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Note

In vitro sulfation of *N*-acetyllactosaminide by soluble recombinant human β-Gal-3′-sulfotransferase

P. Greimel,[a,†] Sabrina Jabs,[b,†] Stefan Storch,[b] Slim Cherif,[a] Koichi Honke,[c] Thomas Braulke[b,*] and Joachim Thiem[a,*]

[a]*Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany*
[b]*University of Hamburg, UKE, Children's Hospital-Biochemistry, Martinistr.52, 20246 Hamburg, Germany*
[c]*Osaka University, Graduate School of Medicine, Department of Biochemistry, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

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Abstract—Membrane-bound β-Gal-3′-sulfotransferase (GP3ST) was expressed and used for in vitro sulfation of Tamm–Horsfall glycoprotein. Further, the regioselective transfer of sulfate to an *N*-acetyllactosamine derivative could be realised with soluble chimeric GP3ST, also in combination with Lac transglycosylation by means of β-galactosidase. Two alternative straightforward chemical syntheses for the target compound could be elaborated.

Keywords: β-Gal-3′-sulfotransferase; Tamm–Horsfall glycoprotein; *N*-Acetyllactosaminide-3′-sulfate; Chemoenzymatic synthesis; Glycosylation

Sulfation of glycans by Golgi-resident sulfotransferases is a widespread modification of glycosaminoglycans, glycoproteins and glycolipids regulating their functional activities such as hormone pharmacokinetics, growth factor action, leukocyte rolling or bacterial adhesion.[1–4] All sulfotransferases utilise the sulfate donor 5′-phosphoadenosin-3′-phosphosulfate (PAPS, 5) for transfer to an acceptor molecule thereby releasing 5′-phosphoadenosine-3′-phosphate (PAP, 6).[5,6] Recently, a novel β-Gal-3′-sulfotransferase (GP3ST) has been cloned and characterised transferring a sulfate group to C3 of a non-reducing terminal β-galactose unit in oligosaccharides that were not associated with lipids.[7]

In the present study the capability of recombinant membrane-bound GP3ST for sulfation of the Tamm–Horsfall protein (THP) was examined. THP is the most abundant urinary proteins in mammals and can bind to type 1 fimbriated *E. coli* implying a protective role for THP in urinary tract infections.[8,9] THP is equipped with N-linked glycans of di-, tri- and tetra-antennary-type and one high mannose-type oligosaccharide.[10] Variable sulfate groups on terminal galactose residues in the glycans of THP have been reported.[11] Furthermore, to establish conditions for large-scale generation of sulfated oligosaccharides in vitro, the usage of a soluble GP3ST fusion protein was studied.

In a previous analytical study, the membrane-bound GP3ST has been reported to catalyse the transfer of sulfate to LacNAc, lactose and lacto-*N*-tetraose.[7] It was uncertain whether GP3ST would act on *N*- or *O*-glycans. Here two GP3ST proteins, the C-terminally myc-tagged GP3ST and a soluble, secreted chimeric ProtA-GP3ST (Fig. 1) were transiently and stably expressed, respectively, in baby hamster kidney (BHK) cells. pcDNA3.1 vector transfected BHK cells were used as controls.

Western blot analysis using anti-myc antibodies revealed a single prominent immunoreactive band of 60 kDa in cell extracts of GP3STmyc expressing cells (Fig. 2A). No specific cross-reacting material was found

* Corresponding authors. Tel.: +49 40 428035947; fax: +49 40428038504 (T.B.); tel.: +49 40 428384241; fax: +49 40 428384325 (J.T.); e-mail addresses: braulke@uke.uni-hamburg.de; joachim.thiem@chemie.uni-hamburg.de
† Both authors contributed equally to the work.

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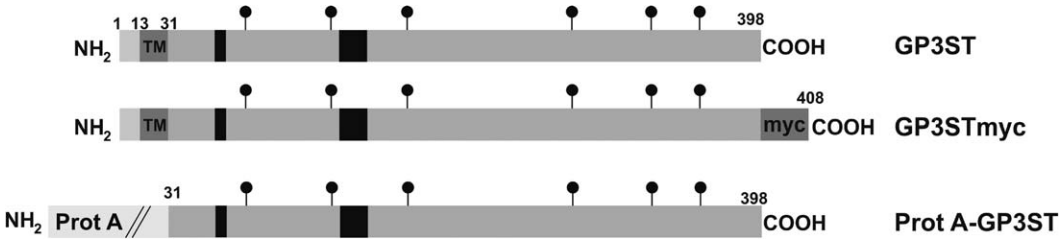


Figure 1. Schematic representation of β-Gal-3′-sulfotransferase (GP3ST) constructs. The numbering of the type 2 membrane protein starts at the first methionine of the GP3ST coding sequence. The transmembrane domain (TM), PAPS binding sites (black area) and the six potential N-glycosylation sites are indicated. The C-terminal location of the myc-tag in the GP3STmyc construct is given. In the soluble secretory ProtA-GP3ST chimera the *N*-terminal cytoplasmatic and transmembrane domain of GP3ST are substituted by a protein A-tag containing signal peptide sequence.

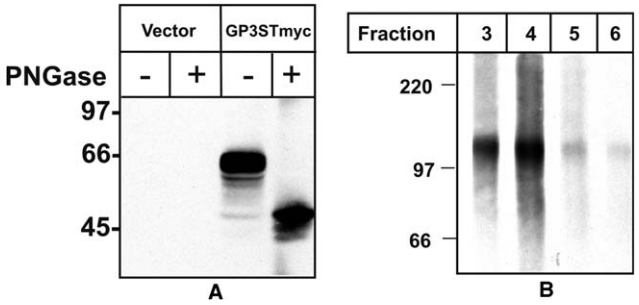


Figure 2. Expression analysis of GP3Stmyc and ProtA-GP3ST. (A) Cell extracts of vector-transfected and GP3STmyc overexpressing BHK cells were incubated in the presence (+) or absence (−) of PNGase F for 16 h at 37 °C. The samples were separated by SDS-PAGE and analysed by Western blotting using anti-myc antibodies (1:1000). (B) The positions of the molecular mass marker proteins (in kDa) are indicated.

