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Note

Synthesis of Lewis A trisaccharide analogues in which D-glucose and L-rhamnose replace D-galactose and L-fucose, respectively

Liang Liao and France-Isabelle Auzanneau*

Department of Chemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Abstract—In our effort to design a safe anti-cancer vaccine based on the tumor associated carbohydrate antigen Le^aLe^x, we are studying the cross-reactivity between the Le^a natural trisaccharide antigen and analogues in which the L-fucose, D-galactose, and/or D-glucosamine residues are replaced by L-rhamnose or D-glucose, respectively. We describe here the chemical synthesis of two such Le^a trisaccharide analogues. In one trisaccharide, D-glucose replaces D-galactose and in the second analogue L-rhamnose and D-glucose replace L-fucose and D-galactose, respectively. Introduction of the rhamnose and fucose moiety onto the poorly reactive 4-OH group of the *N*-acetylglucosamine residue in a disaccharide acceptor was successful after bis-N-acetylation of the amine group. These analogues will be used in competitive binding experiments with anti-Le^a antibodies and their solution conformations will be studied.

Keywords: Synthetic carbohydrate-based anti-cancer vaccines; Cross-reactivity; Auto-immune reactions; Le^aLe^x analogues; Le^a analogues; GlcNAc-containing acceptors

Synthetic anti-cancer vaccines based on carbohydrates have been shown to hold great promise as immunotherapeutics against cancer.[1–3] In fact, candidates such as Globo-H,[4–6] Le^y,[7] and sTn-[8,9] based vaccines are now being investigated in clinical trials. Vaccination therapy to treat cancer takes advantage of the abnormal glycosylation[10] on tumor cell surfaces. Indeed, since the early reports by Hakomori and co-workers[11,12] that large quantities of fucose-containing blood group antigens accumulated in human adenocarcinoma, tumor associated carbohydrate antigens (TACAs) have been extensively studied and reviewed.[10,13,14] The TACA Le^aLe^x (**1**) is a hexasaccharide that displays the Le^a trisaccharide linked to O-3 of the galactose residue of a reducing end Le^x trisaccharide.

Originally found in feces from preterm infants fed on breast milk[15] and later isolated from human milk,[16] Le^aLe^x was shown to bind strongly to a monoclonal antibody (mAb) 43-9F that had been selected after immunization of mice with biopsied human squamous lung carcinoma (SLC) cells.[17] Interestingly, although the mAb 43-9F also showed some cross-reactivity with the Le^a antigen, the hexasaccharide Le^aLe^x was shown to bind mAb 43-9F over 100 times more tightly than the Le^a antigen.[18] Further studies by Hakomori and co-workers[19] using a synthetic glycosphingolipid displaying Le^aLe^x confirmed that the epitope recognized by mAb 43-9F was indeed presented most efficiently by this hexasaccharide. Moreover, it was also shown that this epitope was largely associated with lung cancer[17,20–22] and tumorigenicity of SLC cells,[23,24] while it was only found in normal tissues on a subset of seromucous glands (trachea-bronchial tree) and on limited populations of epithelial cells in the digestive and urogenital systems.[17] Therefore, Le^aLe^x is an interesting target for the development of anti-cancer vaccines.

However, it is well known since Lemieux's pioneering work[25] that the Le^a trisaccharide may be converted to an immunogenic determinant through its association with an immunostimulant carrier protein. Indeed such a Le^a glycoconjugate has been used to raise an immune

* Corresponding author. E-mail: fauzanne@uoguelph.ca

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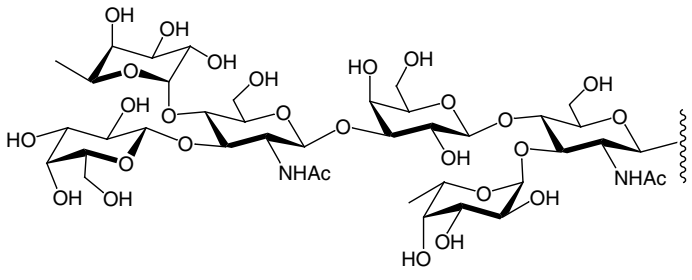
response in mice and the Abs obtained were shown, in turn, to recognize Le^a expressed at the surface of normal human cells and tissues.[26] Therefore, attempts to use Le^aLe^x for the development of anti-cancer vaccines may lead to the production of anti-Le^a antibodies that could ultimately lead to auto-immune reactions that will destroy normal tissues and cells expressing Le^a. Thus, our research program aims at discovering analogues of Le^aLe^x that could be used as anti-cancer vaccines, that is, cross-react with the natural antigen, but would not induce the production of anti-Le^a antibodies. We postulate that replacing any of the L-fucose, D-galactose, or *N*-acetyl D-glucosamine residues displayed by the nonreducing end Le^a trisaccharide by L-rhamnose or D-glucose units, respectively, might abolish the secretion of such anti-Le^a antibodies while still allowing that of anti-Le^aLe^x specific antibodies such as mAb 43-9F.

