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Synthesis, Biological Evaluation and Docking Studies of Casuarine Analogues: Effects of Structural Modifications at Ring B on Inhibitory Activity Towards Glucoamylase

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We report the total synthesis of a series of pyrrolizidine analogues of casuarine (**1**) and their 6-*O*-α-glucoside derivatives. The synthetic strategy is based on a totally regio- and stereoselective 1,3-dipolar cycloaddition of suitably substituted alkenes and a carbohydrate-based nitrone. We also report the evaluation of the biological activity of casuarine and its derivatives towards a wide range of glycosidases and a molecular modeling study focused on glucoamylase (GA) in which the binding modes of the newly synthesized compounds within the enzyme cavity are investigated. The results highlight the prominent structural features of casuarine and its derivatives that make them selective glucoamylase inhibitors.

Introduction

Iminosugars are very attractive carbohydrate mimics in which the endocyclic oxygen atom is replaced by the more basic, trivalent nitrogen atom.[1] In their protonated form, iminosugars resemble the transition state or intermediate generated during the hydrolysis reaction catalysed by glycosidases, key hydrolytic enzymes involved in many physiological functions. Since the discovery of the inhibitory properties of iminoalditols towards glycosidases, they have received increasing attention as diagnostic compounds as well as tools for the investigation of the structures, functions and catalytic mechanisms of carbohydrate-processing enzymes.[2,3] Furthermore, given the important role of glycosidases and glycosyltransferases in controlling the structures and functions of carbohydrates at the cell surface, competitive inhibitors of these classes of enzymes are potential antidiabetes, anti-viral and anti-cancer agents.[1,2] Recently, interesting immunosuppressive activities have been discovered for this class of compounds.[4] In the past 40 years, more than 100 polyhydroxylated alkaloids have been isolated from plants and microorganisms[5] with structures that include polyhydroxylated piperidines, pyrrolidines, indolizidines, pyrrolizidines and nortropanes. For instance, the piperidine alkaloid 1-deoxynojirimycin (DNJ, Scheme 1), prepared first by Paulsen et al. in 1967[6a] and then isolated from a species of *Moris* (Moraceae),[6b] was found to strongly inhibit α-glucosidases.[5a] *N*-Alkylated derivatives of DNJ have found applications as anti-diabetic drugs (i.e., Miglitol, Glyset) or anti-HIV agents (Glycovir, SC 49483).[2a] The indolizidine alkaloid (+)-lentiginosine (Scheme 1) was isolated in 1990 from the leaves of *Astragalus lentiginosus* and was found to inhibit amyloglucosidases.[7] Its non-natural enantiomer (–)-lentiginosine was recently discovered to possess proapoptotic activity towards tumoral cells.[8] Castanospermine (Scheme 1), isolated in 1981 from the seeds, leaves and barks of *Castanospermum australe* and in 1988 from the seeds, leaves and barks of *Alexa sp.*,[5a] and its ester and salt derivatives are able to inhibit tumour growth and metastasis.[9]

Casuarine (**1**, Scheme 2) and its 6-*O*-α-glucoside, casuarine-6-*O*-α-glucopyranoside (**2**, Scheme 2), have been isolated from the bark of *Casuarina equisetifolia* L. (Casuarinaceae) and from the leaves of *Eugenia jambolana* Lam. (Myrtaceae).[10] We recently reported that casuarine (**1**) is able to inhibit a human maltase-glucoamylase (MGAM,

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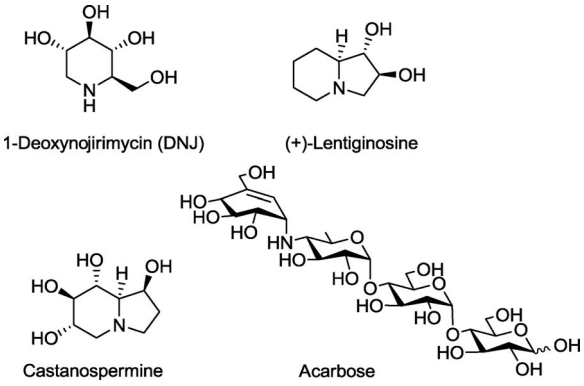
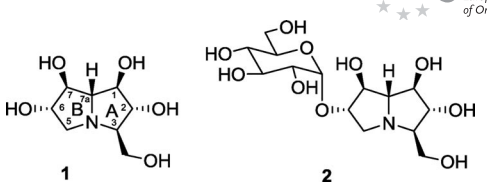


Scheme 1. Glycosidase inhibitors.

EC 3.2.1.20) more strongly than the pseudo-tetrasaccharide acarbose (Scheme 1) currently on the market as an anti-diabetic drug (Glucobay, Precose) and thus has promise for the development of novel anti-diabetic drugs.[11]

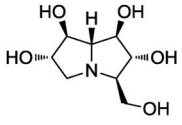
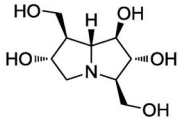
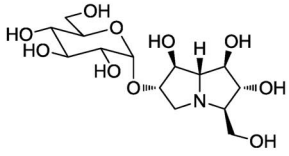
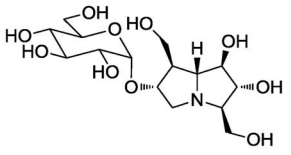
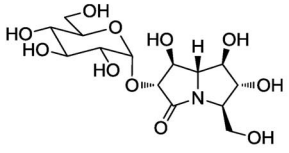
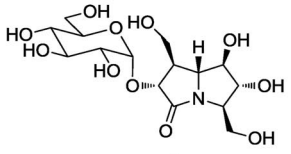
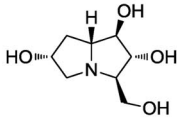
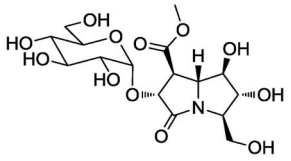
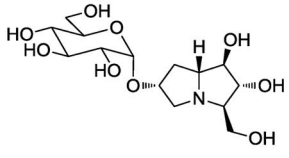
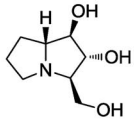
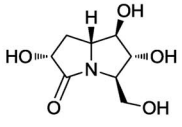


Scheme 2. Casuarine and its 6-*O*-α-D-glucoside.

Our total synthesis of casuarine (**1**) and its 6-*O*-α-glucoside (**2**) took advantage of a complete stereoselective nitrone cycloaddition strategy with Tamao–Fleming oxidation and selective α-glucosylation as the key steps.[11]

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 catalyses 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 at the anomeric carbon atom. Glucoamylases are also able to hydrolyse α-1,6 linkages, but the specific activity is

Table 1. Structures and inhibition activities (IC_{50}) of compounds **1**–**11** towards glucoamylase from *aspergillus niger*.

Molecule	Structure	IC_{50} [μM]	Molecule	Structure	IC_{50} [μM]
1 casuarine		1.9	**7** 7-homocasuarine		8.1
2		4.4	**8**		23.7
3		0%[a]	**9**		25%[a]
4 7-deoxycasuarine		4.5	**10**		76%[a]
5		7.7	**11** hyacinthacine A_2 (6,7-dideoxycasuarine)		1.9
6		214			

[a] For compounds with an inhibition percentage less than 90% at 1 mM concentration, the IC_{50} values were not determined, and the percentage inhibition is reported.

