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Chemo-enzymatic synthesis of a divalent sialyl Lewis^x ligand with restricted flexibility

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Abstract

To study the influence of the entropic factor in cluster cooperative effects, a divalent sialyl Lewis^x ligand with restricted flexilbility was chemo-enzymatically synthesized. First, a cyclized precursor with both glucosamine residues bridged together by a succinyl group was readily obtained in 42% yield by treatment of 2,2-bis(benzyloxymethyl)-1,3-bis(3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyloxy)-propane with succinyl chloride. After deacetylation, this precursor was subjected to stepwise enzymatic elongation utilizing successively, soluble galactosyltransferase, then recombinant sialyltransferase and fucosyltransferase; the latter enzymes immobilized on Ni^{2+}-Agarose, to afford, after debenzylation, a divalent sialyl Lewis^x ligand of restricted flexibility, in 45% overall yield. Following the same enzymatic sequence, a totally flexible ligand, required as a reference compound for evaluation of inhibitory activity toward selectins, was also prepared from 2,2(bis-benzyloxymethyl)-1,3-bis(2-acetamido-2-deoxy-β-D-glucopyranosyloxy)-propane, as well as both related divalent Lewis^x molecules lacking the sialic acids, the rigid one and the flexible one.

Keywords: Chemo-enzymatic synthesis; Divalent sialyl Lewis^x; Selectin ligand; Cooperativity; Cluster effect

1. Introduction

It is now well established that leukocyte adhesion to the vascular endothelium is mediated in the early steps of the inflammatory cascade, by selectins, a family of calcium-dependent carbohydrate-binding cell adhesion molecules. E-Selectin is induced on the vascular endothelium after stimulation by cytokines released from damaged tissues, P-selectin is immediately expressed on the cell surface of platelets and endothelial cells in response to inflammatory signals, while L-selectin is constitutively expressed on the surface of leukocytes. The tetrasaccharides sialyl Lewis^x, sialyl Lewis^a and sulfated analogues thereof have been identified as minimal carbohydrate epitopes recognized by the selectins.[1] However, the affinity of selectins for these monovalent epitopes is usually weak with dissociation constants typically in the millimolar range, except for the sulfate Lewis^a pentasaccharide, the most potent monovalent E-selectin ligand so far, showing an inhibitory activity at least 15-fold greater than that of the sialyl Lewis^x trisaccharide.[2] In fact, physiological ligands on cell surfaces like GlyCAM-1 and PSGL-1 have been shown to bind with high affinity to individual selectins, probably because, as mucin-like molecules, they are highly glycosylated and carry carbohydrates displayed in clusters. The requirement of multivalency for strong binding has stimulated the synthesis of glyco-dendrimers,[3] -polymers[4] and liposomes,[5] and also of several types of glycoconjugates with multiple carbohydrate binding sites.[6–12] When these multivalent displays were tested as inhibitors of selectins, the most powerful inhibitors turned out to be five to six times more active than sialyl Lewis x, only showing a moderate improvement in binding as compared to the monovalent counterpart.[1] Interestingly, remarkable IC50 values of 1 nanomolar against L-selectin have been reported for branched oligosaccharides containing four sialyl Lewis^x epitopes.[13] The strong binding of these ligands to their receptor could be explained by the enhanced cluster cooperative effects in these oligosaccharides, of limited mobility, like in mucins.[14]

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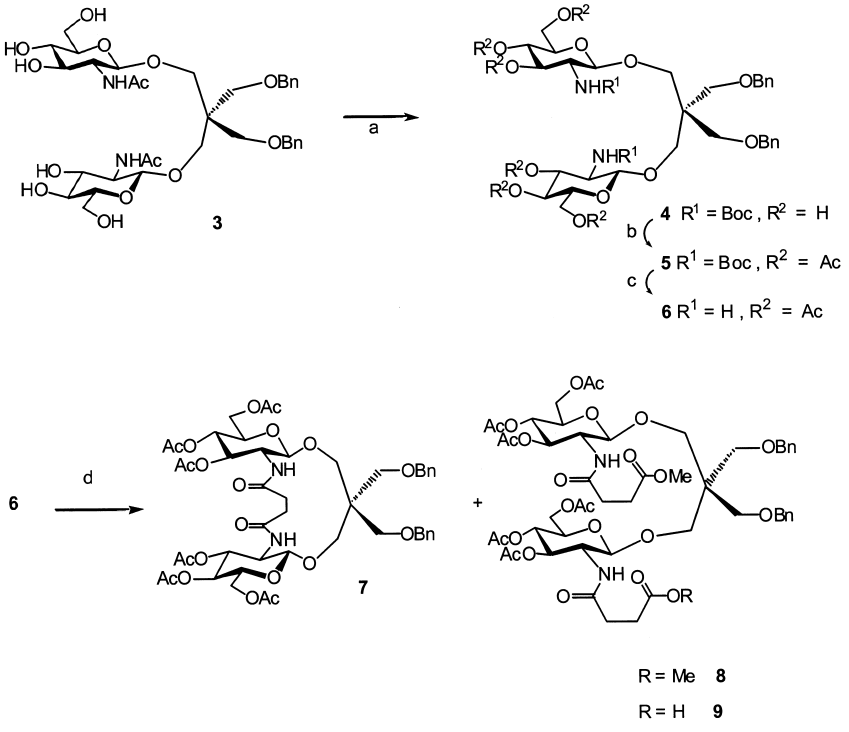
These findings prompted us to consider the synthesis of selectin ligands with restricted conformational flexibility. We herein wish to report on the chemo-enzymatic synthesis of the divalent sialyl Lewis^x molecule **1** constructed on pentaerythritol with both amine functions of the glucosamine residues bridged by a succinyl group. For the synthesis of the sialyl Lewis^x epitopes, we turned to the well-known strategy based on the use of glycosyltransferases, bringing thus new insights into the specificity of these enzymes.

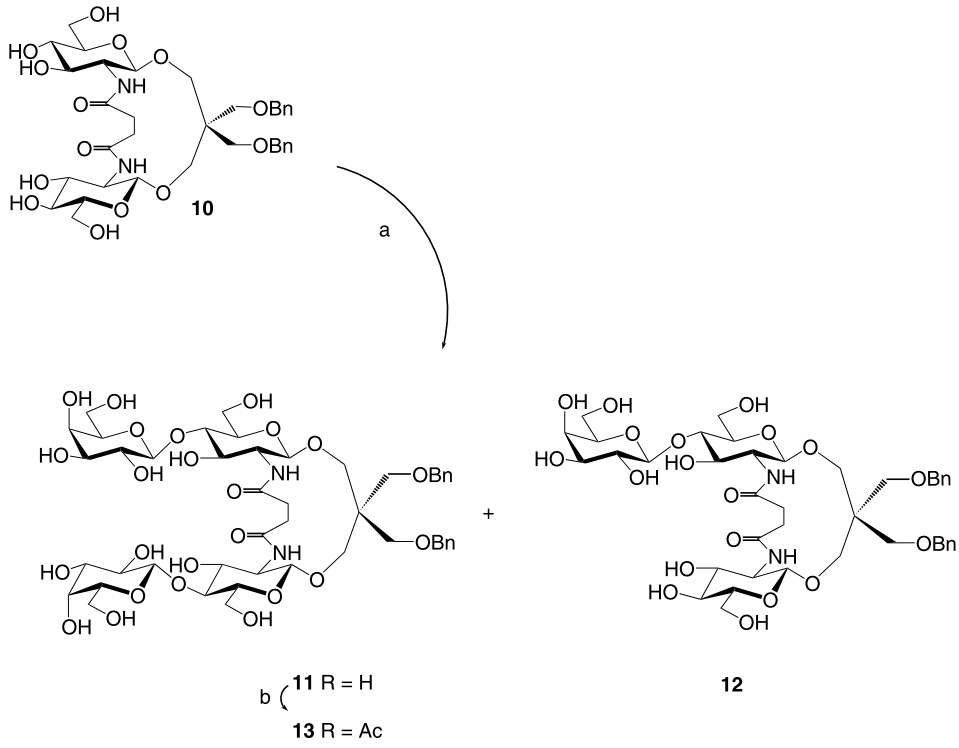
Both carbohydrate moieties of ligand **1** linked by two amide bonds spaced by three single bonds have obviously limited mobility. To our knowledge, this is the first synthesis of a divalent selectin ligand of low entropy. Moreover, the divalent ligand **2** built on pentaerythritol but totally flexible was also prepared in order to allow the direct comparison of the inhibitory activity of both ligands.

