Synthesis and Investigation of Polyhydroxylated Pyrrolidine Derivatives as Novel Chemotypes Showing Dual Activity as Glucosidase and Aldose Reductase Inhibitors.

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Abstract

Diabetes is a multi-factorial disorder that should be treated with multi-effective compounds. Here we describe the access to polyhydroxylated pyrrolidines, belonging to the D-*gluco* and D-*galacto* series, through aminocyclization reactions of two differentially protected D-*xylo*-hexos-4-ulose derivatives. The prepared compounds proved to inhibit both alpha-glucosidase, responsible for the emergence of hyperglycemic spikes, and aldose reductase, accountable for the development of abnormalities in diabetic tissues. Accordingly, they show the dual inhibitory profile deemed as ideal for diabetes treatment. Significantly, compound **17b** reduced the process of cell death and restored the physiological levels of oxidative stress when tested in the photoreceptor-like 661w cell line, thus proving to be effective in an in vitro model of diabetic retinopathy.

Keywords

Diabetes, aldose reductase, aldose reductase inhibitors, glucosidase, glucosidase inhibitors, azafuranose, pyrrolidine, aminocyclization.

Abbreviations

AG, alpha-glucosidase; ALR2, aldose reductase; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; Sod1, Cu-Zn-superoxide dismutase.

1. Introduction

Diabetes mellitus is a multi-factorial metabolic disorder characterized by high levels of blood glucose, resulting from defects in insulin production, insulin action, or both. It is recognized as a public health problem, as it affects a significant portion of the population worldwide and is expanding to pandemic dimensions. Indeed, according to epidemiological studies, approximately 415 million of adults worldwide were diagnosed with diabetes in 2015, and this number is expected to rise to at least 640 million by 2040 [1].

Despite the administration of insulin and/or potent oral hypoglycemics, diabetes still remains a significant cause of morbidity and mortality, especially due to the development of related disabling complications. Patients with diabetes have a 2-4 times higher risk for cardiovascular pathologies, including strokes, and show accelerated formation of severe atherosclerotic lesions in peripheral, coronary, and cerebral arteries. Diabetes is also the leading cause of visual impairment and blindness, due to severe retinopathy and cataract, as well as terminal renal diseases, which requires renal dialysis or even kidney transplantation. Moreover, a great proportion of people with diabetes show different forms of nervous system damage, including impaired sensation of pain and physical disability. Severe forms of diabetic nerve diseases are the major contributing cause of lower-extremity amputations, more than 60% of which occur among people affected by this disease [2].

While research on the prevention of diabetes itself is promising, efforts to control hyperglycemic spikes and prevent the resulting onset of chronic functional alterations are still unfruitful. Therefore, the research of novel and ground-breaking therapeutic solutions for diabetic people is more relevant than ever.

An attractive strategy to address diabetes is the development of a multi-effective compound, able to combine a hypoglycaemic effect with the ability to prevent long term diabetic complications.

The straightforward way to control sugar plasma concentration is to take action on enzymes managing its availability, being alpha-glucosidase (AG) the most relevant among others [3]. Up to 70% of the daily

glucose load results from carbohydrate intake through the diet. Indeed, sugars are digested on the brush border of the small intestine and AG provides glucose for intestinal absorption through the regulation of the speed and the extent of post-prandial hyperglycaemia. On the other hand, the onset of pathological changes affecting the nervous, renal, vascular and ocular systems of diabetic patients are generated by the activation of aldose reductase (ALR2), which has been finally accepted as the key and critical checkpoint of the main biochemical abnormalities affecting diabetic tissues [4].

On these grounds, the ideal anti-diabetic agent should be able to simultaneously block the catalytic activity of both AG and ALR2.

Broadly speaking, developing a dual-target agent is a challenging task, as it is considerably hard to optimize multiple, selective and potent activities within the same chemical structure. However, with regard to compounds able to take action on glucose metabolism, a privileged class of derivatives do exist, which might come to the aid of medicinal chemists for the design of novel effective agents.

This is represented by the naturally-derived carbohydrate analogues iminosugars, characterized by the presence of a nitrogen atom replacing the endocyclic oxygen of carbohydrates. Thanks to their structural similarity to natural sugars, iminosugars are able to modulate carbohydrate-processing enzymes and are therefore particularly suitable for the development of the aforementioned bi-functional anti-diabetic agents.

Several iminosugars are already used as effective AG inhibitors, including the oligosaccharide Acarbose [3] (**1**, Chart 1) and the piperidine derivative Miglitol or Glyset [5] (**2**, Chart 1), both marketed worldwide as the standard of care approach to control glucose plasma concentration. Their ability of inhibit ALR2 has never been thoroughly investigated but it is a fact that polyhydroxylated compounds, often of natural origin, can be effective ALR2 inhibitors [6]. These findings suggest that iminosugars might represent the reference chemotype for the discovery of dual AG/ALR2 inhibitors.

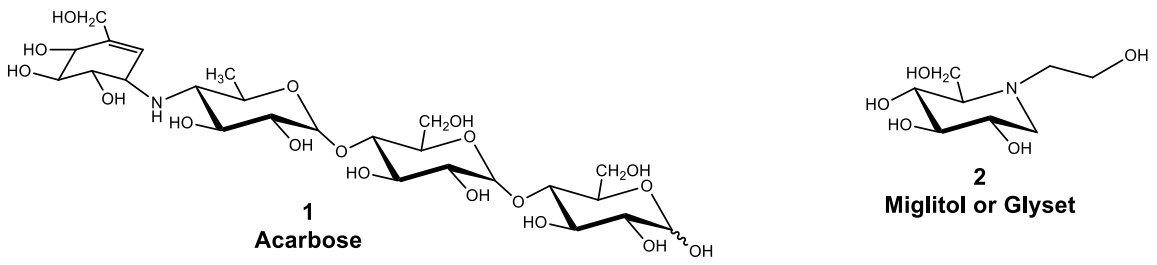


Chart 1. Oligosaccharide Acarbose and Miglitol or Glyset.

With this in mind, we designed a series of novel polyhydroxylated pyrrolidine derivatives as possible bi-functional compounds, able to both delay glucose absorption and prevent long term diabetic complications. Our strategy involved a combination of our synthetic expertise in transforming common sugars into complex architectures, [7-9] in particular into iminosugars derivatives *via* dicarbonyl compounds, [10-12] with the long-lasting experience in the ALR2 inhibitors field [13,14]. Results obtained are reported here below.

2. Results and Discussion

2.1. Chemistry

The retrosynthetic approach to prepare structurally-related families of polyhydroxylated pyrrolidine derivatives is depicted in Figure 1. The double reductive amination reaction (aminocyclization) of 1,4-dicarbonyl derivatives, which in turn can be obtained from a readily available enol ether intermediate **3**, represents the key step.

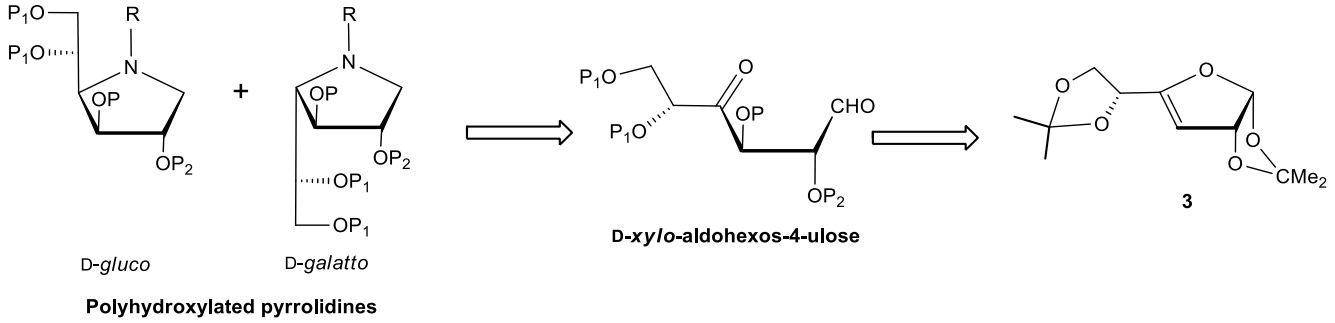
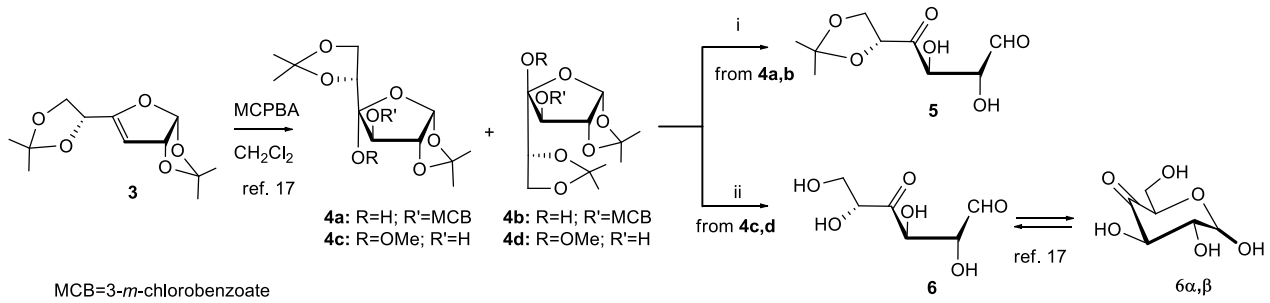


Figure 1. Retrosynthetic approach to the polyhydroxylated pyrrolidine.

The known enol ether **3** [15-16] was subjected to the epoxidation reaction with meta-chloroperoxybenzoic acid (MCPBA) in CH_2Cl_2 affording the reported 7:3 mixture (81%) of C-4 epimeric 3-*m*-chlorobenzoates **4a,b** [17] (Scheme 1). When the epoxidation reaction of **3** was carried in MeOH, a 3:2 mixture of 4-*C*-methoxy derivatives **4c,d** was obtained [17] (Scheme 1). By treating the mixture **4c,d** with CF_3COOH in 2:1 MeCN-water, hydrolysis of all the acetal groups was observed and D-*xylo*-hexos-4-ulose **6** was isolated [17]. However, by employing milder reaction conditions (Et_3N in 5:1 MeOH-H_2O), it was possible to selectively cleave the *m*-chlorobenzoate protecting group (MCB) from **4a,b** and unmask the carbonyl groups while leaving the lateral chain isopropylidene moiety untouched. In doing so, the partially protected 5,6-*O*-isopropylidene-D-*xylo*-hexos-4-ulose (**5**) was obtained.

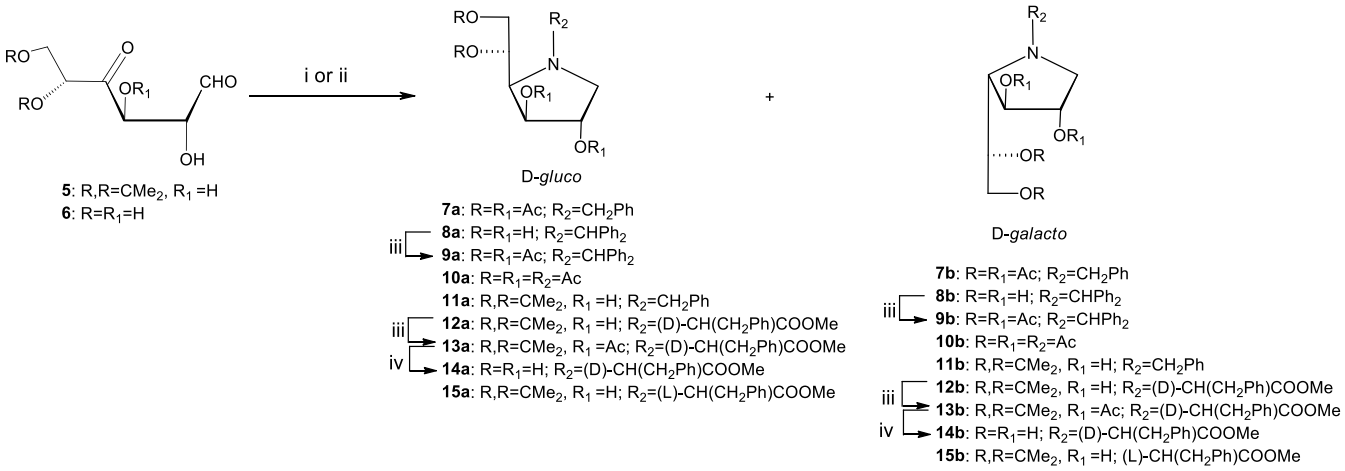


Scheme 1. Synthesis of 4-uloside derivatives **5** and **6**. *Reagents and conditions*: (i) Et_3N, 5:1 MeOH-H_2O (quantitative yield); (ii) CF_3COOH, 2:1 CH_3CN-H_2O (quantitative yield).

1,4-Dicarbonyl hexose **6** exists predominantly as the two anomeric α- and β- 4-*keto* pyranose forms **6α,β** (Scheme 1), deriving from hemiacetalyzation of the C-5 hydroxyl group onto the carbon of the aldehyde group [17]. In the case of **5**, a rather complex mixture of tautomeric forms (as shown by NMR analysis) was instead observed as the 5-OH is blocked into the isopropylidene acetal protecting group.

The two dicarbonyl compounds **5** and **6** were subjected without further purification to the aminocyclization reaction. To a solution of either **5** or **6** and the selected ammonium salt (1 eq) in MeOH, $NaBH_3CN$ (2.2 eq) was added and the mixture was stirred at 60 °C for 24-48 hours (Scheme 2). Overall,

the yields of the aminocyclization reactions were quite satisfactory, spanning from 37% to 50% (Table 1-SI, Supporting Information), although somewhat lower than those usually obtained in the D-*xylo*-hexos-5-ulose series [11,18,19]. Particularly frustrating were instead the difficulties encountered when trying to purify the pyrrolidine diastereoisomers *via* chromatography on silica gel. Only in the case of the **11a,b** mixture it was possible to separate the D-*gluco* and D-*galacto* diastereoisomers. For **7a,b** and **15a,b** mixtures, a partial separation was achieved and pure samples of only **7b** and **15a** were obtained. Some of the pyrrolidine mixtures were also acetylated, in order to facilitate NMR assignment (Scheme 2).



