H, Ar), 4.58-4.43 (m, 6 H, Bn), 4.00-3.96 (m, 3 H, H-3, H-4, H-2'), 3.72 (d, *J* = 5.6 Hz, 2 H, Ha,b-1'), 3.53 (d, *J* = 4.3 Hz, 2 H, Ha,b-6), 3.46 (dd, *J* = 9.6, 2.5 Hz, 1 H, H-3'), 3.40 (s, 3 H, Me), 3.36 (dd, *J* = 9.6, 3.8 Hz, 1 H, H-2), 3.28 (dt, *J* = 5.8, 4.3 Hz, 1H, H-5). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.1, 138.0, 137.8 (s, Ar), 128.5-127.8 (d, 12 C, Ar), 87.2 (d, C-3), 85.8 (d, C-4), 78.6 (d, C-2), 73.2, 72.3, 71.4 (t, Bn), 71.6 (d, C-2'), 68.3 (t, C-6), 64.0 (d, C-3'), 63.4 (t, C-1'), 62.1 (d, C-5), 58.2 (q, Me). IR (KBr): 3318, 2926, 2829, 1090, 1072, 734, 696 cm^{-1}. HRMS (ESI) for $C_{30}H_{38}NO_6$ $[M+H]^+$ calculated: 508.2654; found: 508.2701; Anal. Calcd for $C_{30}H_{37}NO_6$ (506.62): C, 70.98; H, 7.35; N, 2.76. Found: C, 69.34; H, 2.55; N, 7.24.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-1,2-bis(benzyloxy)-3-[(benzyloxy)methyl]-7-methoxyhexahydro-1H-pyrrolizin-6-yl methanesulfonate (**97**) To a stirred solution of **96** (196 mg, 0.39 mmol) in dry DCM (29 mL), TEA (163 µL, 1.17 mmol) and MsCl (67 µL, 0.86 mmol) were added at rt under argon atmosphere. The solution was stirred at rt for 1.5 h. A TLC control (DCM/MeOH 9:1) showed the disappearance of the starting material (R_f =

0.55) and the appearance of a new product (R_f = 0.78), then H_2O (7.0 mL) was added and the mixture was extracted with DCM (3 x 20 mL) and the combined organic layers were dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 1:1) afforded pure **97** (R_f=0.43, 189 mg, 0.33 mmol, 85 % yield) a colourless oil. $[\alpha]_D^{20}$ = + 16.2 (c 0.37, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.37-7.24 (m, 15 H, Ar), 5.05 (dt, *J* = 5.2, 3.3 Hz, 1 H, H-6), 4.65-4.52 (m, 6 H, Bn), 4.07 (t, *J* = 5.3 Hz, 1 H, H-1), 3.98 (dd, *J* = 6.8, 5.3 Hz, 1 H, H-2), 3.94 (t, *J* = 3.8 Hz, 1 H, H-7), 3.55 (dd, *J* = 9.5, 4.6 Hz, 1 H, Ha-8), 3.46 (dd, *J* = 9.5, 6.4 Hz, 1 H, Hb-8), 3.44-3.39 (m, 2H, Ha-5, H-7a), 3.41 (s, 3 H, Me), 3.30 (dd, *J* = 13.0, 3.4 Hz, 1 H, Hb-5), 3.23 (ddd, *J* = 11.3, 6.5, 4.7 Hz, 1H, H-3), 2.93 (s, 3 H, Ms). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.3, 138.2, 137.9 (s, Ar), 128.6-127.7 (d, 12 C, Ar), 88.7 (d, C-7), 86.2 (d, C-1), 85.4 (d, C-2), 84.9 (d, C-6), 73.5, 72.6 (t, Bn), 72.4 (d, C-7a), 72.3 (t, C-8), 72.0 (t, Bn), 68.1 (d, C-3), 58.2 (q, Me), 57.0 (t, C-5), 38.7 (q, Ms). IR (KBr): 2930, 2904, 2862, 1359, 1341, 1174, 1092, 950, 885, 734, 696 cm^{-1}. HRMS (ESI) for $C_{31}H_{38}NO_7S$ $[M+H]^+$ calculated: 568.2324; found: 568.2369. Anal. Calcd for $C_{31}H_{37}NO_7S$ (567.69): C, 65.59; H, 6.57; N, 2.47. Found: C, 65.62; H, 6.68; N, 2.56.

(1*R*,2*R*,3*R*,7*R*,7a*R*)-2-(benzyloxy)-3-[(benzyloxy)methyl]hezahydro-1H-pyrrolizine-1,7-diol (**98**). To a stirred solution of **97** (92 mg, 0.16 mmol) in dry toluene (5 mL), DIBAL-H 1.2 M in toluene (1.33 mmol, 1.60 mL) was added at 0°C under argon atmosphere. The solution was stirred at reflux for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.25) and the appearance of a new product (R_f = 0.01), then a saturated aqueous solution of Na_2SO_4 (0.7 mL) was added and the mixture was filtered over Celite® and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (dichloromethane/methanol 15:1) afforded pure **98** (R_f=0.24, 33 mg, 0.07 mmol, 44 % yield) as a colourless oil. $[\alpha]_D^{20}$ = − 10.0 (c 0.19, $CHCl_3$). ^{1}H-NMR

(500 MHz, $CDCl_3$) δ 7.35-7.25 (m, 10 H, Ar), 4.72-4.57 (m, 4 H, Bn), 4.06 (dd, *J* = 7.0, 6.0 Hz, 1 H, H-2), 3.80 (t, *J* = 6.0 Hz, 1 H, H-1), 3.72 (dt, *J* = 5.5, 3.7 Hz, 1 H, H-7), 3.58-3.52 (m, 2 H, Ha,b-8), 3.38 (dd, *J* = 6.0, 3.4 Hz, 1 H, H-7a), 3.33 (s, 3 H, H-Me), 3.10-3.05 (m, 1H, Ha-5), 2.80 (dt, *J* = 7.3, 3.6 Hz, 1 H, H-3), 2.76-2.71 (m, 1H, Hb-5), 1.98-1.92 (m, 1 H, Ha-6), 1.84-1.79 (m, 1 H, Hb-6). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.2, 138.1 (s, Ar), 128.5-127.8 (d, 10 C, Ar), 86.5 (d, C-1), 85.9 (d, C-7), 84.1 (d, C-2), 73.0 (t, Bn), 72.9 (d, C-7a), 72.0 (t, Bn), 69.4 (d, C-3), 60.6 (t, C-8), 57.1 (q, C-Me), 51.7 (t, C-5), 30.1 (t, C-6). HRMS (ESI) for $C_{23}H_{30}NO_4$ $[M+H]^+$ calculated: 384.2169; found: 384.2173.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-1,2-bis(benzyloxy)-3-[(benzyloxy)methyl]-7-methoxyhexahydro-1H-pyrrolizin-6-ol (**99**). To a stirred solution of **97** (149 mg, 0.26 mmol) in dry THF (5 mL), $LiAlH_4$ 1M in THF (1.04 mmol, 1.04 mL) was added at 0°C under argon atmosphere. The solution was stirred at reflux for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.45) and the appearance of a new product (R_f = 0.06), then a saturated aqueous solution of Na_2SO_4 (0.4 mL) was added and the mixture was filtered over Celite® and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 1:3) afforded pure **99** (R_f=0.17, 102 mg, 0.22 mmol, 85 % yield) as colourless oil. $[\alpha]_D^{20}$ = + 4.5 (c 0.91, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 15 H, Ar), 4.62-4.49 (m, 6 H, Bn), 4.19 (dt, *J* = 4.7, 3.5 Hz, 1 H, H-6), 4.15 (t, *J* = 4.3 Hz, 1H, H-1), 4.06 (t, *J* = 4.3 Hz, 1 H, H-2), 3.64 (t, *J* = 3.4 Hz, 1 H, H-7), 3.52-3.47 (m, 2 H, Ha-8, OH), 3.43 (t, *J* = 4.2 Hz, 1 H, H-7a), 3.38 (s, 3H, Me), 3.40-3.34 (m, 3 H, H-3, Ha-5, Hb-8), 2.98 (dd, *J* = 12.0, 3.7 Hz, 1 H, Hb-5). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.5, 138.1, 137.7 (s, Ar), 128.5-127.6 (d, 12 C, Ar), 91.1 (d, C-7), 86.9 (d, C-1), 85.8 (d, C-2), 77.2 (d, C-6), 73.5 (d, C-7a), 73.3 (t, C-8), 72.4, 72.1, 71.9 (t, Bn), 70.4 (d, C-3), 61.1 (t, C-5), 57.7 (q, Me). IR (KBr): 3407, 3029, 2895, 2863, 1452, 1098, 1067, 733, 695 cm^{-1}. HRMS (ESI) for

$C_{30}H_{36}NO_5$ $[M+H]^+$ calculated: 490.2549; found: 490.2564. Anal. Calcd for $C_{30}H_{35}NO_5$ (489.60): C, 73.59; H, 7.21; N, 2.86. Found: C, 73.59; H, 7.24; N, 2.88.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*S*)-3-(hydroxymethyl)-7-methoxyhexahydro-1H-pyrrolizine-1,2,6-triol (7-methoxy-casuarine, **94**). To a stirred solution of **99** (182 mg, 0.38 mmol) in MeOH (15 mL), 4-5 drops of conc. HCl and 304 mg of Pd (10% on C) were added. The suspension was stirred under hydrogen atmosphere for 4 d, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute 7-methoxycasuarine (**94**). Evaporation of the solvent afforded 7-methoxycasuarine as a yellow solid (52 mg, 0.24 mmol, 62%), m.p. 146-148 °C. $[\alpha]_D^{20}$ = + 36.9 (*c* 0.18, H_2O). ^{1}H-NMR (400 MHz, D_2O, 25°C) δ 4.30 (q, *J* = 4.4 Hz, 1 H, H-6), 4.12 (t, *J* = 8.4 Hz, 1 H, H-1), 3.84 (t, *J* = 3.3, 1 H, H-7) 3.76 (t, *J* = 8.7 Hz, 1 H, H-2), 3.74 (dd, *J* = 11.7, 3.7 Hz, 1H, Ha-8), 3.58 (dd, *J* = 11.7, 6.7 Hz, 1H, Hb-8), 3.41 (s, 3 H, Me), 3.17 (dd, *J* = 12.1, 4.8 Hz, 1 H, Ha-5) 3.08 (dd, *J* = 8.4, 3.1 Hz, 1 H, H-7a), 3.03-2.99 (m, 2 H, H-3), 2.90 (dd, *J* = 12.4, 4.3 Hz, 1 H, Hb-5). ^{13}C-NMR (50 MHz, D_2O, 25°C) δ 89.0 (d, C-7), 78.2 (d, C-1), 77.2 (d, C-2), 76.1 (d, C-6), 70.8 (d, C-7a), 70.3 (d, C-3), 62.9 (t, C-8), 58.8 (t, C-5), 57.3 (q, Me). IR (KBr): 3392, 3292, 3226, 2892, 1447, 1436, 1395, 1336, 1332, 1323, 1133, 1106, 1095, 1030, 952 cm^{-1}. MS (70 eV): *m/z* 205 (M^+, 0.9), 188 ($M^+ - H_2O$, 0.2), 174 ($M^+ - CH_2OH$, 100), 128 (15), 102 (17), 70 (40). Anal. Calcd for $C_9H_{17}NO_5$ (219.24): C, 49.31; H, 7.82; N, 6.39. Found: C, 49.29; H, 7.85; N, 6.43.

4.5.5 Syntheses of casuarine and 7-*epi*australine

(4a*S*,5*R*,6*R*,7*R*)-4,5,6-tris(benzyloxy)-7-[(benzyloxy)methyl]-4a,5,6,7-tetrahydro-2H-pyrrolo[1,2-b][1,2]oxazine (**86**). To a stirred solution of benzyloxyallene (572 mg, 3.91 mmol) in dry THF (15 mL), a solution of BuLi 2.5

M in THF (1.44 mL, 3.61 mmol) was added at -40°C under argon atmosphere. The mixture was stirring at -40°C for 10 minutes under argon atmosphere, then a solution of nitrone **1** (860 mg, 2.10 mmol) in dry THF (15 mL) were added at -78°C. The mixture was stirring at -78°C under argon atmosphere for 2 hours. A TLC control (DCM/AcOEt 8:1) showed the disappearance of the starting material (R_f = 0.11) and the appearance of a new product (R_f = 0.82) then H_2O (30 mL) was added dropwise then the mixture was warm up to rt. The mixture was extracted with AcOEt (3 x 20 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 4:1) afforded pure **86** (R_f=0.26, 892 mg, 1.58 mmol, 75 % yield) as a yellow oil. $[\alpha]_D^{20}$ = – 20.7 (c 1.4, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.46-7.25 (m, 20 H, Ar), 4.93-4.87 (m, 3 H, H-3, Bn), 4.74-4.53 (m, 6 H, Bn), 4.51-4.49 (m, 2 H, H-5, Ha-2), 4.42 (dd, *J* = 14.7, 3.3 Hz, 1 H, Hb-2), 4.23 (t, *J* = 4.2 Hz, 1 H, H-6), 3.97 (d, *J* = 6.3 Hz, 1 H, H-4a), 3.78-3.74 (m, 2 H, H-7, Ha-8), 3.66 (dd, *J* = 11.3, 7.7 Hz, 1 H, Hb-8). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 151.2 (s, C-4), 138.4-136.9 (s, 4 C, Ar), 128.7-127.7 (d, 16 C, Ar), 93.0 (d, C-3), 87.9 (d, C-5), 86.3 (d, C-6), 73.6, 72.4, 72.1 (t, Bn), 69.6 (t, C-8), 69.4 (t, Bn), 68.3 (d, C-7), 66.3 (d, C-4a), 63.6 (t, C-2). IR (KBr): 3029, 2897, 1673, 1496, 1453, 1361, 1349, 1216, 1199, 1074, 732, 694 cm^{-1}. HRMS (ESI) for $C_{36}H_{38}NO_5$ $[M+H]^+$ calculated: 564.2705; found: 564.2742. Anal. Calcd for $C_{36}H_{37}NO_5$ (563.68): C, 76.71; H, 6.62; N, 2.48. Found: C, 76.76; H, 6.58; N, 2.50.

(3*S*,4*S*,4a*S*,5*R*,6*R*,7*R*)-4,5,6-tris(benzyloxy)-7-[(benzyloxy)methyl]-hexahydro-2H-pyrrolo[1,2-b][1,2]oxazin-3-ol (**101**). To a stirred solution of **86** (410 mg, 0.73 mmol) in dry THF (15 mL), a solution of BH_3·THF 1M in THF (2.92 mmol, 2.92 mL) was added at -30°C under argon atmosphere. The solution was stirred at -30°C for 5 minutes and then at rt for 2 h. A TLC control (Hex/AcOEt 2:1) showed the disappearance of the starting material (R_f = 0.35) and the appearance of a new product (R_f = 0.63), then a solution of NaOH 2N (4.38 mL)

and H_2O_2 (1.46 mL) were added dropwise at -10°C and the mixture was stirring overnight. Then a saturated aqueous solution of $Na_2S_2O_3$ (5 mL) was added dropwise and the mixture was stirring for few minutes. The mixture was extracted with Et_2O (3 x 20 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (hexane/ethyl acetate 2:1) afforded pure **101** (R_f=0.16, 345 mg, 0.59 mmol, 81 % yield) as a colourless oil. $[\alpha]_D^{20} = -25.5$ (c 0.41, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.38-7.21 (m, 20 H, Ar), 4.70-4.44 (m, 8 H, Bn), 4.31-4.25 (m, 1 H, H-5), 4.06 (t, *J* = 2.8 Hz, 1 H, H-6), 4.03 (d, *J* = 3.3 Hz, 1 H, Ha-2), 3.77-3.69 (m, 4H, Hb-2, H-3, H-7, Ha-8), 3.59 (t, *J* = 6.1 Hz, 1 H, H-4), 3.54 (dd, *J* = 8.9, 7.4 Hz, 1 H, Hb-8), 3.35 (t, *J* = 6.5 Hz, 1 H, H-4a), 2.39 (bs, 1 H, OH). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 138.3, 138.2, 138.0, 137.9 (s, Ar), 128.6-127.8 (d, 16 C, Ar), 85.4 (d, C-6), 84.7 (d, C-5), 76.9 (d, C-4), 73.6, 73.1, 71.9, 71.7 (t, Bn), 69.0 (d, C-3), 68.9, 68.8 (t, C-2, C-8), 67.9 (d, 2 C, C-7, C-4a). IR (KBr): 3432, 3029, 2910, 2864, 1495, 1453, 1362, 1206, 1093, 750, 734 cm^{-1}. HRMS (ESI) for $C_{36}H_{40}NO_6$ $[M+H]^+$ calculated: 582.2811; found: 582.2861. Anal. Calcd for $C_{36}H_{39}NO_6$ (581.69): C, 74.33; H, 6.76; N, 2.41. Found: C, 74.17; H, 6.98; N, 2.42.