Prior to embarking on the cumbersome synthesis of such hexasaccharide analogues, it seemed reasonable to first study the cross-reactivity of the natural Le^a antigen with trisaccharide analogues in which the individual sugar residues have been substituted, as mentioned above, by other monosaccharide units (D-Gal and/or D-GlcNAc by D-Glu, and/or L-Fuc by L-Rha). These analogues will be compared to the natural antigen not only in terms of cross-reactivity with anti-Le^a antibodies, but also in terms of conformation using a combination of both NMR and molecular modeling experiments. Only those analogues that do not bind to anti-Le^a antibodies even though they display a three-dimensional conformation similar to the Le^a trisaccharide will be selected and further investigated as hexasaccharides. In line with this approach, we have reported[27,28] the synthesis of a Le^a analogue in which the L-fucose moiety is replaced by L-rhamnose as well as the synthesis of trisaccharide Le^a. We report here the synthesis of the Le^a analogues **2** and **3**. In analogue **2** the D-galactose unit is replaced by a D-glucose residue, while in analogue **3** both D-galactose and L-fucose are substituted by D-glucose and L-rhamnose, respectively.

The known[29] monosaccharide glycosyl acceptor **4** (Scheme 1) was obtained according to the reported procedures. Coupling of acceptor **4** with peracetylated glucosyl bromide **5**[30] under Helferich conditions proceeded uneventfully to afford disaccharide **6** in 74% yield. Although the benzylidene acetal in **6** could be reductively opened to afford a disaccharide acceptor with a free C-4 hydroxyl group, it is well known[31,32] that the 4-OH group in *N*-acetylglucosamine derivatives is poorly reactive toward glycosylation. In addition to steric hindrance at this position,[31] it has been demonstrated that the reactivity of this hydroxyl group was greatly influenced by the presence of the *N*-acetyl group at C-2 of the glucosamine residue.[28,32] A similar observation was made in sialic acid glycosyl acceptors in which the reactivity of OH-8 is affected by the presence of the *N*-acetyl group at C-5 of the sialic acid residue.[33] Thus, Demchenko and Boons[33] established a synthetic strategy for the glycosylation of such acceptors in which the amino group of sialic acid was bis-acetylated prior to glycosylation. This strategy was later applied successfully to achieve glycosylation of the 4-OH group of N-acetylated glucosamine acceptors.[28,32] Interestingly, di-N,N-acetylated glucosamine derivatives have also shown improved reactivity when used as glycosyl donors.[34] Applying this strategy to the synthesis of our target trisaccharides, disaccharide **6** was treated with acetyl chloride in the presence of excess Hünig's base and gave bis-N-acetylated disaccharide **7** in 89% yield. The benzylidene acetal was then reductively opened in standard conditions ($NaBH_3CN$–HCl/diethyl ether) and the desired di-*N*,*N*-acetate disaccharide glycosyl acceptor **8** was isolated in 62% yield (Scheme 1).



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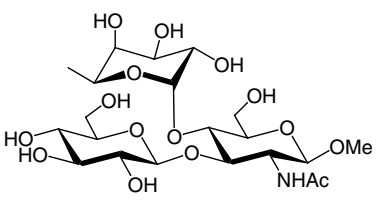

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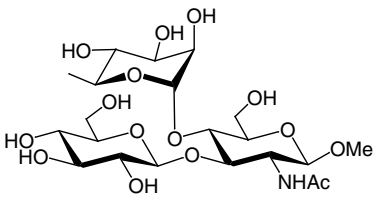

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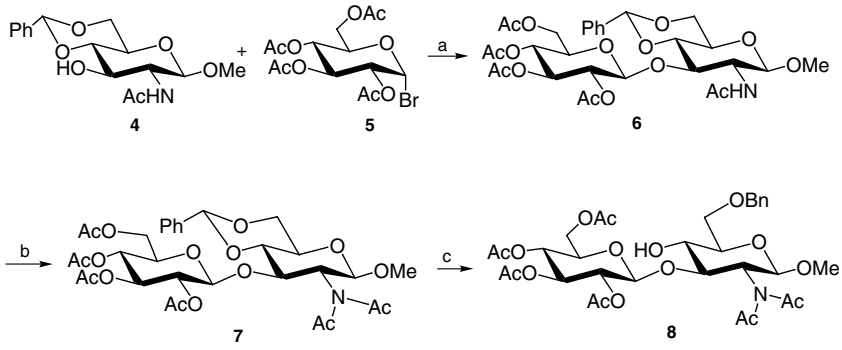
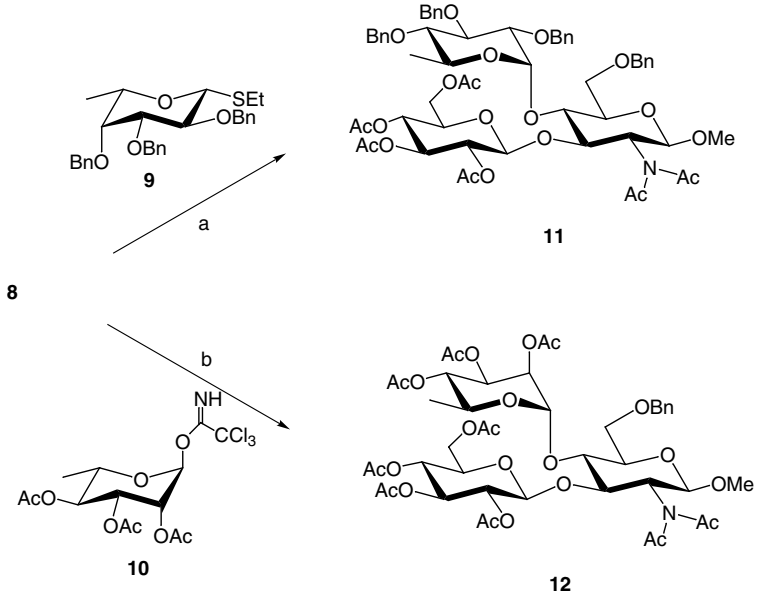