Scheme 2. Reagents and conditions: (i) $PhCH_2NH_2 \cdot HCl$ (for **7a,b**, 50% yield), or $NH_3 \cdot AcOH \cdot$(for **10a,b**, 55% yield), $NaBH_3CN$, MeOH, 60 °C, 24-48 h, followed by 1:2 Ac_2O-Py, rt, 12 h; (ii) $Ph_2CHNH_2 \cdot AcOH$ (for **8a,b**, 52% yield), $PhCH_2NH_2 \cdot HCl$ (**11a,b**, 37% yield), D-Phe-OMe·HCl (for **12a,b**, 42% yield) or L-Phe-OMe·HCl (for **15a,b**, 44% yield), $NaBH_3CN$, MeOH, 60 °C, 24-48 h; (iii) 1:2 Ac_2O-Py, rt, 12 h (for **9a,b**, 71% yield; for **13a,b**, 75% yield); (iv) 80% aq AcOH, 40 °C, 15 min (for **14a,b**, 46% yield).

Conversely, really intriguing were the results in terms of the stereoselectivity of the reaction. The configuration of the C-4 furanoside epimers was deduced by NMR analysis. In the ^{13}C spectra, signals of the carbons adjacent to the nitrogen (C-1 and C-4), which are shielded and easily recognizable by

DEPT-135 experiments, were diagnostic for the two epimers. In particular, in the D-*gluco* derivatives C-4 was always shifted by about 3-6 ppm towards higher fields than in the D-*galacto* epimers (see experimental section). Very different ratios of the two diastereoisomers were obtained by using diverse amine partners: from 15:85 to 70:30 D-*gluco*:D-*galacto* ratios (Table 1-SI, entries 3 and 6). The results suggest that the stereo outcome of the reaction is particularly sensitive to the steric hindrance of the amine; the D-*galacto* diastereoisomer is favored with NH_3 and $PhCH_2NH_2$ (Table 1-SI, entries 1 and 3), while the D-*gluco* diastereoisomer becomes the major reaction product with Ph_2CHNH_2 and D/L-Phe-OMe (Table 1-SI, entries 4-6). This hypothesis works for both dicarbonyl derivatives **5** and **6** tested in the aminocyclization reaction.

The aminocyclization of aldohexos-4-ulose derivatives is far less studied than that of the more common aldohexos-5-uloses. For the latter, in the case of compounds of the D-*xylo* series, a strong preference for the "D-*gluco*" product is observed regardless of the structure of the amine reacting partner [20]. Therefore, there is a huge difference in the stereochemical outcome between the two dicarbonyl isomers of the same D-*xylo* stereochemical series, even when the same reaction conditions are applied. It is commonly accepted [11] that the reaction proceeds through two consecutive steps, the fast reductive amination of the *aldehydo* function, followed by a second, slower intramolecular condensation of the resulting amine with the *keto* group. In this way a cyclic iminium ion is formed, on which the final hydride attack determines the stereochemical outcome of the reaction [21].

For D-*xylo* aldohexos-5-uloses, the two chair-like transition states deriving from the two possible 6-membered half-chair iminium ions have such a different energy that practically only one of them is accessible, hence the high if not complete stereoselectivity of the reaction. In the cases under investigation here, the formation of 5-membered envelope iminium ions (^2E and E_2, Figure 2) can be envisaged. Based on the results, it is likely that the E_2 iminium ion gives rise to the most stable transition state and thus preferentially affords the D-*galacto* diastereoisomer as long as the amine partner has a

reduced steric hindrance. If the steric hindrance of the amine comes into play, than the ^{2}E cyclic iminium ion becomes the preferential one for the hydride attack. Studies on this mechanism are currently underway ad will be presented in due course.

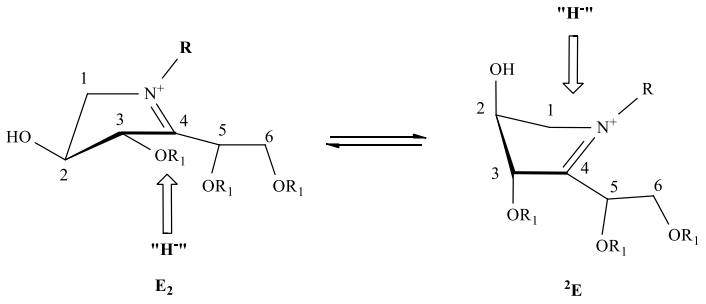
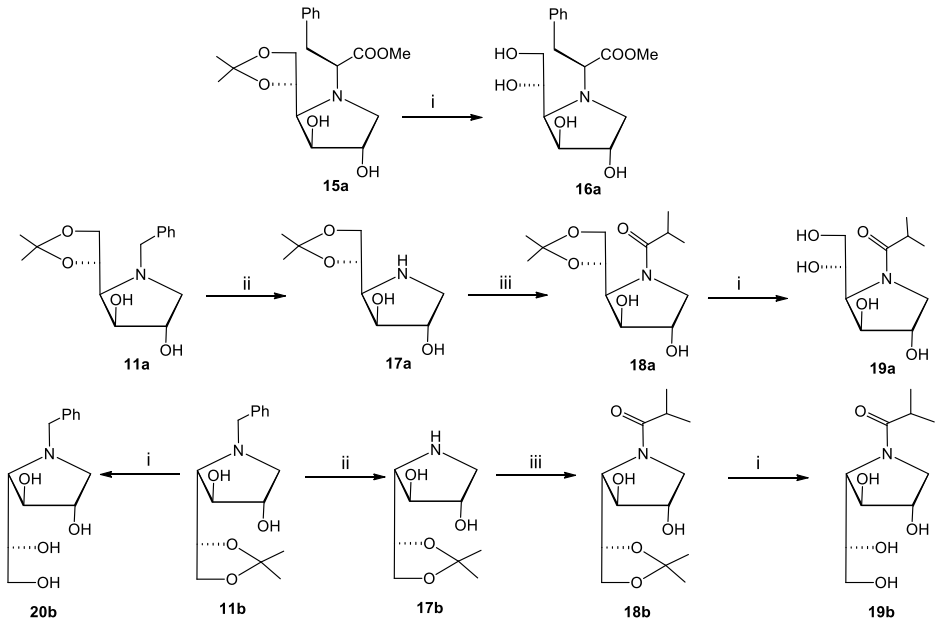


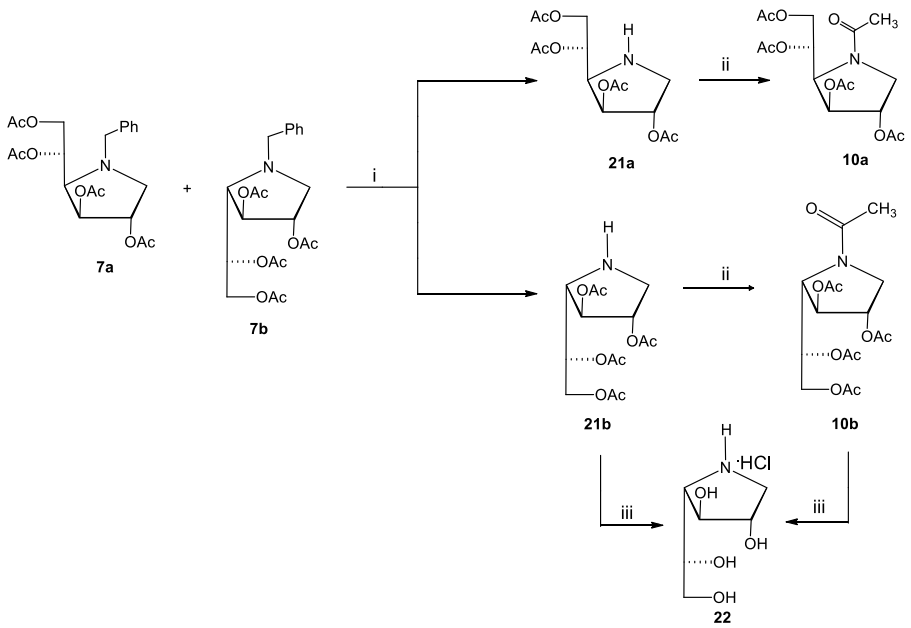
Figure 2. ^{2}E and E_2 cyclic iminium ions.

In order to increase the number of structurally-related compounds for biological evaluations, a few different protecting group manipulations were carried out on those structures that were obtained as pure compounds (**11a**, **11b** and **15a**) from the aminocyclization reaction (Scheme 3). In particular, the benzyl group on the nitrogen was removed by hydrogenolysis (**11a→17a**, **11b→17b**) and the resulting compounds were reacted with isobutyric anhydride (**17a→18a**, **17b→18b**). Finally, the 5,6-*O*-isopropylidene protecting group was removed from compounds **11b, 15a, 18a** and **18b** by acid hydrolysis to afford a series of pyrrolydine derivatives characterized by an enhanced hydrophilic character (**11b→20b**, **15a→16a**, **18a→19a**, **18b→19b**).



Scheme 3. Reagents and conditions: (i) 80% aq AcOH, 40 °C, 0.5-1 h (**16a**: 89%; **19a**: 83%; **19b**: 85%; **20b**: 90%); (ii) H_2, 10% Pd/C, absolute EtOH, 48 h (**17a**: 85%; **17b**: 87%); (iii) isobutyric anhydride, Et_3N, 3:1 MeOH-H_2O, rt, 2 h (**18a**: 85%; **18b**: 75%).

In the case of the mixture of **7a** and **7b**, for which it was only possible to obtain a small sample of pure **7b** by chromatographic purification on silica gel, an attempt to verify whether the removal of the nitrogen benzyl group would favour the diastereoisomeric separation was performed (Scheme 4). By treating **7a** and **7b** with H_2 and Pd/C, the corresponding mixture of **21a** and **21b** was obtained and it was possible to isolate both pyrrolidines derivatives as pure compounds by chromatographic purification and further transform them into the known peracetylated derivatives **10a** [22] and **10b** [23] (Scheme 4). The most abundant *galacto*-diastereoisomer was also converted into the known deprotected hydrochloride salt **22** [24] both from **21b** and **10b** by treatment with the acid conditions (2M HCl).



Scheme 4. Reagents and conditions: (i) H_2, 10% Pd/C, absolute EtOH, 2 h (**21a**: 30%; **21b**: 52%); (ii) 1:2 Ac_2O-Py, rt, 12 h (**10a**: 91%; **10b**: 90%); (iii) 2M HCl, 100 °C, 15 min (87%).

It is worth mentioning that NMR analysis of pyrrolidine amides **18a,b**, **19a,b** and **10a,b** was complicated by the presence of C-N amide bond rotational isomers (see Experimental Section). It is well known that the rotation energy barrier could determine the presence of rotational isomers for amides and these have been reported before for piperidine [25, 26] and pyrrolidine [27, 28] amide derivatives. To the best of our knowledge, this is the first time that this hindered rotation has been observed for polyhydroxylated *N*-acyl-pyrrolidine derivatives of the D-*gluco* and D-*galacto* series (azafuranose). Rotational isomers were found for all the synthesized *N*-acyl-compounds (**18a,b**, **19a,b** and **10a,b**) and their ratio depended on both the substitution pattern and the configuration of the furanoside epimers. For example, in the case of **18a** and **18b**, two rotational isomers (ratios 75:25 and 60:40 respectively) were observed by recording the NMR spectra at 20 °C. The signals coalesced and sharpened when the temperature was increased to 80 °C (see experimental section).

2.2. Biological activity

2.2.1. Aldose Reductase and alpha-Glucosidase Inhibitory assays.

To get some insight into the bi-functional activity of the novel pyrrolidine derivatives, compounds were tested as both ALR2 and AG inhibitors. Results obtained are listed in Table 1.

Within the *D-gluco* subseries, the presence of the 5,6-*O*-isopropylidene group was detrimental for the activity, being the protected compounds generally less effective than the hydrophilic counterparts. Compare for example the isopropylidene derivative **18a**, devoid of any significant inhibitory efficacy, with the hydrophilic parent **19a**, which proved to halve ALR2 activity when tested at the same concentration. Similar results were also achieved testing the *D-gluco* compounds against AG, thus rewarding the 1,4-immino-D-glucitol **19a** as the best dual inhibitor of the whole subseries.

As for the *D-galacto* subseries, an opposite inhibitory trend was observed. Actually, in the case of ALR2, the best functional efficacy was displayed by the 5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol **17b**, and any attempt to modify its chemical structure, by removing the protecting group as well as inserting suitable substituents on both the hydroxy residues and the nitrogen atom, resulted in less effective analogues. On the contrary, the aforementioned structural modifications allowed to get promising AG inhibitors. Indeed, removal of the isopropylidene bridge from **17b** led to compound **22**, which displayed an IC_{50} value of 62.4 μM against the target enzyme. An even more marked increase of efficacy was obtained by inserting a benzyl residue on the nitrogen atom of the de-protected **22**, as in **20b** (IC_{50}: 40.64 μM).

On the whole, *D-galacto* derivatives turned out to be more effective than the *D-gluco* isomers against the two enzymes, thus proving to have the molecular geometry that best fits the catalytic binding sites of both ALR2 and AG at the same time. The observed inhibitory effects were generally more marked toward AG rather than ALR2, nonetheless suggestive for the intended dual functional profile.

Table 1. Aldose Reductase (ALR2) and α-Glucosidase (AG) Inhibitory Data of Polyhydroxylated Pyrrolidine Derivatives.

		% Inhibition[a]	
Entry	**Compound**	**ALR2**	**AG**
D-*Gluco Derivatives*			
1	**18a**	n.a.[b]	33.3
2	**19a**	49.0%	54.5
3	**11a**	n.a.[b]	21.0
4	**17a**	n.a.[b]	34.0
5	**15a**	27.2	35.6
6	**16a**	15.8	n.a.[b]
D-*Galacto Derivatives*			
7	**18b**	18.8%	31.4
8	**19b**	32.2	62.1
9	**20b**	32.6	93.2[c]
10	**17b**	57.1	30.2
11	**22**	35.2	89.4[d]
12	**7b**	29.6	n.a.[b]
13	**10b**	42.8	33.3
Epalrestat		98.7	n.t.[e]
DNJ·HCl		29.4	72.3

[a]Percentage of enzyme inhibition at 100 μM test compound, obtained as mean of at least three determinations. Standard errors of the means (SEMs) are ≤10%. [b]Not active. No inhibition was observed at 100 μM test compound. [c]IC_{50}: 40.6±3.80 μM. [d]IC_{50}: 62.4±5.11 μM. [e]Not tested.

2.2.2. *In vitro* assessment of ALR2 inhibition

Compound **17b**, showing the best inhibitory profile against ALR2, was tested on the murine retinoblastoma photoreceptor-like cell line 661w, [29] in order to evaluate its efficacy in vitro. The 661w cell line is characterized by both the presence of ALR2, as evidenced in Figure 3A, and the high sensitivity to hyperglycemic conditions, thus turning out to be an excellent in vitro model of diabetic retinopathy. [30] Tested at the concentration of 100 μM, compound **17b** proved to inhibit the activity of

the target enzyme by approximately 90% with respect to the activity shown by the enzyme in the untreated hyperglycemic cells (Figure 3B).