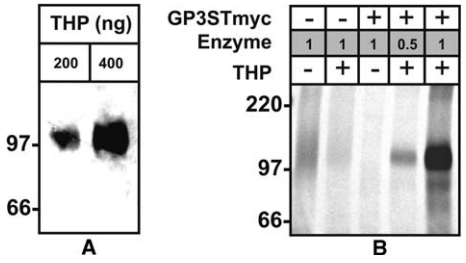


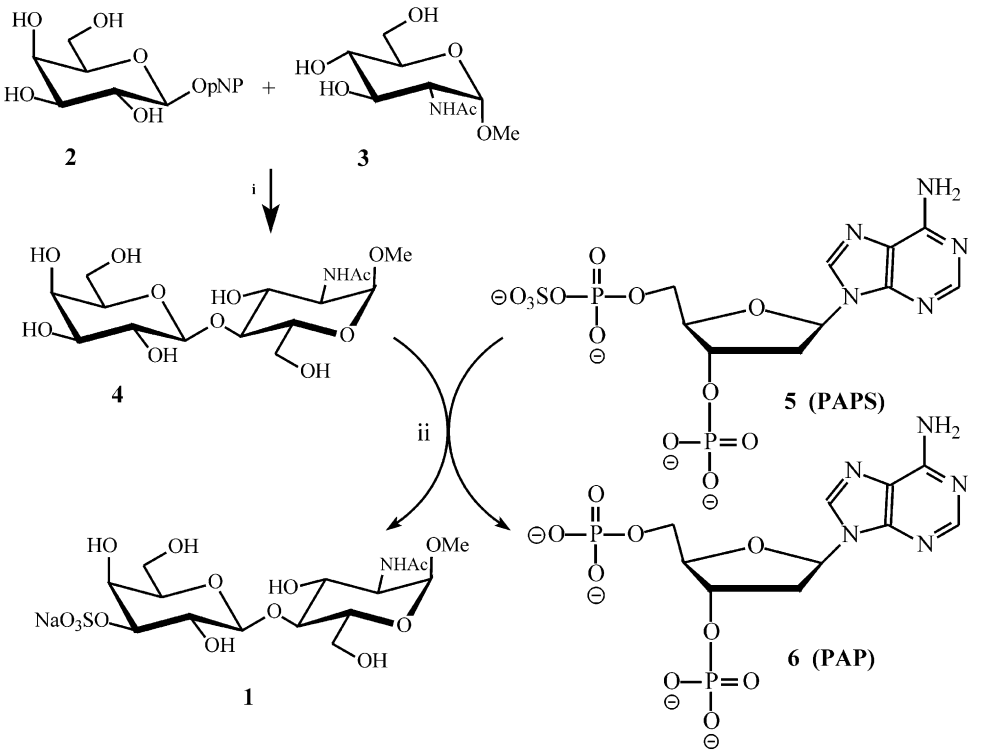
Figure 3. Purification and sulfation of THP by GP3STmyc. (A) THP was purified from human urine, separated by SDS-PAGE and analysed by Western blotting using anti-THP antibodies (1:250). (B) THP (1 μg) was incubated with cell extracts vector (−) and GP3STmyc overexpressing BHK cells (+) and [^{35}S]-PAPS as described in the Experimental section. In this experiment 4 (=0.5) or 8 μg (=1) cell extract protein was used for the assay. The reaction mixtures were subjected to SDS-PAGE and fluorography.

in cells transfected with the vector only. After deglycosylation with PNGase F the apparent molecular mass of GP3STmyc was reduced to approximately 46 kDa (Fig. 2A). Due to the reported masses of a single N-linked oligosaccharide chain varying between 2 to 5 kDa[12–14] it remains uncertain whether all of the six potential N-glycosylation sites of GP3ST are used. The chimeric ProtA-GP3ST was purified from overexpressing BHK cell media by IgG-affinity chromatography. Eluted fractions containing the GP3ST fusion protein (Fig. 2B) were combined, dialysed and used for in vitro sulfation of oligosaccharides.

The sulfotransferase activity was first determined in extracts of vector transfected and GP3STmyc overexpressing cells using the 100 kDa THP purified from human urine (Fig. 3A) as acceptor. Carbohydrates account for approximately 30% of the mass of human THP and consist mainly of N-linked glycans of di-, tri- and tetra-antennary type modified by sulfation.[11] As shown in Figure 3B, $^{35}SO_4$ was incorporated into the 100 kDa THP depending on the concentration of the protein extract from GP3STmyc overexpressing cells. In the presence of an excess of unlabelled PAPS, no $^{35}SO_4$ incorporation into THP was detectable. When extracts from vector transfected cells were used as enzyme source, no ^{35}S-labelled THP was observed.

For formation of methyl *N*-acetyllactosaminide (**4**),[15,16] the transgalactosylation of the acceptor methyl *N*-acetyl-glucosaminide (**3**) with *p*-nitrophenyl β-D-galactopyranoside (**2**) as donor and β-galactosidase (*Bacillus circulans*) was advantageous[17] and proceeded with 61% yield. The subsequent enzymatic sulfation of **4** was performed in phosphate buffer with PAPS (**5**) as donor substrate and the soluble chimeric ProtA-GP3ST for 6 h at 37 °C. After termination by lyophilisation HPLC revealed the 3′-sulfated derivative **1** in about 14% yield. Under comparable conditions, both enzymatic processes could be coupled efficiently. HPLC calibrated with synthetic 3SLacNAcOMc (**1**) showed yields of 9% at 20 °C and 37% at 37 °C (Scheme 1).