(2'*S*,3'*S*)-3'-(benzyloxy)-3'-{(2*R*,3*R*,4*R*,5*R*)-3,4-bis(benzyloxy)-5-[(benzyloxy)methyl]pyrrolidin-2-yl}-1',2'-diol (**103**). To a stirred solution of 1,2-diiodoethane (376 mg, 1.33 mmol) in dry THF (9 mL), Sm (232 mg, 1.54 mmol) was added at rt under argon atmosphere. The solution was stirred at rt for 2 h, then a solution of **101** (216 mg, 0.37 mmol) in dry THF (9 mL) was added and the mixture was stirring at rt for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.55) and the appearance of a new product (R_f = 0.02), then a saturated aqueous solution $NaHCO_3$ (6.5 mL) was added and the mixture was extracted with Et_2O (3 x 20 mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Purification of the residue by flash column

chromatography on silica gel (dichloromethane/methanol 20:1) afforded pure **103** (R_f=0.38, 173 mg, 0.30 mmol, 81 % yield) as an orange oil. $[\alpha]_D^{20} = -7.14$ (c 0.49, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.37-7.22 (m, 20 H, Ar), 4.66 (d, *J* = 11.4 Hz, 1H, Bn), 4.54-4.42 (m, 7 H, Bn), 4.05-4.01 (m, 2 H, H-3, H-2'), 3.98 (dd, *J* = 5.7, 2.7 Hz, 1 H, H-4), 3.78 (d, *J* = 5.4 Hz, 2 H, Ha,b-1'), 3.68 (dd, *J* = 9.5, 3.8 Hz, 1 H, H-3'), 3.57-3.56 (m, 1 H, H-2), 3.56 (d, *J* = 4.5 Hz, 2 H, Ha,b-6), 3.36 (dd, *J* = 10.0, 4.6 Hz, 1H, H-5). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.2, 138.1, 137.9, 137.8 (s, Ar), 128.6-127.8 (d, 16 C, Ar), 86.9 (d, C-3), 88.8 (d, C-4), 76.4 (d, C-3'), 73.3, 72.9 (t, Bn), 72.2 (d, C-2'), 72.1, 71.5 (t, Bn), 68.7 (t, C-6), 64.4 (d, C-2), 63.4 (t, C-1'), 62.4 (d, C-5). IR (KBr): 3312, 3029, 2862, 1495, 1452, 1070, 695 cm^{-1}. HRMS (ESI) for $C_{36}H_{42}NO_6$ $[M+H]^+$ calculated: 584.2967; found: 584.3011. Anal. Calcd for $C_{36}H_{41}NO_6$ (583.71): C, 74.04; H, 7.08; N, 2.40. Found: C, 73.61; H, 7.01; N, 2.33.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*S*)-1,2,7-tris(benzyloxy)-3-[(benzyloxy)methyl] hexahydro-1H-pyrrolizin-6-yl methanesulfonate (**104**). To a stirred solution of **103** (151 mg, 0.26 mmol) in dry DCM (20 mL), TEA (108 µL, 0.78 mmol) and MsCl (44 µL, 0.57 mmol) were added at rt under argon atmosphere. The solution was stirred at rt for 1.5 h. A TLC control (DCM/MeOH 9:1) showed the disappearance of the starting material (R_f = 0.54) and the appearance of a new product (R_f = 0.88), then H_2O (5.0 mL) was added and the mixture was extracted with DCM (3 x 20 mL) and the combined organic layers were dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 1:1) afforded pure **104** (R_f=0.54, 158 mg, 0.25 mmol, 94 % yield) as a colourless oil. $[\alpha]_D^{20} = -7.08$ (c 1.3, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.38-7.25 (m, 20 H, Ar), 5.17 (dt, *J* = 5.3, 3.1 Hz, 1 H, H-6), 4.69-4.46 (m, 8 H, Bn), 4.22 (dd, *J* = 3.8, 3.5 Hz, 1 H, H-7), 4.04 (t, *J* = 5.2 Hz, 1 H, H-1), 4.01 (dd, *J* = 12.2, 5.8 Hz, 1 H, H-2), 3.59 (dd, *J* = 9.5, 4.6 Hz, 1 H, Ha-8), 3.54-3.49 (m, 3 H, Hb-8, H-7a, Ha-5), 3.37 (dd, *J* =13.2, 3.1 Hz, 1 H, Hb-5), 3.28 (td, *J*

= 6.4, 4.8 Hz, 1 H, H-3), 2.89 (s, 3 H, Ms). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.4, 138.2, 138.0, 137.5 (s, Ar), 128.6-127.7 (d, 16 C, Ar), 85.9 (d, C-7), 85.8 (d, C-1), 85.3 (d, C-2), 85.3 (d, C-6), 73.5, 72.8 (t, Bn), 72.5 (t, C-8), 72.4, 72.1 (t, Bn), 68.2 (d, C-3), 57.2 (t, C-5), 38.6 (q, Ms). IR ($CDCl_3$): 3029, 2893, 2863, 1342, 1175, 1090, 1071, 750, 696 cm^{-1}. HRMS (ESI) for $C_{37}H_{42}NO_7S$ $[M+H]^+$ calculated: 644.2637; found: 644. 2678. Anal. Calcd for $C_{37}H_{41}NO_7S$ (643.79): C, 69.03; H, 6.42; N, 2.18. Found: C, 69.50; H, 6.03; N, 2.60.

(1*R*,2*R*,3*R*,7*R*,7a*R*)-1,2-bis(benzyloxy)-3-[(benzyloxy)methyl]hexahydro-1H-pyrrolizin-7-ol (**105**). To a stirred solution of **104** (67 mg, 0.10 mmol) in dry toluene (4 mL), DIBAL-H 1.2 M in toluene (0.83 mmol, 1.00 mL) was added at 0°C under argon atmosphere. The solution was stirred at reflux for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.64) and the appearance of a new product (R_f = 0.01), then a saturated aqueous solution of Na_2SO_4 (0.5 mL) was added and the mixture was filtered over Celite® and evaporated under reduced pressure. Purification of the residue by preparative TLC on silica gel (ethyl acetate/methanol 30:1) afforded pure **105** (R_f=0.50, 24 mg, 0.05 mmol, 50 % yield) as a colourless oil. $[\alpha]_D^{20}$ = – 27.0 (c 0.33, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 15 H, Ar), 4.70-4.48 (m, 6 H, Bn), 4.07 (dd, *J* = 6.9, 6.2 Hz, 1 H, H-2), 3.94 (dt, *J* = 5.2, 4.0 Hz, 1 H, H-7), 3.78 (t, *J* = 5.9 Hz, 1 H, H-1), 3.60-3.56 (m, 2 H, Ha,b-8), 3.46 (dd, *J* = 5.9, 3.5 Hz, 1 H, H-7a), 3.15-3.10 (m, 1 H, Ha-5), 2.79 (dt, *J* = 7.1, 3.6 Hz, 1 H, H-3), 2.77-2.72 (m, 1H, Hb-5), 2.00-1.93 (m, 1 H, Ha-6), 1.91-1.86 (m, 1 H, Hb-6). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 138.3, 138.2, 138.1 (s, Ar), 128.5-127.8 (d, 12 C, Ar), 86.4 (d, C-1), 84.1 (d, C-2), 83.4 (d, C-7), 73.3 (d, C-7a), 73.0, 71.9, 71.3 (t, Bn), 69.3 (d, C-3), 60.6 (t, C-8), 51.7 (t, C-5), 30.5 (t, C-6). IR (KBr): 3029, 2922, 2867, 1453, 1361, 1108, 734, 696 cm^{-1}. HRMS (ESI) for $C_{29}H_{34}NO_4$ $[M+H]^+$ calculated: 460.2443; found: 460.2495.

(1*R*,2*R*,3*R*,7*R*,7a*R*)-3-(hydroxymethyl)hexahydro-1H-pyrrolizine-1,2,7-triol (7-*epi*australine, **17**). To a stirred solution of **105** (58 mg, 0.13 mmol) in

MeOH (6 mL), 4-5 drops of concentrated HCl and 104 mg of Pd (10% on C) were added. The suspension was stirred under hydrogen atmosphere overnight, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a viscous oil that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (20 mL), H_2O (20 mL) to remove non amine containing products and then with 7% NH_4OH (30 mL) to elute 7-*epi*australine (**17**). Evaporation of the solvent afforded 7-*epi*australine as a yellow solid (24 mg, 0.13 mmol, 100% yield). $[\alpha]_D^{20} = -6.3$ (c 0.49, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 4.25 (dt, *J* = 4.6, 2.6 Hz, 1 H, H-7), 3.69-3.59 (m, 3 H, H-1, H-2, Ha-8), 3.53 (dd, *J* = 11.7, 6.3 Hz, 1 H, Hb-8) 3.00 (dddd, *J* = 16.5, 11.4, 7.7 Hz, 1 H, Ha-5), 2.95 (dd, *J* = 7.7, 1.7 Hz, 1H, H-7a), 2.80 (dddd, *J* = 11.1, 7.1, 3.4 Hz, 1H, Hb-5), 2.60 (dddd, *J* = 9.2, 6.2, 3.7 Hz, 1 H, H-3), 2.02-1.93 (m, 1 H, Ha-6), 1.71-1.65 (m, 1 H, Hb-6). ^{13}C-NMR (50 MHz, D_2O): δ 77.1 (d, C-1), 75.5 (d, C-2), 74.4 (d, C-7), 73.9 (d, C-7a), 68.2 (d, C-3), 61.5 (t, C-8), 51.8 (t, C-5), 31.3 (t, C-6). IR (KBr): 3357, 3307, 3243, 2912, 1443, 1340, 1316, 1261, 1035, 978, 530 cm^{-1}. HRMS (ESI) for $C_8H_{16}NO_4$ $[M+H]^+$ calculated: 190.1074; found: 190.1087.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-1,2,7-tris(benzyloxy)-3-[(benzyloxy)methyl] hexahydro-1H-pyrrolizin-6-ol (**106**). To a stirred solution of **104** (140 mg, 0.22 mmol) in dry THF (4 mL), $LiAlH_4$ 1M in THF (0.88 mmol, 0.88 mL) was added at 0°C under argon atmosphere. The solution was stirred at reflux for 2 h. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.44) and the appearance of a new product (R_f = 0.16), then a saturated aqueous solution of Na_2SO_4 (0.7 mL) was added and the mixture was filtered over Celite® and evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (Hexane/ethyl acetate 1:3) afforded pure **106** (R_f=0.43, 105 mg, 0.19 mmol, 86 % yield) as a colourless oil. $[\alpha]_D^{20} = -7.14$ (c 0.14, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.36-7.25 (m, 20 H, Ar), 4.60-4.48 (m, 8 H, Bn), 4.26 (q, *J* = 4.0 Hz, 1 H, H-6), 4.12 (t, *J* = 4.4 Hz, 1

H, H-1), 4.08 (t, *J* = 4.6 Hz, 1 H, H-2), 3.87 (t, *J* = 3.7 Hz, 1 H, H-7), 3.56-3.50 (m, 3 H, H-7a Ha,b-8), 3.43-3.38 (m, 2 H, H-3, Ha-5), 2.98 (dd, *J* = 12.0, 3.9 Hz, 1 H, Hb-5). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 138.5, 138.3, 138.2, 137.8 (s, Ar), 128.6-127.6 (d, 16 C, Ar), 88.6 (d, C-7), 86.5 (d, C-1), 85.7 (d, C-2), 77.6 (d, C-6), 73.6 (d, C-7a), 73.4, 72.4 (t, Bn), 72.2 (t, C-8), 71.8, 71.1 (t, Bn), 70.3 (d, C-3), 61.1 (t, C-5). IR (KBr): 3029, 2862, 1452, 1363, 1205, 1067, 732, 694 cm^{-1}. HRMS (ESI) for $C_{36}H_{40}NO_5$ $[M+H]^+$ calculated: 566.2901; found: 566.2904. Anal. Calcd for $C_{36}H_{39}NO_5$ (565.69): C, 76.43; H, 6.95, N, 2.48. Found: C, 75.79; H, 7.00; N, 2.43.

(1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-3-(hydroxymethyl)hexahydro-1H-pyrrolizine-1,2,6,7-tetrol (casuarine, **11**). To a stirred solution of **106** (160 mg, 0.10 mmol) in MeOH (10 mL), 4-5 drops of concentrated HCl and 84 mg of Pd (10% on C) were added. The suspension was stirred under hydrogen atmosphere overnight, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a viscous oil that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (20 mL), H_2O (20 mL) to remove non amine containing products and then with 7% NH_4OH (30 mL) to elute casuarine (**11**). Evaporation of the solvent afforded casuarine (**11**) as a white solid (21 mg, 0.10 mmol, 100% yield). $[\alpha]_D^{20}$ = + 13.6 (c 0.66, MeOH). ^{1}H-NMR (500 MHz, D_2O) δ 4.20-4.17 (m, 2 H, H-6, H-7), 4.14 (t, *J* = 8.2 Hz, 1 H, H-1), 3.79-3-74 (m, 2 H, H-2, Ha-8) 3.60 (dd, *J* = 11.8, 6.6 Hz, 1 H, Hb-8), 3.25 (dd, *J* = 12.3, 4.4 Hz, 1H, Ha-5), 3.06 (dd, *J* = 8.2, 3.3 Hz, 1H, H-7a), 3.04-3.01 (m, 1 H, H-3), 2.91 (dd, *J* = 12.3, 3.8 Hz, 1 H, Hb-5). ^{13}C-NMR (50 MHz, D_2O) δ 79.3 (d, C-7), 78.2 (d, C-1), 77.9 (d, C-6), 77.1 (d, C-2), 72.7 (d, C-7a), 70.5 (d, C-3), 62.7 (t, C-8), 58.6 (t, C-5).

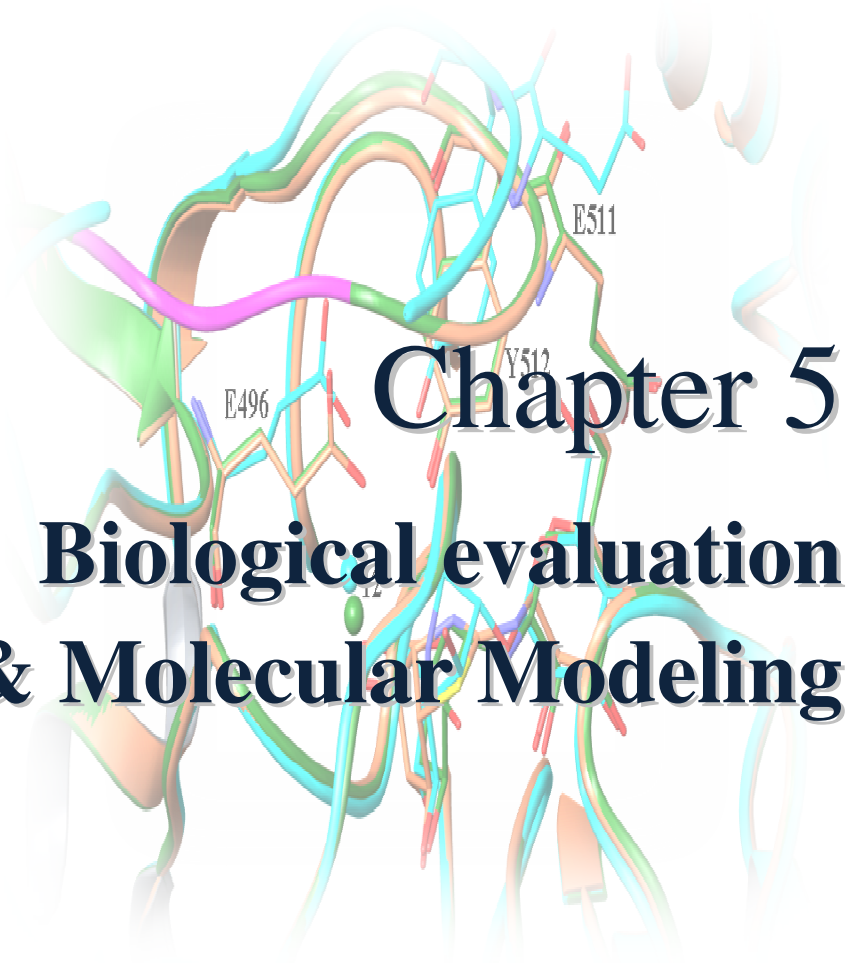
(1R,2R,3R,6R,7S,7aR)-6-azido-1,2,7-tris(benzyloxy)-3-[(benzyloxy) methyl]hexahydro-1H-pyrrolizine (**110**). To a stirred solution of **104** (81 mg, 0.13 mmol) in dry DMF (4 mL), NaN_3 (85 mg, 1.30 mmol) were added under argon atmosphere. The solution was stirred at 100°C for 2 days. A TLC control (Hex/AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.53)

and the appearance of a new product (R_f = 0.78), then the suspension was cooled at rt, extract with AcOEt and the organic layers were washed with water and brine, dried on Na_2SO_4, filtered and evaporated. Purification of the residue by preparative TLC on silica gel (hexane/ethyl acetate 3:1) afforded pure **110** (R_f=0.38, 46 mg, 0.08 mmol, 62 % yield) as a colourless oil. $[\alpha]_D^{20}$ = – 39.8 (c 0.43, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.38-7.21 (m, 20 H, Ar), 4.73 (d, *J* = 11.9 Hz, 1 H, Bn) 4.63-4.44 (m, 7 H, Bn), 4.05-4.02 (m, 2 H, H-6, H-2), 3.94 (dd, *J* = 7.4, 4.7 Hz, 1 H, H-7), 3.88 (t, *J* = 3.9 Hz, 1 H, H-1), 3.64 (dd, *J* = 7.4, 3.7 Hz, 1 H, H-7a), 3.58 (dd, *J* = 9.4, 6.2 Hz, 1 H, Ha-8), 3.51 (dd, *J* = 9.4, 6.5 Hz, 1 H, Hb-8), 3.36 (dd, *J* = 11.7, 3.0 Hz, 1 H, Ha-5), 3.09-3.02 (m, 2H, H-3, Hb-5). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 138.5, 138.2, 138.1, 137.5 (s, Ar), 128.7-127.6 (d, 16 C, Ar), 85.9 (d, C-2), 85.7 (d, C-1), 82.9 (d, C-7), 73.4, 72.5 (t, Bn), 72.4 (t, C-8), 72.2, 71.6 (t, Bn), 71.1 (d, C-7a), 70.1 (d, C-3), 61.6 (d, C-6), 57.8 (t, C-5). IR (KBr): 3031, 2864, 2129, 2102, 2077, 1496, 1452, 1311, 1255, 1116, 733, 692 cm^{-1}.HRMS (ESI) for $C_{36}H_{39}N_4O_4$ $[M+H]^+$ calculated: 591.2927; found: 591.2968.