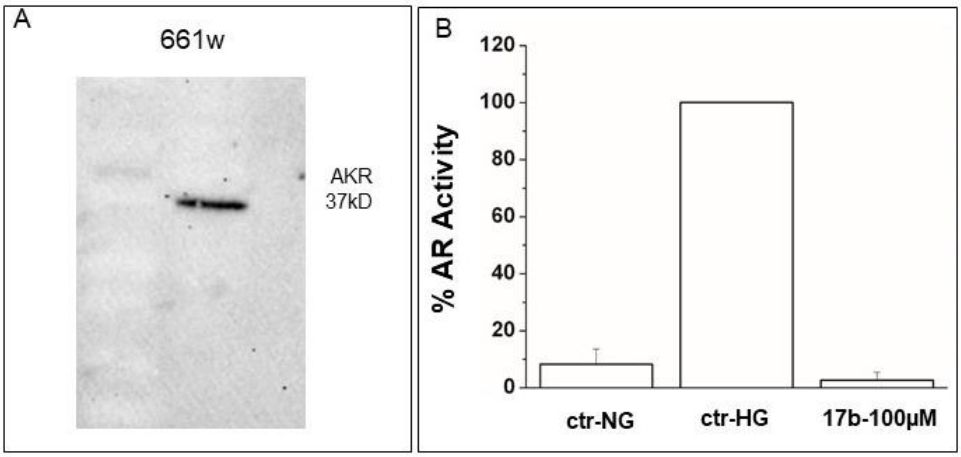


Figure 3. A) Representative western blot confirming the presence of ALR2 in the experimental cell model; B) Results obtained in the presence of **17b**. Inhibition of ALR2 was assessed by using a specific kit assay. Values are calculated as percentage of maximum activity measured in hyperglycemic control (ctr-HG), and are reported as mean ± SEM (n=3, independent experiments).

Once verified the ability of compound **17b** to inhibit the enzymatic activity of ALR2, its efficacy in both increasing cell viability and reducing the biological pathways that trigger hyperglycemia-induced cell death was also evaluated. Figure 4A shows how the treatment at two concentrations (50 and 100 μM) of the test compound is able to increase, although not significantly, the levels of cellular vitality. Moreover, Figure 4B indicates the ability of **17b** to reduce significantly the apoptotic process, the major pathway of death of retinal neurons.

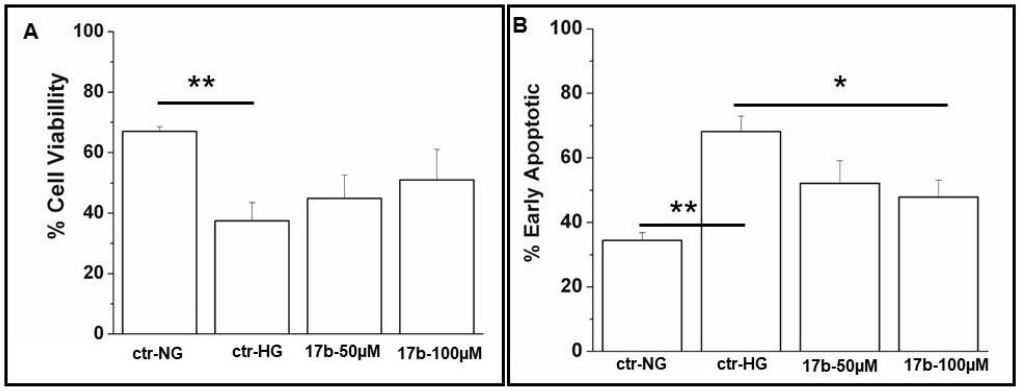


Figure 4. Effectiveness of compound **17b** in increasing cell viability. A) the histogram shows that while the hyperglycemic condition impairs significantly the cell viability, compared to the normoglycemic control (ctr-NG), the treatment with **17b** at both 50 and 100 μM concentration partially recovers the level of cell viability; B) The histograms show results obtained for apoptotic pathway inhibition. It is important to note that the level of apoptotic process is significantly reduced after the treatment with 100μM of **17b**. The values are reported as mean ± SEM (n=3, independent experiments).

Finally, the ability of **17b** to act also against oxidative stress triggered by hyperglycemic conditions was evaluated. Figure 5A shows the immunocytochemistry for Nrf2 as a transcription factor for phase 2 antioxidant enzymes. Under physiological conditions, Nrf2 is found in the cytosol complex with the inhibitory factor Keap1. Under high oxidative stress, like in hyperglycemic conditions, ROS induce the disruption of the Nrf2-Keap1 complex and while Keap1 is degraded, Nrf2 translocates to the nucleus where it interacts with the specific DNA sequence ARE, thus inducing the increase of the synthesis of antioxidant enzymes such as Sod1. As can be seen from Figure 5A, in the ctr-NG, the localization of Nrf2 is cytosolic (green staining) and well separated from the red nuclear staining, but in the ctr-HG the green cytosolic staining disappears and a yellow-orange color becomes visible of the nucleus, indicating that Nrf2 translocation occurred in the nucleus in response to the increase in ROS. Treatment with **17b** at a concentration of 100 μM restores the localization of cytosolic Nrf2, and results obtained are fully comparable to those achieved in ctr-NG. This result is also confirmed by data obtained by biochemical

evaluation of Sod1 levels. Indeed, Figure 5B shows how the 100 µM dose of **17b** is able to reduce Sod1 levels, indicating a reduction of ROS levels.

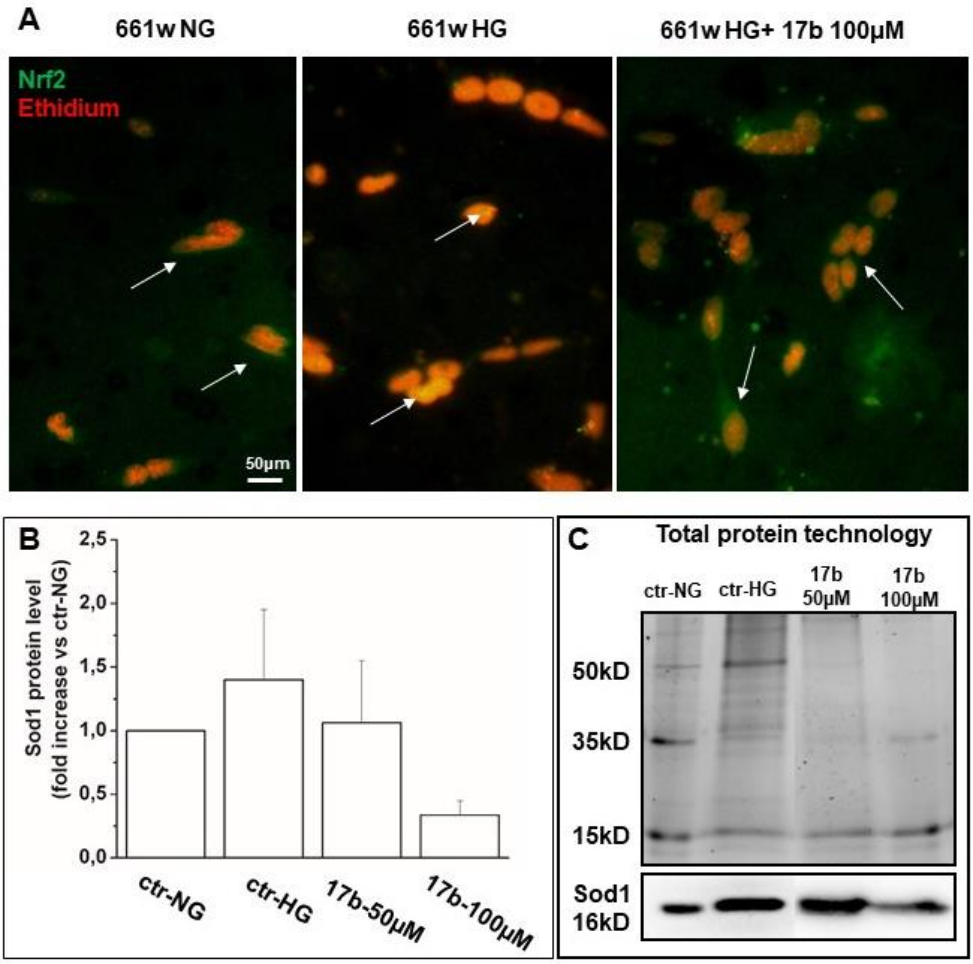


Figure 5. Antioxidant activity of **17b**. A) The image obtained with the florescence microscope shows the specific staining for Nrf20 (green) and the nuclear staining obtained with ethidium bromide (red). The arrows indicate the different localization of Nrf2, cytosolic in normoglycemic conditions and after treatment with **17b**, while it is nuclear (yellow-orange marking, co-localization) in hyperglycemic conditions. B) The histogram shows the protein levels of the antioxidant enzyme Sod1. To be noted that the treatment with **17b** is effective in restoring protein levels in a way comparable to those of ctr-NG. The values are reported as mean ± SEM (n=3, independent experiments). C) Representative image of a

western blot experiment, the content of Sod1 was normalized for total protein content by using the Stain Free Technology (BioRad) [31].

3. Conclusions

Polyhydroxylated pyrrolidine derivatives of the D-*gluco* and D-*galacto* series have been prepared, exploiting the aminocyclization reaction of two differentially protected D-*xylo*-hexos-4-ulose derivatives. The stereoselection of the reaction was affected by the steric hindrance of the amine partner employed. This stereochemical outcome strongly differs from what has been reported before for D-*xylo*-hexos-5-ulose derivatives and is probably related to the different size of the cyclic iminium ion intermediate (6-membered *versus* 5-membered) as well as the stabilities of the conformers involved in the stereodetermining step. These compounds, belonging to both D-*gluco* and D-*galacto* stereochemical series, were tested both as AG and as ALR2 inhibitors. Intriguing structure-related activity trends were observed, which, far from being conclusive, suggest that subtle interactions between the enzymes and the iminosugar are present and that the development of bi-functional molecules of this kind deserves to be further investigated. Moreover, compound **17b**, emerged as the one showing the best inhibitory activity against ALR2, has been shown to have activity also on an in vitro model of diabetic retinopathy. Indeed, once tested in photoreceptor-like 661w hyperglycemic cell line, **17b** reduced the processes of cell death and restored the physiological levels of oxidative stress, probably reducing the production of ROS mediated by ALR2.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 20±2 °C. Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded in

appropriate solvents with a Bruker AC 200 (200.13 MHz) or Bruker Avance II (250.13 MHz or 400 MHz). ^{13}C NMR spectra were recorded with the spectrometers operating at 50.33 or 62.9 MHz. The assignments were made, when possible, with the aid of DEPT-135, HETCOR, HSQC and COSY experiments and by comparison of values for known compounds and applying the additivity rules [32]. In the case of mixtures, assignments were made by referring to the differences in the peak intensities. The first order proton chemical shifts δ are referenced to either residual CD_3CN (δ_H 1.94, δ_C 1.28) or residual CD_3OD (δ_H 3.31 ppm, δ_C 49.0 ppm) and *J*-values are given in Hz. All reactions were followed by TLC on Kieselgel 60 F_{254} with detection by UV light and/or with ethanolic 10% phosphomolybdic or sulfuric acid, and heating or for exposure to iodine vapors. Kieselgel 60 (E. Merck, 70-230 and 230-400 mesh, respectively) was used for column and flash chromatography. Some of flash chromatography were conducted by the automated system Isolera Four SVTM (Biotage®), equipped with UV detector with variable wavelength (200-400 nm). All reactions involving air- or moisture-sensitive reagents were performed under an argon atmosphere using anhydrous solvents. Anhydrous methanol (MeOH) and pyridine were purchased from Sigma-Aldrich. Other dried solvents were obtained by distillation according to standard procedure [33] and stored over 4Å molecular sieves activated at least 12 h at 200 °C. All reagents were purchased from Aldrich Chemical Co. and were used without further purification. $MgSO_4$ was used as the drying agent for solutions. Elemental analysis was used to determine the purity of compounds. Analytical results are within ± 0.40% of the theoretical values.

The following standard procedure was used for acetylation: a solution of the compound (1.0 mmol) in a 2:1 (v/v) mixture (6 mL) of pyridine and Ac_2O was stirred at room temperature for 12-24 h, and then repeatedly co-evaporated under diminished pressure with toluene, and the residue was purified by flash chromatography on silica. "Solid foam" refers to amorphous compounds, recovered pure by chromatography for which all attempts to crystallize failed. 3-*deoxy*-1,2:5,6-di-*O*-isopropylidene-D-*erythro*-hex-3-enofuranose (**3**) [15, 16], (4R)- and (4S)-3-*O*-*m*-chlorobenzoyl-4-*C*-hydroxy-1,2:5,6-di-

O-isopropylidene-α-D-*xylo*-hexofuranose (**4a** and **4b**) [17] and D-*xylo*-hexos-4-ulose (**6**) [17] were prepared according to the reported procedure.

4.1.2. 5,6-*O*-isopropylidene-D-xylo-hexos-4-ulose (5). The known mixture of 3-*O*-*m*-chlorobenzoyl derivatives **4a,b** [17], (1.00 g, 2.42 mmol) was dissolved in 5:1 (v/v) MeOH-H_2O (50 mL), treated with Et_3N (0.85 mL) and the solution was stirred at room temperature until TLC analysis (EtOAc) revealed the complete disappearance of the starting material (R_f 0.72) and the formation of a more retained product. After 15 min the mixture was concentrated under diminished pressure and repeatedly co-evaporated with toluene (4×15 mL). The crude product (1.16 g) was a syrup and the NMR analysis (^{1}H and ^{13}C) showed the presence of 1,4-dicarbonyl derivative **5** as a complex mixture of tautomeric forms that was submitted without further purification to a double-reductive aminocyclisation.