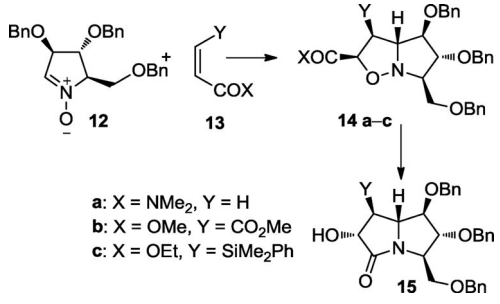
only 0.2% with respect to α-1,4 hydrolysis.[12] The interest in glucoamylase is related to its use in the industrial production of bioethanol, glucose and fructose syrups.[13] Furthermore, given the presence of these enzymes in a wide variety of organisms and their quite simple obtainment in a pure form, GA has been extensively studied as a model for other members of the glycosyl hydrolase family.[14]

In this paper we report the total synthesis of the pyrrolizidine analogues of casuarine **4**, **6**, **7** and **11** (see Table 1), all bearing the same stereochemical pattern at the more substituted five-membered ring A as that of casuarine, and their 6-*O*-α-glucoside derivatives **3**, **5** and **8**–**10**. We also report the evaluation of the inhibitory activities of compounds **1**–**11** towards a wide range of commercially available glycosidases and a molecular modelling study on glucoamylase (GA) from *Aspergillus awamori* as a result of the selective inhibitory activity towards GA shown by the tested compounds.

Results and Discussion

Synthesis

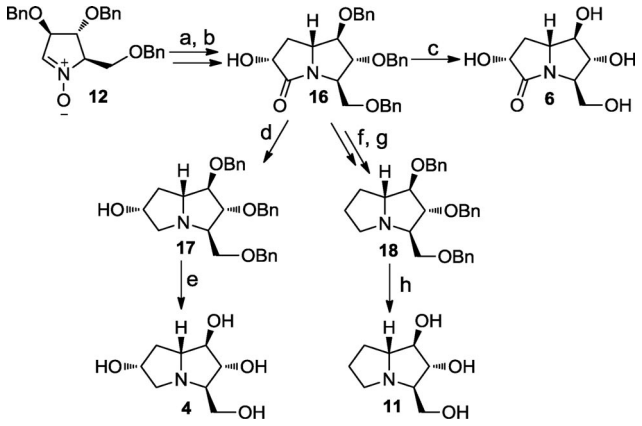
The general strategy followed for the synthesis of the pyrrolizidine alkaloids is outlined in Scheme 3. We took advantage of a stereocontrolled cyclic nitrone cycloaddition strategy[15] employing polyfunctionalized nitrone **12** and suitable dipolarophiles **13**, which afforded regio- and stereoselectively isoxazolidines **14**. These were then converted into pyrrolizidinone derivatives **15** by reductive ring-opening/cyclization. Intermediates **15** bear a free hydroxy group at C6 of the pyrrolizidine ring, which allows selective glucosylation at this position. A good choice of dipolarophile is crucial for the success of the strategy. For instance, for the synthesis of casuarine (**1**) a good regioselectivity of the cycloaddition was assured by using dipolarophile **13** with Y = $SiMe_2Ph$ and X = OEt.[11]



Scheme 3. General procedure for the synthesis of the pyrrolizidine alkaloids.

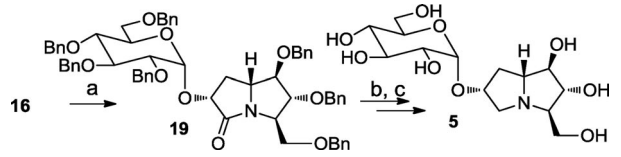
Nitrone **12** was conveniently prepared on a multigram scale by starting from commercially available tribenzyl D-arabinose.[16] It has the absolute configuration of the stereogenic centres at C1, C2 and C3 required for casuarine and its analogues such as non-natural 7-deoxycasuarine (**4**),[16a,17] its lactam derivative **6** and hyacinthacine A_2 (**11**).[16a,18]

The first key step in the synthesis of 7-deoxycasuarine (**4**), its lactam derivative **6** and hyacinthacine A_2 (**11**) is the 1,3-dipolar cycloaddition of nitrone **12** to dimethylacrylamide, which, after N–O bond cleavage of the isoxazolidine **14a** with Zn in acetic acid gave lactam **16** in 68% yield over two steps (Scheme 4). Compound **16** is the key intermediate for the total synthesis of all three target molecules. Hydrogenation in EtOH catalysed by Pd/C afforded lactam **6** in 88% yield. Reduction of the C=O bond with $LiAlH_4$ in THF at reflux gave compound **17** in 75% yield, which, after catalytic hydrogenation in EtOH, afforded 7-deoxycasuarine (**4**) in 88% yield (Scheme 4). Deoxygenation at C6 was achieved through the mesylation of **16** followed by reduction with $LiAlH_4$ in THF at reflux. This gave **18** in 80% yield over two steps. Finally, catalytic hydrogenation gave hyacinthacine A_2 (**11**) in 72% yield (Scheme 4).



Scheme 4. Syntheses of 7-deoxycasuarine (**4**), its lactam derivative **6** and hyacinthacine A_2 (**11**): Reagents and conditions: (a) dimethylacrylamide, CH_2Cl_2, room temp., 3 d, 85%; (b) Zn, AcOH/H_2O, 50 °C, 4 h, 80%; (c) H_2, Pd/C, EtOH, 3 d, 88%; (d) $LiAlH_4$, THF, reflux, 3 h, 75%; (e) H_2, Pd/C, HCl, EtOH, 3 d, 88%; (f) MsCl, NEt_3, CH_2Cl_2, room temp., 2 h, 100%; (g) $LiAlH_4$, THF, reflux, 1.5 h, 80%; (h) H_2, Pd/C, HCl, MeOH, 3 d, 72%.

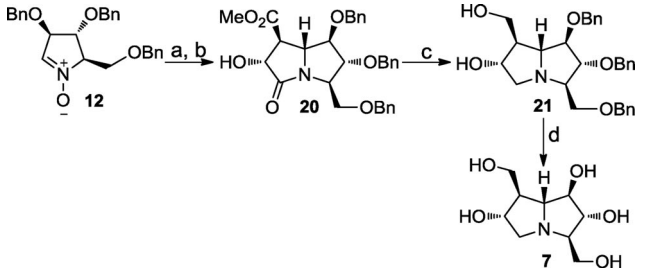
The lactam intermediate **16** was also employed in the synthesis of glucoside **5**; the initial selective α-glucosylation provided compound **19**.[19] Reduction of its amide moiety with $LiAlH_4$ followed by catalytic hydrogenolysis gave **5** in 45% yield over two steps (Scheme 5).



Scheme 5. Synthesis of 7-deoxycasuarine glucosyl derivative **5**: Reagents and conditions: (a) 2,3,4,6-tetra-*O*-benzylglucopyranosyl trichloroacetimidate, TMSOTf, Et_2O, 1 h, 88%; (b) $LiAlH_4$, THF, room temp., 1 h, 58%; (c) H_2, Pd/C, HCl, MeOH, room temp., 18 h, 77%.

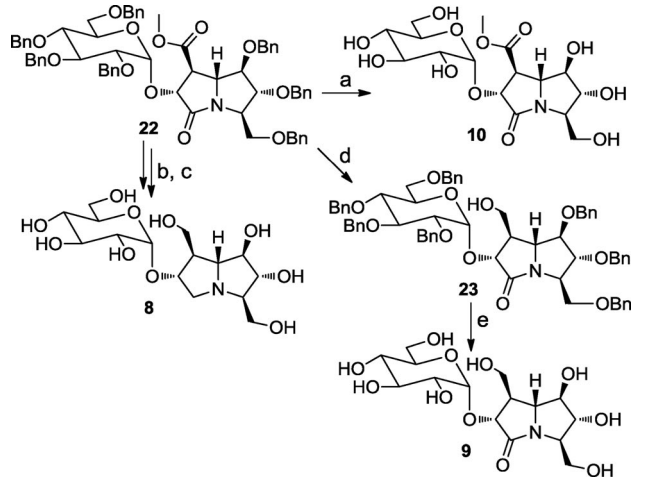
For the synthesis of the non-natural 7-homocasuarine (**7**) and of its glucosyl derivatives **8**–**10**, dimethyl maleate was chosen as the dipolarophile. Treatment of the isoxazolidine

14b with Zn in acetic acid at 50 °C for 3 h gave lactam **20** in 70% yield over two steps (Scheme 6). Reduction of both the ester and lactam moieties with excess $LiAlH_4$ in THF at reflux provided diol **21** quantitatively. Finally, catalytic hydrogenolysis with Pd/C as catalyst in the presence of HCl gave non-natural 7-homocasuarine (**7**) in 89% yield (Scheme 6).