(1*R*,2*R*,3*R*,6*R*,7*R*,7a*R*)-6-amino-3-(hydroxymethyl)hexahydro-1H-pyrrolizine-1,2,7-triol (7-*epi*aminocasuarine, **109**). To a stirred solution of **110** (86 mg, 0.15 mmol) in MeOH (6 mL), 4-5 drops of concentrated HCl and 80 mg of Pd (10% on C) were added. The suspension was stirred under hydrogen atmosphere for 2 days, then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a viscous oil that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (20 mL), H_2O (20 mL) to remove non amine containing products and then with 7% NH_4OH (30 mL) to elute 7-*epi*aminocasuarine (**109**). Evaporation of the solvent afforded 7-*epi*aminocasuarine as a transparent oil (20 mg, 0.10 mmol, 67% yield). ^{1}H-NMR (400 MHz, D_2O) δ 3.94 (bd, *J* = 3.4 Hz, 1 H, H-7), 3.67-3.56 (m, 3 H, H-1, H-2, Ha-8), 3.45 (dd, *J* = 11.6, 6.2 Hz, 1 H, Hb-8) 3.35-3.30 (m, 1 H, H-6), 2.97 (d, *J* = 7.7 1H, H-7a), 2.80 (dd, *J* = 11.6, 6.8, 3.4 Hz, 1H, Ha-5), 2.61-2.53

(m, 2 H, Hb-5. H-3). ^{13}C-NMR (50 MHz, D_2O): δ 76.9, 75.2 (d, C-1, C-2), 74.1 (d, C-7), 72.3 (d, C-7a), 68.0 (d, C-3), 61.9 (t, C-8), 55.8 (t, C-5), 50.7.

4-(benzyloxy)-4-{(4a*S*,5*R*,6*R*,7*R*)-5,6-bis(benzyloxy)-7-[(benzyloxy) methyl]pyrrolidin-4a-yl}prop-3-en-2-ol (**111**). To a solution of **86** (153 mg, 0.27 mmol) in CH_3CN/H_2O 7/1 (4 mL), $Mo(CO)_6$ (112 mg, 0.42 mmol) and $NaBH_4$ (5 mg, 0.14 mmol) were added. The reaction mixture was stirred at reflux for 3 h. The mixture was then filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (EtOAc/hexane 2:3) to afford pure **111** (R_f=0.32) as a yellow oil (68 mg, 45%). $[\alpha]_D^{23}$ = + 14.6 (c 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.34-7.22 (m, 20 H, Ar), 5.13 (t, *J* = 8.0 Hz, 1 H, H-3), 4.75-4.69 (AB system, *J* = 11.5 Hz, 2 H, Bn), 4.59-4.48 (m, 6 H, Bn), 4.18-3.99 (m, 4 H, Ha-2, Hb-2, H-4a, H-5, H-6), 3.56-3.49 (m, 2 H, Ha-8, Hb-8), 3.49-3.42 (m, 1 H, H-7). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 156.7 (s, C-4), 138.0, 137.4, 136.4 (s, Ar.), 128.4-127.5 (d, 20 C, Ar), 101.4 (d, C-3), 87.1 (d, C-5), 86.6 (d, C-6), 73.4, 72.6, 72.1 (t, Bn), 71.4 (t, C-8), 69.4 (t, Bn), 61.7 (d, C-7), 60.2 (d, C-4a), 57.2 (t, C-2). IR ($CDCl_3$): 3450, 3350, 3066, 3031, 2932, 2867, 2243, 1654, 1495, 1362, 1220, 1094, 1076 cm^{-1}. MS (ESI) for $C_{36}H_{39}NO_5Na$ $[M+Na]^+$ calculated: 588.70; found: 588.45. Anal. Calcd for $C_{36}H_{39}NO_5$ (565.7) C, 76.43; H, 6.95; N, 2.48; Found: C, 76.00 ; H, 7.47; N, 2.94.



Chapter 5

Biological evaluation & Molecular Modeling

5.1 Biological evaluation and modeling studies

As previously mentioned, all the synthesized molecules were tested, or are currently under biological evaluation, towards a wide range of glycosidases. For some enzymes, molecular modelling studies have also been performed.

5.1.1 Inhibition of glycosidases

This study has been done in collaboration with Prof. Pierre Vogel of the University of Lausanne. In particular, we thank Dr. Claudia Bello for inhibition studies and mass spectrometry.

Compounds **11**, **14**-**16**, **50**-**52**, **57**, **60** and **63** (Figure 5.1) were assayed toward a panel of fourteen commercially available glycosidases: α-L-fucosidase (EC 3.2.1.51) from bovine kidney; α-galactosidase (EC 3.2.1.22) from coffee bean; β-galactosidases (EC 3.2.1.23) from *Escherichia coli*, bovine liver and *Aspergillus oryzae*; α-glucosidases (EC 3.2.1.20) from yeast and rice; amylogucosidase (EC 3.2.1.3) from *Aspergillus niger*; β-glucosidase (EC 3.2.1.21) from almond; α-mannosidase (EC 3.2.1.24) from jack bean; β-mannosidase (EC 3.2.1.25) from snail; β-xylosidase (EC 3.2.1.37) from *Aspergillus niger*; β-N-acetylglucosaminidases (EC 3.2.1.30) from jack bean and bovine kidney with appropriate *p*-nitrophenyl glycoside substrates.[135]

The errors were estimated to be around 20% on the measurements (statistical study carried out with model compounds) and around 10-15% on the concentrations (errors on sample weight).

[135] Brandi, A.; Cicchi, S.; Cordero, F. M.; Frignoli, R.; Goti, A.; Picasso, M.; Vogel, P. *J. Org. Chem.* **1995**, *60*,6806 and references therein.

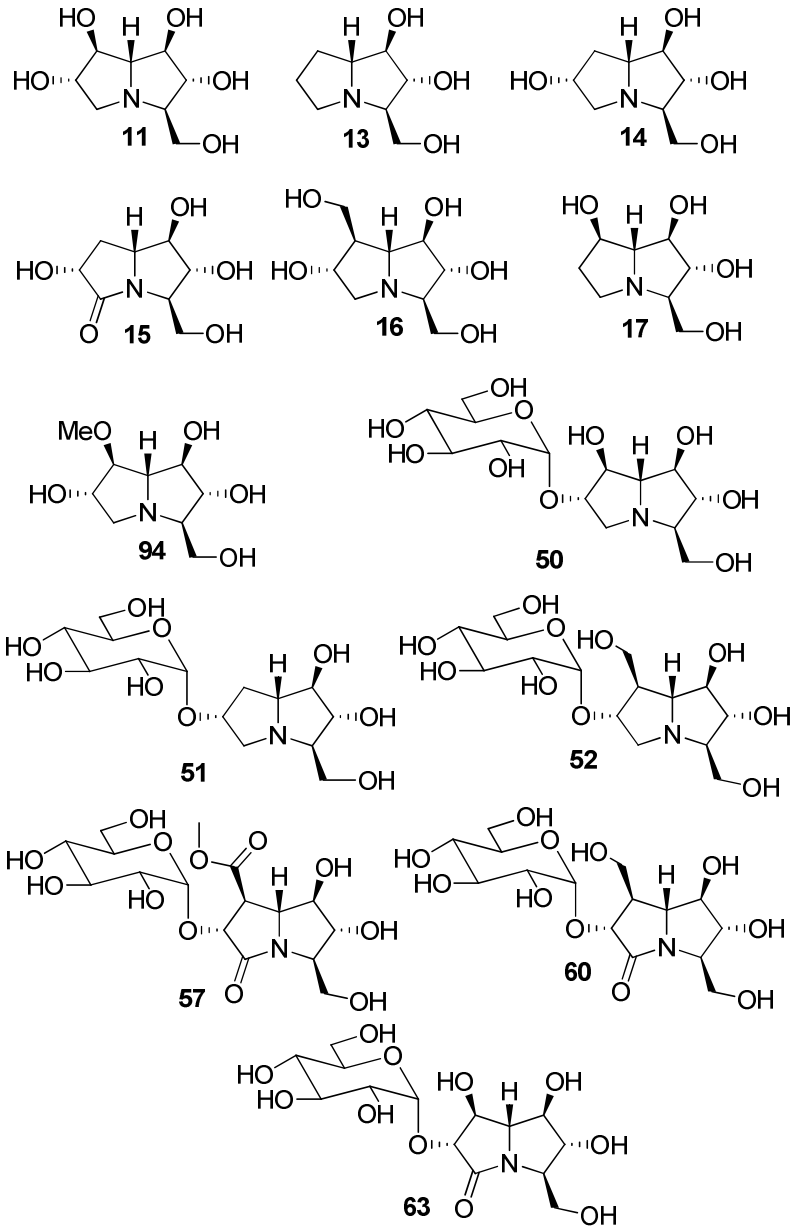


Figure 5.1. Molecules tested

Casuarine (**11**), Table 5.1, showed to be a potent and competitive inhibitor of amyloglucosidase from *Aspergillus niger* (IC_{50} = 1.9 ± 0.4 µM, K_i = 2.0 ± 0.4 µM). It also inhibits α-glucosidases from yeast and from rice (91% and

94% at 1mM, respectively) and, to a lesser extent, β-glucosidase from almonds, α-mannosidase from jack bean, β-xylosidase from *Aspergillus niger* and β-N-acetylglucosaminidase from jack bean (46%, 21%, 24% and 16% at 1mM, respectively).

Casuarine-6-*O*-α-glucoside (**50**, Table 5.1) was slightly less potent, with IC_{50} and K_i in the same order of magnitude (IC_{50} = 4.4 ± 0.9 μM, K_i = 3.9 ± 0.8 μM, mixed type inhibition), but more selective than casuarine: indeed at 1mM concentration it gave only 20% inhibition toward α-glucosidase from yeast and did not inhibit at all α-glucosidase from rice. Moreover it showed only 19% inhibition toward α-L-fucosidase from bovine kidney (1mM).

Glucoside **51** (Table 5.1), 7-*homo*casuarine **16** and glucoside **52** (Table 5.2) were good and selective competitive inhibitors of amyloglucosidase from *Aspergillus niger* (IC_{50} = 7.7 ± 1.5, 8.1± 1.6 and 24 ± 4.7 μM, respectively; K_i = 7.4 ± 1.2, 11± 2.1 and 23 ± 4.6 μM, respectively). They inhibited α-glucosidase from yeast weakly (29%, 45% and 55% at 1mM, respectively) and did not inhibit α-glucosidase from rice.

7-Deoxycasuarine (**14**), Table 5.1, was a very potent and selective inhibitor of amyloglucosidase from *Aspergillus niger* (IC_{50} = 4.5 ± 0.9 μM, K_i = 3.5 ± 0.7 μM). Among the other glycosidases assayed, only α-glucosidase from rice was also inhibited weakly (36% at 1 mM).

Lactam **15** (Table 5.2) exhibited weak (IC_{50} = 210 ± 43 μM) but very selective inhibitory activity toward amyloglucosidase from *Aspergillus niger*.

	Percentage of inhibition at 1mM				
Compound / Enzyme (pH)	**11**	**50**	**63**	**14**	**51**
α-L-fucosidase EC 3.2.1.51 1-bovine kydney (6)	n.i.	1.9	n.i.	n.i.	n.t.
α-galactosidase EC 3.2.1.22 2-coffe bean (6)	n.i.	n.i.	n.i.	n.i.	n.t.
β-galactosidase EC 3.2.1.23 5-*Escherichia coli* (7)	n.i.	n.i.	n.i.	n.i.	n.t.
8-*Aspergillus oryzae*(4)	n.i.	n.i.	n.i.	n.i.	n.i.
α-glucosidase EC 3.2.1.20 10-yeast (7)	91	20	n.i.	n.i.	29
11-rice (4)	94	n.i.	n.i.	36	n.i.
amyloglucosidase EC 3.2.1.3 13-*Aspergillus niger*(5)	**98** IC_{50}=1.9μM K_i=2.0μM	**97** IC_{50}=4.4μM K_i=3.9μM	n.i.	**100** IC_{50}=4.5μM K_i=3.5μM	**97** IC_{50}=7.7μM K_i=7.4μM
β-glucosidase EC 3.2.1.21 15-almond (5)	46	n.i.	n.i.	n.i.	n.t.
α-mannosidase EC 3.2.1.24 16-jack bean (5)	21	n.i.	n.t.	n.i.	n.t.
β-mannosidase EC 3.2.1.25 18-snail (4)	n.i.	n.t.	n.t.	n.i.	n.t.
β-xylosidase EC 3.2.1.37 19-*Aspergillus niger*(5)	24	n.t.	n.t.	n.i.	n.t.
β-N-acetylglucosaminidase EC 3.2.1.30 21-jack bean (5)	16	n.i.	n.i.	n.i.	n.i.
22-bovine kidney (4)	n.i.	n.t.	n.i.	n.t.	n.t.

Table 5.1. Inhibition for compounds 11, 50, 63, 14 and 51 toward commercially available glycosidases. n.i. = no inhibition, n.t. = test not performed.

Among the remaining compounds, **63** was not active toward all the glycosidases assayed, **60** and **57** showed weak and very selective inhibitory activity (25% and 76% inhibition at 1mM, respectively) toward amyloglucosidase from *Aspergillus niger*.

	Percentage of inhibition at 1mM				
Compound / Enzyme (pH)	**15**	**16**	**52**	**60**	**57**
α-L-fucosidase EC 3.2.1.51 1-bovine kydney (6)	n.i.	n.i.	n.t.	n.i.	n.i.
α-galactosidase EC 3.2.1.22 2-coffe bean (6)	n.i.	n.i.	n.t.	n.i.	n.i.
β-galactosidase EC 3.2.1.23 5-*Escherichia coli* (7)	n.i.	n.i.	n.t.	n.i.	n.i.
8-*Aspergillus oryzae*(4)	n.i.	n.i.	n.t.	n.i.	n.i.
α-glucosidase EC 3.2.1.20 10-yeast (7)	n.i.	45	55	n.i.	n.i.
11-rice (4)	n.i.	n.i.	n.i.	n.i.	n.i.
amyloglucosidase EC 3.2.1.3 13-*Aspergillus niger*(5)	**91** IC_{50}=210μM	**99** IC_{50}=8.1μM K_i=11 μM	**92** IC_{50}=24μM K_i=23 μM	25	76
β-glucosidase EC 3.2.1.21 15-almond (5)	42	n.t	n.t.	n.i.	n.i.
α-mannosidase EC 3.2.1.24 16-jack bean (5)	n.i.	n.t	n.t.	n.i.	n.i.
β-mannosidase EC 3.2.1.25 18-snail (4)	n.i.	n.t	n.t.	n.t.	n.i.
β-xylosidase EC 3.2.1.37 19-*Aspergillus niger*(5)	n.i.	n.t	n.t.	n.t.	n.i.
β-N-acetylglucosaminidase EC 3.2.1.30 21-jack bean (5)	44	n.t	n.t.	n.t.	n.i.
22-bovine kidney (4)	20	n.t	n.t.	n.t.	n.i.

Table 5.2. Inhibition of compounds 15, 16, 52, 60, 57 toward commercially available glycosidases. n.i. = no inhibition, n.t. = test not performed.

As **50** is a glucoside, we verified whether amyloglucosidase was able to hydrolyze its glycosidic bond under the test conditions. Indeed, we had to consider the possibility that the inhibitory activities observed for **50** were due to the casuarine liberated by the hydrolytic reaction catalyzed by amyloglucosidase.

Thus, a series of mass spectra analyses using HR-ESI-TOF-MS (high resolution ESI mass spectrometry, positive ionization mode) were performed: as shown in Figure 5.2 the peak assigned to protonation of the glucoside (A), MW = 367.35, was detected in the solution when the measurement was performed immediately after the addition of the enzyme (C) as well as after 20 minutes of incubation at room temperature (D) and after 20 minutes of incubation at 37 °C (E). We verified that the mixture of enzyme and buffer did not give similar signals (B).

The spectra of the different assays performed with the buffered solution (phosphate) of the inhibitor in the presence of the enzyme neither showed peaks corresponding to the aglycone (MW = 205.21), nor to glucose (180.16) alone (Figure 5.3). This demonstrates that glucoside **50** is not hydrolyzed by the enzyme (amyloglucosidase from *Aspergillus niger*) under the conditions of our test.