4.1.3. N-*benzyl-2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-glucitol (7a) and* N-*benzyl-2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-galactitol (7b).* A solution of the crude D-*xylo*-esos-4-ulose (**6**) [17] (1.10 g, 4.16 mmol, 1.0 eq) in dry MeOH (12 mL) was treatment with a solution of $BnNH_2$ (0.51 mL, 4.58 mmol, 1.1 eq) and AcOH (0.38 mL) in dry MeOH (23 mL) and stirred at room temperature. After 5 min $NaBH_3CN$ (576 mg, 9.16 mmol, 2.2 eq) was added, the mixture was heated to 60 °C and stirred until the starting material was completely disappeared (48 h, TLC, 1:1 EtOAc-MeOH). The solution was cooled to room temperature, excess $NaBH_3CN$ was destroyed by adding methanol HCl (1.3 N) until pH 1-2 and stirred at room temperature for 1 h. The solution was neutralized by addition of solid $NaHCO_3$, stirred for 30 min and filtered over a short pad of Celite®, washed with MeOH, and the combined organic phases were concentrated at diminished pressure. The crude residue was dissolved in a mixture of pyridine and Ac_2O (2:1 v/v, 90 mL) and stirred at room temperature until TLC analysis (EtOAc, 20 h) revealed the formation of a single spot (R_f 0.57). The solution was co-evaporated with toluene (4×30 mL) under diminished pressure and the crude residue was partitioned between CH_2Cl_2 (80 mL) and satd aq $NaHCO_3$ (25 mL) and the aqueous phase extracted with CH_2Cl_2 (4×40 mL). The organic

layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure to leave a crude residue (1.73 g) constituted (NMR, $CDCl_3$) by a mixture of 1-deoxyazafuranose derivatives **7a** and **7b** in the ratio of 4:6, measured on the relative intensities of two C-4 signals at δ 65.6 and 68.3 respectively. Purification of crude product by flash chromatography on silica gel (first 7:3 hexane-EtOAc + 0.1% Et_3N and then 3:7 hexane-EtOAc + 0.1% Et_3N) gave a mixture of pure **7a** and **7b** (872 mg, 50%) as a clear syrup, R_f 0.57 (EtOAc). Compounds **7a** and **7b** were inseparable by TLC with several elution systems and their structures were unequivocally inferred from their NMR data. A second purification of a mixture of **7a** and **7b** by flash-chromatography on silica gel (1:9 hexane-EtOAc) afforded a sample of pure **7b**, as a clear syrup, $[\alpha]_D$ -31.8 (*c* 0.9 in $CHCl_3$), ^{1}H NMR (250.13 MHz, $CDCl_3$): δ 7.28-7.14 (m, 5H, Ar-*H*), 5.20 (ddd, 1H, $J_{5,6a}$ 8.2 Hz, $J_{4,5}$ 6.2 Hz, $J_{5,6b}$ 2.5 Hz, H-5), 5.14 (m, 1H, H-3), 4.89 (m, 1H, H-2), 4.58 (dd, 1H, $J_{6a,6b}$ 12.1 Hz, H-6b), 4.11 (dd, 1H, H-6a), 4.03, 3.46 (AB system, 2H, J_{AB} 13.5 Hz, CH_2Ph), 2.96-2.89 (m, 2H, H-4, H-1α), 2.65 (dd, 1H, $J_{1\beta,1\alpha}$ 11.6 Hz, $J_{1\beta,2}$ 4.7 Hz, H-1β), 2.14-1.96 (m, 12H, 4×*Me*CO); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 170.8-169.1 (4×C=O), 137.8 (Ar-*C*), 128.5-127.3 (Ar-*C*H), 77.2 (C-3), 76.0 (C-2), 70.9 (C-5), 68.3 (C-4), 63.1 (C-6), 59.1 (*C*H_2Ph), 56.6 (C-1), 20.9-20.8 (4×*Me*CO). Anal. Calcd for $C_{21}H_{27}NO_8$: C, 59.85; H, 6.43; N, 3.32. Found: C, 59.78; H, 6.39; N, 3.27.

The NMR analysis of the mixture of **7a** and **7b** allowed assigning the chemical shifts of the proton and carbon related to the azafuranosic structure **7a.** ^{1}H NMR (200.13 MHz, $CDCl_3$): δ 7.37-7.19 (m, 5H, Ar-*H*), 5.48 (dd, 1H, $J_{3,4}$ 6.5 Hz, $J_{2,3}$ 3.1 Hz, H-3), 5.22 (m, 1H, H-5), 5.00 (ddd, 1H, $J_{1\beta,2}$ 5.3 Hz, $J_{1\alpha,2}$ 4.5 Hz, H-2), 4.26-4.20 (m, 2H, H-6a, H-6b), 4.18, 3.54 (AB system, 2H, J_{AB} 13.4 Hz, CH_2Ph), 3.40 (dd, 1H, $J_{4,5}$ 5.1 Hz, H-4), 3.29 (dd, 1H, $J_{1\beta,1\alpha}$ 11.5 Hz, H-1β),2.40 (dd, 1H, H-1α), 2.11-2.04 (m, 12H, 4×*Me*CO); ^{13}C NMR (50.33 MHz, $CDCl_3$) δ 170.4-168.8 (C=O), 138.1 (Ar-*C*), 128.6-126.1 (Ar-*C*H), 76.6, 76.3 (C-2, C-3), 70.9 (C-5), 65.6 (C-4), 63.3 (C-6), 60.9 (*C*H_2Ph), 55.9 (C-1), 20.6 (4×*Me*CO). Anal. Calcd for $C_{21}H_{27}NO_8$: C, 59.85; H, 6.43; N, 3.32. Found: C, 59.75; H, 6.35; N, 3.28.

4.1.4. N-*benzhydryl-1,4-dideoxy-1,4-immino-D-glucitol (8a) and* N-*benzhydryl-1,4-dideoxy-1,4-immino-D-galactitol (8b).* A solution of the crude D-*xylo*-esos-4-ulose (**6**) [17] (253 mg, 1.38 mmol, 1.0 eq) in dry MeOH (12 mL) was treatment with a solution of Ph_2CHNH_2 (0.24 mL, 1.38 mmol, 1 eq) and AcOH (0.11 mL) in dry MeOH (7 mL) and stirred at room temperature. After 5 min $NaBH_3CN$ (173 mg, 2.76 mmol, 2 eq) was added, the mixture was heated to 60 °C and stirred until TLC analysis (10:3 EtOAc-MeOH) showed the complete disappearance of the starting material (5 h) and the formation of a single spot (R_f 0.42). The solution was cooled to room temperature, excess $NaCNBH_3$ was destroyed by adding methanol HCl (1.3 N) until pH 1-2 and stirred at room temperature for 1 h. The solution was neutralized by addition of solid $NaHCO_3$, stirred for 30 min and filtered over a short pad of Celite®, washed with MeOH, and the combined organic phases were concentrated at diminished pressure. The crude residue (625 mg) constituted (NMR, CD_3CN-D_2O) by a mixture of azafuranose derivatives **8a** and **8b** in the ratio of 60:40, measured on the relative intensities of two C-4 signals at δ 60.0 and 64.6 respectively. Purification of crude product by flash chromatography on silica gel (9:1 EtOAc-MeOH + 0.1% Et_3N) gave pure a mixture of **8a** and **8b** (237 mg, 52%) as a yellow foam, R_f 0.42 (10:3 EtOAc-MeOH). Compounds **8a** and **8b** were inseparable by TLC with several elution systems and their structures were unequivocally inferred from their NMR data. ^{1}H NMR (200.13 MHz, CD_3CN-D_2O) of **8a**: δ 5.01 (s, 1H, C*H*Ph_2), 3.20 (dd, 1H, $J_{1\beta,1\alpha}$ 10.8 Hz, $J_{1\beta,2}$ 4.8 Hz, H-1β), 3.06 (dd, 1H, $J_{4,5}$ 5.5 Hz, $J_{3,4}$ 3.2 Hz, H-4), 2.33 (dd, 1H, $J_{1\alpha,2}$ 4.1 Hz H-1α); of **8b**: δ 4.94 (s, 1H, C*H*Ph_2), 2.90 (m, 1H, H-4), 2.87 (dd, 1H, $J_{1\beta,1\alpha}$ 11.4 Hz, $J_{1\alpha,2}$ 3.0 Hz H-1α), 2.71 (dd, 1H, $J_{1\beta,2}$ 5.1 Hz, H-1β); cluster of signals for **8a** and **8b**: δ 7.63-7.19 (m, 10H, Ar-*H*), 4.04 (m, 4H, 2×H-2, 2×H-3), 3.40 (m, 6H, 2×H-5, 2×H-6a, 2×H-6b); ^{13}C NMR (50.33 MHz, CD_3CN-D_2O) of **8a**: δ 79.4, 76.6 (C-2, C-3), 73.4 (C-5), 71.6 ($CHPh_2$), 64.5 (C-6), 60.0 (C-4), 56.2 (C-1); of **8b**: δ 80.1, 73.9 (C-2, C-3), 73.4 ($CHPh_2$), 71.6 (C-5), 64.6 (C-4), 64.5 (C-6), 57.7 (C-1);

cluster of signals for **8a** and **8b**: δ 143.8, 142.8 (2×Ar-*C*), 129.6-126.6 (Ar-*C*H). Anal. Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.22; H, 6.99; N, 4.21.

4.1.5. N-*benzhydryl-2,3,5,6-tetra*-O-*acetyl-1,4-dideoxy-1,4-immino-D-glucitol (9a) and* N-*benzhydryl-2,3,5,6-tetra*-O-*acetyl-1,4-dideoxy-1,4-immino-D-galactitol (9b).* Routine acetylation of a mixture of **8a** and **8b** (237 mg, 0.72 mmol) with 1:2 Ac_2O-Py mixture (9.0 mL) gave after flash chromatography (7:3 hexane-EtOAc+ 0.1% Et_3N) pure a 6:4 mixture of tetra-*O*-acetyl azafuranose derivatives **9a** and **9b** (250 mg, 71%) as a syrup, R_f 0.36 (1:1 hexane- EtOAc). Compounds **9a** and **9b** were inseparable by TLC with several elution systems and their structures were in equivocally inferred from their NMR data. ^{1}H NMR (200.13 MHz, $CDCl_3$) of **9a**: δ 5.30 (m, 2H, H-3, H-5), 5.23 (s 1H, C*H*Ph_2), 5.12 (m, 1H, H-2), 4.53 (dd, 1H, $J_{6a,6b}$ 12.0 Hz, $J_{5,6b}$ 3.5 Hz, H-6b), 4.08 (dd, 1H, $J_{5,6a}$ 7.7 Hz, H-6a), 3.61 (dd, 1H, $J_{4,5}$ 7.0 Hz, $J_{3,4}$ 3.2 Hz, H-4), 3.34 (dd, 1H, $J_{1\beta,1\alpha}$ 11.6 Hz, $J_{1\beta,2}$ 6.1 Hz, H-1β), 2.63 (dd, 1H, $J_{1\alpha,2}$ 4.2 Hz H-1α), 2.13, 2.12, 1.97, 1.83 (4s, each 3H, 4×*Me*CO); of **9b**: δ 5.12 (m, 3H, H-2, H-3, H-5), 5.08 (s 1H, C*H*Ph_2), 4.62 (dd, 1H, $J_{6a,6b}$ 12.2 Hz, $J_{5,6b}$ 2.7 Hz, H-6b), 4.18 (dd, 1H, $J_{5,6a}$ 8.2 Hz, H-6a), 3.20 (dd, 1H, $J_{4,5}$ 6.7 Hz, $J_{3,4}$ 2.0 Hz, H-4), 3.10 (dd, 1H, $J_{1\beta,1\alpha}$ 12.0 Hz, $J_{1\alpha,2}$ 2.3 Hz H-1α), 2.84 (dd, 1H, $J_{1\beta,2}$ 5.4 Hz, H-1β), 2.17, 2.13, 2.01, 1.94 (4s, each 3H, 4×*Me*CO); cluster of signals for **9a** and **9b**: δ 7.38-7.26 (m, 10H, Ar-*H*); ^{13}C NMR (50.33 MHz, $CDCl_3$) of **9a**: δ 141.3, 139.2 (2×Ar-*C*), 75.7, 75.6 (C-2, C-3), 70.7 (*C*HPh_2), 68.4 (C-5), 63.4 (C-6), 60.7 (C-4), 51.3 (C-1); of **9b**: δ 141.4, 139.0 (2×Ar-*C*), 78.0, 75.9 (C-2, C-3), 71.0 (C-5), 70.5 (*C*HPh_2), 66.2 (C-4), 63.4 (C-6), 53.3 (C-1); cluster of signals for **9a** and **9b**: δ 170.2-169.6 (C=O), 129.2-126.9 (Ar-*C*H), 21.0-20.3 (4×*Me*CO). Calcd for $C_{27}H_{31}NO_8$: C, 65.18; H, 6.28; N, 2.83. Found: C, 65.14; H, 6.25; N, 2.80.

4.1.6.* N-*acetyl-2,3,5,6-tetra*-O-*acetyl-1,4-dideoxy-1,4-immino-D-glucitol (10a) and* N-*acetyl-2,3,5,6-tetra*-O-*acetyl-1,4-dideoxy-1,4-immino-D-galactitol (10b). A solution of the crude D-*xylo*-esos-4-ulose (**6**) [17] (560 mg, 1.65 mmol, 1.0 eq) in dry MeOH (23 mL) was treatment with a solution of ammonium

acetate ($NH_4^+AcO^-$, 1.37 g, 17.8 mmol, 10.8 eq) in dry MeOH (39 mL) and stirred at room temperature. After 5 min $NaBH_3CN$ (228 mg, 3.62 mmol, 2.2 eq) was added, the mixture was heated to 60 °C and stirred until the starting material was completely disappeared (90 h, TLC, 1:1 EtOAc-MeOH). The solution was cooled to room temperature, excess $NaCNBH_3$ was destroyed by adding methanol HCl (1.3 N) until pH 1-2 and stirred at room temperature for 1 h. The solution was neutralized by addition of solid $NaHCO_3$, stirred for 30 min and filtered over a short pad of Celite®, washed with MeOH, and the combined organic phases were concentrated at diminished pressure. Routine acetylation of crude residue with 1:2 Ac_2O-Py (10.0 mL) gave after work-up the reaction a crude syrup (714 mg) constituted (NMR) by a mixture of azafuranose derivatives **10a** and **10b** in the ratio of 15:85, measured on the relative intensities of two C-4 signals at δ 56.1 (for **10a**) and 61.6 and 63.5 (for rotational isomers amide **10b**). Purification of crude product by flash chromatography on silica gel (7:2:1 Et_2O-EtOAc-^{i}PrOH) gave pure a mixture of **10a** and **10b** (338 mg, 55%). Compounds **10a** and **10b** were inseparable by TLC with several elution systems and their NMR spectroscopic data of **10a** and **10b** was described below.

