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Chemoenzymatic synthesis of sulfated *O*-linked oligosaccharides: epitopes for MECA-79

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Abstract—Sulfated oligosaccharides **I**, **II** and **III**, representatives of those found on L-selectin counterreceptors, have been efficiently prepared employing a combination of chemical and enzymatic synthetic methods.

1. Introduction

Sulfated oligosaccharides are implicated in a number of biological roles, including development, differentiation, and homeostasis. Those present on L-selectin counterreceptors play a critical role in lymphocyte homing and recirculation.[1] L-Selectin counterreceptors on peripheral lymph node high endothelial venules in humans and rodents include a heterogeneous group of glycoproteins termed peripheral node addressins (PNAd) which are recognized by the MECA-79 antibody.[2] The MECA-79 antibody can inhibit lymphocyte adhesion in vivo, indicating that the MECA-79 epitope binds to the functional L-selectin ligand.[2] The MECA-79 epitope had been suggested to be a sulfated *O*-linked oligosaccharide.[3,4] Only recently has the minimum epitope been identified as a sulfated extended core 1 mucin type *O*-glycan, β-D-Gal-(1→4)-β-D-GlcNAc6SO_3Na-(1→3)-β-D-Gal-(1→3)-α-D-GalNAc-(1→R) which is a backbone structure for PNAd that is detected by MECA-79.[5] We report herein the chemical and enzymatic synthesis of novel sulfated *O*-linked oligosaccharides **I**–**III** recognized by MECA-79 of which **I** was found to be the minimum required epitope (Fig. 1).

Our synthetic scheme was substantially modified from previous work dedicated to the synthesis of such sulfated oligosaccharides.[6] During the last decade, a number of glycosyltransferases have become available[7] which lead us to consider the use of sialyltransferase and fucosyltransferase as an alternative way to synthesize the desired hexasaccharide. Therefore a chemoenzymatic strategy was envisaged to deliver the target oligosaccharides in a more efficient way than the conventional strategy.

We planned that the tetrasaccharide **I** would be the key intermediate of our synthesis as it could be prepared chemically and then also used as a substrate for the enzymatic reactions leading to the target oligosaccharides **II** and **III**. **I** could be prepared through the coupling of two disaccharides, and the sulfation at the 6-*O*-GlcNAc position was accomplished by employing a benzyl ether as a temporary protecting group (Fig. 2).

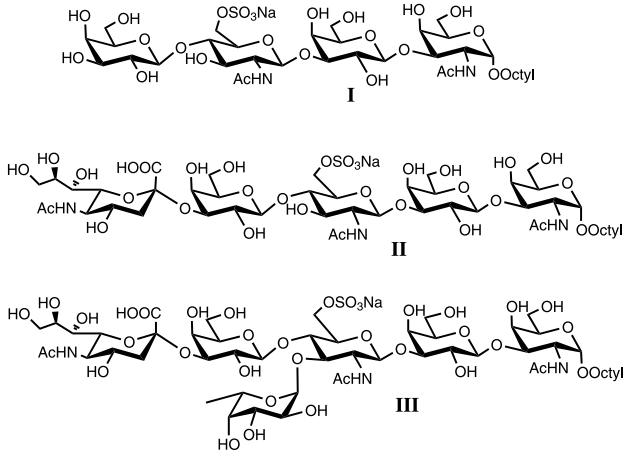

Figure 1. Target oligosaccharides.

