The Glc₂Man₂-fragment of the *N*-glycan precursor – a novel ligand for the glycan-binding protein *malectin*? \dagger ‡

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The Glc $\alpha(1\rightarrow 3)$ Glc $\alpha(1\rightarrow 3)$ Man $\alpha(1\rightarrow 2)$ Man tetrasaccharide (Glc₂Man₂-fragment), a substructure of the natural *N*-glycan precursor, was synthesized. The interaction of this fragment with the protein *malectin*, a carbohydrate binding protein localized in the endoplasmatic reticulum, was investigated by ¹H¹⁵N HSQC experiments and isothermal calorimetry. The chemical shift perturbations of nuclei in the protein's backbone caused by the binding of the Glc₂Man₂-fragment to *malectin* suggest a binding mode like the known ligand nigerose.

Introduction

The majority of all proteins expressed in living organisms is subjected to posttranslational modifications; among the most widely spread of those modifications is the addition of carbohydrate moieties to the protein's side chains to form glycoproteins.¹ Over 90% of the glycan structures present in eukaryotic proteins are glycosidically linked to the side chains of asparagines (\rightarrow *N*-glycans). These *N*-glycan structures participate in a multitude of functions *e.g.* as ligands in various recognition processes, mediators in pathogen interactions, stabilizers against enzymatic degradation, solubility enhancers, or modifiers of an enzyme's charge, structure or orientation. This plethora of adopted roles is matched by an equally diverse multitude of *N*-glycan structures.²

While the glycan structures present in an organism are highly diverse, the first steps of their biogenesis are highly conserved. *N*-Glycan structures in eukaryotic organisms are derived from a common lipid-bound, tetradecasaccharidic precursor (Glc₃Man₉GlcNAc₂-glycan; **A** in Fig. 1A), that is cotranslationally transferred onto the nascent polypeptide chain while still in the endoplasmic reticulum (ER).

Subsequently the glycan moiety is trimmed to a common core region present in all *N*-glycans. The early stages of this trimming process occur in the ER and the resultant *N*-glycan structures are closely associated with the correct folding process of the newly formed protein.³ Only recently *malectin*, a membranebound ER protein, was identified to exhibit preferential binding to an early stage form of *N*-glycan trimming. By carbohydrate microarray experiments a selective binding of *malectin* to the Glc₂Man₇GlcNAc₂-form (Glc₂-*N*-glycan; **A** without the residues G1, D2 and D3) was detected. Furthermore, the structure of a

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Fig. 1 (A) Lipid-bound Glc₃Man₉GlcNAc₂-*N*-glycan precursor A (dol = dolichol); (B) Glc₂Man₂-fragment, assumed binding region for the carbohydrate binding protein *malectin*; (C) known *malectin* ligand nigerose (Glc α 1 \rightarrow 3Glc).

complex of *malectin* with nigerose (Glc α 1-3Glc; Fig. 1C), was determined by NMR experiments. *Malectin's* location in the ER, its remarkable selectivity for the Glc₂-*N*-glycan and its wide conservation across different species, has led to the assumption that *malectin* is closely involved in the quality assurance of *N*-glycan biogenesis.⁴ However, the exact mode of *malectin's* binding to the Glc₂-*N*-glycan has not yet been determined. Despite the fact that a binding of *malectin* to mannose could not be observed, the interaction with the Glc₂-*N*-glycan appeared to be much stronger than only with nigerose (deduced from the carbohydrate microarray data). This leads to the assumption that a cooperative binding effect to one or several of the adjacent mannose residues of the Glc₂-*N*-glycan takes place.

While the synthesis of fully processed *N*-glycan structures has been of interest for the last 30 years, the synthesis of early stage *N*-glycan trimming products or substructures of these has not received such widespread attention. Some syntheses in this field have been reported, *e.g.* the group of Ito has recently reported on their synthesis of the complete Glc₃Man₉GlcNAc₂-glycan, as well as the trimmed Glc₂Man₉GlcNAc₂-glycan,⁵ and Fairbanks *et al.* have recently accomplished a synthesis of the 1,3-arm's terminal Glc₃Man moiety.⁶ However, to our knowledge, there is no literature precedent for the preparation of small substructures of the trimmed 1,3-arm.

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 $[\]dagger$ Dedicated to Professor Dr Richard R. Schmidt on the occasion of his $75^{\rm th}$ birthday.