4.1.7.* N-*benzyl-5,6*-O-*isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (11a) and* N-*benzyl-5,6*-O-*isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (11b). The crude 5,6-*O*-isopropylidene-D-*xylo*-hexos-4-ulose (**5**) (1.16 g) obtained from **4a,b** (2.42 mmol) was dissolved in dry MeOH (50 mL), and a solution of $BnNH_2$·HCl (346 mg, 2.42 mmol, 1 eq) in dry MeOH (20 mL) and $NaBH_3CN$ (303 mg, 4.84 mmol, 2.2 equiv.) were added. The mixture was heated to 60 °C and stirred until TLC analysis (9:1 EtOAc-MeOH) showed the complete disappearance of the starting material (40 h) and the formation of a two spot (R_f 0.52 and 0.38) visible under UV light. The solution was cooled to room temperature and concentrated under diminished pressure. The crude residue was partitioned between CH_2Cl_2 (70 mL) and satd aq $NaHCO_3$ (50 mL) and the aqueous phase was extracted with CH_2Cl_2 (4×50 mL). The organic layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure to leave a crude syrup (680 mg) constituted (NMR) by a mixture of azafuranose derivatives **11a** and **11b** in the ratio of 1:1,

measured on the relative intensities of two C-4 signals at δ 66.7 and 73.7 respectively. Purification of crude product by flash chromatography on silica gel (1:1 Et_2O-EtOAc + 0.1% Et_3N) gave pure **11a** (90 mg, calculated from **4a,b** 13%) and **11b** (168 mg, 24% calculated from **4a,b**).

N-benzyl-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (**11a**) as a white solid, R_f 0.52 (9:1 EtOAc-MeOH); $[\alpha]_D$ -23.9 (c 1.1 in $CHCl_3$), mp 83-85 °C (from EtOAc-hexane), ^{1}H NMR (250.13 MHz, $CDCl_3$): δ 7.39-7.24 (m, 5H, Ar-*H*), 4.27 (m, 1H, H-5), 4.20 (dd, 1H, $J_{3,4}$ 5.9 Hz, $J_{2,3}$ 3.7 Hz, H-3), 4.15-4.08 (m, 2H, H-2, H-6b), 4.13, 3.58 (AB system, 2H, J_{AB} 13.2 Hz, CH_2Ph), 3.96 (dd, 1H, $J_{6a,6b}$ 8.0 Hz, $J_{5,6a}$=7.6 Hz,H-6a), 3.28 (dd, 1H, $J_{1\beta,1\alpha}$ 10.5 Hz, $J_{1\beta,2}$ 5.7 Hz, H-1β), 3.22 (bt, 1H, $J_{4,5}$ $J_{3,4}$=5.9 Hz, H-4), 3.50.3.30 (bs, 2H, 2×OH), 2.35 (dd, 1H, $J_{1\alpha,2}$ 5.5 Hz, H-1α),1.44, 1.37 (2s, each 3H, C*Me*$_2$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 138.0 (Ar-*C*), 128.9-127.3 (Ar-*C*H), 108.5 (*C*Me$_2$), 78.9 (C-3), 76.1, 76.0 (C-2, C-5), 67.1 (*C*H$_2$Ph), 66.5 (C-4), 60.4 (C-6), 58.3 (C-1), 26.4, 25.0 (C*Me*$_2$). Anal. Calcd for $C_{16}H_{23}NO_4$: C, 65.51; H, 7.90%; N, 4.77%; Found: C, 65.49; H, 7.85%; N, 4.74%.

N-benzyl-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (**11b**) as a colourless syrup, R_f 0.38 (9:1 EtOAc-MeOH); $[\alpha]_D$ +52.2 (c 1.2 in $CHCl_3$), ^{1}H NMR (200.13 MHz, CD_3CN): δ 7.37-7.24 (m, 5H, Ar-*H*), 4.29 (ddd, 1H, $J_{4,5}$ 6.6 Hz, $J_{5,6a}$ 7.2 Hz, $J_{5,6b}$ 6.4 Hz, H-5), 4.24, 3.42 (AB system, 2H, J_{AB} 13.6 Hz, CH_2Ph), 4.03 (dd, 1H, $J_{6a,6b}$ 8.4 Hz, H-6b), 3.93 (dd, 1H, H-6a), 3.82 (m, 1H, H-2), 3.76 (m, 1H, H-3), 3.39 (bs, 2H, 2×OH), 2.61 (dd, 1H, $J_{3,4}$ 4.1 Hz, H-4), 2.54 (dd, 1H, $J_{1\alpha,1\beta}$ 10.4 Hz, $J_{1\beta,2}$ 4.8 Hz, H-1β), 2.70 (bd, 1H, H-1α),1.42, 1.33 (2s, each 3H, (C*Me*$_2$); ^{13}C NMR (50.33 MHz, CD_3CN): δ 140.3 (Ar-*C*), 129.6-127.7 (Ar-*C*H), 109.6 (*C*Me$_2$), 80.9, 78.8 (C-2, C-3), 77.4 (C-5), 73.7 (C-4), 67.0 (C-6), 60.3 (*C*H$_2$Ph), 60.0 (C-1).27.0, 25.6 (C*Me*$_2$). Anal. Calcd for $C_{16}H_{23}NO_4$: C, 65.51; H, 7.90%; N, 4.77%; Found: C, 65.48; H, 7.86%; N, 4.75%.

4.1.8.* N-*[(2'R)-1'-carbomethoxy-2'-benzylethyl]-2,3-di-*O*-acetyl-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (13a) and N-*[(2'R)-1'-carbomethoxy-2'-benzylethyl]-2,3-di-*O*-acetyl-5,6-*O*-

isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (13b). The crude 5,6-*O*-isopropylidene-D-*xylo*-hexos-4-ulose (**5**) (534 mg, 2.45 mmol) obtained from **4a,b** was dissolved in dry MeOH (30 mL), and a solution of D-Phe-OMe·HCl (580.7 mg, 2.69 mmol, 1.1 eq) in dry MeOH (6.0 mL) and a solution of $NaBH_3CN$ (338 mg, 5.38 mmol, 2.2 equiv.) in dry MeOH (6.0 mL) were added. The mixture was heated to 65 °C and stirred until TLC analysis (1:1 Et_2O-EtOAc + Et_3N) showed the complete disappearance of the starting material (48 h) and the formation of a major spot (R_f 0.50) visible under UV light. The solution was cooled to room temperature and concentrated under diminished pressure. The crude residue was partitioned between CH_2Cl_2 (30 mL) and satd aq $NaHCO_3$ (30 mL) and the aqueous phase was extracted with CH_2Cl_2 (4×35 mL). The organic layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure to leave a crude syrup (772 mg) constituted (NMR) by a mixture of azafuranose derivatives **12a** and **12b** in the ratio of 6:4, measured on the relative intensities of two C-4 signals at δ 64.5 and 68.4 respectively. Purification of crude product by flash chromatography on silica gel (3:1 Et_2O-EtOAc + Et_3N) gave pure a 7:3 mixture of **12a** and **12b** (375 mg, 42% calculated from **4a,b**), impure for the excess of D-Phe-OMe. Compounds **12a** and **12b** were inseparable by TLC with several elution systems and the NMR analysis of the isolated fraction allowed assigning only the chemical shifts of the carbon related to the azafuranosic structures. ^{13}C NMR (62.9 MHz, $CDCl_3$) of **12a**: δ 173.6 (C=O), 138.2 (Ar-*C*), 109.0 (*C*Me_2), 79.3, 78.1 (C-3, C-2), 75.3 (C-5), 65.6 (C-6), 64.5 (C-4, *C*HN), 52.1 (C-1), 51.4 (OMe), 36.8 (*C*H_2Ph), 26.2, 24.9 (*C*Me_2); of **12b**: δ 175.1 (C=O), 138.0 (Ar-*C*), 108.0 (*C*Me_2), 79.2, 77.0 (C-3, C-2), 75.8 (C-5), 68.9 (C-4), 66.1 (C-6), 63.7 (*C*HN), 52.7 (C-1), 51.5 (OMe), 32.4 (CH_2Ph), 26.1, 25.3 (*C*Me_2); cluster of signals for **12a** and **12b**: δ 129.1-126.2 (Ar-*C*H).

Routine acetylation of a mixture of **12a** and **12b** (200 mg, 0.55 mmol) with 1:2 Ac_2O-Py mixture (10.0 mL) gave after flash chromatography (6:4 hexane-EtOAc) pure a 1:1 mixture of di-*O*-acetyl azafuranose derivatives **13a** and **13b** (185 mg, 75%) as a colourless syrup, R_f 0.62 (6:4 hexane-EtOAc), Compounds **13a** and **13b** were inseparable by TLC with several elution systems and their structures were in

equivocally inferred from their NMR data. ^{1}H NMR (250.13 MHz, $CDCl_3$) of **13a**: δ 5.24 (dd, 1H, $J_{3,4}$ 6.3 Hz, $J_{2,3}$ 3.7 Hz, H-3), 5.01 (m, 1H, H-2), 4.20-4.06 (m, 3H, H-5, H-6b, C*H*N), 3.90 (dd, 1H, $J_{6a,6b}$ 7.4 Hz, $J_{5,6b}$ 6.4 Hz, H-6b), 3.76 (dd, 1H, $J_{4,5}$ 3.3 Hz, H-4), 3.56 (s, 3H, OMe), 3.45 3.34 (dd, 1H, $J_{1\beta,1\alpha}$ 11.4 Hz, $J_{1\beta,2}$ 5.8 Hz, H-1β), 3.15 (m, 1H, H-1α), 1.36, 1.27 (2s, each 3H, C*Me*$_2$); of **13b**: δ 4.95 (m, 1H, H-2), 4.88 (bd, 1H, $J_{3,4}$ 3.3 Hz, H-3), 4.04-4.00 (m, 2H, H-5, C*H*N), 3.76 (dd, 1H, $J_{4,5}$ 7.2 Hz, H-4), 3.68 (s, 3H, OMe), 3.63 (m, 2H, H-6a, H-6b), 3.45 (dd, 1H, $J_{1\beta,1\alpha}$ 11.0 Hz, $J_{1\beta,2}$ 4.8 Hz, H-1β), 3.15 (dd, 1 H, $J_{1\alpha,2}$ 6.8 Hz, H-1α,), 1.37, 1.31 (2s, each 3H, C*Me*$_2$), cluster of signals for **13a** and **13b**: δ 7.29-7.16 (m, 5 H, Ar-*H*), 3.12-2.90 (m, 2 H, CH_2Ph), 2.06-2.04 (2s, each 3H, 2×*Me*CO); ^{13}C NMR (62.9 MHz, $CDCl_3$) of **13a**: δ 172.2 (*C*OOMe), 137.5 (Ar-*C*), 108.0 (*C*Me$_2$), 77.0 (C-3), 75.8 (C-5), 75.1 (C-2), 65.4 (C-6), 64.1 (*C*HN), 61.0 (C-4), 51.5 (OMe), 50.2 (C-1), 36.3 (*C*H_2Ph), 26.1, 24.7 (C*Me*$_2$); of **13b**: δ 172.0 (COOMe), 138.0 (Ar-*C*), 109.2 (*C*Me$_2$), 77.1 (C-3), 76.1 (C-5), 75.9 (C-2), 67.1 (C-4), 65.7 (C-6), 62.3 (*C*HN), 51.1 (OMe), 50.6 (C-1), 31.2 (*C*H_2Ph), 26.3, 24.9 (C*Me*$_2$); cluster of signals for **13a** and **13b**: δ 169.9-169.3 (C=O), 129.1-126.3 (Ar-*C*H), 20.8 (4×*Me*CO). Anal. Calcd for $C_{23}H_{31}NO_8$: C, 61.46; H, 6.95%; N, 3.12%; Found: C, 61.43; H, 6.90%; N, 3.09%.

4.1.9.* N-*[(2'R)-1'-carbomethoxy-2'-benzylethyl]-2,3-di-*O*-acetyl-1,4-dideoxy-1,4-immino-D-glucitol (14a) and* N-*[(2'R)-1'-carbomethoxy-2'-benzylethyl]-2,3-di-*O*-acetyl-1,4-dideoxy-1,4-immino-D-galactitol (14b). A solution of 1:1 mixture of di-*O*-acetyl azafuranose derivatives **13a** and **13b (**81 mg, 0.18 mmol) in 80% aq AcOH (v/v,3.5 mL) was stirred at 40 °C until the TLC analysis (2:8 hexane-EtOAc) revealed the complete reaction of the starting material with formation of a major slower moving products (R_f 0.35). The solution was then cooled to room temperature and co-evaporated with toluene (6×5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (3:7 hexane-EtOAc) gave pure a mixture of azafuranose derivatives **14a** and **14b** (34 mg, 46%) in the ratio of 65:35, measured on the relative intensities of two *C*H_2Ph signals at δ 37.5 and 33.0

respectively. Compounds **14a** and **14b** as a colourless syrup, R_f 0.35 (2:8 hexane-EtOAc), were unseparable by TLC with several elution systems and the NMR analysis of the isolated fraction allows to assign only the chemical shifts of the carbon related to the azafuranosic structures. ^{13}C NMR (62.9 MHz, CD_3CN) of **14a**: δ 139.4 (Ar-*C*), 79.3, 78.9, 77.2 (C-3, C-5, C-2), 62.2 (C-6), 61.5 (C-4), 59.7 (*C*HN), 56.3 (C-1), 52.1 (OMe), 37.5 (CH_2Ph); of **14b**: δ 139.9 (Ar-*C*), 79.0 (C-3), 76.3 (C-2), 70.8 (C-5), 67.0 (C-6), 64.9 (C-4), 63.9 (*C*HN), 52.1 (OMe), 50.6 (C-1), 33.0 (CH_2Ph); cluster of signals for **14a** and **14b**: δ 171.1-171.0 (C=O), 130.9-127.3 (Ar-*C*H), 21.2-21.0 (*Me*CO). Anal. Calcd for $C_{20}H_{27}NO_8$: C, 58.67; H, 6.65%; N, 3.42%; Found: C, 58.64; H, 6.61%; N, 3.39%.