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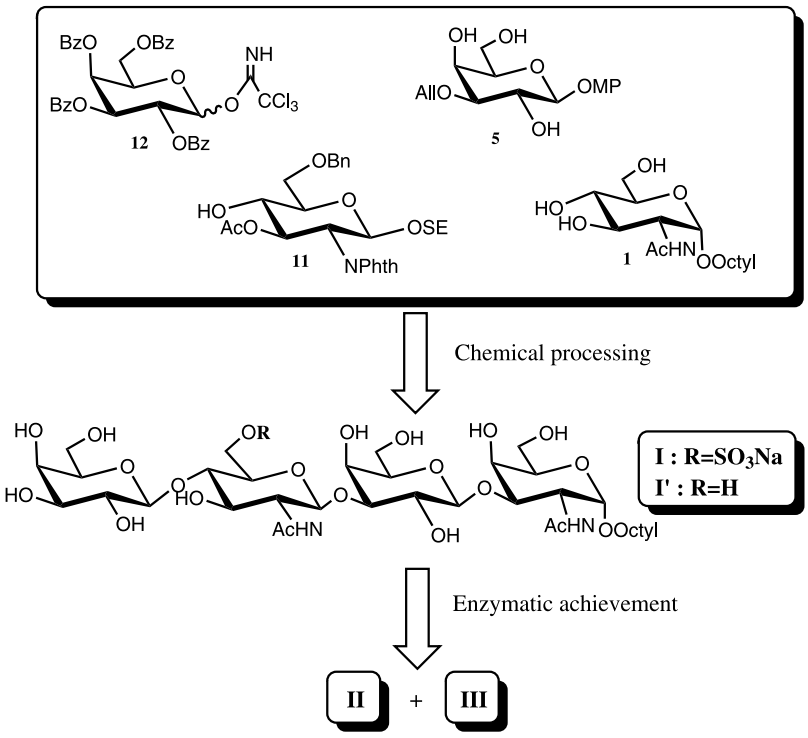


Figure 2. Key features of the chemoenzymatic approach to **I**, **II** and **III**.

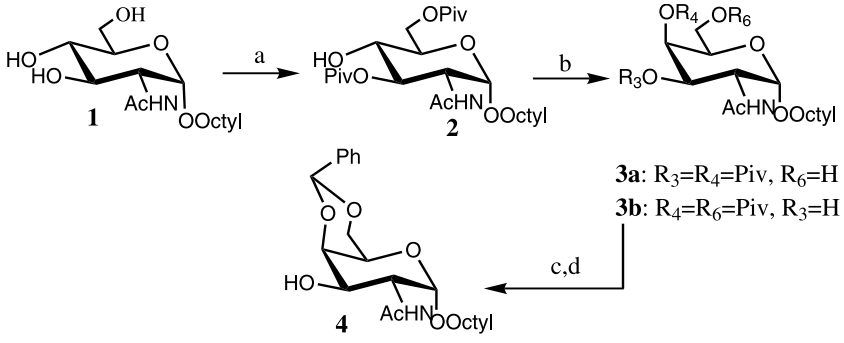
2. Synthesis of the acceptor 9

Two major difficulties were envisioned in the synthesis of the acceptor **9**. The starting material GalNAc is very costly and therefore the desired GalNAc would be synthesized conveniently and cost effectively through readily available GlcNAc. The other major difficulty in the synthesis would be the Gal(β1→3)GalNAc linkage.

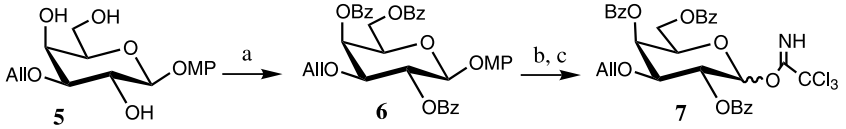
The GalNAc derivative **3** was prepared from compound **1**[9] by a literature procedure[10] using intramolecular S_N2 displacement of the 4-*O*-triflate by the 3-*O*-pivaloyl group. The correct *galacto* structure was deduced from the ^1H NMR spectra where H-4 signals resonated at 5.30 ppm ($J_{3,4}=3.2$ Hz, $J_{4,5}<1.0$ Hz) for **3a** and 5.32 ppm ($J_{3,4}=3.1$ Hz, $J_{4,5}<1.0$ Hz) for **3b**. Both compounds **3a** and **3b** were *O*-depivaloylated using Zemplen conditions, and the 4 and 6 positions of the residue were masked by a benzylidene acetal with TFA and benzaldehyde. The acceptor **4** was obtained in an overall yield of 69% (Scheme 1).