In parallel, the synthesis of the sulfated disaccharide **1** following classical glycosylation pathways with a terminal sulfation step was approached. Peracetylated phenyl thiogalactopyranoside **7**[18] was deacetylated,[18] then transferred into the dibutyltin derivative and in situ benzylated to give regiospecifically the 3-*O*-benzyl component,[19,20] which in turn was peracetylated to give **8**[19] which served as donor structure for the glycosylation.

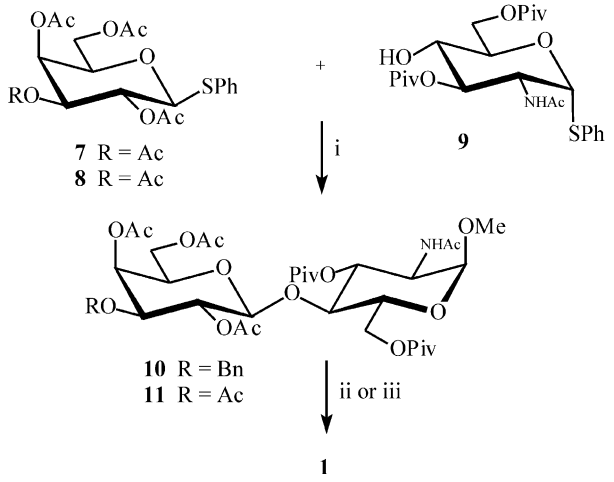


Scheme 1. Reagents and conditions: (i) β-galactosidase (*B. circulans*); (ii) β-Gal-3′-sulfotransferase, $MnCl_2$, phosphate buffer.

As acceptor component the 3,6-dipivoylated glycoside **9**[21] could be obtained from **3**. NIS-promoted galactosylation with donor **8** resulted in formation of the desired disaccharide **10**. By hydrogenolysis in ethanol, a 45:55 mixture as evident from ^{1}H NMR of both the regioisomeric tri-*O*-acetates was obtained. Their reaction with the sulfor trioxide pyridinium complex in DMF and chromatographic purification gave a single monosulfated derivative which by treatment with an excess of sodium methoxide in methanol led to the title compound **1** (Scheme 2).

A superior pathway did not require the regiospecific protection of the 3′-position. Starting with the simple peracetylated phenyl thiogalactopyranoside **7** as donor and again the acceptor **9**, the NIS-promoted glycosylation gave straightforwardly the disaccharide **11**. With excess of sodium methoxide in methanol, the fully deprotected disaccharide **4** (MeLacNAc) was obtained.[15,16] Again, the regiospecific oxygen activation at position 3′ could be achieved via intermediate 3′,4′-dibutylstannylation.[22] Further treatment with the sulfur trioxide–triethylamine complex directly resulted in formation of the 3′-sulfated compound **1**.

For structural assignment of the target compound **1** comparison with the literature data with its precursor **4** is useful. The ^{1}H NMR reveal a significant downfield shift for H-3′ (**4**: δ 3.51→**1**: δ 4.30) and noticeable shifts ($\Delta\delta$ 0.2 and 0.4) for H-2′ and H-4′, respectively. In the ^{13}C NMR this reflects in a large downfield shift for



Scheme 2. Reagents and conditions: (i) NIS, TfOH; (ii) H_2/Pd–C, then NaOMe, then SO_3–Py, DMF; (iii) NaOMe then Bn_2SnO, DMF, SO_3–Et_3N.

C-3′ (**4**: δ 73.7→**1**: δ 80.6) and obvious upfield shifts for C-2′ and C-4′ ($\Delta\delta$ −1.7 and −1.9). The NMR and MALDI-TOF data could also be related to data of compounds containing comparable functional groups.[23,24]

Thus, both the sulfation of N-linked oligosaccharides of Tamm–Horsfall glycoprotein and of disaccharides by membrane-bound and soluble recombinant GP3ST could be demonstrated. Therefore, GP3ST can be used to produce substantial amounts of sulfated glyco-

proteins allowing more detailed studies of the biological role of this modification. The regiospecific sulfation of a LacNAc derivative by a combination of a transglycosylating hydrolase and GP3ST requires four to five steps whereas twelve to nine steps were necessary for chemical synthesis depending on the precursor used. The experimental approach via stannylene activation for sulfation proved to be of considerable advantage.

1. Experimental

1.1. Biochemical procedures

1.1.1. Materials. [^{35}S] 3′-Phosphoadenosine-5′-phosphosulfate ([^{35}S]-PAPS) was obtained from Perkin–Elmer Life Sciences. OptiMEM, Lipofectamine 2000 and antibiotics (penicillin/streptomycin) and molecular biology reagents are purchased from Invitrogen. Bradford reagent and Trans-Blot nitrocellulose membranes were from BioRad.

1.1.2. Antibodies. Polyclonal antibodies against human Tamm–Horsfall Protein (THP) and the anti-myc antibody were obtained from Harbor Bioproducts and SantaCruz Biotechnology, respectively. Peroxidase-conjugated goat anti-rabbit and rabbit IgG were from Jackson ImmunoResearch Laboratories.

1.1.2.1. GP3ST cDNA constructs. Two GP3ST expression constructs were generated (Fig. 1). First, the β-Gal-3′-sulfotransferase (GP3ST; Accession-No.: AB040610) cDNA was subcloned in frame with the C-terminal myc-his tag of the pcDNA3.1/myc-his (−) A expression vector (Invitrogen). Second, the cDNA coding for the luminal, catalytically active domain of GP3ST and 80 base pairs of the 3′ untranslated cDNA were amplified by PCR using Pfu Turbo polymerase, a set of primers GP3-ST-For: G GAA TTC G CAC TCG GAC TTA GAG CTG GAC and GP3-ST-Rev: GC TCT AGA GGA TCT ACT AGT CAT ATG, and the construct pSV-GP3ST as a template. After separation on an agarose gel the 1193 bp PCR product was extracted and sequentially cleaved by Eco *RI* and Xba*I*. The product was cloned in frame with the N-terminally placed transin signal sequence and the protein A-tag into the corresponding restriction sites of vector pProtA-EK-IRES (kindly provided by Dr. Gieselmann, University of Bonn). The corresponding expression construct was verified by sequencing and named ProtA-GP3ST.