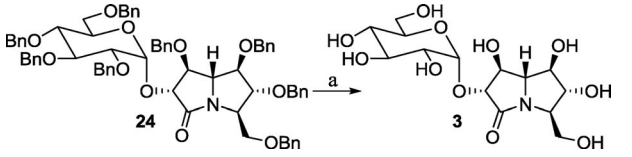
Scheme 6. Synthesis of 7-homocasuarine (**7**). Reagents and conditions: (a) dimethyl maleate, CH_2Cl_2, room temp., 4 d, 78%; (b) Zn, AcOH/H_2O, 50 °C, 3 h, 90%; (c) $LiAlH_4$, THF, reflux, 2 h, 100%; (d) H_2, Pd/C, HCl, EtOH, room temp., 4 d, 89%.

Lactam **20** was also selectively α-glucosylated to give α-glucoside **22** in 75% yield.[19] Intermediate **22** could be manipulated in different ways allowing us to obtain the three glucosyl derivatives **8**–**10**. Catalytic hydrogenolysis of **22** afforded compound **10** (96%), which retained both the ester and lactam moieties. Treatment of **22** with excess $LiBH_4$ and BH_3·THF[20] led to the complete reduction of both the ester and lactam moieties, and subsequent catalytic hydrogenolysis afforded the target glucosylated 7-homocasuarine **8** in 77% yield over two steps. Selective reduction of the ester moiety was achieved by treatment of **22** with $LiBH_4$ in THF at room temperature for 18 h. This afforded **23** in 62% yield. Finally, catalytic hydrogenolysis of **23** provided glucoside **9** in 72% yield (Scheme 7).



Scheme 7. Synthesis of 7-homocasuarine glucosyl derivatives **8**–**10**. Reagents and conditions: (a) H_2, Pd/C, MeOH/EtOAc, room temp., 4 d, 96%; (b) $LiBH_4$, BH_3·THF, THF, room temp., 11 d, 98%; (c) H_2, Pd/C, HCl, MeOH, room temp., 12 h, 79%; (d) $LiBH_4$, THF, room temp., 18 h, 62%; (e) H_2, Pd/C, MeOH, 24 h, 72%.

We recently presented the total synthesis of casuarine (**1**) and its 6-*O*-α-glucoside **2**,[11,21] as well as its epimer at C6, namely uniflorine A,[22] obtained from a derivative of lactam **15** (Y = $SiMe_2Ph$) by inversion of the configuration at C6. We have now also synthesized the lactam derivative **3** in 77% yield by hydrogenolysis of the fully protected lactam **24** (Scheme 8).[11]



Scheme 8. Synthesis of casuarine 6-*O*-α-glucoside derivative **3**. Reagents and conditions: (a) H_2, Pd/C, MeOH/EtOAc, room temp., 24 h, 77%.

The lactams **10**, **9** and **3** were synthesized to investigate the importance of the basic nitrogen atom in glycosidase inhibition. With all these compounds in hand, we investigated their inhibitory activity towards a wide range of commercially available glycosidases.

Glycosidase Inhibitory Activities

Compounds **1**–**11** were assayed with respect to a panel of 13 commercially available glycosidases (Table 2): α-L-fucosidase (EC 3.2.1.51) from bovine kidney, α-galactosidase (EC 3.2.1.22) from coffee beans, β-galactosidase (EC 3.2.1.23) from *Escherichia coli* and *Aspergillus orizae*, α-glucosidase (EC 3.2.1.20) from yeast and rice, amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*, β-glucosidase (EC 3.2.1.21) from almonds, α-mannosidase (EC 3.2.1.24) from jack beans, β-mannosidase (EC 3.2.1.25) from snails, β-xylosidase (EC 3.2.1.37) from *Aspergillus niger*, β-*N*-acetylglucosaminidase (EC 3.2.1.30) from jack beans and bovine kidney with appropriate *p*-nitrophenyl glycoside substrates.[23] The errors in the measurements were estimated to be around 20% (statistical study carried out with model compounds) and in the concentrations to be around 10–15% (errors in sample weight). Casuarine (**1**) was found 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; ref.[24] IC_{50} = 0.7 μM). It also inhibits α-glucosidase from yeast and rice (91 and 94% at 1 mM, respectively) and, to a lesser extent, β-glucosidase from almonds, α-mannosidase from jack beans, β-xylosidase from *Aspergillus niger* and β-*N*-acetylglucosaminidase from jack beans (46, 21, 24 and 16% at 1 mM, respectively). Glucoside **2** was slightly less potent with the IC_{50} and K_i values in the same order of magnitude (IC_{50} = 4.4 ± 0.9 μM, K_i = 3.9 ± 0.8 μM, mixed-type inhibition; ref.[24] IC_{50} = 1.1 μM), but more selective than casuarine: indeed at 1 mM concentration it gave only 20% inhibition towards α-glucosidase from yeast and did not inhibit at all α-glucosidase from rice. Moreover, it showed only 19% inhibition towards α-L-fucosidase from bovine kidney (1 mM). Glucosides **5** and **8** and 7-homocasu-

arine (**7**) 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 and 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 1 mM, respectively) and did not inhibit α-glucosidase from rice. 7-Deoxycasuarine (**4**) was a very potent and selective competitive 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 **6** exhibited weak (IC_{50} = 0.21 ± 0.04 mM) but very selective inhibitory activity towards amyloglucosidase from *Aspergillus niger*. Hyacinthacine A_2 (6,7-dideoxycasuarine, **11**) was also a very strong inhibitor of this enzyme (IC_{50} = 1.9 μM ± 0.4 μM, K_i = 2.6 μM ± 0.5 μM, non-competitive inhibition; ref.[25] IC_{50} = 8.6 μM). Among the remaining compounds, **3** was not active towards any of the glycosidases assayed, **9** and **10** showed weak and very selective inhibitory activity (25 and 76% inhibition at 1 mM, respectively) towards amyloglucosidase from *Aspergillus niger*. Glucosides **5** and **8** together with the parent compound **2** have also been found to be potent inhibitors of bacterial and insect trehalases.[19]

Table 2. Inhibitory activity of compounds **1**–**11** towards commercially available glycosidases.[a]

Enzyme (pH)	**1**	**2**	**3**	**4**	**5**	**6**
α-L-Fucosidase Bovine kidney (6)	n.i.	19	n.i.	n.i.	n.t.	n.i.
α-Galactosidase Coffee beans (6)	n.i.	n.i.	n.i.	n.i.	n.t.	n.i.
β-Galactosidase *Escherichia coli* (7)	n.i.	n.i.	n.i.	n.i.	n.t.	n.i.
Aspergillus orizae (4)	n.i.	n.i.	n.i.	n.i.	n.t.	n.i.
α-Glucosidase Yeast (7)	91	20	n.i.	n.i.	29	n.i.
Rice (4)	94	n.i.	n.i.	36	n.i.	n.i.
Amyloglucosidase *Aspergillus niger* (5)	98 IC_{50} = 1.9 μM K_i = 2.0 μM	97 IC_{50} = 4.4 M K_i = 3.9 μM	n.i.	100 IC_{50} = 4.5 μM K_i = 3.5 μM	97 IC_{50} = 7.7 μM K_i = 7.4 μM	91 IC_{50} = 0.21 mM
β-Glucosidase Almonds (5)	46	n.i.	n.i.	n.i.	n.t.	42
α-Mannosidase Jack beans (5)	21	n.i.	n.t.	n.i.	n.t.	n.i.
β-Mannosidase Snails (4)	n.i.	n.t.	n.t.	n.i.	n.t.	n.i.
β-Xylosidase *Aspergillus niger* (5)	24	n.t.	n.t.	n.i.	n.t.	n.i.
β-*N*-Acetylglucosaminidase Jack beans (5)	16	n.t.	n.i.	n.i.	n.t.	44
Bovine kidney (4)	n.i.	n.t.	n.i.	n.t.	n.t.	20

