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Recombinant (2→3)-α-sialyltransferase immobilized on nickel-Agarose for preparative synthesis of sialyl Lewis^x and Lewis^a precursor oligosaccharides

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Abstract

The specificity of recombinant (2→3)-α-sialyltransferase (ST3Gal-III), expressed in baculovirus-infected insect cells, has been determined with various oligosaccharide acceptors and sugar-nucleotide donors using a fluorescence based assay. Recombinant ST3Gal-III tagged with a polyhistidine tail was immobilized on Ni^{2+}-NTA-Agarose as an active enzyme for use in the synthesis of three sialylated oligosaccharides: (i) the divalent molecule [α-Neu5Ac-(2→3)-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-O-CH_2]$_2$-C-(CH_2OBn)$_2$ (**12**); (ii) the dansylated derivative, α-Neu5Ac-(2→3)-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-O-$(CH_2)_6$-NH-dansyl and; (iii) the tetrasacharide α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-O-CH_3. Compound **12** was itself prepared from the divalent *N*-acetyllactosamine molecule built on pentaerythritol by a chemo-enzymatic route.

Keywords: Chemo-enzymatic synthesis; Recombinant sialyltransferase; Baculovirus-infected insect cells; Enzyme immobilization

1. Introduction

Sialyltransferases catalyzing the transfer of *N*-acetylneuraminic acid at the nonreducing end of oligosaccharides with complete regio- and stereoselectivity have been widely used over the past decade in the synthesis of bioactive oligosaccharides related to selectin-mediated cell adhesion. These enzymes have become increasing available by genetic engineering, and soluble forms of glycosyltransferases, originally membrane-bound proteins, have been generated by removal of the membrane spanning region. A soluble form of CMP-Neu5Ac:β-D-Gal*p*-(1→3/4)-β-D-Glc*p*NAc-(2→3′)-α-sialyltransferase (ST3Gal-III according to Tsuji's nomenclature[1]), first purified from rat liver,[2] was cloned 10 years ago[3] and the recombinant enzyme expressed in insect cells allowed the enzymatic synthesis of sialyl Lewis^x tetrasaccharide with in situ regeneration of CMP-Neu5Ac.[4] Another construct of recombinant ST3Gal-III was used for the efficient production of sialyl Lewis^x and Lewis^a libraries[5–7] and saccharopeptides.[8,9]

In this report, the cDNA coding for the rat liver ST3Gal-III starting at amino acid 34 with a hexahistidine tag added to the N-terminus, was expressed in baculovirus-infected insect cells. Here we wish to report on: (i) the specificity of the recombinant ST3Gal-III based on a novel fluorescent assay; (ii) enzyme immobilization on Nickel charged beads via the His_6 tag; (iii) the use of the immobilized enzyme in the synthesis of

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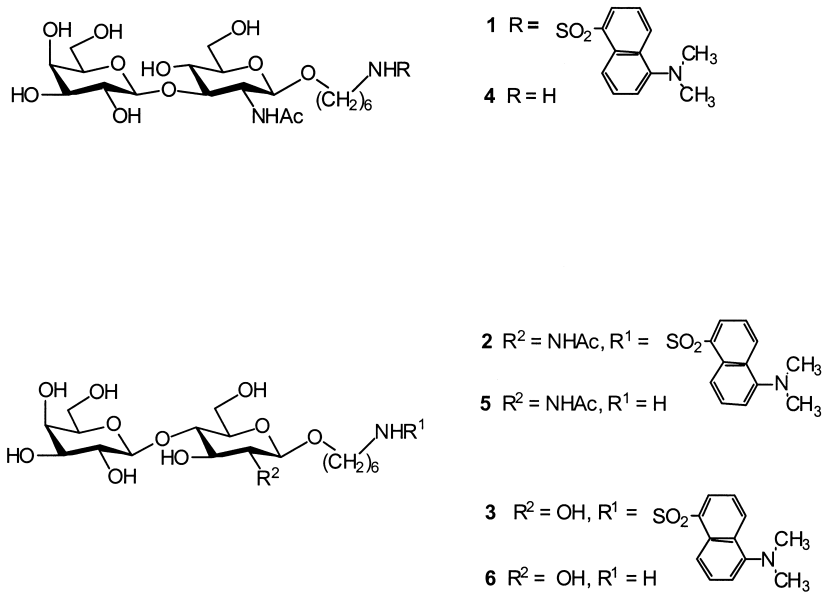


Scheme 1. Fluorescent substrates for ST3Gal-III.

new sialylated oligosaccharides belonging to Type 2 (β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc) or Type 1 (β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc) series.

2. Results and discussion

2.1. Expression of soluble ST3Gal-III in Sf9

Ten recombinant viral clones were used to infect *Sf*9 cell cultures and the conditioned culture media were tested for (2→3)-α-sialyltransferase activity 5 days after infection using β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-O-$(CH_2)_7$-CH_3 as an acceptor substrate. For two independent clones (#6703 and #6708), a functional soluble form of ST3Gal-III (EC 2.4.99.5) was produced based on enzymatic assay of the culture supernatant and by anti-His mAb detection (Qiagen, France) (data not shown). Clone #6703 was chosen for large scale production of ST3Gal-III in 400 mL roller bottles. The medium was collected and the recombinant His_6-tagged ST3Gal-III was visualized by Western blotting as a single 60 kDa band using anti-His mAb that is in agreement with the theoretical molecular weight of the His_6-tagged ST3Gal-III of 39 kDa (data not shown).