1.1.3. Cell culture and transfection. Baby hamster kidney (BHK) cells were cultured in DMEM containing 5% FCS and antibiotics at 37 °C and 5% CO_2. Cells were transiently transfected with the GP3STmyc cDNA and Lipofectamine 2000 in Opti-MEM medium according to the manufacturer's instructions. Control cells were transfected with the pcDNA3.1/myc-his (−) A vector alone. For stable transfections, 1.2×10^6 BHK-21 cells were grown on 60 mm plates and transfected with 5 μg of ProtA-GP3ST cDNA and 10 μL Lipofectamine 2000. Twenty four hours after start of transfection, cells were trypsinised and diluted 1:10 in DMEM, 5% FCS containing antibiotics. The next day the medium was supplemented with puromycin. Cell clones stably secreting ProtA-GP3ST fusion proteins were detected by affinity purification of the media over IgG-Sepharose 6 Fast Flow (Amersham Biosciences). Cell clones expressing highest levels of protein A fusion proteins were chosen for further analyses.

1.1.4. Preparation of cell extracts. Twenty-four hours after transfection each three 10 cm dishes of vector or GP3STmyc overexpressing BHK cells were washed, harvested with 10 mL of phosphate-buffered saline using a rubber scraper, centrifuged at 1000 rpm for 5 min, and resuspended in 1 mL ice-cold 0.1 M MES buffer, pH 6.2 containing 0.1% Triton X-100. After sonification the cell extracts were incubated for 10 min on ice followed by ultracentifugation for 15 min at 100,000*g* (Sorvall, Discovery M120). The supernatant was used for sulfation assays using THP as substrate.

1.1.5. SDS-gelelectrophoresis and westernblotting. Solubilised proteins from transfected cells or eluated fractions from IgG-Sepharose columns were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and examined for myc-immunoreactivity as described previously[25] or for peroxidase coupled IgG-binding ability followed by enhanced chemiluminescence detection (Pierce).

1.1.6. Affinity purification of ProtA-GP3ST fusion proteins. For production of ProtA-GP3ST fusion proteins, stably transfected BHK cells were cultivated in DMEM, 0.05% BSA at 37 °C, 5% CO_2 and media conditioned for 48–72 h were collected. The media were centrifuged for 5 min at 1000*g* to remove cells and the supernatants were adjusted to 0.05% Tween 20. IgG sepharose (2 mL) equilibrated with 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20 (TST buffer) incubated with the media for 4 h at 4 °C under constant rotation. The flow through was collected and the column was washed with 20 mL of TST buffer followed by 4 mL of 5 mM ammonium acetate buffer, pH 5.5. Bound proteins were eluted with 10 mL of 0.5 M acetic acid, pH 3.4 and collected in 0.5 mL fractions. The optical density of each fraction was measured and aliquots of the peak fractions were separated by SDS-PAGE and tested for the presence of protein A-fusion protein as described above.

1.1.7. GP3ST activity assay using THP. The standard incubation mixture contained the following components in a total volume of 50 μL: 0.1 M MES (pH 6.2), 10 mM $MnCl_2$, 40 mM mercaptoethanol, 1% Triton X-100, 10 mM NaF, 10% glycerol, 1 mM ATP, protease inhibitor cocktail, 1.3 nmol [^{35}S]-PAPS (8×10^6 cpm), 0.5–1 μg THP and 12 μL cell extract. The reaction mixtures were incubated at 28 °C for 3 h, terminated by boiling for 5 min in SDS sample buffer, separated by SDS-PAGE (10% acrylamide) and visualised by fluorography.[26]

1.1.8. Other methods. Protein concentrations were determined using the Bradford protein assay with bovine serum albumin as standard. Deglycosylation experiments with total BHK cell extracts using peptide N-glycosidase F (PNGase F) were carried out as described.[27] THP was isolated from human urine as reported previously.[28]

1.1.9. Methyl *N*-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-α D-glucopyranoside (4). *para*-Nitrophenyl β-D-galactopyranoside (**2**, 31 mg, 0.103 mmol, 1.0 equiv) and methyl *N*-acetamido-2-deoxy-α-D-glucopyranoside (**3**, 235 mg, 1.00 mmol, 10.0 equiv) were dissolved in a mixture of CH_3CN (1.5 mL) and potassium phosphate buffer (1.5 mL, 50 mM in Milli-Q water, pH 7.0). β-Galactosidase (*B. circulans*, 150 μL, 8.0 U; in potassium phosphate buffer, 50 mM in Milli-Q water at pH 7.0) was added and the mixture stirred for 1.5 d at room temperature. Subsequently, the reaction mixture was lyophilised and the residue was subjected to a silica gel chromatography to give compound **4** (25 mg, 0.063 mmol, 61% yield) as a white amorphous solid. Physical data were identical to synthesised (**4**).[22]

1.1.10. Sulfation of disaccharide 4 with ProtA-GP3ST to give 1. The standard incubation mixture contained the following components in a total volume of 50 μL: 25 mM potassium phosphate buffer (pH 7.0), 0.5 mM $MnCl_2$, 1.6 mM mercaptoethanol, 11 μL CH_3CN, 3 mM PAPS, 0.04 mM LacNAcOMe **4** 10 μL ProtA-GP3ST. After incubation for 6 h at 37 °C, the reaction was terminated by lyophilisation. The sample was resuspended in Milli-Q water (30 μL), injected (20 μL) to a Polysphere CH CA column equipped Hitachi-Merck HPLC system (eluent: water, column temperature: 90 °C) and monitored with a RI-detector. The approximate yield of **1** (0.14 μg, 14%) was calculated from HPLC data. Calibration was carried out with synthetic compound **1**.