2. Results and discussion

We chose the commercially available pentaerythritol as the backbone for our divalent sialyl Lewis x cluster, according to a research program initiated some years ago.[15] Two of the hydroxymethyl groups were substituted with benzyl groups and the other with glucosamine units affording the symmetrical molecule **3**, the synthesis of which has been already described.[16] A drastic treatment of **3** with barium hydroxide[17] afforded the intermediate N-de-acetylated derivative which was reprotected in situ with a *tert*-butyloxycarbonyl group by treatment of the neutralized reaction mixture with di-*tert*-butyl dicarbonate in the presence of triethylamine,[18] to give compound **4** in 82% overall yield. Subsequent conventional acetylation led to compound **5** (Scheme 1). The inherent C_2 symmetry of the molecule was evidenced in the NMR spectrum of **5** in deuterated dimethyl sulfoxide. The amine functions of the di-glucosamine derivative **5** were deprotected by treatment with trifluoroacetic acid affording compound **6** in 92% yield. Then, the acetylated derivative **6** was reacted with succinyl chloride in dichloromethane in the presence of an excess of triethylamine. Reaction conditions were optimized to favour intramolecular reaction leading to the expected cyclization product, rather than the intermolecular reaction affording the di-substituted derivative. Thus, a satisfactory yield of 42% for the desired compound **7** was reached by high dilution condensation of compound **6** (1 mM) with a slight excess of succinyl chloride (1.4 equiv). Yields could not be increased by lowering the temperature to 0 °C or by progressive addition of the reagent. However, a significant proportion (32%) of the di-succinylated products, isolated as the dimethyl ester **8** or the mixed mono-ester and mono-acid **9**, was also obtained, in addition to 15% of the starting material **6** (Scheme 1). Deacetylation of compound **7** afforded the precursor **10** that was elabo-



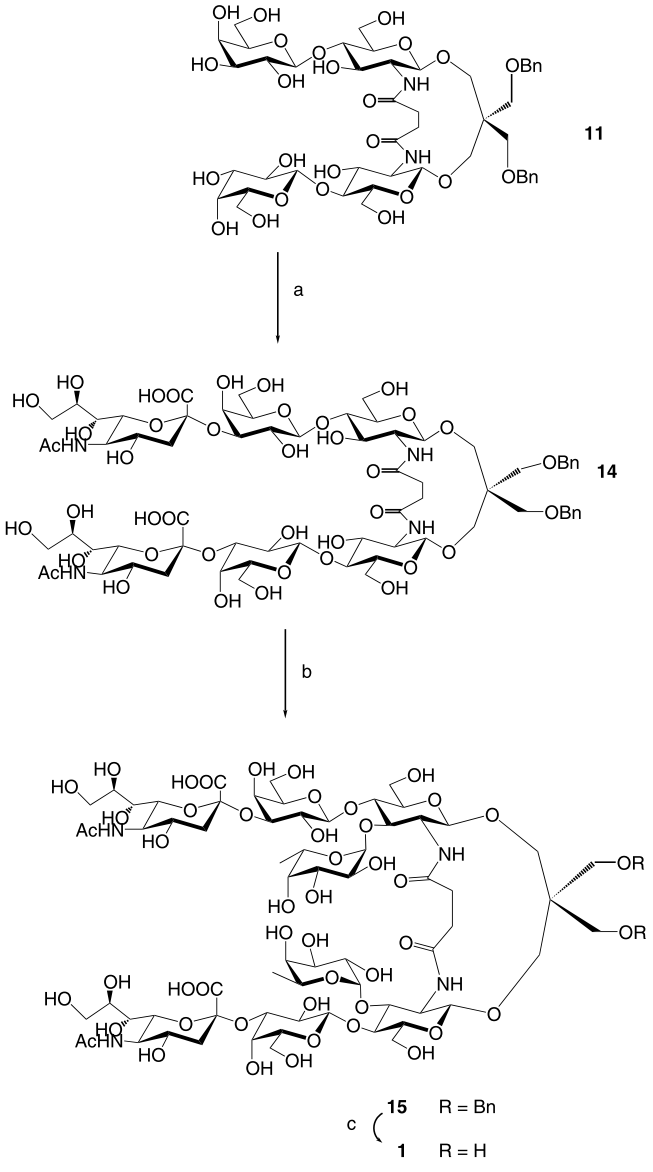
Scheme 1. *Reagents and conditions*: (a) $Ba(OH)_2$, 110 °C, 20 h; then M $H_2SO_4 \rightarrow$ pH 2; $(Boc)_2O$, water–dioxane, Et_3N, 18 h, rt, 82%; (b) Ac_2O, pyridine, 16 h, rt, 90%; (c) CF_3COOH, CH_2Cl_2, 1 h, rt, 92%; (d) ClCO–$(CH_2)_2$–COCl 1.4 equiv, CH_2Cl_2, Et_3N, 13 h, rt, 42% for **7**.



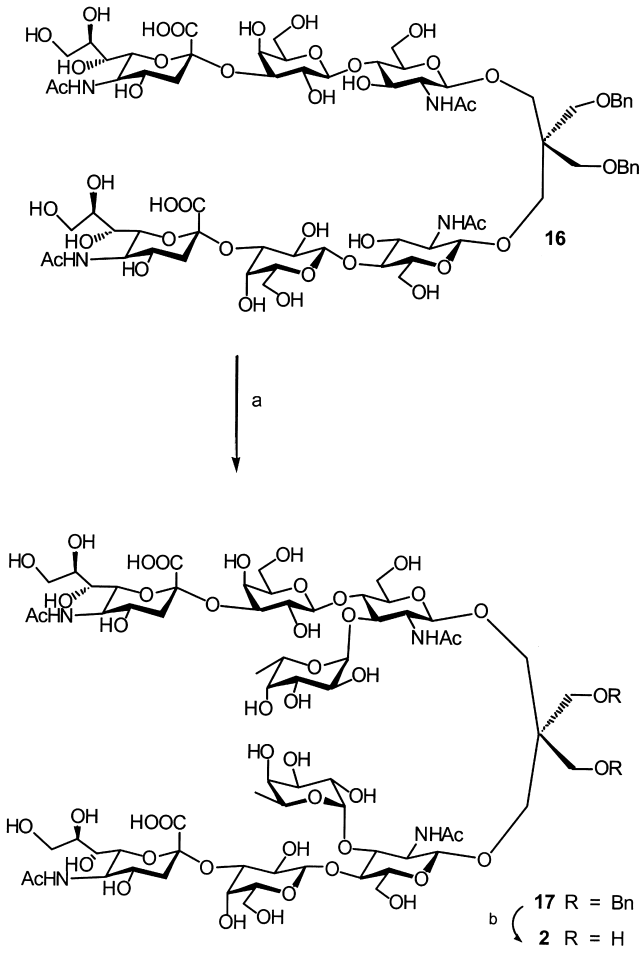
Scheme 2. *Reagents and conditions*: (a) GalT, UDPGE, UDP-Glc 2 equiv, AP, 15 mM $MnCl_2$, 25 mM sodium cacodylate buffer pH 7.4, 37 °C, 4 days, 70% (for **11**), 20% (for **12**); (b) Ac_2O, pyridine, 16 h, rt, 81%.

rated, using glycosyltransferases[19–21] that catalyse the regio- and stereospecific transfer of a monosaccharide to oligosaccharide acceptors from a sugar-nucleotide donor. Compound **10** was first incubated with commercial β-(1→4)-galactosyltransferase (E.C.2.4.1.22), manganese chloride, alkaline phosphatase[22] together with UDP-Glc and UDP-Glc epimerase that epimerize in situ UDP-Glc into UDP-Gal. The glucosamine derivative with restricted flexibility could be galactosylated very efficiently, a result in accordance with previous reports on enzymatic galactosylation of non-natural glucosamine acceptors.[23] After 6 days of incubation, the di-galactosylated compound **11** was isolated in 70% yield, along with the mono-galactosylated compound **12** (20%) (Scheme 2). The structure of **11** was unambiguously assigned from the ^{1}H NMR spectrum of the peracetylated derivative **13**, readily obtained by treatment of **11** with acetic anhydride and pyridine, which clearly showed a two-proton integrating signal at 5.34 ppm, with a small coupling constant, characteristic of the most deshielded H-4 equatorial galactose protons. The following two enzymatic steps were achieved with recombinant mammalian glycosyltransferases produced in baculovirus-infected insect cells. The recombinant enzymes tagged with a six histidine tail were immobilized on nickel-Agarose, a procedure that proved to be of benefit for synthetic purposes.[24] Mammalian biosynthesis requires that sialylation precedes fucosylation. Thus first, compound **11** was incubated with the recombinant CMP-Neu5Ac:β-D-Gal*p*-(1→3/4)-β-D-Glc*p*NAc-(2→3′)-α-sialyltransferase (EC 2.4.99.5, ST3Gal-III) adsorbed on Ni^{2+}-Agarose and CMP-Neu5Ac in 25 mM sodium cacodylate buffer, to afford the disialylated derivative **14** in excellent yield (85%) (Scheme 3). The downfield shift for both galactose H-3 at 4.11 ppm in the ^{1}H NMR spectrum of **14** confirmed the structure. The next step utilized recombinant GDP-Fuc:β-D-Gal*p*-(1→3/4)-β-D-Glc*p*NAc-(2→3′)-α-fucosyl-transferase (EC 2.4.1.65, FucT-III), the only recombinant fucosyltransferase at our disposal,[24] that is known to exhibit a lower affinity for β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc (Type 2) than for Gal*p*-(1→3)-β-D-Glc*p*NAc (Type 1) sequences. Nevertheless, provided that high substrate concentration and a larger amount of enzyme were used, fucosylation of compound **14** could be achieved in good yield (80%), by incubation with FucT-III expressed in insect cells and adsorbed on Ni^{2+}-Agarose, GDP-Fuc, manganese chloride and alkaline phosphatase. Finally, hydrogenolytic debenzylation led to the target di-sialyl Lewis^x molecule **1** in 95% yield. The flexible divalent ligand **2**, required as a reference compound for evaluation of its inhibitory activity towards selectins, was obtained from the disialylated compound **16**[16] through the same sequence, fucosylation leading to compound **17** and debenzylation (Scheme 4). Intermediates lacking sialic acids, both divalent Lewis^x molecules, the rigid **19** and the

flexible analog **22** were also prepared. Fucosylation was achieved with another source of FucT-III: we used the recombinant enzyme expressed in Chinese Hamster Ovary (CHO) cells and immobilized on Ni^{2+}-Agarose,[24] an enzymatic preparation unsuitable for fucosylation of sialylated oligosaccharides because it contains residual sialidase activity. The fucosylated intermediates **18** and **21** were not obtained in as good yields as compounds **15** and **17**, probably because of the lower enzyme affinity for these nonsialylated oligosaccharide acceptors than for these sialylated ones.



