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A preparation and screening strategy for glycosidase inhibitors

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Abstract—Here we present a one-pot procedure for the synthesis of a library of carbohydrate compounds belonging to four classes of glycosidase inhibitors: glycosides, aldonic acid lactams, aldonic acid lactones and dideoxy-iminoalditols. The complex mixture was separated into individual components that were screened for activity against α- and β-glucosidases. Among these were two 2-*O*-methyl glycosides that reduced the rate of the enzymatically-catalyzed hydrolysis of the *p*-nitrophenyl glucosides used in the assay. Relative inhibition indices normalized to the inhibition observed with deoxynojirimycin under similar conditions were determined. This methodology provides a straightforward, general and time-efficient way to synthesize, test and analyze an extensive number of glycosidase inhibitor candidates from several carbohydrate compound classes in one scheme. The direct reference to deoxynojirimycin gives an immediate indication of the concentration range in which a significant inhibitory activity can be obtained.

1. Introduction

Glycosidase inhibitors have the potential to produce several beneficial therapeutic effects including reduction of the blood glucose levels,[1] inhibition of tumor metastasis[2] and inhibition of viral replication.[3–5] Among the most common and potent classes of glycosidase inhibitors are glycosides, aldono-1,5-lactones, aldono-1,5-lactams, and dideoxy-imino alditols (aza-sugars) (Fig. 1). There are several authoritative reviews on this area.[6–10] These classes of inhibitory molecules are usually prepared using unrelated synthetic routes although one way of preparing δ-lactams has been to oxidize 1,5-dideoxy-1,5-iminosugars such as nojirimycin.[11] Because of the high cost and instability of nojirimycin, this method of preparing lactams is not very appealing.

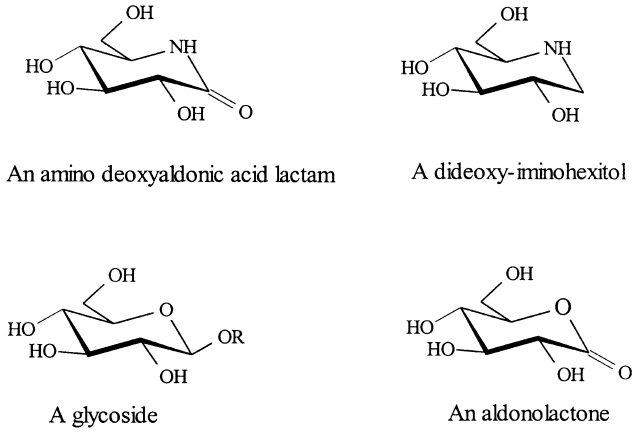

Figure 1. Important classes of glycosidase inhibitors.

Tests have shown that the inhibitory activity of these classes of compounds can be modulated by substitution. For instance, some *O*-alkylated and -arylated δ-lactams have been found to markedly inhibit metastasis of lung cancer cells while having very low cytotoxicity.[12] In addition to this, an important number of *N*-substituted derivatives of deoxynojirimycin (D-1,5-dideoxy-1,5-iminoglucitol) have anti-HIV activity and at least one has been in clinical trials for this indication.[13] Substitution therefore affords a way of influencing the specificity, efficacy, potency and bio-availability of drug targets based on these molecules.

Routes for the syntheses of carbohydrate glycosidase inhibitors, especially with O-substituents, are very involved. Because of this, it is not possible to routinely prepare and screen a significant number of candidates. An integrated combinatorial approach that gives access to candidates from all four classes mentioned above and with variations in substitution would be an ideal and valuable strategy.

The primary goal of the work described here was the rapid synthesis and testing of a library of glucosidase inhibitors. We have recently developed a procedure for the transformation of methyl-β-D-glucopyranoside **1** into 5-amino-D-gluconic acid lactams **5**, and **8** and then to dideoxynojirimycin **6** and deoxynojirimycin **9** (Scheme 1).[14] This involves the chromium trioxide oxidation of methyl-β-D-glucopyranoside to form 5-keto aldonic acid methyl ester, which is then converted to a cyclic aminosugar by reductive

Keywords: aldonic acid lactams; aldonic acid lactones; 1,5-dideoxy-1,5-iminosugars; glucosidase inhibitors.

