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Chemoenzymatic Synthesis of L-Galactosylated Dimeric Sialyl Lewis X Structures Employing α-1,3-Fucosyltransferase V

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Abstract—L-Galactosylated dimeric sialyl Lewis X (SLe^x) has been prepared employing a combination of chemical and enzymatic synthetic methods. GDP-L-galactose has been chemically synthesised. Enzymatic transfer of L-galactose onto the acceptor (Sia-α2,3-Gal-β1,4-GlcNAc-β1,3/6)$_2$-Man-α1-OMe was achieved using the human α-1,3-fucosyltransferase V.

Introduction

White blood cells, or leukocytes, are important species for the repair of tissue damage and defense against microbial infection. Leukocytes are brought to the site of injury by a complex series of steps, referred to as the inflammatory cascade. The initial events in this cell–cell recognition process, the tethering and rolling of leukocytes, are mediated by selectins, a family of calcium dependent carbohydrate binding cell adhesion molecules.[1] SLe^x, among others, has been recognised as a small molecule ligand for the selectins.[1] An attractive strategy for treating inflammation-related diseases, such as rheumatoid arthritis, is to inhibit the selectin–ligand interaction that initiates these processes. Consequently, attempts have been made to develop SLe^x inhibitors and much recent effort has gone into preparing multimers of SLe^x in hope of mimicking nature's use of polyvalency.[1]

Since Whitesides' pioneering work 20 years ago, chemoenzymatic methods in carbohydrate chemistry have undergone extensive development and have proven to be very attractive for the synthesis of natural and unnatural oligosaccharides.[2–4] The most important strategy for in vitro enzymatic oligosaccharide synthesis is the use of the glycosyltransferases of the Leloir pathway, which require sugar nucleotides as donors.[5,6]

The use of these 'Leloir' glycosyltransferases in preparative enzymatic glycosylation to date has mainly focused on the transferases involved in the biosynthesis of sialyl Lewis X (SLe^x), namely β-1,4-galactosyltransferase (β-1,4-GalT), α-2,3-sialyltransferase (α-2,3-SiaT) and α-1,3-fucosyltransferase (α-1,3-FucT).[7–9] In addition α-2,6-sialyltransferase (α-2,6-SiaT) has been frequently used.[10,11]

In order to extend the synthetic applicability of the limited number of glycosyltransferases available, intensive studies towards the substrate specificity of these enzymes have been carried out. A number of unnatural acceptor and donor substrates have been employed, disproving the original 'one enzyme–one linkage' conception and broadening the scope of glycosyltransferases in synthetic chemistry. In this context derivatives of GDP-L-fucose have been chemically synthesised and examined as donor substrates for various fucosyltransferases.[12]

In earlier work concerning the *N*-glycans from the Glycodelins, human glycoproteins with immunosuppressive and contraceptive activity,[13,14] we reported the chemoenzymatic synthesis of monomeric SLe^x structures

Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; GDP, guanidine 5′-diphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; MES, 2-(*N*-morpholine)ethane sulfonic acid; MK, myokinase; NMK, nucleoside monophosphate kinase; PEP, phosphoenol pyruvate; PK, pyruvate kinase; PPase, inorganic pyrophosphorylase.

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containing the L-fucose and the L-galactose motif.[15] Additionally the preparation of dimeric SLe^x was presented.[15] Encouraged by these results, we attempted the synthesis of the dimeric SLe^x undecasaccharide **1** (Fig. 1) using α-1,3-FucT V to achieve enzymatic *bis*-L-galactosylation of a suitable precursor.

Results and Discussion

Synthesis of GDP-L-galactose

The synthesis of GDP-L-galactose was achieved in six steps and 57% overall yield starting from L-galactose employing an improved procedure for the coupling of sugar diphosphate nucleosides (Scheme 1).[16] Glycosylation of the galactosyl bromide **2**[17] with dibenzyl phosphate in the presence of silver carbonate gave the protected β-L-galactopyranosyl phosphate **3** as the sole product. The phosphate group was deprotected by hydrogenation in a mixture of toluene, pyridine and triethylamine using Pd/C as a catalyst to give galactopyranosyl phosphate **4**. The solvent system was chosen to keep the product in solution. Subsequent debenzoylation was achieved by refluxing a solution of **4** in methanol and cyclohexylamine (1:1) giving access to the desired β-L-fucopyranosyl dicyclohexylammonium phosphate **5** in crystalline form. The best results for subsequent coupling of **5** with GDP-morpholidate are usually obtained if the reaction is carried out in pyridine.[18,19] In order to make the β-L-galactopyranosyl phosphate soluble in pyridine, the counterion has to be changed from cyclohexylammonium to triethylammonium. The final coupling reaction was carried out under strictly anhydrous conditions using 1*H*-tetrazole as a catalyst.[16] GDP-L-galactose **6** was isolated in 74% yield after purification by size exclusion chromatography.