Scheme 3. *Reagents and conditions*: (a) ST3Gal-III immobilized on Ni^{2+}-NTA-Agarose, CMP-Neu5Ac 3.2 equiv, 25 mM sodium cacodylate buffer pH 7.1, 37 °C, 4 days, 85%; FucT-III immobilized on Ni^{2+}-NTA-Agarose, GDP-Fuc 2.8 equiv, AP, 15 mM $MnCl_2$, 25 mM sodium cacodylate buffer pH 6.6, 30 °C, 5 days, 80%; (c) 20% $Pd(OH)_2$–C, H_2, MeOH–water, 16 h, 95%.



Scheme 4. *Reagents and conditions*: (a) FucT-III immobilized on Ni^{2+}-NTA-Agarose, GDP-Fuc 2.8 equiv, AP, 15 mM $MnCl_2$, 25 mM sodium cacodylate buffer pH 6.6, 30 °C, 5 days, 82%; (b) 20% $Pd(OH)_2$–C, H_2, MeOH–water, 16 h, 95%.

Again debenzylation led to the target molecules **19** and **22** (Scheme 5).

All of these divalent derivatives are now under biological evaluation towards E, L and P-selectins. It will be most interesting to compare the inhibitory activity of sialyl Lewis^x ligands, **1** and **2**. Compound **1**, the first divalent sialyl Lewis^x with restricted flexibility is expected to have much better inhibition effect than divalent sialyl Lewis^x **2**, in which the six single bonds between both sialyl Lewis^x epitopes allow for considerable conformational flexibility.

3. Experimental

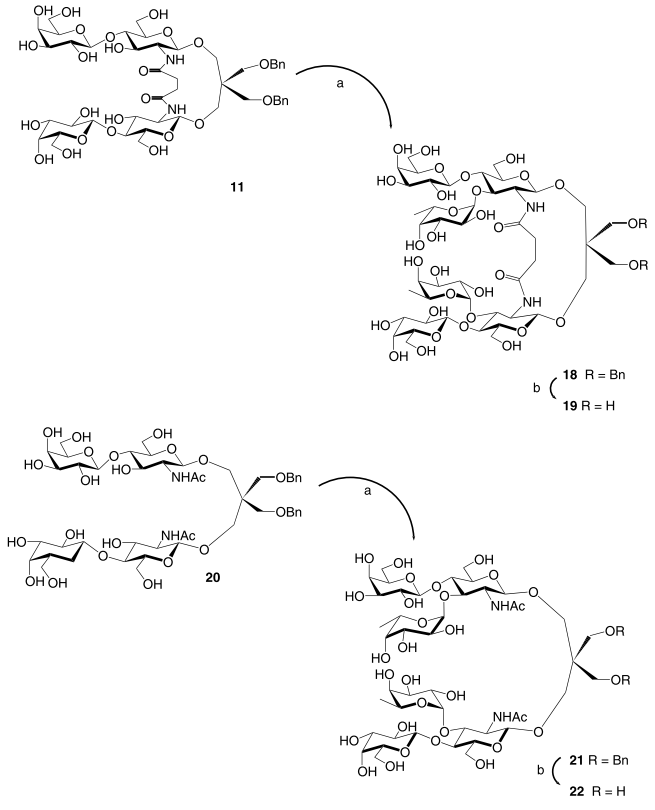
3.1. General

NMR spectra were recorded with Bruker AC-200 or AC-250 spectrometers; the chemical shifts are given relative to the signal of tetramethylsilane in $CDCl_3$; for

^{1}H and ^{13}C NMR spectra in D_2O, acetone (δ 2.22 and 30.5 ppm) was used as an internal reference. Optical rotations were measured with a JASCO digital micropolarimeter. Elemental analyses were performed at the Service Central de Microanalyse, CNRS, Gif sur Yvette, France. Mass spectra were performed on a Finigan MATT 95 apparatus using ESI. Reactions were monitored by TLC on Silica Gel $60F_{254}$ with detection by charring with 10% H_2SO_4 in EtOH or 2% orcinol in 10% H_2SO_4. C_{18} Sep-Pak cartridges were from Millipore-Waters. CMP-Neu5Ac and GDP-Fuc were purchased from Kyowa Hakko, UDP-Glc from Sigma, calf intestine alkaline phosphatase from Roche Molecular Biochemicals, bovine milk D-GlcNAc-β-(1→4)-galactosyltransferase and UDP-glucose 4-epimerase from Calbiochem.

3.2. 2,2-Bis-(benzyloxymethyl)-1,3-bis[2-*tert*-(butyloxycarbonyl-amino)-2-deoxy-β-D-glucopyranosyloxy]-propane (4)

To a solution of **3** (1.496 g, 2.07 mmol) in degassed water (14 mL) was added barium hydroxide (7.84 g, 24.84 mmol) and the mixture stirred at 110 °C for 20 h under Ag. The solution was acidified to pH 2 with M sulfuric acid, filtered and the remaining solution evaporated to dryness. The residue was dissolved in 1:1 water–dioxane (16 mL), triethylamine (0.60 mL, 4.14 mmol) and di-*tert*-butyl dicarbonate (1.8 g, 8.28 mmol) were added and the mixture was stirred for 18 h at room temperature (rt). Then solvents were evaporated and the residue was purified by flash chromatography on silica gel (95:5 EtOAc–MeOH) to afford compound **4** (1.42 g, 82%); $[\alpha]_D^{29}$ −13° (*c* 1, MeOH); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.30–7.20 (m, 10 H, Ph), 6.4 (d, 2 H, $J_{NH,2}$ 6.4 Hz, NH), 4.44 (s, 4 H, 2 CH_2Ph), 4.19 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 3.95 (d, J_{gem} 9.8 Hz, 2 CH), 3.83 (d, 2 H, $J_{6,6'}$ 11.7 Hz, 2 H-6), 3.67 (dd, 2 H, $J_{6',5}$ 5.3 Hz, 2 H-6′), 3.57–3.39 (m, 6 H, 2 H-3, 2 H-4, 2 CH), 3.37–3.16 (m, 8 H, 2 CH_2, 2 H-5, 2 H-2) and 1.40 (s, 18 H, 2 $(CH_3)_3$C); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 158.3 (NHCO), 140.1, 129.3, 128.6, (C–Ar), 104.3 (C-1), 80.2 ($(CH_3)_3C$), 77.6, 75.8, 72.1 (C-3, C-4, C-5), 74.4 (CH_2Ph), 69.9, 69.4, (CH_2), 62.7 (C-6), 58.4 (C-2), 46.7 (Cq) and 29.0 (CH_3); ES$^+$MS: *m*/*z* 861.3 $[M + Na]^+$. Anal. Calcd for $C_{41}H_{62}N_2O_{16}\cdot 0.5\ H_2O$: C, 58.07; H, 7.49; N, 3.30; O, 31.13. Found: C, 58.23; H, 8.02; N, 3.08; O, 30.91.



Scheme 5. *Reagents and conditions*: (a) FucT-III immobilized on Ni^{2+}-NTA-Agarose, GDP-Fuc 2.8 equiv, AP, 15 mM $MnCl_2$, 25 mM sodium cacodylate buffer pH 6.6, 30 °C, 8 days, 74% (for **18**), 50% (for **21**); (b) 20% $Pd(OH)_2$–C, H_2, MeOH–water, 16 h, 95%.

