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Synthesis of the O-linked pentasaccharide in glycoproteins of *Trypanosoma cruzi* and selective sialylation by recombinant *trans*-sialidase

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Abstract—The mucin-like glycoproteins of *Trypanosoma cruzi* have novel O-linked oligosaccharides that are acceptors of sialic acid in the *trans*-sialidase (TcTS) reaction. The transference of sialic acid from host glycoconjugates to the mucins is involved in infection and pathogenesis. The synthesis of the pentasaccharide, β-D-Gal*p*-(1→2)-[β-D-Gal*p*-(1→3)]-β-D-Gal*p*-(1→6)-[β-D-Gal*f*-(1→4)]-D-Glc*p*NAc and the corresponding alditol, previously isolated by reductive β-elimination of the mucins, is described. The key step was the 6-O-glycosylation of a easily accessible derivative of β-D-Gal*f*-(1→4)-D-Glc*p*NAc with a β-D-Gal*p*-(1→2)-[β-D-Gal*p*-(1→3)]-D-Gal*p* donor using the trichloroacetimidate method. The β-linkage was diastereoselectively obtained by the nitrile effect. The pentasaccharide is the major oligosaccharide in the mucins of *T. cruzi*, G strain and presents two terminal β-D-Gal*p* residues for possible sialylation by TcTS. A preparative sialylation reaction was performed with its benzyl glycoside and the sialylated product was isolated and characterized. NMR spectroscopic analysis showed that selective monosialylation occurred at the terminal (1→3) linked galactopyranose.

Keywords: *Trypanosoma cruzi*; Pentasaccharide; Trichloroacetimidate glycosylation; *trans*-Sialidase; Sialic acid; Galactofuranose

1. Introduction

Trypanosoma cruzi is the agent of Chagas' disease, which currently affects approximately 20 million people in Central and South America.[1] On the basis of biochemical and molecular studies, *T. cruzi* strains may be grouped into two divergent genetic divisions designated as lineages 1 and 2. Lineage 1 is related with the sylvatic cycle and lineage 2 is associated with the domestic cycle, involved in human infection.[2,3]

The surface of *T. cruzi* is dominated by glycoinositolphospholipids (GIPLs)[4,5] and mucin-like glycoproteins anchored to the membrane by a glycosylphosphatidylinositol moiety.[6,7] The O-linked chains in these mucin-like, sialic acid acceptors are linked to the protein by α-Glc*p*NAc and may be derived from two cores, β-D-Gal*p*-(1→4)-Glc*p*NAc or β-D-Gal*f*-(1→4)-Glc*p*NAc. The cores are further branched with various units of Gal*f* and/or Gal*p*. Thus far, the galactofuranose form was found in the mucins of strains belonging to lineage 1 (Fig. 1),[8–11] whereas in the more infective Y[12] and CL[13–15] strains, galactose in the mucins is only present in the pyranose form. The mechanism by which the presence of Gal*f* correlates with the parasite inactivation has not been fully elucidated. Gal*f* is an antigenic epitope[16] and an immunological reaction could influence the infection. On the other hand, as the Gal*p* units are the acceptors of the sialic acid in the *trans*-sialidase reaction