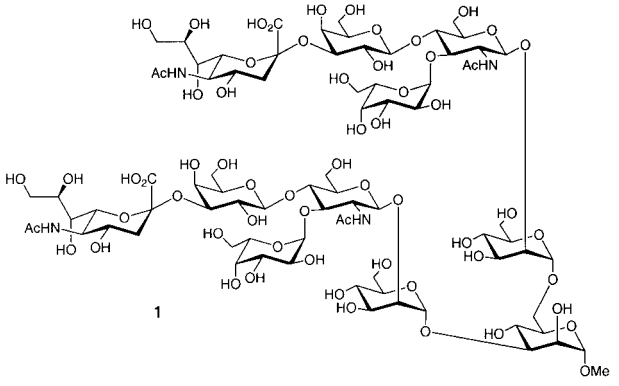
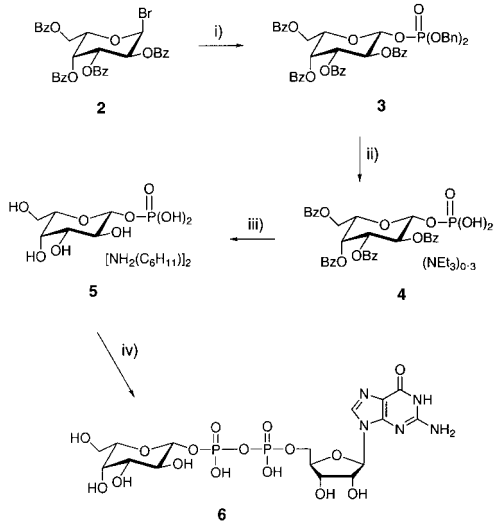


Figure 1. L-Galactosylated SLe^x undecasaccharide.



Scheme 1. (i) Ag_2CO_3, $(Bn)_2P(O)OH$, CH_2Cl_2, Et_2O, CH_3CN, 93% over two steps; (ii) Pd/C, H_2, toluene, pyridine, NEt_3, 97%; (iii) MeOH, cyclohexylamine, 90%; (iv) BioRad AG 50W-X2 (NEt_3-form), quant, then guanosinemorpholidate, tetrazole, pyridine, 3 days, 74%.

Synthesis of the undecasaccharide 1

Our synthesis of undecasaccharide **1** is based on the enzymatic decoration of pentasaccharide precursor **7** (Fig. 2). A similar strategy has been employed by Unverzagt in their synthesis of complex *N*-glycans[20] and presented an alternative to our route in the synthesis of complex *N*-glycans from the human glycoproteins Glycodelin A and Glycodelin S reported earlier.[15]

Double glycosylation of the readily available diol acceptor **9**[15] with three equivalents of the β-thioglycoside **8**[21] in the presence of silver(I) triflate/NIS as activator system gave the symmetrical pentasaccharide **10** in 85% yield (Scheme 2). A four step deprotection sequence finally gave pentasaccharide **7** as a colourless amorphous powder after size exclusion chromatography. ^1H NMR analysis revealed that **7** existed as a 40:1 β:α mixture of anomers. This result reflects a slight improvement over a 30:1 β:α ratio for the double glycosylation of the diol acceptor **9** with a fully acetylated lactosamine bromide donor reported earlier.[15]

With pentasaccharide **7** in hand, selective galactosylation was achieved in a single step using β-1,4-GalT and the donor substrate UDP-D-galactose in the presence of alkaline phosphatase.[10] Heptasaccharide **11** was isolated in 69% yield. In our hands the purification of **11** by size exclusion chromatography proved difficult, restricting this synthesis to a small preparative scale (10 mg). Incubation of **11** with α-2,3-sialyltransferase and CMP-NeuAc gave the *bis*-sialylated nonasaccharide **12** in nearly quantitative yield (Scheme 3). When nonasaccharide **12** was incubated with GDP-L-galactose and α-1,3-FucT V the bivalent SLe^x analogue **1** was isolated

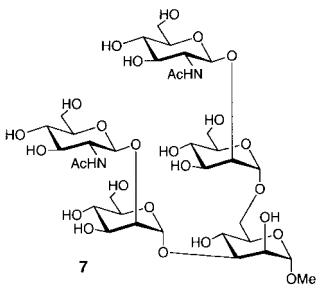
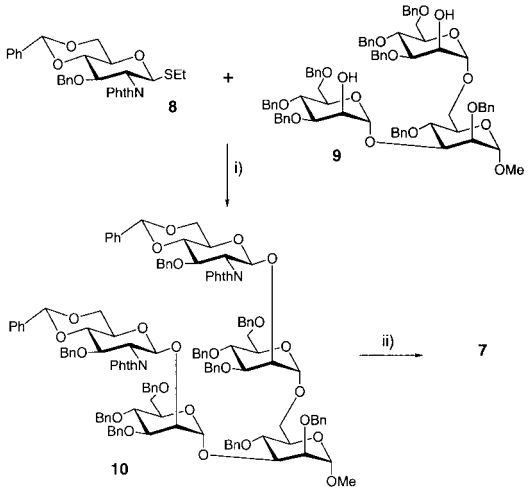
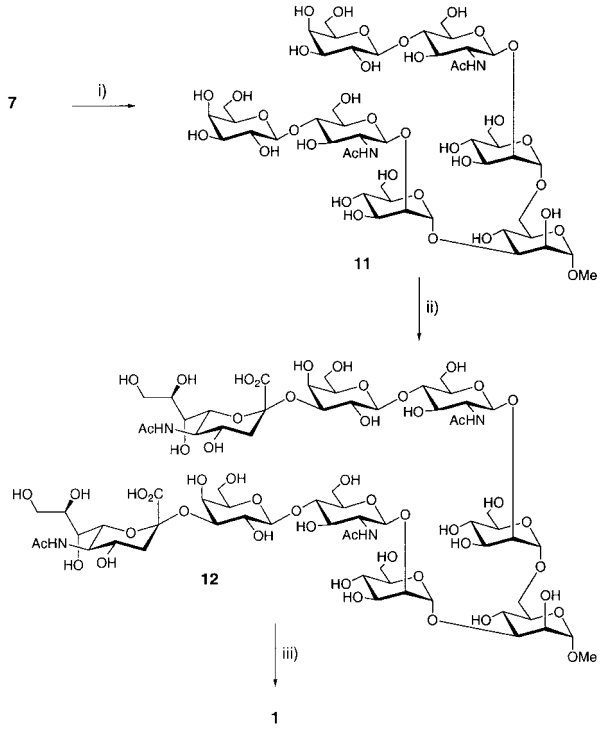


Figure 2. Pentasaccharide precursor for enzymatic decoration.