3.3. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[3,4,6-tri-*O*-acetyl-2-(*tert*-butyloxycarbonyl)-amino-2-deoxy-β-D-glucopyranosyloxy]-propane (5)

To a solution of **4** (0.70 g, 0.84 mmol) in pyridine (10 mL) cooled to 0 °C was added acetic anhydride (8 mL) and the mixture was stirred at rt for 16 h. Then solvents were evaporated, co-evaporated with toluene and the residue was chromatographed on silica gel (2:1 petroleum ether–EtOAc) to give pure **5** (0.808 g, 90%); $[\alpha]_D^{31}$ −26° (*c* 0.5, CH_2Cl_2); ^{1}H NMR (Me_2SO-d_6, 250 MHz): δ 7.35–7.24 (m, 10 H, Ph), 6.97 (d, $J_{NH,2}$ 10 Hz, NH), 5.06 (t, 2 H, $J_{4,5} = J_{4,3}$ 10 Hz, 2 H-4), 4.82 (t, 2 H, $J_{3,2}$ 10 Hz, 2 H-3), 4.48–4.35 (m, 6 H, J_{gem} 12, $J_{1,2}$ 8.3 Hz, 2 CH_2Ph, 2 H-1), 4.20 (dd, 2 H, $J_{6',6}$ 12, $J_{6,5}$ 4.5 Hz, 2 H-6), 3.98 (dd, 2 H, $J_{6',5}$ 2 Hz, 2 H-6′), 3.78 (d, 1 H, J_{gem} 10 Hz, CH), 3.71 (m, 2 H, 2 H-2), 3.52–3.25 (m, 9 H, 2 H-5, 3 CH_2, CH), 2.02 (2 s, 18 H, 6 OAc) and 1.45 (s, 18 H, 2 $(CH_3)_3$C); ^{13}C NMR (Me_2SO-d_6, 50.3 MHz): δ 169.9, 169.7, 169.3 (CO), 155.1 (NHCO), 138.4, 128.1, 127.1 (C–Ar), 101.7 (C-1), 72.5 (CH_2Ph), 77.9, 72.4, 70.7 (C-3, C-4, C-5), 68.7, 68.3, 68.1 (CH_2O), 61.8 (C-6), 54.7 (C-2), 44.9 (Cq), 28.0 $(CH_3)_3$) and 20.4 ($COCH_3$); HRMS: Calcd for $C_{53}H_{74}N_2NaO_{22}$ $[M + Na]^+$ 1113.4630. Found: *m*/*z* 1113.4629.

3.4. 2,2-Bis-(benzyloxymethyl-1,3-bis(3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyloxy)-propane (6)

To a solution of **5** (0.608 g, 0.56 mmol) in CH_2Cl_2 (2.8 mL) was added trifluoroacetic acid (2.7 mL) and the

solution was stirred for 1 h at rt. Then solvents were evaporated and flash chromatography on silica gel of the residue (97:3 CH_2Cl_2–MeOH) gave pure **6** (0.573 g, 92%); $[\alpha]_D^{27}$ −3.5° (*c* 0.38, CH_2Cl_2) ^{1}H NMR ($CDCl_3$, 200 MHz): δ 8.2–7.9 (m, 6 H, 2 NH_3), 7.28 (s, 10 H, Ph), 5.30 (t, 2 H, $J_{4,3} = J_{4,5}$ 9.2 Hz, 2 H-4), 5.03 (t, 2 H, $J_{3,2}$ 9.2 Hz, 2 H-3), 4.55 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 4.5 (s, 4 H, 2 CH_2Ph), 4.33 (dd, 2 H, $J_{6',6}$ 11.7, $J_{6,5}$ 3 Hz, 2 H-6), 4.07 (d, 2 H, 2 H-6′), 3.92 (d, 2 H, J_{gem} 8.3 Hz, 2 CH), 3.78 (d, 2 H, 2 CH), 3.65 (m, 2 H, 2 H-5), 3.54 (2 d, 4 H, 4 CH), 3.31 (dd, 2 H, 2 H-2) and 2.05 (s, 18 H, 6 OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 171.8, 170.1 (CO), 138.0, 128.2, 127.4 (C–Ar), 98.5 (C-1), 73.5 (CH_2Ph), 73.5, 71.6, 71.2 (C-3, C-4, C-5), 68.3 (CH_2O), 61.7 (C-6), 54.8 (C-2), 44.5 (Cq), 20.6, 20.5 and 20.3 (CO*C*H$_3$); HRMS: Calcd for $C_{43}H_{59}N_2O_{18}$ $[M+H]^+$ 891.3763. Found: *m*/*z* 891.3765. Anal. Calcd for $C_{43}H_{58}N_2O_{18}$: C, 57.90; H, 6.67; N, 3.14. Found: C, 58.02; H, 7.02; N, 3.21.

3.5. 2,2-Bis(benzyloxymethyl)-1,3-bis[3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyloxy]-propane butane 1*N*,4*N*′-dioyl amide (7), 2,2-bis-(benzyloxymethyl)-1,3-bis-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(3-methyloxycarbonyl-propanamido)-β-D-glucopyranosyloxy]-propane (8) and 2,2-bis-(benzyloxymethyl)-1-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(3-methyloxycarbonyl-propanamido)-β-D-glucopyranosyloxy]-3-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(3-carboxyl-propanamido)-β-D-glucopyranosyloxy]-propane (9)

To a solution of **6** (150 mg, 0.134 mmol) in CH_2Cl_2 (130 mL) was added succinyl chloride (0.015 mL, 0.134 mmol) dissolved in CH_2Cl_2 (1 mL). After stirring for 5 min, triethylamine (0.113 mL, 0.67 mmol) was added and the mixture was stirred for 10 h at rt. Succinyl chloride (0.006 mL, 0.054 mmol) in CH_2Cl_2 (0.4 mL) and triethylamine (0.023 mL, 0.134 mmol) were added again and the solution was stirred for 3 h. The reaction mixture was quenched with MeOH and solvents were evaporated; the residue dissolved in CH_2Cl_2, washed with water, concentrated to dryness, was purified by flash chromatography on silica gel (97:3 CH_2Cl_2–MeOH) to give in the order of elution: **8** (12 mg, 8%), remaining starting material **6** (22 mg, 15%), **7** (55 mg, 42%), and **9** (35 mg, 24%).

Compound **7**: $[\alpha]_D^{24}$ −5° (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.40–7.20 (m, 10 H, Ph), 6.10 (d, 2 H, $J_{NH,2}$ 8.8 Hz, NH), 5.24 (t, 2 H, $J_{4,3} = J_{4,5}$ 9.8 Hz, 2 H-4), 5.04 (t, 2 H, $J_{3,2}$ 9.8 Hz, 2 H-3), 4.57 (d, 2 H, $J_{1,2}$ 8.8 Hz, 2 H-1), 4.46 (2 d, 4 H, J_{gem} 12.2 Hz, 2 CH_2Ph), 4.26 (dd, 2 H, $J_{6',6}$ 12.2, $J_{6,5}$ 4.4 Hz, 2 H-6), 4.04 (d, 2 H, 2 H-6′), 3.82–3.58 (m, 8 H, J_{gem} 9.3 Hz, 4 CH, 2 H-2, 2 H-5), 3.54 (d, 2 CH), 3.40 (d, 2 CH), 2.38 (2 d, 4 H, J_{gem} 13.2 Hz, 2 CH_2) and 2.05–1.95 (m, 18 H, 6 OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 172.1, 170.7, 169.5 (CO), 138.8, 128.3, 127.6 (C–Ar), 101.1 (C-1), 73.3 (CH_2Ph), 72.2, 71.5, 69.4 (C-3, C-4, C-5), 68.8, 68.7 (CH_2O), 62.1 (C-6), 55.1 (C-2), 45.0 (Cq), 31.6 (CO*C*H$_2$) and 20.7 (CO*C*H$_3$); HRMS: Calcd for $C_{47}H_{60}N_2NaO_{20}$ $[M+Na]^+$ 995.3637. Found: *m*/*z* 995.3636.

Compound **8**: $[\alpha]_D^{26}$ −11° (*c* 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6, 250 MHz): δ 7.99 (d, 2 H, $J_{NH,2}$ 8.8 Hz, NH), 7.35–7.2 (m, 10 H, Bn), 5.07 (t, 2 H, $J_{4,3} = J_{4,5}$ 9.8 Hz, 2 H-4), 4.82 (t, 2 H, $J_{3,2}$ 9.8 Hz, 2 H-3), 4.47 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 4.43 (d, 2 H, J_{gem} 12.2 Hz, 2 CHPh), 4.37 (d, 2 H, 2 CHPh), 4.21 (dd, 2 H, $J_{6,5}$ 4.3, $J_{6,6'}$ 12.2 Hz, 2 H-6), 3.96 (dd, 2 H, $J_{6',5}$ 2 Hz, 2H-6′), 3.87–3.70 (m, 6 H, 2 H-5, 2 CH_2), 3.53 (s, 6 H, 2 OCH_3), 3.35–3.25 (m, 6 H, 2 H-2, 2 CH_2), 2.46–2.12 (m, 8 H, 4 CH_2), 1.97 (s, 6 H, 2 OAc), 1.95 (s, 6 H, 2 OAc) and 1.91 (s, 6 H, 2 OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 173.7 (CO_2Me), 171.3, 170.7, 169.4 (CO), 138.7, 128.4, 128.2, 127.7, 127.4 (C–Ar), 101.4 (C-1), 73.4 (CH_2Ph), 72.5, 71.7, 71.5 (C-3, C-4, C-5), 68.8, 68.7 (CH_2O), 62.2 (C-6), 54.2 (C-2), 52.0 (CH_3), 45.2 (Cq), 30.5 (*C*H$_2$CO), 28.8 (*C*H$_2$CO) and 20.6 (*C*H$_3$CO); ES$^+$MS: *m*/*z* 1141.3 $[M+Na]^+$. Anal. Calcd for $C_{53}H_{70}N_2O_{24}$: C, 56.88; H, 6.30. Found: C, 56.81; H, 6.56.

