Pergamon

Tetrahedron: *Asymmetry* 11 (2000) 621–628

TETRAHEDRON:
ASYMMETRY

The preparation of deoxy derivatives of mannose-1-phosphate and their substrate specificity towards recombinant GDP-mannose pyrophosphorylase from *Salmonella enterica*, group B

Gregory M. Watt,[a] Sabine L. Flitsch,[a,*] Sven Fey,[b] Lothar Elling [c] and Udo Kragl [b,†]

[a]*The Edinburgh Centre for Protein Technology, Department of Chemistry, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JJ, UK*

[b]*Forschungzentrum Jülich, Institut für Biotechnologie 2, D-52425 Jülich, Germany*

[c]*Heinrich-Heine-Universität Düsseldorf, Institut für Enzymtechnologie, D-52426 Jülich, Germany*

Received 5 November 1999; accepted 2 December 1999

Abstract

2-Deoxy-α-D-glucose-1-phosphate, 3-deoxy-α-D-*arabino*-hexose-1-phosphate, 4-deoxy-α-D-*lyxo*-hexose-1-phosphate, and α-D-lyxose-1-phosphate were synthesised chemically, and evaluated as substrates for a recombinant GDP-mannose pyrophosphorylase (*Salmonella enterica*, group B, cloned in *Escherichia coli*). The deoxy derivatives were all substrates for the enzyme, with slightly reduced V_{max} values but significantly higher K_m values than those recorded for the native substrate, mannose-1-phosphate. The pyrophosphorylase was used for the synthesis of GDP-mannose analogues GDP-2-deoxy-glucose and GDP-lyxose on a milligram scale.

1. Introduction

Oligosaccharides are ubiquitous in Nature and can display many biological activities, which makes them important synthetic targets.[1] However, chemical synthesis of oligosaccharides is difficult due to multi-step protection/deprotection strategies, and in some cases low stereoselectivity of chemical glycosidation reactions. Glycosyltransferases catalyse the formation of glycosidic linkages in Nature, and have become major tools for the synthesis of complex oligosaccharides.[2] Catalysis by glycosyltransferases is both regio- and stereoselective, allowing relatively straightforward purifications of glycosylated products. We are particularly interested in the application of mannosyltransferases, which we have used for the selective formation of the core β-mannosidic linkage of *N*-glycans.[3,4]

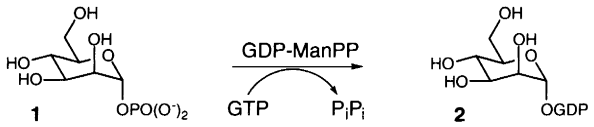
* Corresponding author.

† New address: Universität Rostock, Fachbereich Chemie, Buchbinderstraße 9, D-18055 Rostock, Germany.

0957-4166/00/$ - see front matter
PII: S0957-4166(99)00556-X

The requirement of expensive sugar nucleotides as glycosyl donor substrates for glycosyltransferases is a disadvantage, which has largely been overcome with the development of in situ sugar nucleotide recycling systems.[5,6] This has allowed the large-scale production of carbohydrate-based drugs for the pharmaceutical industry;[7,8] two important examples are the selectin inhibitor sialyl Lewis X and the microbial adhesion inhibitor 3′-sialyllactose.

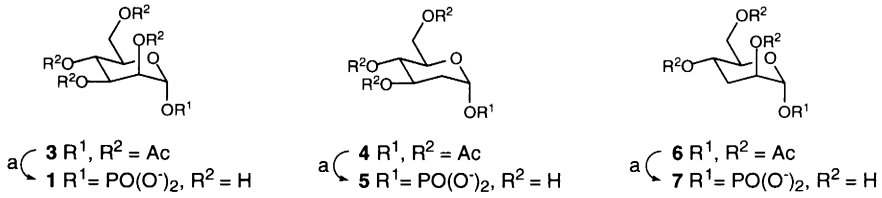
An efficient enzymatic synthesis of GDP-mannose **2** from mannose-1-phosphate **1** and GTP using GDP-mannose pyrophosphorylase from *Salmonella enterica*, group B (Scheme 1) has been developed[9,10] with the aim of producing this sugar nucleotide on a large scale at low cost. Here we report the substrate specificity of this GDP-mannose pyrophosphorylase for chemically synthesised deoxy-derivatives of mannose-1-phosphate. In addition, we show that the enzyme can be used for the preparation of GDP-2-deoxy-α-D-glucose **18** and GDP-α-D-lyxose **19** on a milligram scale.