 $[\]ddagger$ Electronic supplementary information (ESI) available: ${}^{1}H^{15}N$ HSQC spectra of *malectin* with/without 1, experimental details for compounds 2c, 4c and 6–11, and NMR spectra for compounds 1–3, 4c and 6–12a. See DOI: 10.1039/c004502k

Table 1 Formation of disaccharide $8\alpha/\beta$ from different donors

		BnO BnO 4a : X = 4b : X = Se	$\begin{array}{cccc} & & & & & & & & & & & & & & & & & $		
	Donor ^a	Acceptor	Conditions	Yield ^b	α/β ratio ^c
1	4a	5	TMSOTf, Et ₂ O, RT, 25 min	~65%	2:1–3:1
2 ^d	4b	5	NIS, AgOTf, 4 Å MS, CH ₂ Cl ₂ , Et ₂ O, RT, 30 min	~75%	1.2:1–1.5:1
3 ^{<i>d</i>}	4c	5	NIS, AgOTf, 4 Å MS, CH ₂ Cl ₂ , Et ₂ O, RT, 30 min	70–80%	1 : 1
4	4c	5	CuBr ₂ , "Bu ₄ NBr, 4 Å MS, DCE, DMF, RT, 3–5 d	65–75%	6 : 1–8 : 1

^{*a*} **4a**: trichloroacetimidyl α-glucoside; **4b**: phenyl β-selenoglucoside; **4c**: methyl β-thioglucoside. ^{*b*} Determined from the isolated mass of epimers 8α/β. ^{*c*} Estimated from ¹H NMR spectra. ^{*d*} Substitution of Et₂O by THF did not lead to an increase in diastereoselectivity.

Here, we report on our chemical synthesis of the G2-G3-D1-C motif of the *N*-glycan precursor (Glc₂Man₂-fragment; highlighted region in Fig. 1A or chemical structure depicted in Fig. 1B). Using this defined carbohydrate structure in NMR-based chemical shift perturbation experiments, we hope to gain further information on the Glc₂-*N*-glycan's binding epitope with respect to *malectin* and a possible participation of the adjacent mannose units.

Results and discussion

Synthesis of the Glc₂Man₂-fragment

Inspecting the Glc₂Man₂-fragment **1** (Scheme 1) it is evident that the main challenge in preparing **1** resides in the glucose $\alpha 1 \rightarrow 3$ -linkages between the G2- and G3-residues and the G3- and D1-residues of the *N*-glycan precursor, respectively.



Scheme 1 Retrosynthetic analysis of the *N*-glycan precursor's Glc_2Man_2 -fragment 1, which can be traced to the monosaccharide building blocks 4–7 (X = OC(NH)CCl₃ (4a), SePh (4b), SMe (4c)).

The formation of these 1,2-*cis*-configured glycosides cannot be aided by classical neighbouring group assistance.⁷ However, the α -1 \rightarrow 2-linkage between the mannose residues D1 and C is readily accessible by making use of the participation of an *O*-acyl residue located at the C2-position of the D1-building block during the glycosylation. Thus, our approach to the tetrasaccharide **1** was based on a block glycosylation strategy relying on building blocks carrying a general *O*-benzyl based protecting group scheme (Scheme 1). This leads to nigerose building block **2**⁸ and dimannose **3** as key intermediates in the synthesis. Retrosynthetically these disaccharides can be traced to the corresponding monosaccharide derivatives **4**–**7**, which are accessible from D-glucose and D-mannose, respectively. The preparation of the building blocks **4**–**7** closely followed known procedures and is detailed in the ESI.‡

In our initial attempts to efficiently prepare the diglucose donor 2, we investigated a variety of glucose donors (4a–c; *cf.* Table 1) in the coupling of 4 and 5 with regard to the yields and the ratio of α/β -epimers obtained. The trichloroacetimidate donor 4a⁹ and the seleno glycoside 4b^{6,10} afforded the desired disaccharide 8 in useful yields of 65–75% upon rapid activation of the glycoside donor. However, the selectivity with regard to the α/β -configuration of the newly formed glycosidic bond was moderate (at best α/β ratios of 3:1 were obtained); the use of different coordinating solvents¹¹ (*e.g.* diethylether or THF) did not increase the diastereoselectivity of the reaction (Table 1 entries 1 and 2).

When thioglycoside **4c** was used under reaction conditions as employed for glycosylations with **4a** or **4b**, that is a rapid activation of the donor and reliance on the solvent effect to ensure a stereoselective reaction, disaccharide **8a/β** was obtained in good yields with no diastereoselectivity observed (Table 1 entry 3). Based on literature precedent⁸ we switched the mode of activation to a metal bromide mediated variant¹² of Lemieux's *in situ* anomerisation methodology,¹³ thus we were able to obtain the desired disaccharide **8a/β** in yields of 65–75% and with diastereoselectivities of $\alpha/\beta > 7:1$ (Table 1 entry 4). To our delight, the epimers could easily be separated at this stage by conventional chromatography on silica gel, allowing an efficient access of up to several tens of grams of **8a**.

