



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.

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

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

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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.

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 Fig. 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.

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.

1,4-Dicarbonyl hexose 6 exists predominantly as the two anomeric α - and β - 4-*keto* pyranose forms 6a,b (Scheme 1), deriving from hemiacetalization 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 h (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).

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

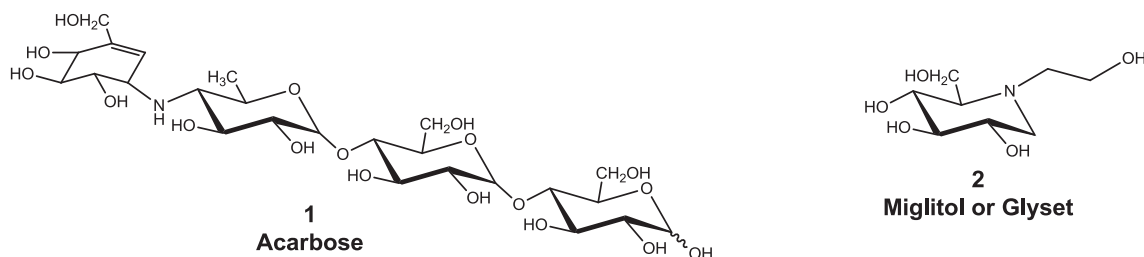


Chart 1. Oligosaccharide Acarbose and Miglitol or Glyset.

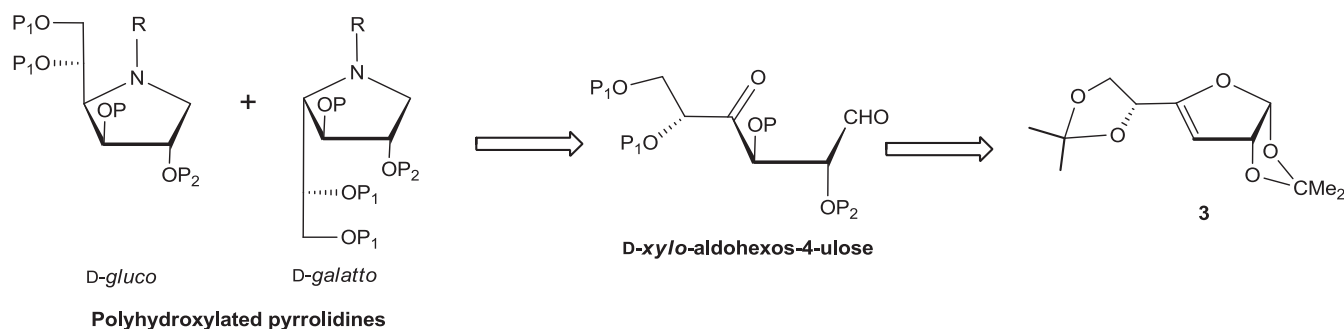


Fig. 1. Retrosynthetic approach to the polyhydroxylated pyrrolidine.

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 *aldehyde* 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 E₂, Fig. 2) can be envisaged. Based on the results, it is likely that the E₂ 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 ²E cyclic iminium ion becomes the preferential one for the hydride attack. Studies on this mechanism are currently underway and will be presented in due course.

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 pyrrolidine derivatives characterized by an enhanced hydrophilic character (11b → 20b, 15a → 16a, 18a → 19a, 18b → 19b).

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 H₂ 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 (2 M HCl).

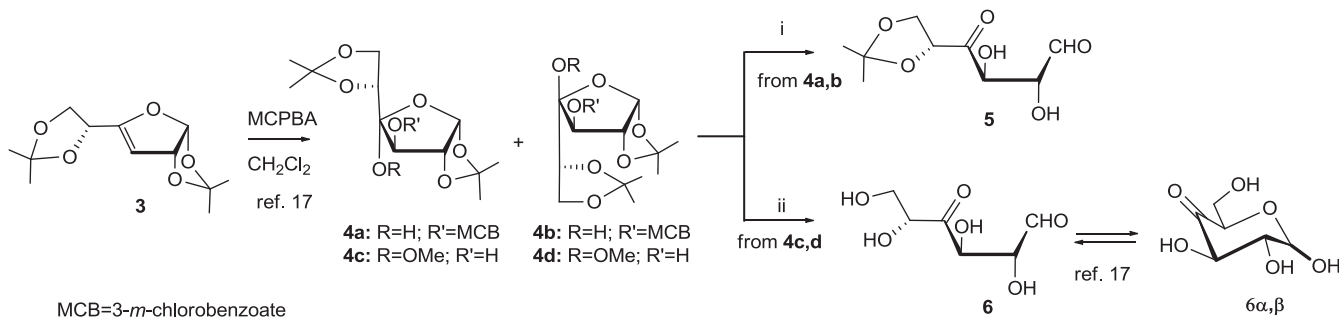
It is worth mentioning that NMR analysis of pyrrolidine amides 18a,b, 19a,b and 10a,b was complicated by the presence of C–N amide bond rotational isomers (see Experimental Section). It is well known that the rotation energy barrier could determine the presence of rotational isomers for amides and these 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

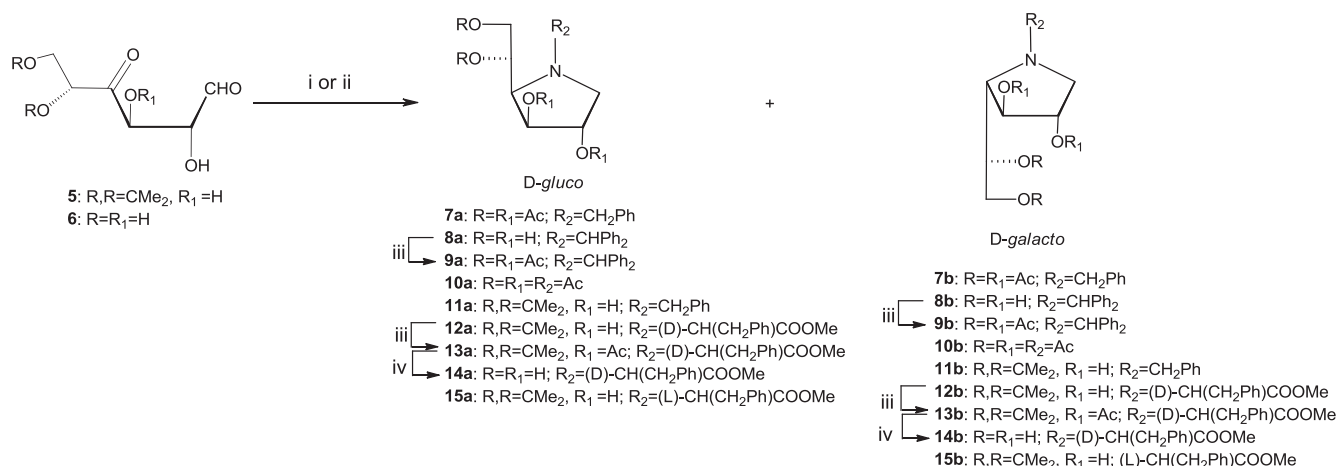
2.2.1. Aldose reductase and alpha-glucosidase inhibitory assays

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



Scheme 1. Synthesis of 4-ulose derivatives 5 and 6. Reagents and conditions: (i) Et₃N, 5:1 MeOH-H₂O (quantitative yield); (ii) CF₃COOH, 2:1 CH₃CN-H₂O (quantitative yield).