As described previously,[8] 4-methoxyphenyl 3-*O*-ally-β-D-galactopyranoside **5** was prepared from a commercially available galactose penta-acetate. After benzoylation of **5** (BzCl, pyridine, 80%), anomeric position of **6** was deblocked (CAN, toluene, acetonitrile, water) and subsequently converted to a trichloroacetimidate **7** (CCl_3CN, DBU, DCM) in 83% overall yield (two steps) (Scheme 2).

Use of benzoyl ester as neighboring group at the position 2 was preferred to acetyl group because, in our hands, the desired 1,2-*trans* glycosidic linkage was preferably obtained with the former (details not presented).



Scheme 1. *Reagents and conditions*: (a) PivCl, Py, DCM, 67% (two steps from 2-deoxy-2-acetamido-glucopyrannose); (b) Tf_2O, Py, DCE then H_2O, 90°C; (c) MeONa, MeOH, 80% (two steps); (d) PhCHO, TFA, 76%.



Scheme 2. *Reagents and conditions*: (a) BzCl, Py, 80%; (b) CAN, Tol, CH_3CN, H_2O; (c) CCl_3CN, DBU, DCM, 83% (two steps).

Coupling of **4** and **7** was accomplished in slightly harsher conditions (DCE, TfOH cat., molecular sieves, 55°C)[15] that afforded the Gal-GalNAc disaccharide **8** in good and reproducible yields of 69% (^{1}H NMR data: Ref. 14). These unusual coupling conditions helped us to avoid orthoester as a side product. These conditions were also used for two other glycosylations in our goal to furnish the synthesis of tetrasaccharide **I**.

Removal of the benzylidene group of **8** (AcOH/water) followed by treatment with Ac_2O, pyridine (81% two steps), afforded the ester protected intermediate, which was then treated with $Pd(Cl)_2$ in MeOH to afford the disaccharide acceptor **9** (80%) (Scheme 3).

3. Synthesis of the donor 14

According to our synthetic strategy, a benzyl group was introduced at the 6 position of the GlcNAc residue to temporarily mask the position to be sulfated, and the desired GlcNAc derivative **11** was readily obtained from the known 2-(trimethylsilyl)ethyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside **10**[11] by reductive opening of the benzylidene group, by treatment with Et_3SiH, and $BF_3{\cdot}Et_2O$ in DCM.[12] Under the normal conditions we found that the glycosylation of **11** and **12**[13] gave a very poor yield; however, when new conditions were employed[15] disaccharide **13** was obtained in a good yield of 85%. The ^{1}H NMR spectrum confirmed unambiguously the correct β(1→4) substitution.[14] The anomeric position was deblocked by TFA/water and was subsequently converted to the imidate **14** (74%, two steps) (Scheme 4).

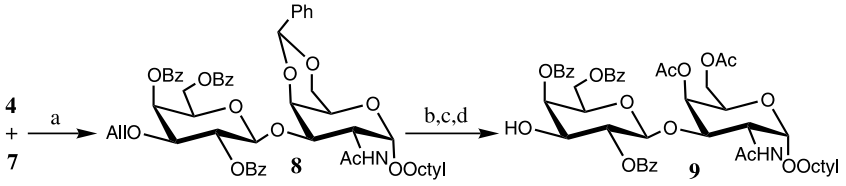
4. Synthesis of the sulfotetrasaccharide I and its unsulfated analogue I′

Glycosylation between **14** and **9** used the previously mentioned conditions,[15] which gave the fully protected tetrasaccharide **15**[14] in a satisfactory yield (50%). The phthalimido group was replaced by acetamido group to afford **16** (72%) (Scheme 5).