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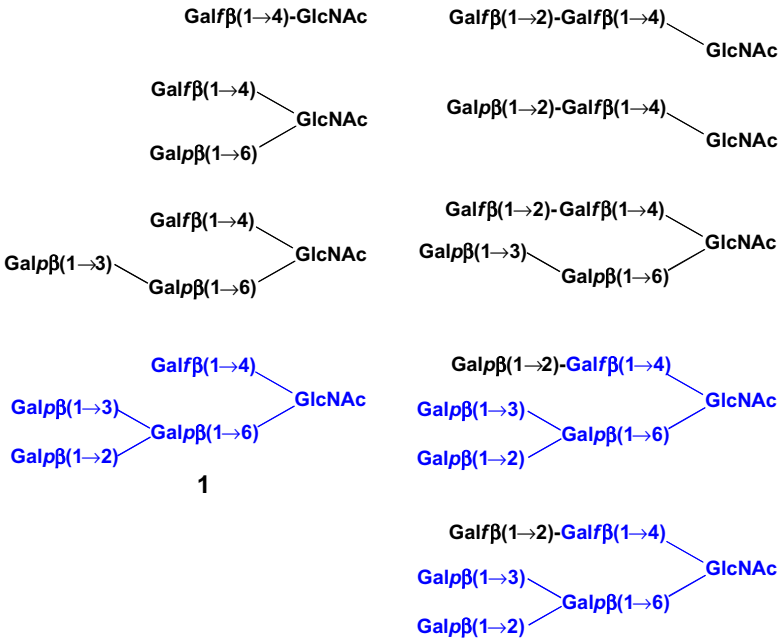


Figure 1. Oligosaccharides in mucins of *Trypanosoma cruzi*, lineage 1 (Refs. 8–11); pentasaccharide **1** is shown in blue.

that involves the host glycoproteins, it is interesting to study the influence of the coexistence of Gal*f* on these properties.

In our laboratory, we have undertaken the chemical synthesis of the parasite mucin oligosaccharides, in particular of those containing galactofuranose.[17–19] The metabolic pathways involved in the transference of Gal*f* in *T. cruzi* have not been elucidated, thus, these oligosaccharides could be useful tools for biosynthetic studies. Here, we report the first synthesis of free β-D-Gal*p*-(1→2)-[β-D-Gal*p*-(1→3)]-β-D-Gal*p*-(1→6)-[β-D-Gal*f*-(1→4)]-D-Glc*p*NAc (**1**, Scheme 1) and the corresponding alditol. Pentasaccharide **1** is the major oligosaccharide in the mucins of the G strain[9] and presents two terminal β-D-Gal*p* residues for possible sialylation in the TcTS reaction. We have recently demonstrated selective mono sialylation of 2,3-di-*O*-(β-D-Gal*p*)-D-Gal*p*, the external unit in the pentasaccharide **1**.[20] The acceptor substrate selectivity was now studied for compound **1**, its benzyl glycoside and the corresponding alditol.