Scheme 2. (i) NIS, AgOTf, 4 Å ms, CH_2Cl_2, toluene, −50 °C to rt, 85%; (ii) (a) NH_2NH_2, EtOH; (b) Ac_2O, pyridine; (c) K_2CO_3, MeOH; (d) Pd/C, CH_2Cl_2:EtOH:H_2O 3:3:1, 79% over four steps.



Scheme 3. (i) UDP-galactose, β-1,4-galactosyltransferase, alkaline phosphatase, BSA, HEPES (50 mM, pH 7.2), 69%; (ii) CMP-NeuAc, α-2,3-sialyltransferase, $MnCl_2$, $MgCl_2$, alkaline phosphatase, BSA, HEPES (200 mM, pH 7.5), 99%; (iii) GDP-L-galactose, α-1,3-fucosyltransferase V, $MnCl_2$, alkaline phosphatase, BSA, MES (50 mM, pH 6.0), 56%.

as a colourless solid after size exclusion chromatography in 56% yield.

A prolonged reaction time in comparison with the synthesis of the L-fucosylated analogue of **1**[15] indicated the decreased donor efficiency of GDP-L-galactose compared to GDP-L-fucose. This observation is consistent with kinetic data reported by Hindsgaul et al. for the enzymatic transfer of L-galactose using an α-1,3/4 FucT.[22] Similar experiments employing GDP-L-galactose as an unnatural substrate for α-1,3-fucosyltransferase III have been reported by Öhrlein et al.[23]

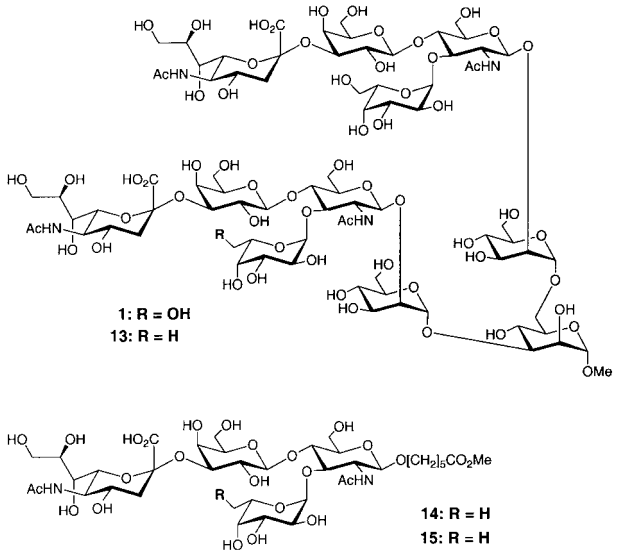


Figure 3. Monomeric and dimeric SLe^x analogues undergoing inhibition assays.

Conclusion

The high yielding synthesis of the L-galactosylated undecasaccharide **1** gives testimony to the broad applicability of glycosyltransferases and the power of the chemoenzymatic approach chosen.

On a small preparative scale the chemoenzymatic synthesis of heptasaccharide **11** presents a viable alternative to the purely chemical synthesis reported earlier.[15]

Also noteworthy is the high yield observed in the enzymatic sialylation. These results are of particular interest as the chemical glycosylation of *N*-acetylneuraminic acid suffers from complex problems such as the stereochemical outcome of the reaction and the formation of by-products by elimination.[24,25]

Although in the final step GDP-L-galactose showed a decrease in donor efficiency when compared with the natural substrate GDP-L-fucose, L-galactosylation of the acceptor saccharide **12** was achieved in good yield.

The dimeric SLe^x analogue **1** and the related structures **13**–**15**[15] (Fig. 3) are currently undergoing tests concerning their binding affinity towards E-selectin.

Experimental

^1H NMR spectra were recorded in $CDCl_3$ or D_2O on Bruker DRX-600, DRX-500 and DPX-200 spectrometers at 300 K. Residual protic solvent $CHCl_3$ (δ_H = 7.26 ppm) was used as the external reference. ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O at 150 or 100 MHz on Bruker DRX-600 and AC400 spectrometers, respectively, using the central resonance of $CDCl_3$

($\delta_C = 77.0$ ppm) as the external reference. DQF-COSY, HMQC, decoupled-HMQC, HMBC, TOCSY and 1-D TOCSY experiments were used to assist assignment of the products. NMR assignments are as indicated in Figure 3. Infra-red spectra were recorded as thin films between sodium chloride plates, deposited from chloroform solution on an FTIR 1620 spectrometer. Mass spectra were obtained on Micromass Platform LC–MS and Q-Tof, Kratos MS890MS and Kompact 4, Brucker Daltonics Bio-Apex II (FTICR) spectrometers at the Department of Chemistry, University of Cambridge and on a Voyager STR spectrometer at M-Scan, Silwood Park, Ascot.