Scheme 2. Reagents and conditions: (i) $\text{PhCH}_2\text{NH}_2\cdot\text{HCl}$ (for **7a,b**, 50% yield), or $\text{NH}_3\cdot\text{AcOH}$ (for **10a,b**, 55% yield), NaBH_3CN , MeOH , 60°C , 24–48 h, followed by 1:2 $\text{Ac}_2\text{O}\cdot\text{Py}$, rt, 12 h; (ii) $\text{Ph}_2\text{CHNH}_2\cdot\text{AcOH}$ (for **8a,b**, 52% yield), $\text{PhCH}_2\text{NH}_2\cdot\text{HCl}$ (**11a,b**, 37% yield), $\text{D-Phe-OMe}\cdot\text{HCl}$ (for **12a,b**, 42% yield) or $\text{L-Phe-OMe}\cdot\text{HCl}$ (for **15a,b**, 44% yield), NaBH_3CN , MeOH , 60°C , 24–48 h; (iii) 1:2 $\text{Ac}_2\text{O}\cdot\text{Py}$, 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).

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 $62.4\ \mu\text{M}$ 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} : $40.64\ \mu\text{M}$).

On the whole, *D-galacto* derivatives turned out to be more effective than the *D-glucito* 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.

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 Fig. 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 $100\ \mu\text{M}$, 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 (Fig. 3B).

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. Fig. 4A shows how the treatment at two concentrations (50 and $100\ \mu\text{M}$) of the test compound is able to increase, although not significantly, the levels of cellular vitality. Moreover, Fig. 4B indicates the ability of **17b** to reduce significantly the apoptotic process, the major pathway of death of retinal neurons.

Finally, the ability of **17b** to act also against oxidative stress triggered by hyperglycemic conditions was evaluated. Fig. 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 Fig. 5A, in the ctr-NG, the localization of Nrf2 is cytosolic (green staining) and well separated

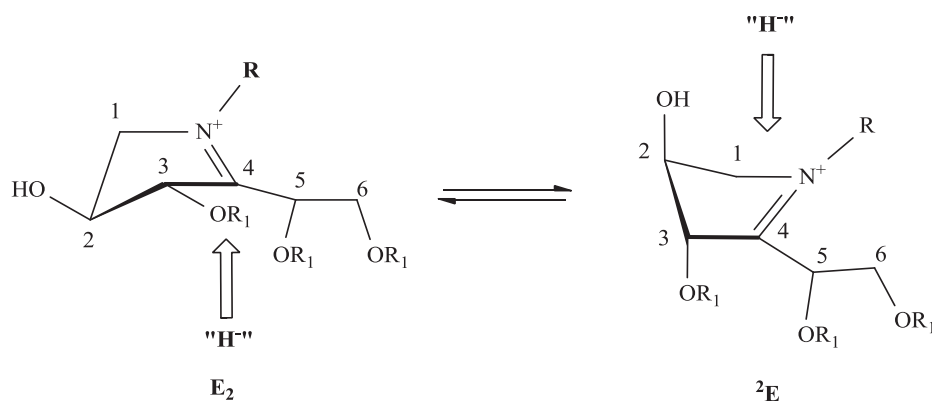
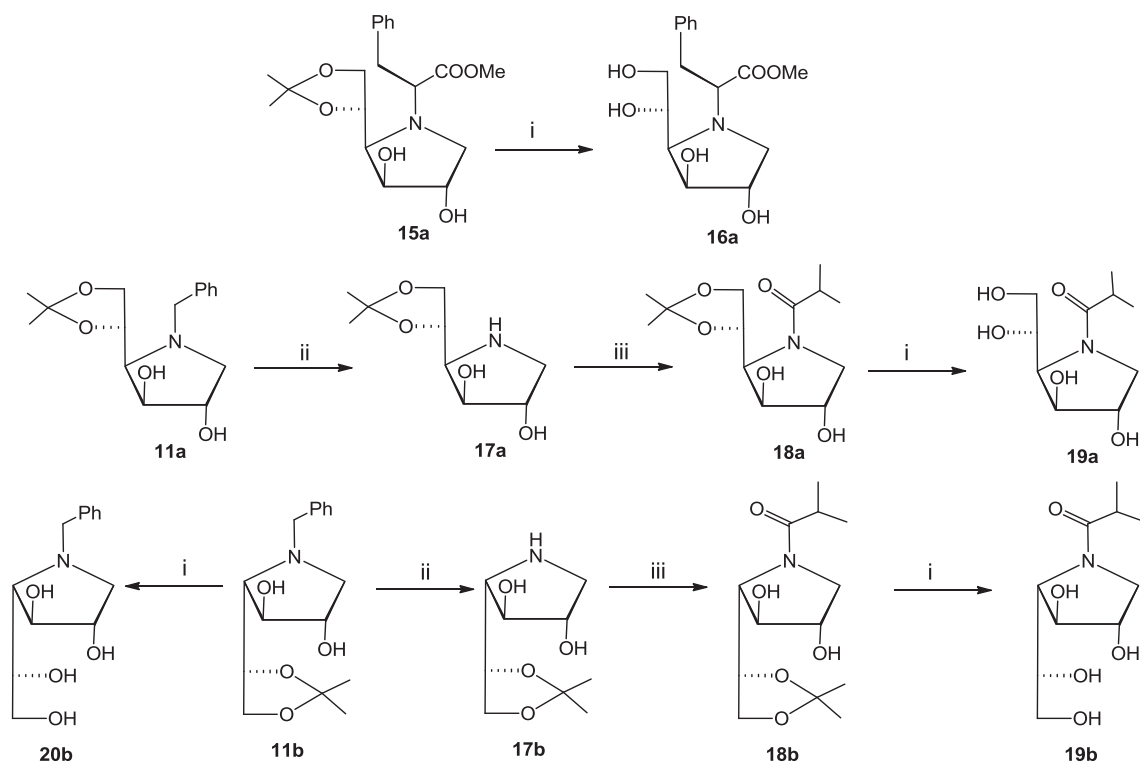