Enzyme (pH)	**7**	**8**	**9**	**10**	**11**
α-L-Fucosidase Bovine kidney (6)	n.i.	n.t.	n.i.	n.i.	n.t.
α-Galactosidase Coffee beans (6)	n.i.	n.t.	n.i.	n.i.	n.i.
β-Galactosidase *Escherichia coli* (7)	n.i.	n.t.	n.i.	n.i.	n.i.
Aspergillus orizae (4)	n.i.	n.t.	n.i.	n.i.	n.i.
α-Glucosidase Yeast (7)	45	55	n.i.	n.i.	72
Rice (4)	n.i.	n.i.	n.i.	n.i.	n.i.
Amyloglucosidase *Aspergillus niger* (5)	99 IC_{50} = 8.1 μM K_i = 11 μM	92 IC_{50} = 24 μM K_i = 23 μM	25	76	97 IC_{50} = 1.9 μM K_i = 2.6 μM
β-Glucosidase Almonds (5)	n.t.	n.t.	n.i.	n.i.	n.i.
α-Mannosidase Jack beans (5)	n.t.	n.t.	n.i.	n.i.	n.i.
β-Mannosidase Snails (4)	n.t.	n.t.	n.t.	n.i.	n.i.
β-Xylosidase *Aspergillus niger* (5)	n.t.	n.t.	n.t.	n.i.	n.i.
β-*N*-Acetylglucosaminidase Jack beans (5)	n.t.	n.t.	n.t.	n.i.	n.i.
Bovine kidney (4)	n.t.	n.t.	n.t.	n.i.	n.i.

[a] Percentage inhibition at a concentration of 1 mM. n.i. = no inhibition, n.t. = test not performed.

As **2** is a glucoside we verified whether the amyloglucosidase was able to hydrolyse its glucosidic bond or not under our test conditions. Indeed, we had to consider the possibility that the inhibitory activities observed for **2** were due to the casuarine liberated by the hydrolysis reaction catalysed by amyloglucosidase. Thus, we performed a series of mass spectral analyses using HR-ESI-TOF-MS (high-resolution ESI mass spectrometry, positive ionization mode). As shown in Figure S48 (see the Supporting Information) the peak assigned to the glucoside (A), MW = 367.35, was detected in solution when the measurement was performed immediately after the addition of the enzyme (C) as well as after 20 min of incubation at room temperature (D) and after 20 min 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 aglycon (MW = 205.21) nor to glucose (180.16) alone (see Figure S48 of the Supporting Information). This indicates that glucoside **2** is not hydrolysed significantly by the enzyme (amyloglucosidase from *Aspergillus niger*) under the conditions of our test.

Computational Studies

The data reported in Table 2 show that the casuarine derivatives presented here, with the exception of glycosylated lactams **3**, **9** and **10**, all inhibit more than 90% of *Aspergillus niger* amyloglucosidase activity with IC_{50} values ranging from 1.9 μM for pyrrolizidines **1** and **11** to 24 μM for glucoside **8**, with lactam **6** showing the weakest activity (0.21 mM). Furthermore, the active compounds showed a competitive inhibition profile, thus indicating a similar binding mode within the enzyme. Nevertheless, no significant differences were found in the inhibition activity of glucosides and their parent compounds, which clearly indicates a lack of correlation between inhibition and the ability to occupy a second subsite.

In the past years, several crystallographic structures of the proteolytic fragment of glucoamylase G2 from *Aspergillus awamori* (95% sequence identity with the *A. niger* protein) bound to different inhibitors have been reported,[26] which has made it possible to investigate the nature of the interaction between glucoamylase and its ligands in detail. The enzyme active site is characterized by an excess of negative charge, which has been principally ascribed to residues D55, E179 and E400 from the –1 subsite. Furthermore, E179 and E400 have been shown to be the putative catalytic acid and base, respectively, and the hydrolysis reaction was hypothesized to proceed through the formation of a glucopyranosyl cation intermediate after nucleophilic attack by a water molecule. With this information in mind and with the aim to interpret the biological data in a structural way, we decided to study the docking on the glucoamylase structure to investigate the possible binding mode of the casuarine derivatives presented here.

After completion of docking calculations, ring A of casuarine (**1**) was found to be deeply located within the –1 site where it is involved in an optimal hydrogen-bonding network involving C8–OH and C2–OH of the ligand and active-site residues R54 and D55 together with the nucleophilic water Wat501. In addition, C1–OH is positioned at a hydrogen-bond distance from both the carbonyl oxygen atom of residue L177 and Wat501 (Figure 1). All other pyrrolizidine molecules were oriented in a similar way and conserved these interactions, which have also been observed in the crystallographic complexes with both DNJ and acarbose.[26a,26b]

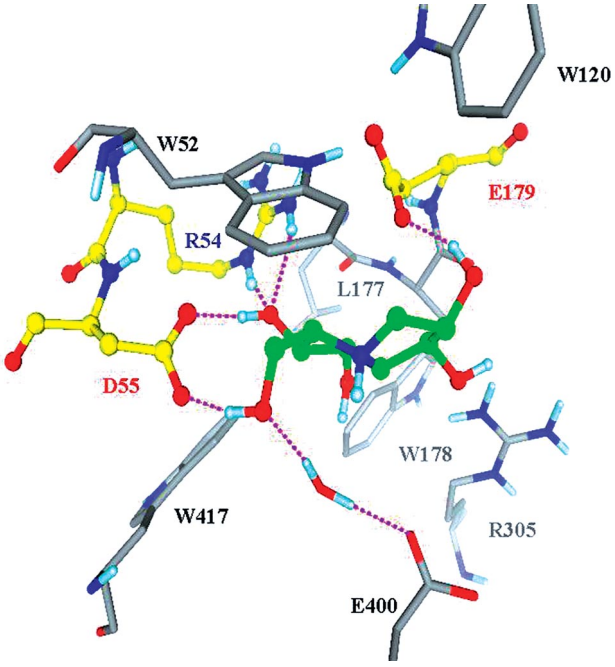


Figure 1. Docked orientation of molecule **1** within the glucoamylase active site. Hydrogen-bonds are depicted as magenta dashed lines.

As a consequence of this orientation, the protonated nitrogen atom of pyrrolizidine derivatives is not involved in the strong hydrogen-bond interaction with E179 observed for the nitrogen atom in the acarbose complex,[26b] but is oriented towards the region in which the nucleophile Wat501 lies, analogous to what was observed for the iminosugar-type inhibitor DNJ.[26a] We could hypothesize that the high affinity of acarbose (K_i = 10^{-12} M)[26b] for glucoamylase is due, at least in part, to the presence of the charged hydrogen-bond interaction with E179 given that it has been suggested that charged hydrogen bonds can be responsible for a change in the binding constant by a factor of 1000.[27] Indeed, a maltoside hetero-analogue carrying a nitrogen atom at the interglycosidic linkage, which enables it to establish a charged hydrogen bond with E179, showed a 1000-fold stronger competitive inhibition than the analogue in which the interglycosidic atom is sulfur.[28] In contrast, a comparison of the crystal complexes of GA with acarbose and its weaker D-*gluco*-dihydro derivative (K_i = 10^{-8} M) shows that the two molecules bind in a very similar

way (including the charged hydrogen bond with E179), and the 6 kcal/mol difference in binding energies could be largely attributed to unfavourable steric interactions between the hydrogen atoms at C7A of D-*gluco*-dihydroacarbose and the catalytic water,[26b] thus highlighting a comparable effect of electrostatic and hydrophobic interactions in guiding interactions with GA. Molecules **1**, **4**, **6** and **7** are able to bind E179 through a neutral hydrogen bond involving C6–OH, whereas the 6-deoxy derivative **11** clearly lacks this interaction. Anyway, apart from C6–OH when present, pyrrolizidine ligands are oriented towards the negatively charged E179, a very hydrophobic portion of the molecule, which could contribute negatively to the binding.