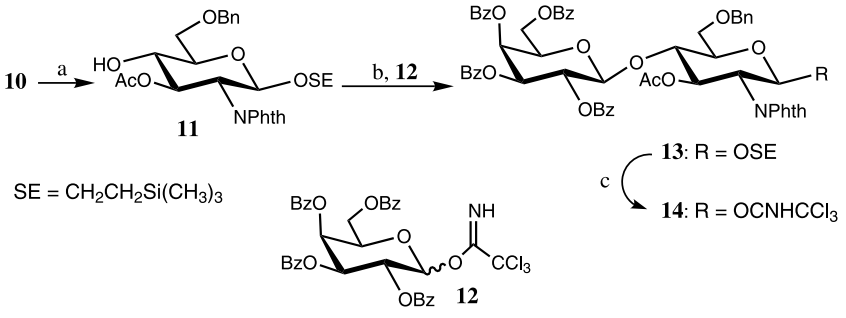
Treatment with Pd/C and H_2 selectively cleaved the benzyl ether to give **17** and sulfation was carried out using $Me_3N{\cdot}SO_3$ in DMF to give the 6-*O*-sulfated tetrasaccharide **18** in 76% overall yield. Final saponification (MeOH, MeONa) yielded quantitatively the target sulfated oligosaccharide **I** (Scheme 6). In addition, saponification of **17** led quantitatively to the analogue non-sulfated tetrasaccharide **I′**.[18]

5. Enzymatic sialylation and fucosylation of sulfonato-pentasaccharide II and sulfonato-hexasaccharide III

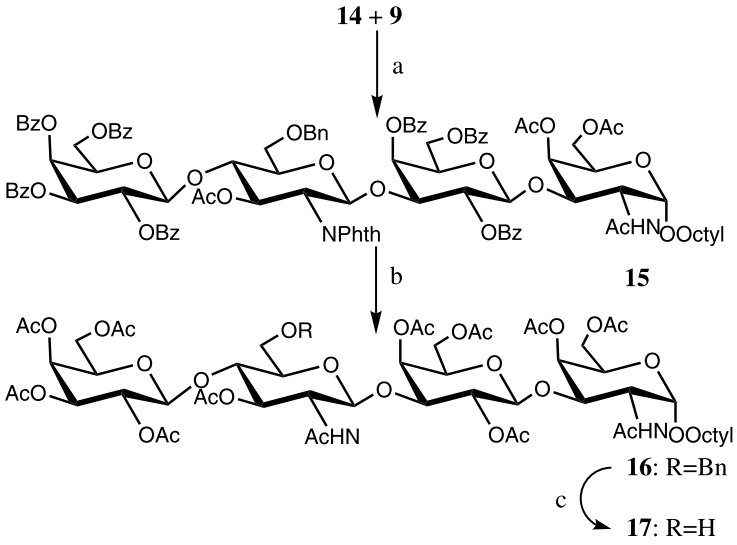
We planned to use enzymes for introducing key sialic acid and fucose residues to the sulfated hexasaccharide.



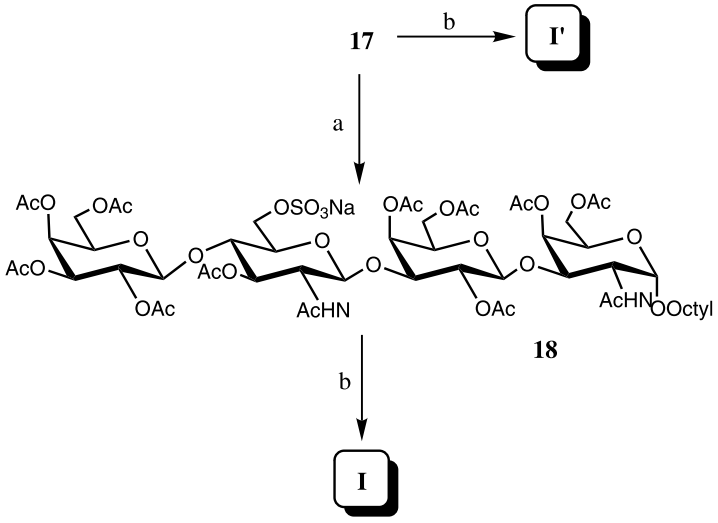
Scheme 3. *Reagents and conditions*: (a) cat. TfOH, DCE, 4 Å molecular sieves, 55°C, 69%; (b) $AcOH/H_2O$, 90°C; (c) Ac_2O, Py, 81% (two steps); (d) $Pd(Cl)_2$, MeOH, 80%.