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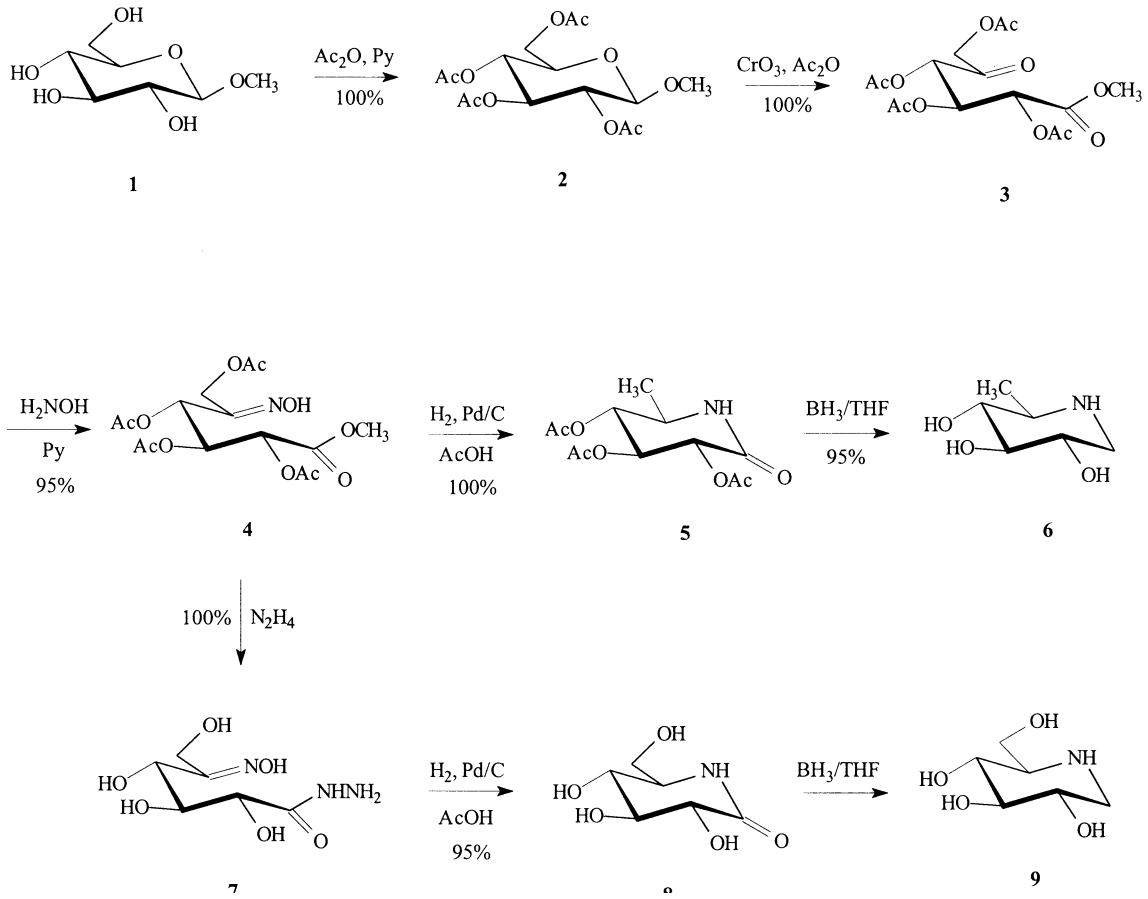