The conversion of disaccharide 8α to the desired thioglycosyl donor **2** has previously been described by Takeo *et al.*,⁸ however, that route requires the handling of toxic tin species to introduce the methyl thioglycoside. Thus we decided to investigate an alternative approach to accomplish the conversion of the *O*-acetyl group at the reducing end into an *S*-methyl group. Our approach (Scheme 2) requires the installation of a uniform *O*-acetyl protecting group



Scheme 2 Preparation of the G2-G3-glycosyl donor 2 from D-Glucose precursors 4c and 5; (a) CuBr₂, "Bu₄NBr, 4 Å MS, DCE, DMF, RT, 5 d, 68%; (b) i. H₂, Pd(OH)₂/C, MeOH, RT, 12 h; ii. Ac₂O, pyridine, RT, over night, 88% over 2 steps; (c) i. HBr, HOAc, 0 °C \rightarrow RT, 120 min; ii. thiourea, acetone, reflux, 60 min; iii. Na₂SO₃, K₂CO₃, CHCl₃–H₂O, RT, 60 min; iv. Et'Pr₂N, MeI, CH₂Cl₂, RT, 60 min, 90% over 4 steps; (d) i. NaOMe (~0.02 M), MeOH, RT, 4 h; ii. BnBr, NaH, DMF, RT, over night, 71% over two steps.

pattern as a first step. This was achieved by catalytic cleavage of the O-benzyl groups by hydrogenolysis in the presence of Pearlman's catalyst¹⁴ and subsequent treatment with acetic anhydride in pyridine to afford the fully O-acetylated **9** in 88% yield in two synthetic steps.

An easily scalable route was chosen to form the S-methyl thioglycoside **10** in a 4-step sequence¹⁵ from **9** in 90% yield (the purification of intermediates was not required). This approach compares favourably with Takeo's tin-based methodology, for which yields of ~85% were reported. At this stage the final O-benzyl protecting groups in **2** are installed in 71% yield by removing the ester groups in fully O-acetylated **10** under Zemplén conditions,¹⁶ followed by exhaustive O-benzylation.

The synthesis of the glycosyl acceptor, dimannose **3** (Scheme 3), was achieved by glycosylating the D-mannose acceptor **7** carrying a free 2-OH group with the trichloroacetimidate donor **6**, which carried a 2-*O*-acetyl group to ensure the formation of the desired 1,2-*trans*-configured glycosidic bond.



Scheme 3 Preparation of the D1-C-glycosyl acceptor 3 from the mannose building blocks 6 and 7; (a) TMSOTf, CH_2Cl_2 , 0 °C \rightarrow RT, 30 min, 90%; (b) (HF)_x.pyr, pyridine, RT, overnight, 79%.

This glycosylation was effected by the action of trimethylsilyl triflate as a promoter in dichloromethane to afford the disaccharide **11** in excellent yield (90%). Subsequent cleavage of the 3-*O-tert*-butyldimethylsilyl protecting group from the terminal mannose moiety with hydrogen fluoride-pyridine complex in pyridine allowed an efficient access to the 3-OH free glycosyl acceptor **3** in 79% yield.

The glycosylation of the G2-G3 moiety **2** with the D1-C moiety **3** to afford the fully protected tetrasaccharide **12** was anticipated to proceed under conditions similar to those reported for the glycosylation of **4c** and **5** (*vide supra*). While the formation of a

mixture of diastereoisomeric tetrasaccharides $12\alpha/\beta$ was observed under these reaction conditions in moderate yields of <50% (ratio of the diastereoisomers was not determined), we were not able to isolate the desired tetrasaccharide 12α as a uniform compound. Instead we observed that the glycosyl acceptor **3** is consumed slower than in the corresponding glycosylation of the monosaccharide **5** with the *S*-methyl thioglycoside **4c** to afford **8a**/ β (Scheme 2). After seven days some amounts of the acceptor **3** still remained, while the donor **2** had partially decomposed.

To overcome this mismatch of reactivities, we reconsidered the activation of the S-methyl thioglycoside **2** with methyl triflate.¹⁷ These reactions were carried out in the presence of diethylether and 1,2-dimethoxyethane as coordinating solvents¹¹ to effect preferential formation of the 1,2-*cis* glycosidic linkage. This more S_N1-like glycosylation reaction proceeded decidedly faster and afforded a mixture of the diastereoisomers **12a/β** (*e.g.* 61% yield, α/β ratio 2.5:1; Scheme 4). Fortunately a separation of the obtained mixture of isomers was possible by standard column chromatography on silica gel, directly affording **12a** in amounts of several hundred milligrams.



Scheme 4 Glycosylation towards the fully protected G2-G3-D1-C moiety and liberation of title tetrasaccharide 1; (a) MeOTf, 4 Å MS, Et₂O/1,2-dimethoxyethane, 0 °C \rightarrow RT, 3.5 h, 61% ($\alpha/\beta = 2.5:1$); (b) i. Na, NH₃/THF, -78 °C, 120 min; ii. NH₄Cl, NH₃ -78 °C \rightarrow RT; iii. gel filtration, >90% from 12*a*.

Subjecting fully protected 12a to Birch-like conditions¹⁸ removed the *O*-benzyl groups as well as the *O*-benzylidene group. Under these reaction conditions the concomitant cleavage of the 3-*O*-acetyl group on the D1-mannose moiety allowed complete deprotection in one step to afford the fully deprotected tetrasaccharide. After purification by desalting and gel filtration of the title compound 1, the Glc₂Man₂-fragment, was afforded in over 90% yield as a mixture of the anomers at the reducing end.