Compound **9**: $[\alpha]_D^{24}$ −14° (*c* 1, $CHCl_3$); ^{1}H NMR (Me_2SO-d_6, 250 MHz): δ 8.04 (d, 1 H, $J_{NH,2}$ 10.3 Hz, NH), 8.0 (d, 1 H, $J_{NH,2}$ 10.3 Hz, NH), 7.35–7.2 (m, 10 H, Ph), 5.07 (t, 2 H, $J_{4,5} = J_{4,3}$ 9.8 Hz, 2 H-4), 4.82 (t, 2 H, $J_{3,4} = J_{3,2}$ 9.8 Hz, 2 H-3), 4.48 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 4.43 (d, 2 H, J_{gem} 12.2 Hz, 2 CHPh), 4.37 (d, 2 H, 2 CHPh), 4.21 (dd, 2 H, $J_{6,5}$ 3, $J_{6,6'}$ 12.2 Hz, 2 H-6), 3.97 (d, 2 H, 2 H-6′), 3.87–3.69 (m, 6 H, 2 H-5, 2 CH_2O), 3.54 (s, 3 H, OCH_3), 3.34–3.18 (m, 6 H, 2 H-2, 2 CH_2O), 2.46–2.12 (m, 8 H, 4 CH_2), 1.97 (s, 6 H, 2 OAc), 1.95 (s, 6 H, 2 OAc) and 1.91 (s, 6 H, 2 OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 172.3, 172.0 (CO_2Me, CO_2H), 171.0, 169.6 (CO), 138.4, 128.1, 127.4 (C–Ar), 101.2 (C-1), 73.3 (CH_2Ph), 72.7, 72.4, 71.3 (C-3, C-4, C-5), 68.6 (CH_2O), 62.0 (C-6), 54.0, 53.8 (2 C-2), 51.6 (OCH_3), 45.1 (Cq), 30.6, 30.3 (*C*H$_2$CONH), 29.0, 28.7 (*C*H$_2$COO) and 20.3 (*C*H$_3$CO); ES$^+$MS: *m*/*z* 575.1 $[(M+2\ Na)/2]^+$, 1127.4 $[M+Na]^+$. Anal. Calcd for $C_{52}H_{68}N_2O_{24}$: C, 56.52; H, 6.20; N, 2.53; O, 34.75. Found: C, 56.67; H, 6.46; N, 2.51; O, 34.48.

3.6. 2,2-Bis-(benzyloxymethyl-1,3-bis-[2-amino-2-deoxy-β-D-glucopyranosyloxy]-propane butane 1*N*,4*N*′-dioyl amide (10)

Compound **7** (90 mg, 0.092 mmol) dissolved in 8:2:1 MeOH–triethylamine–water (5.5 mL) was stirred at rt for 24 h, then concentrated to dryness. The residue was chromatographed on silica gel (8:3:1 EtOAc–2-propanol–water) to afford **10** (65 mg, 92%); $[\alpha]_D^{31}$ −3° (*c* 1, MeOH); ^{1}H NMR (D_2O, 200 MHz): δ 7.20 (s, 10

H, Ph), 4.37 (s, 4 H, CH_2Ph), 4.22 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1) and 2.5 (2 d, 4 H, J_{gem} 11.7 Hz, 2 CH_2CO); ^{13}C NMR (D_2O, 62.9 MHz): δ 175.5 (NHCO), 138.9, 129.5, 129.1 (C–Ar), 102.9 (C-1), 74.1 (CH_2Ph), 76.6, 74.7, 70.8 (C-3, C-4, C-5), 69.8, 69.2 (CH_2O), 61.7 (C-6), 56.7 (C-2), 45.6 (Cq) and 31.9 ($COCH_2$); HRMS: Calcd for $C_{35}H_{48}N_2O_{14}Na$ $[M+Na]^+$ 743.3003. Found: m/z 743.3003. Anal. Calcd for $C_{35}H_{48}N_2O_{14}\cdot 1\ H_2O$: C, 56.89; H, 6.83; N, 3.79; O, 32.59. Found: C, 56.62; H, 6.81; N, 3.69; O, 32.17.

3.7. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[*O*-β-D-galactopyranosyl-(1→4)-(2-amino-2-deoxy-β-D-glucopyranosyloxy)]-propane butane-1*N*,4*N*′-dioyl amide (11) and 2,2-bis-benzyloxymethyl-1-(*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-oxy)-3-[*O*-β-D-galactopyranosyl-(1→4)-(2-amino-2-deoxy-β-D-glucopyranosyloxy)]-propane butane-1*N*,4*N*′-dioyl amide (12)

Compound **10** (60 mg, 0.083 mmol), UDP-Glc (51 mg, 0.083 mmol), bovine milk D-GlcNAc β-(1→4)-galactosyltransferase (0.3U), UDP-glucose-4-epimerase (1.5 U), were incubated at 37 °C in 25 mM sodium cacodylate buffer pH 7.4 (6 mL) containing 15 mM $MnCl_2$. The reaction was monitored by TLC on silica gel (3:3:1 EtOAc–2-propanol–water). After 48 h UDP-Glc (51 mg, 0.083 mmol), bovine milk D-GlcNAc β-(1→4)-galactosyltransferase (0.2 U), UDP-glucose-4-epimerase (1 U) and $MnCl_2$ (10 mg, 0.05 mmol) were added again together with alkaline phosphatase (2 U) and the incubation was continued for 48 h. The reaction mixture divided into five portions was applied to Sep-Pak C_{18} cartridges; the combined MeOH eluates were evaporated to dryness and the residue was purified by flash chromatography on silica gel (6:4:1 EtOAc–2-propanol–water) to give the di-galactosylated compound **11** (60 mg, 70%) and the mono-galactosylated compound **12** (15 mg, 20%).

Compound **11**: $[\alpha]_D^{27}$ −4° (*c* 1, water); ^1H NMR (D_2O, 200 MHz): δ 7.4 (s, 10 H, Ph), 4.60–4.43 (m, 6 H, $J_{1',2'}$ 8.8, J_{gem} 12.2 Hz, 2 H-1′, 2 CH_2Ph), 4.36 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1) and 2.62 (2 d, 4 H, J_{gem} 12.7 Hz, 2 CH_2CO); ^{13}C NMR (D_2O, 50.3 MHz): δ 175.5 (NHCO), 138.7, 129.6, 129.2 (C–Ar), 103.9 (C-1′), 102.9 (C-1), 79.4 (C-4), 76.3, 75.6, 74.2, 73.5, 73.2, 71.9, 69.6 (C-2′, C-3′, C-4′, C-5′, C-3, C-5, $PhCH_2$), 69.5, 69.3 (CH_2O), 62.0, 61.0 (C-6, C-6′), 56.2 (C-2), 45.5 (Cq) and 31.9 (CH_2CO); HRMS: Calcd for $C_{47}H_{68}N_2O_{24}Na$ $[M+Na]^+$ 1067.4076. Found: m/z 1067.4060.

Compound **12**: $[\alpha]_D^{27}$ +1° (*c* 1, MeOH); ^1H NMR (D_2O, 250 MHz): δ 7.4–7.3 (m, 10 H, Bn), 4.55–4.41 (m, 5 H, $J_{1',2'}$ 8.8 Hz, H-1′, 2 CH_2Ph), 4.31 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1) and 2.60 (2 d, 4 H, J_{gem} 11.7 Hz, 2 CH_2CO); ^{13}C NMR (D_2O, 50.3 MHz): δ 175.4, 175.3 (NHCO), 138.8, 129.6, 129.1 (C–Ar), 103.9 (C-1′), 102.9 (C-1), 79.4 (C-4), 76.7, 76.4, 75.6, 74.7, 73.6, 73.4, 72.0, 70.9, 69.6 (C-2′, C-3′, C-4′, C-5′, 2 C-3, C-4, 2 C-5), 74.2 (CH_2Ph), 69.8, 69.5 (CH_2O), 62.0, 61.7, 61.1 (2 C-6, C-6′), 56.7, 56.2 (2 C-2), 45.6 (Cq) and 31.9 (CH_2CO); ES^+MS: m/z 905.2 $[M+Na]^+$. Anal. Calcd for $C_{41}H_{58}N_2O_{19}\cdot 2\ H_2O$: C, 53.58; H, 6.80; N, 3.05. Found: C, 53.34; H, 6.64; N, 2.89.