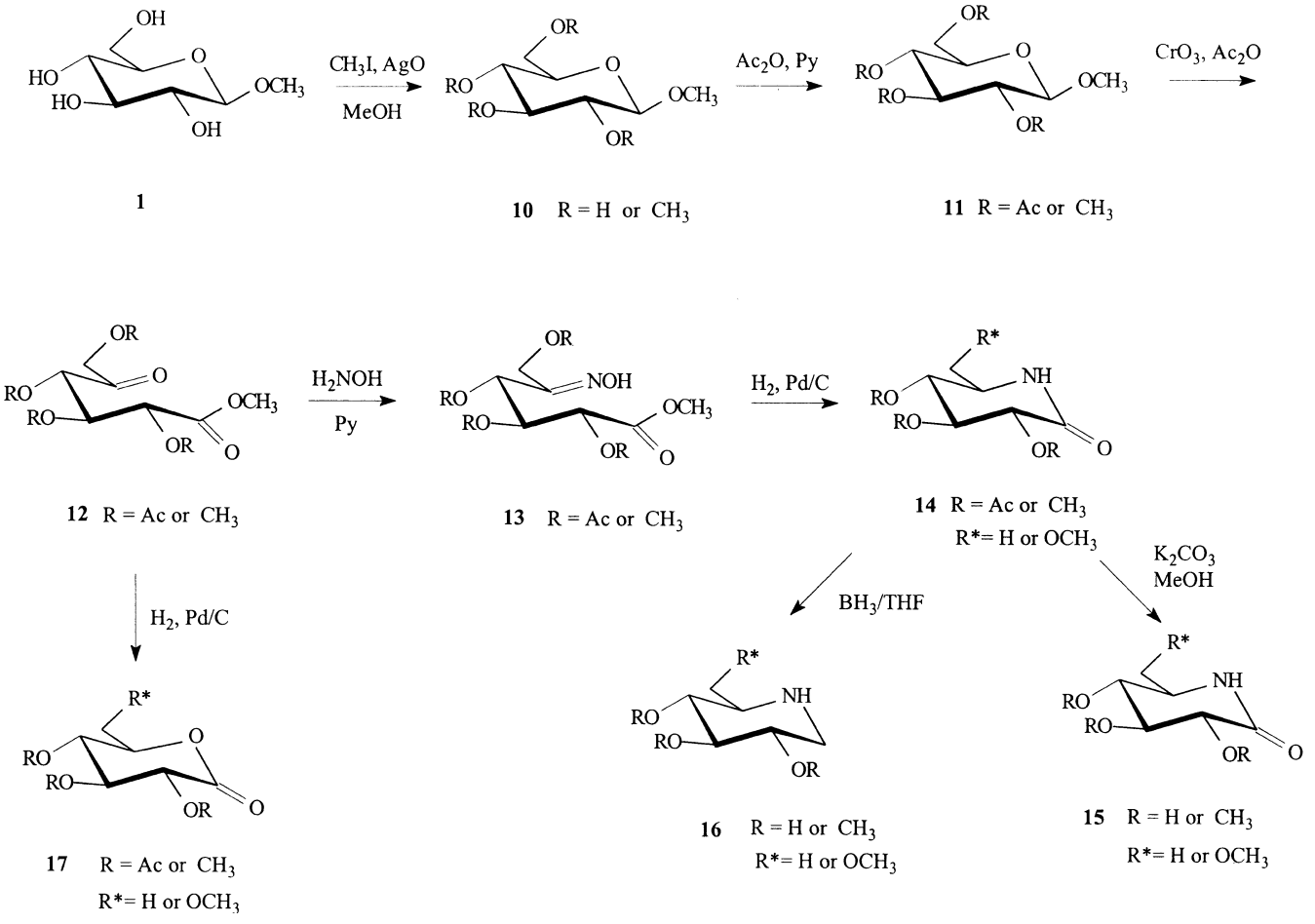
Scheme 1. Synthesis of δ-gluconolactam, dideoxynojirimycin and deoxynojirimycin.

ring closure with a nitrogen species. One important feature of this method is that it can potentially afford access to all four of these classes of molecules. Since all conversions are nearly quantitative, we used this procedure as the basis for the design of a combinatorial approach to partially methylated methyl-β-D-glucopyranosides, 5-amino-5-deoxy-D-gluconic acid lactams, 5-amino-5-deoxy-D-gluconic acid lactones and 1,5,6-trideoxy-1,5-imino glucitols. This method has the advantage of producing structural diversity and a comprehensive spectrum of molecules through a few simple and high yield reactions. The strategy is illustrated in Scheme 2.