With respect to the crystallographic orientation of acarbose, the ring B atoms of the pyrrolizidines extend towards the +1 site, with C6 and C7 of **1** almost perfectly overlapped with C5B and N4B of the acarbose ring B, respectively. C7–OH of **1** is thus in close proximity to both R305 and the carbonyl oxygen atom of W178, but it does not present the correct geometry for hydrogen-bond formation, in contrast to what has been observed for C3B–OH of acarbose. The elimination of the C7 substituent (molecule **4**) led to a slight decrease in the IC_{50} value as well as its elongation (molecule **7**), although this latter modification allowed the molecule to hydrogen-bond to both W178 and R305. Molecule **6**, which shows a very weak inhibition, is the only non-glycosylated lactam of this series, and after docking it is oriented like molecule **4**. Anyway, given the limited conformational flexibility imposed on molecule **6** by the presence of the lactam structure, the conformation of ring B is clearly influenced. Indeed, molecule **6** is also able to hydrogen-bond E179 through C6–OH, but the absence of the positive charge on the nitrogen atom eliminates the possibility of molecule **6** compensating the excess negative charge present in the glucoamylase active site, which has been hypothesized as one of the mechanisms involved in complex stabilisation.[26a,26b] Finally, the unfavourable effects due to the positioning of a hydrophobic portion of the ligand close to a charged amino acid (E179) is even more pronounced here given the higher hydrophobicity of **6** relative to **4**. All these considerations are in agreement with the very low IC_{50} value found for **6**.

As far as the glucoside derivatives are concerned, the results of the docking calculations are comparable for all molecules, with ligands showing two possible binding modes in which either the pyrrolizidine or the glucose moiety is oriented in the –1 site; we call these two orientations CAS-IN and GLU-IN, respectively. In the CAS-IN orientation, the position of the pyrrolizidine nucleus is almost coincident with the docked pose of the corresponding unglucosylated compounds, except for the conformation of ring B, which is influenced by the positioning of the glucose moiety, and for the lack of a hydrogen-bond donor for E179 at the C6 atom. The glucose moiety is oriented in the outer part of the +1 site, where it can assume different conformations that allow it to hydrogen-bond E180 and/or Y311. When the molecules adopt the GLU-IN orientation, the glucose in the –1 site overlaps well with DNJ and with ring A of acarbose. In all the selected poses showing this orientation the pyrrolizidine nucleus orients the $N–H^+$ towards the aromatic ring of Y311 in the +1 site with a geometry compatible with an NH···π interaction. Furthermore, C8–OH is able to donate one hydrogen bond to E180, thus contributing to the stabilization of this binding mode. Anyway, by comparison of the GLU-IN orientation of **2** with the docked orientation of the hydrolysable maltose, which perfectly overlaps the glucose moiety of **2** with its non-reducing end, it is not clear how the casuarine glucoside could resist hydrolysis, as we observed, because the nucleophile Wat501 is perfectly oriented towards the anomeric carbon atom of the ligand. In contrast, the CAS-IN binding mode of glucosides explains the resistance to hydrolysis that we observed for molecule **2** because the glucose moiety is located in the +1 site, far from the nucleophile Wat501. Moreover, given the longer C2–C6 distance of pyrrolizidine (4.9 Å) with respect to the C1–C4 distance of glucose (2.9 Å), the CAS-IN orientation allows ring B of pyrrolizidine to extend to the +1 site such that the glucose moiety is projected towards the third GA subsite (see Figure S49 of the Supporting Information). Interestingly, after docking, the lactam derivatives of glucosides **3**, **9** and **10**, which did not show inhibitory activity, all adopted the same GLU-IN orientation. The interactions of glucose at the –1 site are the same as those observed for active glucosides, and at the level of the +1 site the results of docking converged to a unique solution, which differ from the active glucosides for the aglycon conformation clearly influenced by the presence/absence of the lactam structure. Furthermore, the absence of charge on the nitrogen atom prevents lactam derivatives in the GLU-IN orientation from reinforcing the interaction at the +1 site through NH···π interactions. These observations on lactam molecules highlight some interesting features of the GA interaction: the fact that the CAS-IN orientation was never found for lactam-glucoside, together with the observations we made on the structural features of the interaction with molecule **6**, clearly indicate a poor binding of the lactam moiety to the –1 site due to both the absence of positive charge on the ligand and the steric effects associated with the lactam structure. Given also that at the +1 site the lack of charge on the ligand could be detrimental to the binding affinity and considering that the GLU-IN orientation of glucoside molecules could be associated with the hydrolysis of the molecule, it is not surprising that lactam molecules are not able to inhibit glucoamylase.

In summary, we have analysed the binding features of competitive pyrrolizidine inhibitors of glucoamylase as determined by docking simulations. None of the molecules we considered in this study presented structural variations in ring A, which in casuarine perfectly mimics the stereochemical arrangement of glucose. As far as ring B is concerned, none of the structural variations introduced at the 6- (including glycosylation) and 7-positions seem to significantly influence the inhibitory activity. In contrast, the presence of the lactam structure at the 4- and 5-positions has a very dramatic effect on the activity, and this could be due to

both the lack of a positive charge, whose role in stabilizing the complex has been already highlighted, and the limited conformational flexibility, which could determine unfavourable steric contacts.

Conclusions

We have reported a novel and efficient strategy for the synthesis of casuarine-like pyrrolizidines and their 6-*O*-α-glucoside derivatives. Our methodology was based on a totally regio- and stereoselective 1,3-dipolar cycloaddition of suitably substituted alkenes with a carbohydrate-based nitrone. After N–O bond cleavage of the cycloadducts thus obtained, the lactams were used as key intermediates in the synthesis of all the target compounds, including the glucosyl derivatives that were obtained by selective α-glucosylation. Evaluation of the inhibitory activity of casuarine and its derivatives towards a wide range of commercially available glycosidases was undertaken, and several new inhibitors of glucoamylase from *Aspergillus niger* were discovered. Docking experiments performed on pyrrolizidine derivatives allowed us to investigate the binding mode of the competitive inhibitors. It is evident that an optimal network of hydrogen-bonding interactions at the inner –1 site has to be achieved for a ligand to bind. The presence of a positive charge on the ligand is helpful for the stabilization of the complex, independently of the possibility that a ligand has to hydrogen-bond E179 through the charged atom. Finally, interactions at the +1 site also seem to have an important role in modulating the affinity of more extended molecules, but the comparison of **2** with acarbose clearly shows that to increase the affinity for GA it is necessary to bind subsites outside of the +1 site.

Experimental Section

General: Commercial reagents were used as received. All reactions were carried with magnetic stirring and were monitored by TLC on 0.25 mm silica gel plates (Merck F_{254}). Column chromatography was carried out on silica gel 60 (32–63 mm). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. ^{1}H NMR spectra were recorded with a Varian Mercury-400 spectrometer. ^{13}C NMR spectra were recorded with a Varian Gemini-200 spectrometer. Infrared spectra were recorded with a Perkin–Elmer Spectrum BX FT-IR System spectrophotometer. Mass spectra were recorded with a QMD 1000 Carlo Erba instrument by direct inlet injection; relative percentages are shown in parentheses. ESI full mass spectra were recorded with a Thermo LTQ instrument by direct inlet injection; relative percentages are shown in parentheses. HR-ESI-TOF-MS experiments were performed with a Q-Tof Ultima mass spectrometer (Waters) fitted with a standard Z-spray ion source and operated in the positive ionization mode. Elemental analyses were performed with a Perkin–Elmer 2400 analyser. Optical rotation measurements were performed with a JASCO DIP-370 polarimeter.