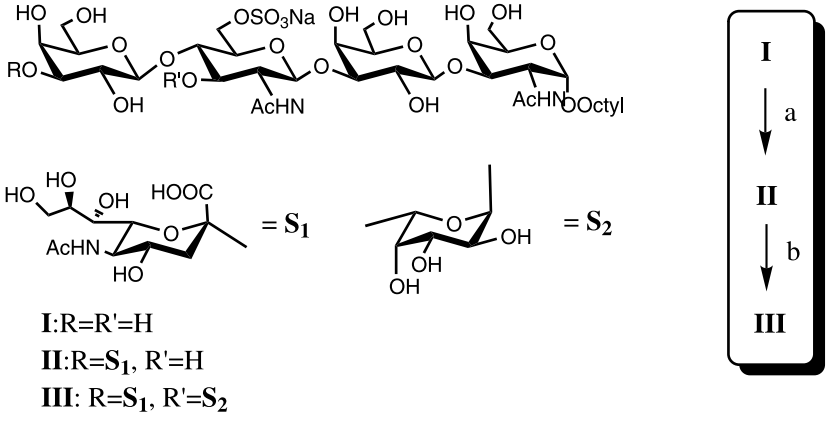
Scheme 4. *Reagents and conditions*: (a) Et_3SiH, $BF_3{\cdot}Et_2O$, DCM, 66%; (b) cat. TfOH, DCE, 4 Å molecular sieves, 55°C, 85%; (c) TFA, DCM, then CCl_3CN, DBU, DCM, 74% (two steps).



Scheme 5. *Reagents and conditions*: (a) cat. TfOH, DCE, 4 Å molecular sieves, 55°C, 50%; (b) ethylene diamine, *t*-BuOH, 90°C, 12 h, then Ac_2O, MeOH, Et_3N, 72% (two steps); (c) Pd/C, H_2, EtOH, 80%.



Scheme 6. *Reagents and conditions*: (a) $(CH_3)_3N{\cdot}SO_3$, DMF, 95%; (b) MeOH, NaOMe, quant.



Scheme 7. *Reagents and conditions*: (a) SAT(N), CMP-NANA, MOPS (pH 7.4), Triton S4, BSA, H_2O, CIP, 55%; (b) FucT(V), GDP-Fuc, $MnCl_2$, DTT, Tris–HCl (pH 7.4), quant.

The sulfation is thought to be the final step in the proposed pathway of 6-*O*-sulfated Sialyl Lewis^x.[16,17] We were therefore interested in determining whether sialyltransferase and/or fucosylfransferase could take the sulfated oligosaccharide as a substrate. With tetrasaccharide **I** in hand, selective sialylation was achieved

in a single step using α2,3-sialyl transferase and CMP-NANA. The reaction did not go to completion, however, ion exchange chromatography enabled us to isolate the desired pentasaccharide **II** in 55% yield. For fucosylation, incubation of **II** with FucT-V and GDP-Fuc gave the sulfonato-hexasaccharide **III** in a nearly quantitative yield.[18,19] Both enzymes demonstrated a tolerance for sulfated acceptor substrates as the enzymes in the biosynthetic route for the MECA-79 epitope (Scheme 7).