1.1.11. One-pot transglycosylation with β-galactosidase from *B. circulans* and enzymatic sulfation of LacNAcOMe to give 1. The standard incubation mixture for the initial step contained the following components in a total volume of 0.91 mL: potassium phosphate buffer (0.45 mL, 50 mM, pH 7.0), CH_3CN (0.45 mL), *para*-nitrophenyl β-D-galactopyranoside (**2**, 0 mg, 0.033 mmol, 1.0 equiv), methyl *N*-acetamido-2-deoxy-α-D-glucopyranoside (**3**, 78 mg, 0.330 mmol, 10.0 equiv), β-galactosidase from *B. circulans* (10 μL, 3.0 U; in potassium phosphate buffer, 50 mM at pH 7.0). The reaction mixture was incubated for 1 d at 20 °C. Subsequently, the standard incubation mixture for the second step contained the following components in a total volume of 79.8 μL: a 2 μL aliquot of the initial step, 7.5 μL CH_3CN 0.29 mM $MnCl_2$, 0.25 mM mercaptoethanol, 31 mM PAPS (**5**), 13 μL ProtA-GP3ST. The reaction mixture was incubated at 20 °C, or 37 °C, respectively. Subsequently, an aliquot (25 μL) was removed from the reaction mixture after 2 h and 25 h, respectively. The reactions were terminated by lyophilisation. The samples were resuspended in Milli-Q water (30 μL), injected (20 μL) onto a Polysphere CH CA column equipped Hitachi-Merck HPLC system (eluent: water, column temperature: 90 °C) and monitored with a RI-detector. Yield of 3SLacNAcOMe (**1**) calculated as above: 6.2 μg (37%) at 37 °C and 1.6 μg (9%) at 20 °C.

1.2. Synthetic procedures

1.2.1. General methods. All reactions requiring anhydrous conditions were carried out under argon. All other solvents were purified and dried by standard procedures. Analytic thin-layer chromatography was performed using silica gel 60 F_{254} precoated plates (E. Merck 5554). Compounds were detected by quenching of UV fluorescence and by spraying with ethanol/sulfuric acid (95:5, vv) and subsequent heating. Flash chromatography was performed with the indicated solvent system on Silica Gel 60, 230–400 mesh (E. Merck 9385). Melting points were determined either with a Reichert Heiztischmikroskop or an ST-apotec and are uncorrected. Optical rotations were determined at room temperature with a Perkin–Elmer 341 polarimeter. Mass spectra were recorded on a Bruker Biflex-III MALDI-TOF, using positive reflector mode and 2,5-dihydroxybenzoic acid (DHB) as matrix. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer at 400.14 MHz and 100.04 MHz, respectively, and on a Bruker DRX 500 MHz spectrometer at 500.13 MHz and 125.03 MHz, respectively.

1.2.2. Methyl *N*-acetamido-2-deoxy-4-*O*-(2′,4′,6′-tri-*O*-acetyl-3′-*O*-benzyl-β-D-galacto pyranosyl)-3,6-di-*O*-pivaloyl-α-D-glucopyranoside (10). A solution of compound 8 (129 mg, 0.26 mmol, 1.0 equiv) and methyl *N*-acetamido-2-deoxy-3,6-di-*O*-pivaloyl-α-D-glucopyranoside (9, 117 mg, 0.29 mmol, 1.1 equiv) in anhydrous CH_2Cl_2 (20 mL) with molecular sieve (3 Å) under argon atmosphere was cooled to −30 °C and under stirring treated