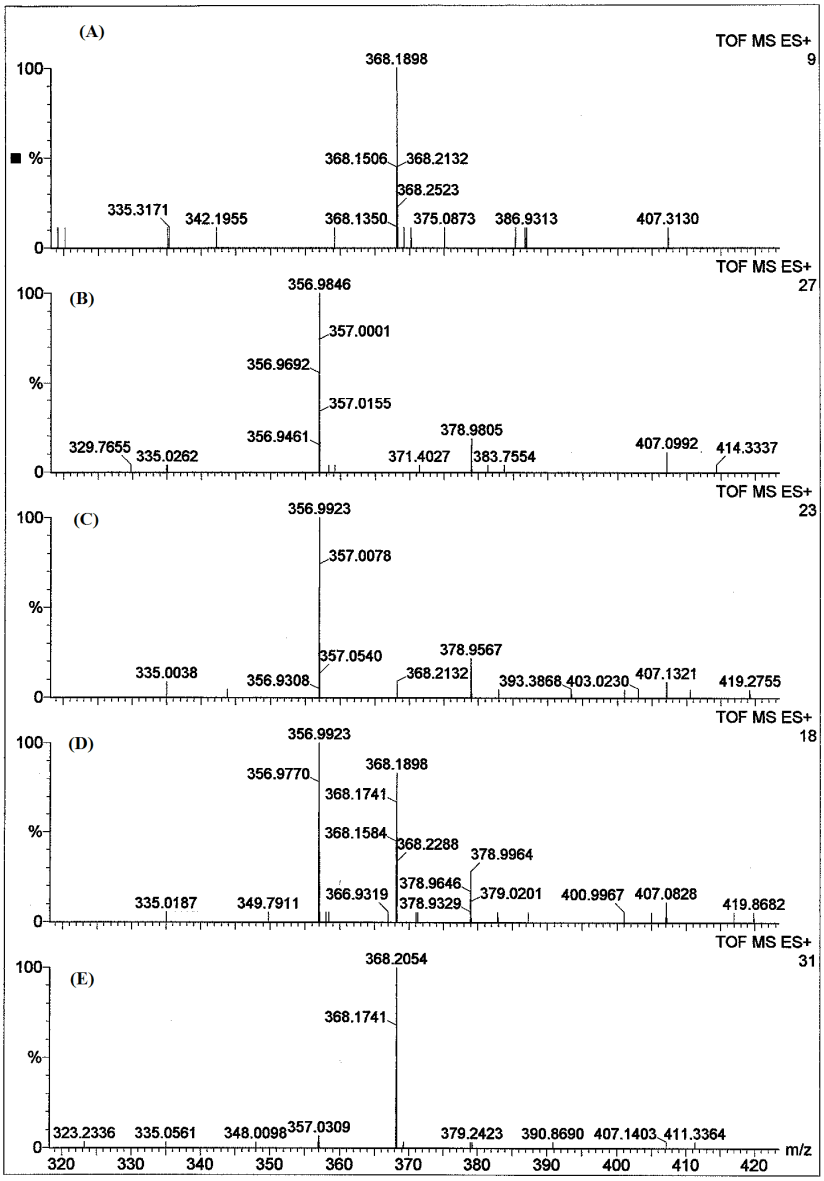


Figure 5.2. (A): HR-ESI-TOF-MS of an aqueous solution of 50; (B): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger*; (C): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed at t = 0 min and T = 25°C; (D): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed at t = 20 min and T = 25°C; (E): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed after 20 min incubation at 37°C.

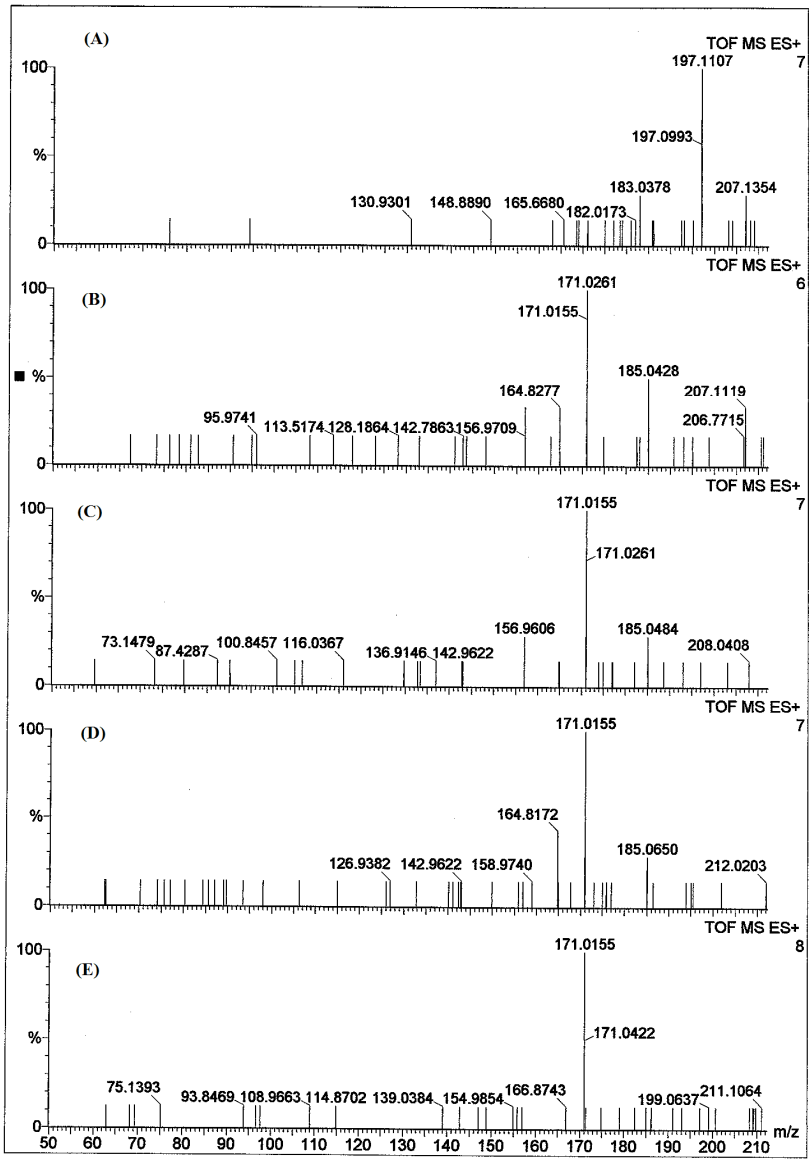


Figure 5.3. (A): HR-ESI-TOF-MS of an aqueous solution of 50; (B): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger*; (C): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed t = 0 and T = 25°C; (D): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed t = 20 min and T = 25°C; (E): HR-ESI-TOF-MS of a phosphate buffer solution (pH = 5) of amyloglucosidase from *Aspergillus niger* in the presence of 50, measurement performed after 20 min incubation at 37°C.

Compounds **13**, **17** and **94** were assayed toward a panel of fourteen commercially available glycosidases: α-L-fucosidase (EC 3.2.1.51) from bovine kidney; α-galactosidase (EC 3.2.1.22) from coffee bean; β-galactosidases (EC 3.2.1.23) from *Escherichia coli* and *Aspergillus oryzae*; α-glucosidases (EC 3.2.1.20) from yeast and rice; amylogucosidase (EC 3.2.1.3) from *Aspergillus niger*; β-glucosidase (EC 3.2.1.21) from almond; α-mannosidase (EC 3.2.1.24) from jack bean; β-mannosidase (EC 3.2.1.25) from snail; β-xylosidase (EC 3.2.1.37) from *Aspergillus niger*; β-N-acetylglucosaminidases (EC 3.2.1.30) from jack bean and bovine kidney with appropriate *p*-nitrophenyl glycoside substrates (Table 5.3).

The errors were estimated to be around 20% on the measurements (statistical study carried out with model compounds) and around 10-15% on the concentrations (errors on sample weight).

Hyacinthacine A_2 (**13**), Table 5.3, showed to be a potent and non competitive inhibitor of amyloglucosidase from *Aspergillus niger* (IC_{50} = 1.9 ± 0.4 μM). It also inhibits α-glucosidase from yeast (72% at 1mM).

7-*Epi*australine (**17**) (Table 5.3) was slightly less potent and competitive inhibitor of amyloglucosidase from *Aspergillus niger*, with IC_{50} in the same order of magnitude (IC_{50} = 3.5 ± 0.9 μM, mixed type inhibition). It also inhibits α-glucosidase from yeast (80% at 1mM) and, to a lesser extent, β-N-acetylglucosaminidase from jack bean and α-glucosidase from rice (38% and 16% at 1mM respectively).

7-Methoxycasuarine **94** (Table 5.3), was quite good and selective inhibitor of amyloglucosidase from *Aspergillus niger* (IC_{50} = 9.7 ± 1.5 μM, mixed type inhibition). It also inhibits β-glucosidase from almond (34% at 1mM) and did not inhibit α-glucosidase from rice or from yeast.

	Percentage of inhibition at 1mM		
Compound / Enzyme (pH)	**13**	**17**	**94**
α-L-fucosidase EC 3.2.1.51 1-bovine kydney (6)	n.t.	n.t.	n.t.
α-galactosidase EC 3.2.1.22 2-coffe bean (6)	n.i.	n.i.	n.i.
β-galactosidase EC 3.2.1.23 5-*Escherichia coli* (7)	n.i.	n.i.	n.i.
8-*Aspergillus oryzae* (4)	n.i.	n.i.	n.i.
α-glucosidase EC 3.2.1.20 10-yeast (7)	72	80	n.i.
11-rice (4)	n.i.	16	n.i.
amyloglucosidase EC 3.2.1.3 13-*Aspergillus niger* (5)	**97** IC_{50}=1.9μM	**95** IC_{50}=3.5μM	**97** IC_{50}=9.7μM
β-glucosidase EC 3.2.1.21 15-almond (5)	n.i.	n.i.	34
α-mannosidase EC 3.2.1.24 16-jack bean (5)	n.i.	n.i.	n.i.
β-mannosidase EC 3.2.1.25 18-snail (4)	n.i.	n.i.	n.i.
β-xylosidase EC 3.2.1.37 19-*Aspergillus niger* (5)	n.i.	n.i.	n.i.
β-N-acetylglucosaminidase EC 3.2.1.30 21-jack bean (5)	n.i.	n.i.	n.i.
22-bovine kidney (4)	n.i.	38	n.i.

Table 5.3. Inhibition of compounds 14, 17, and 94 toward commercially available glycosidases. n.i. = no inhibition, n.t. = test not performed.

In summary, evaluation of the inhibitory activity of casuarine and its derivatives towards a wide range of commercially available glycosidases allowed us to discover several new inhibitors of glucoamylase from *Aspergillus niger*.

Glucoamylase (1,4-α-D-glucan glucohydrolase, GA; EC 3.2.1.3; glycoside hydrolase family GH15, www.cazy.org) is an *exo*-hydrolase that catalyzes the removal of glucose units from the non-reducing end of starch and related oligosaccharides. The hydrolytic reaction, which preferentially occurs at α-1,4 linkages, proceeds with inversion of configuration. Glucoamylases are also able to hydrolyze α-1,6 linkages, but the specific activity is only 0.2% with respect to α-1,4 hydrolysis.[136] The interest in glucoamylase is related to its use in the industrial production of bioethanol, glucose and fructose syrups.[137] Furthermore, given the presence of these enzymes in a wide variety of organisms and their quite simple obtainment in a pure form, GA have been extensively studied as a model for other glucosyl-hydrolase family members.[138]

5.1.2 Modeling studies of the amyloglucosidase/inhibitors interactions

This study has been performed in collaboration with Prof. Paola Gratteri (Department of Pharmaceutical Sciences, University of Florence). In particular, we thank Dr. Claudia Bonaccini and Matteo Chioccioli for docking studies.

For the discussion of the modeling, we report a more convenient numeration for the studied molecules (Table 5.4) together with their inhibition values towards glucoamylase from *Aspergillus niger.*

Based on the inhibition results, our molecular study was focused on glucoamylase from *Aspergillus niger*, and it was aimed at correlating experimental affinity with binding energy predictions obtained through molecular docking and MM-GBSA rescoring techniques.

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[136] Hiromi, K.; Hamauzu, Z. I.; Takahashi, K.; Ono, S. *J. Biochem. (Tokio)* **1966**, *59*, 411.
[137] Saha, B. C.; Zeikus, J. G. *Starch/Staerke* **1989**, *41*, 57.
[138] Sauer, J.; Sigurskjold, B. W.; Christensen, U.; Frandsen, T. P.; Mirgrorodskaya, E.; Harrison, M.; Roepstorff, P.; Svensson, B. *Biochim. Biophys. Acta* **2000**, *1543*, 275.

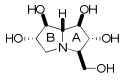
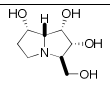
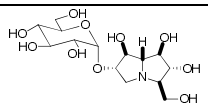
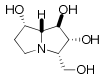
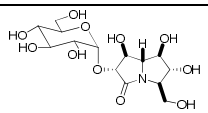
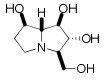
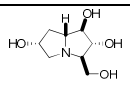
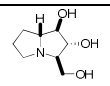
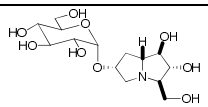
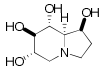
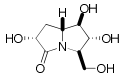
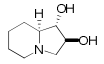
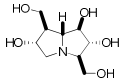
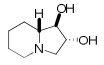
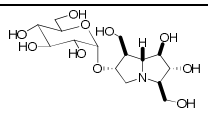
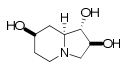
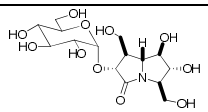
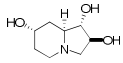
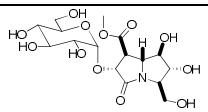
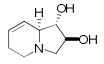
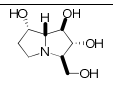
Molecule	Structure	IC_{50} (μM)	Molecule	Structure	IC_{50} (μM)
I		1.9	**XII**		300[b]
II		4.4	**XIII**		420[b]
III		0%[a]	**XIV**		3.5
IV		4.5	**XV**		1.9
V		7.7	**XVI**		4.3[c]
VI		214	**XVII**		2.7[c]
VII		8.1	**XVIII**		108[d]
VIII		23.7	**XIX**		9.8[d]
IX		25%[a]	**XX**		290[d]
X		76%[a]	**XXI**		63[e]
XI		28[b]			

Table 5.4. Structures and inhibition activities (IC_{50}) towards glucoamylase from *Aspergillus niger* for the compounds used in the molecular modeling study. [a]For molecules with percentage of inhibition less than 90% IC_{50} values were not calculated [b]Values from Ref. 58. [c]Values from Ref. 138. [d]Values from Ref. 139. [e]Values from Ref. 112g.

[139] Goti, A.; Cardona, F.; Brandi, A.; Picasso, S.; Vogel, P. *Tetrahedron: Asymmetry* **1996**, *7*, 1659.

Furthermore, the docking/rescoring procedure tested on glucoamylase shows promising ability in discriminating active and inactive molecules, and thus it can be used for the rational design of new glucosidase inhibitors.

5.1.2.a Docking studies

As a first stage of this molecular modeling study we performed a series of docking runs using **i)** two available crystallographic structures of complexes of glucoamylase from *Aspergillus awamori* with ligands (PDB ID: 1DOG and 1GAI)[140,141] and **ii)** considering as part of the target one or two water molecules. The choice of using more than one structure has been made assuming that the structural alignment of the two complexes did not reveal any significant conformational difference and thus making it difficult to influence ligand posing. We experienced that such "invisible" differences could greatly influence the scoring of docked poses. As far as the presence of water molecules is concerned, the two solvent molecules considered here, Wat501 and Wat534 in 1DOG structure, are located in close proximity to the ligands. In particular Wat501 is directly engaged in H-bonding interaction with ligands, while Wat534 seems to have a structural role, since it is coordinated by three residues, one of which (D55) has been demonstrated to be required for substrate binding and catalysis.[142] Preliminary analysis of docking accuracy performances highlighted the need to consider at least Wat501 in order to reproduce the correct binding mode of co-complexed ligands, especially for 1-deoxynojirimycin. Moreover, the comparison of the crystal structures of the complexes with acarbose and D-gluco-dihydroacarbose suggested a fundamental role of Wat501 in modulating the affinity of ligands, given that a

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[141] Aleshin, A. E.; Stoffer, B.; Firsov, L. M.; Svensson, B.; Honzatko, R. B. *Biochemistry* **1996**, *35*, 8319.
[142] Itoh, T.; Sakata, Y.; Akada, R.; Nimi, O.; Yamashita, I. *Agric. Biol. Chem.*, **1989**, *53*, 3159.

0.2 Å shift of this water molecule induced by the presence of an additional hydrogen atom on C-7a of D-gluco-dihydroacarbose has been indicated as the most apparent cause for the 10000-fold drop in activity with respect to acarbose.[141] For these reasons we performed docking calculations in the presence of both the only Wat501 and the two water molecules together. The selection of the best docked orientation for each ligand has been performed on the basis of either Gscore and Emodel values and the Gscore values for the selected poses have been compared to experimental pIC_{50} data. It is important to note that ligand affinity data refer to the inhibition of *Aspergillus niger* glucoamylase, whereas the available 3D structures were obtained with the *Aspergillus awamori* enzyme; nevertheless, the sequence comparison of the proteins from the two strains highlighted more than 95% of sequence identity, with all binding site forming residues completely conserved in the two proteins. This observation supported our choice of correlating *Aspergillus niger* experimental affinities with *Aspergillus awamori* calculated binding energies. Indeed, while this work was in preparation, the crystallographic structure of the catalytic domain of A*spergillus niger* glucoamylase was solved (PDB ID: 3EQA)[143] and the structural comparison with *Aspergillus awamori* protein confirmed they are almost identical. We report the results from the visual inspection of selected docked poses.