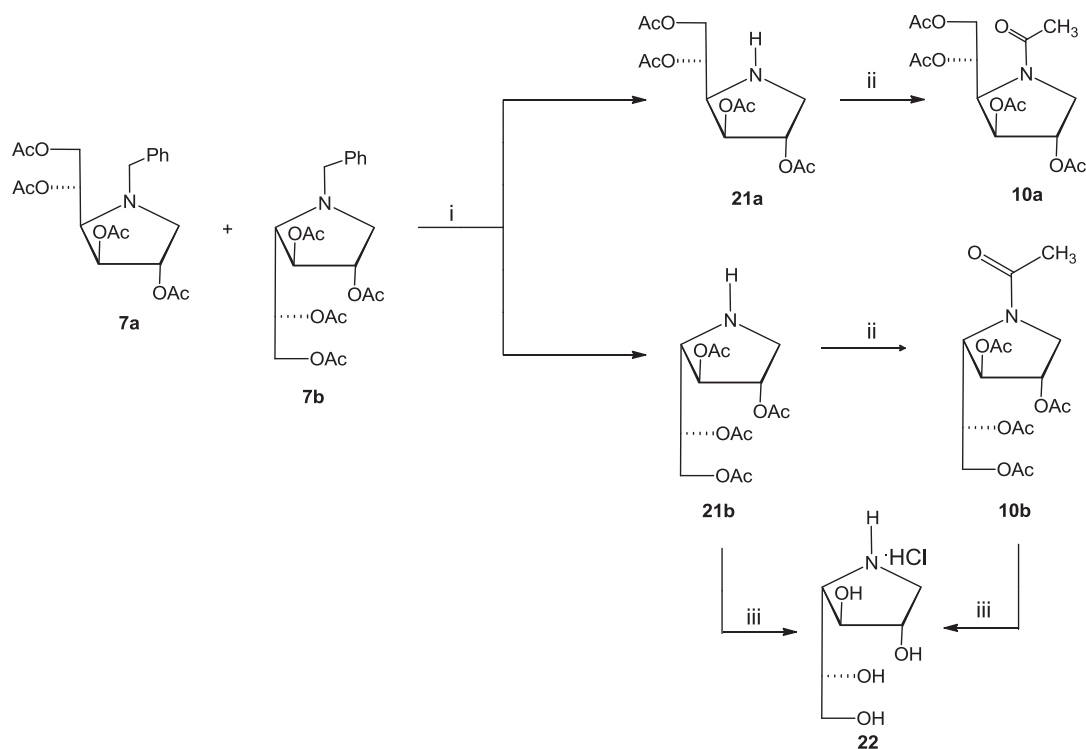
Fig. 2. ^2E and E_2 cyclic iminium ions.



Scheme 3. Reagents and conditions: (i) 80% aq AcOH, 40 °C, 0.5–1 h (**16a**: 89%; **19a**: 83%; **19b**: 85%; **20b**: 90%); (ii) H₂, 10% Pd/C, absolute EtOH, 48 h (**17a**: 85%; **17b**: 87%); (iii) isobutyric anhydride, Et₃N, 3:1 MeOH-H₂O, rt, 2 h (**18a**: 85%; **18b**: 75%).

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 μ M 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, Fig. 5B shows how the 100 μ M dose of **17b** is able to reduce Sod1 levels, indicating a reduction of ROS levels.



Scheme 4. Reagents and conditions: (i) H₂, 10% Pd/C, absolute EtOH, 2 h (**21a**: 30%; **21b**: 52%); (ii) 1:2 Ac₂O-Py, rt, 12 h (**10a**: 91%; **10b**: 90%); (iii) 2 M HCl, 100 °C, 15 min (87%).

Table 1

Aldose Reductase (ALR2) and α -glucosidase (AG) inhibitory data of polyhydroxylated pyrrolidine derivatives.

Entry	Compound	% Inhibition ^a	
		ALR2	AG
D-Glucose 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-Galactose 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 μ M test compound, obtained as mean of at least three determinations. Standard errors of the means (SEMs) are $\leq 10\%$.

^b Not active. No inhibition was observed at 100 μ M test compound.

^c IC₅₀: 40.6 $\pm$ 3.80 μ M.

^d IC₅₀: 62.4 $\pm$ 5.11 μ M.

^e Not tested.

3. Conclusions

Polyhydroxylated pyrrolidine derivatives of the *D-glucose* and *D-galactose* 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-glucose* and *D-galactose* 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 imino-sugar 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 $\pm$ 2 °C. Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. ¹H 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). ¹³C 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 CD₃CN (δ_H 1.94, δ_C 1.28) or residual CD₃OD (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 F₂₅₄ 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 4 Å molecular sieves activated at least 12 h at 200 °C. All reagents were purchased from Aldrich Chemical Co. and were used without further purification. MgSO₄ was used as the drying agent for solutions. Elemental analysis was used to determine the purity of compounds. Analytical results are within $\pm$ 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 Ac₂O 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], (4*R*)- and (4*S*)-

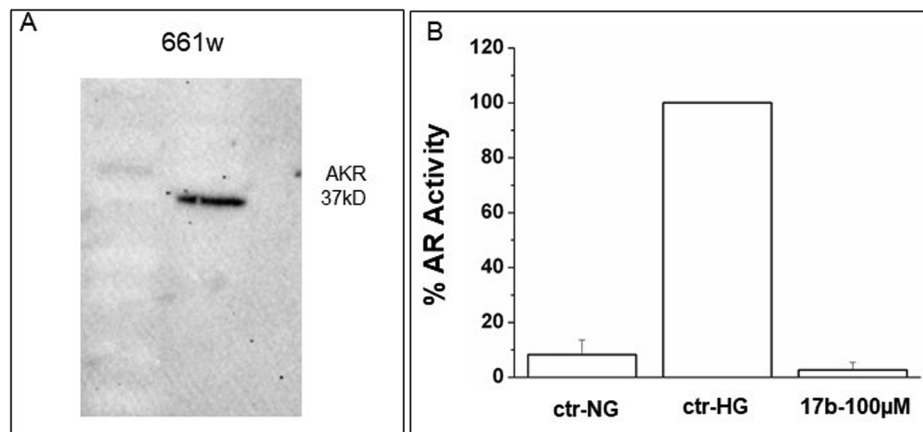


Fig. 3. (A) Representative western blot confirming the presence of ALR2 in the experimental cell model; (B) Results obtained in the presence of **17b**. Inhibition of ALR2 was assessed by using a specific kit assay. Values are calculated as percentage of maximum activity measured in hyperglycemic control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