Interaction studies

The chemical shifts of NMR-active nuclei are sensitive to alterations of their electronic and/or conformational environments as *e.g.* induced by binding of a ligand molecule. Consequently, we investigated the interaction between *malectin* and the

tetrasaccharide 1 by chemical shift perturbation in ${}^{1}H^{15}N$ HSQC spectra. The pattern of the corresponding chemical shift changes per residue is comparable to the one obtained for the known ligand nigerose⁴ (Fig. 2). A superposition of the HSQC spectra of the free and Glc₂Man₂-bound form of *malectin* is depicted in the ESI.‡



Fig. 2 Comparison of the chemical shifts changes $\Delta\delta$ induced by the addition of (A) Glc₂Man₂-fragment 1 and (B) nigerose; abscissa labelled with amino acid numbers in the *malectin* sequence.

It was expected that the additional mannose residues present at the reducing end of the Glc₂Man₂-fragment would mainly affect the region close to the nigerose binding site. According to the determined *malectin*-nigerose complex structure (PBD accession number 2K46)¹⁹ the region following Tyr89 or Tyr185 was most strongly influenced by the binding of nigerose. Consequently, we explicitly inspected the chemical shift changes in these areas (Fig. 2A). However, significant chemical shift changes additional to those already observed for nigerose (Fig. 2B) could not be detected. These observations lead us to the assumption, that the Glc₂Man₂-fragment is bound by *malectin* and it is bound by the same region of *malectin* as nigerose is.

However, earlier results⁴ had shown that at least in the case of the *malectin*-nigerose interaction, large chemical shift changes cannot necessarily be interpreted as a direct interaction with the bound ligand. For example, Phe117 had a very low chemical shift change despite measurable nOE contacts to the glucose residue at the reducing end of the nigerose, whereas Glu114 displayed the highest observed shift change without any direct contact to the bound carbohydrate as evidenced by nOE contacts. The lack of additional chemical shift changes, thus offered no additional information with regard to *malectin's* binding epitope for the Glc₂Man₂-fragment **1**.

To substantiate the results obtained from NMR experiments the binding affinity of *malectin* to tetrasaccharide **1** was determined by isothermal calorimetry. The determined dissociation constant $(K_{\rm D} = 12.3 \,\mu\text{M})$ is comparable to the value previously determined for nigerose $(K_{\rm D} = 26.3 \,\mu\text{M})$.⁴ With observed binding affinities that lie in the same order of magnitude the existence of significant, additional interactions between the D1- and C-residues of the Glc₂Man₂-fragment and *malectin* reliably recognizes only the nigerose motif in the Glc₂Man₂-fragment and that the stronger interaction observed with the Glc₂-*N*-glycan, may be mediated by residues on the 1,6-arms of the Glc₂-*N*-glycan.

Conclusion

In conclusion, we have established a concise, preparative access to the Glc_2Man_2 -fragment 1 of the *N*-glycan precursor. Hallmark features of our synthesis are the successful construction of $Glc\alpha(1\rightarrow 3)$ -linkages between the rings G2-G3 and G3-D1, and the global deprotection of fourteen hydroxyl groups in one single step to afford title compound 1 in high yield and purity.

At the current stage, without further knowledge of the structure of the N-glycan-malectin-complex, we find no evidence for interactions of the additional two D-mannose residues in 1 with the protein malectin.

Experimental section

General methods

Solvents were purified by distillation and dried by standard procedures. Solvents for flash chromatography were purchased in technical quality and redistilled. Organic solvents for HPLC (MeCN, MeOH) were purchased from Fischer Scientific. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 F₂₅₄ plates (0.2 mm) and E. Merck RP-18 F₂₅₄ plates (0.2 mm). The plates were visualized by immersion in mostain $(200 \text{ mL } 10\% \text{ H}_2\text{SO}_4, 10 \text{ g}(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot4\text{H}_2\text{O}, 200 \text{ mg Ce}(\text{SO}_4)_2),$ 10% H₂SO₄ or KMnO₄ solution (1% in water, 1% NaHCO₃) followed by heating (165 °C). Preparative flash chromatography was carried out on Macherey-Nagel Silica Gel 60 (43-60 µm) at a positive pressure of 0.2–0.4 bar. ¹H NMR and ¹³C NMR spectra were recorded on Bruker WM 250 Cryospec, AM 400, DRX 500 or Avance 600 instruments, respectively. Proton chemical shifts are reported in ppm relative to signal from residual solvent protons or Me₄Si as internal standard. Assignments of protons and carbons were carried out with the aid of 400 or 600 MHz spectra: COSY, HMQC, nOESY, ROESY, TOCSY. Multiplicities are reported as they appear in the spectra. They are designated as: s = single, d = doublet, t = triplet, q = quartet, qu = quintet, dd = doubletof doublets, m = multiplet, bs = broad single. Diastereotopic geminal protons are designated with subscripts a and b; nondistinguishable diastereotopic protons are designated n.d. Carbohydrate residues in disaccharides and larger are differentiated with superscripts (e.g. ', ", ...); monosaccharides or the carbohydrate residue at the reducing end carry no superscripts. Measurements of optical rotations were performed on a Perkin-Elmer polarimeter 241 MC (1 dm cell). MALDI-MS were obtained on a Bruker Biflex MALDI-TOF instrument with 2,5-dihydroxybenzoic acid (DHB), 2',4',6'-trihydroxyacetophenone monohydrate (THAP) or α-cyano-4-hydroxycinnamic acid (CHCA) as matrix (positive mode).