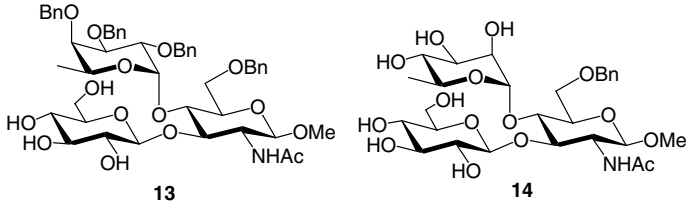
Scheme 1. Reagents and conditions: (a) $Hg(CN)_2$, toluene/CH_3NO_2 (1:1), 40 °C, 16 h, 74%; (b) AcCl, $(i\text{-Pr})_2NEt$, CH_2Cl_2, rt, 89%; (c) $NaBH_3CN$, HCl/Et_2O, THF, 0 °C, 62%.



Scheme 2. Reagents and conditions: (a) CH_3OTf (5 equiv), Et_2O, rt, 88%; (b) TESOTf (0.15 equiv), CH_2Cl_2, −78 °C→rt, 86%.

Glycosylation of acceptor **8** with the known[35] β-thioethyl per-benzylated fucosyl donor **9** went smoothly under CH_3OTf catalysis using diethyl ether as a solvent to assist in the formation of the α-anomer (Scheme 2). Indeed trisaccharide **11** was obtained as the single product and in an excellent 88% yield. Glycosylation of disaccharide **8** with the known[36] trichloroacetimidate donor **10** (Scheme 2) was catalyzed by TESOTf and afforded the desired trisaccharide **12** in excellent 86% yield. The α-(1→4) linkage of the newly introduced L-rhamnose unit was unambiguously confirmed by the one bond coupling constant measured for the rhamnose anomeric center $^1J_{C1-H1}$ (173 Hz).[37]

Trisaccharides **11** and **12** were then submitted to Zemplén de-acetylation to remove the *O*-acetyl groups as well as one of the *N*-acetyl groups, affording polyols **13** and **14** in 78% and 84% yields, respectively. Finally, hydrogenolysis of **13** and **14** over 10% Pd/C gave the desired Le^a trisaccharide analogues **2** and **3** in good yields. Future study will use **2** and **3** as soluble antigens to test their



cross-reactivity to anti-Le[a] antibodies. Conformational analysis of these analogues with a combination of NMR and molecular modeling is also underway.

1. Experimental

1.1. General methods

^{1}H NMR (400.13 and 600.13 MHz) and ^{13}C NMR (75.5, 100.6, and 150.9 MHz) spectra were recorded in $CDCl_3$ (internal standard, for ^{1}H residual $CHCl_3$ δ 7.24; for ^{13}C: $CDCl_3$ δ 77.0), CD_3OD (internal standard, for ^{1}H residual CH_3OD δ 3.30; for ^{13}C: CD_3OD δ 49.0), or D_2O [external standard 3-(trimethylsilyl)-propionic acid-d_4, sodium salt (TSP) for ^{1}H δ 0.00, for ^{13}C δ 0.00]. ^{1}H NMR and ^{13}C NMR chemical shifts are reported in parts per million (ppm). Coupling constants (J) are reported in hertz (Hz). Chemical shifts and coupling constants were obtained from a first-order analysis of one-dimensional spectra. Assignments of proton and carbon resonances were based on two-dimensional ^{1}H–^{1}H and ^{13}C–^{1}H correlation experiments. Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broadened (br). All NMR spectra were recorded at 300 K. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F254 precoated plates (250 μm) with a fluorescent indicator, visualized under UV and charred with 10% sulfuric acid in ethanol. Flash chromatography was performed using silica gel 60 (230–400 mesh) from EM science. All reactions were carried out under nitrogen atmosphere with anhyd solvents freshly distilled according to standard procedures. Commercially available methyl triflate and TESOTf were freshly distilled under reduced pressure prior to their use in glycosylation reactions. All other reagents were purchased from commercial suppliers and used without further purification.