Scheme 1.

2. Results and discussion

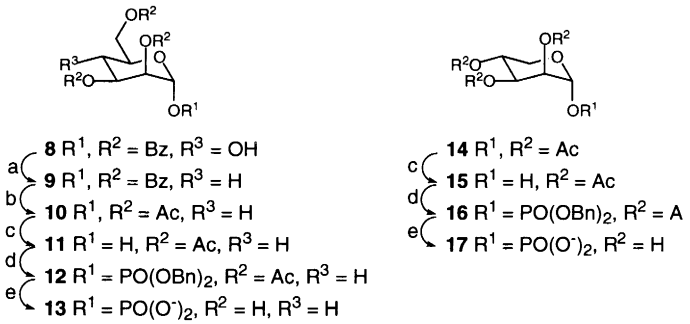
Glycosyl-1-phosphates are biologically important molecules, and much research has been carried out on possible synthetic routes to these compounds. We chose the MacDonald reaction,[11] which involves treatment of peracetylated sugars with phosphoric acid, followed by addition of lithium hydroxide, thus providing a straightforward route to glycosyl-1-phosphates. Mannose-1-phosphate **1** was synthesised from peracetylated mannose **3** on a multi-gram scale using this reaction (Scheme 2). Due the absence of the 2-hydroxyl group, 2-deoxy-α-D-glucose-1-phosphate **5** is more labile to hydrolytic cleavage of the anomeric phosphate, which makes its chemical synthesis difficult.[12–14] However, we found that under carefully controlled reaction conditions both 2-deoxy-glucose-1-phosphate **5**[15] and 3-deoxy-*arabino*-hexose-1-phosphate **7** could be prepared from **4** and peracetylated 3-deoxy-*arabino*-hexose **6**,[16] respectively, using the MacDonald reaction (Scheme 2).[11]



Scheme 2. *Reagents:* (a) (i) H_3PO_4; (ii) LiOH

The preparation of 4-deoxy-*lyxo*-hexose-1-phosphate **13**, shown in Scheme 3, involved selective benzoylation of mannose[17] affording tetra-benzoate **8**, followed by deoxydation of the free 4-position by treatment with thiocarbonyl-dipyridone then tributyltin hydride and AIBN[18] affording perbenzoylated 4-deoxy-*lyxo*-hexose **9** in 54% yield. This was deprotected using sodium methoxide in methanol to give 4-deoxy-*lyxo*-hexose, which was acetylated using acetic anhydride and pyridine to give peracetylated 4-deoxy-*lyxo*-hexose **10** in quantitative yield.

Peracetylated lyxose **14** was subjected to MacDonald reaction conditions[11] but an inseparable 4:1 α:β mixture of lyxose-1-phosphates was obtained. This was possibly due to the high temperatures required



Scheme 3. *Reagents:* (a) (i) *N*,*N*′-thiocarbonyl-dipyridone, toluene; (ii) AIBN, tributyltin hydride; (b) (i) NaOMe, MeOH; (ii) Ac_2O, pyridine; (c) benzylamine, THF; (d) (i) LDA, THF; (ii) tetrabenzyl pyrophosphate; (e) (i) $H_{2(g)}$, Pd/C, EtOH, 10% $NaHCO_3$ solution; (ii) NaOMe, MeOH

for this reaction. Thus, an alternative approach to lyxose-1-phosphate **17**, whereby phosphorylation was carried out at low temperature, was used.

The preparation of 4-deoxy-*lyxo*-hexose-1-phosphate **13** and lyxose-1-phosphate **17** (Scheme 3) started from the peracetylated deoxy sugars **10** and **14**, respectively, which were firstly deprotected at the anomeric centre using benzylamine.[19] The resulting hemiacetals **11** and **15** were each treated with lithium diisopropylamide at low temperature to ensure exclusive formation of the α-anomeric oxides; tetrabenzyl pyrophosphate was added to give the protected sugar phosphates **12** and **16**. Removal of the protecting groups afforded the sugar phosphates **13** and **17** in 54 and 49% yield, respectively.