2. Results and discussion

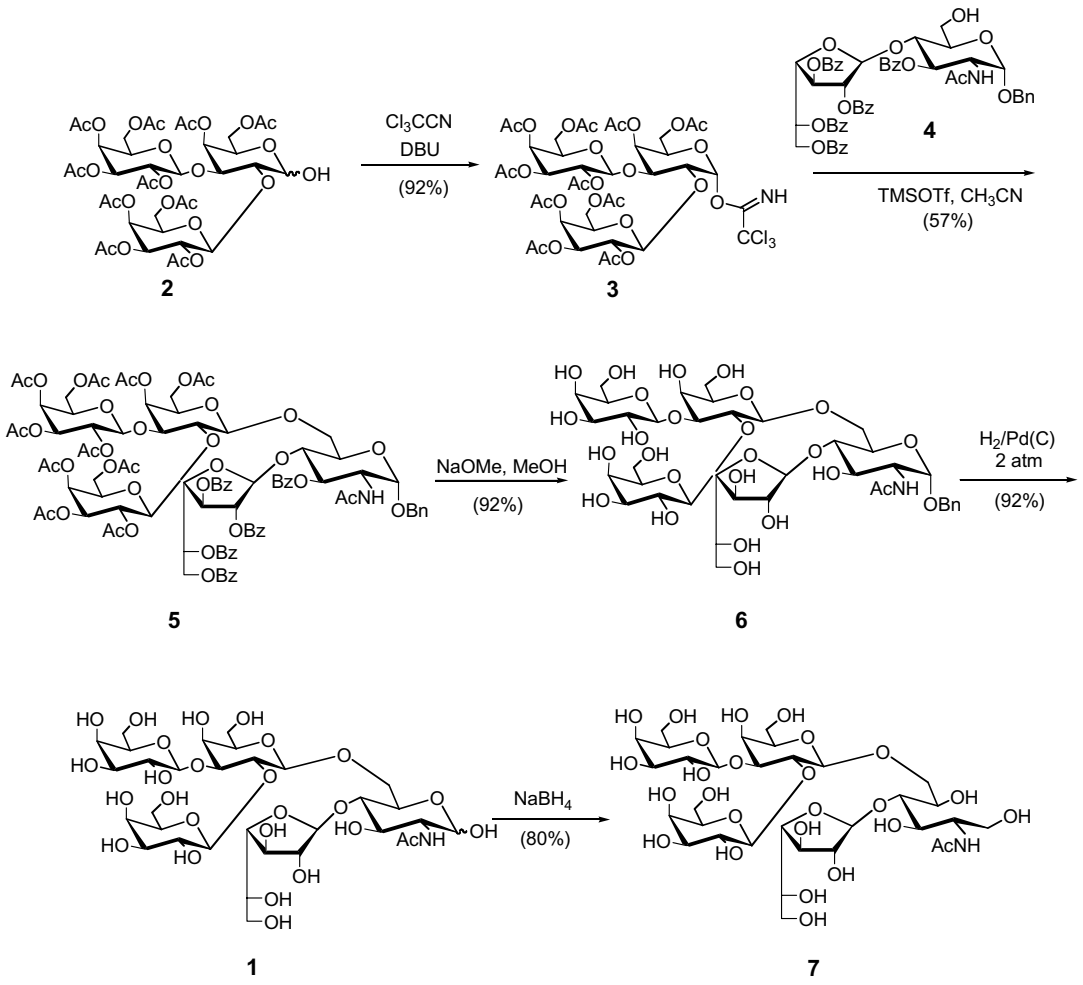
We have previously synthesized the tetrasaccharide β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→6)-[β-D-Gal*f*-(1→4)]-D-Glc*p*NAc via a convergent route, by condensing a β-D-Gal*f*-(1→4)-D-Glc*p*NAc acceptor with a β-D-Gal*p*-(1→3)-D-Gal*p* donor.[19] This strategy was designed taking into account that the 4-hydroxyl group of *N*-acetylglucosamine derivatives is a poor nucleophile in glycosylation reactions.[21] Thus, the last glycosylation step of that synthesis was on the primary 6-hydroxyl group of the Glc*p*NAc moiety.

For the synthesis of target pentasaccharide **1**, a similar convergent route could be employed by glycosylation of the acceptor derivative of β-D-Gal*f*-(1→4)-D-Glc*p*NAc **4** with a derivative of the trisaccharide 2,3-di-*O*-(β-D-Gal*p*)-D-Gal*p*. In this case, the diastereoselectivity of the glycosylation would be affected by the absence of neighbouring group assistance at C-2.

The trichloroacetimidate method was used for the glycosylation step. Reaction of 4,6-di-*O*-acetyl-2,3-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-galactopyranose (**2**)[22] with trichloroacetonitrile and DBU at 0 °C gave the α-trichloroacetimidate **3** in 92% yield (Scheme 1). The configuration at the anomeric centre was confirmed from the ^{1}H NMR spectrum (δ 6.55 ppm $J_{1,2} = 3.8$ Hz for H-1) and ^{13}C NMR spectrum (δ 95.6 ppm for C-1).

Due to the lack of a participating group at C-2 of donor **3**, to obtain the β-(1→6) linkage, the glycosylation was performed in a participating solvent such as acetonitrile taking advantage of the nitrile effect.[23,24] The condensation of benzyl 2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→4)-2-acetamido-3-*O*-benzoyl-2-deoxy-α-D-glucopyranoside (**4**)[19] with imidate **3** in the presence of TMSOTf as catalyst, and employing acetonitrile as solvent at −40 °C, gave pentasaccharide **5** diastereoselectively in 57% yield. The α-glycosylation product was not detected, and unreacted acceptor **4** was also recovered.