2. Results and discussion

Methyl-β-D-glucoside **1** was partially methylated and then acetylated, giving an array of differently substituted glucosides **11** (Scheme 2). This mixture of partially methylated, partially acetylated glucosides was subjected to chromium trioxide oxidation to give 5-keto-gluconic acid methyl ester derivatives **12**. These were reacted with hydroxylamine to produce oximes **13**. The oximes were then converted to δ-lactams **14** by catalytic hydrogenation. Part of this reaction mixture was deacetylated to give mixture A (compound family **15**) while another portion was reduced to the mixture B primarily 1,5-imino-D-glucitols (compound family **16**). None of the intermediate products **11**–**14** were separated or characterized but the rationale for their formation and the expected yields were predicated on our previous work in which the products expected were isolated and rigorously characterized.[14] In this present study, we use this information in a scheme designed to accomplish the rapid generation, separation, characterization and assay of α- and β-glucosidase inhibitor candidates. Once these are found, more targeted synthetic routes can be designed and used. Both final mixtures A and B were subjected to HPLC separation. Twenty-eight fractions were collected from mixture A and 52 from mixture B. All of these were assayed for their inhibitory activity against α- and β-glucosidase. Six compounds proved to be active, two of which appeared in both mixtures A and B.

As it is not possible, or indeed necessary, to determine the inhibition constant (K_i) of each candidate molecule in a screening scheme such as this one, a direct comparison with an equivalent amount of deoxynojirimycin was obtained. We chose deoxynojirimycin as a reference compound because it is a potent glucosidase inhibitor with a K_i value of 8.67×10^{-6} for α-glucosidase (brewers yeast) and 1.8×10^{-5} for α-glucosidase (sweet almond).[11] A relative inhibitory index was calculated. Values greater than 1 represented better inhibition compared to deoxynojirimycin. The purity of these substances has been verified by GC–MS. Structure determination using NMR, IR and high resolution mass spectrometry (HRMS) on the acetylated active substances led to the structures in Table 1. Only the active compounds were characterized.



Scheme 2. Synthesis of partially methylated 5-amino-5-deoxy-gluconic acid lactams, 1,5-dideoxy-1,5-imino-glucitols and gluconic acid lactones.

The presence of 2-*O*-alkylated methyl-β-D-glucopyranosides **11-1** and **11-2** was quite unexpected considering the total oxidation of the peracetylated analogs. It is possible that normally the acetoxy group at C2 may either coordinate to CrO_3 and in this way facilitate oxidation of the anomeric center or might be involved in stabilizing a charged species formed at that center. The absence of this group in the 2-*O*-methylated substrates delays or even prevents oxidation.

Three active lactams were isolated: **15-1**, **15-2** and **15-3**. The unsaturation in compound **15-2** probably arose during the oxime formation step by the abstraction of a proton α to the ester group followed by elimination of the acetoxy group at C3. The ketolactam **15-3** was most probably formed through oxidation of a 2-*O*-methyl aldonic ester, which subsequently underwent α-β-elimination. An interesting compound that was isolated is the vicinal dicarbonyl derivative **17-1**. This is a novel glucosidase inhibitor that we found to be more potent than deoxynojirimycin. NMR spectroscopy and mass spectrometry indicated that it existed preferably as the more stable hydrate form. This lactone most likely was formed from 5-keto-gluconic acid methyl ester that was not oximated and in which the carbonyl group was reduced back to a mixture of D-*gluco* and L-*ido* aldonic esters. These subsequently cyclized to produce the derivatized lactones. In the scheme described here, the proportion of lactam and lactone species can be controlled by varying the amount of hydroxylamine used. For instance, the use of half the required amount of hydroxylamine would increase the proportion of lactone species produced.