2.1. Incubation of deoxy analogues with GDP-mannose pyrophosphorylase

Previous studies have focused on deoxy analogues of GDP-mannose **2**, which were tested as substrates for a GDP-mannose:dolichyl phosphate mannosyltransferase in chick embryo microsomes.[20] The results of this study revealed that GDP-mannose **2** has an identical apparent K_m value to the apparent K_i value measured for GDP-6-deoxymannose, indicating that the 6-hydroxyl group of mannose does not participate in enzyme–substrate recognition. The 3-deoxy, 2-deoxy, and 4-deoxy analogues were worse inhibitors by factors of 2, 2.5, and 6, respectively, indicating that the hydroxyl groups at these positions, in particular position 4, may have some influence in enzyme–substrate recognition. The substrate specificity of GDP-mannose **2** has also been tested on a mannosyltransferase from *Salmonella anatum*,[21] whereby the 6-deoxy, 3-deoxy, and 2-deoxy analogues were shown to be transferred at relative velocities of 0.29, 0.12, and 0.11, respectively, to the native compound.

The present study investigates the role hydroxyl groups play in the biosynthesis of GDP-mannose itself. Thus, mannose-1-phosphate **1**, 2-deoxy-glucose-1-phosphate **5**, 3-deoxy-*arabino*-hexose-1-phosphate **7**, 4-deoxy-*lyxo*-hexose-1-phosphate **13**, and lyxose-1-phosphate **17** were studied in detail with fully purified recombinant GDP-mannose pyrophosphorylase. The apparent K_m and V_{max} values are listed in Table 1. The V_{max} values were least affected by the removal of the 3-hydroxyl group and were similarly affected by removal of the 2-, 4-hydroxyl groups and the hydroxymethylene group in the 5 position. The highest reduction in V_{max} was seen for **17** which had a value of about 10% of that for the natural substrate **2**.

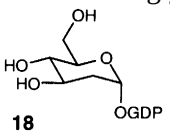
The effect on the K_m values was much more significant, with the 2-deoxy-glucose-1-phosphate **5** being the worst substrate, showing a 300 fold increase in K_m. This would suggest that all four hydroxy groups in mannose-1-phosphate contribute to the binding to the pyrophosphorylase.

It has been shown previously that the present GDP-mannose pyrophosphorylase can be used to catalyse

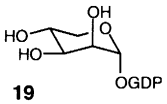
Table 1

Sugar-1-phosphate	Km (mM)	Vmax (U/ml)
Man-1-P **4**	0.014 (±0.002)	4.1. (±0.1)
2-d-Glc-1-P **5**	41.1 (±2.9)	0.6 (±0.04)
3-d-*Arabino*-hex-1-P **7**	15.2 (±2.9)	2.9 (±0.4)
4-d-*Lyxo*-hex-1-P **13**	0.94 (±0.2)	0.9 (±0.1)
Lyx-1-P **17**	13.8 (±2.2)	0.5 (±0.05)

the synthesis of GDP-mannose itself on a large scale.[10] It was, therefore, of interest to see if the enzyme could also be applied to the preparation of deoxy analogues of GDP-mannose, despite their less favourable kinetic constants. We found that even with the poorest substrate in our studies, the 2-deoxy analogue **5**, synthesis on a preparative scale was possible. Thus, GDP-2-deoxy-glucose **18** and GDP-lyxose **19** were synthesised, purified and characterised by NMR spectroscopy. This shows that the GDP-mannose pyrophosphorylase from *S. enterica* can be used for the synthesis of GDP-mannose deoxy derivatives. These are useful probes for monitoring the substrate specificity of mannosyltransferases and will be used by us in further studies of the *N*-glycan biosynthetic pathway in yeast.[22]



18



19

3. Experimental

3.1. General methods

Proton NMR spectra were recorded at 200 MHz on Varian and Bruker spectrometers, at 400 MHz on a Bruker spectrometer, and at 600 MHz on a Varian spectrometer. HPLC[23] was carried out using an Hypersil ODS-5 μm (RP-18) column of dimensions 125×4.8 mm; the eluents were for A: 100 mM KH_2PO_4, 8 mM tetrabutylammonium sulfate (pH 5.3); and for B: MeOH; the gradient employed was for 0 min: 0% B, 2.5 min: 0% B, 16.5 min: 12% B, 17.5 min: 30% B, 29 min: 30% B, 30 min: 0% B, and 35 min 0% B; nucleotide derivatives were detected by UV absorption (253 nm); the injection volume was 20 μl. Enzymes used were: inorganic phosphatase (PPase) from yeast (Boehringer M.) and alkaline phosphatase molecular biology grade 713023 from calf intestine (Boehringer M.). All other chemicals were purchased from Aldrich/Sigma/Fluka or Merck.