(1*R*,2*R*,3*R*,6*R*,7a*R*)-1,2-Bis(benzyloxy)-3-[(benzyloxy)methyl]-6-hydroxyhexahydro-5*H*-pyrrolizin-5-one (16): A mixture of **14a** (816 mg, 1.58 mmol) and Zn dust (407 mg) in CH_3COOH/H_2O (9:1, 12.5 mL) was heated at 50 °C for 4 h and then filtered through cotton. The solution was cooled to 0 °C, and, under vigorous stirring, a saturated aqueous solution of $NaHCO_3$ (100 mL) was added until a basic pH was reached. The aqueous phase was extracted with EtOAc (3 × 20 mL), and the combined organic phases were dried with Na_2SO_4. After filtration and concentration under reduced pressure, **16** was obtained as a yellow oil, pure enough to be used in 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, 6-H), 4.34 (t, *J* = 4.3 Hz, 1 H, 2-H), 4.11 (q, *J* = 4.5 Hz, 1 H, 3-H), 3.87 (br. s, 1 H, OH), 3.79 (dd, *J* = 6.9, 5.0 Hz 1 H, 1-H), 3.73 (dt, *J* = 8.6, 6.5 Hz, 1 H, 7a-H), 3.63 (dd, *J* = 9.8, 5.5 Hz, 1 H, 8-Ha), 3.52 (dd, *J* = 9.8, 4.1 Hz, 1 H, 8-Hb), 2.70 (ddd, *J* = 12.3, 7.8, 6.1 Hz, 1 H, 7-Ha), 1.80 (ddd, *J* = 12.3, 10.4, 8.6 Hz, 1 H, 7-Hb) ppm. ^{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) ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3671, 3373, 3012, 2867, 1697, 1454, 1100 cm^{-1}. MS (EI): *m*/*z* (%) = 381 (12) [M – Bn]$^+$, 336 (20), 275 (32), 180 (98), 153 (100), 88 (100). $C_{29}H_{31}NO_5$ (473.56): calcd. 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 (6): Pd (10% on C, 300 mg) was added to a stirred solution of **16** (150 mg, 0.32 mmol) in EtOH (15 mL) under nitrogen. The suspension was stirred under hydrogen at room temp. for 3 d, then filtered through Celite® and washed with MeOH. Concentration 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) to afford pure **6** 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, 1 H, 6-H), 4.16 (t, *J* = 6.3 Hz, 1 H, 2-H), 3.84 (dd, *J* = 3.0, 8.0 Hz, 1 H, 8-Ha), 3.67–3.53 (m, 4 H, 8-Hb, 1-H, 3-H, 7a-H), 2.78 (ddd, *J* = 12.2, 7.8, 5.9 Hz, 1 H, 7-Ha), 1.74 (ddd, *J* = 12.0, 10.7, 8.3 Hz, 1 H, 7-Hb) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ = 175.3 (s, C-5), 81.8 (d, C-1), 78.3 (d, C-2), 71.7 (d, C-6), 62.0 (d, C-3), 60.2 (t, C-8), 59.8 (d, C-7a), 36.8 (t, C-7) ppm. MS: *m*/*z* (%) = 204 (5) [M + H]$^+$, 203 (3) [M]$^+$, 185 (27) [M – H_2O]$^+$, 172 (52), 144 (74), 126 (51), 100 (87), 86 (100), 72 (59), 57 (38). $C_8H_{13}NO_5$ (203.19): calcd. 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 (17): A 1 M solution of $LiAlH_4$ in THF (1.6 mL, 1.61 mmol) was added to a cooled (0 °C) solution of **16** (255 mg, 0.54 mmol) in dry THF (6 mL) under nitrogen. The mixture was then heated at reflux for 1.5 h. Then, after cooling to 0 °C, an aqueous saturated solution of Na_2SO_4 (560 μL) was added dropwise. The suspension was then filtered through Celite® and washed with EtOAc. Concentration under reduced pressure afforded **17** as a yellow oil pure enough 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, 6-H), 4.11 (t, *J* = 4.6 Hz, 1 H, 1-H), 4.07 (t, *J* = 4.7 Hz, 1 H, 2-H), 3.60-3.44 (m, 4 H, 7a-H, 3-H, 8-Ha, 8-Hb), 3.21 (dd, *J* = 12.3, 4.5 Hz 1 H, 5-Ha), 2.98 (dm, *J* = 12.3 Hz, 1 H, 5-Hb), 2.21 (ddd, *J* = 13.8, 9.0, 5.7 Hz, 1 H, 7-Ha), 1.84 (dm, *J* = 13.8 Hz, 1 H, 7-Hb) ppm. ^{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)

ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3392, 3010, 2927, 2858, 1748, 1710, 1454, 1262 cm^{-1}. MS (EI): *m/z* (%) = 366 (6), 336 (6), 216 (61), 160 (100), 90 (100). $C_{29}H_{33}NO_4$ (459.58): calcd. 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, 4): Concentrated HCl (4–5 drops) and Pd (10% on C, 250 mg) were added to a stirred solution of **17** (120 mg, 0.26 mmol) in EtOH (10.5 mL) under nitrogen. The suspension was stirred at room temp. under hydrogen for 3 d, then filtered through Celite® and washed with MeOH. Concentration 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 (**4**) as a white solid (43 mg, 88% yield), m.p. 205–208 °C. $[\alpha]_D^{20}$ = +19.8 (*c* = 0.4, H_2O) {ref.[17a] $[\alpha]_D^{20}$ = +10.9 (*c* = 0.11, H_2O); ref.[16b] $[\alpha]_D^{25}$ = +23 (*c* = 0.3, MeOH)}. ^1H NMR (400 MHz, D_2O): δ = 4.44–4.38 (m, 1 H, 6-H), 4.05 (t, *J* = 8.2 Hz, 1 H, 1-H), 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, 1 H, 5-Hb), 2.12 (ddd, *J* = 13.6, 8.5, 3.4 Hz, 1 H, 7-Ha), 1.89 (dm, *J* = 13.6 Hz, 1 H, 7-Hb) ppm. ^{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) ppm. MS (EI): *m/z* (%) = 190 (3) $[M + H]^+$, 189 (1) $[M]^+$, 176 (66), 158 (65) $[M - CH_2OH]^+$, 132 (64), 112 (23), 85 (62), 58 (100). $C_8H_{15}NO_4$ (189.21): calcd. 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*,7a*R*)-1,2-Bis(benzyloxy)-3-(benzyloxymethyl)hexahydro-1*H*-pyrrolizine (18): NEt_3 (75 μL, 0.54 mmol) was added to a stirred solution of **16** (95 mg, 0.20 mmol) in dry CH_2Cl_2 (0.45 mL) under nitrogen, and, at 0 °C, MsCl (20 μL, 0.26 mmol) was added dropwise. The solution was stirred at 0 °C for 30 min and at room temp. for 2 h. The mixture was filtered through Celite® and washed with EtOAc. Concentration under reduced pressure afforded the mesylated derivative as a white oil (quantitative yield), which was dissolved in dry THF (2.5 mL). A 1 M solution of $LiAlH_4$ in THF (0.8 mL, 0.8 mmol) was added dropwise at 0 °C under nitrogen. The mixture was heated at reflux for 3 h. An aqueous saturated solution of Na_2SO_4 (280 μL) was added dropwise, and the mixture was stirred at room temp. for 10 min. After filtration through Celite®, a crude residue (109 mg) was obtained that was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate, 1:2, R_f = 0.28) to afford pure **18** (71 mg, 80% yield) as an oil. $[\alpha]_D^{24}$ = –5.1 (*c* = 0.6, $CHCl_3$). ^1H 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, 1 H, 2-H), 3.81 (t, *J* = 5.9 Hz, 1 H, 1-H), 3.60 (dd, *J* = 9.6, 4.7 Hz, 1 H, 8-Ha), 3.54 (dd, *J* = 9.6, 5.7 Hz, 1 H, 7a-H), 3.51–3.46 (m, 1 H, 8-Hb), 3.07 (dt, *J* = 10.5, 6.1 Hz, 1 H, 5-Ha), 2.98–2.94 (m, 1 H, 3-H), 2.78 (dt, *J* = 10.5, 6.6 Hz, 1 H, 5-Hb), 2.03–1.95 (m, 1 H, 7-Ha), 1.91–1.82 (m, 1 H, 6-Ha), 1.81–1.74 (m, 1 H, 6-Hb), 1.72–1.62 (m, 1 H, 7-Hb) ppm. ^{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) ppm.