1.2. Methyl 2-acetamido-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (6)

The known[29] glycosyl acceptor **4** (50 mg, 0.15 mmol) and $Hg(CN)_2$ (88 mg, 0.46 mmol) were mixed in a mixture of anhyd toluene (2.5 mL) and anhyd CH_3NO_2 (2.5 mL) containing activated 3 Å molecular sieves (500 mg). This mixture was stirred at rt for 1 h and heated to 40 °C. 2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyl bromide[30] **5** (95.5 mg, 0.23 mmol) was added and the mixture was stirred at 40 °C for 16 h. The mixture was diluted with CH_2Cl_2 (100 mL) and filtered through Celite®. The solids were washed with CH_2Cl_2 (10 mL × 3) and the combined filtrate and washings were washed sequentially with satd aq $NaHCO_3$ (100 mL) and brine (100 mL). The aqueous phases were re-extracted with CH_2Cl_2 (50 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. Chromatography (98:2, CH_2Cl_2/CH_3OH) of the dry residue gave compound **6** (75 mg, 74%) as a colorless glass. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58–7.35 (m, 5H, Ar), 5.83 (d, 1H, J = 6.6 Hz, N*H*), 5.53 (s, 1H, C*H*Ph), 5.12–5.09 (m, 2H, H-1, H-3′), 5.03 (br t, 1H, J = 9.7 Hz, H-4′), 4.95 (dd, 1H, J = 9.2, 8.1 Hz, H-2′), 4.77 (d, 1H, J = 8.0 Hz, H-1′), 4.71 (br t, 1H, J = 9.5 Hz, H-3), 4.34 (dd, J = 4.9, 10.5 Hz, H-6a), 4.08–3.92 (m, 2H, H-6a′, H-6b′), 3.79 (dd, 1H, J = 10.3, 10.2 Hz, H-6b), 3.68 (br t, 1H, J = 9.2 Hz, H-4), 3.57 (m, 1H, H-5), 3.51 (s, 3H, OCH_3), 3.37 (m, 1H, H-5′), 3.04 (m, 1H, H-2), 2.02–1.92 (5br s, 5 × 3H, *N*-acetyl and *O*-acetyl); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 167.9, 166.8, 166.1 (C=O), 138.5, 138.0, 130.2–128.0 (Ar), 101.6 (*C*HPh), 100.3 (C-1), 99.3 (C-1′), 80.2 (C-4), 76.4 (C-3), 72.9 (C-3′), 71.7, 71.7 (C-2′ and C-5′), 68.8 (C-6′), 67.9 (C-4′), 65.8 (C-5), 62.3 (C-6), 58.5 (C-5′), 57.3 (O*C*H$_3$), 20.7, 20.6 (*O*- and *N*-CO*C*H$_3$); ESIMS: m/z calcd for $C_{30}H_{40}NO_{15}$ $[M+H]^+$: 654.2398. Found: 654.2426.

1.3. Methyl 2-(*N*-acetylacetamido)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (7)

Compound **6** (67 mg, 0.1 mmol), acetyl chloride (374 μL, 5 mmol, 50 equiv), and *N*,*N*-diisopropylethylamine (180 μL, 1 mmol, 10 equiv) were mixed together in CH_2Cl_2 (7 mL) containing 4 Å molecular sieves (670 mg). The reaction mixture was stirred at rt overnight, then diluted with CH_2Cl_2 (50 mL) and washed sequentially with satd aq $NaHCO_3$ (30 mL) and brine (30 mL). The aqueous phases were re-extracted with CH_2Cl_2 (30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. Chromatography (30:70→40:60, EtOAc/hexanes) gave **7** as a colorless glass (69 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51–7.45 (m, 5H, Ar), 5.56 (s, 1H, C*H*Ph), 5.11 (d, 1H, J = 7.7 Hz, H-1), 5.06–5.03 (m, 2H, H-3′, H-4′), 4.95 (br t, 1H, J = 8.2 Hz, H-2), 4.75 (dd, 1H, J = 9.4, 8.5 Hz, H-3), 4.56 (d, 1H, J = 8.0 Hz, H-1′), 4.35 (dd, 1H, J = 10.4, 4.9 Hz, H-6a′), 3.97 (dd, 1H, J = 12.5, 3.5 Hz, H-6a), 3.80 (t, 1H, J = 10.3 Hz, H-6b′), 3.76 (br s, 1H, H-6b), 3.74–3.66 (m, 2H, H-2, H-5), 3.59 (m, 1H, H-5), 3.46 (s, 3H, OCH_3), 3.28 (m, 1H, H-5′), 2.49–1.93 (6s, 6 × 3H, *N*-acetyl and *O*-acetyl); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 170.1 (C=O), 129.4–126.0 (Ar), 101.6 (*C*HPh), 100.3, 100.3 (C-1, C-1′), 81.3 (C-4), 76.7 (C-3), 73.1 (C-3′ or C-4′), 71.5, 71.5 (C-2, C-5), 68.9 (C-6′), 67.7 (C-3′ or C-4′), 65.4 (C-5), 63.3 (C-2), 61.1 (C-6), 57.5 (OCH_3), 25.2-20.5 (*O*- and *N*-CO*C*H$_3$); ESIMS: m/z calcd for $C_{32}H_{42}NO_{16}$ $[M+H]^+$: 696.2504. Found: 696.2535.