*3.2. 2-Deoxy-α-D-glucose-1-phosphate (dilithium salt) **5***

Peracetylated 2-deoxy-α-D-glucose **4** (0.20 g, 0.60 mmol) was dissolved in a 1:10 w/v solution of dry phosphoric acid in THF (5 ml, 6.00 mmol) and the solvent was removed under reduced pressure. The resulting syrup was stirred under reduced pressure (0.01 kPa) at room temperature for 40 min, whereafter the vacuum was removed and a 1 M solution of lithium hydroxide (25 ml) was added. The resulting milky suspension was stirred for 3 h and then filtered through a Whatman® GF/A glass microfibre filter. The filtrate was neutralised with AG® 50W-X2 (H^+ form) ion exchange resin and the aqueous solvent was removed under reduced pressure. The resulting residue was treated with methanol (10 ml) and the resulting suspension was centifuged at 4000 rpm for 5 min, whereafter the supernatant

was removed. After this was repeated three times, the pellet was dried in vacuo to give 2-deoxy-α-D-glucose-1-phosphate **5** (72 mg, 41%) as a white powder; $[\alpha]_D$ +73 (*c* 0.4 in H_2O) [lit.,[15] +72.4 (*c* 0.5 in H_2O)]; NMR (D_2O) (200 MHz) δ_H 1.59 (ddd, 1H, $J_{1,2ax}$ 2 Hz, $J_{2ax,2eq}$ 13 Hz, $J_{2ax,3}$ 9 Hz, H-2ax), 2.12 (ddd, 1H, $J_{1,2eq}$ <1 Hz, $J_{2ax,2eq}$ 13 Hz, $J_{2eq,3}$ 5 Hz, H-2eq), 3.25 (dd, 1H, $J_{3,4}$ 9 Hz, $J_{4,5}$ 9 Hz, H-4), 3.66 (dd, 1H, $J_{5,6a}$ 6 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 3.83 (m, 2H, H-5 and H-6b), 3.95 (ddd, 1H, $J_{2eq,3}$ 5 Hz, $J_{2ax,3}$ 9 Hz, $J_{3,4}$ 9 Hz, H-3), and 5.45 (br-d 1H, $J_{1,P}$ 8 Hz, H-1); ESMS (−) 243.0.

3.3. 3-Deoxy-α-D-arabino-*hexose-1-phosphate (dilithium salt)* ***7***

Peracetylated 3-deoxy-α-D-*arabino*-hexose **6** (0.46 g, 1.37 mmol) was dissolved in anhydrous THF containing 1:10 w/v phosphoric acid (11.5 ml, 11.73 mmol), and the solvent was removed under reduced pressure. The resulting syrup was stirred at room temperature under reduced pressure (0.01 kPa) for 90 min, the temperature was increased to 60°C, and stirring was continued at the same pressure for 50 min. After the reaction had cooled to room temperature, the vacuum was removed and 1 M lithium hydroxide (45 ml) was added. The resulting suspension was stirred for 3 h, filtered through a Whatman® GF/A glass microfibre filter, and the filtrate was neutralised with AG® 50W-X2 (H^+ form) ion exchange resin, then concentrated. The residue was washed with methanol as described for 2-deoxy-glucose-1-phosphate **5**, and dried in vacuo to give 3-deoxy-α-D-*arabino*-hexose-1-phosphate **7** (0.13 g, 38%) as a white powder; $[\alpha]_D$ +53 (*c* 0.4 in H_2O) [lit.,[24] +53.3 (*c* 0.13 in H_2O)]; NMR (D_2O) (200 MHz) δ_H 1.85 (m, 2H, H-3ax, H-3eq), 3.51 (dd, 1H, $J_{5,6a}$ 7 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 3.67 (ddd, 1H, $J_{3eq,4}$ 6 Hz, $J_{3ax,4}$ 10 Hz, $J_{4,5}$ 10 Hz, H-4), 3.63–3.81 (m, 3H, H-2, H-5, and H-6b), and 5.02 (dd, 1H, $J_{1,2}$ <2 Hz, $J_{1,P}$ 9 Hz, H-1); ESMS (−), 243.0.