^{13}C and ^{1}H NMR assignments for pentasaccharide derivative **5** were performed on the basis of ^{1}H–^{1}H



Scheme 1.

COSY and HETCOR experiments (Tables 1 and 2). The ^{13}C NMR spectrum of **5** (Table 2) showed the resonances for the anomeric carbons at 96.1 (Glc*p*NAc), 107.1 (Gal*f*), 102.5 (Gal*p*-β-(1→6)), 99.9 and 99.4 ppm (Gal*p*-β-(1→3) and Gal*p*-β-(1→2)). In the ^{1}H NMR spectrum, the resonance for the newly constructed β-(1→6) glycosidic linkage, appeared at 4.68 ppm with a coupling constant of 7.5 Hz, indicating the β-pyranosidic configuration. Based on the COSY experiment, H-2 and H-3 of this internal β-Gal*p* unit appeared at 3.89 and 3.98 ppm, respectively, shielded compared to the H-2 and H-3 signals in the terminal β-Gal*p* units. The assignment of each spin system of Gal*p*-β-(1→2) and Gal*p*-β-(1→3) could be interchanged.

It is important to point out the influence of the solvent in the glycosylation reaction. When the reaction was performed in CH_2Cl_2 as solvent, at −12 °C for 90 min and then at 5 °C for 16 h, low diastereoselectivity in the glycosidic bond formation was obtained, as two products could be distinguished by TLC, but could not be separated by column chromatography. Analysis of the mixture (37%) by ^{1}H NMR spectroscopy showed the signals for compound **5** together with the signals for the α-(1→6) product in a 1:2.5 β:α ratio as indicated by integration. In the ^{13}C NMR spectrum, the anomeric carbons of the α-(1→6) product appeared at 96.1 (Glc*p*NAc), 106.7 (Gal*f*), 101.9 and 100.5 (Gal*p*-β-(1→3) and Gal*p*-β-(1→2)); and 98.2 ppm (Gal*p*-α-(1→6)), the latter indicating the α-configuration. Moreover, the anomeric proton of the new glycosidic bond appeared at 5.26 ppm as a doublet with a coupling constant of 3.7 Hz. Deacylation of the mixture followed by NMR analysis of the benzyl glycosides confirmed the ratio obtained in the glycosylation reaction.

Deacylation of pentasaccharide **5** with sodium methoxide gave crystalline benzyl glycoside **6** in 92% yield. Taking into account that **6** was used as substrate for a preparative *trans*-sialylation reaction, NMR assignments were needed for comparison with the signals for the sialylated product (Tables 1 and 2), and were performed based on ^{1}H–^{1}H COSY, HETCOR and NOESY experiments.

Table 1. ^{1}H NMR data for **5** and **6** (500 MHz, $CDCl_3$ and D_2O, respectively)