1.4. Methyl 2-(*N*-acetylacetamido)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (8)

Compound **7** (50 mg, 72 μmol), sodium cyanoborohydride (37 mg, 590 μmol, 8 equiv), and powdered activated molecular sieves 4 Å (100 mg) were mixed together in anhyd THF (2 mL). A 2 M solution of HCl in Et_2O (697 μL) was added slowly at 0 °C and stirring was maintained at 0 °C for an additional 30 min, once the addition was complete (~30 min). The reaction mixture was then diluted with CH_2Cl_2 (50 mL), washed sequentially with 0.5 M HCl (30 mL), satd aq $NaHCO_3$ (30 mL), and brine (30 mL). The aqueous phases were re-extracted with CH_2Cl_2 (30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. Chromatography (50:50, EtOAc/hexanes) gave acceptor **8** as a colorless glass (31 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32–7.27 (m, 5H, Ar), 5.15 (br t, 1H, $J = 9.5$ Hz, H-2′), 5.04–4.97 (m, 3H, H-1′, H-3′, H-4′), 4.63 (s, 2H, OCH_2Ph), 4.45 (d, 1H, $J = 8.0$ Hz, H-1), 4.45 (dd, 1H, $J = 10.4$, 7.0 Hz, H-3), 4.24–4.11 (m, 2H, H-6a′, H-6b′), 4.05 (s, 1H, OH), 3.88 (dd, 1H, $J = 11.0$, 1.6 Hz, H-6a), 3.82 (m, 1H, H-5′), 3.71 (dd, 1H, $J = 10.1$, 5.1 Hz, H-6b), 3.61 (dd, 1H, $J = 10.2$, 7.8 Hz, H-2), 3.54 (m, 2H, H-4, H-5), 3.46 (s, 3H, OCH_3), 2.49–1.99 (6s, 6 × 3H, *N*-acetyl and *O*-acetyl); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 175.2, 174.7, 170.5, 170.0, 169.9, 169.3 (C=O), 138.2, 128.3, 127.6 (Ar), 100.9 (C-1), 99.6 (C-1′), 83.8 (C-3), 75.0 (C-4 or C-5), 73.5 (O*C*H$_2$Ph), 72.8 (C-2′), 71.9 (C-5′), 71.2 (C-3′ or C-4′), 70.3 (C-4′ or C-5′), 69.2 (C-6), 68.2 (C-3′ or C-4′), 62.8 (C-2), 61.9 (C-6′), 57.1 (OCH3), 28.4, 25.4, 20.6, 20.4, 20.4 (*O*- and *N*-CO*C*H$_3$); ESIMS: *m*/*z* calcd for $C_{32}H_{47}N_2O_{16}$ $[M+H]^+$: 698.2660. Found: 698.2662.

1.5. Methyl 2-(*N*-acetylacetamido)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-*O*-benzyl-4-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (11)

Glycosyl acceptor **8** (50 mg, 72 μmol) and glycosyl donor ethyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside[35] **9** (107.5 mg, 216 μmol, 3 equiv) were mixed together in anhyd Et_2O (2.5 mL) containing powdered, activated 4 Å molecular sieves (250 mg). The mixture was stirred at rt for 3 h, CH_3OTf (42.5 μL, 0.36 mmol, 5 equiv) was added and the reaction was allowed to proceed at rt for 18 h and was then quenched by the addition of NEt_3 (100 μL). The reaction mixture was diluted with CH_2Cl_2 (50 mL) and filtered through Celite®. The solids were washed with CH_2Cl_2 (10 mL × 3) and the combined filtrate and washings were washed sequentially with satd aq $NaHCO_3$ (50 mL) and brine (50 mL). The aqueous phases were re-extracted with CH_2Cl_2 (30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. Chromatography (3:7, EtOAc/hexanes) gave trisaccharide **11** as a colorless glass (70 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.19 (m, 20H, Ar), 5.15 (d, 1H, $J = 3.7$ Hz, H-1″), 5.01–4.98 (m, 2H, H-3′, OCH_2Ph), 4.92 (br t, 2H, $J = 8.5$ Hz, H-2′, H-4′), 4.86–4.79 (m, 4H, H-1, H-3, OCH_2Ph), 4.72–4.58 (m, 4H, H-5″, OCH_2Ph), 4.50–4.38 (m, 4H, H-1′, H-6a′, OCH_2Ph), 4.12 (d, 1H, $J = 7.0$, 2.7 Hz, H-2″), 3.99 (dd, 1H, $J = 12.4$, 2.0 Hz, H-6b′), 3.96–3.90 (m, 3H, H-4, H-6a, H-3″), 3.77 (br s, 1H, H-4″), 3.68–3.54 (m, 4H, H-6b, H-2, H-2′, H-5′), 3.49 (m, 1H, H-5), 3.41 (s, 3H, OCH_3), 2.50, 2.26 (2br s, 2 × 3H, *N*-acetyl), 2.02, 1.99, 1.97, 1.94 (4s, 4 × 3H, *O*-acetyl), 1.29 (d, 3H, $J = 6.5$ Hz, H-6″); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 170.4, 170.0, 169.5, 169.1 (C=O), 138.8, 138.7, 138.2, 128.4–127.0 (Ar), 99.6 (C-1′), 99.4 (C-1), 97.6 (C-1″), 80.0 (C-3″ or C-4), 78.4 (C-4″), 77.5 (C-2″), 75.4 (C-3), 75.0 (C-2 or C-2′ or C-5′), 74.9, 74.6, 73.2, 72.7 (OCH_2Ph), 74.4 (C-4 or C-3″), 73.6 (C-3′), 72.1 (C-2 or C-2′ or C-5′), 71.4 (C-2′ or C-4′), 68.2 (C-2′ or C-4′), 67.6 (O*C*H$_2$Ph), 66.6 (C-5″), 64.5 (C-2 or C-2′ or C-5′), 61.9 (OCH_2Ph), 57.0 (OCH_3), 20.6 (*O*- and *N*-CO*C*H$_3$), 16.9 (C-6″); ESIMS: *m*/*z* calcd for $C_{59}H_{71}N_2O_{20}$ $[M+H]^+$: 1131.4913. Found: 1136.4845.