with *N*-iodosuccinimide (0.15 mg, 0.66 mmol, 2.5 equiv). The reaction was initiated by the addition of trifluoromethanesulfonic acid (one drop) and allowed to reach 0 °C slowly. After full conversion, the reaction was quenched by the addition of anhydrous pyridine (100 μL) and the molecular sieve was filtered off over Celite. The filtrate was extracted once with satd aq $NaHCO_3$, once with sodium thiosulfate solution, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was subjected to silica gel chromatography, to give **10** (88 mg, 0.11 mmol, 43% yield) as colourless oil. $[\alpha]_D^{20}$ +69 (*c* 0.2, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 7.21–7.04 (m, 5H, Bn), 5.61 (d, 1H, $J_{NH\text{-}2} = 9.6$ Hz, NH), 5.31 (d, 1H, $J_{4'\text{-}3'} = 3.5$ Hz, H-4′), 5.00 (dd, 1H, $J_{3\text{-}2} = 10.7$ Hz, $J_{3\text{-}4} = 9.4$ Hz, H-3), 4.83 (dd, 1H, $J_{2'\text{-}3'} = 9.9$ Hz, $J_{2'\text{-}3'} = 8.1$ Hz, H-2′), 4.52 (d, 1H, $J_{Bn\ a\text{-}b} = 12.0$ Hz, Bn-CH_2^a), 4.78 (d, 1H, $J_{1\text{-}2} = 3.8$ Hz, H-1), 4.33 (dd, 1H, $J_{6a\text{-}6b} = 11.5$ Hz, $J_{6a\text{-}5} = 1.8$ Hz, H-6a), 4.78 (d, 1H, $J_{1'\text{-}2'} = 8.1$ Hz, H-1′), 4.20 (d, 1H, $J_{Bn\ b\text{-}a} = 12.0$ Hz, Bn-CH_2^b), 4.06 (dd, 1H, $J_{2\text{-}3} = 10.7$ Hz, $J_{2\text{-}NH} = 10.0$ Hz, $J_{2\text{-}1} = 3.8$ Hz, H-2), 4.02 (dd, 1H, $J_{6a'\text{-}6b'} = 11.4$ Hz, $J_{6a'\text{-}5} = 6.6$ Hz, H-6a′), 3.94 (dd, 1H, $J_{6b\text{-}6a} = 11.9$ Hz, $J_{6b\text{-}5} = 4.4$ Hz, H-6b), 3.91 (dd, 1H, $J_{6b'\text{-}6a'} = 11.4$ Hz, $J_{6b'\text{-}5} = 6.9$ Hz, H-6b′), 3.72 (dd, 1H, $J_{4\text{-}5} = 9.8$ Hz, $J_{4\text{-}3} = 9.4$ Hz, H-4), 3.64 (ddd, 1H, $J_{5\text{-}4} = 9.8$ Hz, $J_{5\text{-}6b} = 4.4$ Hz, $J_{5\text{-}6a} = 1.8$ Hz, H-5), 3.56 (dd, 1H, $J_{5'\text{-}6b'} = 6.9$ Hz, $J_{5'\text{-}6a'} = 6.0$ Hz, H-5′), 3.30 (dd, 1H, $J_{3'\text{-}2'} = 9.9$ Hz, $J_{3'\text{-}4'} = 3.5$ Hz, H-3′), 3.21 (s, 3H, OMe), 1.94 (s, 3H, Ac), 1.92 (s, 3H, Ac), 1.85 (s, 3H, 2-*O*-Ac), 1.76 (s, 3H, NHAc), 1.08 (s, 9H, 6-*O*-Piv), 1.04 (s, 9H, 3-*O*-Piv); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.4 (C, 1C, 3-*O*-Piv), 178.3 (C, 1C, 6-*O*-Piv), 170.6 (C=O, 2C, 4′-*O*-Ac, 6′-*O*-Ac), 170.3 (C=O, 1C, NHAc), 169.4 (C=O, 1C, 2′-Ac), 137.7 (C, 1C, Bn_{ipso}), 128.8, 128.3, 128.1 (CH, 5C, Bn), 100.4 (CH, 1C, C-1′), 98.6 (CH, 1C, C-1), 77.5 (CH, 1C, C-3), 73.8 (CH, 1C, H-4), 71.9 (CH_2, 1C, Bn), 71.7 (CH, 1C, C-5′), 71.3 (CH, 1C, C-2′), 70.6 (CH, 1C, C-3), 69.6 (CH, 1C, C-5), 66.1 (CH, 1C, C-4′), 62.3 (CH_2, 2C, C-6, C-6′), 55.7 (CH_3, 1C, OMe), 52.9 (CH, 1C, C-2), 27.7 (CH_3, 3C, 6-*O*-Piv), 27.5 (CH_3, 3C, 3-*O*-Piv), 23.6 (CH_3, 1C, NHAc), 21.3, 21.2, 21.1 (CH_3, 3C, 2′-*O*-Ac, 4′-*O*-Ac, 6′-*O*-Ac); MALDI-TOF (DHB, positive mode): $C_{38}H_{55}NO_{16}$ $m/z = 804.5$ $[M+Na]^+$, 820.5 $[M+K]^+$.

1.2.3. Methyl *N*-acetamido-2-deoxy-4-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galacto-pyranosyl)-3,6-di-*O*-pivaloyl-α-D-gluco-pyranoside (11). A solution of compound **7** (997 mg, 2.26 mmol, 1.3 equiv) and **9** (690 mg, 1.71 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (50 mL) with molecular sieve (3 Å) under argon atmosphere was cooled to −30 °C and treated with *N*-iodosuccinimide (1.17 g, 5.25 mmol, 3.0 equiv). The reaction was initiated by the addition of trifluoromethanesulfonic acid (one drop) and stirred for 3 h at −30 °C. Subsequently, the reaction was quenched by the addition of anhydrous pyridine (1 mL), diluted with CH_2Cl_2 (100 mL) and the molecular sieve filtered off over Celite. The filtrate was extracted once with satd aq $NaHCO_3$, once with sodium thiosulfate solution, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was subjected to silica gel chromatography, to give **11** (905 mg, 1.23 mmol, 72% yield) as colourless oil. $[\alpha]_D^{20}$ +21 (*c* 0.5, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 5.94 (d, 1H, $J_{1'\text{-}2'} = 4.7$ Hz, H-1′), 5.47 (dd, 1H, $J_{4'\text{-}5'} = 3.2$ Hz, $J_{3'\text{-}4'} = 3.2$ Hz, H-4′), 5.05 (m, 1H, H-3), 4.98 (dd, 1H, $J_{4'\text{-}3'} = 3.2$ Hz, $J_{3'\text{-}2'} = 6.6$ Hz, H-3′), 4.65 (d, 1H, $J_{1\text{-}2} = 3.5$ Hz, H-1), 4.61 (dd, $J_{6a\text{-}5} = 1.6$ Hz, $J_{6a\text{-}6b} = 10.7$ Hz, 1H, H-6a), 4.57 (dd, 1H, $J_{2'\text{-}3'} = 6.6$ Hz, $J_{2'\text{-}1'} = 4.7$ Hz, H-2′), 4.39 (ddd, 1H, $J_{4'\text{-}5'} = 2.8$ Hz, $J_{6a'\text{-}5'} = 6.6$ Hz, $J_{6b'\text{-}5'} = 6.6$ Hz, H-5′), 4.30 (dd, 1H, $J_{3\text{-}2} = 11.3$ Hz, $J_{1\text{-}2} = 3.2$ Hz, H-2), 4.21 (dd, 1H, $J_{6a'\text{-}5'} = 6.6$ Hz, $J_{6a'\text{-}6b'} = 11.7$ Hz, H-6a′), 4.21 (m, 1H, H-6b) 4.13 (dd, 1H, $J_{6b'\text{-}5'} = 6.6$ Hz, $J_{6a'\text{-}6b'} = 11.7$ Hz, H-6b′), 3.81 (m, 2H, H-4, H-5), 3.44 (s, 3H, OCH_3), 2.15 (s, 3H, O-Ac), 2.06 (s, 3H, O-Ac), 2.04 (s, 3H, O-Ac), 1.92 (s, 3H, O-Ac), 1.65 (s, 3H, N-Ac), 1.27 (s, 9H, O-Piv), 1.19 (s, 9H, O-Piv); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.7 (C=O, 1C, O-Piv), 178.4 (C=O, 1C, O-Piv), 172.1 (C=O, 1C, O-Ac), 171.1 (C=O, 1C, O-Ac), 170.5 (C=O, 1C, O-Ac), 170.2 (C=O, 1C, O-Ac), 122.8 (C=O, 1C, N-Ac), 98.4 (CH, 1C, C-1), 97.3 (CH, 1C, C-1′), 76.2 (CH, 1C, C-2′), 72.3 (CH, 1C, C-3′), 70.3 (CH, 1C, C-3), 69.8 (CH, 1C, C-5′), 69.1 (CH, 2C, C-4, C-5), 66.7 (CH, 1C, C-4′), 63.3 (CH_2, 1C, C-6), 61.3 (CH_2, 1C, C-6′), 54.5 (CH_3, 1C, OCH_3), 51.5 (CH, 1C, C-2), 38.8 (C, 2C, O-Piv), 26.6 (CH_3, 1C, N-Ac), 25.7 (CH_3, 1C, O-Ac), 21.4 (CH_3, 1C, O-Ac), 19.6 (CH_3, 1C, O-Ac), 19.3 (CH_3, 1C, O-Ac); MALDI-TOF (DHB, positive mode): $C_{33}H_{51}NO_{17}$ $m/z = 756.5$ $[M+Na]^+$, 772.4 $[M+K]^+$.