NMR experiments

¹⁵N-labelled His₆-malectin was prepared as previously described.⁴ NMR spectra were acquired at 22 °C in 20 mM potassium phosphate buffer pH 6.8, 150 mM KCl and 2 mM DTT. ¹H¹⁵N HSQC spectra of 250 μM His₆-malectin in the absence or presence of 1 mM Glc₂Man₂-fragment 1 were acquired on a Bruker Avance II 600 spectrometer equipped with a broadband triple resonance probe, with 8 scans per increment and a total of 128 increments in the indirect dimension. Details on the experiments used for chemical shift assignment of the protein have been previously published.⁴ Data were processed with NMRPIPE²⁰ and analyzed using NMRVIEW.²¹ The difference in chemical shift $\Delta\delta$ was calculated with the formula $\Delta\delta = \sqrt{((\Delta\delta_{\rm HN})^2 + (\Delta\delta_{\rm N}/10)^2)}$.

Isothermal microcalorimetry

ITC measurements were carried out using a VP-ITC Microcalorimeter (Microcal, Northhampton, MA, USA) in 20 mM phosphate buffer pH 6.8, 150 mM KCl and 1 mM TCEP. A typical titration consisted of sequential injections (10 μ l each) of a Glc₂Man₂-solution (1.5 mM) into a *malectin* sample (50 μ M), at time intervals of five minutes, to ensure that the titration peak returned to the baseline.

α -D-Glucopyranosyl- $(1 \rightarrow 3)$ - α -D-glucopyranosyl- $(1 \rightarrow 3)$ - α -D-mannopyranosyl- $(1 \rightarrow 2)$ -D-mannopyranose (1)

Into a two-neck Schlenk flask equipped with a glass stirbar and a Dewar-type condenser ammonia (~20 mL) was condensed at -78 °C. Sodium sand (50 mg, 2.18 mmol, 130 eq.) was added, whereupon the solution turned a deep blue colour. It was stirred for 10 min at -78 °C until all sodium was dissolved. Then a solution of tetrasaccharide (30 mg, 17 µmol, 1 eq.) in anhydrous THF (2.00 mL) was added dropwise via a syringe and the reaction was stirred for 2 h at -78 °C. Solid NH₄Cl (200 mg) was added and the solution was stirred until the blue colour disappeared. The cooling bath was removed so that the ammonia could volatilize. The resulting white solid was dried under high vacuum for 1 h and then purified by gel filtration on a Bio-Rad P-2 gel filtration column $(\sim 15 \times 1.5 \text{ cm}, \text{H}_2\text{O}: \text{MeOH } 20\%)$. The remaining salts were removed by precipitating them with MeOH. After lyophilization the unprotected tetrasaccharide 1 (11 mg, 16.5 µmol, 98%) was obtained as a mixture of the anomers at the reducing end; in aqueous solution the α -configured compound predominates (TLC: $R_{\rm f} = 0.03$ (silica gel, EtOAc : MeOH 20%); $[\alpha]_{\rm D}^{20} = +60.9$ (c = 1.2, H₂O); $\delta_{\rm H}$ (600 MHz; D₂O) 5.43 (d, ${}^{3}J_{1,2}$ = 1.9 Hz, 1H, H-1), 5.41 (d, ${}^{3}J_{1,2} = 3.9$ Hz, 1H, H-1""), 5.33 (d, ${}^{3}J_{1,2} = 3.9$ Hz, 1H, H-1"), 5.09 (d, ${}^{3}J_{1,2} = 2.0$ Hz, 1H, H-1'), 4.30 (dd, ${}^{3}J_{2,3} = 3.4$ Hz, ${}^{3}J_{1,2} = 1.9$ Hz, 1H, H-2'), 4.06 (ddd, ${}^{3}J_{4,5} = 10.0$ Hz, ${}^{3}J_{5,6a} = 4.2$ Hz, ³*J*_{5.6b} = 2.0 Hz, 1H, H-5'''), 4.01 (1H, H-2), 4.01 (1H, H-3'), 4.00 (n.d., 2H, H-6_{a,b}), 3.96 (1H, H-3''), 3.95-3.83 (n.d., 2H, H-6'_{a,b}), 3.91 (1H, H-5"), 3.89 (1H, H-3), 3.88 (1H, H-4'), 3.88-3.77 (n.d., 2H, H-6"_{ab}), 3.86 (1H, H-4), 3.84 (n.d., 2H, H-6""_{ab}), 3.82 (1H, H-5'), 3.80 (1H, H-3'"), 3.71 (1H, H-2"), 3.75 (1H, H-5), 3.68 (1H, H-4"), 3.62 (dd, ${}^{3}J_{2,3} = 10.0$ Hz, ${}^{3}J_{1,2} = 4.0$ Hz, 1H, H-2""), 3.50 (t, ${}^{3}J_{3,4} \approx {}^{3}J_{4,5} = 9.7$ Hz, 1H, H-4'''); $\delta_{\rm C}(101$ MHz; D₂O) 102.5 (C1'), 101.0 (C1"), 99.7 (C1""), 93.0 (C1), 80.2, 79.6, 78.8, 73.7, 73.4, 73.0, 72.6, 72.2, 72.2, 70.9, 70.7, 70.5, 70.3, 69.9, 67.6, 66.6, 61.5, 61.4, 61.0, 60.9 ppm; non-decoupled ¹H¹³C HMQC (151 MHz; CDCl₃): $J_{Cl,H-1} = 172$ Hz, $J_{Cl',H-1'} = 172$ Hz, $J_{Cl',H-1'} = 173$ Hz, $J_{C1'',H-1''} = 172$ Hz; MALDI-MS (pos. mode, THAP): [M+Na]⁺ calcd.: 689.2, found: 689.3; [M+K]⁺ calcd.: 705.2, found: 705.2; $C_{24}H_{42}O_{21}$ (666.6)).