Acetylation of compound **11**: to **11** (22 mg, 0.021 mmol) dissolved in pyridine (4 mL) was added acetic anhydride (2 mL); the mixture was stirred overnight at rt, then evaporated to dryness, co-evaporated twice with toluene and the residue was purified by flash chromatography (9:1 EtOAc–petroleum ether) to afford the peracetylated derivative **13** (26 mg, 81%); $[\alpha]_D^{27}$ −12° (*c* 1, CH_2Cl_2); ^1H NMR ($CDCl_3$, 250 MHz): δ 7.38–7.27 (m, 10 H, Ph), 5.80 (d, 2 H, $J_{NH,2}$ 9.3 Hz, 2 NH), 5.34 (d, 2 H, $J_{3',4'}$ 3.5 Hz, 2 H-4′), 5.11 (dd, 2 H, $J_{1',2'}$ 8.3, $J_{2',3'}$ 10.3 Hz, 2 H-2′), 5.02–4.93 (m, 4 H, 2 H-3, 2 H-3′), 4.54–4.35 (m, 8 H, $J_{1',2'}$ 8.3, J_{gem} 12, $J_{6a,6b}$ 12, $J_{6a,5}$ 2 Hz, 2 H-1′, 2 H-6a, 4 CHPh), 4.24 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 4.18–4.02 (m, 6 H, 2-H6b, 2 H-6′a, 2 H-6′b), 3.99–3.83 (m, 4 H, 2 H-2, 2 H-5′), 3.72–3.59 (m, 6 H, J_{gem} 9.3, $J_{3,4}$ 9.8, $J_{4,5}$ 9.3 Hz, 2 H-4, 4 CHO), 3.54–3.46 (m, 4 H, 2 H-5, 2 CHO), 3.37 (d, 2 H, 2 CHO), 2.49 (d, 2 H, J_{gem} 13 Hz, 2 CHCO), 2.20 (d, 2 H, 2 CHCO), 2.15 (s, 6 H, 2 OAc), 2.06 (s, 6 H, 2 OAc), 2.05 (s, 12 H, 4 OAc), 2.01 (s, 6 H, 2 OAc) and 1.97(s, 6 H, 2 OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 172.5 (NHCO), 170.6, 170.4, 170.3, 170.1, 169.4 (CO), 138.5, 128.0, 127.1 (C–Ar), 100.9 (C-1), 100.6 (C-1′), 76.3, 73.0, 72.6, 72.0, 70.8, 70.3, 68.9, 66.6 (C-2′, C-3′, C-4′, C-5′, C-3, C-5, $PhCH_2$, CH_2O), 62.0 (C-6), 60.6 (C-6′), 53.7 (C-2), 44.5 (Cq), 31.0 (CH_2CO), 20.4 and 20.2 (CH_3CO); ES^+MS: m/z 1571.5 $[M+Na]^+$, 797.3 $[(M+2\ Na)/2]^+$.

3.8. 2,2-Bis-(benzyloxymethyl)-1,3-bis[*O*-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-(2-amino-2-deoxy-β-D-glucopyranosyloxy)]-propane butane-1*N*,4*N*′-dioyl amide (14)

Compound **11** (40 mg, 0.038 mmol), CMP-NeuAc (25 mg, 0.038 mmol) and ST3Gal-III adsorbed on Ni^{2+}-Agarose (50 mU) were incubated in 50 mM sodium cacodylate buffer pH 7.1 (12 mL) at 30 °C for 4 days with gentle stirring. Additional CMP-NeuAc (27 mg, 0.041 mmol) was added twice, after 24 and 48 h. At the end of incubation, the gel was filtered off, washed with 10 mM sodium cacodylate buffer pH 7.1 and filtrate and washings were combined, divided into four portions and applied to Sep-Pak C_{18} cartridges; the combined methanol eluates were evaporated to dryness; then the residue taken up in water was purified on Bio-Gel P2 and passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) to give **14** as its sodium salt (54 mg, 85%); $[\alpha]_D^{27}$ −4° (*c* 1, water); ^1H

NMR (D_2O, 250 MHz): δ 7.5–7.3 (m, 10 H, Ph), 4.59–4.51 (m, 4 H, $J_{1',2'}$ 7.3, J_{gem} 12.2 Hz, 2 H-1′, 2 CHPh), 4.48 (d, 2 H, 2 CHPh), 4.36 (d, 2 H, $J_{1,2}$ 7.3 Hz, 2 H-1), 4.11 (dd, 2 H, $J_{3',4'}$ 3, $J_{3',2'}$ 9.8 Hz, 2 H-3′), 3.39 (d, 2 H, J_{gem} 9.7 Hz, 2 CH), 2.8–2.67 (m, 4 H, $J_{3''e,3''a}$ 12.5, $J_{3''a,4''}$ 4.5, J_{gem} 12.7 Hz, 2 H 3-″e, 2 CHCO), 2.5 (d, 2 H, 2 CHCO), 2.02 (s, 6 H, 2 OAc) and 1.79 (t, 2 H, $J_{3''a,4''}$ 12.5 Hz, 2 H-3″a); ^{13}C NMR (D_2O, 50.3 MHz): δ 176.4, 175.9, 175.3 (NHCO, CO_2Na), 139.1, 130.1, 129.7 (C–Ar), 104.0 (C-1′), 103.3 (C-1), 101.2 (C-2″), 74.7 (CH_2Ph), 79.6 (C-4), 76.9, 76.6, 76.0 (C-5, C-5′, C-3′), 74.3 (C-6″), 73.6 (C-3), 73.2 (C-8″), 71.0, 70.8, 70.3, 69.5, 68.9 (CH_2O, C-2′, C-4′, C-4″, C-7″), 64.0 (C-9″), 62.5, 61.4 (C-6, C-6′), 56.6 (C-2), 53.1 (C-5″), 45.9 (Cq), 41.0 (C-3″), 32.3 (CH_2CO) and 23.4 (CH_3CO); HRMS (negative mode): Calcd for $C_{69}H_{100}N_4O_{40}$ $[(M-2\ Na)/2]^-$ 812.2957. Found: m/z 812.2959.

3.9. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[*O*-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-(2-amino-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane butane-1*N*,4*N*′ dioyl amide (15)

Compound **14** (8 mg, 0.0048 mmol), GDP-Fuc (3 mg, 0.0048 mmol), $MnCl_2$ (12 mg, 0.060 mmol) and FucT-III adsorbed on Ni^{2+}-Agarose (190 mU) were incubated in 25 mM sodium cacodylate buffer pH 6.6 (4 mL) at 30 °C for 5 days with gentle stirring. Additional GDP-Fuc was added twice, after 2 days (3 mg, 0.0048 mmol) and after 4 days (2.4 mg, 0.0038 mmol) together with alkaline phosphatase (2 U) and $MnCl_2$ (4 mg). At the end of incubation the gel was filtered off, washed with 10 mM sodium cacodylate buffer pH 6.6 and filtrate and washings were combined and applied to Sep-Pak C_{18} cartridges; the methanol eluate was evaporated to dryness; then the residue dissolved in water was purified on Bio-Gel P2 and passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) affording 7.5 mg of **15** (80%); $[\alpha]_D^{25}$ −32° (*c* 0.75, water); ^1H NMR (D_2O, 200 MHz): δ 7.44–7.32 (m, 10 H, 2 Ph), 5.08 (d, 2 H, $J_{1''',2'''}$ 4 Hz, 2 H-1‴), 4.83 (m, 2 H, 2 H-5‴), 4.60–4.44 (m, 6 H, J_{gem} 11.7, $J_{1',2'}$ 7.3 Hz, 2 H-1′, 2 CH_2Ph), 4.35 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 4.07 (dd, 2 H, $J_{3',2'}$ 9.3, $J_{3',4'}$ 3 Hz, 2 H-3′), 3.38 (d, 2 H, J_{gem} 9.7 Hz, 2 CH), 2.82–2.64 (m, 4 H, $J_{3''e,3''a}$ 12.5, $J_{3''e,4''}$ 4.5, J_{gem} 12.7 Hz, 2 H-3″e, 2 CHCO), 2.46 (d, 2 H, 2 CHCO), 2.02 (s, 6 H, 2 OAc), 1.75 (t, 2 H, $J_{3''a,4''}$ 12.5 Hz, 2 H-3″a) and 1.16 (d, 6 H, $J_{6''',5'''}$ 6.3 Hz, 2 CH_3); ^{13}C NMR (D_2O, 50.3 MHz): δ 176.5, 175.7, 175.4 (NHCO, CO_2Na), 139.1, 130.1, 129.7 (C–Ar), 102.9 (C-1′, C-1), 101.1 (C-2″), 100.0 (C-1‴), 74.6 (CH_2Ph), 77.1, 76.4, 74.5, 74.3, 73.3, 70.6, 70.1, 69.7, 69.5, 69.1, 68.7, 68.1 (C-5, C-5′, C-3′, C-3, C-6″, C-8″, C-2′, CH_2O, C-4, C-4′, C-4″, C-7″, C-2‴, C-3‴, C-4‴, C-5‴), 64.0 (C-9″), 63.0 (C-6), 60.0 (C-6′), 57.4 (C-2), 53.1 (C-5″), 45.9 (Cq), 41.2 (C-3″), 32.2 (CH_2CO), 23.4 (CH_3CO) and 16.7 (CH_3); HRMS (negative mode): Calcd for $C_{81}H_{120}N_4O_{48}$ $[(M-2\ Na)/2]^-$ 958.3536. Found: m/z 958.3531.