5.1.2.b Binding mode analysis

For most of the molecules, almost coincident orientations were found independently on the protein structure, the number of water molecules considered as part of the target during docking and the kind of evaluation function used to select the poses. This observation confirms that Wat534 does not influence ligand posing during docking, but we cannot exclude its role in maintaining the structure of the binding site.

[143] Lee, J.; Paetzel, M. To be published.

For molecules **I**, **IV** and **VII** all the selected poses are almost coincident. In this preferred orientation (Figure 5.4A) ring A of the pyrrolizidine nucleus is located in the inner part of the -1 subsite, finding an optimal H-bonding network in which the OH at C-8 is bonded to D55 (acceptor) and Wat501 (donor), the OH at C-2 is bonded to D55 (acceptor) and R54 (donor) and the OH at C-1 is alternatively oriented toward Wat501 or the backbone carbonyl of L177 (Figure 5.4C) for donating an additional H-bond. Given this orientation, the positively charged nitrogen atom orients the hydrogen toward Wat501 and does not interact with any negatively charged residue of the protein, in contrast to finding obtained for the imino group of acarbose and D-gluco-dihydro-acarbose.[141]

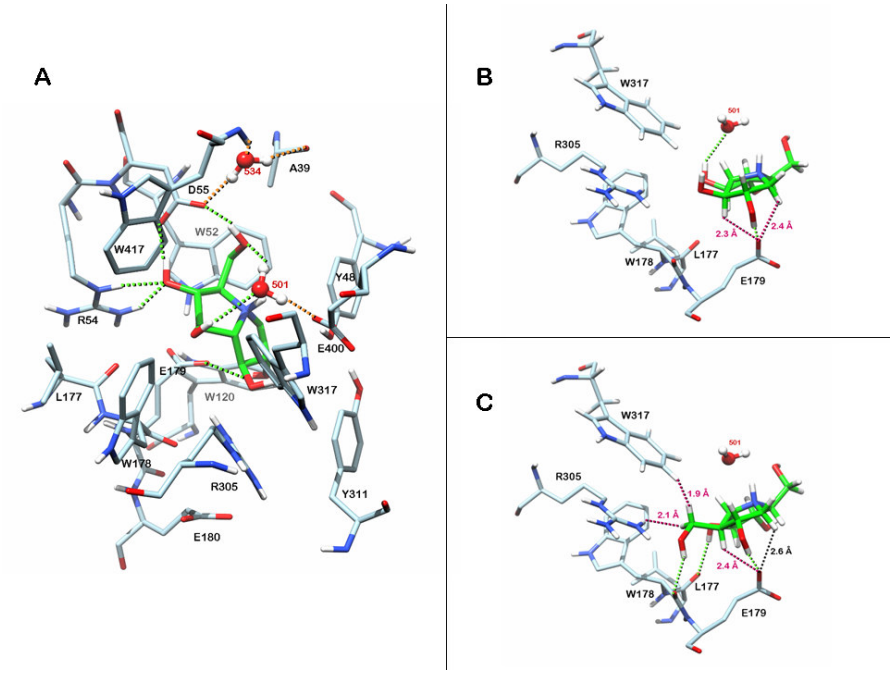

Figure 5.4. Docked orientation of casuarine I and 7-modified derivatives; ligand molecules are shown as stick representation with green coloured carbon atoms. A) Interactions between molecule I and active site residues; the two water molecules considered during docking are also shown. H-bonds are indicated as dashed lines coloured in green or orange if involving the ligand or water molecules, respectively. B) Detailed inspection of steric repulsion (magenta dashed lines) between molecule I and E179. C) Detailed inspection of steric repulsion (magenta dashed lines) between molecule VII and E179, W317 and R305

Indeed, the docked orientation of casuarine and derivatives places the hydrogen atoms of C-5 and C-7 in close contact with E179 (Figure 5.4B).

This could negatively contribute to the affinity of such compounds, but it is partly compensated by the additional H-bond that the OH at C-6 is able to donate to E179. Finally, as far as position 7 is concerned, no H-bond interactions are established by the OH at C-7 of **I**, in that resembling the behaviour of the deoxy-derivative **IV**. For molecule **VII**, instead, the OH at C-9 can form two H-bonds with R305 (donor) and W178 (acceptor), but strong repulsive interactions between the methylene hydrogens and R305 and/or W317 are present, which could explain the relatively lower affinity of **VII** over **I** (Figure 5.4C).

Comparing molecule **IV** and its lactam derivative **VI**, the assumption that repulsive interactions could have a very important role in decreasing binding affinity finds a confirmation: the docked orientations are very similar, but the limited conformational flexibility of **VI** results in stronger repulsive interactions with residues of the binding site, in accordance with the lower pIC_{50} value.

The analysis of the selected poses for the corresponding glucoside derivatives **II**, **V** and **VIII** showed two possible binding modes which alternatively oriented the pyrrolizidine or the glucose moiety in the -1 site, which are defined as CAS-IN and GLU-IN, respectively. Interestingly, the two orientations showed no significant differences in terms of associated Gscore.

When the molecules adopt the GLU-IN orientation (Figure 5.5), which is the more frequently selected by our docking procedure, the glucose in -1 site overlaps to ring A of D-gluco-dihydro-acarbose but lacks the steric repulsions described for C-7a of the acarbose derivative,[141] given the presence in the corresponding position of glucose of the endocyclic oxygen. In all the selected poses showing GLU-IN orientation the pyrrolizidine nucleus, which is able to completely fill the +1 site (Figure 5.5B), orients the $N\text{-}H^+$ toward the aromatic ring of Y311, with a geometry compatible with NH-π interaction. Furthermore,

the OH at C-8 is able to donate one H-bond to E180, thus contributing to the stabilization of this binding mode (Figure 5.5A). Interestingly, all lactam derivatives of glucosides **III**, **IX**, **X** adopt the same GLU-IN orientation and this could confirm the poor binding at -1 site of the lactam moiety with respect to pyrrolizidine and glucose. Also at the level of +1 site the results of docking converged to a unique solution for all the lactam molecules, which is almost coincident with the orientation of the pyrrolizidine moiety of glucoside derivatives except for the ring conformation clearly influenced by the presence of the lactam structure. Furthermore, the absence of the charge on nitrogen atom prevents lactam derivatives to reinforce the interaction at +1 site through NH-π interactions.

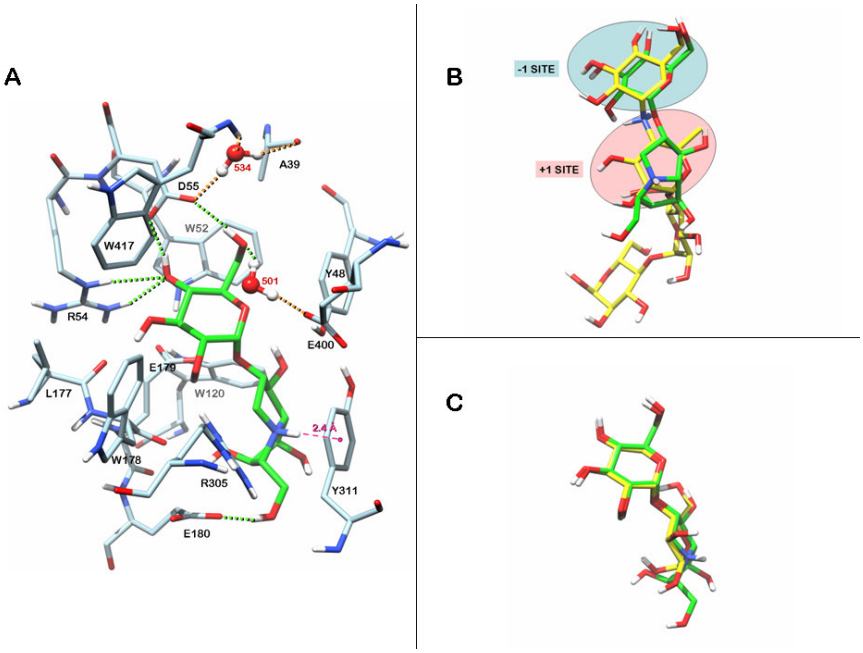

Figure 5.5. GLU-IN orientation of glucoside derivatives; ligand molecules are shown as stick representation with green coloured carbon atoms. A) Interactions between molecule 2 and active site residues; the two water molecules considered during docking are also shown. H-bonds are indicated as dashed lines coloured in green or orange if involving the ligand or water molecules, respectively. The distance between N-H^+ and the centroid of Y311 aromatic ring is also shown. B) Comparison between the GLU-IN orientation of glucoside derivatives and the docked orientation of D-gluco-dihydroacarbose (yellow carbons). C) Comparison between the GLU-IN orientation of glucoside derivatives and the docked orientation of maltose (yellow carbons)

Apart from that, the visual inspection of selected docked poses for corresponding glucoside/lactam molecules in this orientation did not highlight any other significant difference in the interactions which could justify the complete absence of inhibitory properties for lactam compounds.

Moreover, comparing the orientation of **II** with the docked orientation of the hydrolyzable maltose (Figure 5.5C), it is not clear how the casuarine glucoside could resist hydrolysis, as observed, since the nucleophile Wat501 is perfectly oriented toward the anomeric carbon.

In the CAS-IN orientation (Figure 5.6A) the position of pyrrolizidine nucleus of glucoside molecules is almost coincident to the docked pose of the parent unglucosylated compounds, except for the conformation of ring B, which is influenced by the positioning of glucose moiety, and for the lack of the H-bond interaction between the OH at C-6 and E179.

As a consequence, the same repulsive interactions previously described are present. Given that orientation of the pyrrolizidine portion, the glucose moiety is oriented in the outer part of the binding site, but it is not able to completely occupy the +1 site as is the case for ring B of acarbose and its D-gluco-dihydro-derivative[141] due to the longer C-2/C-6 distance of pyrrolizidine (4.9 Å) with respect to the C-1/C-4 distance of ring A of D-gluco-dihydro-acarbose or glucose (2.9 Å) (Figure 5.6B). As a consequence, the C-6 atom of pyrrolizidine is more than 2 Å far from the C-1 atom of glucose in the docked pose of maltose (Figure 5.6C), which is the target of the nucleophilic attack during glucoamylase hydrolysis. With these observations in mind, we could hypothesize a different binding mode for glucosides (CAS-IN) and their lactam derivatives (GLU-IN); as a consequence of that, glucosides strongly bind and resist to hydrolysis, while lactam derivatives are hydrolyzed by glucoamylase soon after binding releasing the corresponding lactam and glucose, that are not good inhibitors.

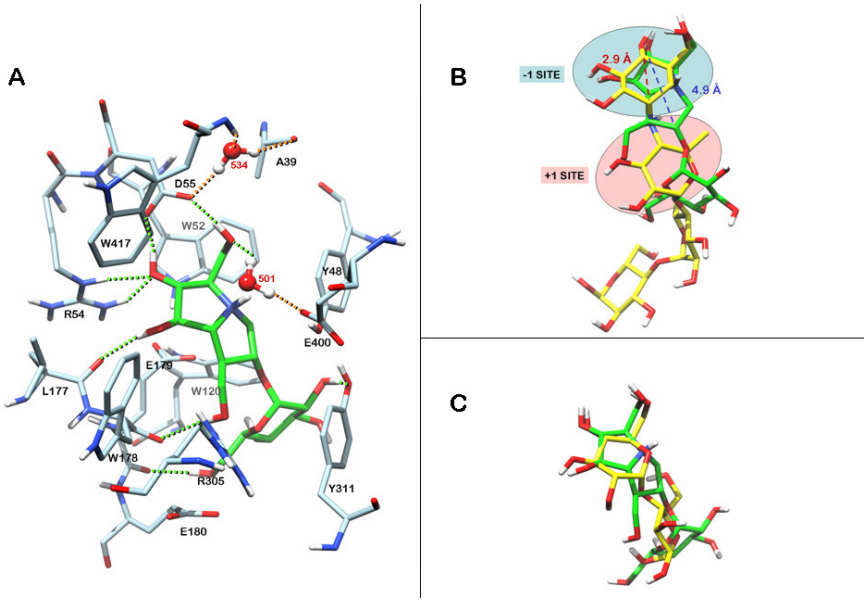

Figure 5.6. CAS-IN orientation of glucoside derivatives; ligand molecules are shown as stick representation with green coloured carbon atoms. A) Interactions between molecule VIII and active site residues; the two water molecules considered during docking are also shown. H-bonds are indicated as dashed lines coloured in green or orange if involving the ligand or water molecules, respectively. B) Comparison between the CAS-IN orientation of glucoside derivatives and the docked orientation of D-gluco-dihydroacarbose (yellow carbons). The C2-C6 distance of pyrrolizidine and the C1-C4 distance of ring A of D-gluco-dihydro-acarbose are also shown as dashed lines coloured in blue and red, respectively C) Comparison between the CAS-IN orientation of glucoside derivatives and the docked orientation of maltose (yellow carbons)

Molecules **XI**-**XV**, the inhibitory activities of which towards glucoamylase were reported previously (See Table 5.4), are pyrrolizidine alkaloids, which differ from casuarine (**I**) in the number of the hydroxyl groups and/or the configuration of stereogenic centres. In particular, **XI**, **XIV** and **XV** present the same ring A substitutions of casuarine but different substituents at C-6 and C-7 of ring B. Interestingly, docking results for **XIV** (the 6-deoxy analog of **I**) and **XV** (the 6,7-di-deoxy analog of **I**) converge to a unique solution almost coincident with the previously described orientation of casuarine derivatives. Molecule **XI**, instead, which lacks the OH at C-6 and presents the inverted configuration at C-7 with respect to **I**, showed also a second binding mode (II) in which N-H^+ is oriented toward D179 (Figure 5.7A). This second binding mode, which

preserves the same H-bonds with R54, D55, L177 and Wat501 already described for casuarine derivatives, allowed the molecule to better fill the -1 site, placing the unsubstitued C-6 close to the aromatic rings of Y48 and W52. It is quite interesting to note that this binding mode is not possible for casuarine given the presence of the hydroxyl substituent at C-6 and the different configuration of the OH at C-7 which are not tolerated by steric repulsions. The lack of the OH at C-7 in molecule **XV** or its different configuration in molecule **XIV**, which results in the loss of H-bonds with D55 and Wat501, is probably the reason why this second binding mode is not observed for these two molecules.

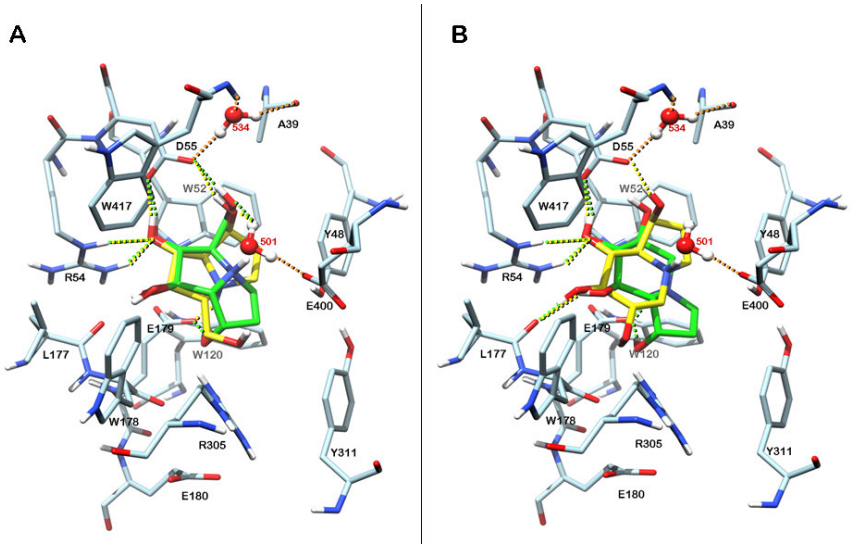

Figure 5.7. Docked orientations of molecule XI (A) and XVI (B). The ligand molecules are shown as stick representation with carbon atoms coloured in green (orientation I) or yellow (orientation II); the two water molecules considered during docking are also shown. H-bonds are indicated as dashed lines coloured in orange if involving water molecules and in green or yellow if involving ligand orientation I or II, respectively

Molecules **XII** and **XIII** present the same ring B substitutions of **XI**, but different configuration at C-1 or C-3 on ring A, respectively. Molecule **XII** shows both the two binding modes described for **XI**, although the presence of the 1-*epi* modification determined ring A distortion and the loss of several favourable interactions. Molecule **XIII**, instead, shows always the second

binding mode which is however characterized by the presence of strong repulsive interactions which could justify the very low affinity of this compound.