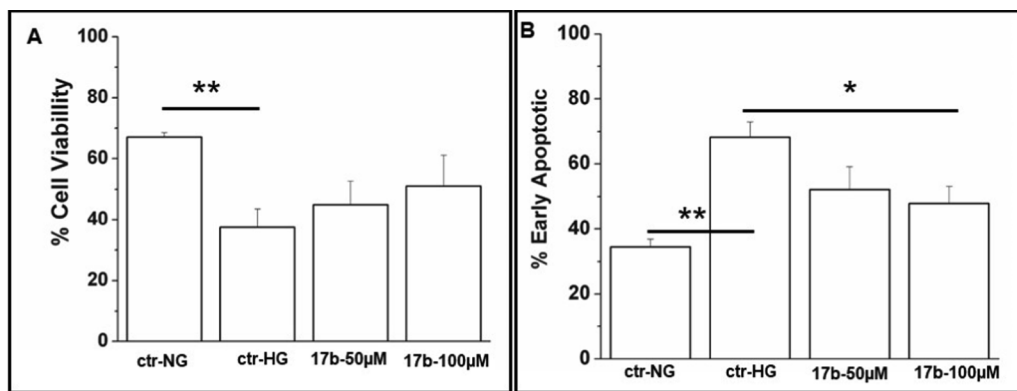


Fig. 4. Effectiveness of compound **17b** in increasing cell viability. (A) The histogram shows that while the hyperglycemic condition impairs significantly the cell viability, compared to the normoglycemic control (ctr-NG), the treatment with **17b** at both 50 and 100 μM concentration partially recovers 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 100 μM of **17b**. The values are reported as mean ± SEM (n = 3, independent experiments).

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-H₂O (50 mL), treated with Et₃N (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 (¹H and ¹³C) 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 BnNH₂ (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 NaBH₃CN (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

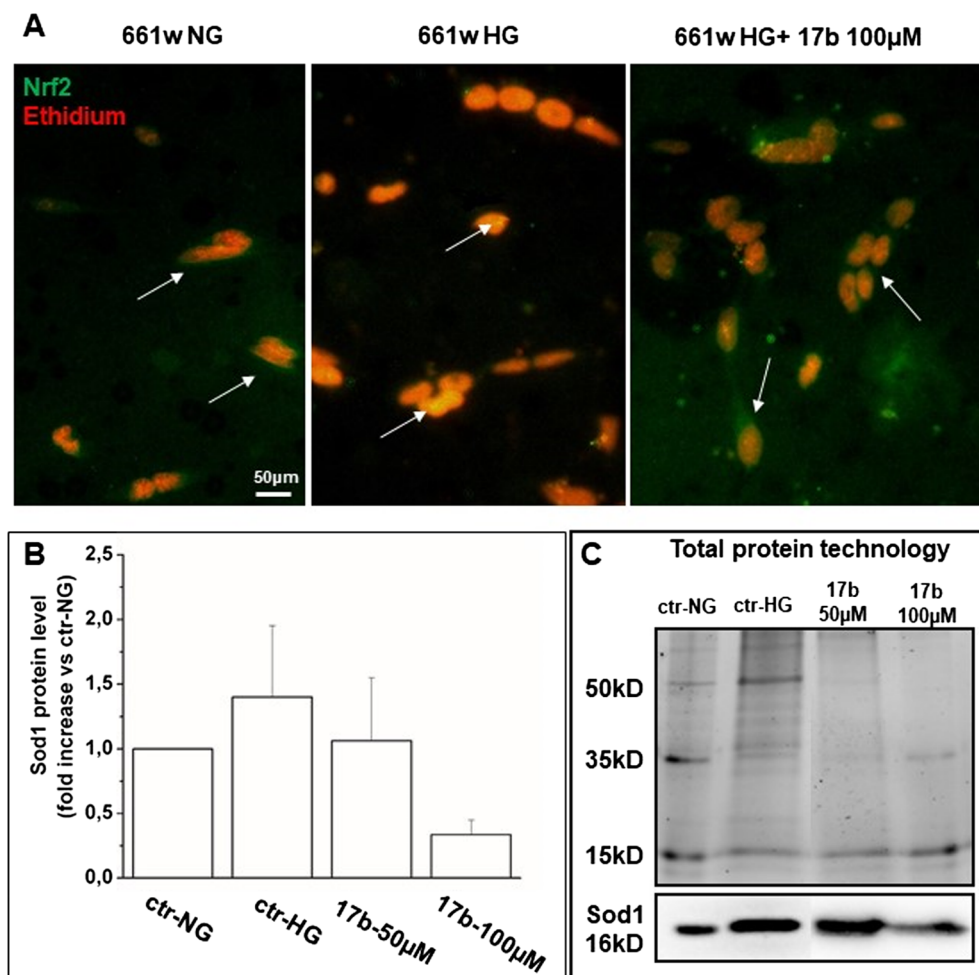


Fig. 5. Antioxidant activity of **17b**. (A) The image obtained with the fluorescence microscope shows the specific staining for Nrf20 (green) and the nuclear staining obtained with ethidium bromide (red). The arrows indicate the different localization of Nrf2, cytosolic in normoglycemic conditions and 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].