Flash column chromatography was carried out using Merck Kieselgel (230–400 mesh). Analytical thin layer chromatography (tlc) and preparative tlc were performed using precoated glass-backed plates (Merck Kieselgel 60 F254) and visualised by uv and acidic ammonium molybdate (IV). Petrol refers to petroleum ether bp 40–60 °C, which was distilled prior to use.

All reactions were carried out under an argon atmosphere in oven-dried glassware unless otherwise stated. Diethyl ether was distilled from sodium benzophenone ketyl, dichloromethane and toluene from calcium hydride. Other reagents and solvents were purified using standard procedures. Aqueous solutions are saturated unless otherwise specified.

α-1,3-Fucosyltransferase V and α-2,3-sialyltransferase were purchased from Calbiochem®. CMP-Sialic acid and GDP-galactose were purchased from Calbiochem® or synthesised using published protocols.[16]

Dibenzylphosphoryl 2,3,4,6-tetra-*O*-benzoyl-β-L-galactopyranoside (3). Ag_2CO_3 (1.08 g 3.94 mmol) was added in one portion to a cooled mixture of **2**[17] (1.30 g, 1.97 mmol), dibenzylphosphate (1.65 g, 5.91 mmol) and 3 Å ms (3.50 g) in CH_2Cl_2, Et_2O, CH_3CN (11 mL each). The mixture was stirred for 24 h at room temperature with exclusion of light, filtered through Celite® and the filtrate was concentrated. The residue was purified by column chromatography (toluene:EtOAc, 2:1) to give **3** as a colourless foam (1.59 g, 95%). $R_f = 0.48$ (silica gel, 1:2 EtOAc:hexane); δ_H (400 MHz, $CDCl_3$) 4.41–4.52 (3H, m, C*H*$_2$Ph), 4.62 (1H, dd, $J = 6.2$ and 10.8 Hz, C*H*$_2$Ph), 4.76 (1H, dd, $J = 7.0$ and 11.6 Hz, H_a-6), 4.86 (1H, dd, $J = 6.8$ and 11.6 Hz, H_b-6), 5.07 (1H, d, $J = 7.9$ Hz, H-1), 5.65 (1H, dd, $J = 3.5$ and 10.5 Hz, H-3), 5.76 (1H, m, H-5), 5.95 (1H, dd, $J = 7.9$ and 10.5 Hz, H-2), 6.05 (1H, d, $J = 3.5$ Hz, H-4), 6.98–8.12 (30H, m, H_{arom}); δ_C (100 MHz, $CDCl_3$) 62.0, 67.8, 69.4 (d, $J = 5.8$ Hz), 69.5 (d, $J = 5.8$ Hz), 69.6 (d, $J = 7.2$ Hz), 71.4, 72.4, 97.0 (d, $J = 4.4$ Hz), 127.4, 127.9, 128.3, 128.4, 128.5, 128.7, 129.7, 129.8, 129.9, 130.0, 133.3, 133.4, 133.5, 133.7, 165.2, 165.4, 165.9; δ_P (162 MHz, $CDCl_3$) −2.22; *m/z* (FAB) found: $[M + Cs]^+$, 989.1306. $C_{48}H_{41}O_{13}P$ requires M + Cs, 989.1339.

Di(triethylammonium)phosphoryl 2,3,4,6-tetra-*O*-benzoyl-β-L-galactopyranoside (4). Compound **3** (1.40 g, 1.64 mmol) was dissolved in toluene (12 mL), triethylamine (1.80 mL) and pyridine (2.5 mL). The mixture was stirred under an H_2 atmosphere with 10% activated Pd/C (90 mg) as catalyst. After 36 h the mixture was filtered and the filtrate was concentrated to give **4** as a colourless foam (1.40 g, 97%). $R_f = 0.52$ (silica gel, ^{i}PrOH:NH_3: δ_H (400 MHz, $CDCl_3$) 1.23 (3H, t, $J = 7.3$ Hz, N(CH$_2$C*H*$_3$)$_3$), 3.03 (2H, q, $J = 7.3$ Hz, N(C*H*$_2$CH$_3$)$_3$), 4.45 (1H, dd, $J = 7.3$ and 10.8 Hz, H_a-6), 4.57–4.63 (1H, m, H-1), 4.69 (1H, dd, $J = 5.4$ and 10.8 Hz, H_b-6), 5.63–5.68 (1H, m, H-3), 5.75–5.84 (2H, m, H-2, H-5), 6.04 (1H, dd, $J = 0.8$ and 3.0 Hz, H-4), 7.20–8.06 (20H, m, H_{arom}); δ_C (100 MHz, $CDCl_3$) 20.8, 42.8, 43.4, 60.5, 67.5, 69.6 (d, $J = 8.7$ Hz), 70.1, 94.8 (d, $J = 4.4$ Hz), 126.8, 126.9, 127.0, 127.2, 127.3, 127.9, 128.8, 128.9, 131.7, 131.8, 131.9, 132.2, 164.0, 164.6, 164.7, 164.8; δ_P (162 MHz, $CDCl_3$) 2.06; *m/z* (FAB) found: $[M + Cs]^+$, 809.0432. $C_{34}H_{29}O_{13}P$ requires M + Cs, 809.0400.