1.6. Methyl 2-(*N*-acetylacetamido)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-4-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (12)

Compound **8** (50 mg, 72 μmol), peracetylated α-L-rhamnopyranosyl trichloroacetimidate[36] **10** (155 mg, 0.36 mmol, 5 equiv), and powdered activated molecular sieves 4 Å (100 mg) were mixed in anhyd CH_2Cl_2 (1.25 mL) and the mixture was stirred at rt for 3 h. The reaction mixture was cooled down to −78 °C and a freshly prepared 0.37 M solution of TESOTf in anhyd CH_2Cl_2 (30 μL, 10.8 μmol, 0.15 equiv) was added. The reaction was then allowed to reach rt slowly (over 2 h) and was quenched with NEt_3 (10 μL). The reaction mixture was diluted with CH_2Cl_2 (50 mL) and filtered through Celite®. The solids were washed with CH_2Cl_2 (10 mL × 3) and the combined filtrate and washings were washed sequentially with satd aq $NaHCO_3$ (50 mL) and brine (50 mL). The aqueous phases were re-extracted with CH_2Cl_2 (30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. Chromatography (40:60→50:50, EtOAc/hexane) gave trisaccharide **12** as a colorless glass (57 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32–7.25 (m, 5H, Ar), 5.28 (dd, 1H, $J = 10.6$, 3.8 Hz, H-3″), 5.21 (m, 1H, H-2″), 5.14 (br t, 1H, $J = 10.0$ Hz, H-4″), 5.07 (br t, 1H, $J = 9.3$ Hz, H-3′), 5.01 (br s, 1H, H-1″), 4.97–4.90 (m, 3H, H-1, H-2′, H-4′), 4.84 (br t, 1H, $J = 10.0$ Hz, H-3), 4.59 (m, 1H, H-5″), 4.54 (2d, 2H, $J = 12.2$ Hz, OCH_2Ph), 4.38 (d, 1H, $J = 8.0$ Hz, H-1′), 4.26 (d, 2H,

$J = 5.3$ Hz, H-6a′, H-6b′), 3.93 (br t, 1H, $J = 9.4$ Hz, H-4), 3.74 (br s, 2H, H-6a, H-6b), 3.67–3.58 (m, 2H, H-5′, H-2), 3.49 (m, 1H, H-5), 3.43 (s, 3H, OCH_3), 2.49, 2.34, 2.18, 2.14, 2.10, 2.03, 2.02, 1.99, 1.96 (9s, 9 × 3H, *N*-acetyl and *O*-acetyl), 1.30 (d, 3H, $J = 6.3$ Hz, H-6″); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 170.4, 169.1 (C=O), 128.2, 127.5 (Ar), 99.5 (C-1), 99.2 (C-1′), 96.0 (C-1″, $^1J_{C-H} = 173$ Hz), 74.4 (C-5), 73.8 (C-3), 73.6 (C-3′), 73.1 (C-4), 73.0 (O*C*H_2Ph), 72.2 (C-2 or C-5′), 71.6 (C-2′ or C-4′), 71.3 (C-4″), 70.3 (C-2″), 69.5 (C-2′ or C-4′), 68.9 (C-3″), 67.9 (C-6), 66.1 (C-5″), 64.2 (C-2 or C-5′), 62.7 (C-6′), 57.1 (OCH_3), 20.9, 20.7, 20.5 (*O*- and *N*-CO*C*H_3), 17.2 (C-6″); ESIMS: *m*/*z* calcd for $C_{44}H_{63}NO_{23}$ $[M+Na]^+$: 992.3418. Found: 992.3376.