Benzyl (2-*O*-acetyl-4,6-*O*-benzylidene- α -D-mannopyranosyl)-(1 \rightarrow 2)-3,4,6-tri-*O*-benzyl- α -D-mannopyranoside (3)

In a Teflon flask disaccharide **11** (1.03 g, 1.09 mmol, 1 eq.) was dissolved in dry pyridine (12 mL). $(HF)_x$ ·pyridine (65–70%)

in pyridine, 1.09 mL, 8.16 mmol) was added and the mixture was stirred overnight. Saturated aqueous NaHCO3 and Et2O were added, the layers were separated and the aqueous layer was extracted with Et₂O (2×30 mL). The combined organic layers were dried over MgSO4, filtered, concentrated in vacuo and purified by column chromatography (silica gel, Hx: EtOAc 33%). The disaccharide 3 (720 mg, 0.87 mmol, 79%) was obtained as colourless oil (TLC: $R_f = 0.49$ (silica gel, Hx: EtOAc 50%); $[\alpha]_{D}^{20} = +58.8 \ (c = 1.0, \text{CHCl}_3); \ \delta_{H}(600 \text{ MHz}; \text{CDCl}_3) \ 7.47-7.44$ (m, 2H, ar), 7.38-7.21 (m, 21H, ar), 7.16-7.13 (m, 2H, ar), 5.54 (s, 1H, benzylidene), 5.42 (dd, ${}^{3}J_{2'3'} = 3.5$ Hz, ${}^{3}J_{1'2'} = 1.3$ Hz, 1H, H-2'), 5.01 (d, ${}^{3}J_{1',2'} = 1.3$ Hz, 1H, H-1'), 4.92 (d, ${}^{3}J_{1,2} = 1.5$ Hz, 1H, H-1), 4.82 (d, ${}^{2}J = 10.7$ Hz, 1H, CH₂Ph), 4.71 (d, ${}^{2}J = 11.8$ Hz, 1H, CH_2Ph), 4.69 (d, ${}^{2}J = 11.7$ Hz, 1H, CH_2Ph), 4.68 (d, ${}^{2}J = 12.1$ Hz, 1H, CH₂Ph), 4.63 (d, ${}^{2}J = 11.7$ Hz, 1H, CH₂Ph), 4.55 (d, ${}^{2}J =$ 12.1 Hz, 1H, CH_2Ph), 4.51 (d, $^2J = 10.7$ Hz, 1H, CH_2Ph), 4.47 (d, ${}^{2}J = 11.8$ Hz, 1H, CH₂Ph), 4.25 (dt, ${}^{3}J_{3',4'} = 9.5$ Hz, ${}^{3}J_{2',3'} \approx$ ${}^{3}J_{3',\text{OH}} = 3.1 \text{ Hz}, 1\text{H}, \text{H-3'}), 4.07 \text{ (dd, } {}^{2}J = 10.3 \text{ Hz}, {}^{3}J_{6a',5'} = 4.7 \text{ Hz},$ 1H, H-6a'), 3.98 (t, ${}^{3}J_{1,2} \approx {}^{3}J_{2,3} \approx 2$ Hz, 1H, H-2), 3.95 (dd, ${}^{3}J_{2,3} =$ 2.8 Hz, ${}^{3}J_{3,4} = 9.2$ Hz, 1H, H-3), 3.93 (t, ${}^{3}J_{3,4} \approx {}^{3}J_{4,5} = 9.2$ Hz Hz, 1H, H-4), 3.90 (dd, ${}^{3}J_{5',4'} = 9.5$ Hz, ${}^{3}J_{5',6a,b'} = 4.6$ Hz, 1H, H-5'), 3.85 $(t, {}^{3}J_{3',4'} \approx {}^{3}J_{4',5'} = 9.5 \text{ Hz}, 1\text{H}, \text{H-4'}), 3.81 \text{ (ddd}, {}^{3}J_{4,5} = 9.3 \text{ Hz}, {}^{3}J_{5,6a} =$ 4.6 Hz, ${}^{3}J_{5,6b} = 1,3$ Hz, 1H, H-5), 3.78 (dd, ${}^{2}J = 10.7$ Hz, ${}^{3}J_{5,6a} =$ 4.7 Hz, 1H, H-6a), $3.72 (d, {}^{2}J = 10.3 \text{ Hz}, 1\text{H}, \text{H-6b'}), 3.69 (dd, {}^{2}J =$ $10.7 \text{ Hz}, {}^{3}J_{5.6b} = 1.3 \text{ Hz}, 1\text{H}, \text{H-6b}, 2.23 \text{ (bs, 1H, OH)}, 2.12 \text{ (s, 3H, }$ $CH_{3}CO$ ppm; $\delta_{C}(151 \text{ MHz}; CDCl_{3})$ 170.2, 138.4, 138.2, 137.1, 137.0, 129.2, 128.4, 128.4, 128.3, 128.3, 128.3, 128.0, 127.9, 127.9, 127.7, 127.6, 127.5, 127.5, 127.5, 126.3, 102.2 (benzylidene), 100.0 (C1'), 97.9 (C1), 79.7, 78.8, 75.2, 75.1, 74.6, 73.3, 72.3, 72.1, 71.8, 69.0, 68.9, 68.5, 67.2, 63.7, 21.0; non-decoupled ¹H¹³C HMQC (151 MHz, CDCl₃): $J_{C,Hbenzylidene} = 161.5$ Hz, $J_{C1,H-1} = 170.1$ Hz, $J_{CI',H-I'} = 173.7$ Hz; MALDI-MS (pos. mode, DHB): [M+Na]⁺ calcd.: 855.3, found: 855.1; [M+K]⁺ calcd.: 871.3, found: 871.0; $C_{49}H_{52}O_{12}$ (832.9)).