(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), ^1H 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-1 α), 2.65 (dd, 1H, $J_{1\beta,1\alpha}$ 11.6 Hz, $J_{1\beta,2}$ 4.7 Hz, H-1 β), 2.14–1.96 (m, 12H, $4 \times \text{MeCO}$); ^{13}C NMR (62.9 MHz, CDCl_3): δ 170.8–169.1 ($4 \times \text{C}=\text{O}$), 137.8 (Ar-C), 128.5–127.3 (Ar-CH), 77.2 (C-3), 76.0 (C-2), 70.9 (C-5), 68.3 (C-4), 63.1 (C-6), 59.1 (CH_2Ph), 56.6 (C-1), 20.9–20.8 ($4 \times \text{MeCO}$). Anal. Calcd for $\text{C}_{21}\text{H}_{27}\text{NO}_8$: 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**. ^1H 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-1 β), 2.40 (dd, 1H, H-1 α), 2.11–2.04 (m, 12H, $4 \times \text{MeCO}$); ^{13}C NMR (50.33 MHz, CDCl_3): δ 170.4–168.8 ($\text{C}=\text{O}$), 138.1 (Ar-C), 128.6–126.1 (Ar-CH), 76.6, 76.3 (C-2, C-3), 70.9 (C-5), 65.6 (C-4), 63.3 (C-6), 60.9 (CH_2Ph), 55.9 (C-1), 20.6 ($4 \times \text{MeCO}$). Anal. Calcd for $\text{C}_{21}\text{H}_{27}\text{NO}_8$: 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-immuno-D-glucitol (**8a**) and *N*-benzhydryl-1,4-dideoxy-1,4-immuno-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, $\text{CD}_3\text{CN-D}_2\text{O}$) 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. ^1H NMR (200.13 MHz, $\text{CD}_3\text{CN-D}_2\text{O}$) of **8a**: δ 5.01 (s, 1H, CHPh_2), 3.20 (dd, 1H, $J_{1\beta,1\alpha}$ 10.8 Hz, $J_{1\beta,2}$ 4.8 Hz, H-1 β), 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-1 α); of **8b**: δ 4.94 (s, 1H, CHPh_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-1 α), 2.71 (dd, 1H, $J_{1\beta,2}$ 5.1 Hz, H-1 β); cluster of signals for **8a** and **8b**: δ 7.63–7.19 (m, 10H, Ar-H), 4.04 (m, 4H, $2 \times \text{H-2}$, $2 \times \text{H-3}$), 3.40 (m, 6H, $2 \times \text{H-5}$, $2 \times \text{H-6a}$, $2 \times \text{H-6b}$); ^{13}C NMR (50.33 MHz, $\text{CD}_3\text{CN-D}_2\text{O}$) 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 ($2 \times \text{Ar-C}$), 129.6–126.6 (Ar-CH). Anal. Calcd for $\text{C}_{19}\text{H}_{23}\text{NO}_4$: 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-immuno-D-glucitol (**9a**) and *N*-benzhydryl-2,3,5,6-tetra-O-acetyl-1,4-dideoxy-1,4-immuno-D-galactitol (**9b**)

Routine acetylation of a mixture of **8a** and **8b** (237 mg, 0.72 mmol) with 1:2 $\text{Ac}_2\text{O-Py}$ 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. ^1H NMR (200.13 MHz, CDCl_3) of **9a**: δ 5.30 (m, 2H, H-3, H-5), 5.23 (s 1H, CHPh_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-1 β), 2.63 (dd, 1H, $J_{1\alpha,2}$ 4.2 Hz H-1 α), 2.13, 2.12, 1.97, 1.83 (4 s, each 3H, $4 \times \text{MeCO}$); of **9b**: δ 5.12 (m, 3H, H-2, H-3, H-5), 5.08 (s 1H, CHPh_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-1 α), 2.84 (dd, 1H, $J_{1\beta,2}$ 5.4 Hz, H-1 β), 2.17, 2.13, 2.01, 1.94 (4 s, each 3H, $4 \times \text{MeCO}$); 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 ($2 \times \text{Ar-C}$), 75.7, 75.6 (C-2, C-3), 70.7 (CHPh_2), 68.4 (C-5), 63.4 (C-6), 60.7 (C-4), 51.3 (C-1); of **9b**: δ 141.4, 139.0 ($2 \times \text{Ar-C}$), 78.0, 75.9 (C-2, C-3), 71.0 (C-5), 70.5 (CHPh_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 ($\text{C}=\text{O}$), 129.2–126.9 (Ar-CH), 21.0–20.3 ($4 \times \text{MeCO}$). Calcd for $\text{C}_{27}\text{H}_{31}\text{NO}_8$: 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-immuno-D-glucitol (**10a**) and *N*-acetyl-2,3,5,6-tetra-O-acetyl-1,4-dideoxy-1,4-immuno-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 ($\text{NH}_4^+ \text{AcO}^-$, 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 $\text{Ac}_2\text{O-Py}$ (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 Et₂O-EtOAc-ⁱPrOH) 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-immuno-D-glucitol (11a) and N-benzyl-5,6-O-isopropylidene-1,4-dideoxy-1,4-immuno-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 BnNH₂HCl (346 mg, 2.42 mmol, 1 eq) in dry MeOH (20 mL) and NaBH₃CN (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 CH₂Cl₂ (70 mL) and satd aq NaHCO₃ (50 mL) and the aqueous phase was extracted with CH₂Cl₂ (4 × 50 mL). The organic layers were collected, dried (MgSO₄) 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 Et₂O-EtOAc + 0.1% Et₃N) 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-immuno-D-glucitol (**11a**) as a white solid, *R_f* 0.52 (9:1 EtOAc-MeOH); [α]_D –23.9 (c 1.1 in CHCl₃), mp 83–85 °C (from EtOAc-hexane), ¹H NMR (250.13 MHz, CDCl₃): 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, CH₂Ph), 3.96 (dd, 1H, *J*_{6a,6b} 8.0 Hz, *J*_{5,6a} 7.6 Hz, H-6a), 3.28 (dd, 1H, *J*_{1β,1α} 10.5 Hz, *J*_{1β,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*_{1α,2} 5.5 Hz, H-1α), 1.44, 1.37 (2 s, each 3H, CMe₂); ¹³C NMR (62.9 MHz, CDCl₃): δ 138.0 (Ar-C), 128.9–127.3 (Ar-CH), 108.5 (CMe₂), 78.9 (C-3), 76.1, 76.0 (C-2, C-5), 67.1 (CH₂Ph), 66.5 (C-4), 60.4 (C-6), 58.3 (C-1), 26.4, 25.0 (CMe₂). Anal. Calcd for C₁₆H₂₃NO₄: 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-immuno-D-galactitol (**11b**) as a colourless syrup, *R_f* 0.38 (9:1 EtOAc-MeOH); [α]_D +52.2 (c 1.2 in CHCl₃), ¹H NMR (200.13 MHz, CD₃CN): 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, CH₂Ph), 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*_{1α,1β} 10.4 Hz, *J*_{1β,2} 4.8 Hz, H-1β), 2.70 (bd, 1H, H-1α), 1.42, 1.33 (2 s, each 3H, CMe₂); ¹³C NMR (50.33 MHz, CD₃CN): δ 140.3 (Ar-C), 129.6–127.7 (Ar-CH), 109.6 (CMe₂), 80.9, 78.8 (C-2, C-3), 77.4 (C-5), 73.7 (C-4), 67.0 (C-6), 60.3 (CH₂Ph), 60.0 (C-1), 27.0, 25.6 (CMe₂). Anal. Calcd for C₁₆H₂₃NO₄: 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-immuno-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-immuno-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 NaBH₃CN (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 Et₂O-EtOAc + Et₃N) 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 CH₂Cl₂ (30 mL) and satd aq NaHCO₃ (30 mL) and the aqueous phase was extracted with CH₂Cl₂ (4 × 35 mL). The organic layers were collected, dried (MgSO₄) 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 Et₂O-EtOAc + Et₃N) 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. ¹³C NMR (62.9 MHz, CDCl₃) of **12a**: δ 173.6 (C=O), 138.2 (Ar-C), 109.0 (CMe₂), 79.3, 78.1 (C-3, C-2), 75.3 (C-5), 65.6 (C-6), 64.5 (C-4, CHN), 52.1 (C-1), 51.4 (OMe), 36.8 (CH₂Ph), 26.2, 24.9 (CMe₂); of **12b**: δ 175.1 (C=O), 138.0 (Ar-C), 108.0 (CMe₂), 79.2, 77.0 (C-3, C-2), 75.8 (C-5), 68.9 (C-4), 66.1 (C-6), 63.7 (CHN), 52.7 (C-1), 51.5 (OMe), 32.4 (CH₂Ph), 26.1, 25.3 (CMe₂); cluster of signals for **12a** and **12b**: δ 129.1–126.2 (Ar-CH).