3.4. 2,3,6-Tri-O-*acetyl-4-deoxy*-D-lyxo-*hexose* ***11***

A solution of the peracetylated compound **10** (860 mg, 2.60 mmol) in THF (8 ml) was treated with benzylamine (0.43 ml, 3.90 mmol) for 20 h. The reaction was diluted with chloroform (100 ml) and washed with cold 0.2 M hydrochloric acid (200 ml), saturated solution of sodium hydrogencarbonate (200 ml) and water (200 ml). The organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. Flash-column chromatography (eluent: 50 to 60% gradient of ethyl acetate in hexane) of the residue afforded the hemiacetal **11** as a syrup (487 mg, 65%); NMR ($CDCl_3$) (200 MHz) δ_H 1.70–1.90 (m, 2H, H-4ax and H-4eq), 2.07, 2.12, and 2.13 (3×s, 9H, 3×$COCH_3$), 4.10–4.40 (m, 3H, H-5, H-6a, and H-6b), and 5.25–5.44 (m, 3H, H-1, H-2, and H-3).

3.5. *Dibenzyl 2,3,6-tri*-O-*acetyl-4-deoxy*-α-D-lyxo-*hexose-1-phosphate* ***12***

A cooled (–60°C) and stirred solution of the hemiacetal **11** (487 mg, 1.68 mmol) in freshly distilled THF (12 ml) was treated with a 2 M solution of lithium diisopropylamide (2.18 mmol) in 1:1:1 heptane:THF:ethylbenzene (1.10 ml) for 45 min at −60°C, whereafter a solution of tetrabenzyl pyrophosphate (1.27 g, 2.35 mmol) in THF (3 ml) was added. Stirring was continued at −60°C for 4 h, after which time the reaction mixture was slowly allowed to attain room temperature. The solvent and diisopropylamine formed in the reaction were removed under reduced pressure. Flash-column chromatography (eluent: 40 to 50% gradient of ethyl acetate in hexane) of the residue afforded the title compound **12** as a white solid (600 mg, 65%); NMR[25] ($CDCl_3$) (200 MHz) δ_H 1.82–1.90 (m, 2H, H-4ax and H-4eq), 2.01, 2.07, and 2.17 (3×s, 9H, 3×$COCH_3$), 4.05–4.18 (m, 3H, H-5, H-6a, and H-6b), 5.11

(m, 1H, H-2), 5.11 and 5.15 (2×s, 4H, 2×CH_2Ph), 5.29 (m, 1H, H-3), 5.71 (dd, 1H, $J_{1,2}$ 2 Hz, $J_{1,P}$ 6 Hz, H-1), and 7.39 (m, 10H, 2×Ph).

3.6. 4-Deoxy-α-D-lyxo-hexose-1-phosphate (disodium salt) **13**

Ethanol (6 ml) and 10% sodium hydrogen carbonate (4 ml) were added to a flask containing the protected deoxy-α-D-*lyxo*-hexose-1-phosphate **12** (193 mg, 0.35 mmol) and 10% palladium on carbon (40 mg). The resulting suspension was stirred vigorously under a slight positive pressure of hydrogen for 15 h, whereafter TLC (eluent: 40% ethyl acetate in hexane) showed that no starting material remained. The mixture was filtered through a Celite pad and washed in turn with 2:1 (20 ml) and 1:1 (20 ml) mixtures of methanol:water. The filtrate and washing were concentrated under reduced pressure and the resulting residue was taken up in a 10:10:1 mixture of methanol:water:triethylamine and left to stand at room temperature for 3 h, whereafter TLC (eluent: 40% 0.1 M ammonium chloride solution in acetonitrile) indicated the formation of a single product. The reaction mixture was concentrated under reduced pressure to give a white solid, which was purified by ion exchange chromatography DOWEX® 50W-X8 (sodium form, Serva Feinbiochemica) to afford 4-deoxy-α-D-lyxo-hexose-1-phosphate **13** (62 mg, 54%) as a white solid; NMR[25] (D_2O) (200 MHz) δ_H 1.40–1.59 (m, 2H, H-4ax and H-4eq), 3.48–4.10 (m, 5H, H-2, H-3, H-5, H-6a and H-6b), and 5.11 (dd, 1H, $J_{1,2}$ 2 Hz, $J_{1,P}$ 9 Hz, H-1); ESMS (−): 243.