1.2.4. Methyl *N*-acetamido-2-deoxy-4-*O*-(3′-*O*-sulfo-β-D-galactopyranosyl)-α-D-glucopyranoside sodium salt (1). (A) A solution of disaccharide **10** (56 mg, 0.07 mmol, 1.0 equiv) in anhydrous EtOH (3 mL) was treated with Pd/C (a tip of a spatula). The reaction mixture was stirred under hydrogen atmosphere (1 bar) for 48 h. Subsequently, the reaction was filtered over Celite, and the solvent was removed under reduced pressure. The residue was subjected to silica gel chromatography, to give an inseparable mixture of regioisomers (43 mg, 0.06 mmol, 88% yield) as colourless oil. MALDI-TOF (DHB, positive mode): $C_{31}H_{49}NO_{16}$ $m/z = 714.4$ $[M+Na]^+$, 730.3 $[M+K]^+$.

(B) A mixture of the regioisomers (19 mg, 0.027 mmol, 1.0 equiv) in anhydrous DMF (5 mL) was treated with sulfur trioxide–pyridine complex (60 mg, 0.380 mmol, 14 equiv) and stirred at room temperature for 1 d. Subsequently, the reaction was quenched with

MeOH (2 mL) and the solvent was removed under reduced pressure. The residue was subjected to silica gel chromatography, to give the monosulfated disaccharide (5 mg, 0.007 mmol, 24% yield) as an amorphous solid.

(C) *Method A:* This was dissolved in anhydrous MeOH (2 mL) under argon atmosphere and treated twice with sodium methoxide (2×4 mg, 0.148 mmol, 24.0 equiv). The mixture was stirred at room temperature for 1 d. Subsequently the reaction mixture was neutralised with Amberlite IR-120 H^+ resin, filtered and the solvent was removed under reduced pressure to give crude **1** (2 mg, 0.004 mmol, 60% yield) as an amorphous solid. *Method B:* A solution of compound **4** obtained by deacylation of **11** (19 mg, 0.047 mmol, 1.0 equiv) in anhydrous MeOH (5 mL) was treated with dibutyltin oxide (13 mg, 0.051 mmol, 1.1 equiv) and refluxed for 1.5 h under argon atmosphere. Subsequently, the solvent was removed under reduced pressure. The residue was suspended in anhydrous THF (5 mL), treated with sulfur trioxide–triethylamine complex (17 mg, 0.094 mmol, 2.0 equiv) and stirred for 48 h at room temperature. Afterwards, the solvent was removed under reduced pressure and the residue subjected to silica gel chromatography to give **1** (10 mg, 0.025 mmol, 53% yield), as an amorphous solid. $[\alpha]_D^{20}$ +71 (*c* 0.2, H_2O); ^1H NMR (500 MHz, MeOH-d_4): δ 4.71 (d, 1H, $J_{1\text{-}2} = 3.8$ Hz, H-1), 4.54 (d, 1H, $J_{1'\text{-}2'} = 7.8$ Hz, H-1′), 4.30 (dd, 1H, $J_{3'\text{-}2'} = 9.7$ Hz, $J_{3'\text{-}4'} = 3.4$ Hz, H-3′), 4.27 (broad d, 1H, $J_{4'\text{-}3'} = 3.4$ Hz, H-4′), 4.00 (dd, 1H, $J_{2\text{-}3} = 10.6$ Hz, $J_{2\text{-}1} = 3.8$ Hz, H-2), 3.96 (dd, 1H, $J_{6a\text{-}6b} = 12.0$ Hz, $J_{6a\text{-}5} = 3.2$ Hz, H-6a), 3.89 (dd, 1H, $J_{6b\text{-}6a} = 12.0$ Hz, $J_{6b\text{-}5} = 1.9$ Hz, H-6b), 3.84 (dd, 1H, $J_{3\text{-}2} = 10.6$ Hz, $J_{3\text{-}4} = 8.2$ Hz, H-3), 3.80 (dd, 1H, $J_{6a'\text{-}6b'} = 11.4$ Hz, $J_{6a'\text{-}5'} = 7.2$ Hz, H-6a′), 3.76 (dd, 1H, $J_{2'\text{-}3} = 9.7$ Hz, $J_{2'\text{-}1'} = 7.8$ Hz, H-2′), 3.74 (m, 1H, H-6b′), 3.71–3.66 (m, 3H, H-4, H-5, H-5′), 3.41 (s, 3H, OMe), 2.02 (s, 3H, NHAc); ^{13}C NMR (100 MHz, MeOH-d_4): δ 172.5 (C=O, 1C, NHAc), 103.8 (CH, 1C, C-1′), 98.5 (CH, 1C, C-1), 80.6 (CH, 1C, C-3′), 80.3 (CH, 1C, C-4), 75.6 (CH, 1C, H-5′), 70.9 (CH, 1C, C-5), 70.1 (CH, 1C, C-3), 69.8 (CH, 1C, C-2′), 67.4 (CH, 1C, C-4′), 61.3 (CH_2, 1C, C-6′), 60.6 (CH_2, 1C, C-6), 54.5 (CH_3, 1C, OMe), 53.7 (CH, 1C, C-2), 21.4 (CH_3, 1C, NHAc); Anal. Calcd for $C_{15}H_{26}NNaO_{14}S$: C, 36.07; H, 5.25; N, 2.80. Found: C, 35.98; H, 5.29; N, 2.72.