4.1.10.* N-*[(2'S)-1'-carbomethoxy-2'-benzylethyl]-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (15a) and* N-*[(2'S)-1'-carbomethoxy-2'-benzylethyl]-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (15b). The crude 5,6-*O*-isopropylidene-D-*xylo*-hexos-4-ulose (**5**) (470 mg) obtained from **4a,b** (2.15 mmol) was dissolved in dry MeOH (25 mL), and a solution of L-Phe-OMe·HCl (511 mg, 2.37 mmol, 1.1 eq) in dry MeOH (7.0 mL) and a solution of $NaBH_3CN$ (298 mg, 4.74 mmol, 2.2 equiv.) in dry MeOH (7.0 mL) were added. The mixture was heated to 65 °C and stirred until TLC analysis (1:2 Et_2O-EtOAc + Et_3N) showed the complete disappearance of the starting material (48 h) and the formation of a major spot (R_f 0.51) visible under UV light. The solution was cooled to room temperature and concentrated under diminished pressure. The crude residue was partitioned between CH_2Cl_2 (30 mL) and satd aq $NaHCO_3$ (30 mL) and the aqueous phase was extracted with CH_2Cl_2 (4×35 mL). The organic layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure to leave a crude syrup (689 mg) constituted (NMR) by a mixture of azafuranose derivatives **15a** and **15b** in the ratio of 7:3, measured on the relative intensities of two C-4 signals at δ 63.4 and 69.6 respectively. Purification of crude product by flash-chromatography on silica gel (3:1 Et_2O-EtOAc + Et_3N) afforded a pure **15a** (166 mg, 21%, calculated from **4a,b**) and 35:65 mixture of **15a** and **15b** (182 mg), impure for the excess of L-Phe-OMe. *N*-[(2'S)-1'-carbomethoxy-2'-benzylethyl]-5,6-*O*-isopropylidene-1,4-dideoxy-

1,4-immino-D-glucitol (**15a**) as colourless syrup, R_f 0.52 (1:2 Et_2O-EtOAc + 0.1% di Et_3N), ^{1}H NMR (250.13 MHz, $CDCl_3$): δ 7.36-7.23 (m, 5 H, Ar-*H*), 4.17-4.06 (m, 2H, H-3, H-3), 3.96-3.89 (m, 2H, H-5, C*H*N), 3.73 (m, 1H, H-6b), 3.71 (s, 3H, OMe), 3.58-3.42 (m, 3H, H-1β, H-4, H-6a), 3.15-2.85 (m, 5H, H-1α, CH_2Ph, OH-2, OH-3), 1.33, 1.26 (2s, each 3H, C*Me₂*); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 175.2 (C=O), 138.1 (Ar-*C*), 129.0-126.5 (Ar-*C*H), 107.4 (*C*Me_2), 78.4, 76.6 (C-3, C-2), 75.7 (C-5), 63.9 (*C*HN), 63.4 (C-4), 66.0 (C-6), 53.0 (C-1), 51.8 (OMe), 37.7 (*C*H_2Ph), 26.2, 24.5 (C*Me₂*). Anal. Calcd for $C_{19}H_{27}NO_6$: C, 62.46; H, 7.45%; N, 3.83%; Found: C, 62.42; H, 7.41%; N, 3.79%.

The NMR analysis of the 35:65 mixture of **15a** and **15b** allows to assign only the chemical shifts of the carbon related to the azafuranosic structure **15b**. ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 173.4 (C=O), 138.4 (Ar-*C*), 129.1-126.1 (Ar-*C*H), 109.1 (*C*Me_2), 79.5, 78.2 (C-3, C-2), 75.6 (C-5), 64.0 (*C*HN), 69.4 (C-4), 66.4 (C-6), 54.2 (C-1), 51.4 (OMe), 32.5 (CH_2Ph), 26.4, 25.5 (C*Me₂*).

4.1.11.* N-*[(2'S)-1'-carbomethoxy-2'-benzylethyl]-1,4-dideoxy-1,4-immino-D-glucitol (16a). A solution of azafuranose derivative **15a** (48 mg, 0.131 mmol) in 80% aq AcOH (v/v, 2.6 mL) was stirred at 40 °C until the TLC analysis (9:1 EtOAc-MeOH) revealed the complete reaction of the starting material with formation of a major slower moving products (R_f 0.40). After 1 h the solution was then cooled to room temperature and co-evaporated with toluene (6×5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (95:15 EtOAc-MeOH) gave pure of azafuranose derivatives **16a** (38 mg, 88.8%) as colourless syrup, R_f 0.30 (95:15 EtOAc-MeOH), $[\alpha]_D$ -23.9 (c 1.1 in $CHCl_3$), ^{1}H NMR (250.13 MHz, CD_3OD): δ 7.28 (m, 5 H, Ar-*H*), 3.16, 2.96 (AB system, 2H, $J_{A,B}$ 13.6 Hz, CH_2Ph), 4.11 (dd, 1H, $J_{3,4}$ 4.8 Hz, $J_{2,3}$ 6.8 Hz, H-3), 4.00 (m, 1H, H-2), 3.99 (dd, 1H, $J_{2',A}$ 7.3 Hz, $J_{2',B}$ 8.2 Hz, C*H*N), 3.78 (m, 1H, H-5), 3.67 (s, 3H, OMe), 3.55 (dd, 1H, $J_{6a,6b}$ 11.2 Hz, $J_{5,6b}$ 4.2 Hz, H-6b), 3.45 (dd, 1H, $J_{5,6a}$ 5.7 Hz, H-6a), 3.40 (m, 1H, H-1β), 3.22 (dd, 1H, $J_{4,5}$ 6.3 Hz, H-4), 2.90 (m, 1H, H-1α); ^{13}C NMR (62.9 MHz, CD_3OD): δ 174.1 (C=O), 139.8 (Ar-*C*), 130.1-127.5 (Ar-*C*H), 79.1 (C-3),

76.4 (C-2), 73.0 (C-5), 64.8 (*C*HN), 64.3 (C-6), 64.0 (C-4), 52.4 (C-1), 51.7 (OMe), 38.0 (CH_2Ph). Anal. Calcd for $C_{16}H_{23}NO_6$: C, 55.06; H, 7.13%; N, 4.31%; Found: C, 55.02; H, 7.10%; N, 4.28%.

4.1.12. 5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (17a). To a solution of azafuranose derivative **11a** (369 mg, 1.26 mmol) in absolute EtOH (60 mL), 10% Pd on charcoal (230 mg) was added and the mixture was stirred at room temperature under H_2 (atmospheric pressure) until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material. After 48 h the suspension was filtered through a small layer of Celite® and evaporated under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (90:10:1 $CHCl_3$-MeOH-H_2O) gave pure of azafuranose derivatives **17a** (218 mg, 85%) as white solid, R_f 0.38 (70:30:3 $CHCl_3$-MeOH-H_2O), mp 160-164 °C (dec.); $[\alpha]_D$ -6.2 (c 1.0 in MeOH), ^{1}H NMR (400 MHz, CD_3CN-D_2O): δ 4.15 (dt, 1H, $J_{5,6a}$ = $J_{5,6b}$ 6.2 Hz, $J_{4,5}$ 86 Hz, H-5), 4.03 (dd, 1H, $J_{6a,6b}$ 8.4 Hz, H-6b), 3.98, 3.93 (2m, each 1H, H-2, H-3), 3.76 (dd, 1H, H-6b), 3.14-3.09 (m, 2H, H-1β, H-4), 2.62 (d, 1H, $J_{1\alpha,1\beta}$ 11.9 Hz, $J_{1\alpha,2}$ 1.5 Hz, H-1α), 1.35, 1.29 (2s, each 3H, C*Me*$_2$); ^{13}C NMR (100 MHz, CD_3CN-D_2O): δ 109.8 (*C*Me$_2$), 78.0, 77.6 (C-2, C-3), 74.8 (C-5), 68.4 (C-6), 63.6 (C-4), 52.7 (C-1), 26.9, 25.6 (C*Me*$_2$). Anal. Calcd for $C_9H_{17}NO_4$: C, 53.19; H, 8.43%; N, 6.89%; Found: C, 53.16; H, 8.39%; N, 6.86%.

4.1.13. 5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (17b). To a solution of azafuranose derivative **11b** (508 mg, 1.73 mmol) in absolute EtOH (60 mL), 10% Pd on charcoal (327 mg) was added and the mixture was stirred at room temperature under H_2 (atmospheric pressure) until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material. After 48 h, the suspension was filtered through a small layer of Celite® and evaporated under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (90:10:1 $CHCl_3$-MeOH-H_2O) gave pure **17b** (306 mg, 87%) as white solid, R_f 0.47 (70:30:3 $CHCl_3$-MeOH-H_2O), mp 129-130 °C (from EtOH), $[\alpha]_D$ -13.9 (c 1.1 in MeOH), ^{1}H NMR (400 MHz, CD_3CN): δ 4.14 (ddd, 1H, $J_{4,5}$ 5.4 Hz, $J_{5,6a}$ 7.00 Hz, $J_{5,6b}$ 6.4

Hz, H-5), 3.98 (dd, 1H, $J_{6a,6b}$ 8.2 Hz, H-6b), 3.85 (m, 1H, H-2), 3.76 (dd, 1H, H-6a), 3.64 (dd, 1H, $J_{2,3}$ 3.3 Hz, $J_{3,4}$ 5.2 Hz, H-3), 3.03 (dd, 1H, $J_{1\alpha,1\beta}$ 11.2 Hz, $J_{1\beta,2}$ 5.4 Hz, H-1β), 3.18 (dd, 1H, H-4), 2.73 (dd, 1H, $J_{1\alpha,2}$ 3.1 Hz, H-1α), 1.36, 1.30 (2s, each 3H, C*Me*$_2$); ^{13}C NMR (62.9 MHz, CD_3CN,): δ 109.7 (*C*Me$_2$), 77.7, 77.3 (C-2, C-3), 74.5 (C-5), 63.7 (C-4), 68.3 (C-6), 52.8 (C-1), 27.0, 25.6 (C*Me*$_2$). Anal. Calcd for $C_9H_{17}NO_4$: C, 53.19; H, 8.43%; N, 6.89%; Found: C, 53.17; H, 8.40%; N, 6.85%.

4.1.14.* N-*(2-methylpropanoyl)-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-glucitol (18a). To a solution of **17a** (180 mg, 0.89 mmol) in 3:1 mixture of MeOH-H_2O (v/v, 2.5 mL), Et_3N (0.16 mL, 1.14 mmol) and isobutyric anhydride (0.30 mL, 1.80 mmol) were added and the solution was stirred at room temperature until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material. After 2 h, satd aq $NaHCO_3$ (30 mL) was added and the mixture was stirred at room temperature for 15 min and the aqueous phase was extracted with EtOAc (4×15 mL). The organic layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure to afford a crude (syrup) which as purified by flash chromatography on silica gel (EtOAc). Pure **18a** (195 mg, 80%) was obtained as white solid, R_f 0.35 (9:1 EtOAc-MeOH), mp = 115-118 °C (chrom); $[\alpha]_D$ -41.5 (c 1.0 in $CHCl_3$). The NMR analysis (^{1}H and ^{13}C) of the **18a** was rather complex due to the presence of the two rotational isomers of the amide bond in the ratio of 70:30, measured on the relative integrals of two *CH*Me$_2$ protons at δ 2.67 and 3.16 respectively. By recording the spectra at 80 °C (CD_3CN-D_2O), coalescence was observed in the ^{13}C spectrum, while **18a-major** and **18a-minor** are still evident in the ^{1}H spectrum. Selected ^{1}H NMR (400 MHz, CD_3CN-D_2O, 20 °C) signals: **18a-major**: δ 3.72 (dd, 1H, $J_{1\alpha,1\beta}$ 11.2 Hz, $J_{1\beta,2}$ 4.3 Hz, H-1β), 2.67 (m, 1H, (C*H*Me$_2$), 1.35,1.28 (2s, each 3H, C*Me*$_2$), 1.01 (d, 3H, J_{vic} 6.5 Hz, CH*Me*$_2$), 0.98 (d, 3H, J_{vic} 6.5 Hz, CH*Me*$_2$); **18a-minor**: δ 4.46 (m, 1H, H-4), 4.36 (m, 1H, H-5), 3.16 (m, 1H, C*H*Me$_2$), 1.37, 1.31 (2s, each 3H, C*Me*$_2$), 1.04 (d, 3H, J_{vic} 5.8 Hz, CH*Me*$_2$), 0.96 (d, 3H, J_{vic} 5.8 Hz, CH*Me*$_2$); ^{13}C NMR (100 MHz, CD_3CN-D_2O, 20 °C): **18a-major**: δ 180.1 (C=O), 109.9 (C*Me*$_2$), 77.5 (C-2), 77.3 (C-3), 74.8 (C-

5), 70.0 (C-6), 62.0 (C-4), 54.0 (C-1), 33.5 (*C*HMe$_2$), 27.1, 26.6 (C*Me$_2$*), 20.3, 19.3 (CH*Me$_2$*); **18a-minor**: δ 180.1 (C=O), 109.5(C*Me$_2$*), 77.3 (C-2), 77.1 (C-3), 74.6 (C-5), 66.4 (C-6), 60.2 (C-4), 52.5 (C-1), 31.9 (*C*HMe$_2$), 27.3, 56.0 (C*Me$_2$*), 20.5, 19.6 (CH*Me$_2$*); ^{13}C NMR (50.33 MHz, CD$_3$CN-D$_2$O, 80 °C): δ 179.8 (C=O), 109.8 (C*Me$_2$*), 78.0 (C-2), 77.4 (C-3), 75.1 (C-5), 69.7 (C-6), 61.7 (C-4), 53.7 (C-1), 33.0 (*C*HMe$_2$), 27.1, 26.1 (C*Me$_2$*), 20.3, 19.2 (CH*Me$_2$*). Anal. Calcd for $C_{13}H_{23}NO_5$: C, 57.13; H, 8.48%; N, 5.18%; Found: C, 57.10; H, 8.45%; N, 5.14%.

4.1.15.* N-*(2-methylpropanoyl)-5,6-*O*-isopropylidene-1,4-dideoxy-1,4-immino-D-galactitol (18b). To a solution of **17b** (250 mg, 1.23 mmol) in 3:1 mixture of MeOH-H$_2$O (v/v, 3.3 mL), Et$_3$N (0.22 mL, 1.60 mmol) and isobutyric anhydride (0.41 mL, 2.46 mmol) were added and the solution was stirred at room temperature until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material. After 2 h, satd aq NaHCO$_3$ (30 mL) was added and the mixture was stirred at room temperature for 15 min and the aqueous phase was extracted with EtOAc (4×15 mL). The organic layers were collected, dried (MgSO$_4$) and evaporated under diminished pressure to afford a crude (syrup) which was purified by flash chromatography on silica gel (EtOAc). Azafuranose derivatives **18b** (252 mg, 75%) as obtained pure as white solid, R_f 0.46 (9:1 EtOAc-MeOH), mp 106-108 °C (chrom); $[\alpha]_D$ +110.5 (c 1.0 in CHCl$_3$). The ^{1}H NMR analysis of **18b** showed the presence of the two rotational isomers of the amide bond in the ratio of 60:40, measured on the relative intensities of two *C-1* signals at δ 53.5 and 55.1 respectively. By recording the spectra at 80 °C (CD$_3$CN-D$_2$O) coalescence was observed in the ^{13}C spectrum. ^{13}C NMR (100 MHz, CD$_3$CN-D$_2$O, 20 °C): **18b-major**: δ 180.1 (C=O), 110.5 (*C*Me$_2$), 78.9, 75.8, 74.8 (C-2, C-3, C-5), 69.6 (C-4), 67.5 (C-6), 53.5 (C-1), 32.3 (*C*HMe$_2$), 26.4, 25.3 (C*Me$_2$*), 20.0, 19.3 (*C*HMe$_2$); **18b-minor**: δ 179.6 (C=O), 109.9 (*C*Me$_2$), 79.2, 77.3, 76.3 (C-2, (C-3, C-5), 67.1 (C-6), 64.5 (C-4), 55.1 (C-1), 32.8 (*C*HMe$_2$), 26.3, 25.7 (C*Me$_2$*), 20.0, 18.7 (*C*HMe$_2$); ^{13}C NMR (50.33 MHz, CD$_3$CN-D$_2$O, 80 °C): δ 179.9 (C=O), 110.5 (*C*Me$_2$), 79.9, 77.2, 76.3 (C-2, C-3, C-5), 69.6 (C-6), 64.5

(C-4), 54.8 (C-1), 32.8 (*C*HMe$_2$), 26.8, 25.9 (C*Me$_2$*), 20.3, 19.3 (*C*HMe$_2$). Anal. Calcd for $C_{13}H_{23}NO_5$: C, 57.13; H, 8.48%; N, 5.18%; Found: C, 57.11; H, 8.44%; N, 5.15%.