Compound	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6a}$)	H-6a ($J_{6b,6a}$)	H-6b ($J_{5,6b}$)	Other signals ($J_{H,H'}$)
5								
Glc*p*NAc-OBn	5.02	4.43	5.64	4.12	4.21	4.36	3.96	5.82 (N*H*)
	(*3.7*)	(*11.0*)	(*9.0*)	(*10.0*)	(*4.3*)	(*11.0*)	(*6.2*)	(*9.5*)
Gal*f*-β-(1→4)	5.43	5.31	5.66	4.40	5.53	4.30	4.24	CH_2Ph
	(*b.s.*)	(*1.4*)	(*4.7*)	(*4.3*)	(*7.8*)	(*12.1*)	(*3.2*)	4.85, 4.57
Gal*p*-β-(1→6)	4.68	3.89	3.98	5.37	4.00	4.35	4.08	(*11.6*)
	(*7.5*)	(*9.3*)	(*3.8*)	(*1.2*)	(*5.5*)	(*10.9*)	(*8.3*)	CH_3CONH
Gal*p*-β-(1→2)[a]	4.72	5.08	5.08	5.35	3.90	4.19	4.16	1.77 (s)
	(*7.7*)	(*n.d.*)	(*3.0*)	(*1.2*)	(*6.8*)	(*11.2*)	(*6.8*)	
Gal*p*-β-(1→3)[a]	4.96	5.21	5.00	5.36	3.81	4.13	4.00	
	(*8.0*)	(*10.5*)	(*3.7*)	(*1.0*)	(*6.5*)	(*11.2*)	(*6.7*)	
6								
Glc*p*NAc-OBn	4.90	3.85	3.79[b,e]	3.89	3.88[b,e]	4.13	3.82[e]	CH_2Ph
	(*3.5*)	(*n.d.*)	(*n.d.*)	(*n.d.*)	(*<1*)	(*10.4*)	(*n.d.*)	4.69, 4.51
Gal*f*-β-(1→4)	5.28		4.07–4.00		3.78[e]	3.64–3.59[e]		(*12.0*)
	(*2.5*)		(*n.d.*)		(*n.d.*)	(*n.d.*)		CH_3CONH
Gal*p*-β-(1→6)	4.49	3.91	3.95	4.15	3.67[e]	3.88–3.65[e]		1.89 (s)
	(*7.5*)	(*9.7*)	(*3.2*)	(*n.d.*)	(*n.d.*)	(*n.d.*)		
Gal*p*-β-(1→2)	4.78	3.57[c]	3.62–3.55[e]	3.83[d,e]	3.60[e]	3.88–3.65[e]		
	(*7.5*)	(*9.9*)	(*n.d.*)	(*n.d.*)	(*n.d.*)	(*n.d.*)		
Gal*p*-β-(1→3)	4.60	3.56[c]	3.62–3.55[e]	3.87[d,e]	3.62[e]	3.88–3.65[e]		
	(*7.1*)	(*10.0*)	(*n.d.*)	(*n.d.*)	(*n.d.*)	(*n.d.*)		

[a] The assignments for the residues could be interchanged.
[b–d] Assignment could be interchanged.
[e] Assigned from two-dimensional spectra.

Table 2. ^{13}C NMR data for compounds **5** and **6** (125 MHz, $CDCl_3$ and D_2O, respectively)

Compound	C-1	C-2	C-3	C-4	C-5	C-6	Other signals
5							
Glc*p*NAc-OBn	96.1	52.4	72.5	76.2	70.9	69.1	69.8 (*C*H_2Ph)
Gal*f*-β-(1→4)	107.1	82.4	76.9	81.4	69.8	63.2	23.0 (*C*H_3CO)
Gal*p*-β-(1→6)	102.5	78.8	75.8	68.8	70.6	60.5	
Gal*p*-β-(1→2)[a]	99.4	70.4[g]	69.8[g]	67.0	70.7	61.0	
Gal*p*-β-(1→3)[a]	99.9	69.8	71.1	66.8	70.8	60.3	
6							
Glc*p*NAc-OBn	96.4	54.2	69.9[b]	78.5	70.3[b]	68.3	70.5 (*C*H_2Ph)
Gal*f*-β-(1→4)	108.3	82.6[c]	76.3	81.8[c]	70.9	63.2	22.4 (*C*H_3CO)
Gal*p*-β-(1→6)	102.4	76.3	83.5	69.4	75.2	61.8[f]	
Gal*p*-β-(1→2)	103.4	70.7	73.5[d]	69.7[e]	76.0	61.5[f]	
Gal*p*-β-(1→3)	104.5	72.0	73.4[d]	69.2[e]	75.7	61.4[f]	

[a] The assignments for the residues could be interchanged.
[b–g] Assignment could be interchanged.