Di(cyclohexylammonium)phosphoryl-β-L-galactopyranoside (5). Cyclohexylamine (5 mL) was added to a solution of compound **4** (1.30 g, 1.48 mmol) in dry MeOH (10 mL). The mixture was heated at reflux for 4 h after which the mixture was concentrated, partitioned between water and $CHCl_3$, and the aqueous phase was washed three times with $CHCl_3$ and then concentrated to dryness by coevaporating with MeOH. The product was dissolved in hot EtOH and triturated with acetone. Filtration gave a colourless solid (610 mg, 90%). $R_f = 0.14$ (silica gel, ^{i}PrOH:NH_3:H_2O, 6:3:1); δ_H (400 MHz, D_2O) 1.06–1.16 (2H, m, $C_6H_{11}NH_2$), 1.20–1.34 (8H, m, $C_6H_{11}NH_2$), 1.55–1.62 (2H, m, $C_6H_{11}NH_2$), 1.69–1.77 (4H, m, $C_6H_{11}NH_2$), 1.90–1.96 (4H, m, $C_6H_{11}NH_2$), 3.03–3.12 (2H, m, $C_6H_{11}NH_2$), 3.48 (1H, dd, $J = 7.6$ and 9.7 Hz), 3.62 (1H, dd, $J = 3.5$ and 10.3 Hz), 3.65–3.68 (2H, m), 3.75 (1H, dd, $J = 9.4$ and 12.4 Hz), 3.83 (1H, d, $J = 3.5$ Hz), 4.77 (1H, t, $J = 7.6$ Hz, H-1); δ_C (100 MHz, D_2O) 26.2, 26.7, 32.8, 52.7, 63.9, 71.3, 74.5 (d, $J = 5.8$ Hz), 75.0, 78.0, 100.0 (d, $J = 4.4$ Hz); *m/z* (FAB) found: $[M + Na]^+$, 283.0187. C6H13O9P requires M + Na, 283.0195.

Guanosine 5′-diphospho-β-L-galactopyranoside (6). Compound **15** (184 mg, 401 μmol) was dissolved in water (3 mL), applied to a Bio-Rad AG 50 W-X2 cation-exchange column (Et_3N^+-form, 2.5×6 cm) and eluted with water (150 mL). The eluent was evaporated, coevaporated with MeOH (2×5 mL) and dried for 3 days under vacuum to give triethylammonium β-L-galactopyranosyl phosphate. After addition of 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidinium guanosine 5′-monophosphomorpholidate (319 mg, 440 μmol) the mixture was coevaporated with dry pyridine (3×5 mL). 1*H*-Tetrazole (56.0 mg, 800 μmol) and dry pyridine (3 mL) were added and the solution was stirred at room temperature. After 2 days, the mixture was diluted with water (3 mL) to become a clear solution, and it was extracted with CH_2Cl_2 (3×5 mL). The aqueous phase was concentrated and applied to a Bio-Gel® P-2 column (2.5×70 cm, eluent: 50 mM NH_4HCO_3) to give the desired product as a colourless solid after lyophilization (180 mg, 74%). $R_f = 0.26$ (silica gel, 2:1 ^{i}PrOH:1 M NH_4OAc); δ_H (400 MHz, D_2O) 1.18 (1H, d, $J = 6.7$ Hz, H-6″), 3.56–3.82 (5H, m), 3.88 (1H, d, $J = 3.2$ Hz), 4.18–4.22 (1H, m), 4.31–4.34 (1H, m), 4.50 (1H, t, $J = 4.2$ Hz), 4.71 (1H, t, $J = 5.4$ Hz, H-2′), 4.93

(1H, t, $J=7.6$ Hz, H-1″), 5.87 (1H, d, $J=5.7$ Hz, H-1′), 8.05 (1H, s, H-8); δ_C (100 MHz, D_2O) 63.6, 67.7 (d, $J=5.8$ Hz), 71.1, 72.8, 73.7 (d, $J=7.3$ Hz), 74.7, 76.2, 78.3, 86.1 (d, $J=8.7$ Hz), 89.3, 100.9 (d, $J=5.8$ Hz), 118.5, 139.9, 154.1, 156.3, 161.2; δ_P (162 MHz, D_2O) −12.1 (d, $J=18.3$), −10.4 (d, $J=18.3$ Hz); *m/z* (ESI) found: $[M-H]^-$, 604. $C_{16}H_{25}N_5O_{16}P_2$ requires M−H, 604.

Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-α-D-mannopyranoside (10). A mixture of glycosyl donor **8** (51.3 mg, 96.6 μmol) and glycosyl acceptor **9** (40.0 mg, 32.2 μmol) was dried by azeotropic distillation with toluene and left under vacuum for 18 h. After addition of NIS (29.0 mg, 129 μmol) and 4 Å molecular sieves (400 mg) the mixture was suspended in CH_2Cl_2 (1 mL) and cooled to −50 °C. AgOTf (8.3 mg, 32.2 μmol) in toluene (0.4 mL) was added over 20 min and the reaction mixture was warmed slowly to −30 °C. After 12 h at −20 °C it was diluted with ether, filtered through Celite®, washed with $Na_2S_2O_3$, concentrated and purified by size exclusion chromatography on Sephadex® LH-20 (CH_2Cl_2:MeOH 1:1). Pentasaccharide **10** was obtained in 85% yield (60.1 mg, 27.4 μmol). $R_f=0.33$ (silica gel, PE:Et_2O, 1:2); δ_H (600 MHz, $CDCl_3$) 2.74–2.78 (1H, m, H-5_c), 2.80 (1H, dd, $J=7.4$ and 10.7 Hz, H$_a$-6_b), 2.99 (1H, dd, $J=6.3$ and 11.0 Hz, H$_a$-$6_{b'}$), 3.21 (3H, s, OCH_3), 3.27–3.32 (2H, m, H$_a$-6_a, H$_b$-$6_{b'}$), 3.39 (1H, t, $J=9.7$ Hz, H-4_b), 3.43–3.47 (2H, m, H$_b$-6_b, H-$5_{b'}$), 3.48–3.53 (2H, m, H-5_a, H-$4_{b'}$), 3.55–3.60 (1H, m, H-$5_{c'}$), 3.63–3.67 (3H, m, H-2_a, H-4_a, H$_b$-6_a), 3.69–3.73 (3H, m, H-5_b, H-4_c, H$_a$-6_c), 3.75 (2H, s, H-2_b), 3.76–3.82 (3H, m, H-3_a, H-3_b, H-$3_{b'}$), 3.84–3.89 (3H, m, H-$4_{c'}$, H$_a$-$6_{c'}$, CH_2Ph), 3.92–3.97 (2H, m, CH_2Ph), 4.01 (1H, d, $J=11.8$, CH_2Ph), 4.05 (1H, dd, $J=4.9$ and 10.1 Hz, H$_b$-6_c), 4.14 (1H, s, H-$2_{b'}$), 4.22–4.29 (2H, m, H-2_c, H-3_c), 4.31–4.34 (1H, m, H$_b$-$6_{c'}$), 4.37–4.52 (11H, m, H-$2_{c'}$, H-$3_{c'}$, CH_2Ph), 4.53–4.55 (2H, m, H-$1_{b'}$, CH_2Ph), 4.59 (1H, s, H-1_a), 4.65 (1H, d, $J=11.6$ Hz, CH_2Ph), 4.71 (1H, s, H-1_b), 4.75 (1H, d, $J=12.3$, CH_2Ph), 4.79 (1H, d, $J=12.3$ Hz, CH_2Ph), 4.83–4.86 (2H, m, CH_2Ph), 4.91 (1H, d, $J=8.1$ Hz, H-1_c), 5.28 (1H, s, H-$1_{c'}$), 5.53 (1H, s, C*H*Ph), 5.62 (1H, s, C*H*Ph), 6.84–7.71 (68H, m, H_{arom}); δ_C (125 MHz, $CDCl_3$) 54.7 (OCH_3), 55.5 (C-2_c), 55.6 (C-$2_{c'}$), 65.6 (C-5_c), 66.1 (C-6_a), 66.2 (C-$5_{c'}$), 68.5 (C-6_c), 68.6 (C-$6_{c'}$), 69.8 (C-$6_{b'}$), 70.5 (C-6_b), 70.8 (C-5_a), (70.9, 71.6, 72.2, 72.5, 72.9, 73.8, 74.0, 74.7, 75.1 (*C*H$_2$Ph)), 71.7 (C-$5_{b'}$), 72.6 (C-5_b), 73.7 (C-2_b, C-$2_{b'}$), 73.9 (C-4_a), 74.1 (C-3_c, C-$3_{c'}$), 74.6 (C-4_b, C-$4_{b'}$), 77.6 (C-$3_{b'}$), 77.7 (C-2_a), 78.1 (C-3_b), 80.2 (C-3_a), 82.8 (C-4_c), 82.9 (C-$4_{c'}$), 96.8 (C-1_c), 97.4 (C-$1_{b'}$), 97.6 (C-$1_{c'}$), 98.3 (C-1_a), 99.2 (C-1_b), 101.3 (*C*HPh), (123.1, 126.1, 127.3, 127.4, 127.6, 128.0, 128.1, 128.2, 128.3, 129.0, 131.8, 133.5, 133.6, 137.4, 137.5, 138.0, 138.1, 138.2, 138.5, 138.6, 138.8 (C_{arom})); *m/z* (ESI) found: $[M + Na]^+$, 2199.8504. $C_{131}H_{128}N_2O_{28}$ requires M + Na, 2199.8546.

Methyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (7). 58 mg (26.6 mmol) of fully protected pentasaccharide **10** was suspended in EtOH (10 mL) and $NH_2NH_2{\cdot}H_2O$ (1 mL) was added. The reaction mixture was heated under reflux for 18 h. The solvent was removed in vacuo and the residue co-evaporated with toluene (3×5 mL). Ac_2O (2 mL) and pyridine (4 mL) were added and the solution was stirred for 18 h. The reaction mixture was concentrated and co-evaporated with toluene to remove traces of pyridine and the residue was purified by size exclusion chromatography on Sephadex® LH-20 (DCM/MeOH 1:1).