3.10. 2,2-Bis-[*O*-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-(2-amino-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane butane-1*N*,4*N*′-dioyl amide]-1,3-diol (1)

A solution of **15** (8 mg, 0.004 mmol) in 1:1 MeOH–water (0.5 mL) was stirred under hydrogen with 20% $Pd(OH)_2$ on charcoal (10 mg) for 16 h at rt. The solution was then filtered through Celite; the filtrate was purified on Bio-Gel P2 and passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) to afford 7 mg of **1** (95%); $[\alpha]_D^{28}$ −17° (*c* 0.83, water); ^1H NMR (D_2O, 400 MHz): δ 5.18 (d, 2 H, $J_{1''',2'''}$ 4 Hz, 2 H-1‴), 4.88 (m, 2 H, $J_{5''',6'''}$ 6.5 Hz, 2 H-5‴), 4.59 (d, 2 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′), 4.53 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 4.07 (dd, 2 H, $J_{3',2'}$ 9.3, $J_{3',4'}$ 3 Hz, 2 H-3′), 4.08 (d, 2 H, J_{gem} 11.5 Hz, 2 CHO), 3.53 (d, 2 H, 2 CHO), 2.87–2.76 (m, 4 H, $J_{3''a,3'''e}$ 12 Hz, $J_{3''e,4''}$ 4.5 Hz, J_{gem} 12.5 Hz, 2 H-3″e, 2 CHCO), 2.46 (d, 2 H, 2 CHCO), 2.04 (s, 6 H, 2 OAc), 1.87 (t, 2 H, $J_{3''a,4}$ 12 Hz, 2 H-3″a) and 1.24 (d, 6 H, $J_{6''',5'''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 62.9 MHz): δ 176, 175.3, 174.9 (NHCO, CO_2Na), 102.6 (C-1′, C-1), 100.6 (C-2″), 99.6 (C-1‴), 76.6, 76.1, 75.9, 74.3, 73.9; 73, 72.9, 70.6, 70.5, 70.2, 69.6, 69.5, 69.3, 69.1, 68.7, 68.3, 67.7 (C-5, C-5′, C-3′, C-3, C-6″, C-8″, C-2′, CH_2O, C-4, C-4′, C-4″, C-7″, C-2‴, C-3‴, C-4‴, C-5‴), 63.6 (C-9″), 62.5, 61.2, 60.6 (CH_2OH, C-6′, C-6), 57.0 (C-2), 52.7 (C-5″), 46.1 (Cq), 40.8 (C-3″), 31.8 (CH_2CO), 23 (CH_3CO) and 16.3 (CH_3); ESMS (negative mode): m/z 868.3 $[(M-2\ Na)/2]^-$ $C_{67}H_{108}N_4O_{48}Na_2$.

3.11. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[*O*-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2 → 3)-O-β-D-galactopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane (17)

Compound **16** (7 mg, 0.0042 mmol), GDP-Fuc (3 mg, 0.0048 mmol), $MnCl_2$ (12 mg, 0.060 mmol) and FucT-III adsorbed on Ni^{2+}-Agarose (50 mU) were incubated in 25 mM sodium cacodylate buffer pH 6.6 (4 mL) at 30 °C for 5 days with gentle stirring. Additional GDP-Fuc was added twice, after 2 days (2.7 mg, 0.0042 mmol) and after 4 days (2.1 mg, 0.0034 mmol) together with alkaline phosphatase (3 U) and $MnCl_2$ (4 mg). At the end of incubation the gel was filtered off, washed with 10 mM sodium cacodylate buffer pH 6.6 and

filtrate and washings were combined and applied to Sep-Pak C_{18} cartridges; the methanol eluate was evaporated to dryness; then the residue dissolved in water, purified on Bio-Gel P2 and passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) affording 7 mg of **17** (82%); $[\alpha]_D^{24}$ −33°(*c* 0.75, water); ^{1}H NMR (D_2O, 200 MHz): δ 7.44–7.29 (m, 10 H, 2 Ph), 5.04 (d, 2 H, $J_{1'''2'''}$ 4 Hz, 2 H-1‴), 4.75 (m, 2 H, 2 H-5‴), 4.51–4.43 (m, 6 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′, 2 CH_2Ph), 4.34 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 4.05 (dd, 2 H, $J_{3',2'}$ 9.3, $J_{3',4'}$ 2.5 Hz, 2 H-3′), 2.72 (dd, 2 H, $J_{3''e,3''a}$ 12.5, $J_{3''e,4''}$ 4.5 Hz, 2 H-3″e), 1.99 (s, 6 H, 2 OAc), 1.89 (s, 6 H, 2 OAc), 1.76 (t, 2 H, $J_{3''a,4''}$ 12.5 Hz, 2 H3-″a) and 1.14 (d, 6 H, $J_{6''',5'''}$ 6.3 Hz, 2 CH_3); ^{13}C NMR (D_2O, 50.3 MHz): δ 176.0, 174.9, 174.7 (NHCO, CO_2Na), 138.6, 129.7, 129.1 (C–Ar), 102.6 (C-1′, C-1), 100.7 (C-2″), 99.5 (C-1‴), 74.4 (CH_2Ph), 76.6, 76.2, 75.8, 75;7, 74.3, 73.9, 72.8, 70.2, 69.8, 69.7, 69.3, 69.1, 68.7, 68.3, 67.6 (C-5, C-5′, C-3′, C-3, C-6″, C-8″, C-2′, CH_2O, C-4, C-4′, C-4″, C-7″, C-2‴, C-3‴, C-4‴, C-5‴), 63.6 (C-9″), 62.4 (C-6), 60.7 (C-6′), 56.8 (C-2), 52.7 (C-5″), 45.7 (Cq), 40.8 (C-3″), 23.3, 23.0 (CH_3CO) and 16.3 (CH_3); ESMS (negative mode): *m*/*z* 959.3 $[(M-2\ Na)/2]^-$ $C_{81}H_{122}N_4O_{48}Na_2$.

3.12. 2,2-Bis-[*O*-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2→3)-*O*-β-D-galactopyranosyl)-(1→4)-(2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane-1,3-diol (2)

A solution of **17** (9 mg, 0.0047 mmol) in 1:1 MeOH–water (0.6 mL) was stirred under hydrogen with 20% $Pd(OH)_2$ on charcoal (12 mg) for 16 h at rt. The solution was then filtered through Celite; the filtrate was purified on Bio-Gel P2 and passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) to afford 8 mg of **2** (95%); $[\alpha]_D^{28}$ −28°(*c* 0.75, water); ^{1}H NMR (D_2O, 400 MHz): δ 5.02 (d, 2 H, $J_{1''',2'''}$ 4 Hz, 2 H-1‴), 4.73 (m, 2 H, 2 H-5‴), 4.43 (d, 2 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′), 4.34 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 3.99 (dd, 2 H, $J_{3',2'}$ 10.2, $J_{3',4'}$ 3 Hz, 2 H-3′), 4.00 (d, 2 H, J_{gem} 9.8 Hz, 2 CH), 2.74 (dd, 2 H, $J_{3''e,3''a}$ 12.5, $J_{3''e,4''}$ 4.5 Hz, 2 H-3″e), 2.05 (s, 6 H, 2 OAc), 2.03 (s, 6 H, 2 OAc), 1.78 (t, 2 H, $J_{3''a,4'''}$ 2.5 Hz, 2 H-3″a) and 1.16 (d, 6 H′, $J_{6''',5'''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 62.9 MHz): δ 176.0, 175.1, 174.9 (NHCO, CO_2Na), 102.7, 102.6 (C-1′, C-1), 100.7 (C-2″), 99.6 (C-1‴), 76.7, 76.2, 75.9, 75.7, 74.3, 73.9, 72.9, 70.2, 70.0, 69.3, 69.1, 68.8, 68.3, 67.7 (C-5, C-5′, C-3′, C-3, C-6″, C-8″, C-2′, CH_2O, C-4, C-4′, C-4″, C-7″, C-2‴, C-3‴, C-4‴, C-5‴), 63.6 (C-9″), 62.5 (C-6), 61.6, 60.6 (CH_2OH, C-6′), 56.9 (C-2), 52.7 (C-5″), 46.1 (Cq), 40.8 (C-3″), 23.2, 23.0 (CH_3CO) and 16.3 (CH_3); ESMS (negative mode): *m*/*z* 869.3 $[(M-2Na)/2]^-$ $C_{67}H_{110}N_4O_{48}Na_2$.