Hydrogenolysis of the anomeric benzyl group of **6** either with hydrogen and palladium-on-carbon or ammonium formate, 10% palladium-on-carbon in CH_3OH at 65 °C gave the pure free pentasaccharide β-D-Gal*p*-(1→2)-[β-D-Gal*p*-(1→3)]-β-D-Gal*p*-(1→6)-[β-D-Gal*f*-(1→4)]-D-Glc*p*NAc (**1**) in high yield as a α:β mixture in a 4:1 ratio. Further reduction of **1** with $NaBH_4$ gave the corresponding alditol **7**. The chemical shifts in the ^{1}H and ^{13}C NMR spectra matched with those reported for the alditol released by reductive β-elimination from mucins of *T. cruzi* (G strain).[8]

Acosta-Serrano et al.[9] described that the pentasaccharide alditol **7**, obtained by reductive β-elimination from mucins of *T. cruzi*, accepted only one sialic acid molecule when incubated with native *trans*-sialidase and 3′-sialyllactose as donor; however, the site of sialylation was not determined. We have previously shown[20] that selective sialylation of benzyl 2,3-di-*O*-(β-D-Gal*p*)-β-D-Gal*p*, the terminal constituent of pentasaccharide **1**, occurred at the less hindered (1→3) linked Gal*p* unit. Compounds **1**, **6** and **7** were assayed as acceptor substrates for TcTS, using conditions previously described for incubation with the donor 3′-sialyllactose[25] (Fig. 2). In all cases, the reaction was fast and reached equilibrium after about 30 min. The progress of the transference of sialic acid was followed by HPAEC. As expected, sialylated pentasaccharide **S-1** (Fig. 2A) was eluted later (CarboPac PA-100 column) than