3. Conclusion

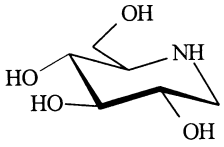
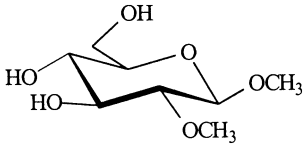
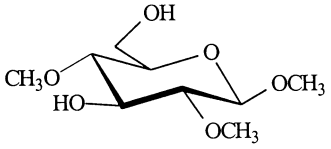
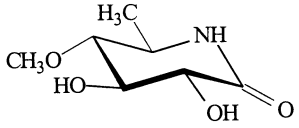
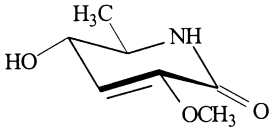
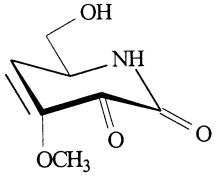
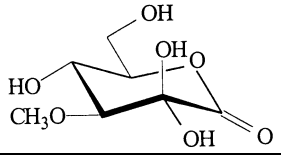
Using a combinatorial library approach towards synthesis and screening, we identified several compounds that exhibited inhibitory activity towards α- and β-glucosidases. In the case of two 2-*O*-methyl glycosides, it was not determined whether they were true inhibitors or just competing substrates. The method, which is primarily a discovery and screening routine, is very convenient and practical. It circumvents the complexity and tremendous effort needed to access carbohydrate candidates with glycosidase inhibitory activity and also provides a wide cross section of candidates. This procedure is general and can be used to prepare, isolate and screen other classes of *O*-alkylated and *O*-arylated carbohydrate derivatives.

4. Experimental

4.1. Synthesis

In this procedure, the products of each reaction were used, after workup, without further purification or separation. In a

Table 1. Structure and inhibitory activity (relative to deoxynojirimycin) of some synthesized glucosidase inhibitors

Compound	Structure	Relative inhibitory index[a]
DNJ		1(α) 1(β)
11-1		1.20(β)
11-2		0.28(β) 0.51(α)
15-1		0.35(β)
15-2		0.31(α) 0.35(β)
15-3		1.84(α)
17-1		1.72(α)

[a] % Inhibition by candidate molecules relative to the one obtained by an equivalent amount of deoxynojirimycin (DNJ). In brackets, the enzyme type is indicated.

200 mL round-bottom flask, equipped with a condenser and drying tube, methyl-β-D-glucopyranoside **1** (10.0 g, 49.2 mmol) was dissolved in methyl iodide (30.0 mL, 492.0 mmol) and methanol (40 mL) and heated under reflux for 6 h. Ag_2O (25.0 g, 107.8 mmol) was added to the solution in 10 equal portions at 0.5 h intervals. After the final addition, heating was continued for 4 h, followed by stirring at room temperature for another 12 h. The suspension was then filtered, extracted with chloroform and dried on the rotary evaporator to give **10**.

Acetylation of the partially methylated methyl-β-D-glucopyranoside was performed by dissolving **10** in a mixture of pyridine (50 mL) and acetic anhydride (40 mL) and stirring the solution for 4 h at room temperature. The reaction mixture was poured over ice and water (500 mL) and then extracted three times with dichloromethane (200 mL). The combined organic layers were dried with Na_2SO_4 and then rotary evaporated, affording **11**.