Finally, indolizidines **XVI-XXI**, the inhibitory activities of which towards glucoamylase were reported previously (See Table 5.4), present a 6-membered ring B with a similar stereochemical arrangement (molecule **XVI**) or a low degree of hydroxylation with respect to ring A of **I** and different substitutions on C-1 and C-2 of ring A. Most of the selected poses for **XVI** oriented ring B in a very similar way as ring A of pyrrolizidine, with C-6, C-7 and C-8 of **XVI** almost coincident to C-1, C-2 and C-3 of **XI**, respectively (Figure 5.7B). Contrarily to pyrrolizidines, given the presence in C-6 position of compound **XVI** of a hydroxy group instead of the C-3 hydroxymethyl group of pyrrolizidine derivatives, **XVI** is unable to H-bond both D55 and Wat501. Anyway, in that pose both $N\text{-}H^+$ and the OH at C-1 orient toward E179 establishing strong H-bonding interactions. The second binding mode which we observed for this molecule is very similar to orientation II of pyrrolizidine **XI**, with the full H-bonding network involving Wat501, D55 and R54, but orienting $N\text{-}H^+$ toward Wat501.

Molecules **XVII-XXI** present a low degree of hydroxylation and, interestingly, very different activities associated to quite small structural variations. The two enantiomers of lentiginosine, **XVII** and **XVIII**, showed a different binding mode with respect to **XVI** given the total absence of hydroxyl groups on ring B. In fact, all the selected poses for the two compounds orient ring A in the inner portion of -1 site; for molecule **XVIII**, probably due to the stereochemical arrangement of ring A substituents, all the selected poses converged to a unique orientation in which the OH at C-2 is always engaged in H-bonding interactions with R54 and D55, the positively charged nitrogen orients the hydrogen toward E179 with the possibility of charge-reinforced H-bond formation and the OH at C-1 alternatively orients toward Wat501 or the

carbonyl oxygen of L177, depending on the conformation adopted by the indolizidine nucleus (Figure 5.8A). The (+)-enantiomer **XVII** showed two possible binding modes, which always form H-bonds with R54, D55 and Wat501 through the two hydroxy groups, but alternatively orient N-H^+ toward Wat501 (I) or E179 (II) (Figure 5.8B).

The introduction of 7(*R*)-OH on (+)-lentiginosine (compound **XIX**) led to almost the same binding mode of **XVII** with N-H^+ oriented toward Wat501 and the formation of an additional H-bond between the OH at C-1 and the carbonyl oxygen of W178 which causes the movement of the indolizidine nucleus in closer contact to Y48. The 7(*S*)-OH derivative **XX**, instead, showed the two binding modes described for **XVII**, with orientation I showing the same shift towards Y48 previously described for **XIX**, but with a very distorted geometry of ring B. Finally, the more rigid analog **XXI** always showed the same orientation I of **XVII** with subtle differences in the positioning of carbon atoms of ring B due to the presence of the double bond between C-7 and C-8.

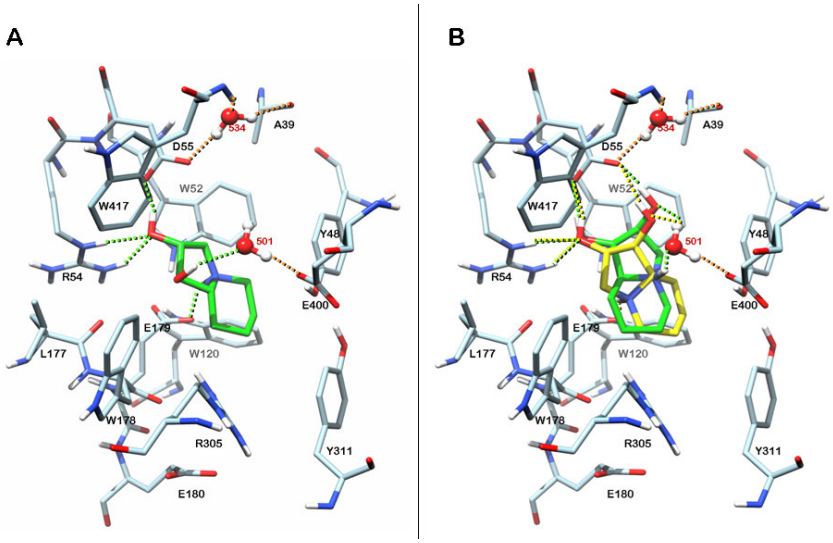

Figure 5.8. Docked orientations of molecule XVIII (A) and XVII (B). The ligand molecules are shown as stick representation with carbon atoms coloured in green (orientation I) or yellow (orientation II); the two water molecules considered during docking are also shown. H-bonds are indicated as dashed lines coloured in orange if involving water molecules and in green or yellow if involving ligand orientation I or II, respectively

In conclusion, docking experiments performed on pyrrolizidine and indolizidine derivatives allowed one to hypothesize preferential binding modes for all the studied compounds. It is evident that an optimal network of H-bonding interaction at the level of the inner -1 site has to be found for a ligand to bind, but also interactions at the level of +1 site seem to have an important role in modulating the affinity of more extended molecules. Furthermore, another important contribution to the stability of protein-ligand complexes could be represented by steric repulsions which are especially found for analogues with reduced flexibility (lactam derivatives).

5.1.3 Inhibition of a human maltase glucoamylase

This study has been performed in collaboration with Prof. David Rose (University of Toronto, Canada). In particular, we thank Lyann Sim for the inhibition tests and X-ray analysis.

Human maltase-glucoamylase (MGAM, EC 3.2.1.20) falls into family GH31 of the Carbohydrate Active enZyme (CAZy) classification.[144] It is one of the two enzymes (together with sucrase-isomaltase SI, EC 3.2.1.48 and 3.2.1.10) responsible for catalysing the last step in starch digestion, by hydrolyzing mixtures of dextrins, at the non-reducing end, into glucose with net retention of anomeric configuration.[145] Inhibitors of enzymes involved in the starch-digestion pathway are used to delay glucose production and thus aid in the treatment of type II diabetes.[84b] This therapeutic potential would be encouraged by an in-depth study of their mode of action.[146] Rose et al.

[144] Coutinho, P. M.; Henrissat, B. in *Recent Advances in Carbohydrate Bioengineering* (Eds.: H. J. Gilbert, G. J. Davies, B. Henrissat, B. Svensson), Royal Society of Chemistry, Cambridge, **1999**, pp. 3.

[145] a) Nichols, B. L.; Eldering, J.; Avery, S.; Hahn, D.; Quaroni, A.; Sterchi, E. E. *J. Biol. Chem.* **1998**, *273*, 3076; b) Nichols, B. L.; Avery, S.; Sen, P.; Swallow, D. M.; Hahn, D.; Sterchi, E. E. *Proc. Natl. Acad. Sci USA* **2003**, *100*, 1432.

[146] For reviews on glycosidase mechanisms, see: a) Heightman, T. D.; Vasella, A. T. *Angew. Chem.* **1999**, *111*, 795; *Angew. Chem. Int. Ed.* **1999**, *38*, 750; b) Zechel, D. L.; Withers, S. G. *Acc. Chem. Res.* **2000**, *33*, 11; c) Ref. 2.

recently reported[147] the first crystal structure of the human *N*-terminal subunit of MGAM (NtMGAM) in its apo form and in complex with acarbose (**113**) (Figure 5.9), a tetrasaccharide analogue currently on the market as an antidiabetic drug (Glucobay, Precose).

NtMGAM inhibition studies revealed that casuarine (**11**), with a K_i of 0.45 μM, displayed over 600 fold greater inhibition against NtMGAM compared to its glucoside **50**, which inhibited with a K_i of 280 μM.

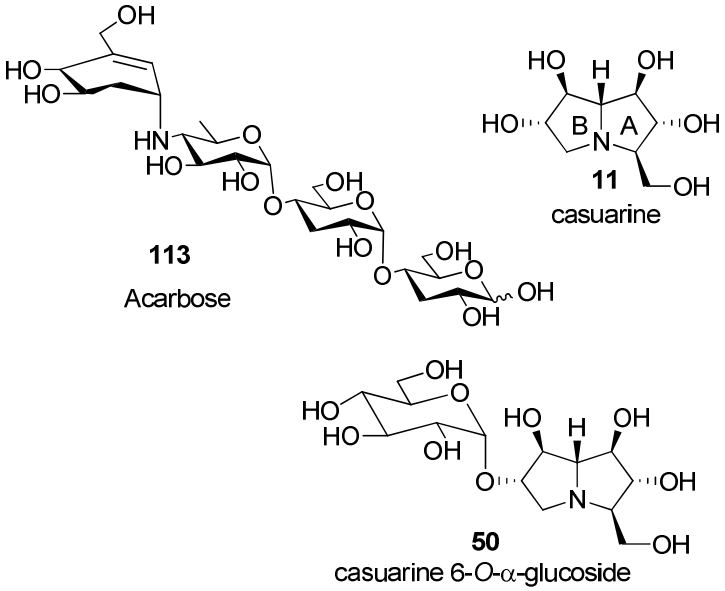

Figure 5.9. Acarbose (113), casuarine (11) and its glucoside 50

These results are similar to the inhibition profile of rat intestinal maltase where **11** and **50** inhibit with IC_{50} values of 0.7 μM and 260 μM, respectively, but are in contrast to the inhibition profile of fungal glucoamylases where **11** and **50** display similar and potent inhibitory activities.[58]

[147] Sim, L.; Quezada-Calvillo, R.; Sterchi, E. E.; Nichols, B. L.; Rose, D. R. *J. Mol. Biol.* **2008**, *375*, 782.

The similarity in inhibition profiles between NtMGAM and the rat intestinal maltase is consistent with previous kinetic studies,[147,148] which proposed that the *N*-terminal NtMGAM domain of human maltase-glucoamylase displays maltase activity whereas its *C*-terminal catalytic domain displays glucoamylase activity.

The crystal structure of NtMGAM in complex with **11** was solved to 2.1 Å (see Experimental Section for details and data processing and refinement statistics). The electron density clearly revealed one molecule of **11** (Figure 5.10) bound in the –1 subsite and one glycerol molecule (originating from the cryoprotection solution) bound in the +1 subsite.

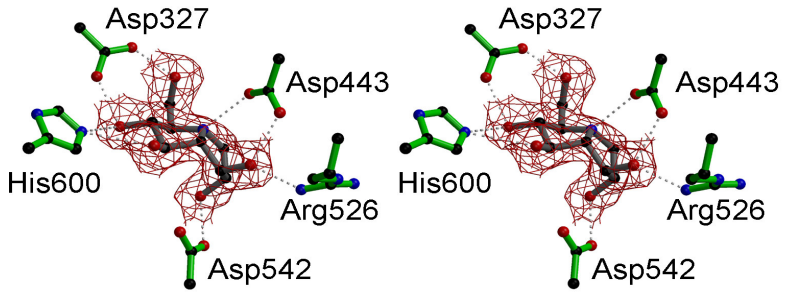

Figure 5.10. Stereo (divergent) ball-and-stick representation of casuarine (11) in complex with NtMGAM. Observed electron density for the maximum likelihood weighted $2F_{obs}$-F_{calc} map is contoured at 1σ. Figure were drawn using BOBSCRIPT[149]

The two pyrrolidine rings, named 'A' and 'B' in Figure 5.9, both adopt an envelope configuration; ring A is in a ^{2}E conformation, and ring B in an E_6 conformation.

With its heavily hydroxylated rings, **11** binds tightly to the NtMGAM active site primarily through hydrogen bonding interactions with the side chains lining the –1 subsite (Figure 5.11).

[148] Quezada-Calvillo, R.; Sim, L.; Ao, Z.; Hamaker, B. R.; Quadroni, A.; Brayer, G. D.; Sterchi, E. E.; Robayo-Torres, C. C.; Rose, D. R.; Nichols, B. L. *J. Nutr.* **2008**, *138*, 685.
[149] Esnouf, R. M. *J. Mol. Graph. Model.* **1997**, *15*, 132.

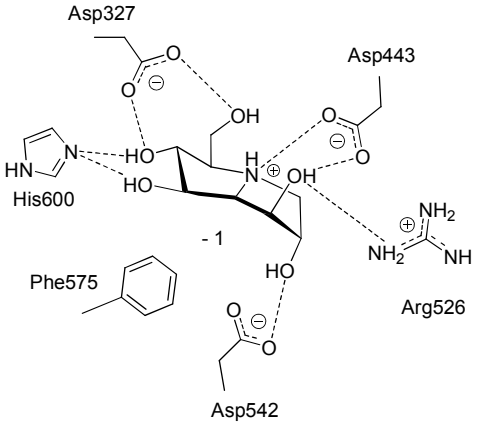

Figure 5.11. Interactions between 11 and NtMGAM (D443 is the catalytic nucleophile, D542 the catalytic acid/base). Hydrogen bonds are shown by dashed lines

These include hydrogen bonding between D327 and the hydroxyl groups at C-8 and C-2, H600 with the OH at C-1 and the OH at C-2, D443 with the OH at C-7 and the pyrrolidine nitrogen, R526 with the OH at C-7 and D542 with the OH at C-6. The weaker inhibition of **50** compared to **11** leads us to believe that the addition of glucoside group of **50** makes unfavourable interactions with the +1 subsite, or weakly competes with the casuarine group to occupy the –1 subsite, both of which may decrease the inhibition properties of the casuarine group.

Indeed, molecule **50** is a mimic of a α-1,1-glucoside while the enzyme hydrolyses 1,4-glucosides. Work is underway in our laboratory in order to synthesize mimetics of 1,4-glucosides based on casuarine (Figure 5.12).

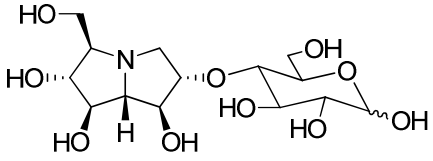

Figure 5.12. 1,4-Glucoside based on casuarine

5.1.4 Inhibition on trehalases

5.1.4.a Trehalase inhibition

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) (**114**, Figure 5.13) has a multifunctional physiological role in various organisms ranging from bacteria and fungi to invertebrates and higher plants. Many of these lower organisms produce and store trehalose, sometimes in amounts as high as 10% to 20% of their dry weight.[75b] Trehalose is particularly important for insects (it constitutes the major blood sugar of most insects)[150] as it is hydrolysed into glucose, which is vital for insect flight. Inhibition of the hydrolysis of trehalose is thus an interesting target for novel insecticides.

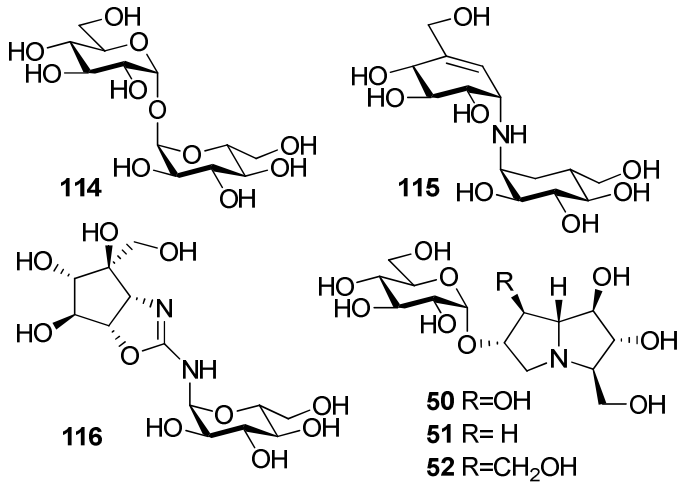

Figure 5.13. Trehalose (114) and trehalase inhibitors

In order to utilise trehalose, insect tissues have the enzyme α-trehalase (EC 3.2.1.28), which promotes the irreversible hydrolysis of one of the two glycosidic bonds in trehalose with inversion at the anomeric configuration.[150,151] Trehalose does not occur in mammalian cells, although

[150] Becker, A.; Schlöder, P.; Steele, J. E.; Wegener, G. *Experientia*, 1996, **52**, 433.
[151] Defaye, J.; Driguez, H.; Henrissat, B. *Carbohydr. Res.*, 1983, **124**, 265.

humans have the enzyme trehalase in intestinal villae cells and in kidney brush border cells, probably to handle ingested trehalose.[75b]

Although insects possess two types of treahalases, a soluble one (localized in haemolymph) and a membrane-bound trehalase (localized in tissues), very little is known about this latter and the difference in function about soluble and membrane-bound trehalases.[152,153]

Trehalases inhibitors are valuable tools for studying the molecular physiology of trehalase function and sugar metabolism in insects.[154] The evaluation of the enzymatic activity helps in ensuring clarification of the key role of this class of enzymes in insects and in vertebrates. Together with the selection of new potential insecticides, inhibition studies have been proved useful for elucidating structural features of the active site.[155]

Among the most powerful inhibitors of trehalases there are some natural pseudodisaccharides, such as validoxylamine A (**115**),[156] trehazolin (**116**),[156] and casuarine-6-*O*-α-D-glucoside (**50**) (Figure 5.13).[58]

5.1.4.b Inhibition of trehalase from Escherichia coli (Tre37A)

Structural determination of complexes of enzymes with strong inhibitors is able to provide molecular insights into enzyme mechanism and features of the active site. Davies et al. recently presented the first three-dimensional structure of a periplasmic trehalase from *Escherichia coli* (Tre37A), which belongs to family GH37 of the CAZy classification, in complex with some potent