The resulting oil was dissolved in CH_2Cl_2 (1 mL) and MeOH (2 mL) and a catalytic amount of K_2CO_3 was added. The mixture was stirred overnight and Amberlite IR-120 was added to neutralise the reaction mixture. After filtration and concentration the resulting material was dissolved in CH_2Cl_2 (6 mL), MeOH (6 mL) and H_2O (2 mL), and $Pd(OH)_2$/C was added. The reaction mixture was stirred under an atmosphere of H_2 for 24 h and filtered through Celite®. Size exclusion chromatography on Sephadex® G-15 gave pentasaccharide **7** in 79% (19.5 mg, 21.0 mmol) over four steps. δ_H (600 MHz, D_2O) 1.98 (3H, s, NHC(O)CH_3), 1.99 (3H, s, NHC(O)CH_3), 3.35 (3H, s, OCH_3), 3.36–3.41 (4H, m, H-4_c, H-5_c, H-$4_{c'}$, H-$5_{c'}$), 3.43–3.51 (4H, m, H-4_b, H-3_c, H-$4_{b'}$, H-$3_{c'}$), 3.55–3.60 (3H, m, H$_a$-6_b, H-$5_{b'}$, H$_a$-$6_{b'}$), 3.61–3.66 (4H, m, H$_a$-6_a, H-5_b, H-2_c, H-$2_{c'}$), 3.67–3.74 (3H, m, H-5_a, H$_a$-6_c, H$_a$-$6_{c'}$), 3.77–3.87 (8H, m, H-3_a, H-4_a, H-3_b, H$_b$-6_b, H$_b$-6_c, H-$3_{b'}$, H$_b$-$6_{b'}$, H$_b$-$6_{c'}$), 3.98 (1H, dd, $J=4.0$ and 11.4 Hz, H$_b$-6_a), 4.02 (1H, dd, $J=1.7$ and 3.1 Hz, H-2_a), 4.06 (1H, dd, $J=1.6$ and 3.1 Hz, H-$2_{b'}$), 4.11 (1H, dd, $J=1.6$ and 3.2, H-2_b), 4.48 (1H, d, $J=8.4$ Hz, H-$1_{c'}$), 4.50 (1H, d, $J=8.4$ Hz, H-1_c), 4.67 (1H, s, H-1_a), 4.85 (1H, s, H-$1_{b'}$), 5.05 (1H, s, H-1_b); δ_C (125 MHz, D_2O) 22.3 (NHC(O)*C*H$_3$), 54.9 (O*C*H$_3$), 55.3 (C-$2_{c'}$), 55.4 (C-2_c), 60.6 (C-6_c, C-$6_{c'}$), 61.6 (C-6_b, C-$6_{b'}$), 65.4 (C-6_a), 65.6 (C-4_a), (67.2, 67.3 (C-4_b, C-$4_{b'}$)), (69.4, 69.5, 69.6 (C-2_a, C-3_b, C-$3_{b'}$)), 69.9 (C-4_c, C-$4_{c'}$), 71.0 (C-5_a), 71.9 (C-$5_{b'}$), (73.3, 73.4 (C-3_c, C-$3_{c'}$)), 73.5 (C-5_b), (75.8, 75.9 (C-5_c, C-$5_{c'}$)), 76.4, (C-$2_{b'}$), 76.6 (C-2_b), 78.6 (C-3_a), 96.8 (C-$1_{b'}$), 99.5 (C-1_b), 99.6 (C-$1_{c'}$), 99.7 (C-1_c), 101.4 (C-1_a); *m/z* (FAB) found: $[M + Na]^+$, 947.3319. $C_{35}H_{60}N_2O_{26}$ requires M + Na, 947.3326.

Methyl β-D-galactopyranosyl-(1→4)-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)]-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (11). β-1,4-Galactosyltransferase (300 mU, 438 μL) was added to a solution of pentasaccharide **7** (5.0 mg, 5.4 μmol) and UDP-D-galactose (8.2 mg, 13.5 μmol) in HEPES buffer (0.5 mL, 50 mM, pH 7.2) containing 1 mg BSA and 7 U alkaline phosphatase. The mixture was shaken at 37 °C for 5 days. The reaction mixture was centrifuged, the supernatant was concentrated and purified on Sephadex® G-15 (100 mM NH_4HCO_3) to give **11** as a white solid after lyophilization (4.6 mg, 5.0 μmol, 69%). δ_H (600 MHz, D_2O) 1.98 (3H, s, NHC(O)CH_3), 1.99 (6H, s, NHC(O)CH_3), 3.34 (3H, s OCH_3), 3.42–3.87 (33H, m), 3.90 (2H, d, $J=11.7$ Hz, H$_b$-6_c, H$_b$-$6_{c'}$), 3.98 (1H, d, $J=11.1$ Hz, H$_b$-6_a), 4.02 (1H, s, H-2_a), 4.06 (1H, s, H-$2_{b'}$), 4.11 (1H, s, H-2_b), 4.40 (2H, d, $J=7.8$ Hz, H-1_d, $1_{d'}$), 4.51 (1H, d, $J=7.5$ Hz,

H-1_c), 4.53 (1H, d, J = 7.7 Hz, H-$1_{c'}$), 4.66 (s, 1H, H-1_a), 4.86 (1H, s, H-$1_{b'}$) 5.04 (1H, s, H-1_b); δ_C (125 MHz, D_2O, selected data) 22.3 (NHC(O)CH_3), 54.9 (OCH_3), 60.0 (C-6_c, $6_{c'}$), 65.4 (C-6_a), 69.5 (C-2_a), 76.3 (C-$2_{b'}$), 76.5 (C-2_b), 96.8 (C-$1_{b'}$), 99.4 (C-1_b, C-$1_{c'}$), 99.5 (C-1_c), 101.0 (C-1_a), 102.9 (C-1_d, $1_{d'}$); m/z (ESI) found: $[M + Na]^+$, 1271.4355. $C_{47}H_{80}N_2O_{26}$ requires M + Na, 1271.4388.