3.13. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[*O*-β-D-galactopyranosyl-(1→4)-(2-amino-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane butane 1*N*,4*N*′-dioyl amide (18)

Compound **11** (8.5 mg, 0.008 mmol), GDP-Fuc (5.4 mg, 0.008 mmol), $MnCl_2$ (12 mg, 0.060 mmol) and FucT-III adsorbed on Ni^{2+}-Agarose (50 mU) were incubated in 25 mM sodium cacodylate buffer pH 6.6 (4 mL) at 30 °C for 7 days with gentle stirring. Additional GDP-Fuc was added three times, after 2 days (5.4 mg, 0.008 mmol) and after 4 days (2.1 mg, 0.0032 mmol), after 6 days ((2.1 mg, 0.0032 mmol) together with alkaline phosphatase (2 U) and $MnCl_2$ (4 mg). At the end of incubation the gel was filtered off, washed with 10 mM sodium cacodylate buffer pH 6.6 and filtrate and washings were combined and applied to Sep-Pak C_{18} cartridges; the MeOH eluate was evaporated to dryness; then the residue taken up in water was purified on Bio-Gel P2 to give pure **18** (8 mg, 75%) and a mixture of **18** and the monofucosylated product (1.5 mg); $[\alpha]_D^{27}$ −43° (*c* 0.67, water); ^{1}H NMR (D_2O, 400 MHz): δ 7.44–7.33 (m, 10 H, 2 Ph), 5.1 (d, 2 H, $J_{1'',2''}$ 4 Hz, 2 H-1″), 4.84 (m, 2 H, 2 H-5″), 4.54 (d, 2 H, J_{gem} 12 Hz, 2 CHPh), 4.48 (d, 2 H, 2 CHPh), 4.44 (d, 2 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′), 4.36 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 3.38 (d, 2 H, J_{gem} 9.8 Hz, 2 CH), 2.7 (d, 2 H, J_{gem} 13 Hz, 2 CHCO), 2.48 (d, 2 H, 2 CHCO) and 1.16 (d, 6 H, $J_{6'',5''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 62.9 MHz): δ 175.2 (NHCO), 138.8, 129.6, 129.2 (Ph), 102.8, 102.6 (C-1′, C-1), 99.7 (C-1″), 74.2 ($PhCH_2$), 76.1, 76, 73.5, 73.1, 73, 70.3, 69.7, 69.4, 69.2, 68.8, 67.7, 62.6, 62.1, 60.8 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″, C-6, C-6′, CH_2O), 57.0 (C-2), 45.5 (Cq), 32.0 (CH_2CO) and 16.4 (CH_3); HRMS: Calcd for $C_{59}H_{88}N_2O_{32}Na_2$ $[(M+Na)/2]^+$ 691.2558. Found: *m*/*z* 691.2555.

3.14. 2,2-Bis-[*O*-β-D-galactopyranosyl-(1→4)-(2-amino-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)-propane butane-1*N*,4*N*′-dioyl amide]-1,3-diol (19)

A solution of **18** (9 mg, 0.0063 mmol) in 1:1 MeOH–water (0.9 mL) was stirred under hydrogen with 20% $Pd(OH)_2$ on charcoal (18 mg) for 16 h at rt. The solution was then filtered through Celite; the filtrate was purified on Bio-Gel P2 to afford 8 mg of **19** (quantitative yield); $[\alpha]_D^{31}$ 0° (*c* 0.25, water); ^{1}H NMR (D_2O, 250 MHz): δ 5.14 (d, 2 H, $J_{1'',2''}$ 4.5 Hz, 2 H-1″), 4.8 (m, 2 H, 2 H-5″), 4.35 (d, 4 H, $J_{1',2'}$ 7.8, $J_{1,2}$ 7.8 Hz, 2 H-1, 2 H-1, 2 H-1′), 3.99 (d, 2 H, J_{gem} 12 Hz, 2 CH), 3.44 (d, 2 H, J_{gem} 12 Hz, 2 CH), 2.74 (d, 2 H, J_{gem} 13 Hz, 2 CHCO), 2.51 (d, 2 H, 2 CHCO) and 1.17 (d, 2 H, $J_{6'',5''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 50.3 MHz): δ 175.3 (NHCO), 102.8, 102.6 (C-1, C-1′), 99.6 (C-1″), 76.1, 76.0, 75.9, 74.3, 73.5, 72.9, 72.0, 70.2, 69.6, 69.3,

68.7, 67.7 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″, CH_2O), 62.5 (C-6), 61.2, 60.7 (C-6′, CH_2OH), 57.0 (C-2), 46.1 (Cq), 31.8 (CH_2CO) and 16.3 (CH_3); HRMS: Calcd for $C_{45}H_{76}N_2O_{32}$ $[(M+2\ Na)/2]^+$ 601.2088. Found: m/z 601.2087.

3.15. 2,2-Bis-(benzyloxymethyl)-1,3-bis-[*O*-β-D-galactopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane (21)

Compound **20** (8 mg, 0.0076 mmol), GDP-Fuc (5.1 mg, 0.0076 mmol), $MnCl_2$ (12 mg, 0.060 mmol) and FucT-III adsorbed on Ni^{2+}-Agarose (60 mU) were incubated in 25 mM sodium cacodylate buffer pH 6.6 (4 mL) at 30 °C for 7 days with gentle stirring. Additional GDP-Fuc was added three times, after 2 days (5.1 mg, 0.0076 mmol), after 4 days (2.1 mg, 0.0032 mmol), after 6 days (2.1 mg, 0.0032 mmol) together with alkaline phosphatase (2 U) and $MnCl_2$ (4 mg). At the end of incubation the gel was filtered off, washed with 10 mM sodium cacodylate buffer pH 6.6 and filtrate and washings were combined and applied to Sep-Pak C_{18} cartridges; the methanol eluate was evaporated to dryness; then the residue taken up in water was purified on Bio-Gel P2 to afford pure **21** (5 mg, 50%), and a mixture of **21** and the monofucosylated product (30%); $[\alpha]_D^{25}$ −31° (*c* 0.67, water); ^{1}H NMR (D_2O, 250 MHz): δ 7.43–7.29 (m, 10 H, 2 Ph), 5.04 (d, 2 H, $J_{1''2''}$ 4 Hz, 2 H-1″), 4.77 (m, 2 H, 2 H-5″), 4.45 (s, 4 H, 2 CH_2Ph), 4.39 (d, 2 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′), 4.33 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1), 1.88 (s, 6 H, 2 OAc) and 1.12 (d, 6 H, $J_{6'',5''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 62.9 MHz): δ 174.8 (NHCO), 138.6, 129.7, 129.2, (C–Ar), 102.8, 102.5 (C-1′, C-1), 99.6 (C-1″), 74.4 (CH_2Ph), 76.2, 75.9, 74.3, 73.5, 72.9, 72.0, 70.2, 69.9, 69.6, 69.4, 68.7, 67.7 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″, CH_2O), 62.5, 60.7 (C-6, C-6′), 56.9 (C-2), 45.6 (Cq), 23.3 (CH_3CO) and 16.3 (CH_3); HRMS: Calcd for $C_{59}H_{90}N_2O_{32}Na_2$ $[(M+Na)/2]^+$ 692.2636. Found: m/z 692.2615.

3.16. 2,2-Bis-[*O*-β-D-galactopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-β-D-glucopyranosyloxy)]-propane-1,3-diol (22)

A solution of **21** (4.5 mg, 0.0034 mmol) in 1:1 MeOH–water (0.6 mL) was stirred under hydrogen with 20% $Pd(OH)_2$ on charcoal (8 mg) for 16 h at rt. The solution was then filtered through Celite; the filtrate was purified on Bio-Gel P2 to afford 4 mg of **22** (quant. yield); $[\alpha]_D^{30}$ −76° (*c* 0.25, water); ^{1}H NMR (D_2O, 200 MHz): δ 5.12 (d, 2 H, $J_{1'',2''}$ 4.5 Hz, 2 H-1″), 4.90 (m, 2 H, 2 H-5‴), 4.45 (d, 4 H, $J_{1',2'}$ 7.8, $J_{1,2}$ 7.8 Hz, 2 H-1, 2 H-1′), 3.98 (d, 2 H, J_{gem} 12 Hz, 2 CH), 3.42 (d, 2 H, 2 CH), 2.04 (s, 6 H, 2 OAc) and 1.17 (d, 6 H, $J_{6'',5''}$ 6.5 Hz, 2 CH_3); ^{13}C NMR (D_2O, 50.3 MHz): δ 175.1, (NHCO), 102.8, 102.7 (C-1, C-1′), 99.6 (C-1″), 76.3, 75.9, 75.7, 74.3, 73.4, 72.9, 72.0, 70.2, 70.1, 69.3, 68.7, 67.7 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″, CH_2O), 62.5 (C-6), 61.6, 60.7 (C-6′, CH_2OH), 56.8 (C-2), 46.1 (Cq), 23.2 (CH_3CO) and 16.2 (CH_3); HRMS: Calcd for $C_{45}H_{78}N_2O_{32}$ $[(M+2\ Na)/2]^+$ 602.2166. Found: m/z 602.2167.

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