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[152] Lee, J.-H.; Saito, S.; Mori, H.; Nishimoto, M.; Okuyama, M.; Kim, D.; Wongchawalit, J.; Kimura, A.; Chiba, S. *Biosci. Biotechnol. Biochem.*, 2007, **71**, 2256.
[153] Tang, B.; Chen, X.; Liu, Y.; Tian, H.; Liu, J.; Hu, J.; Xu, W.; Zhang, W. *BMC Mol. Biol.*, 2008, **9**, 51.
[154] Wegener, G.; Tschiedel, V.; Schlöder, P.; Ando, O. *J. Experim. Biol.*, 2003, **206**, 1233.
[155] Asano, N.; Takeuchi, M.; Kameda, Y.; Matsui, K.; Kono, Y. *J. Antibiot.* , 1990, **43**, 722.
[156] Kyosseva, S. V.; Kyossev, Z. N.; Elbein, A. D. *Arch. Biochem. Biophys.*, 1995, **316**, 821.

inhibitors, 1-thiatrehazolin (**116**) and validoxylamine A (**115**, Figure 5.13).[157] Casuarine (**11**) and its glucoside **50** are known to inhibit porcine kidney trehalase (IC_{50} = 12 μM and IC_{50} = 0.34 μM, respectively);[5] consequentially, the measurement of their inhibitory activity towards Tre37A was of high interest. Kinetic data were collected on Tre37A using an assay where glucose was detected by glucose oxidase/peroxidase linking enzymes following trehalose hydrolysis (see Experimental Section for details). K_i values for **11** and **50** were determined at 37 °C and pH 5.5, and included a 20 minute pre-incubation of the inhibitor with the enzyme to prevent any complications in the data analysis from slow onset inhibition. Compound **50**, with a K_i = 12 nM, was shown to be around a thousand fold more potent than **11** (K_i = 17 μM), indicating that the glucose moiety in the +1 subsite (see below) contributes considerably to binding. This fits with the fact that Tre37A hydrolyses disaccharide substrates and therefore presumably has evolved to optimise interactions in both the –1 and +1 subsites.[157]

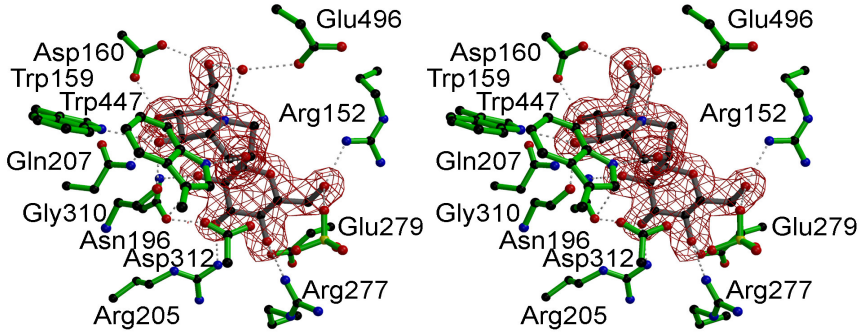


Figure 5.14. Stereo (divergent) ball-and-stick representation of casuarine-6-*O*-α-glucoside (50) in complex with Tre37A. Observed electron density for the maximum likelihood weighted $2F_{obs}$-F_{calc} map is contoured at 1σ. Figures were drawn using BOBSCRIPT.[148]

X-ray data for Tre37A in complex with **50** were collected to 1.9 Å (see Experimental Section for details and data processing and refinement statistics).

[157] Gibson, R. P.; Gloster, T. M.; Roberts, S.; Warren, R. A. J.; de Gracia, I. S.; García, A.; Chiara, J. L.; Davies, G. J. *Angew. Chem.* **2007**, *119*, 4193; *Angew. Chem. Int. Ed.* **2007**, *46*, 4115.

There was clear electron density in each of the four molecules of the asymmetric unit corresponding to a molecule of **50**, Figure 5.14.

The casuarine moiety of **50** is bound in the –1 subsite of Tre37A (Figure 5.15).

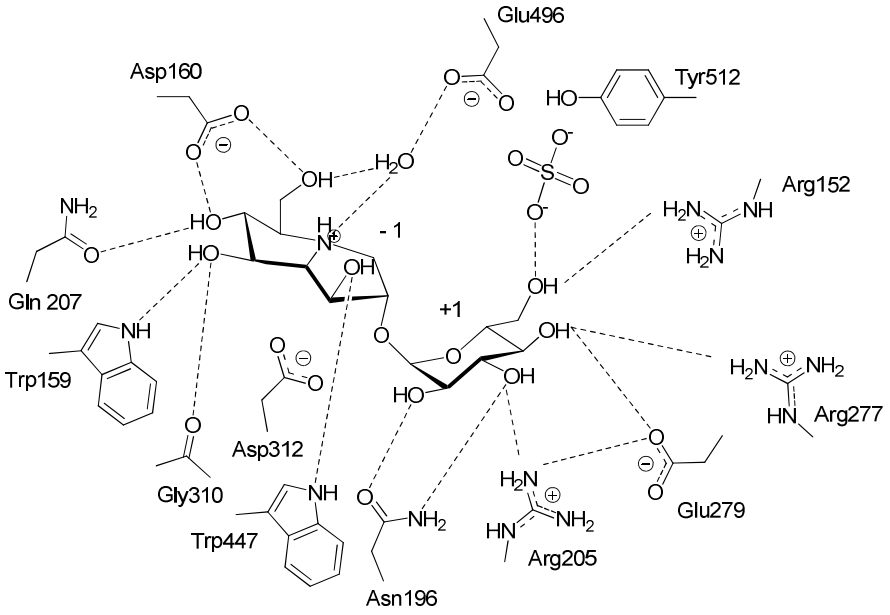


Figure 5.15. Interactions between 50 and Tre37A (E496 is the catalytic base, D312 the catalytic acid). Hydrogen bonds are shown by dashed lines

The two pyrrolidine rings are both found in an envelope conformation; ring A is in an ^{2}E conformation, and ring B in an ^{5}E conformation. The glucose moiety of **50**, in the +1 subsite, is bound in a relaxed $^{4}C_{1}$ conformation. The majority of the interactions between the glucose and the active site residues of Tre37A are as described previously;[157] the exception to this is the hydroxyl group at the C-6 position, which hydrogen bonds with a sulfate group from the crystallisation conditions, and appears to cause a large movement of E511 away from the active site.

The hydroxyl group at C-8 of the casuarine moiety hydrogen bonds with D160 and a water molecule, the OH at C-2 interacts with D160 and the oxygen of Q207 and the OH at C-1 with W159 and the backbone carbonyl of G310.

The nitrogen atom in the pyrrolizidine ring interacts with the same water molecule. In ring B, the hydroxyl group at C-7 interacts with W447 and a different water molecule (not shown).

In order to generate highly potent trehalase inhibitors with potential insecticide activity, we studied the inhibition activity of two casuarine-6-*O*-α-D-glucoside analogues **51** and **52** (Figure 5.13). Compounds **51** and **52** bear a hydrogen atom and a hydroxymethyl group in place of the hydroxy group at C-7 of casuarine-6-*O*-α-D-glucoside, respectively. We analysed the inhibitory potency of the compounds towards Tre37A and commercial porcine kidney trehalases, obtaining the K_i values in Table 5.5. Moreover, we succeeded in crystallizing and solving the structure of Tre37A in complex with **52** to gain insights into the interactions important for binding.

	Porcine Kidney K_i	*E. coli* Tre 37A K_i
50	11 nM	12nM
51	138 nM	86 nM
52	>10 μM	2.8 μM

Table 5.5. Inhibition of porcine kidney trehalase, and Tre37A by compounds 50, 51 and 52

The potency of **50, 51** and **52** towards the two trehalases showed a similar trend, with the strongest inhibition obtained using compound **50**. These direct comparisons demonstrate that the functional group at the C-7 position has a significant effect on the inhibition of the casuarine-6-*O*-α-D-glucoside analogues; a hydroxyl group appears to be preferential to a hydrogen atom, which in turn are both more potent than a hydroxymethyl group.

Molecular insights into casuarine-6-*O*-α-D-glucoside analogue inhibition can be obtained from the three-dimensional structure of the trehalase in complex with the inhibitor. Although it would be ideal to obtain both the structure of studied trehalase to demonstrate the basis for selectivity, to date only the trehalase from *E. coli* has been amenable to crystallization. We solved the structure of Tre37A in complex with **52**, with the aim of dissecting which

features make the inhibitor less favourable to this trehalase compared to **50**. X-ray data for Tre37A in complex with **52** were collected to 2.1 Å (see Supplementary Material for data collection and refinement statistics). Electron density clearly showed the presence of a molecule of **52** bound in the -1 (casuarine analogue moiety) and +1 (glucose) subsites of each molecule of Tre37A in the asymmetric unit (Figure 5.16a).

Compound **52** and active site residues overlap perfectly with Tre37A in complex with **50** (Figure 5.16b).

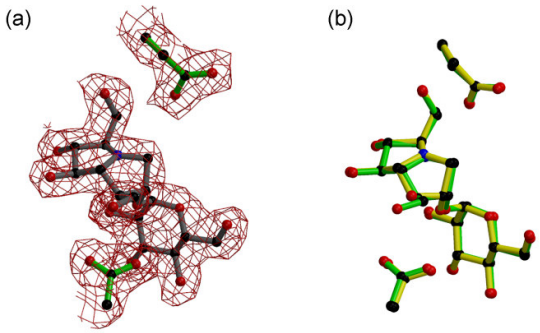


Figure 5.16. (a) Ball-and-stick representation of Tre37A in complex with 52. Glu496 (above), the catalytic acid and Asp312 (below), the catalytic base are shown. Observed electron density, contoured at 1σ (0.44 electrons /Å^3) for the maximum likelihood weighted $2F_{obs}$-F_{calc} map is shown. (b) Overlap between Tre37A in complex with 52 (green) and 50 (yellow)

As observed with the Tre37A complex with **50**, both pyrrolidine rings were found in an envelope conformation. The majority of the interactions with active site residues are the same as described previously and can be seen in Figure 5.17.

The only differences lie with the hydroxymethyl group at C-7. Whereas previously **50** was observed to make a weak (3.1 Å) hydrogen bond interaction with Trp447 and with a water molecule, **52** forms two hydrogen bonds with active site residues: Trp447 (at a distance of 2.7 Å) and the peptide carbonyl group of Asp312 (which incidentally is the catalytic base). The observation of the formation of additional interactions with active site residues with **52** compared to **50** makes it difficult to rationalize why it binds more weakly.

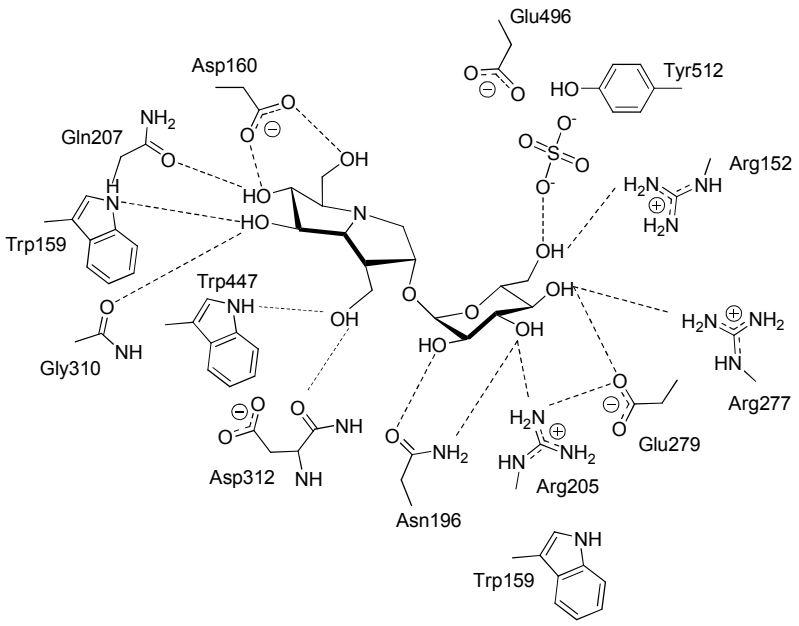


Figure 5.17. Active site interactions between Tre37A and 52

5.1.4.c Inhibition of trehalase from Chironomus riparius

This study has been performed in collaboration with Prof. Paolo Parenti (University of Milano-Bicocca). In particular, we thank Dr. Matilde Forcella for biological evaluation.

As with the design of any inhibitor, specificity is a significant concern. The design of a viable insecticide which targets a trehalase would therefore require it to be "mammal-safe" and thus only specific for insect trehalases.

A membrane-bound isoform of trehalase from midge larvae (*Chironomus riparius*) has been recently purified.[158] *C. riparius* represents a good model for biochemical studies and it was demonstrated that its resistance to stress factors is highly dependent on trehalose catabolism.[159] These studies revealed that trehalase from *C. riparius* is specific for trehalose

[158] Forcella, M.; Cardona, F.; Goti, A.; Parmeggiani, C.; Cipolla, L.; Gregori, M.; Schirone, R.; Fusi, P.; Parenti, P. *Glycobiology* **2009**, submitted.
[159] Forcella, M.; Berra, E.; Giacchini, R.; Parenti, P. *Arch. Insect Biochem. Physiol.*, 2007, **65**, 181.

and is competitively inhibited by known trehalase inhibitors. It can be considered a target for new and specific molecules having insecticide activity.

Compound **50** is the most potent trehalase inhibitor described to date with a K_i value against *C. riparius* of 0.66 nM, more potent than that displayed by validoxylamine A[156,160] and an order of magnitude lower than determined for trehazolin in other insect species.[154,156] However, compound **51** display a K_i value of 22 nM and in terms of selectivity, as well as potency, it is perhaps **52**, K_i = 157 nM, that is the most interesting compound of this set of inhibitors, as far their selectivity with respect to mammals trehalases and to other human/mammals glycosidases, is taken into account. Indeed, **52** displays nearly 20-fold selectivity for the insect trehalase over the *E. coli* enzyme, and is at least 100-fold selective over the porcine kidney trehalase (see Table 5.5). Additionally and more importantly, it is essentially inactive towards relevant α-glucosidases, such as NtMGAM and HPA.

[160] Asano, N.; Ymaguchi, T.; Kameda, Y.; Matsui. K. *J. Antibiotics*, 1987, **40**, 526.

5.2 Conclusion

In conclusion, the biological tests described in this chapter showed that several new inhibitors of glucoamylase from *Aspergillus Niger* were discovered and new potent and selective inhibitors of trehalases were synthesized.

The study toward commercially available glycosidases shows an interesting selective inhibitory activity of several molecules towards amyloglucosidase from *Aspergillus Niger,* and only a slightly diminished activity between casuarine (**11**) and its methoxy derivative (**94**) which opens the way to the synthesis of a new class of glycosidases inhibitors. The modelling study gave us useful suggestions for the rational design of new glucosidase inhibitors.

The study on NtMGAM and Tre37A, demonstrates that among glycoside hydrolase enzymes that belong to different families and clans, and which differ in mechanism of action, there is a degree of conservation of the analogous active site residues reflecting the convergent evolution of optimised non-covalent interactions. The binding of inhibitors **11** and **50** were investigated with NtMGAM and Tre37A by kinetic and structural methods, which provided information on the relevant interactions in the active site and could form the basis for the synthesis of analogues. This study shows that casuarine can be envisaged as a lead compound for the construction of novel disaccharide and polysaccharide type glycoside hydrolase inhibitors, the syntheses of which are currently ongoing in our laboratories.

Comparison of the inhibitor activity of target molecules on different trehalases showed that compounds **50** and **52** are at least one order of magnitude more potent towards the *C. riparius* trehalase than both the *E. coli* and porcine trehalases (Table 5.6).

	Porcine Kidney, K_i	*E. coli* Tre37A, K_i	*C. riparius,* K_i
50	11 nM	12nM	0.66 nM
51	138 nM	86 nM	22 nM
52	>10 μM	2.8 μM	157 nM

Table 5.6. Inhibition of porcine kidney trehalase, Tre37A and *C. riparius* trehalase by compounds 50, 51 and 52

As promiscuous inhibition is a potential problem for agricultural applications of trehalase inhibitors, the specificity of these compounds for trehalases towards other glycosidases was also addressed. The inhibition of **50**, **51** and **52** towards two human glycosidases, N-terminal human maltase glucoamylase (NtMGAM)[147] and human pancreatic amylase (HPA) was assessed.[161,162] All compounds were found not to inhibit HPA at a concentration of 100 μM. Of the three compounds, only **50** inhibits NtMGAM to any significant extent, with a K_i of 280 μM;[72] a value that is around 20,000-fold less than with the insect trehalase.

For all these reasons the tested compounds are especially attractive since they bind only weakly to relevant human enzymes displaying significant selectivity for the insect enzyme.

[161] Brayer, G. D.; Sidhu, G.; Maurus, R.; Rydberg, E. H.; Braun, C.; Wang, Y. L.; Nguyen, N. T.; Overall, C. M.; Withers, S. G. *Biochemistry*, 2000, **39**, 4778.
[162] Numao, S.; Maurus, R.; Sidhu, G.; Wang, Y.; Overall, C. M.; Brayer, G. D.; Withers, S. G. *Biochemistry*, 2002, **41**, 215.