4.1.16. Preparation of azafuranose derivatives 18a and 18b from mixture of 11a and 11b. To a solution of 2:3 mixture of azafuranose derivatives **11a** and **11b** (757 mg, 2.58 mmol) in absolute EtOH (80 mL), 10% Pd on charcoal (450 mg) was added and the mixture was stirred at room temperature under H_2 (atmospheric pressure) until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material (48 h). The suspension was filtered through a small layer of Celite®, evaporated under diminished pressure gave a crude product (525 mg) constituted (NMR) by a mixture of azafuranose derivatives **17a** and **17b** in the ratio of 2:3. To a solution of crude mixture of **17a** and **17b** (530 mg) in 3:1 mixture of MeOH-H_2O (v/v, 7.0 mL), Et_3N (0.47 mL, 1.60 mmol) and isobutyric anhydride (0.41 mL, 2.46 mmol) were added and the solution was stirred at room temperature until TLC analysis (8:2 EtOAc-MeOH) showed the complete disappearance of the starting material (2 h). Satd aq $NaHCO_3$ (15 mL) was added, stirred at room temperature (15 min), the aqueous phase was extracted with CH_2Cl_2 (4×15 mL) and the organic layers were collected, dried ($MgSO_4$) and evaporated under diminished pressure. Purification of the crude residue (596 mg) by flash chromatography on silica gel (EtOAc) gave pure of azafuranose derivatives **18a** (263 mg, 37% calculated from **11a**+**11b**) and **18b** (281 mg, 40% calculated from **11a**+**11b**) having NMR parameters identical to those of the sample prepared above.

4.1.17.* N-*(2-methylpropanoyl)-1,4-dideoxy-1,4-immino-D-glucitol (19a). A solution of azafuranose derivative **18a** (55.2 mg, 0.20 mmol) in 80% aq AcOH (v/v, 3.0 mL) was stirred at 40 °C until the TLC analysis (9:1 EtOAc-MeOH) revealed the complete reaction of the starting material with formation of a major slower moving products (R_f 0.18). After 40 min the solution was then cooled to room temperature and co-evaporated with toluene (6×5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (9:1 EtOAc-MeOH) gave pure of azafuranose derivative **19a** (38.7mg, 83%) as white foam, R_f 0.18 (9:1 EtOAc-MeOH). The NMR analysis (^{1}H and ^{13}C, CD_3OD) of

19a, showed the presence of the two rotational isomers of the amide bond **19a-major** and **19a-minor** in the ratio 9:1 calculated on the basis of the signals related to the C-6 carbons at δ 64.9 and 64.6 respectively. The ^{1}H NMR analysis of the **19a** allows to assign only the chemical shifts of the proton related to the rotational isomer **19a-major.** ^{1}H NMR (250.13 MHz, CD_3OD, at 20 °C) of **19a-major**: δ 4.28-4.00 (m, 4 H, H-2, H-3, H-4, H-5), 3.75 (dd, 1H, $J_{1\alpha,1\beta}$ 11.3 Hz, $J_{1\beta,2}$ 4.1 Hz, H-1β), 3.63 (dd, 1H, $J_{6a,6b}$ 11.3 Hz, $J_{5,6b}$ 8.5 Hz, H-6b), 3.56 (dd, 1H, $J_{1\alpha,2}$ 2.0 Hz, H-1α), 3.50 (dd, 1H, $J_{5,6a}$ 6.5 Hz, H-6b), 2.46 (m, 1H, C*H*Me$_2$), 1.13 (d, 3H, J_{vic} 6.8 Hz, CH*Me$_2$*), 1.09 (d, 3H, J_{vic} 6.7 Hz, CH*Me$_2$*); ^{13}C NMR (62.9 MHz, CD_3OD, at 20 °C) of **19a-major**: δ 180.3 (C=O), 77.5, 75.6, 72.7 (C-2, C-3, C-5), 63.2 (C-4), 64.9 (C-6), 54.0 (C-1), 33.6 (*C*HMe$_2$), 19.7, 18.9 (CH*Me$_2$*); of **19a-minor**: δ 180.3 (C=O), 79.4, 76.2 74.9 (C-2, C-3, C-5), 64.6 (C-6), 61.8 (C-4), 52.3 (C-1), 33.4 (*C*HMe$_2$), 20.1, 19.6 (CH*Me$_2$*). Anal. Calcd for $C_{10}H_{19}NO_5$: C, 51.49; H, 8.21%; N, 6.00%; Found: C, 51.46; H, 8.17%; N, 5.97%.

4.1.18.* N-*(2-methylpropanoyl)-1,4-dideoxy-1,4-immino-D-galactitol (19b). A solution of azafuranose derivative **18b** (58.9 mg, 0.22 mmol) in 80% aq AcOH (v/v, 3.2 mL) was stirred at 40 °C until the TLC analysis (9:1 EtOAc-MeOH) revealed the complete reaction of the starting material with formation of a major slower moving products (R_f 0.23). After 40 min the solution was then cooled to room temperature and co-evaporated with toluene (6×5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (9:1 EtOAc-MeOH) gave pure of azafuranose derivative **19b** (43 mg, 85%) as white foam, R_f 0.23 (9:1 EtOAc-MeOH). The NMR analysis (^{1}H and ^{13}C, CD_3OD) of **19a**, showed the presence of the two rotational isomers of the amide bond **19b-major** and **19b-minor** in the ratio 75:25 calculated on the basis of the signals related to the C-1 carbons at δ 55.8 and 54.1 respectively. ^{1}H NMR (250.13 MHz, CD_3OD, at 20 °C) of **19b-major**: δ 4.17 (m, 1H, H-4), 4.14 (m, 1H, H-3), 4.10 (m, 1H, H-2), 4.02 (m, 1H, H-1β), 3.95 (m, 1 H, H-5), 3.53-3.45 (m, 3H, H 1α, H-6a, H 6b), 2.82 (m, 1H, C*H*Me$_2$), 1.12 (d, 3H, J_{vic} 6.8 Hz, CH*Me$_2$*), 1.08 (d, 3H, J_{vic} 6.7 Hz, CH*Me$_2$*); of **19b-minor**: δ 4.05

(m, 1H, H-1β), 4.10 (m, 1H, H-2), 4.05 (m, 1H, H-3), 3.95 (m, 2H, H-4, H-5), 3.65 (dd, 1H, $J_{6a,6b}$ 12.1 Hz, $J_{5,6b}$ 3.5 Hz, H-6b), 3.58 (dd, 1H, $J_{5,6a}$ 4.4 Hz, H-6a), 3.22 (bd, 1H, $J_{1\alpha,1\beta}$ 12.2 Hz, H-1a), 3.17 (m, 1H, C*H*Me$_2$), 1.10 (d, 3H, J_{vic} 6.5 Hz, CH*Me$_2$*), 1.04 (d, 3H, J_{vic} 7.0 Hz, CH*Me$_2$*); ^{13}C NMR (62.9 MHz, CD_3OD, at 20 °C) of **19b-major**: δ 181.2 (C=O), 76.5 (C-2), 75.6 (C-3), 74.7 (C-5), 67.7 (C-4), 65.0 (C 6), 55.8 (C 1), 33.4 (*C*HMe$_2$), 20.0, 18.7 (CH*Me$_2$*); of **19b-minor**: δ 180.2 (C=O), 79.8 (C-3), 75.8 (C-2), 71.5 (C-5), 68.8 (C-4), 64.6 (C 6), 54.1 (C 1), 32.8 (*C*HMe$_2$), 19.3, 19.9 (*C*HMe$_2$). Anal. Calcd for $C_{10}H_{19}NO_5$: C, 51.49; H, 8.21%; N, 6.00%; Found: C, 51.47; H, 8.18%; N, 5.96%.

4.1.19.* N-*benzyl-1,4-dideoxy-1,4-immino-D-galactitol (20b). A solution of azafuranose derivative **11b** (58.9 mg, 0.20 mmol) in 80% aq AcOH (v/v, 3.2 mL) was stirred at 40 °C until the TLC analysis (8:2 EtOAc-MeOH) revealed the complete reaction of the starting material with formation of a major slower moving products (R_f 0.40). After 40 min the solution was then cooled to room temperature and co-evaporated with toluene (6×5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (8:2 EtOAc-MeOH) gave pure of azafuranose derivative **20b** (47 mg, 90%) as white solid, R_f 0.40 (8:2 EtOAc-MeOH); mp 133-135 °C (from EtOH); $[\alpha]_D$ -25.5 (c 1.0 in $CHCl_3$). ^{1}H NMR (200.13 MHz, CD_3OD): δ 7.39-7.20 (m, 5 H, Ar-*H*), 4.20, 3.52 (AB system, 2H, $J_{A,B}$= 13.6 Hz, C*H$_2$*Ph), 4.09 (m, 1H, H-3), 3.95-3.84 (m, 2H, H-2, H-5), 3.72 (dd, 1H, $J_{6a,6b}$ 11.1 Hz, $J_{5,6b}$ 5.6 Hz, H-6b), 3.68 (dd, 1H, $J_{5,6a}$ 6.2 Hz, H-6a), 2.91 (dd, 1H, $J_{4,5}$ 4.6 Hz, $J_{3,4}$ 2.7 Hz, H-4), 2.86 (m, 1H, H-1β), 2.71 (dd, 1H, $J_{1\alpha,1\beta}$ 10.7 Hz, $J_{1\alpha,2}$ 4.4 Hz, H-1α); ^{13}C NMR (62.9 MHz, CD_3OD,): δ 138.6 (Ar-*C*), 128.4, 127.9, 126.7 (Ar-C*H*), 79.2 (C-3), 75.7 (C-2), 73.3 (C-4), 71.2 (C-5), 63.7 (C-6), 60.7 (*C*H$_2$Ph), 58.9 (C-1). Anal. Calcd for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56%; N, 5.53%; Found: C, 61.61; H, 7.52%; N, 5.50%.

4.1.20. 2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-glucitol (21a) and 2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-galactitol (21b). To a solution of mixture of azafuranose derivatives **7a** and **7b** (402 mg, 0.96 mmol) in absolute EtOH (30 mL), 10% Pd on charcoal (180 mg) was added and the

mixture was stirred at room temperature under H_2 (atmospheric pressure) until the starting compound was completely reacted (TLC, EtOAc, 2 h). The suspension was diluted with MeOH (20 mL), filtered over a pad of Celite®, washed with MeOH, and the combined organic phases were concentrated at diminished pressure. Purification of crude syrup (332 mg) by flash chromatography on silica gel (7:2:1 Et_2O-EtOAc- ^{i}PrOH + 0.1% Et_3N) gave pure **21a** (99 mg, 30%) and **21b** (171 mg, 52%).

2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-glucitol **(21a**) as a clear syrup, R_f 0.32 (7:2:1 Et_2O-EtOAc- ^{i}PrOH + 0.1% Et_3N), $[\alpha]_D$ -18.3 (*c* 1.0 in $CHCl_3$), ^{1}H NMR (200.13 MHz, C_6D_6): δ 5.60 (bd, 1H, $J_{3,4}$ 4.2 Hz, H-3), 5.24 (ddd, 1H, $J_{4,5}$ 7.1 Hz, $J_{5,6a}$ 5.4 Hz, $J_{5,6b}$ 2.5 Hz, H-5), 4.98 (ddd, 1H, $J_{1\beta,2}$ 5.2 Hz, $J_{1\alpha,2}$ 2.3 Hz, H-2), 4.61 (dd, 1H, $J_{6a,6b}$ 12.2 Hz, H-6b), 4.12 (dd, 1H, H-6a), 3.42 (dd, 1H, H-4), 3.17 (dd, 1H, $J_{1\beta,1\alpha}$ 12.9 Hz, H-1β), 2.66 (dd, 1H, H-1α),1.79, 1.75, 1.67, 1.53 (4s, each 3H, 4×*Me*CO); ^{13}C NMR (50.33 MHz, C_6D_6): δ 170.2, 169.7, 169.1, 169.0 (4×C=O), 78.5, 75.9 (C-2, C-3), 69.8 (C-5), 64.4 (C-6), 60.3 (C-4), 51.8 (C-1), 20.6, 20.4, 20.3, 20.2 (4×*Me*CO). Anal. Calcd for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39%; N, 4.23%; Found: C, 50.72; H, 6.36%; N, 4.20%.

2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-galactitol (**21b**) as colourless syrup, R_f 0.24 (7:2:1 Et_2O-EtOAc- ^{i}PrOH + 0.1% Et_3N), $[\alpha]_D$ +44.3 (*c* 1.0 in $CHCl_3$); ^{1}H NMR (200.13 MHz, C_6D_6): δ 5.41 (m, 1H, H-3), 5.02 (m, 1H, H-5), 4.54 (dd, 1H, $J_{6a,6b}$ 11.6 Hz, $J_{5,6b}$ 4.2 Hz, H-6b), 4.48 (m, 1H, H-2), 4.37 (dd, 1H, $J_{5,6a}$ 5.0 Hz, H-6a), 4.27 (m, 1H, H-4), 3.30 (dd, 1H, $J_{1\alpha,2}$ 2.3 Hz H-1α), 3.06 (dd, 1H, $J_{1\beta,1\alpha}$ 12.1 Hz, $J_{1\beta,2}$ 5.8 Hz, H-1β),1.80, 1.71, 1.58, 1.54 (4s, each 3H,4×*Me*CO); ^{13}C NMR (50.33 MHz, C_6D_6): δ 172.6, 170.6, 169.4, 169.3 (4×C=O), 76.5, 75.4 (C-2, C-3), 71.7 (C-5), 67.1 (C-6), 66.7 (C-4), 52.3 (C-1), 21.9, 20.5, 20.2, 20.1 (4×*Me*CO). Anal. Calcd for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39%; N, 4.23%; Found: C, 50.74; H, 6.37%; N, 4.21%.