2.2. Substrate specificity of ST3Gal-III

As an alternative to radioactive assays routinely used for glycosyltransferases, a novel fluorescent assay was developed. Novel disaccharide derivatives **1**, **2**, **3**, bearing an aminohexyl chain substituted by a dansyl group at the anomeric center were prepared, from glycosides **4**, **5**, **6**, respectively by reaction with dansyl chloride in sodium carbonate (Scheme 1). In evaluation as substrates, acceptor **1** exhibited the highest activity, whereas the relative rate with acceptors **2** and **3** were, 64 and 30%, respectively of the rate observed with acceptor **1** (Table 1). These results are in agreement with the specificity studies reported both for the natural[10] and recombinant enzyme.[11] The cation Mn^{2+} has no effect on enzyme activity. From 1 L of insect cells culture, about 5 U of ST3Gal-III could be reproducibly obtained (1 U of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of Neu5Ac from CMP-Neu5Ac to β-D-Gal*p*-(1→

Table 1
Activity of recombinant ST3Gal-III expressed in baculovirus-infected insect cells on various oligosaccharide acceptors and sugar-nucleotide donors, both tested at 0.8 mM concentration

Donor	Acceptor	Relative velocities [a]
CMP-Neu5Ac	β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-OR [b] (**1**)	100
CMP-Neu5Ac	β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-OR (**4**)	64
CMP-Neu5Ac	β-D-Gal*p*-(1→4)-β-D-Glc*p*-OR (**6**)	30
CMP-Neu5Gc	β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-OR (**1**)	28
CMP-KDN	β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-OR (**1**)	26

[a] Relative velocities with acceptor and donor substrates tested at 0.8 mM.

[b] R = $(CH_2)_6$-NH-dansyl.

Table 2
Immobilization of His-tagged recombinant ST3Gal-III on Ni^{2+}-NTA-Agarose

Initial soluble enzyme activity (mU mL^{-1})	Vol of culture supernatant (mL)	Vol of Ni^{2+}-NTA-Agarose (mL)	Gel-bound enzyme activity (mU)	Remaining soluble enzyme activity (mU)	Immobilization yield [c] (%)
6 [a]	5	1	1	22.5	<5
32 [a]	2	1	38	18.5	59
10 [a]	15	6	66	66	44
13.5 [b]	6	2	44	12	54
29 [b]	5	2	58	32	40

[a] Culture supernatant free of fetal calf serum.
[b] Culture supernatant containing 5% fetal calf serum.
[c] Immobilization yield is expressed as the ratio of immobilized activity to that initially present in solution.

3)-β-D-Glc*p*NAc-O-$(CH_2)_6$-NH-dansyl). The fluorescent assay was used to measure enzyme activity with CMP-sialic acids other than CMP-Neu5Ac, the donor commonly used in sialyltransferase-catalyzed reactions, and the only radiolabeled CMP-donor that is commercially available. Both donors CMP-Neu5Gc and CMP-KDN were recognized by ST3Gal-III but with a lower rate of transfer, 28 and 26%, respectively, as compared to CMP-Neu5Ac (Table 1).

2.3. Enzyme immobilization

Recombinant ST3Gal-III was tagged with a polyhistidine tail. A stretch of His_6 is now commonly added to the primary sequence of recombinant proteins in order to facilitate their purification by Ni^{2+} affinity chromatography, based on strong interactions between Ni^{2+} immobilized on Agarose through nitriloacetic acid (NTA) and the polyhistidine tag.[12] We previously reported that the His_6 tag of recombinant FucT-III could be exploited for enzyme immobilization and that FucT-III immobilized onto nickel-nitriloacetate beads exhibited enhanced stability compared to the soluble enzyme.[13] This result prompted us to examine immobilization for ST3Gal-III secreted in the culture supernatant of baculovirus-infected insect cells. Prior to the binding step, the culture media must be ultrafiltrated to adjust the pH to 8 and to remove any interfering components. Then the His_6 tagged ST3Gal-III was immobilized on Ni^{2+}-NTA-Agarose under native conditions at 4 °C using a batch procedure. In order to optimise the immobilization yield, several parameters were tested (Table 2). For optimal immobilization, the culture supernatant must be concentrated with a ratio of the volume of the soluble enzyme to that of the gel of $\approx 2/1$. ST3Gal-III was immobilized, in an active form, in 40–59% yield depending on the experiments. The soluble enzymatic activity remaining after the immobilization step could not be re-immobilized when again treated with a new batch of nickel-Agarose, probably due to the loss of the His_6 tag owing to enzyme proteolysis in the course of concentration and immobilization steps. No significant difference in immobilization yields was observed between culture media with or without fetal calf serum. The enzymatic activity of the gel stored at 4 °C was stable for at least 5 months, whereas crude culture supernatants were inactivated in 2 weeks of storage at 4 °C. By avoiding the lengthy procedures of enzyme purification, immobilization on Ni^{2+}-Agarose appears to be an ideal method for general use of recombinant glycosyltransferases in synthesis. In addition to the earliest report on immobilization of recombinant mannosyltransferase,[14] there is a recent example describing the use of immobilized enzymes for the efficient production of UDP-Gal from UDP.[15]