5.3 Experimental section

5.3.1 Inhibition of glycosidases

The experiments were performed essentially as follows:0.01-0.5 unit/mL of enzyme (1 unit = 1 mol of glycoside hydrolyzed/min), preincubated for 5 min at 20 °C with the inhibitor, and increasing concentration of aq. sol. of the appropriate p-nitrophenyl glycoside substrates (buffered to the optimum pH of the enzyme) were incubated for 20 min at 37 °C. The reaction was stopped by the addition of 100 µL of 0.3 M sodium borate buffer pH 9.8. The p-nitrophenolate formed was quantified at 405 nm and IC_{50} values were calculated. Double-reciprocal (Lineweaver-Burk) plots were used to determine the inhibition characteristics and the K_i of each compound.

5.3.2. Modeling studies of the amyloglucosidase/inhibitors interactions

The ligand structures (Table 5.4) were built using Maestro v8.5.[163] All the molecules were subjected to conformational search and clusterization with Macromodel 9.6[164] in order to sample the most accessible conformations for both the aglyconic and glucose moieties. The bridgehead nitrogen atoms were treated as ionized to better simulate the physiological conditions, except for lactam intermediates.

5.3.3 Inhibition of a human maltase glucoamylase

Inhibition assays of NtMGAM were carried out in 96-well microtitre plates containing 100 mM MES buffer, pH 6.5, inhibitor and *p*-nitrophenol-D-glucopyranoside (pNP-glucose, Sigma) as substrate (2.5-30 mM). The reaction

[163] Maestro v. 8.5, Schrödinger, L. L. C., New York, NY, 2008, available at: http://www.schrodinger.com.
[164] Macromodel v. 9.6, Schrödinger, L. L. C., New York, NY, 2008, available at: http://www.schrodinger.com.

was allowed to proceed for 50 minutes at 37 °C before quenching with 0.5 M sodium carbonate and measuring the release of the *p*-nitrophenolate ion at 405 nm.

The K_i values of **11** and **50** against NtMGAM were calculated by determining the reaction rates of the enzymes in the absence and presence of inhibitor. Concentrations of 0.2-0.6 µM **11** or 0.4-1.2 mM **50** were used to inhibit NtMGAM Rates were determined and the data fitted to the Michaelis-Menten equation in GRAFIT (Erithacus Software Ltd., Horley, UK) to obtain a K_M (in absence of inhibitor) or apparent K_M (K_M^{app}) (in presence of inhibitor). K_i values were determined using the equation $K_M^{app} = K_M (1+[I]/K_i)$.

The crystallization of NtMGAM was previously reported by Sim *et al.*[147] The complex of NtMGAM with **11** was obtained by soaking NtMGAM crystals for ~12 hrs in mother liquor supplemented with 200 µM **11**. X-ray diffraction data were collected on an ADSC Quantum-4 CCD detector at beamline F1 at the Cornell High Energy Synchrotron Source (CHESS) and were processed with HKL2000.[165] Since the crystal of NtMGAM in complex with **11** was isomorphous to the crystal of NtMGAM in complex with acarbose,[147] this structure was used as an initial model to calculate F_o-F_c maps. These difference maps clearly revealed the position of casuarine in the NtMGAM active site. Inhibitor topologies and restraints were generated using the PRODRG server (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/) and the model was subsequently modified and refined using COOT[166] and REFMAC.[167]

[165] Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307.
[166] Emsley, P.; Cowtan, K. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60*, 2126.
[167] Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 240.

5.3.4 Inhibition of trehalases

5.3.4.a Inhibition of trehalase from Escherichia coli (Tre 37A)

Inhibition of Tre37A were determined using a stopped assay, where glucose was detected using glucose oxidase/peroxidase linking enzymes (Megazyme, Bray, Eire) in the same way as described previously.[156] Measurements were made at trehalose concentrations between 0.05 and 6 mM and Tre37A was present at a final concentration of 0.7 nM.

The K_i values of **11** and **50** against Tre37A were calculated by determining the reaction rates of the enzymes in the absence and presence of inhibitor. Concentrations of 10-40 µM **11** or 10-20 nM **50** were used to inhibit Tre37A. Rates were determined and the data fitted to the Michaelis-Menten equation in GRAFIT (Erithacus Software Ltd., Horley, UK) to obtain a K_M (in absence of inhibitor) or apparent K_M (K_M^{app}) (in presence of inhibitor). K_i values were determined using the equation $K_M^{app} = K_M (1+[I]/K_i)$.

Gene expression and purification of Tre37A was carried out as described previously.[157] Protein, at a concentration of 10-12 mg/mL, was co-crystallized with 5 mM 2 from 1.7 M ammonium sulfate and 0.1 M citric acid buffer, pH 3.5. Crystals were cryoprotected in the mother liquor supplemented with 25% ethylene glycol and flash frozen. Data were collected on beamline ID29 at the European Synchrotron Radiation Facility (ESRF), and were processed with the HKL2000 suite.[165] As the cell dimensions differed from previous complexes of Tre37A, structure solution required molecular replacement in PHASER[168] using a monomer of 2JF4 as the search model. The model was refined with manual building in COOT[166] interspersed with refinement of geometric restraints in REFMAC.[167]

[168] McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. *J. Appl. Cryst.* **2007**, *40*, 658.

5.3.4.b Inhibition of trehalase from Chironomus riparius

Activity for the trehalases from *C. riparius* and porcine kidney was measured using a coupled assay with glucose-6-phosphate dehydrogenase and hexokinase according to the methods used by Wegener et al.[169] Enzyme assays were performed in triplicate at 30 °C using a Cary3 UV-Vis spectrophotometer. Enzyme activity was analyzed by Cary Win UV application software for Windows XP. The effect of compounds **50**-**52** on enzyme activity was evaluated at a fixed substrate concentration in the presence of increasing inhibitor concentrations. Substrate concentrations were 0.5 mM and 2.5 mM for *C. riparius* trehalase and porcine kidney trehalase, respectively: these values are the corresponding K_m values as determined by kinetic studies. The assay was performed in sodium acetate buffer at pH 6.5 as described by Wegener et al.[169] Previous studies[158] revealed that the optimum pH is 6.5. Enzyme concentration in the text tube was 4 nM and 37 nM for *C. riparius* and pig kidney trehalase, respectively. Initial rates as a function of inhibitor concentration were fitted in Sigma Plot (Jandel, CA) to the following equation:

$$\frac{v_i}{v_0} = \frac{1}{1 + \frac{[I]}{IC_{50}}}$$

where v_i and v_0 are the initial rates in the presence and in the absence of inhibitor, respectively, [*I*] the inhibitor concentration, and IC_{50} the inhibitor concentration producing half-maximal inhibition. Enzyme activities were determined in the presence of 1-1000 nM **50,** 0.01-100 µM **51** and 0.1-1000 µM **52**. K_i values were calculated using the Cheng-Prusoff relationship:

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_M} \right)$$

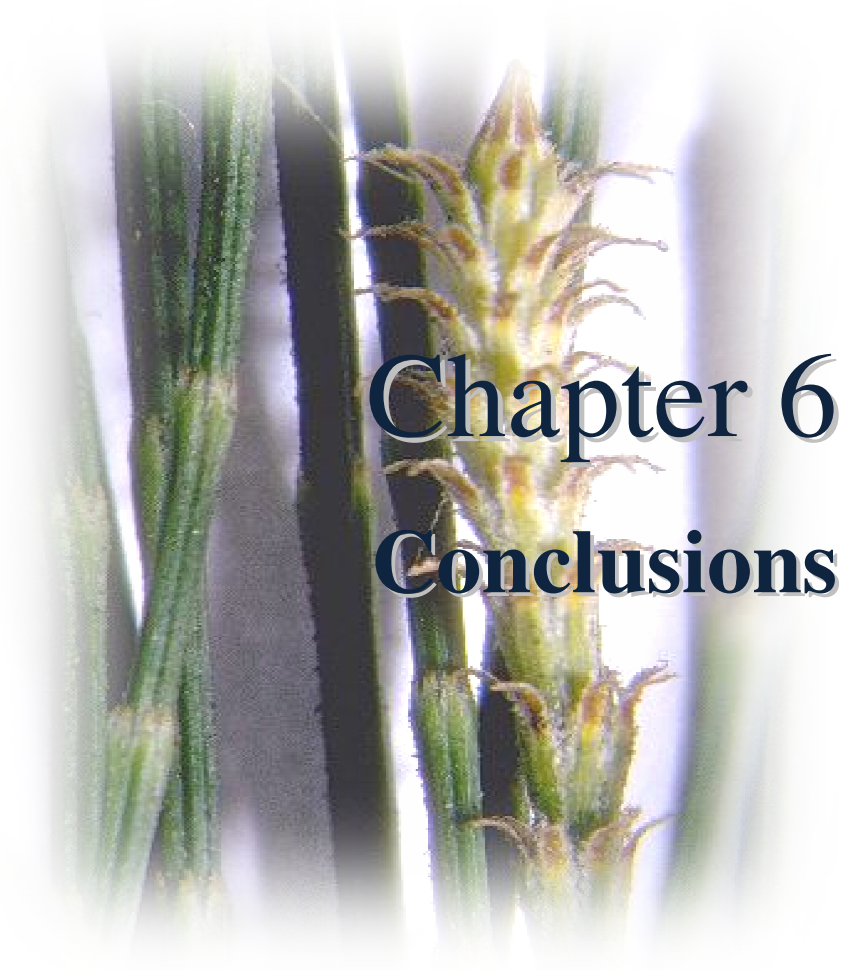
[169] Wegener, G.; Tschiedel, V.; Schlöder, P.; Ando, O. *J. Experim. Biol.*, **2003**, *206*, 1233.

assuming competitive mechanism of inhibition. Competition was assessed by measuring enzyme activity at fixed inhibitor concentration and increasing trehalose concentrations. As the estimated IC_{50} values of **50** and **51** approached the enzyme concentration (E_T), to calculate the corresponding K_i values data were fitted to the Morrison equation for tight binding inhibition:[170]

$$\frac{v_i}{v_0} = 1 - \frac{\left([E]_T + [I]_T + K_i^{app}\right) - \sqrt{\left([E]_T + [I]_T + K_i^{app}\right)^2 - 4[E]_T [I]_T}}{2[E]_T}$$

where K_i^{app} is equivalent to the IC_{50}.

[170] Copeland, R.A. *Evaluation of Enzyme Inhibitors in Drug Discovery,* **2005**, Wiley & Sons, NJ.



Chapter 6

Conclusions

6.1 Summary and Conclusion

In this work we accomplished the total syntheses of a variety of natural and unnatural pyrrolidine alkaloids (Figure 6.1), pyrrolizidine alkaloids (Figure 6.2) and their glucosyl derivatives (Figure 6.3).

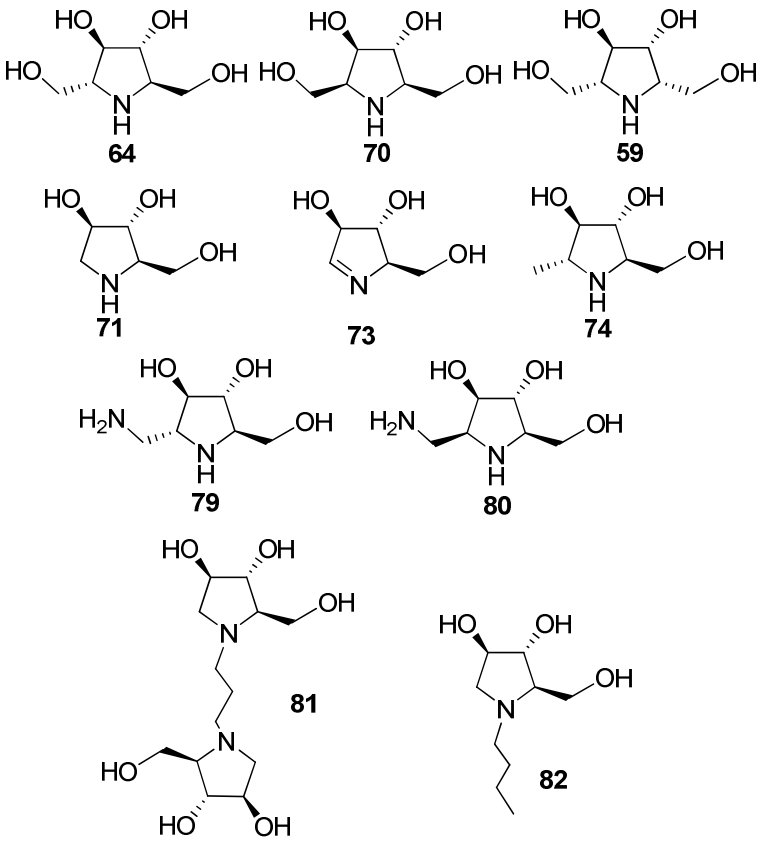


Figure 6.1. Pyrrolidine alkaloids synthesized

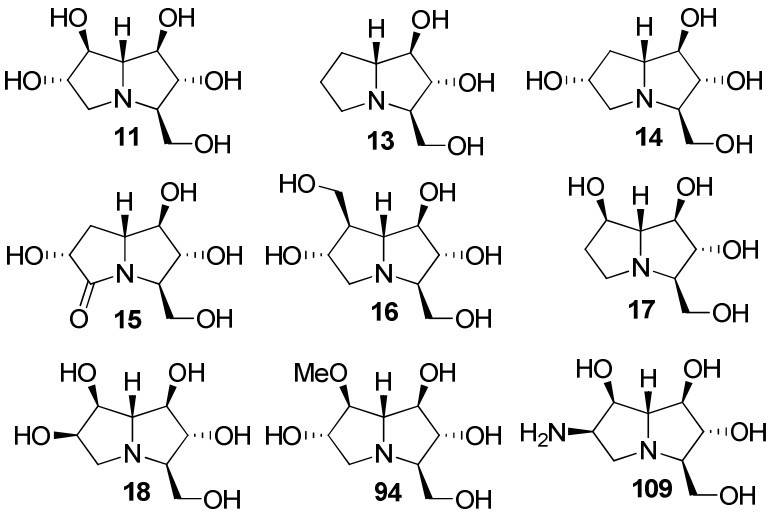


Figure 6.2. Pyrrolizidine alkaloids synthesized

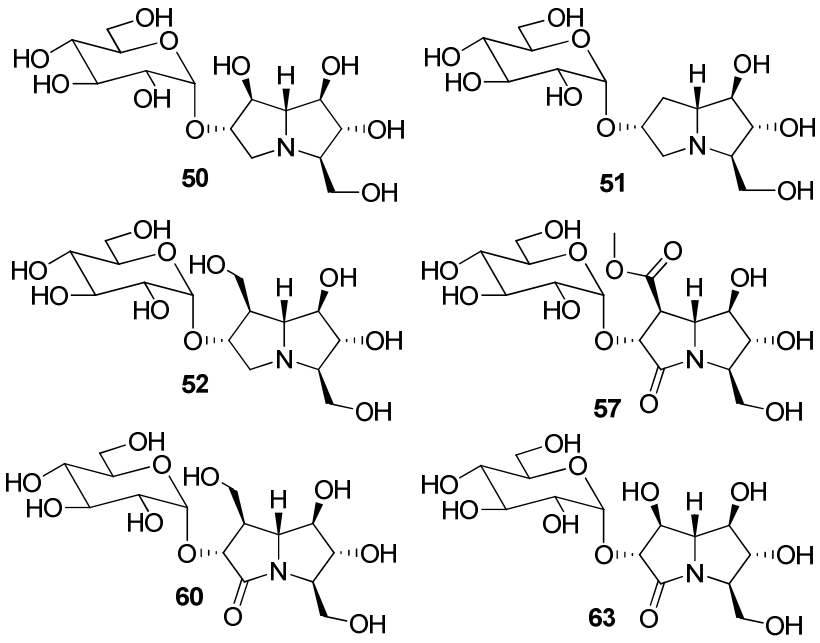


Figure 6.3. Glucosidic derivatives synthesized

The targets were obtained by two different synthetic strategies: the first one is based on a highly regio- and totally stereoselective 1,3 dipolar cycloaddition of suitable substituted alkenes to a carbohydrate-derived nitrone; and the second one is based on a nucleophilic addition of organometallic reagents or allenes to the same carbohydrate-derived nitrone.

All the target molecules were tested towards a wide range of glycosidases (see Chapter 5) like commercially available glycosidases, trehalases and human maltase-glucoamylase and many of them showed interesting inhibition properties.

The synthesis of other natural pyrrolizidine alkaloids such as australine and 6,7-di*epi*casuarine underway in our research group, as well as the synthesis of other unnatural derivatives.

RINGRAZIAMENTI

Il primo, doveroso, ringraziamento è per la mia famiglia, che in questi anni mi ha supportata, sostentata e, sopratutto, sopportata!!!! So che è stata dura... GRAZIE!!!

Vorrei ringraziare Andrea per avermi dato la possibilità (ed aver avuto la pazienza) di farmi fare questo dottorato...e per avermi comprato il rotavapor!!!!

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Quiero agradecer a Pedro y Tomas por aceptarme en sus laboratorios, y a Inaki companiero de trabajo y ademas amigo... GRACIAS!

I really want to thank Prof. Reissing and his research group for the enjoyable hospitality he gave me in his laboratory.

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Un grazie speciale alle coinquiline (& Co.) di via del Risorgimento: Cri, Eli, Mari e Silvi avete reso quest'anno e mezzo indimenticabile... MI MANCHERETE!

Un grazie a Babi, Gulli e Clèo che hanno reso il mio soggiorno berlinese irripetibile.

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