To a solution of **11** (14.0 g) in acetic acid (280 mL) and acetic anhydride (24 mL), CrO_3 (9.0 g) was added and the suspension was stirred at 50°C for 2 h. The mixture was then poured slowly, with stirring into cold water (1000 mL). The product was extracted three times with chloroform. The combined chloroform layers were decolorized with activated charcoal, filtered and washed with saturated $NaHCO_3$ solution and then with water. After drying with Na_2SO_4 and rotary evaporation of the solvent, the ketone **12** was obtained.

This reaction mixture (4.0 g) was dissolved in pyridine (40 mL) and the solution cooled to 0°C. Hydroxylamine hydrochloride (5.0 g) was then added and the solution stirred at 0°C for 15 min and then for another 2 h at room temperature. The mixture was poured onto ice and water and then extracted three times with chloroform. The combined chloroform layers were subsequently washed with water, dried with Na_2SO_4 and then evaporated to give oxime **13**.

A solution of **13** (12.5 g) in glacial acetic acid (300 mL), containing 10% Pd/C (5 g) was hydrogenated in a Parr reactor under a H_2 pressure of 300–400 psi for 40 h at 55°C. The reaction mixture was filtered through Celite and washed with ethanol. The solvent was rotary-evaporated and the lactam **14** (15.0 g) was obtained.

Deacetylation was performed by dissolving **14** (1.0 g) into methanol (20 mL) and water (3 mL) to which K_2CO_3 (1.0 g) was added and stirred overnight at room temperature. The suspension was evaporated, partially re-dissolved in ethanol and filtered. The filtrate was then dried by rotary evaporation, after which the crude reaction mixture A (compound family **15**) was subjected to HPLC separation.

A solution of the lactam **14** (1.2 g) in THF (10 mL) was reduced with 1 M BH_3/THF (13 mL) by stirring at room temperature for 1.5 h and then refluxing for another 1.5 h. After cooling to room temperature 9% methanolic HCl (10 mL) was carefully added and the resulting solution was refluxed for 30 min. The THF was removed by rotary evaporation and the reaction mixture was dissolved repeatedly in methanol, followed by evaporation to remove borates to produce mixture B (compound family **16**) which was then separated by HPLC.

4.2. Separation of the reaction mixtures

The crude reaction mixtures A and B were separated by reverse-phase high performance liquid chromatography (HPLC) using a Waters 600 multisolvent delivery system with an ODS Ultrasphere 4.6×250 mm column, a Spectroflow 783 programmable absorbance detector and a Waters 740 Data Module. The reaction mixture was dissolved in a 5% solution of acetonitrile in water and 70 μL (20 mg) loaded on the column. Running time was 75 min, with a flow rate of 1.5 mL/min. A linear gradient from 5% acetonitrile in water to 100% acetonitrile was used. The column

effluent was monitored by UV absorbance at 219 nm. 28 fractions were collected from reaction mixture A, and 52 fractions from reaction mixture B. These were subjected to inhibition studies against α- and β-glucosidase.

4.3. Inhibition studies

4.3.1. Materials. The enzyme, buffers and substrates were purchased from Sigma. These include: α-D-glucosidase (maltase, 45 U/mg), phosphate buffer, *p*-nitrophenyl α-D-glucoside, β-D-glucosidase (from Almonds, 30 U/mg), citrate buffer, *p*-nitrophenyl β-D-glucoside, deoxynojirimycin.

The solutions were prepared as follows: (a) α-D-glucosidase: the stock enzyme solution was prepared by dissolving 0.2 mg of solid protein in 1 mL of buffer solution. This enzyme solution was diluted 10-fold for the enzymatic assay. (b) β-D-Glucosidase: The stock enzyme solution was prepared by dissolving 0.4 mg of solid protein in 1 mL of buffer solution. This enzyme solution was diluted 10-fold for the enzymatic assay. (c) Phosphate buffer (0.5 M with 0.244 M K_2HPO_4 and 0.256 M KH_2PO_4, pH 6.5): 24.4 mL of 1 M K_2HPO_4 and 25.6 mL of 1 M KH_2PO_4 were mixed and diluted to 100 mL. (d) Citrate buffer (0.3 M, pH 5) (e) *p*-nitrophenyl α-D-glucoside (0.5 mM) and *p*-nitrophenyl β-D-glucoside (0.05 M) were dissolved in the corresponding buffer solutions. (f) 1 mM solutions of inhibitors were used.