2.4. Sialylation with immobilized ST3Gal-III

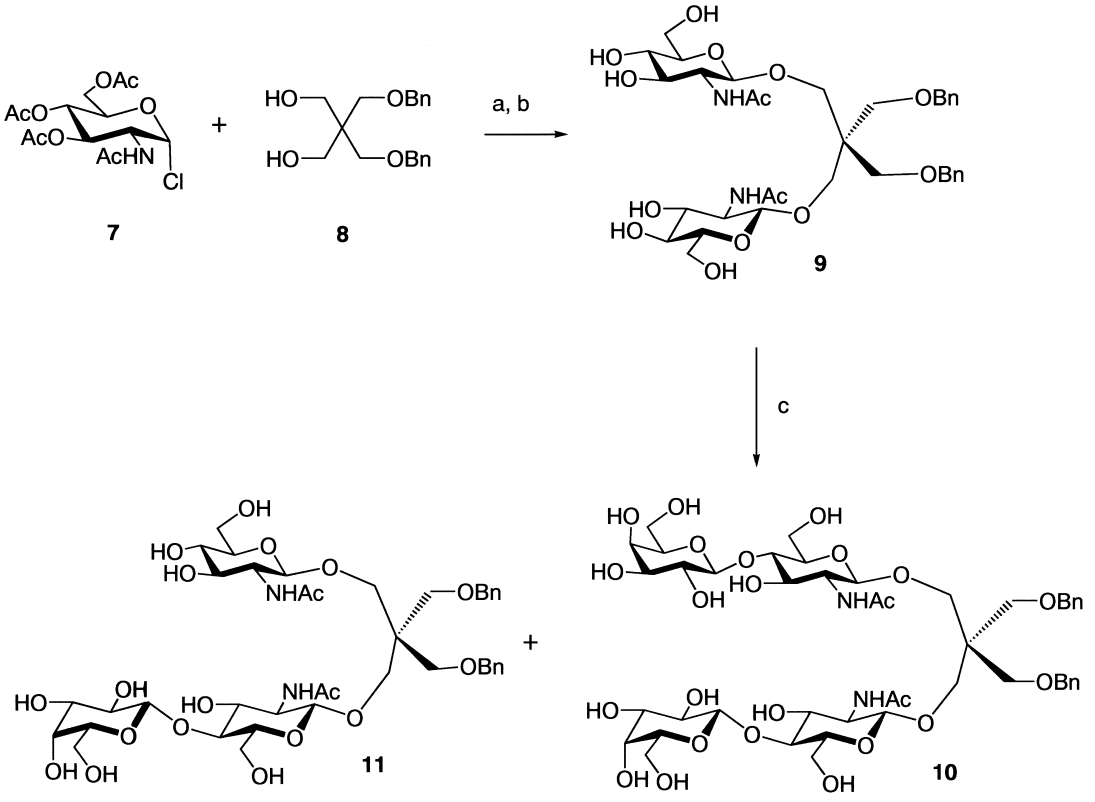
The immobilized enzyme preparation was first used to sialylate compound **10**, comprised of two *N*-acetyllactosamine residues built on the pentaerythritol molecule according to previous work on the preparation of divalent selectin ligands.[16] Compound **10** was prepared by a chemo-enzymatic route. First, condensation of the chloride **7** with the dibenzyl ether of pentaerythritol **8**[17] in toluene–nitromethane at 45 °C in the presence of mercuric cyanide as the promotor, afforded after deacetylation the starting divalent molecule **9** in 68% yield (Scheme 2). Then, enzymatic galactosylation was achieved with β-(1→4)-galactosyltransferase (EC 2.4.1.22), together with UDP-glucose, UDP-glucose epimerase, manganese chloride and alkaline phosphatase, leading to the di-galactosylated derivative **10** in 65% isolated yield, in addition to the mono-galactosylated derivative **11** obtained in 24% yield. Compound **10** was sialylated by incubation with recombinant ST3Gal-III adsorbed on Ni^{2+}-Agarose in 25 mM sodium cacodylate buffer pH 7.1 and CMP-Neu5Ac added in three portions during the 40-h incubation time (Scheme 3). Unlike CDP and CTP, CMP released in the

reaction was not a strong inhibitor, therefore the addition of alkaline phosphatase was unnecessary. The bis-sialylated lactosamine derivative **12** was obtained in 73% yield by methanol elution from C_{18} Sep-Pak cartridges and gel permeation on Biogel P-2. This derivative has been further used for the synthesis of a divalent sialyl Lewis^x molecule, required as a reference compound in the study of low entropy ligands.[18] At the end of incubation, about 75% of the enzymatic activity still remained on the gel. The same immobilized enzyme preparation could then be used for sialylation of the dansylated disaccharide **1**. This substrate was incubated like compound **10** with CMP-Neu5Ac and immobilized ST3Gal-III, and gave trisaccharide **13** in 65% isolated yield. This trisaccharide may serve as a substrate for testing endogenous sialidase activity present in culture of Chinese hamster ovary (CHO) cells.[19] The preparation of immobilized sialyltransferase was also utilized for sialylation of trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-O-CH_3 (**14**), previously synthesized by a chemo-enzymatic method.[20] The sialylated tetrasaccharide **15**, together with its regio-isomer α-Neu5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-O-CH_3[20], are expected to be useful substrates to define the specificity of new (2→8)-α-sialyltransferases.