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References

1. Habuchi, H.; Habuchi, O.; Kimata, K. *Glycoconjugate J.* **2004**, *21*, 47–52.
2. Honke, K.; Taniguchi, N. *Med. Res. Rev.* **2002**, *22*, 637–654.
3. Honke, K.; Zhang, Y.; Cheng, X.; Kotani, N.; Taniguchi, N. *Glycoconjugate J.* **2004**, *21*, 59–62.
4. Rosen, S. D. *Annu. Rev. Immunol.* **2004**, *22*, 129–156.
5. Robbins, P. W.; Lipman, F. *J. Am. Chem. Soc.* **1956**, *78*, 2652–2653.
6. Chapman, E.; Best, M. D.; Hanson, S. R.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2004**, *43*, 3526–3548.
7. Honke, K.; Tsuda, M.; Koyota, S.; Wada, Y.; Iida-Tanaka, N.; Ishizuka, I.; Nakayama, J.; Taniguchi, N. *J. Biol. Chem.* **2001**, *276*, 267–274.
8. Tamm, I.; Horsfall, F. L. *Proc. Soc. Exp. Biol. Med.* **1950**, *74*, 108–114.
9. Tamm, I.; Horsfall, F. L. *J. Exp. Med.* **1952**, *95*, 71–79.
10. Afonso, A. M. *Carbohydr. Res.* **1981**, *89*, 309–319.
11. van Rooijen, J. J. M.; Kamerling, J. P.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1998**, *256*, 471–487.
12. Yamashita, K.; Ohkura, T.; Ideo, H.; Ohno, K.; Kanai, M. *J. Biochem. (Tokyo)* **1993**, *114*, 766–769.
13. Millat, G.; Froissart, R.; Maise, I.; Bozon, D. *Biochem. J.* **1997**, *326*, 243–247.
14. Tsiakas, K.; Steinfeld, R.; Storch, S.; Ezaki, J.; Lukacs, Z.; Komiami, E.; Kohlschuetter, A.; Ullrich, K.; Braulke, T. *Glycobiology* **2004**, *14*, 1C–5C.
15. Dahmer, J.; Gnosspelius, G.; Larsson, A. C.; Lave, T.; Noori, G.; Pålsson, K.; Frjed, T.; Magnusson, G. *Carbohydr. Res.* **1985**, *138*, 17–28.
16. Rana, S. S.; Barlow, J. J.; Matta, K. L. *Carbohydr. Res.* **1983**, *113*, 257–271.
17. Farkas, E.; Thiem, J. *Eur. J. Org. Chem.* **1999**, 3073–3077.
18. Ohlsson, J.; Magnusson, G. *Carbohydr. Res.* **2000**, *329*, 49–55.
19. Riley, J. G.; Grindley, T. B. *J. Carbohydr. Chem.* **2001**, *20*, 159–169.
20. Malet, C.; Hindsgaul, O. *Carbohydr. Res.* **1997**, *303*, 51–65.
21. Ljevakovic, D.; Tomic, S.; Tomašic, J. *Carbohydr. Res.* **1988**, *182*, 197–205.
22. Guilbert, B.; Davis, N. J.; Pearce, M.; Aplin, R. T.; Flitsch, S. L. *Tetrahedron: Asymmetry* **1994**, *5*, 2163–2178.
23. Field, R. A.; Otter, A.; Fu, W.; Hindsgaul, O. *Carbohydr. Res.* **1995**, *276*, 347–363.
24. Dai, J.; Whittal, R. M.; Bridges, C. A.; Isogai, Y.; Hindsgaul, O.; Li, L. *Carbohydr. Res.* **1997**, *304*, 1–9.
25. Muschol, N.; Storch, S.; Ballhausen, D.; Beesley, C.; Westermann, J.-C.; Gal, A.; Ullrich, K.; Hopwood, J. J.; Winchester, B.; Braulke, T. *Hum. Mutat.* **2004**, *23*, 559–566.
26. Bonner, W. M.; Laskey, R. A. *Eur. J. Biochem.* **1975**, *56*, 335–341.
27. Heine, C.; Koch, B.; Storch, S.; Kohlschütter, A.; Palmer, D. N.; Braulke, T. *J. Biol. Chem.* **2004**, *279*, 22347–22352.
28. Cavallone, D.; Malagolini, N.; Frasca, G. M.; Stefoni, S.; Serafini-Cessi, F. *Clin. Biochem.* **2002**, *35*, 405–410.