3.7. 2,3,4-Tri-O-acetyl-D-lyxose **15**

Peracetylated α-D-lyxose **16** (0.73 g, 2.31 mmol) in THF (6 ml) was treated with benzylamine (0.38 ml, 3.47 mmol) as described for 2,3,6-tri-*O*-acetyl-4-deoxy-D-*lyxo*-hexose **11** to afford the hemiacetal **15** (0.46 g, 72%) as a syrup; $[\alpha]_D$ −12 (c 0.7 in $CHCl_3$); λ_{max}(MeOH)/nm 213 and 265 (ε/dm^3 mol^{-1} cm^{-1} 91 and 33); ν_{max}/cm^{-1} 3450m (OH) and 1750s (CO); NMR ($CHCl_3$) δ_H 2.04, 2.06, and 2.11 (3×s, 9H, 3×$COCH_3$), 3.88 (m, 2H, H-5ax and H-5eq) 5.08 (d, 1H, $J_{1,2}$ 3 Hz, H-1), 5.08 (m, 1H, H-4), 5.17 (dd, 1H, $J_{1,2}$ 3 Hz, $J_{2,3}$ 3 Hz, H-2), and 5.38 (dd, 1H, $J_{2,3}$ 3 Hz, $J_{3,4}$ 8 Hz, H-3); δ_C 20.72, 20.80, and 20.83 (3C, 3×CO*C*H_3), 60.59 (C-5), 67.26 (C-4), 68.27 (C-3), 70.03 (C-2), 92.37 (C-1), 169.86 (*C*OCH_3), and 170.38 (2C, 2×*C*OCH_3); *m/z* (EI) 170 (34), 157 (43), 128 (100), 115 (56), 103 (12), 86 (28), and 73 (16).

3.8. Dibenzyl 2,3,4-tri-O-acetyl-α-D-lyxose-1-phosphate **16**

Hemiacetal **15** (0.30 g, 1.08 mmol) was subjected to analogous conditions used for the preparation of dibenzyl 2,3,6-tri-*O*-acetyl-4-deoxy-α-D-mannose-1-phosphate **12** to afford the title compound **16** (0.30 g, 52%) as a syrup; $[\alpha]_D$ +22 (c 0.3 in $CHCl_3$); λ_{max}(MeOH)/nm 213 and 265 (ε/dm^3 mol^{-1} cm^{-1} 4390 and 523); ν_{max}/cm^{-1} 1750s (CO), 1280s (PO), 740 and 700 (CH (Ar)); NMR ($CHCl_3$) (200 MHz) δ_H 2.01, 2.03, and 2.07 (3×s, 9H, 3×$COCH_3$), 3.67 (dd, 1H, $J_{4,5ax}$ 9 Hz, $J_{5ax,5eq}$ 11 Hz, H-5ax), 3.90 (dd, 1H, $J_{4,5eq}$ 5 Hz, $J_{5ax,5eq}$ 11 Hz, H-5eq), 5.02 and 5.09 (2×s, 4H, 2×OCH_2Ph), 5.13 (m, 1H, H-4), 5.21 (dd, 1H, $J_{1,2}$ 3 Hz, $J_{2,3}$ 3 Hz, H-2), and 5.29 (dd, 1H, $J_{2,3}$ 3 Hz, $J_{3,4}$ 9 Hz, H-3), 7.33 and 7.34 (2×s, 10H, 2×OCH_2*Ph*); *m/z* (EI) 259.0 (65, M−277.1 $OPO(OCH_2Ph)_2$), 186.9 (43), 157.0 (37), 107.0 (13, PhCHOH), and 91.0 (100, $PhCH_2$).

3.9. α-D-*Lyxose-1-phosphate (disodium salt)* ***17***

Dibenzyl 2,3,4-tri-*O*-acetyl-α-D-lyxose-1-phosphate **16** was subjected to analogous hydrogenation conditions used for the preparation of 4-deoxy-α-D-*lyxo*-hexose-1-phosphate **13**. The debenzylated material was taken up in methanol (5 ml), filtered to remove any sodium hydrogen carbonate present, and washed with methanol (7 ml). The combined filtrate and washings were treated with a 0.5 M solution of sodium methoxide in methanol (0.3 ml) at room temperature for 2 h, whereafter the reaction mixture was neutralised with AG® 50W-X2 (H^+ form) ion exchange resin and the solvent removed under reduced pressure. The residue was taken up in a minimum amount of water and methanol was added, whereupon a precipitate formed, which was collected by filtration to give the title compound **17** (76 mg, 49%) as a white solid; $[\alpha]_D$ +11 (c 0.3 in H_2O); NMR (D_2O) (200 MHz) δ_H 3.57–3.90 (m, 5H, H-2, H-3, H-4, H-5ax, and H-5eq) and 5.15 (dd, 1H, $J_{1,2}$ 2 Hz, $J_{1,P}$ 8 Hz, H-1); ESMS (−) 229.1.