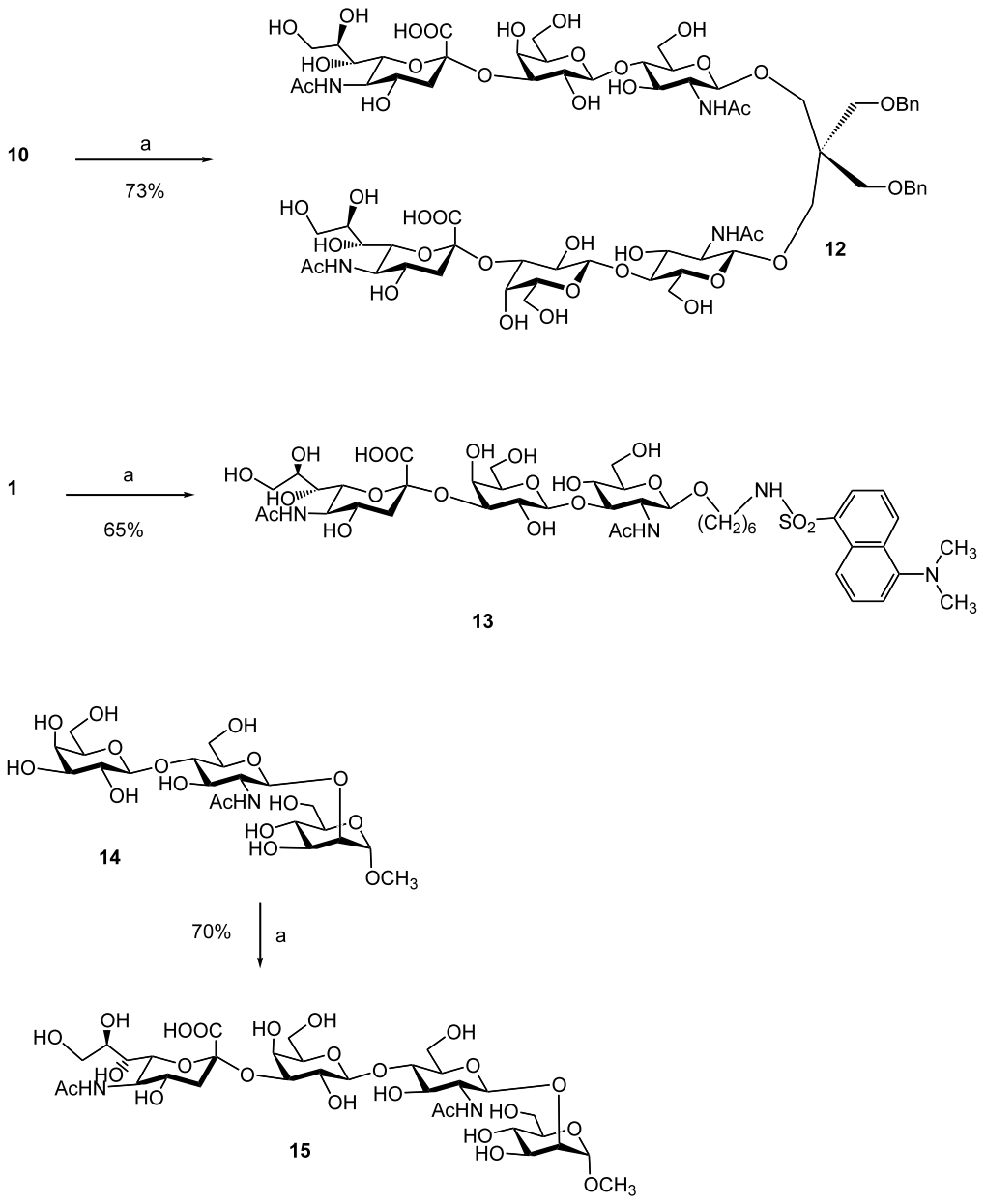
3. Experimental

3.1. General

NMR spectra were recorded with a Bruker AC-200 or AC-250 spectrometer; chemical shifts are given relative to the signal of tetramethylsilane in $CDCl_3$; for ^1H 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. Mass spectra were performed either on a Finigan MATT 95 apparatus using the ESI technique, or on a VOYAGER DE STR Pro instrument (Perseptive Biosystem) using MALDI-TOF-MS with 2,5-dihydroxybenzoic acid as the matrix. 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. Fluorescence measurements were performed with a Perkin–Elmer LS50B fluorimeter, controlled by a PC and equipped with a plate reader allowing TLC plates scanning. Fluorescence was read at 385 nM excitation/540 nM emission. CMP-Neu5Ac was purchased from Kyowa Hakko; CMP-Neu5Gc and CMP-KDN were synthesized according to Lubineau and co-workers[21,22] Bovine milk D-GlcNAc-β-(1→4)-



Scheme 2. *Reagents and conditions*: (a) $HgCN_2$, $CaSO_4$, 1:1 $PhCH_3$–CH_3NO_2, 45 °C, 16 h; (b) 8:1:1 MeOH–water–Et_3N, rt, 16 h, 68% for two steps; (c) GalT, UDPGE, UDP-Glc 2 equiv, AP, 15 mM $MnCl_2$, 25 mM sodium cacodylate buffer pH 7.4, 37 °C, 3 days, 65%.



Scheme 3. *Reagents and conditions*: (a) ST3Gal-III immobilized on Ni^{2+}-NTA-Agarose, CMP-Neu5Ac 1.7 equiv, 25 mM sodium cacodylate buffer pH 7.1, 37 °C, 4 days.

galactosyltransferase and UDP-glucose 4-epimerase were from Calbiochem, UDP-Glc was from Sigma, protease inhibitor cocktail and calf intestine alkaline phosphatase from Roche Molecular Biochemicals.

3.2. Construction of a recombinant baculovirus and expression of a soluble ST3Gal III in Sf9

To express a soluble and His_6-tagged form of the rat ST3Gal-III, the *N*-terminal sequence (aa 9–33), including the transmembrane domain, was eliminated and replaced by the signal peptide sequence of the viral EGT gene. [23]A 78 bp fragment encoding the signal peptide sequence of the EGT gene was obtained after the annealing of the following two synthetic oligonucleotides: EGT 5′-GA-TCC-GCC-ACC-ATG-ACC-ATC-TTA-TGT-TGG-CTC-GCT-CTC-CTG-AGC-ACA-CTC-ACA-GCT-GTT-AAC-GCT-GAC-ATC-A-3′, and Back EGT 5′-GA-TCT-GAT-GTC-AGC-GTT-AAC-AGC-TGT-GAG-TGT-GCT-CAG-GAG-AGC-GAG-CCA-ACA-TAA-GAT-GGT-CAT-GGT-GGC-G-3′ to generate pUC-PS-EGT. A 108 bp DNA fragment containing an *Hpa*I site, the last codon of the EGT signal peptide sequence, 6 histidine codons and 19 codons corresponding to aa 34–52 of the N-terminal domain of the rat ST3Gal-III was reconstituted using a