Methyl α-D-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulo-pyranuronic acid-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[α-D-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulo-pyranuronic acid-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (12). α-2,3-Sialyltransferase (60 mU, 60 μL) was added to a solution of the heptasaccharide **11** (10.0 mg, 8.0 μmol) and CMP-sialic acid (10.0 mg, 16.0 μmol) in HEPES buffer (2.0 mL, 100 mM, pH 7.5) containing 4 mg BSA, 14 U alkaline phosphatase, $MnCl_2{\cdot}4H_2O$ (10 μL of a 1 M solution) and $MgCl_2$ (40 μL of a 1 M solution). The mixture was shaken at 37 °C for 9 days (additions of identical amounts of CMP-sialic acid, α-2,3-sialyltransferase and alkaline phosphatase were repeated after 2, 4, and 7 days). The reaction mixture was centrifuged, the supernatant was concentrated and purified with Bio Gel® P-4 (150 mM NH_4HCO_3) to give **12** as a colourless solid after lyophilization (14.7 mg, 7.8 μmol, 98%). δ_H (600 MHz, D_2O) 1.73 (2H, t, J 12.2 Hz, H_{ax}-3_e, H_{ax}-$3_{e'}$), 1.96 (6H, s, HNC(O)CH_3), 1.98 (3H, s, HNC(O)CH_3), 1.99 (3H, s, HNC(O)CH_3), 2.69 (2H, dd, J = 4.4 and 12.2 Hz, H_{eq}-3_e, H_{eq}-$3_{e'}$), 3.34 (3H, s, OCH_3), 3.43–3.96 (50H, m), 3.97–3.99 (1H, m, H-6_a), 4.02–4.08 (4H, m, H-2_a, H-$2_{b'}$, H-3_d, H-$3_{d'}$), 4.11 (1H, s, H-2_b), 4.46–4.49 (2H, m, H-1_d, H-$1_{d'}$), 4.50 (1H, d, J = 7.9 Hz, H-1_c), 4.52 (1H, d, J = 7.9 Hz, H-$1_{c'}$), 4.67 (1H, s, H-1_a), 4.86 (1H, s, H-$1_{b'}$), 5.05 (1H, s, H-1_b); δ_C (125 MHz, D_2O, selected data according to HMQC) 22.1 (NHC(O)CH_3), 22.3 (NHC(O)CH_3), 39.6 (C-3_e, C-$3_{e'}$), 54.7 (OCH_3), 65.4 (C-6_a), (69.6, 75.5, 75.6, 76.4 (C-2_a, C-2_b, C-3_d, C-$3_{d'}$)), 76.6 (C-2_b), 96.7 (C-$1_{b'}$), 99.3 (C-1_b), 99.4 (C-$1_{c'}$), 99.6 (C-1_c), 101.0 (C-1_a), 102.6 (C-$1_{d/d'}$), 102.7 (C-$1_{d/d'}$); m/z (ESI after permethylation) found: $[M + Na]^+$, 2304.80. $C_{101}H_{178}N_4O_{52}$ requires M + Na, 2303.48.

Methyl α-L-galactopyranosyl-(1→3)-[α-D-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulo-pyranuronic acid-(2→3)-β-D-galactopyranosyl-(1→4)]-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-{α-L-galactopyranosyl-(1→3)-[α-D-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulo-pyranuronic acid-(2→3)-β-D-galactopyranosyl-(1→4)]-β-D-2-acetamido-2-deoxy-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)}-α-D-mannopyranoside (1). α-1,3-Fucosyltransferase V (50 mU, 100 μL) was added to a solution of **12** (5.0 mg, 2.7 μmol) and GDP-L-galactose (4.0 mg, 6.5 μmol) in MES buffer (1.0 mL, 50 mM, pH 6.0) containing 1 mg BSA, 7 U alkaline phosphatase and $MnCl_2{\cdot}4H_2O$ (20 μL of a 1 M solution). The mixture was shaken at 37 °C for 12 days (the addition of identical amounts of GDP-L-galactose, α-1,3-fucosyltransferase V and alkaline phosphatase was repeated after 3, 6 and 9 days). The reaction mixture was centrifuged, the supernatant was concentrated and purified with Sephadex® G-15 (150 mM NH_4HCO_3) to give **1** as a colourless solid after lyophilization in 56% yield (3.3 mg, 1.5 μmol). δ_H (600 MHz, D_2O) 1.70 (2H, t, J 12.2 Hz, H_{ax}-3_e, H_{ax}-$3_{e'}$), 1.92 (3H, s, HNC(O)CH_3), 1.94–1.97 (9H, m, HNC(O)CH_3), 2.64 (2H, dd, J = 4.5 and 12.2 Hz, H_{eq}-3_e, H_{eq}-$3_{e'}$), 3.32 (3H, s, OCH_3), 3.38–3.96 (60H, m), 3.98–4.01 (3H, m, H-2_a, H-3_d, H-$3_{d'}$), 4.04 (1H, s, H-$2_{b'}$), 4.08 (1H, m, H-2_b), 4.34–4.38 (1H, m, H-$1_{d/d'}$), 4.40–4.44 (1H, m, H-$1_{d/d'}$), 4.47–4.52 (2H, m, H-1_c, H-$1_{c'}$), 4.64 (1H, s, H-1_a), 4.82 (1H, s, H-$1_{b'}$), 5.01 (1H, s, H-1_b), 5.09–5.12 (2H, m, H-1_f, H-$1_{f'}$); δ_C (125 MHz, D_2O, selected data according to HMQC) 22.0 (NHC(O)CH_3), 22.5 (NHC(O)CH_3), 39.7 (C-3_e, C-$3_{e'}$), 54.8 (OCH_3), [69.4, 75.7, 75.8, 76.4 (C-2_a, C-2_b, C-$2_{b'}$, C-3_d, C-$3_{d'}$)], 96.7 (C-$1_{b'}$), 98.2 (C-1_f, C-$1_{f'}$), 99.2 (C-1_c, C-$1_{c'}$), 99.4 (C-1_b), 101.1 (C-1_a), 102.0 (C-1_d, C-$1_{d'}$).

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