1.7. Methyl 2-acetamido-6-*O*-benzyl-4-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (13)

Compound **11** (63 mg, 57 μmol) was dissolved in CH_3OH (2.5 mL) and sodium (1 mg) was added. The mixture was stirred at rt for 30 min and neutralized with Dowex® 50WX8-100 H^+ resin. The resin was filtered off, rinsed with CH_3OH and the combined filtrate and washings were concentrated. Column chromatography (9:1, $CHCl_3/CH_3OH$) gave **13** as a pure, colorless glass (40 mg, 78%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.49–7.15 (m, 20H, Ar), 4.99 (d, 1H, $J = 3.6$ Hz, H-1″), 4.95–4.82 (m, 2H, 2 × OC*H*Ph), 4.72 (m, 1H, H-5″), 4.75–4.57 (m, 4H, 4 × OC*H*Ph), 4.46–4.38 (m, 3H, H-1′, 2 × OC*H*Ph), 4.30 (d, 1H, $J = 7.8$ Hz, H-1), 3.98 (dd, 1H, $J = 13.3$, 2.6 Hz, H-2″), 3.96–3.81 (m, 7H, H-2, H-3, H-4, H-6a, H-6a′, H3″, H-4″), 3.67 (m, 1H, H-6b), 3.58 (m, 1H, H-6b′), 3.46 (m, 1H, H-5), 3.45 (s, 3H, OCH_3), 3.28 (m, 2H, H-3′, H-5′), 3.15 (m, 2H, H-2′, H-4′), 1.94 (s, 3H, *N*-acetyl), 1.16 (d, 3H, $J = 6.5$ Hz, H-6″); ^{13}C NMR (100.6 MHz, CD_3OD): δ 173.9 (C=O), 140.2, 139.4, 129.8–128.6 (Ar), 104.4 (C-1′), 103.2 (C-1), 98.5 (C-1″), 81.1 (C-2″), 79.5 (C-2 or C-3 or C-4 or C-3″ or C-4″), 78.5 (C-3′ or C-5′), 78.5 (C2 or C-3 or C-4 or C-3″ or C-4″), 77.8 (C-3′ or C-5′), 77.3 (C-2 or C-3 or C-4 or C-3″ or C-4″), 76.4 (C-5), 76.3, 75.8, 74.5, 73.4 (4 × O*C*H_2Ph), 74.9 (C-2′ or C-4′), 74.2 (C-2 or C-3 or C-4 or C-3″ or C-4″), 74.2 (C-2′ or C-4′), 73.4 (C-6), 71.9 (C-5″), 69.1 (C-6′), 67.9 (OCH_3), 67.9 (C-2 or C-3 or C-4 or C-3″ or C-4″), 23.1 (*N*-CO*C*H_3), 16.7 (C-6″); ESIMS: *m*/*z* calcd for $C_{49}H_{62}NO_{15}$ $[M+H]^+$: 904.4119. Found: 904.4123.

1.8. Methyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-[α-L-fucopyranosyl-(1→4)]-β-D-glucopyranoside (2)

Trisaccharide **13** (33 mg, 37 μmol) was dissolved in CH_3OH (5 mL) and 10% Pd/C (50 mg) was added. The mixture was stirred under H_2 for 24 h and then diluted with CH_3OH, filtered, and concentrated. Chromatography with $CHCl_3/CH_3OH$ (1:1) gave **2** that was lyophilized from H_2O after filtration on glass wool and pure amorphous powder (15 mg, 75%) was obtained. $[\alpha]_D$ −73 (*c* 0.4, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 4.94 (d, 1H, $J = 3.9$ Hz, H-1″), 4.70 (m, 1H, H-5″, overlap with solvent signal), 4.47 (d, 1H, $J = 7.8$ Hz, H-1′), 4.37 (d, 1H, $J = 8.5$ Hz, H-1), 3.97 (br t, 1H, $J = 9.8$ Hz, H-3), 3.92 (dd, 1H, $J = 12.4$, 2.2 Hz, H-6a), 3.87 (dd, 1H, $J = 12.2$, 2.0 Hz, H-6a′), 3.80 (m, 3H, H-2, H-6b, H-3″), 3.73 (dd, 1H, $J = 10.5$, 4.0 Hz, H-2″), 3.69 (br d, 1H, $J = 2.8$ Hz, H-4″), 3.65 (br t, 1H, $J = 9.4$ Hz, H-4), 3.48 (m, 2H, H-5, H-6b′), 3.42 (s, 3H, OCH_3), 3.38 (br t, 1H, $J = 9.2$ Hz, H-3′), 3.31 (m, 2H, H-2′, H-4′), 2.06 (s, 3H, *N*-acetyl), 1.10 (d, 3H, $J = 6.7$ Hz, H-6″); ^{13}C NMR (150.9 MHz, D_2O): δ 174.6 (C=O), 102.4 (C-1′), 101.8 (C-1), 98.1 (C-1″), 76.6 (C-3), 76.1 (C-5′), 75.5 (C-5), 75.4 (C-3′), 73.1 (C-2′ or C-4′), 72.9 (C-4), 72.0 (C-4″), 70.5 (C-2′ or C-4′), 69.2 (C-3″), 67.8 (C-2″), 61.8 (C-6′), 59.8 (C-6), 57.1 (OCH_3), 55.6 (C-2), 22.3 (*N*-CO*C*H_3), 15.5 (C-6″); ESIMS: *m*/*z* calcd for $C_{21}H_{38}NO_{15}$ $[M+H]^+$: 544.2241. Found: 544.2259.