set of nine overlapping synthetic oligonucleotides. This DNA fragment was subcloned and inserted into the *Hpa*I–*Sac*I site of pUC-PS-EGT.[23] In order to avoid homologous recombination between virus and vector encoded EGT, codons were made degenerate. The resulting construct was digested with *Avr*II–*Mun*I to receive the 1900 bp *Avr*II–*Eco*RI fragment prepared from pBS-SK-ST3Gal-III (clone ST3N-1[3]). The full-length modified rat ST3Gal-III gene was then excised by digestion with *Bam*HI and *Hind*III and inserted into the *Bgl*II–*Hind*III site of the p119 transfer vector[24] designed for recombination into the p10 locus of the baculovirus, giving p119-PS-ST3Gal-III. Sf9 Cells (ATCC CRL1711) were cotransfected by lipofection[25] using DOTAP (Roche Applied Science, Germany) with p119-PS-ST3Gal-III and purified viral DNA. The ST3Gal-III recombinant baculoviruses were purified by plaque assay and viral clones were tested in vitro by assaying for a α-(2→3)-sialyltransferase activity using 2 mM β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-O-$(CH_2)_7$-CH_3 as acceptor and 50 μM CMP-[^{14}C]-Neu5Ac (1.85 KBq) as donor.[26] Large scale production of ST3Gal-III was performed in *Sf*9 cells (growing temperature 28 °C), infected with the recombinant baculovirus clone 6703 at a multiplicity of infection of five PFU/cell. This production was undertaken under two different conditions. The first was done in a 400 mL roller bottle using EXTRA-1X medium (Eurobio, France) supplemented with 5% heat-inactivated fetal calf serum (Life Technologies, France). The second one was performed in 4× 400 mL roller bottles using only serum-free medium EXTRA-SB5 (Eurobio, France).

3.3. Enzymatic assay

Enzyme assay with fluorescent acceptors: 50 mM sodium cacodylate buffer pH 6.6, 5 μg bovine serum albumin, 0.8 mM CMP-Neu5Ac (or CMP-Neu5Gc or CMP-KDN when other donors were tested), 0.8 mM fluorescent disaccharide acceptors. The mixture was incubated at 37 °C under stirring for 15–60 min with 10–20 μL of 10-fold concentrated culture supernatant or a Nickel bead suspension containing ST3Gal-III. Incubation mixtures were analyzed by TLC on silica gel (3:3:2 EtOAc–2-propanol–water) and the percentage of conversion was evaluated from the fluorescence intensity of the spots referring to substrate and product, which was directly quantified on the plate.

3.4. Immobilization of ST3Gal-III

To 10 vol of culture medium containing the His_6-tagged soluble form of ST3Gal-III from baculovirus-infected insect cells, 1 vol of 0.2 M Tris pH 8 containing 2 M NaCl, 50 mM imidazole, 10 mM mercaptoethanol, 0.1% NaN_3 and a protease inhibitor cocktail was added, and the solution was ultrafiltered 10-fold. The concentrated solution was diluted in 9 vol of 20 mM Tris pH 8 containing 0.2 M NaCl, 5 mM imidazole, 1 mM mercaptoethanol, 0.01% NaN_3, and again ultrafiltered. The resulting solution was incubated with Ni^{2+}-NTA-Agarose (Qiagen, France) at 4 °C on a rotary shaker overnight. Then the gel was centrifuged at 3000 rpm for 10 min, the supernatant was removed and the gel washed with 0.25 mM sodium cacodylate buffer pH 6.6 containing 0.01% NaN_3 and again centrifuged. The gel was resuspended in the same buffer (5 mL); the enzymatic activity was measured in the supernatant and on aliquots of the gel suspension.

3.5. General procedure for dansylation

The 6-aminohexyl β-D-glycoside[27] (0.5 mmol) was dissolved in water (32 mL) containing sodium carbonate (0.11 g, 1.03 mmol). A solution of dansyl chloride (148 mg, 0.55 mmol) in acetone was added dropwise and the mixture was stirred at room temperature (rt) for 1 h. Solvents were then evaporated and the residual mixture was purified by HPLC on a Waters 600 equipped with a preparative reversed-phase column (Nucleosil C-18) using elution with a solvent gradient of water–MeOH.

3.6. 6-(Dansyl)-aminohexyl *O*-β-D-galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (1)

Disaccharide **4** was treated as described above affording disaccharide **1** (0.22 g, 64%); $[\alpha]_D^{29}$ −19° (*c* 0.98, MeOH);^{1}H NMR (250 MHz, CD_3OD): δ 8.55 (d, *J* 7 Hz 1 H, dansyl), 8.30 (d, 1 H, dansyl), 8.19 (d, 1 H, dansyl), 7.62 (d, 1 H, dansyl), 7.55 (d, 1 H, dansyl), 7.25 (d, 1 H, dansyl), 4.45 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 4.25 (d, 1 H, $J_{1',2'}$ 6 Hz, H-1′), 2.89 (s, 6 H, $N(CH_3)_2$), 1.92 (s, 3 H, NAc) and 1.35–0.90 (m, 8 H, 4 CH_2); ^{13}C NMR (62.9 MHz, CD_3OD) δ: 173.9 (CO), 153.0, 137.0, 131.0, 130.0, 128.9, 124.1, 120.4, 116.3 (C–Ar), 105.4 (C-1′), 102.1 (C-1), 84.8 (C-3) 77.3, 76.9, 74.5, 72.2, 70.4, 70.3, 70.0 (C-5, C-5′, C-4′, C-4, C-3′, C-2′, OCH_2), 62.6, 62.3 (C-6, C-6′), 56.2 (C-2), 45.8 ($N(CH_3)_2$), 43.7 (NCH_2), 30.4, 30.1, 27.0, 26.3 (CH_2), 23.1 (CH_3CO); HRMS Calcd for $C_{32}H_{49}N_3NaO_{13}S$ $[M+Na]^+$: 738.2883. Found: *m*/*z* 738.2882.