4.3.2. General procedure for enzyme assay. To a 96-well disposable microtiter plate with a maximum volume per well being 300 μL, the assay solutions and the potential inhibitors collected from reaction mixtures A and B were added as follows: 20 μL of buffer, 20 μL inhibitor, 10 μL enzyme and 40 μL of water. The solutions were mixed and then incubated at 37°C for 15 min. 10 μL of substrate were then added and the plate was incubated at 37°C for another 35 min. After this, the molar extinction coefficient was read in a spectrophotometer with a microtiter plate reader set at 420 nm. This procedure has been used for both α- and β-glucosidase assays, with the only difference being basification of the β-glucosidase solutions with Na_2CO_3 prior to readings.

4.4. Structure identification

The ^{1}H NMR spectra of the acetylated active compounds were recorded at 500 MHz on a Varian VXR spectrometer. IR spectra were recorded on a Nicolet 710 FT-IR Spectrometer.

The fast atom bombardment high resolution mass spectra (FABHRMS) were obtained using a JEOL HX 110 double focusing mass spectrometer operating in positive ion mode. For clarity, the letter 'Acet' after each characterized compound number denotes peracetylation.

4.4.1. Methyl 2-*O*-methyl-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (11-1Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 1.99 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.06 (s, 3H, OAc), 3.15 (dd, 1H, $J_{1,2}$=7.57 Hz, $J_{2,3}$=9.28 Hz, H_2), 3.48 (s, 3H, OCH_3), 3.54 (s, 3H, OCH_3), 3.63 (m, 1H, H_5), 4.09 (dd, 1H, $J_{6a,6b}$=12.2 Hz, $J_{6a,5}$=1.95 Hz, H_{6a}), 4.26 (dd, 1H, $J_{6a,6b}$=12.2 Hz, $J_{6b,5}$=4.64 Hz, H_{6b}), 4.98 (t, 1H, $J_{3,2}$=$J_{3,4}$=9.28 Hz, H_3), 5.076 (t, 1H, $J_{3,4}$=$J_{4,5}$=9.28 Hz, H_4); IR (film): 1753 (s), 1230 (s), 1048 (s) cm^{-1}; HRFABMS (M+H$^+$) calcd 335.1264, found 335.1353.

4.4.2. Methyl 3,6-di-*O*-acetyl-2,4-di-*O*-methyl-β-D-glucopyranoside (11-2Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 2.10 (s, 3H, OAc), 2.13 (s, 3H, OAc), 3.05 (dd, 1H, $J_{1,2}$=$J_{2,3}$=9.5 Hz, H_2), 3.26 (t, 1H, $J_{3,4}$=$J_{4,5}$=9.5 Hz, H_4), 3.40 (s, 3H, OCH_3), 3.48 (s, 3H, OCH_3), 3.51 (m, 1H, H_5), 3.54(s, 3H, OCH_3), 4.24 (dd, 1H, $J_{6a,6b}$=11.93 Hz, $J_{6a,5}$=5.08 Hz, H_{6a}), 4.27 (d, 1H, $J_{1,2}$=7.73 Hz, H_1), 4.36 (dd, 1H, $J_{6a,b}$=11.93 Hz, $J_{6b,5}$=2.21 Hz, H_{6b}), 5.09 (t, 1H, $J_{2,3}$=$J_{3,4}$ 9.5 Hz, H_3); IR (film): 1744 (m), 1379 (m), 1216 (m) cm^{-1}; HRFABMS (M+H$^+$) calcd 307.1393, found 307.1407.