3.10. *Kinetic measurements of GDP-mannose pyrophosphorylase with glycosyl-1-phosphates as substrates*

To Eppendorf tubes containing GTP (2 mM), $MgCl_2$ (2 mM), Tris® buffer (50 mM, pH 8), and the appropriate concentration of glycosyl-1-phosphate was added GDP-mannose pyrophosphorylase (100 μl). After 5 min at room temperature the tubes were heated at 100°C for 5 min to stop the reaction by denaturation of the enzyme. The tubes were centrifuged at 14 000 rpm for 5 min and the supernatant subjected to HPLC analysis,[23] whereby the concentation of GDP-sugar was calculated by integration of the peak areas using commercially available GDP-mannose as a standard.

3.11. *Synthesis of GDP-sugars using GDP-mannose pyrophosphorylase*

A solution of DTT (5 mM), BSA (1 mg/ml), $MgCl_2$ (4 mM), GTP (4 mM), and the respective glycosyl phosphate (4 mM) in water (19.5 ml) was adjusted to pH 8.0 using 4 M sodium hydroxide. GDP-Man pyrophosphorylase (250 μl of 1.65 U/ml) and inorganic phosphatase (250 μl of 200 U/ml) were added (final volume 20 ml) and the mixture was passed through a sterilizing filter. The pH of 8.0 was maintained by the addition of 4 M sodium hydroxide using Metrohm pH-Stat. apparatus, and the progress of the reaction was monitored by subjecting aliquotes of the reaction mixture (10 μl) diluted with water (490 μl) to HPLC analysis. After 60 h the reaction mixture was treated with alkaline phosphatase for 24 h, whereafter any protein was removed by filtration through Centriplus-10™ ultrafiltration concentraters (centrifugation at 4000 g for 1 h). The filtrate was concentrated to 2 ml under reduced pressure and purified by semi-preparative HPLC. Fractions containing the GDP-sugar derivative were pooled, neutralised (2 M NaOH), washed three times with 1:1 chloroform:acetonitrile (10 ml), and concentrated under reduced pressure to give the GDP-deoxy sugar (contaminated with phosphate salt).

3.11.1. *GDP-2-deoxy-α-D-glucose* ***18***

NMR (D_2O) (600 MHz) δ_H 1.63 (ddd, 1H, $J_{1'',2ax''}$ 3 Hz, $J_{2ax'',2eq''}$ 13 Hz, $J_{2ax'',3''}$ 9 Hz, H-2ax″), 2.12 (ddd, 1H, $J_{1'',2eq''}$ <1 Hz, $J_{2ax'',2eq''}$ 13 Hz, $J_{2eq'',3''}$ 5 Hz, H-2eq″), 3.34 (dd, 1H, $J_{3'',4''}$ 10 Hz, $J_{4'',5''}$ 10 Hz, H-4″), 3.71 (dd, 1H, $J_{5'',3''}$ 5 Hz, $J_{6a'',6b''}$ 13 Hz, H-6a″), 3.77 (dd, 1H, $J_{5'',6b''}$ 2 Hz, $J_{3'',6b''}$ 13 Hz, H-6b″), 3.79 (m, 1H, H-5), 3.91 (ddd, 1H, $J_{2eq'',3''}$ 5 Hz, $J_{2ax'',3''}$ 9 Hz, $J_{3'',4''}$ 10 Hz, H-3″), 4.16 (m, 2H, H-5a′ and H-5b′), 4.31 (m, H1, H-4′), 4.46 (dd, 1H, $J_{2',3'}$ 5 Hz, $J_{3',4'}$ 4 Hz, H-3′), 4.71 (dd, 1H, $J_{1',2'}$ 6 Hz, $J_{2',3'}$ 5 Hz, H-2′), 5.63 (br-d 1H, $J_{1'',P}$ 7 Hz, H-$_{1''}$), 5.90 (d, 1H, $J_{1',2'}$ 6 Hz, H-1′), and 8.07 (s, 1H, H-8).