Benzyl (2,3,4,6-tetra-*O*-benzyl- α -D-glucopyranosyl)-(1 \rightarrow 3)-(2,4,6-tri-*O*-benzyl- α -D-glucopyranosyl)-(1 \rightarrow 3)-(2-*O*-acetyl-4,6-*O*-benzylidene- α -D-mannopyranosyl)-(1 \rightarrow 2)-3,4,6-tri-*O*-benzyl- α -D-mannopyranoside (12 α)

Donor 2 (668 mg, 0.67 mmol, 1.15 eq.) and acceptor 3 (482 mg, 0.58 mmol, 1 eq.) were dried together under high vacuum. After dissolving under argon atmosphere in anhydrous diethyl ether (20 mL) and anhydrous 1,2-dimethoxyethane (1.33 mL) 4 Å molecular sieve (3 g) was added and the mixture was stirred for 30 min. The solution was cooled to 0 °C and methyl triflate (459 µL, 4.05 mmol, 7 eq.) was added. The ice bath was removed and the mixture was stirred for 3.5 h at room temperature. Et₃N (1 mL) was added and the solution was diluted with diethyl ether. After stirring for a few minutes the mixture was filtered over a pad of Celite. The solids were washed successively with ether and the combined filtrates were concentrated in vacuo. The crude product was purified by column chromatography on (silica gel, Hx: EtOAc 16%). Three fractions were collected: pure β -epimer (80 mg, 47 μmol), followed by a mixture of the epimers (120 mg, 67 μmol, $\alpha/\beta \sim 1:7$) and finally the pure α -epimer (430 mg, 240 μ mol) as colourless oils. In total the glycosylation reaction afforded $12\alpha/\beta$ (630 mg, 352 μ mol, 61% $\alpha/\beta \sim 2.5$:1). (TLC: 12 α : $R_{\rm f} = 0.53$; **12β**: $R_{\rm f} = 0.56$ (silica gel, Hx : EtOAc 33%); **12α**: ([α]_D²⁰ = + 49.1