3.7. 6-(Dansyl)-aminohexyl *O*-β-D-galactopyranosyl-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranoside) (2)

Disaccharide **5** was treated as described above affording disaccharide **2** (0.148 g, 43%); $[\alpha]_D^{29}$ −11° (*c* 1.01, MeOH); ^{1}H NMR (200 MHz, CD_3OD): δ 8.56 (d, *J* 7 Hz 1 H, dansyl), 8.35 (d, 1 H, dansyl), 8.19 (d, 1 H, dansyl), 7.61 (d, 1 H, dansyl), 7.54 (d, 1 H, dansyl), 7.25 (d, 1 H, dansyl), 4.38 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.36 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 2.90 (s, 6 H, $N(CH_3)_2$), 2.84 (t, 2

H, NCH_2),1.92 (s, 3 H, NAc) and 1.5–1.1 (m, 8 H, 4 CH_2); ^{13}C NMR (50.2 MHz, CD_3OD) δ: 173.2 (CO),152.97, 136.9, 131.0, 130.0, 128.9, 124.1, 120.4, 116.3 (C-Ar), 104.9 (C-1′), 102.5 (C-1), 81.0 (C-4) 76.8, 76.2, 73.9, 72.3, 70.2, 70.1 (C-5, C-5′, C-4′, C-3, C-3′, C-2′, OCH_2), 62.3, 61.9 (C-6, C-6′), 56.5 (C-2), 45.7 ($N(CH_3)_2$), 43.6 (NCH_2), 30.3, 30.1, 26.9, 26.2 (CH_2)) and 22.9 (CH_3CO); HRMS Calcd for $C_{32}H_{49}N_3NaO_{13}S$ $[M + Na]^+$: 738.2883. Found: m/z 738.2882.

3.8. 6-(Dansyl)-aminohexyl *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (3)

Disaccharide **6** was treated as described above affording disaccharide **3** (0.141 g, 42%); $[\alpha]_D^{29}$ −10° (*c* 0.99, MeOH); ^1H NMR (250 MHz, $CDCl_3$–CD_3OD): δ 8.54 (d, 1 H, *J* 7 Hz, dansyl), 8.32 (d, 1 H, dansyl), 8.21 (d, 1 H, dansyl), 7.60 (d, 1 H, dansyl), 7.53 (d, 1 H, dansyl), 7.22 (d, 2 H, dansyl), 4.37 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.26 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 2.90 (s, 6 H, $N(CH_3)_2$), 2.86 (t, 2 H, *J* 7 Hz, NCH_2) and 1.54–1.15 (m, 8 H, 4 CH_2); ^{13}C NMR (62.9 MHz, CD_3OD): δ 153.1, 137.2, 131.0, 130.1, 129.0, 124.3, 120.6, 116.4 (C–Ar), 105.0 (C-1′), 104.1 (C-1), 80.7 (C-4) 77.0, 76.4, 74.7, 72.5, 70.6, 70.3 (C-5, C-5′, C-4′, C-3, C-3′, C-2, C-2′, OCH_2), 62.5, 62.0 (C-6, C-6′), 45.8 ($N(CH_3)_2$), 43.7 (NCH_2) and 30.4, 27.2, 26.3 (CH_2); HRMS Calcd for $C_{30}H_{46}N_2NaO_{13}S$ $[M + Na]^+$: 697.2618. Found: m/z 697.2619.

3.9. 2,2-Bis-(benzyloxymethyl)-1,3-bis(2-acetamido-2-deoxy-β-D-glucopyranosyl-oxy)-propane (9)

To a mixture of diol **8** (0.438 mg, 1.39 mmol), chloride **7** (1.52 g, 4.16 mmol), calcium sulfate (0.566 g, 4.16 mmol) suspended in 1:1 toluene–nitromethane (10 mL) was added mercuric cyanide (1.05 g, 4.16 mmol). The mixture was stirred for 16 h at 45 °C under Ag, then solvents were evaporated and the residue dissolved in CH_2Cl_2 and filtered through Celite. The filtrate was washed with saturated aq $NaHCO_3$, water, 20% aq potassium iodide and water again, dried ($MgSO_4$) and concentrated. Flash chromatography (1:3 petroleum ether-EtOAc) of the residue afforded the diglycosylation product slightly contaminated by the monoglycosylation product. This mixture (1.04 g), dissolved in 8:1:1 MeOH–water–Et_3N (100 mL), was stirred overnight at rt, then concentrated to dryness and co-evaporated several times with toluene. The residue, chromatographed on silica gel (4:1 EtOAc–MeOH), afforded **9** (0.683 g, 68%); $[\alpha]_D^{27}$ −12.6° (*c* 1, CH_3OH); ^1H NMR (250 MHz, CD_3OD): δ 7.30–7.10 (m, 10 H, Ph), 4.31 (s, 4 H, 2 $PhCH_2$), 4.19 (d, 2 H, $J_{1,2}$ 8.3 Hz, 2 H-1), 3.84 (d, 2 H, J_{gem} 9.8 Hz, 2 CH), 3.72 (d, 2 H, $J_{6,6'}$ 11.7 Hz, 2 H-6), 3.6–3.45 (m, $J_{6,6'}$ 11.7, $J_{6',5}$ 4.9 Hz, 4 H, 2 H-6′, 2 CH), 3.40–3.27 (m, 8 H, 2 CH_2, 2 H-3, 2 H-4), 3.24–3.07 (m, 4 H, $J_{1,2}$ 8.3 Hz, 2 H-2, 2 H-5) and 1.8 (s, 6 H, 2 OAc); ^{13}C NMR (62.9 MHz, CD_3OD): δ 173.5 (CO), 140.1, 129.3, 128.5 (Ar–C), 103.5 (C-1), 77.9, 75.7, 72.1 (C-3, C-4 and C-5), 74.5 (CH_2–Ph), 70.3, 69.8 (C–CH_2), 62.7 (C-6), 57.4 (C-2), 46.5 ($C(CH_2)_4$) and 23.3 (CH_3CO); ES + MS m/z 745.5 $[M + Na]^+$; Anal. Calcd for $C_{35}H_{50}N_2O_{14}\cdot 0.5\ H_2O$: C, 57.44; H, 7.02; N, 3.83. Found: C, 57.76; H, 7.32; N, 3.51.