Routine acetylation of a mixture of **12a** and **12b** (200 mg, 0.55 mmol) with 1:2 Ac₂O-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. ¹H NMR (250.13 MHz, CDCl₃) 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, CHN), 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*_{1β,1α} 11.4 Hz, *J*_{1β,2} 5.8 Hz, H-1β), 3.15 (m, 1H, H-1α), 1.36, 1.27 (2 s, each 3H, CMe₂); 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, CHN), 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*_{1β,1α} 11.0 Hz, *J*_{1β,2} 4.8 Hz, H-1β), 3.15 (dd, 1H, *J*_{1α,2} 6.8 Hz, H-1α), 1.37, 1.31 (2 s, each 3H, CMe₂), cluster of signals for **13a** and **13b**: δ 7.29–7.16 (m, 5H, Ar-H), 3.12–2.90 (m, 2H, CH₂Ph), 2.06–2.04 (2 s, each 3H, 2 × MeCO); ¹³C NMR (62.9 MHz, CDCl₃) of **13a**: δ 172.2 (COOMe), 137.5 (Ar-C), 108.0 (CMe₂), 77.0 (C-3), 75.8 (C-5), 75.1 (C-2), 65.4 (C-6), 64.1 (CHN), 61.0 (C-4), 51.5 (OMe), 50.2 (C-1), 36.3 (CH₂Ph), 26.1, 24.7 (CMe₂); of **13b**: δ 172.0 (COOMe), 138.0 (Ar-C), 109.2 (CMe₂), 77.1 (C-3), 76.1 (C-5), 75.9 (C-2), 67.1 (C-4), 65.7 (C-6), 62.3 (CHN), 51.1 (OMe), 50.6 (C-1), 31.2 (CH₂Ph), 26.3, 24.9 (CMe₂); cluster of signals for **13a** and **13b**: δ 169.9–169.3 (C=O), 129.1–126.3 (Ar-CH), 20.8 (4 × MeCO). Anal. Calcd for C₂₃H₃₁NO₈: 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-immuno-D-glucitol (14a) and N-[(2'R)-1'-carbomethoxy-2'-benzylethyl]-2,3-di-O-acetyl-1,4-dideoxy-1,4-immuno-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 CH₂Ph 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 (CHN), 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 (CHN), 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-CH), 21.2–21.0 (MeCO). Anal. Calcd for $\text{C}_{20}\text{H}_{27}\text{NO}_8$: 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-immuno-D-glucitol (15a) and N-[(2'S)-1'-carbomethoxy-2'-benzylethyl]-5,6-O-isopropylidene-1,4-dideoxy-1,4-immuno-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 Et₂O-EtOAc + Et₃N) 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 $\times$ 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 Et₂O-EtOAc + Et₃N) 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-immuno-D-glucitol (**15a**) as colourless syrup, R_f 0.52 (1:2 Et₂O-EtOAc + 0.1% di Et₃N), ^1H NMR (250.13 MHz, CDCl_3): δ 7.36–7.23 (m, 5H, Ar-H), 4.17–4.06 (m, 2H, H-3, H-3), 3.96–3.89 (m, 2H, H-5, CHN), 3.73 (m, 1H, H-6b), 3.71 (s, 3H, OMe), 3.58–3.42 (m, 3H, H-1 β , H-4, H-6a), 3.15–2.85 (m, 5H, H-1 α , CH_2Ph , OH-2, OH-3), 1.33, 1.26 (2 s, each 3H, CMe_2); ^{13}C NMR (62.9 MHz, CDCl_3): δ 175.2 (C=O), 138.1 (Ar-C), 129.0–126.5 (Ar-CH), 107.4 (CMe_2), 78.4, 76.6 (C-3, C-2), 75.7 (C-5), 63.9 (CHN), 63.4 (C-4), 66.0 (C-6), 53.0 (C-1), 51.8 (OMe), 37.7 (CH_2Ph), 26.2, 24.5 (CMe_2). Anal. Calcd for $\text{C}_{19}\text{H}_{27}\text{NO}_6$: 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-CH), 109.1 (CMe_2), 79.5, 78.2 (C-3, C-2), 75.6 (C-5), 64.0 (CHN), 69.4 (C-4), 66.4 (C-6), 54.2 (C-1), 51.4 (OMe), 32.5 (CH_2Ph), 26.4, 25.5 (CMe_2).