 $(c = 1.1, \text{CHCl}_3); \delta_{\text{H}}(600 \text{ MHz}; \text{CDCl}_3) 7.46-7.02 \text{ (m, 60H, ar)},$ 5.58 (d, ${}^{3}J_{1'',2''} = 3.3$ Hz, 1H, H-1'''), 5.55 (d, ${}^{3}J_{2',3'} = 3.4$ Hz, ${}^{3}J_{1',2'} = 1.1$ Hz, 1H, H-2'), 5.51 (dd, ${}^{3}J_{1',2''} = 3.4$ Hz, 1H, H-1''), 5.30 (s, 1H, benzylidene), 5.13 (d, ${}^{3}J_{1',2'} = 1.1$ Hz, 1H, H-1'), 5.02 $(d, {}^{3}J_{1,2} = 1.7 \text{ Hz}, 1\text{H}, \text{H-1}), 4.96-4.90 \text{ (m, 3H, } CH_2\text{Ph}), 4.85 \text{ (d,}$ $^{2}J = 10.4$ Hz, 1H, CH₂Ph), 4.84 (d, $^{2}J = 10.6$ Hz, 1H CH₂Ph), 4.79 (d, ${}^{2}J = 12.0$ Hz, 1H, CH₂Ph), 4.79 (d, ${}^{2}J = 11.2$ Hz, 1H, CH_2Ph), 4.74-4.70 (m, 3H, CH_2Ph), 4.67 (d, $^2J = 12.5$ Hz, 1H, CH_2Ph), 4.62 (m, 2H, CH_2Ph), 4.55 (d, $^2J = 11.6$ Hz, 1H, CH_2Ph), 4.55 (d, ${}^{2}J = 10.6$ Hz, 1H, CH₂Ph), 4.54 (d, ${}^{2}J = 12.0$ Hz, 1H, CH_2Ph), 4.53 (d, ${}^{2}J = 9.3$ Hz, 1H, CH_2Ph), 4.51-4.45 (m, 2H, CH_2Ph , H-3'), 4.45-4.35 (m, 3H, CH_2Ph , H-5""), 4.30 (d, $^2J =$ 12.2 Hz, 1H, CH_2Ph), 4.25 (d, $^2J = 11.6$ Hz, 1H, CH_2Ph), 4.24 (d, ${}^{2}J = 11.2$ Hz, 1H, CH₂Ph), 4.19 (t, ${}^{3}J_{2^{\prime\prime},3^{\prime\prime}} \approx {}^{3}J_{3^{\prime\prime},4^{\prime\prime}} = 9.3$ Hz, 1H, H-3"), 4.15 (t, ${}^{3}J_{3',4'} \approx {}^{3}J_{4',5'} = 9.9$ Hz, 1H, H-4'), 4.12 (m, 2H, H-6_{a,b}), 4.10 (d, ${}^{3}J_{1,2} = 1.9$ Hz, 1H, H-2), 4.03 (m, 1H, H-3), 4.02 (m, 1H, H-3'"), 4.00 (m, 1H, H-5'), 3.93-3.89 (m, 2H, H-4, H-5"), 3.87 (dd, ${}^{3}J_{4^{\prime\prime},5^{\prime\prime}} = 10.1$ Hz, ${}^{3}J_{3^{\prime\prime},4^{\prime\prime}} = 9.3$ Hz, 1H, H-4''), 3.78 (m, 1H, H-5), 3.76 (m, 2H, H-6_{a,b}'), 3.66 (t, ${}^{3}J_{3'',4''} \approx {}^{3}J_{4'',5''} = 9.4$ Hz, 1H, H-4'''), 3.60 (dd, ${}^{2}J = 10.9$ Hz, ${}^{3}J_{5'',6a''} = 2.1$ Hz, 1H, H-6_a''), 3.56 (m, 1H, H-6_b"), 3.53 (dd, ${}^{3}J_{2^{\prime\prime},3^{\prime\prime}} = 9.8$ Hz, ${}^{3}J_{1^{\prime\prime},2^{\prime\prime}} = 3.7$ Hz, 1H, H-2'''), 3.47 (dd, ${}^{3}J_{2',3''} = 9.6$ Hz, ${}^{3}J_{1'',2''} = 3.5$ Hz, 1H, H-2''), 3.25 (dd, ${}^{2}J = 11.0$ Hz, ${}^{3}J_{5,..,6a,..} = 1.8$ Hz, 1H, H-6a,...), 3.23 (dd, ${}^{2}J = 10.9 \text{ Hz}, {}^{3}J_{5,..,6a,..} = 2.5 \text{ Hz}, 1\text{ H}, \text{H-6}, ...), 2.21 (s, 3\text{H}, CH_{3}\text{CO});$ $\delta_{\rm C}(151 \text{ MHz}; {\rm CDCl}_3)$ 169.6, 138.8 (2C), 138.7, 138.2, 138.1, 138.1, 138.0, 137.9, 137.9, 137.8, 136.9 (2C), 129.1, 128.4, 128.3, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.5, 127.4, 127.4, 127.3, 127.2, 127.2, 126.8, 126.3, 126.2, 102.2 (C_{benzylidene}), 100.1 (C1'), 97.8 (C1), 97.4 (C1'"), 96.3 (C1"), 82.2, 79.7, 79.4, 79.2, 78.0, 77.9, 76.7, 75.4, 75.2, 75.1, 74.8, 74.7, 74.4, 73.3, 73.3, 73.2, 72.6, 72.1, 71.9, 70.2, 70.2, 70.0, 69.6, 69.0, 68.9, 68.5, 68.0, 63.7, 20.8 ppm; non-decoupled ${}^{1}\text{H}, {}^{13}\text{C} \text{HMQC} (151 \text{ MHz}, \text{CDCl}_{3}): J_{\text{C,Hbenzylidene}} = 163 \text{ Hz}, J_{\text{C1,H-1}} =$ 172 Hz, $J_{Cl',H-l'} = 175$ Hz, $J_{Cl',H-l''} = 173$ Hz, $J_{Cl''-H-l'''} = 173$ 175 Hz; MALDI-MS (pos. mode, DHB): [M+Na]⁺ calcd.: 1809.8, found: 1809.8; [M+K]⁺ calcd.: 1825.7, found: 1825.8; C₁₁₀H₁₁₄O₂₂ (1788.1)).

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