6. Conclusion

Sulfated oligosaccharides were efficiently synthesized using chemoenzymatic synthetic scheme with the enzymes utilizing the sulfated substrates. In addition, new glycosylations conditions were tested with success when standard conditions were unsuitable, and it was demonstrated that controlled heating of glycosylation reaction mixtures was a way to activate acceptors without degradation.

Acknowledgements

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14. Partial ^{1}H NMR ($CDCl_3$): **8**: 5.85 (dd, 1H, $J_{3,4}=3.1$ Hz, $J_{4,5}<1.0$ Hz, Gal H-4), 5.60 (m, 1H, OAll), 5.52 (d, 1H, $J_{2,NH}=7.6$ Hz, GalNAc N-H), 5.48 (dd, 1H, $J_{1,2}=7.8$ Hz, $J_{2,3}=10.0$ Hz, Gal H-2), 5.39 (s, 1H, PhC*H*), 5.10 (m, 2H, OAll), 5.06 (d, 1H, $J_{1,2}=3.3$ Hz GalNAc H-1), 5.04 (d, 1H, Gal H-1), 4.60 (dd, 1H, $J_{5,6a}=7.3$ Hz, $J_{6a,6b}=11.5$ Hz, Gal H-6a), 4.51 (m, 1H, $J_{2,3}=11.3$ Hz, GalNAc H-2), 4.42 (dd, 1H, $J_{5,6b}=5.1$ Hz, Gal H-6b), 4.40 (dd, 1H, $J_{3,4}=3.0$ Hz, $J_{4,5}<1.0$ Hz, GalNAc H-4), 4.20 (m, 1H, Gal H-5), 4.15–3.70 (m, 6H, GalNAc H-6a, H-6b, H-3, Gal H-3, OAll), 3.60–3.30 (m, 3H, GalNAc H-5, *O*-Octyl), 1.60 (s, 3H, Ac). **13**: 5.90 (dd, 1H, $J_{3,4}=3.4$ Hz, $J_{4,5}<1$ Hz, Gal H-4), 5.80 (dd, 1H, $J_{2,3}=10.8$ Hz, $J_{3,4}=8.9$ Hz, GlcNAc H-3), 5.68 (dd, 1H, $J_{1,2}=7.9$ Hz, $J_{2,3}=10.4$ Hz, Gal H-2), 5.40 (dd, 1H, Gal H-3), 5.32 (d, 1H, $J_{1,2}=8.5$ Hz, GlcNAc H-1), 4.80 (d, 1H, Gal H-1), 4.85 and 4.40 (2d, 2H, PhCH_2), 4.43 (m, 2H, Gal H-6a H-6b), 4.25 (dd, 1H, GlcNAc H-2), 4.17 (t, 1H, $J_{4,5}=10.5$ Hz, GlcNAc H-4), 4.06 (m, 1H, Gal H-5), 3.90 (m, 1H, OSE), 3.71 (dd, 1H, GlcNAc H-6a), 3.60–3.50 (m, 2H, GlcNAc H-5 H-6b), 3.42 (m, 1H, OSE), 1.92 (s, 3H, OAc), 0.80 (m, 2H, OSE) and −0.1 (s, 9H, OSE). **15**: 5.82 (dd, 1H, $J_{3,4}=3.2$ Hz, $J_{4,5}<1$ Hz, Gal H-4), 5.80 (dd, 1H, $J_{3,4}=3.0$ Hz, $J_{4,5}<1.0$ Hz, Gal H-4), 5.60–5.30 (m, 6H, GlcNAc H-3, GalNAc H-4 and N-H, Gal 2H-2 H-3), 5.02, 4.80 and 4.65 (3d, 3H, $J_{1,2}=8.2$ Hz, $J_{1,2}=7.9$ Hz, $J_{1,2}=8.0$ Hz, Gal 2H-1, GlcNAc H-1), 4.76 (d, 1H, $J_{1,2}=3.3$ Hz, GalNAc H-1), 4.54 and 4.22 (2d, 2H, PhCH_2), 4.46–4.24 (m, 7H, GlcNAc H-2 H-4, GalNAc H-2, Gal 4×H-6), 4.10–3.50 (m, 9H, Gal 2×H-5 H-3, GlcNAc 2×H-6, H-5, GalNAc H-5, 2xH-6), 1.97, 1.93, 1.70, 1.60 (4s, 12H, Ac).