3.10. 2,2-Bis(benzyloxymethyl)-1,3-bis[*O*-β-D-galactopyranosyl-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl-oxy)]-propane (10) and 2,2-bis(benzyloxymethyl)-1-(2-acetamido-2-deoxy-β-D-glucopyranosyl-oxy)-3-[*O*-β-D-galactopyranosyl-(1→4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl-oxy)]-propane (11)

Compound **9** (50 mg, 0.07 mmol), UDP-Glc (45 mg, 0.07 mmol), bovine milk D-GlcNAc β-(1→4)-galactosyltransferase (0.3 U) and UDP-glucose-4-epimerase (1.5 U), were incubated at 37 °C in 25 mM sodium cacodylate buffer pH 7.4 (5 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 (45 mg, 0.07 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 incubation was continued for 24 h. The reaction mixture was divided into four portions and 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 **10** (45 mg, 65%) and the mono-galactosylated compound **11** (15 mg, 24%).

Compound 10: $[\alpha]_D^{27}$ −30° (*c* 0.5, water); ^1H NMR (200 MHz, D_2O): δ 7.5–7.3 (m, 10 H, Ph), 4.45 (m, 6 H, $J_{1',2'}$ 8.8 Hz, 2 H-1′, 2 CH_2Ph), 4.34 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1) and 1.95 (s, 6 H, 2 OAc); ^{13}C NMR (50.3 MHz, D_2O): δ 174.9 (CO), 138.7, 129.7, 129.1 (Ar–C), 103.9 (C-1′), 102.8 (C-1), 79.6, 76.3, 75.7, 74.3, 73.5, 73.2, 69.6 (C-2′, C-3′, C-4′, C-5′, C-3, C-4 and C-5), 74.4 ($PhCH_2$), 69.5 (CH_2), 62.0, 61.1 (C-6, C-6′), 56.1 (C-2), 45.7 ($C(CH_2)_4$ and 23.2 (CH_3CO); HRMS: Calcd for $C_{47}H_{70}N_2NaO_{24}$ $[M + Na]^+$: 1069.4216. Found: m/z 1069.4239.

Compound **11**: $[\alpha]_D^{27}$ −29° (*c* 0.5, MeOH); ^1H NMR (200 MHz, D_2O): δ 7.5–7.3 (m, 10 H, Ph), 4.50–4.41 (m, 5 H, $J_{1',2'}$ 8.8 Hz, H-1′, 2 CH_2Ph), 4.34 (d, 2 H, $J_{1,2}$ 7.8 Hz, 2 H-1) and 1.95 (s, 6 H, 2 OAc); ^{13}C NMR (50.3 MHz, D_2O): δ 173.3 (CO), 140.1, 129.3, 128.6 (Ar–C), 105.1 (C-1′), 103.5 (C-1), 81.1, 78.0, 77.1, 76.5, 75.8, 74.9, 73.9, 72.6, 72.2, 70.4 (C-2′, C-3′, C-4′, C-5′, 2 C-3, 2 C-4, 2 C-5), 74.5 ($PhCH_2$), 70.3, 68.8, 68.7 (CH_2), 62.8, 62.5, 61.9 (2 C-6, C-6′), 57.4, 56.8 (2 C-2),

46.6 ($C(CH_2)_4$ and 23.2 (CH_3CO); HRMS Calcd for $C_{41}H_{60}N_2NaO_{19}$ $[M+Na]^+$: 907.3688. Found: m/z 907.3684.

3.11. 2,2-Bis(benzyloxymethyl)-1,3-bis-[5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyloxy]-propane (12)

Compound **10** (20 mg, 0.019 mmol), CMP-NeuAc (12.6 mg, 0.02 mmol) and ST3Gal-III adsorbed on Ni^{2+}-Agarose (45 mU) were incubated in 25 mM sodium cacodylate buffer pH 7.1 (6 mL) at 30 °C for 4 days with gentle stirring. Additional CMP-NeuAc (15.1 mg, 0.022 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 two portions and applied to Sep-Pak C_{18} cartridges; the combined methanol eluates were 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) to give **12** as its sodium salt (23 mg, 73%); $[\alpha]_D^{27}$ −18° (*c* 1, water); ^{1}H NMR (D_2O, 250 MHz): δ 7.5–7.3 (m, 10 H, Ph), 4.5 (d, 2 H, $J_{1',2'}$ 7.8 Hz, 2 H-1′), 4.46 (s, 4 H, 2 CH_2Ph), 4.32 (d, 2 H, $J_{1,2}$ 7.3 Hz, 2 H-1), 4.08 (dd, 2 H, $J_{3',4'}$ 3, $J_{3',2'}$ 8.8 Hz, 2 H-3′), 2.72 (dd, 2 H, $J_{3''e,3''a}$ 11.7, $J_{3''e,4''}$ 4 Hz, 2 H-3″e), 2.02 (s, 6 H, 2 OAc), 1.90 (s, 6H, 2 OAc) and 1.75 (t, 2 H, $J_{3''a,3''e}$ 11.7 Hz, 2 H-3″a); ^{13}C NMR (D_2O, 62.9 MHz): δ 175.9 (CO_2Na), 175.0, 174.9 (CO), 138.6, 129.7, 129.2 (Ph), 103.6 (C-1′), 102.9 (C-1), 100.8 (C-2″), 79.4 (C-4), 76.5, 76.1, 75.6 (C-5, C-5′, C-3′), 74.4, 73.8, 73.1, 72.7 ($PhCH_2$, C-3, C-6″, C-8″), 70.7, 69.7, 69.3, 69.1, 68.5 (C-2′, C-4′, C-4″, C-7″, CH_2), 63.6 (C-9″), 62.0, 61.0 (C-6, C-6′), 56.1 (C-2), 52.7 (C-5″), 45.7 (Cq), 40.6 (C-3″), 23.2 and 23.0 (CH_3CO); HRMS (negative mode): Calcd for $C_{69}H_{102}N_4O_{40}\cdot Na_2$ $[(M-2\ Na^+)/2]^-$ 813.3035. Found: m/z 813.3031.