(1*R*,2*R*,3*R*,7a*R*)-3-(Hydroxymethyl)hexahydro-1*H*-pyrrolizine-1,2-diol (Hyacinthacine A_2, 11): Concentrated HCl (3 drops) and Pd (10% on C, 45 mg) were added to a stirred solution of **18** (25 mg, 0.056 mmol) in MeOH (2.5 mL). The mixture was stirred at room temp. under hydrogen for 3 d. The mixture was then filtered through Celite® and washed with MeOH. The solvent was evaporated under reduced pressure to afford 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 (**11**) as a white solid (7 mg, 72% yield). $[\alpha]_D^{24}$ = +12.4 (*c* = 0.2, H_2O); {ref.[16a] $[\alpha]_D^{24}$ = +12.7 (*c* = 0.13, H_2O); ref.[18a] $[\alpha]_D$ = +12.5 (*c* = 0.4, H_2O); ref.[16c] $[\alpha]_D^{20}$ = +19.9 (*c* = 0.97, MeOH); ref.[18b] $[\alpha]_D^{25}$ = +10.5 (*c* = 0.6, H_2O); ref.[18f] $[\alpha]_D$ = +12.1 (*c* = 0.3, H_2O); ref.[18g] $[\alpha]_D^{26}$ = +12 (*c* = 0.4, H_2O); ref.[25] $[\alpha]_D$ = +20.1 (*c* = 0.44, H_2O)}. ^1H 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) ppm. ^{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) ppm. $C_8H_{15}NO_3$ (173.21): calcd. C 55.47, H 8.73, N 8.09; found C 55.49, H 8.61, N 8.10.

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 (20): A mixture of **14b** (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 at 50 °C for 3 h and then filtered through cotton. The solution was cooled to 0 °C, and, under vigorous stirring, a saturated aqueous solution of $NaHCO_3$ (100 mL) was added until a basic pH was reached. The aqueous phase was extracted with EtOAc (3 × 60 mL), and the combined organic phases were dried with Na_2SO_4. After filtration and concentration under reduced pressure, **20** was obtained pure as a white solid (743 mg, 90% yield), m.p. 111–113 °C. $[\alpha]_D^{20}$ = –28.7 (*c* = 0.64, $CHCl_3$). ^1H 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, 2-H), 4.57–4.44 (m, 6 H, Bn), 4.31–4.28 (m, 1 H, 5-H), 4.23–4.22 (m, 1 H, 6-H), 3.98–3.95 (m, 1 H, 7a-H), 3.92–3.90 (m, 1 H, 7-H), 3.79 (s, 3 H, Me), 3.58–3.50 (m, 2 H, 8-H), 3.44 (d, *J* = 3.1 Hz, 1 H, OH), 3.03 (t, *J* = 9.3 Hz, 1 H, 1-H) ppm. ^{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) ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3690, 3600–3500 (br), 3027, 2920, 1708, 1601, 1155, 1070 cm^{-1}. MS (EI): *m/z* (%) = 513 (0.6) $[M - H_2O]^+$, 212 (4), 91 (100), 69 (14). $C_{31}H_{33}NO_7$ (531.6): calcd. 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 (21): A 1 M solution of $LiAlH_4$ in THF (1.1 mL, 1.1 mmol) was added to a cooled (0 °C) solution of **20** (115 mg, 0.22 mmol) in dry THF (3 mL) under nitrogen. The mixture was then heated at reflux for 2 h. Then, after cooling at 0 °C, an aqueous saturated solution of Na_2SO_4 (700 μL) was added dropwise. The suspension was then filtered through Celite® and washed with EtOAc. Concentration under reduced pressure afforded solid **21** pure enough to be used in the next step (104 mg, quantitative yield). An analytically pure sample was obtained by filtration through a short pad of silica gel (eluent: EtOAc then EtOAc/MeOH, 5:1), m.p. 85–87 °C. $[\alpha]_D^{20}$ = +3.58 (*c* = 1.18, $CHCl_3$). ^1H 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, 1 H, 6-H), 4.13–4.09 (m, 2 H, 1-H, 2-H), 3.68 (d, *J* = 6.8 Hz, 2 H, 8-Ha, 8-Hb), 3.53 (d, *J* = 6.4 Hz, 2 H, 9-Ha, 9-Hb) 3.43–3.37 (m, 2 H, 3-H, 5-Ha) 3.27 (dd, *J* = 7.0, 3.9 Hz, 1 H, 7a-H), 2.95 (dd, *J* = 10.8, 5.6 Hz, 1 H, 5-Hb), 2.24 (quint., *J* = 6.6 Hz, 1 H, 7-H) ppm. ^{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), 86.1 (d), 76.6 (d), 73.3 (t), 72.4 (t), 72.0 (t), 71.5 (t), 70.4 (d), 70.4 (d), 64.2 (t), 62.4 (t), 54.3 (d) ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3412, 3031, 3010, 2866, 1496, 1454, 1363, 1216, 1212, 1211, 1097 cm^{-1}. MS (EI): *m/z* (%) = 398 (11) $[M - C_7H_7]^+$, 368 (85), 248 (11), 186 (27), 160 (30), 142 (25), 116 (22), 91 (100), 64

(21). $C_{30}H_{35}NO_5$ (489.6): calcd. 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-Homocasuarine, 7): Concentrated HCl (4–5 drops) and Pd (10% on C, 230 mg) were added to a stirred solution of **21** (106 mg, 0.22 mmol) in EtOH (14 mL). The suspension was stirred at room temp. under hydrogen for 4 d, 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-homocasuarine (**7**). Evaporation of the solvent afforded 7-homocasuarine 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, 6-H), 4.04 (t, *J* = 7.8 Hz, 1 H, 1-H), 3.71 (t, *J* = 8.5 Hz, 1 H, 2-H), 3.67 (dd, *J* = 11.7, 3.5 Hz, 1 H, 8-Ha), 3.55-3.44 (m, 3 H, 8-Hb, 9-Ha, 9-Hb), 3.19 (dd, *J* = 10.0, 5.2 Hz, 1 H, 5-Ha), 3.02–2.95 (m, 2 H, 3-H, 7a-H), 2.80 (dd, *J* = 10.0, 5.2 Hz, 1 H, 5-Hb), 2.21 (q, *J* = 5.9 Hz, 1 H, 7-H) ppm. ^{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) ppm. MS (EI): *m*/*z* (%) = 188 (100), 170 (10), 159 (13), 142 (14), 128 (83), 116 (26), 68 (38), 55 (24). $C_9H_{17}NO_5$ (219.23): calcd. C 49.31, H 7.82, N 6.39; found C 49.06, H 7.43, N 6.54.

7-Deoxy-6-*O*-(α-D-glucopyranosyl)-7-(methoxycarbonyl)-5-oxocasuarine (10): Pd (10% on C, 150 mg) was added to a stirred solution of **22** (125 mg, 0.118 mmol) in MeOH/AcOEt (3:1, 12 mL). The suspension was stirred at room temp. under hydrogen for 4 d, then filtered through Celite® and washed with MeOH. Concentration under reduced pressure afforded pure **10** 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, 1-H), 4.97 (d, *J* = 9.6 Hz, 1 H, 6′-H), 4.08 (dd, *J* = 6.8, 6.4 Hz, 1 H, 2′-H), 3.88 (dd, *J* = 7.6, 7.2 Hz, 1 H, 1′-H), 3.80–3.55 (m, 11 H, 5-H, OCH_3, 4-H, 3-H, 6-Ha, 6-Hb, 8-Ha′, 7a′-H, 3′-H), 3.41 (dd, *J* = 9.6, 3.7 Hz, 1 H, 2-H), 3.37 (t, *J* = 9.6 Hz, 1 H, 8-Hb′), 3.28 (dd, *J* = 9.4, 8.2 Hz, 1 H, 7′-H) ppm. ^{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) ppm. IR (KBr): $\tilde{\nu}$ = 3420 (OH), 1710 (C=O), 1684 (C=O), 1205, 1143, 1024 cm^{-1}. HRMS (ESI): calcd. for $C_{16}H_{25}NO_{12}Na$ [M + Na]$^+$ 446.1269; found 446.1266. $C_{16}H_{25}NO_{12}$ (423.37): calcd. C 45.39, H 5.95, N 3.31; found C 44,93, H 6.27, N 3,37.