15. *Standard procedure*: A mixture of donor (*case 1*: **7**, 1.7 equiv.; *case 2*: **12**, 2 equiv.; *case 3*: **14**, 1.5 equiv.), acceptor (*case 1*: **4**, 1 equiv.; *case 2*: **11**, 1 equiv.; *case 3*: **9**, 1 equiv.) and 4 Å powdered molecular sieves in anhydrous 1,2-dichloroethane was stirred for 1 h at rt under dry Ar. Triflic acid was added dropwise and the mixture was stirred for 1 h 30 min at 55°C. After cooling to rt, Et_3N was added, and the mixture was filtered and concentrated. The residue was eluted from a column of silica gel to provide the desired compound (*case 1*: **8**, 69%; *case 2*: **13**, 85%; *case 3*: **15**, 50%).
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18. Partial NMR and MS data for final products: **I′**: ^{1}H NMR (D_2O): δ: 4.89 (d, 1H, $J_{1,2}=3.6$ Hz, H-1_{GalNAc}), 4.72 (d, 1H, $J_{1,2}=8.3$ Hz, H-1_{GlcNAc}), 4.47 (d, 1H, $J_{1,2}=7.3$ Hz, H-1_{Gal}), 4.44 (d, 1H, $J_{1,2}=8.0$ Hz, H-1_{Gal}), 2.04 and 2.03 (2s, 6H, 2 NHAc); ^{13}C NMR: 105.55, 103.69, 103.48, 97.80 (4 C-1); HRMS calcd for $C_{36}H_{64}N_2NaO_{21}$ (M+Na^+): 883.389, found 883.388. **I**: ^{1}H NMR (D_2O): δ: 4.88 (d, 1H, $J_{1,2}=3.6$ Hz, H-1_{GalNAc}), 4.75 (d, 1H, $J_{1,2}=8.2$ Hz, H-1_{GlcNAc}), 4.53 (d, 1H, $J_{1,2}=7.5$ Hz, H-1_{Gal}), 4.48 (d, 1H, $J_{1,2}=7.9$ Hz, H-1_{Gal}), 2.05 and 2.02 (2s, 6H, 2 NHAc); ^{13}C NMR: 105.52, 103.62, 103.44, 97.65 (4 C-1); HRMS calcd for $C_{36}H_{63}N_2Na_2O_{24}S$ (M+Na^+): 985.328, found 985.325. **III**: ^{1}H NMR (D_2O): δ: 5.10 (d, 1H, $J_{1,2}=3.9$ Hz, H-1_{Fuc}), 4.72 (d, 1H, $J_{1,2}=3.5$ Hz, H-1_{GalNAc}), 4.59 (d, 1H, $J_{1,2}=8.0$ Hz, H-1_{GlcNAc}), 4.42 (m, 2H, 2 H-1_{Gal}), 2.72 (dd, 1H, H-3e), 2.02–1.98 (3s, 9H, 3 NHAc), 1.78 (t, 1H, H-3a), 1.18 (d, 3H, $J_{5,6}=6.5$ Hz, H-6_{Fuc}); MS calcd for $C_{53}H_{90}N_3NaO_{36}S$ (M^+): 1399.49, found 1399.4.
19. Sialyltransferase: ST3GalIII, Calbiochem (566218) and Fucosyltransferase: FucT-V, Calbiochem (344320).