3.12. 6-(Dansyl)-aminohexyl (5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (13)

The dansylated disaccharide **1** (14 mg, 0.0195 mmol) and CMP-NeuAc (6.9 mg, 0.011 mmol) were incubated in 25 mM sodium cacodylate buffer pH 7.1 (5 mL) at 30 °C for 4 days with gentle stirring in the presence of ST3Gal-III adsorbed on Ni^{2+}-Agarose (35 mU). Additional CMP-NeuAc (6.9 mg) 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 applied onto a Sep-Pak C_{18} cartridge; the methanol eluate was evaporated to dryness and the residue dissolved in water was purified by chromatography on DEAE-Sephadex A-25 (HCO_3^- form); elution with a gradient of 0–0.4 M triethylammonium hydrogen carbonate (pH 8.0) gave **13** as its triethylammonium salt (14 mg, 65%); $[\alpha]_D^{27}$ −19° (*c* 0.44, H_2O); ^{1}H NMR (250 MHz, D_2O) δ: 8.45 (d, 1 H, *J* 7 Hz, dansyl), 8.29 (d, 1 H, dansyl), 8.21 (d, 1 H, dansyl), 7.68 (d, 1 H, dansyl), 7.61 (d, 1 H, dansyl), 7.39 (d, 1 H, dansyl), 4.47 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.37 (d, 1 H, broad, H-1), 4.09 (dd, 1 H, $J_{3',4'}$ 3, $J_{3',2'}$ 10 Hz, H-3′), 3.26 (q, 6 H, 3 CH_2CH_3), 2.84 (m, 10 H, 2 NCH_3, OCH_2, NCH_2), 2.76 (dd, 1 H, $J_{3''e,3''a}$ 12.5, $J_{3''e,4''}$ 4 Hz, H-3″e), 2.02 (s, 3 H, NAc), 1.92 (s, 3 H, NAc), 1.79 (t, 1 H, H-3″a), 1.24 (t, 9 H, 3 CH_2CH_3) and 1.15–0.7 (m, 8 H, 4 CH_2); ^{13}C NMR (62.9 MHz, D_2O) δ: 177.4, 176.5, 176.3 (CO), 156.0, 153.1, 136.5, 132.4, 132.04, 131.3, 131.1, 118.4 (C–Ar), 105.8 (C-1′), 103.1 (C-1), 102.0 (C-2″), 84.9 (C-3), 78.0, 77.7, 77.4 (C-5, C-5′, C-3′), 75.2 (C-6″), 74.2 (C-8″), 72.4, 71.45, 71.1, 70.7, 70.4, 69.6 (C-2′, C-4, C-4′, C-4″, C-7″, CH_2), 64.8 (C-9″), 63.4, 63.10 (C-6, C-6′), 61.3 (CH_2), 56.8 (C-2), 54.1 (C-5″), 47.3 ($N(CH_2CH_3)_3$), 44.7 ($N(CH_3)$), 42.1 (C-3″, $N(CH_2)$), 30.5, 27.3, 26.5 (CH_2), 24.6, 24.4 (CH_3CO) and 9.8 ($N(CH_2CH_3)_3$); MALDI-HRMS Calcd for $C_{43}H_{65}N_4Na_2O_{21}S$ $[M+2\ Na-H]^+$: 1051.366. Found: m/z 1051.365.

3.13. Methyl (5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2→3)-(*O*-β-D-galacto-pyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-gluco-pyranosyl)-(1→2)-α-D-mannopyranoside (15)

Trisaccharide **14** (8.5 mg, 0.015 mol) and CMP-NeuAc (6.9 mg, 0.011 mmol) were incubated in 25 mM sodium cacodylate buffer pH 7.1 (5 mL) at 30 °C for 4 days with gentle stirring in the presence of ST3Gal-III adsorbed on Ni^{2+}-Agarose (25 mU). Additional CMP-NeuAc (6.9 mg) 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 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 **15** as its sodium salt (9 mg, 69%); $[\alpha]_D^{27}$ +0.5° (*c* 0.3, water); ^{1}H NMR (D_2O, 250 MHz): δ 4.88 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 4.75 (d, 1 H, $J_{1'',2''}$ 8.5 Hz), 4.54 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.11 (dd, $J_{3'',4''}$ 3, $J_{3'',2''}$ 10 Hz, H-3″), 3.40 (s, 3 H, CH_3), 2.75 (dd, 1 H, $J_{3'''e,3'''a}$ 12.5, $J_{3'''e,4''}$ 4 Hz, H-3‴e), 2.05 (s, 3 H, NAc), 2.03 (s, 3 H, NAc) and 1.80 (t, 1 H, H-3‴a); ^{13}C RMN (D_2O, 62.9 MHz): δ 175.3 (CO_2Na), 175.0, 174.2 (CO), 102.9 (C-1″), 100.1 (C-2‴), 99.7 (C-1′), 98.2 (C-1), 78.5 (C-4′), 76.4, 75.7, 75.4, 75.0 (C-2, C-3″, C-5′, C-5″), 73.2, 72.9, 72.2, 72.0 (C-3′, C-5, C-6‴, C-8‴), 69.8, 69.7, 68.7, 68.4, 67.7, 67.7 (C-4, C-4′, C-2″, C-4″, C-4‴, C-7‴), 62.8 (C-9‴), 61.9, 61.3, 60.2 (C-6, C-6′, C-6″, 55.1 (C-2′, OCH_3), 51.9 (C-5‴), 39.9 (C-3‴), 22.6

and 22.3 (CH_3CO); MALDI-HRMS: Calcd for $C_{32}H_{53}N_2Na_2O_{24}$ $[M+2\ Na-H]^+$: 895.278. Found: m/z 895.279.

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