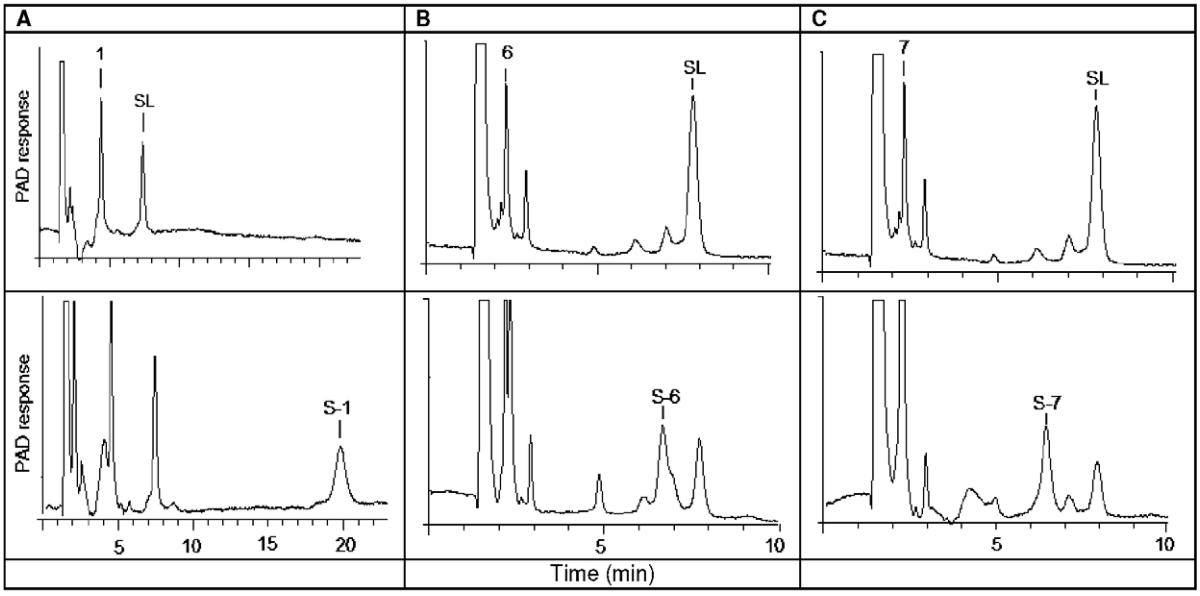


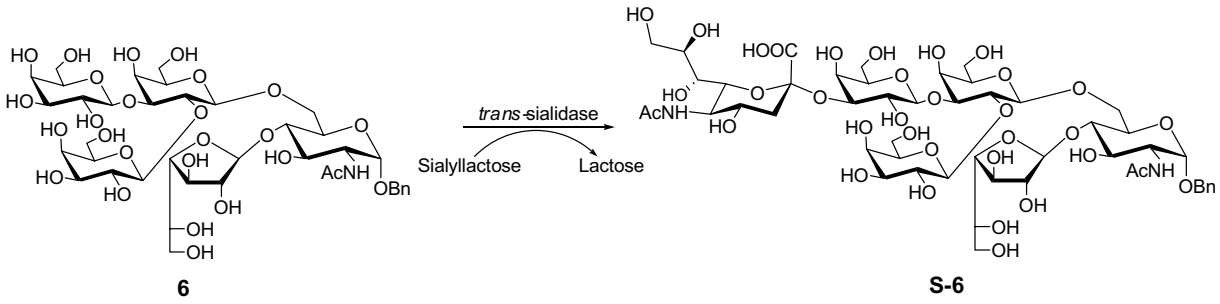
Figure 2. Analysis of compounds **1**, **6** and **7** as substrates of the *trans*-sialidase of *Trypanosoma cruzi*. Compounds **1** (A), **6** (B), **7** (C) at 1 mM concentrations were incubated for 15 min at 25 °C with 1 mM 3′-sialyllactose (**SL**) and 40 ng *trans*-sialidase in 20 mM Tris buffer (pH 7) and analyzed by HPAEC-PAD. For all panels, the upper chromatogram represents incubation in the absence of enzyme. A CarboPac PA-100 column was used and eluted with 70 mM NaOAc in 100 mM NaOH. Compounds **S1**, **S6** and **S7** are the sialylated derivatives of **1**, **6** and **7**, respectively.

sialyllactose. The nonreducing sialylated benzyl glycoside **S-6** (Fig. 2B) and sialylated pentasaccharide alditol **S-7** (Fig. 2C) were less retained on the column than sialyllactose. The three compounds were shown to be good acceptors of sialic acid, with transference values of 60–70%. Similar values were previously obtained for the 2,3-di-*O*-(β-D-Gal*p*)-D-Gal*p* trisaccharide and homologous derivatives. These results indicated that the presence of the Gal*f* branch does not affect the acceptor properties of the pentasaccharide.

With the aim of confirming the site of sialylation in the pentasaccharide, a preparative sialylation reaction was performed using benzyl glycoside **6** as the substrate (Scheme 2). The benzyl glycoside **6** was selected because the hydrophobic aglycon allowed easier purification, by exchange chromatography, of the product from the remaining sialyllactose that was used as donor. Moreover, the absence of anomeric mixtures facilitated analysis of the results by NMR spectroscopy. The reaction was monitored by HPAEC and, under the used conditions, 68% sialylation of compound **6** was obtained. No evidence of the presence of the other monosialylated or disialylated compounds was found. The reaction product was purified using an AG1X2 (acetate form) resin column. After elution of neutral sugars with water, the acidic components were eluted with 200 mM pyridinium acetate buffer, pH 5.0.

The sialyl derivative **S-6** was characterized on the basis of mass spectrometry and NMR spectroscopy. The expected molecular mass of the *trans*-sialylation product was confirmed by Q-TOF positive ion spray mass spectrometry. Peaks at 1251.46, 1273.43 and 1289.42, assigned to $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ established that monosialylation had occurred. The peak corresponding to the loss of sialic acid at 960.34 was observed.

The ^{1}H NMR spectrum (Fig. 3) of the purified sialylated oligosaccharide **S-6** showed, inter alia, the presence



Scheme 2.