4.1.11. N-[(2'S)-1'-carbomethoxy-2'-benzylethyl]-1,4-dideoxy-1,4-immuno-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 $\times$ 5 mL) under diminished pressure. Purification of the crude residue by flash chromatography on silica gel (95:5 EtOAc-MeOH) gave pure of azafuranose derivatives **16a** (38 mg, 88.8%) as colourless syrup, R_f 0.30 (95:5 EtOAc-MeOH), $[\alpha]_D$ –23.9 (c 1.1 in CHCl_3), ^1H NMR (250.13 MHz, CD_3OD): δ 7.28 (m, 5H, 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, CHN), 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-1 β), 3.22 (dd, 1H, $J_{4,5}$ 6.3 Hz, H-4), 2.90 (m, 1H, H-1 α); ^{13}C NMR (62.9 MHz, CD_3OD): δ 174.1 (C=O), 139.8 (Ar-C), 130.1–127.5 (Ar-CH), 79.1 (C-3), 76.4 (C-2), 73.0 (C-5), 64.8 (CHN), 64.3 (C-6), 64.0 (C-4), 52.4 (C-1), 51.7 (OMe), 38.0 (CH_2Ph). Anal. Calcd for $\text{C}_{16}\text{H}_{23}\text{NO}_6$: 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-immuno-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), ^1H NMR (400 MHz, $\text{CD}_3\text{CN-D}_2\text{O}$): 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 (2 m, each 1H, H-2, H-3), 3.76 (dd, 1H, H-6b), 3.14–3.09 (m, 2H, H-1 β , H-4), 2.62 (d, 1H, $J_{1\alpha,1\beta}$ 11.9 Hz, $J_{1\alpha,2}$ 1.5 Hz, H-1 α), 1.35, 1.29 (2 s, each 3H, CMe_2); ^{13}C NMR (100 MHz, $\text{CD}_3\text{CN-D}_2\text{O}$): δ 109.8 (CMe_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 (CMe_2). Anal. Calcd for $\text{C}_9\text{H}_{17}\text{NO}_4$: 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-immuno-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), ^1H 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-1 β), 3.18 (dd, 1H, H-4), 2.73 (dd, 1H, $J_{1\alpha,2}$ 3.1 Hz, H-1 α), 1.36, 1.30 (2 s, each 3H, CMe_2); ^{13}C NMR (62.9 MHz, CD_3CN): δ 109.7 (CMe_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 (CMe_2). Anal. Calcd for $\text{C}_9\text{H}_{17}\text{NO}_4$: 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-immuno-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), Et₃N (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 $\times$ 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 (^1H 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 CHMe_2 protons at δ 2.67 and 3.16 respectively.

By recording the spectra at 80 °C (CD₃CN-D₂O), coalescence was observed in the ¹³C spectrum, while **18a-major** and **18a-minor** are still evident in the ¹H spectrum. Selected ¹H NMR (400 MHz, CD₃CN-D₂O, 20 °C) signals: **18a-major**: δ 3.72 (dd, 1H, *J*_{1α,1β} 11.2 Hz, *J*_{1β,2} 4.3 Hz, H-1β), 2.67 (m, 1H, (CHMe₂)), 1.35, 1.28 (2 s, each 3H, CMe₂), 1.01 (d, 3H, *J*_{vic} 6.5 Hz, CHMe₂), 0.98 (d, 3H, *J*_{vic} 6.5 Hz, CHMe₂); **18a-minor**: δ 4.46 (m, 1H, H-4), 4.36 (m, 1H, H-5), 3.16 (m, 1H, CHMe₂), 1.37, 1.31 (2 s, each 3H, CMe₂), 1.04 (d, 3H, *J*_{vic} 5.8 Hz, CHMe₂), 0.96 (d, 3H, *J*_{vic} 5.8 Hz, CHMe₂); ¹³C NMR (100 MHz, CD₃CN-D₂O, 20 °C): **18a-major**: δ 180.1 (C=O), 109.9 (CMe₂), 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 (CHMe₂), 27.1, 26.6 (CMe₂), 20.3, 19.3 (CHMe₂); **18a-minor**: δ 180.1 (C=O), 109.5 (CMe₂), 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 (CHMe₂), 27.3, 56.0 (CMe₂), 20.5, 19.6 (CHMe₂); ¹³C NMR (50.33 MHz, CD₃CN-D₂O, 80 °C): δ 179.8 (C=O), 109.8 (CMe₂), 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 (CHMe₂), 27.1, 26.1 (CMe₂), 20.3, 19.2 (CHMe₂). Anal. Calcd for C₁₃H₂₃NO₅: 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-H₂O (v/v, 3.3 mL), Et₃N (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 NaHCO₃ (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 (MgSO₄) 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); [α]_D + 110.5 (c 1.0 in CHCl₃). The ¹H 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 (CD₃CN-D₂O) coalescence was observed in the ¹³C spectrum. ¹³C NMR (100 MHz, CD₃CN-D₂O, 20 °C): **18b-major**: δ 180.1 (C=O), 110.5 (CMe₂), 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 (CHMe₂), 26.4, 25.3 (CMe₂), 20.0, 19.3 (CHMe₂); **18b-minor**: δ 179.6 (C=O), 109.9 (CMe₂), 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 (CHMe₂), 26.3, 25.7 (CMe₂), 20.0, 18.7 (CHMe₂); ¹³C NMR (50.33 MHz, CD₃CN-D₂O, 80 °C): δ 179.9 (C=O), 110.5 (CMe₂), 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 (CHMe₂), 26.8, 25.9 (CMe₂), 20.3, 19.3 (CHMe₂). Anal. Calcd for C₁₃H₂₃NO₅: 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 H₂ (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-H₂O (v/v, 7.0 mL), Et₃N (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 NaHCO₃ (15 mL) was added, stirred at room temperature (15 min), the aqueous phase was extracted with CH₂Cl₂ (4 × 15 mL) and the organic layers were collected, dried (MgSO₄) 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** (38.7 mg, 83%) as white foam, *R*_f 0.18 (9:1 EtOAc-MeOH). The NMR analysis (¹H and ¹³C, CD₃OD) 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 ¹H NMR analysis of the **19a** allows to assign only the chemical shifts of the proton related to the rotational isomer **19a-major**. ¹H NMR (250.13 MHz, CD₃OD, at 20 °C) of **19a-major**: δ 4.28–4.00 (m, 4H, H-2, H-3, H-4, H-5), 3.75 (dd, 1H, *J*_{1α,1β} 11.3 Hz, *J*_{1β,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*_{1α,2} 2.0 Hz, H-1α), 3.50 (dd, 1H, *J*_{5,6a} 6.5 Hz, H-6a), 2.46 (m, 1H, CHMe₂), 1.13 (d, 3H, *J*_{vic} 6.8 Hz, CHMe₂), 1.09 (d, 3H, *J*_{vic} 6.7 Hz, CHMe₂); ¹³C NMR (62.9 MHz, CD₃OD, 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 (CHMe₂), 19.7, 18.9 (CHMe₂); 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 (CHMe₂), 20.1, 19.6 (CHMe₂). Anal. Calcd for C₁₀H₁₉NO₅: 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 (¹H and ¹³C, CD₃OD) 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. ¹H NMR (250.13 MHz, CD₃OD, 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, 1H, H-5), 3.53–3.45 (m, 3H, H 1α, H-6a, H 6b), 2.82 (m, 1H, CHMe₂), 1.12 (d, 3H, *J*_{vic} 6.8 Hz, CHMe₂), 1.08 (d, 3H, *J*_{vic} 6.7 Hz, CHMe₂); 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*_{1α,1β} 12.2 Hz, H-1α), 3.17 (m, 1H, CHMe₂), 1.10 (d, 3H, *J*_{vic} 6.5 Hz, CHMe₂), 1.04 (d, 3H, *J*_{vic} 7.0 Hz, CHMe₂); ¹³C NMR (62.9 MHz, CD₃OD, 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 (CHMe₂), 20.0, 18.7 (CHMe₂); 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 (CHMe₂), 19.3, 19.9 (CHMe₂). Anal. Calcd for C₁₀H₁₉NO₅: 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). ^1H NMR (200.13 MHz, CD_3OD): δ 7.39–7.20 (m, 5H, Ar-H), 4.20, 3.52 (AB system, 2H, $J_{A,B} = 13.6$ Hz, CH_2Ph), 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-1 β), 2.71 (dd, 1H, $J_{1\alpha,1\beta} 10.7$ Hz, $J_{1\alpha,2} 4.4$ Hz, H-1 α); ^{13}C NMR (62.9 MHz, CD_3OD): δ 138.6 (Ar-C), 128.4, 127.9, 126.7 (Ar-CH), 79.2 (C-3), 75.7 (C-2), 73.3 (C-4), 71.2 (C-5), 63.7 (C-6), 60.7 (CH_2Ph), 58.9 (C-1). Anal. Calcd for $\text{C}_{13}\text{H}_{19}\text{NO}_4$: 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-immuno-D-glucitol (**21a**) and 2,3,5,6-tetra-O-acetyl-1,4-dideoxy-1,4-immuno-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 Et₂O-EtOAc-ⁱPrOH + 0.1% Et₃N) gave pure **21a** (99 mg, 30%) and **21b** (171 mg, 52%).