1.9. Methyl 2-acetamido-6-*O*-benzyl-2-deoxy-3-*O*-(β-D-galactopyranosyl)-4-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (14)

Compound **12** (47 mg, 0.049 mmol) was dissolved in CH_3OH (2.5 mL) and sodium (1 mg) was added. The mixture was stirred at rt for 30 min and neutralized with Dowex® 50WX8-100 H^+ resin. The resin was filtered, rinsed with CH_3OH and the combined filtrate and washings were concentrated. Column chromatography (9:1, $CHCl_3/CH_3OH$) gave **14** as a pure, colorless glass (26 mg, 84%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.48–7.25 (m, 5H, Ar), 4.95 (br d, 1H, $J = 1.4$ Hz, H-1″), 4.59 (br s, 2H, OCH_2Ph), 4.45 (d, 1H, $J = 7.6$ Hz, H-1′), 4.44 (m, 1H, H-5″), 4.38 (d, 1H, $J = 8.2$ Hz, H-1), 3.95–3.68 (m, 9H, H-2, H-3, H-4, H-6a, H-6b, H-4′, H-6a′, H-6b′, H-2″), 3.48–3.42 (m, 5H, H-3′, H-5′, and OCH_3), 3.35–3.27 (m, 2H, H-5, H-4″), 3.17 (m, 2H, H-2′, H-3″), 1.95 (s, 3H, *N*-acetyl), 1.25 (d, 3H, $J = 6.3$ Hz, H-6″); ^{13}C NMR (100.6 MHz, CD_3OD): δ 174.0 (C=O), 139.4, 129.4, 129.0, 128.9, 128.7 (Ar), 104.7 (C-1′), 103.0 (C-1), 101.3 (C-1″), 79.0 (C-2 or C-3 or C-4 or C-4′ or C-2″), 78.0 (C-2′ or C-3″), 78.0 (C-5 or C-4″), 76.5 (C-5′ or C-3′), 74.9 (C-2 or C-3 or C-4 or C-4′ or C-2″), 74.8 (C-2′ or C-3″), 74.5 (O*C*H_2Ph), 74.1 (C-5 or C-4″), 72.4 (C-2 or C-3 or C-4 or C-4′ or C-2″), 72.1 (C-2 or C-3 or C-4 or C-4′ or C-2″), 70.5 (C-5 or C-3′), 69.7 (C-6′), 69.6 (C-5″), 62.0 (C-6), 57.4 (C-2 or C-3 or C-4 or C-4′ or C-2″), 56.9 (OCH_3), 23.2 (*N*-CO*C*H_3), 18.0 (C-6″); CIMS: *m*/*z* calcd for $C_{28}H_{44}NO_{15}$ $[M+H]^+$: 634.2711. Found: 634.2727.

1.10. Methyl 2-acetamido-β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→4)]-2-deoxy-β-D-glucopyranoside (3)

Trisaccharide **14** (19 mg, 30 μmol) was dissolved in CH_3OH (5 mL) and 10% Pd/C (50 mg) was added. The mixture was stirred under H_2 for 24 h. The reaction mixture was diluted with CH_3OH, filtered, and concentrated. Column chromatography with $CHCl_3/CH_3OH$ (1:1) gave **3** that was lyophilized from H_2O after filtration on glass wool and obtained pure as an amorphous powder (12 mg, 74%). $[\alpha]_D$ −122 (*c* 0.4, H_2O); ^1H NMR (600 MHz, D_2O): δ 4.87 (br s, 1H, H-1″), 4.43 (d, 1H, $J = 7.8$ Hz, H-1′), 4.38 (d, 1H, $J = 8.4$ Hz, H-1), 4.30 (m, 1H, H-5″), 3.93 (br t, 1H, $J = 9.6$ Hz, H-3), 3.91 (dd, 1H, $J = 3.0$, 1.4 Hz, H-2″), 3.84 (br d, 2H, $J = 12.1$ Hz, H-6a, H-6a′), 3.81 (br t, 1H, $J = 9.6$ Hz, H-2), 3.72 (m, 2H, H-4, H-6b′), 3.69 (m, 1H, H-4′), 3.67 (m, 1H, H-6b), 3.47 (m, 1H, H-5), 3.42 (s, 3H, OCH_3), 3.37 (m, 2H, H-3′, H-4″), 3.28 (m, 2H, H-5′, H-3″), 3.16 (dd, 1H, $J = 8.9$, 8.1 Hz, H-2′), 1.96 (s, 3H, *N*-acetyl), 1.17 (d, 3H, $J = 6.3$ Hz, H-6″); ^{13}C NMR (150.9 MHz, D_2O): δ 174.6 (C=O), 102.5 (C-1′), 101.8 (C-1), 100.2 (C-1″), 76.4 (C-3), 76.1 (C-5′ or C-3″), 75.5 (C-3′ or C-4″), 75.2 (C-5), 73.9 (C-4), 73.1 (C-2′), 71.9 (C-3′ or C-4″), 70.2 (C-2″), 70.1 (C-4′), 69.7 (C-3″ or C-5′), 68.9 (C-5″), 61.1 (C-6′), 60.1 (C-6), 57.2 (OCH_3), 55.6 (C-2), 22.4 (*N*-CO*C*H_3), 16.8 (C-6″); ESIMS: *m*/*z* calcd for $C_{21}H_{38}NO_{15}$ $[M+H]^+$: 544.2241. Found: 544.2259.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.07.006.

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