*3.11.2. GDP-α-D-lyxose **19***

NMR (D_2O) (400 MHz) δ_H 3.62–3.95 (m, 5H, H-2′′, H-3′′, H-4′′, H-5ax′′, and H-5eq′′), 4.15 (m, 2H, H-5a′ and H-5b′), 4.31 (m, 1H, H-4′), 4.45 (dd, 1H, $J_{2',3'}$ 5 Hz, $J_{3',4'}$ 4 Hz, H-3′), 4.71 (dd, 1H, $J_{1',2'}$ 6 Hz, $J_{2',3'}$ 5 Hz, H-2′), 5.38 (dd, 1H, $J_{1'',2''}$ 2 Hz, $J_{1'',P''}$ 8 Hz, H-1′′), 5.92 (d, 1H, $J_{1',2'}$ 6 Hz, H-1′), and 8.22 (s, 1H, H-8).

Acknowledgements

The authors are grateful to the BBSRC and the British Council who supported the work carried out by G.M.W. in Edinburgh and Jülich, respectively. We also thank Deutschen Akademischen Austauschdienst for giving S.F. the opportunity to study in Edinburgh. Finally, we thank the technical staff at the University of Edinburgh and Forschungzentrum Jülich for their help regarding the analysis of our products.

References

1. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
2. Palcic, M. M. *Methods Enzymol*. **1994**, *230*, 300–360.
3. Watt, G. M.; Revers, L.; Webberley, M. C.; Wilson, I. B. H.; Flitsch, S. L. *Carbohydr. Res*. **1998**, *305*, 533–541.
4. Watt, G. M.; Revers, L.; Webberley, M. C.; Wilson, I. B. H.; Flitsch, S. L. *Angew. Chem., Int. Ed. Engl*. **1997**, *36*, 2354–2356.
5. Ichikawa, Y.; Wang, R.; Wong, C.-H. *Methods Enzymol*. **1994**, *247*, 107–127.
6. Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem*. **1982**, *47*, 5416–5418.
7. McAuliffe, J. C.; Hindsgaul, O. *Chem. Ind*. **1997**, *3*, 170–174.
8. Simon, P. M. *Drug Discovery Today* **1996**, *1*, 522–528.
9. Elling, L.; Ritter, J. E.; Verseck, S. *Glycobiology* **1996**, *6*, 591–597.
10. Fey, S.; Elling, L.; Kragl, U. *Carbohydr. Res*. **1998**, *305*, 475–481.
11. MacDonald, D. L. *J. Org. Chem*. **1962**, *27*, 1107.
12. Percival, M. D.; Withers, S. G. *Can. J. Chem*. **1988**, *66*, 1970–1972.
13. Niggemann, J.; Lindhorst, T. K.; Walfort, M.; Laupichler, L.; Sajus, H.; Thiem, J. *Carbohydr. Res*. **1993**, *246*, 173–183.
14. Shibaev, V. N.; Kusov, Y. Y.; Kucár, S.; Kochetkov, N. K. *Izv. Akad. Nauk. SSSR* **1973**, *4*, 922–926.
15. Kucár, S.; Zámocky, J.; Bauer, S. *Chem. Zvesti* **1974**, *28*, 115–119.
16. Christian, R.; Schreiner, E.; Zbiral, E.; Schulz, G. *Carbohydr. Res*. **1989**, *194*, 49–61.
17. Williams, J. M.; Richardson, A. C. *Tetrahedron* **1967**, *23*, 1369.
18. Kim, S.; Yi, K. Y. *J. Org. Chem*. **1986**, *51*, 2613–2615.
19. Sim, M.; Kondo, H.; Wong, C. *J. Am. Chem. Soc*. **1993**, *115*, 2260–2267.
20. McDowell, W.; Schwartz, R. T. *FEBS Lett*. **1989**, *243*, 413–416.
21. Shibaev, V. N. *Pure Appl. Chem*. **1978**, *50*, 1421–1436.
22. Wilson, I. B. H.; Webberley, M. C.; Revers, L.; Flitsch, S. L. *Biochem. J*. **1995**, *310*, 909–916.
23. Ryll, T.; Wagner, R. *J. Chromatography* **1991**, *570*, 77–88.
24. Kucár, S.; Zámocky, J.; Zemek, J.; Bauer, S. *Chem. Zvesti* **1976**, *32*, 414–419.
25. Niggemann, J.; Thiem, J. *Liebigs Ann. Chem.* **1992**, 535–358.