2,3,5,6-tetra-O-acetyl-1,4-dideoxy-1,4-immuno-D-glucitol (**21a**) as a clear syrup, R_f 0.32 (7:2:1 Et₂O-EtOAc-ⁱPrOH + 0.1% Et₃N), $[\alpha]_D -18.3$ (c 1.0 in CHCl_3), ^1H 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-1 β), 2.66 (dd, 1H, H-1 α), 1.79, 1.75, 1.67, 1.53 (4 s, each 3H, $4 \times \text{MeCO}$); ^{13}C NMR (50.33 MHz, C_6D_6): δ 170.2, 169.7, 169.1, 169.0 ($4 \times \text{C=O}$), 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 ($4 \times \text{MeCO}$). Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{NO}_8$: 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-immuno-D-galactitol (**21b**) as colourless syrup, R_f 0.24 (7:2:1 Et₂O-EtOAc-ⁱPrOH + 0.1% Et₃N), $[\alpha]_D +44.3$ (c 1.0 in CHCl_3); ^1H 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-1 α), 3.06 (dd, 1H, $J_{1\beta,1\alpha} 12.1$ Hz, $J_{1\beta,2} 5.8$ Hz, H-1 β), 1.80, 1.71, 1.58, 1.54 (4 s, each 3H, $4 \times \text{MeCO}$); ^{13}C NMR (50.33 MHz, C_6D_6): δ 172.6, 170.6, 169.4, 169.3 ($4 \times \text{C=O}$), 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 ($4 \times \text{MeCO}$). Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{NO}_8$: 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-immuno-D-glucitol (**10a**)

Routine acetylation of azafuranose derivative **21a** (80 mg, 0.23 mmol) with 1:2 Ac₂O-Py mixture (9.0 mL) gave after flash chromatography (7:2:1 Et₂O-EtOAc-ⁱPrOH + 0.1% Et₃N) 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 ^1H NMR analysis of the **10a** allows to assign only the chemical shifts of the proton related to the rotational isomer **10a-major**. ^1H 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-1 β), 3.39 (dd, 1H, $J_{1\alpha,2} 4.0$ Hz, H-1 α), 2.04, 2.02, 2.00, 1.97, 1.96 (5 s, each 3H, $5 \times \text{MeCO}$); ^{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 (MeCON), 20.9–20.6 (MeCOO).

4.1.22. N-acetyl-2,3,5,6-tetra-O-acetyl-1,4-dideoxy-1,4-immuno-D-galactitol (**10b**)

Routine acetylation of azafuranose derivative **21b** (140 mg, 0.41 mmol) with 1:2 Ac₂O-Py mixture (12.0 mL) gave after flash chromatography (7:2:1 Et₂O-EtOAc-ⁱPrOH + 0.1% Et₃N) 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. ^1H 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-1 β); 3.43 (dd, 1H, $J_{1\alpha,2} 3.4$ Hz, H-1 α); 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-1 β), 4.05 (m, 1H, H-6a), 3.94 (d, 1H, H-4), 3.23 (dd, 1H, $J_{1\alpha,2} 2.0$ Hz, H-1 α); 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, $5 \times 15\text{H}$, MeCO); ^{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 (MeCON); 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 (MeCON); cluster of signals for **10b-minor** and **10b-major**: δ 170.4–169.5 (C=O), 20.8–20.5 (MeCOO). Anal. Calcd for $\text{C}_{16}\text{H}_{23}\text{NO}_9$: 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-immuno-D-galactitol hydrochloride (**22**)

A solution of **10b** (56 mg, 0.15 mmol) or **21b** (52 mg, 0.15 mmol) in aq HCl (2 M, 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 titration of the crude product with Et₂O afforded pure the 1,4-dideoxy-1,4-immuno-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 *Saccharomyces 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 min 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 μ L, 100 μ M) 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*-nitrophenoxide by reaction with the target enzyme. The reaction mixture contained 100 μ L sodium phosphate buffer pH = 6.8 (0.1 M), 25 μ L enzyme (0.2 U/mL) and 25 μ L of *p*-nitrophenyl α -D-glucopyranoside (0.375 mM), in a 96-multiwell plate. All the reagents were incubated at 37 °C for 10 min. The reaction was then stopped with 100 μ L of saturated Na₂CO₃. 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 μ L, 100 μ M) 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). IC₅₀ data were obtained by linear regression analysis of the log dose-response curve.

4.2.5. 661w cell culture

The 661 W 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% Lglutamine 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 24 h 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% CO₂ 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 24 h; 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 2 h, 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% CO₂ 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 24 h. 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, KH₂PO₄ 1.47 mM, NaCl 139.9 mM, Na₂HPO₄ 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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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2019.103298>.

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