4.4.3. 2,3-Di-*O*-acetyl-4-*O*-methyl-5-amino-5,6-dideoxy-D-glucono-1,5-lactam (15-1Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 1.21 (d, 3H, $J_{5,6}$=6.62 Hz, CH_3), 2.12 (s, 3H, OAc), 2.17 (s, 3H, OAc), 3.45 (s, 3H, OCH_3), 3.52 (m, 1H, H_5), 3.72 (t, 1H, $J_{4,5}$=$J_{3,4}$ 8.61 Hz, H_4), 4.96 (t, 1H, $J_{2,3}$=$J_{3,4}$=8.61 Hz, H_3), 5.16 (d, 1H, $J_{2,3}$=8.61 Hz, H_2), 5.45 (br s, 1H, NH); IR (film): 1751 (s), 1684 (m), 1457 (s), 1222 (s), 1041 (m) cm^{-1}; HRFABMS (M+H$^+$) calcd 260.1134, found 260.1136.

4.4.4. 4-*O*-Acetyl-2-*O*-methyl-2,3-dehydro-3,5,6-trideoxy-5-amino-D-glucono-1,5-lactam (15-2Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 1.25 (d, 3H, $J_{5,6}$=6.84 Hz, CH_3), 2.054 (s, 3H, OAc), 3.66 (s, 3H, OCH_3), 3.88 (m, 1H, H_5), 5.19 (m, 1H, H_4), 5.36 (br s, 1H, NH), 5.58 (d, 1H, $J_{3,4}$=6.35 Hz, H3); IR (film): 1735 (s), 1652 (m), 1378 (m), 1259 (m) cm^{-1}; HRFABMS (M+H$^+$) calcd 200.0923, found 200.0923.

4.4.5. 6-*O*-Acetyl-3-*O*-methyl-5-amino-4,5-dideoxy-3,4-dehydro-D-*gluco*-2-ulosonic acid lactam (15-3Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 2.07 (s, 3H, OAc), 3.80 (s, 3H, OCH_3), 4.19 (dd, 1H, $J_{6a,b}$=12.15 Hz, $J_{6a,5}$=5.74 Hz, H_{6a}), 4.30 (dd, 1H, $J_{6a,b}$=12.15 Hz, $J_{6b,5}$=3.53 Hz, H_{6b}), 5.10 (ddd, 1H, $J_{5,4}$=1.99 Hz, $J_{6a,5}$=5.74 Hz, $J_{6b,5}$=3.54 Hz, H_5), 5.98 (d, 1H, $J_{4,5}$=1.99 Hz, H_4); IR (film): 1740 (s), 1636 (m), 1376 (m), 1237 (s), 1046 (m) cm$^{-1.}$

4.4.6. 4,6-Di-*O*-acetyl-3-*O*-methyl-D-*gluco*-2-ulosonic acid lactone hydrate (17-1Acet). ^{1}H NMR (500 MHz, $CDCl_3$); δ 2.10 (s, 3H, OAc), 2.18 (s, 3H, OAc), 3.74 (s, 3H, OCH_3), 4.46 (dd, 1H, $J_{6a,b}$=9.06 Hz, $J_{6a,5}$=6.63 Hz, H_{6a}), 4.50 (dd, 1H, $J_{6a,b}$=9.06 Hz, $J_{6b,5}$=7.95 Hz, H_{6b}), 4.89 (ddd, 1H, $J_{6a,5}$=6.63 Hz, $J_{5,6b}$=7.95 Hz, $J_{4,5}$=5.3 Hz, H_5), 5.20 (d, 1H, $J_{3,4}$=2.87 Hz, H_3), 5.62 (dd, 1H, $J_{3,4}$=2.87 Hz, $J_{4,5}$=5.3 Hz, H_4); IR (film): 1811 (m), 1753 (s), 1374 (m), 1211 (s), 1085 (m) cm^{-1}; HRMS (FAB M$^+$) calcd 291.0954, found 291.0726.

Acknowledgements

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