6-*O*-α-D-Tris(benzyloxy)-5-oxo-6-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-7-homocasuarine (23): A 2 M solution of $LiBH_4$ in THF (0.42 mL) was added dropwise to a cooled (0 °C) solution of **22** (221 mg, 0.21 mmol) in dry THF (1.5 mL). The reaction mixture was stirred at room temp. 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 **23** (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, 1-H), 5.09 (d, *J* = 11.7 Hz, 1 H, Bn), 5.02 (d, *J* = 10.9 Hz, 1 H, Bn), 4.81–4.75 (m, 3 H, Bn), 4.72 (d, *J* = 7.6 Hz, 1 H, 6′-H), 4.60–4.40 (m, 9 H, Bn), 4.26 (dd, *J* = 3.6, 4 Hz, 1 H, 2′-H), 4.21 (m, 1 H, 3′-H), 3.94 (t, *J* = 9.2 Hz, 1 H, 3-H), 3.87–3.66 (m, 6 H, 5-H, 6-Ha, 7a′-H, 8′-Ha,b, 1′-H), 3.60 (dd, *J* = 9.2, 3.6 Hz, 1 H, 2-H), 3.45 (d, *J* = 5.2 Hz, 2 H, 9′-Ha,b), 3.37 (dd, *J* = 9.8, 7.6 Hz, 1 H, 6-Hb), 3.26 (dd, *J* = 10.8, 9.2 Hz, 1 H, 4-H), 3.13 (t, 1 H, OH), 2.44 (m, 1 H, 7′-H) ppm. ^{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′) ppm. IR ($CDCl_3$): $\tilde{\nu}$ = 3463 (OH), 3032, 2925, 2870, 1703 (C=O), 1454, 1078 cm^{-1}. HRMS (ESI): calcd. for $C_{64}H_{67}NO_{11}Na$ [M + Na]$^+$ 1048.4606; found 1048.4602. $C_{64}H_{67}NO_{11}$ (1026.22): calcd. C 74.90, H 6.58, N 1.36; found C 74.65, H 6.69, N 1.53.

6-*O*-(α-D-Glucopyranosyl)-5-oxo-7-homocasuarine (9): Pd (10% on C, 230 mg) was added to a stirred solution of **23** (134 mg, 0.13 mmol) in MeOH (13 mL). The suspension was stirred at room temp. under hydrogen for 24 h, then filtered through Celite® and washed with MeOH. Concentration under reduced pressure afforded pure **9** 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, 1-H), 4.57 (d, *J* = 9.6 Hz, 1 H, 6′-H), 4.09 (dd, *J* = 6.8, 6.4 Hz, 1 H, 2′-H), 3.82–3.58 (m, 10 H, 1′-H, 3′-H, 8′-Ha,b, 9′-Ha, 3-H, 4-H, 5-H, 6-Ha,b), 3.50–3.45 (m, 2 H, 7a′-H, 2-H), 3.34 (dd, *J* = 9.6, 9.2 Hz, 1 H, 9′-Hb), 2.50–2.44 (m, 1 H, 7′-H) ppm. ^{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) ppm. IR (KBr): $\tilde{\nu}$ = 3378 (OH), 1689 (C=O) cm^{-1}. HRMS (ESI): calcd. for $C_{65}H_{67}NO_{12}Na$ [M + Na]$^+$ 418.1320; found 418.1312. $C_{15}H_{25}NO_{11}$ (395.36): calcd. C 45.57, H 6.37, N 3.54; found C 45.25, H 6.28, N 3.04.

6-*O*-(α-D-Glucopyranosyl)-5-oxocasuarine (3): Pd (10% on C, 180 mg) was added to a stirred solution of **24** (159 mg, 0.144 mmol) in MeOH/EtOAc (7:1, 12 mL). The suspension was stirred at room temp. under hydrogen for 24 h, then filtered through Celite® and washed with MeOH. Concentration under reduced pressure afforded pure **3** (42 mg, 0.110 mmol, 77% yield) as a 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, 1-H), 4.53 (d, *J* = 8.4 Hz, 1 H, 6′-H), 4.27 (dd, *J* = 8.4, 7.0 Hz, 1 H, 7′-H), 4.08 (t, *J* = 6.1 Hz, 1 H, 2′-H), 3.90 (t, *J* = 6.8 Hz, 1 H, 1′-H), 3.78–3.58 (m, 7 H, 3-H, 3′-H, 5-H, 6-Ha,b, 8′-Ha,b), 3.50–3.44 (m, 2 H, 2-H, 7a′-H), 3.34 (t, *J* = 9.5 Hz, 1 H, 4-H) ppm. ^{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), 71.5 (d), 70.5 (d), 68.6 (d), 64.7 (d), 60.7 (d), 59.6 (t), 58.8 (t) ppm. MS (ESI): *m*/*z* (%) = 404 (100) [M + Na]$^+$. $C_{14}H_{23}NO_{11}$ (381.33): calcd. C 44.10, H 6.08, N 3.67; found C 43.96, H 6.19, N 3.42.

Enzymatic Assays: The experiments were performed essentially as follows: 0.01–0.5 unit/mL of enzyme (1 unit = 1 mol of glycoside hydrolysed/min), preincubated at 20 °C with the inhibitor for 5 min, and increasing concentrations of an aqueous solution of the appropriate *p*-nitrophenyl glycoside substrates (buffered at the optimum pH of the enzyme) were incubated at 37 °C for 20 min. The reactions were stopped by the addition of 0.3 M sodium borate buffer (100 μL, 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 values for each compound.

Molecular Modelling: The ligand structures (Table 1) were constructed by using Maestro v8.5.[29] All the molecules were subjected to conformational search and clusterization with Macromodel 9.6[30] in order to sample the most accessible conformations of both the aglyconic and glucose moieties. The bridgehead nitrogen atoms were treated as ionized to better simulate the physiological condi-

tions, except for the lactam intermediates. All the docking calculations were performed by using Glide 5.0.[31] The crystal structure of glucoamylase-471 from *Aspergillus awamori* complexed with 1-deoxynojirimycin (PDB ID: 1DOG)[26a] was downloaded from the PDB and prepared according to the recommended Protein Preparation module in Maestro 8.5 by using default input parameters (no scaling factors for the van der Waals radii of non-polar protein atoms, 0.8 scaling factor for non-polar ligand atoms). This procedure was used to remove water molecules (except for molecule Wat501, which is considered as part of the target structure), to assign missing hydrogen atoms, to optimize hydrogen-bonding interactions and to reduce structural problems. The grids were prepared with the centre of the site defined by the centre of the complexed ligand. All the relevant conformations for the ligands in Table 1 were docked in the binding site by using the SP scoring function to score the ligand poses. The docking calculations were performed in the presence of Wat501. After completion of each docking run, one pose per ligand conformation was saved. Finally, for each ligand, the poses (conformations) with the lowest (best) value of either the model energy score (Emodel)[32] or the Glide score were chosen.

Supporting Information (see footnote on the first page of this article): Syntheses of **5**, **8**, **8a**, **10a**, **16a**, **19**, **19a**,**b**, **22** and **22a**, copies of the NMR spectra of **3**–**11**, **8a**, **10a**, **16**–**23**, **16a**, **19a**,**b** and **22a**, HR-ESI-TOF-MS spectra and the docking results for **2**.

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