4.1.21.* N-*Acetyl-2,3,5,6-tetra-*O*-acetyl-1,4-dideoxy-1,4-immino-D-glucitol (10a). Routine acetylation of azafuranose derivative **21a** (80 mg, 0.23 mmol) with 1:2 Ac_2O-Py mixture (9.0 mL) gave after flash

chromatography (7:2:1 Et_2O-EtOAc- ^iPrOH + 0.1% Et_3N) pure **10a** (79 mg, 91%) as a clear syrup, R_f 0.36 (EtOAc), $[\alpha]_D$ +52.6 (*c* 0.8 in $CHCl_3$); Lit. [22] +53 ($CHCl_3$). The NMR analysis ($CDCl_3$) of **10a**, showed the presence of the two rotational isomers of the amide bond in the ratio 9:1 calculated on the basis of the signals related to the C-1 carbons at δ 50.6 and 48.2 respectively. The ^{1}H NMR analysis of the **10a** allows to assign only the chemical shifts of the proton related to the rotational isomer **10a-major.** ^{1}H NMR (400 MHz, $CDCl_3$ at 20 °C) of **10a-major**: δ 5.40 (m, 2H, H-3, H-5), 5.21 (m, 1H, H-2), 4.61 (dd, 1H, $J_{4,5}$ 7.1 Hz, $J_{3,4}$ 5.9 Hz, H-4), 4.18 (dd, 1H, $J_{6a,6b}$ 11.9 Hz, $J_{5,6b}$ 3.8 Hz, H-6b), 4.09 (dd, 1H, $J_{5,6a}$ 8.1 Hz, H-6a), 3.70 (dd, 1H, $J_{1\beta,1\alpha}$ 11.8 Hz, $J_{1\beta,2}$ 6.0 Hz, H-1β), 3.39 (dd, 1H, $J_{1\alpha,2}$ 4.0 Hz, H-1α), 2.04, 2.02, 2.00, 1.97, 1.96 (5s, each 3H, 5×*Me*CO); ^{13}C NMR (50.33 MHz, $CDCl_3$ at 20 °C) of **10a-major**: δ 74.3, 73.3 69.8 (C-2, C-3, C-5), 63.5 (C-6), 56.1 (C-4), 50.6 (C-1), of **10a-minor**: δ 74.6, 73.38, 70.9 (C-2, C-3, C-5), 62.3 (C-6), 58.2 (C-4), 48.2 (C-1); cluster of signals for **10a-minor** and **10a-major** 170.6-169.4 (C=O), 22.2 (*Me*CON), 20.9-20.6 (*Me*COO).

4.1.22.* N-*acetyl-2,3,5,6-tetra*-O-*acetyl-1,4-dideoxy-1,4-immino-D-galactitol (10b). Routine acetylation of azafuranose derivative **21b** (140 mg, 0.41 mmol) with 1:2 Ac_2O-Py mixture (12.0 mL) gave after flash chromatography (7:2:1 Et_2O-EtOAc- ^iPrOH + 0.1% Et_3N) pure **10b** (139 mg, 90%) as a clear syrup, R_f 0.32 (EtOAc), $[\alpha]_D$ +77.5 (*c* 1.07 in $CHCl_3$). The NMR analysis carried out in various solvents ($CDCl_3$ or CD_3OD or C_6D_6) showed a mixture of the two rotational isomers of the amide bond **10b-minor** and **10b-major** in the ratio of 4:6, measured in $CDCl_3$ on the relative intensities of the H-1 signals at δ 3.28 and 3.51 respectively. ^{1}H NMR (400 MHz, $CDCl_3$) of **10b-major**: δ 5.43 (bt, 1H, $J_{4,5} = J_{5,6a}$ 7.3 Hz, $J_{5,6b}$ 3.2 Hz, H-5), 4.40 (d, 1H, H-4), 4.35 (dd, 1H, $J_{6a,6b}$ 12.3 Hz, H-6b), 4.07 (dd, 1H, H-6a), 4.01 (dd, 1H, $J_{1\beta,1\alpha}$ 12.1 Hz, $J_{1\beta,2}$ 6.7 Hz, H-1β); 3.43 (dd, 1H, $J_{1\alpha,2}$ 3.4 Hz, H-1α); of **10b-minor**: δ 5.31 (ddd, 1H, $J_{4,5}$ 8.3 Hz, $J_{5,6a}$ 5.9 Hz, $J_{5,6b}$ 4.4 Hz, H-5), 4.34 (dd, 1H, $J_{6a,6b}$ 12.5 Hz, H-6b), 4.20 (dd, 1H, $J_{1\beta,1\alpha}$ 14.0 Hz, $J_{1\beta,2}$ 7.0 Hz, H-1β), 4.05 (m, 1H, H-6a), 3.94 (d, 1H, H-4), 3.23 (dd, 1H, $J_{1\alpha,2}$ 2.0 Hz, H-1α); cluster of

signals for **10b-minor** and **10b-major**: δ 5.11-5.02 (m, 1H, H-2, H-3), 2.05-2.1.97 (m, 5×15H, *Me*CO); ^{13}C NMR (100 MHz, $CDCl_3$) of **10b-major**: δ 77.3, 75.7 (C-2, C-3), 70.3 (C-5), 63.5 (C-6), 61.6 (C-4), 52.4 (C-1), 22.3 (*Me*CON); of **10b-minor**: δ 77.9, 74.3 (C-2, C-3), 68.6 (C-5), 63.5 (C-4), 62.9 (C-6), 50.6 (C-1), 22.0 (*Me*CON); cluster of signals for **10b-minor** and **10b-major**: δ170.4-169.5 (C=O), 20.8-20.5 (*Me*COO). Anal. Calcd for $C_{16}H_{23}NO_9$: C, 51.47; H, 6.21%; N, 3.75%; Found: C, 51.45; H, 6.17%; N, 3.73%.

4.1.23. 1,4-dideoxy-1,4-immino-D-galactitol hydrochloride (22). A solution of **10b** (56 mg, 0.15 mmol) or **21b** (52 mg, 0.15 mmol) in aq HCl (2M, 5 mL) was stirred at 100 °C until the TLC analysis (EtOAc) revealed the complete disappearance of the starting material (R_f 0.32). After 15 min the mixture was repeatedly co-evaporated with toluene (4×20 mL) under diminished pressure. The trituration of the crude product with Et_2O afforded pure the 1,4-dideoxy-1,4-immino-D-galactitol hydrochloride (**22**) (22 mg, 87%), as a white solid; mp 98-101 °C; $[\alpha]_D$ -24.1 (*c* 0.8 in MeOH). Lit. [24] $[\alpha]_D$ -25.3 (*c* 1.0 in MeOH), mp 102 °C (from $CHCl_3$-MeOH). The NMR spectroscopic data was completely agreed with those reported in literature [24].

4.2. Biological Experimental

Human recombinant aldose reductase from *Escherichia coli*, alpha-glucosidase from *Saccharomices cerevisiae*, NADPH, D,L-glyceraldehyde, *p*-nitrophenyl-α-D-glucopyranoside and phosphate buffer were purchased from Sigma Aldrich. Epalrestat was obtained from Haorui Pharma-Chem Inc., NJ, USA; 1-deoxynojirimycin chlorohydrate (DNJ·HCl) was purchased from Carbosynth, UK.

4.2.1. ALR2 enzymatic assay. The enzyme activity was determined spectrophotometrically at 340 nm by monitoring the change in absorbance of the NADPH cofactor, according to a previously reported procedure [34]. The reaction mixture contained 100 µL sodium phosphate buffer pH=6.2 (0.1 M), 50 µL NADPH (0.15 mM), 50 µL ALR2 (0.50 µM) and 50 µL D,L-glyceraldehyde (10 mM) in a 96-multiwell

plate. The reaction was monitored for 5 minutes at 30 °C, and the variation of pyridine coenzyme concentration versus time was analysed through the WorkOut program from PerkinElmer.

***4.2.2. ALR2 enzymatic inhibition*.** Inhibition studies were performed by adding the test compounds (25 μL, 100 μM) to the above mentioned reaction mixture. All the compounds were initially solubilized in DMSO, and then diluted with water, before the addition to the assay test. DMSO concentration in the test solutions never exceed 4%, and proved to have no inhibitory effects on the target enzyme. In order to avoid absorption due to the tested molecules, a blank containing all the components, with the exception of the substrate, was analysed. Assays were conducted in triplicate. Epalrestat was used as the reference compound.

***4.2.3. AG enzymatic assay*.** The enzyme activity was determined spectrophotometrically at 405 nm following a literature procedure with some modification [35], by monitoring the change in absorbance of *p*-nitrophenyl α-D-glucopyranoside, which is converted into sodium *p*-nitrophenoxyde by reaction with the target enzyme. The reaction mixture contained 100 μL sodium phosphate buffer pH=6.8 (0.1 M), 25 μL enzyme (0.2 U/mL) and 25 μL of *p*-nitrophenyl α-D-glucopyranoside (0.375 mM), in a 96-multiwell plate. All the reagents were incubated at 37 °C for 10 minutes. The reaction was then stopped with 100 μL of saturated Na_2CO_3. Absorbance of the test solutions were analysed with the program Wallac 1420 of PerkinElmer.

***4.2.4. AG enzymatic inhibition*.** Inhibition studies were performed by adding the test compounds (25 μL, 100 μM) to the above mentioned reaction mixture. Compounds found to be active were tested at additional concentrations between 10^{-5} and 10^{-7} M. All the compounds were initially solubilized in DMSO, and then diluted with water, before the addition to the assay test. DMSO concentration in the test solutions never exceed 4%, and proved to have no inhibitory effects on the target enzyme. In order to avoid absorption due to the tested molecules, a blank containing all the components, with the exception

of the substrate, was analysed. Assays were conducted in triplicate. Positive control was represented by 1-deoxynojirimycin chlorohydrate (DNJ·HCl). IC_{50} data were obtained by linear regression analysis of the log dose-response curve.

4.2.5. 661w cell culture. The 661W cell line, derived from immortalized cone photoreceptors (provided by Muayyad Al-Ubaidi, University of Oklahoma), was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, ThermoFisher Scientific, Waltham, MA) containing 4.5 g/l (24.5 mM) glucose and supplemented with 10% (v/v) heat-inactivated FBS, 1% Lglutammine and 1% pen/strep. For the experiments, confluent cells were maintained in 24.5 mM glucose (normoglycemic condition) or cultured in 55 mM glucose (hyperglycemic condition) during 24h in the presence or absence of **17b** (10^{-6} M).

4.2.6. In vitro ALR2 enzymatic inhibition. Cells were seeded in 24-well tissue culture plates and allowed to stabilize overnight in a 5% CO_2 incubator at 37 °C. The cells were then cultured in 24.5 mM (NG) or 55 mM (HG) glucose-containing medium in the absence or presence of **17b** (10^{-6} M) for 24h; after that the cells were mechanically lysate and the supernatant were collected by centrifugation and incubate with Aldo-Keto Reductase (AKR) Activity Assay Kit (AbCam) in a 96-well for 2h, and absorbance was measured with a spectrophotometer (NanoQuant) at 450 nm. The kit works by measuring the absorbance of the NADH cofactor, which is consumed during the glucose conversion to sorbitol mediated by the ALR2 enzyme. Therefore, a reduction in the absorbance value corresponds to an increased activity of the enzyme itself.

4.2.7. Analysis of cellular viability and detection of apoptosis. Quantification of cellular viability and apoptosis were performed using flow cytometry methods. Cells were seeded in 24-well tissue culture plates and allowed to stabilize overnight in a 5% CO_2 incubator at 37 °C. The cells were then cultured in 24.5 mM (NG) or 55 mM (HG) glucose-containing medium in the absence or presence of **17b** (10^{-6} M)

for 24h. After the induction of HG condition, adherent and non-adherent cells were collected by centrifugation and incubated with Guava® ViaCount® Reagent or Muse™ Annexin V & Dead Cell Kit (Millipore), the reagent distinguishes viable and non-viable cells based on differential permeabilities of two DNA-binding dyes in the Guava ViaCount Reagent. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. This proprietary combination of dyes enables the Guava ViaCount Assay to distinguish viable, apoptotic, and dead cells. Debris is excluded from results based on negative staining with the nuclear dye. The data collected were analyzing with Muse 1.5 Software (Millipore).

4.2.8. Immunocytochemistry. The cells were directly washed in PBS (1X; KCl 2.67 mM, KH_2PO_4 1.47 mM, NaCl 139.9 mM, Na_2HPO_4, 8.1 mM, pH 7.4) and fixed in PAF for 1 min. Once the cells were fixed, they were washed in PBS twice for 10 min and incubated in with PBS containing 0.03% Triton and 5% of bovine serum albumin (1 h at room temperature), and incubated with the primary antibody anti-Nrf2 (1:50; Sigma-Aldrich) overnight at 4°C. After incubation with the primary antibody, the samples were washed three times in PBS and subsequently incubated with the secondary goat anti-rabbit immunoglobulin G (IgG; 1:1000; Vector Laboratories) 488-Alexa fluor conjugated for 2 h at room temperature. After washing in PBS, the samples were incubated with Ethidium Bromide (1:5000, Sigma-Aldrich) for 5 min. Finally, after washing twice in PBS, the chambers were detached from the slide and mounted with Vectashield mounting medium (Vector Laboratories). This immunolabeling protocol was modified respect to Wang et al., 2019 [36]. The slides were examined with a fluorescent microscope (Nikon E-Ri). Images were processed with ImageJ software.

4.2.9. Western blot. For western blot analysis, the proteins were resolved using denaturing sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad, Madrid, Spain). Levels of Sod1 proteins were normalized by the levels of their corresponding total proteins by using the Stain Free Technology (BioRad) [31]. Antibody anti-Sod1 was purchased

from Santa Cruz Biotechnology (Palo Alto, CA) and secondary antibody anti-mouse HRP conjugated was purchased from (Sigma-Aldrich). The chemiluminescence was analyzed using the Chemidoc Quantified (Bio-Rad) and the images obtained analyzed with ImageLAb Software.

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