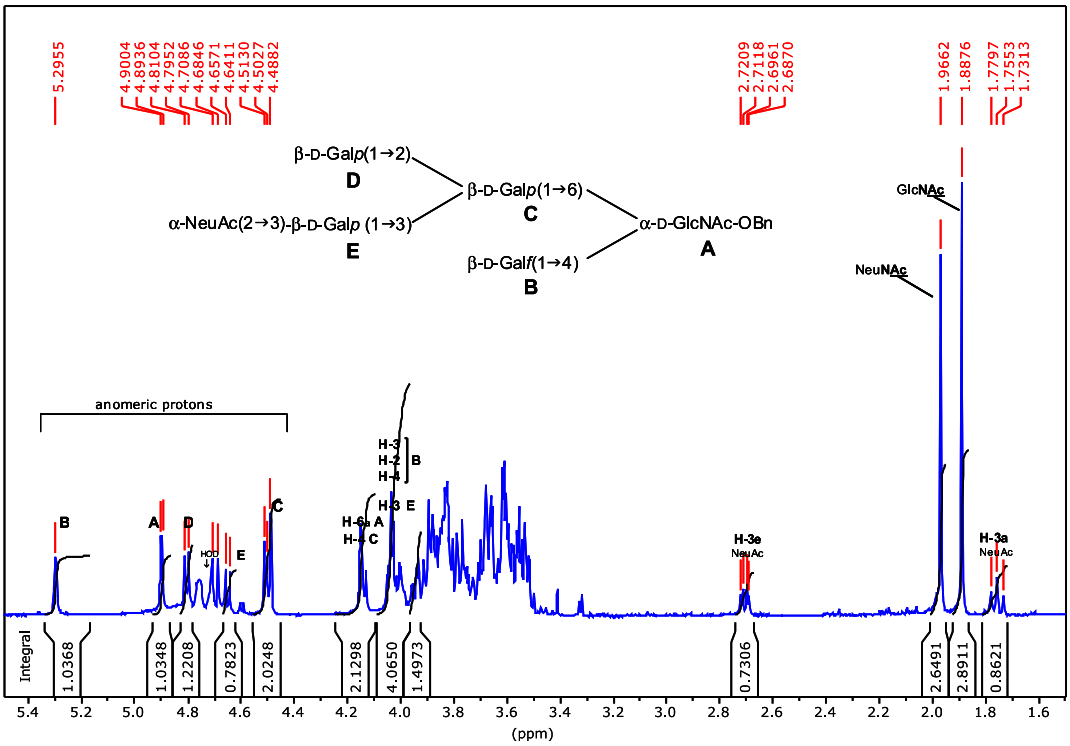


Figure 3. ^{1}H NMR spectrum of sialylated compound **6** (D_2O, 500 MHz).

of the diagnostic signals for the sialyl residue at 1.75 ppm (H-3a) and 2.70 ppm (H-3e). Integration of these signals confirmed that only one sialic acid unit has been introduced. Also, one new singlet appeared at δ 1.97 corresponding to the NAc group of neuraminic acid.

The selectivity of the transfer reaction with respect to the two terminal β-D-Gal*p* units was studied by two-dimensional NMR experiments (COSY, HETCOR and NOESY). The signal of H-3 at the site of sialylation was now at δ 4.04 ppm appearing as the characteristic doublet of doublets ($J = 10$, 3.2 Hz). The signal for the H-2 (COSY spectrum) that correlates with this signal was also shifted downfield (δ 3.59) and correlates with H-1 at δ 4.65 ppm assigned to the Gal*p*-β-(1→3) linked unit (E). NOESY correlation (Fig. 4) between this H-1 (unit E) and H-3 of the internal Gal*p*-β-(1→6) (unit C) confirmed selective sialylation of the terminal Gal*p*-β-(1→3) linked unit. On the other hand, the chemical

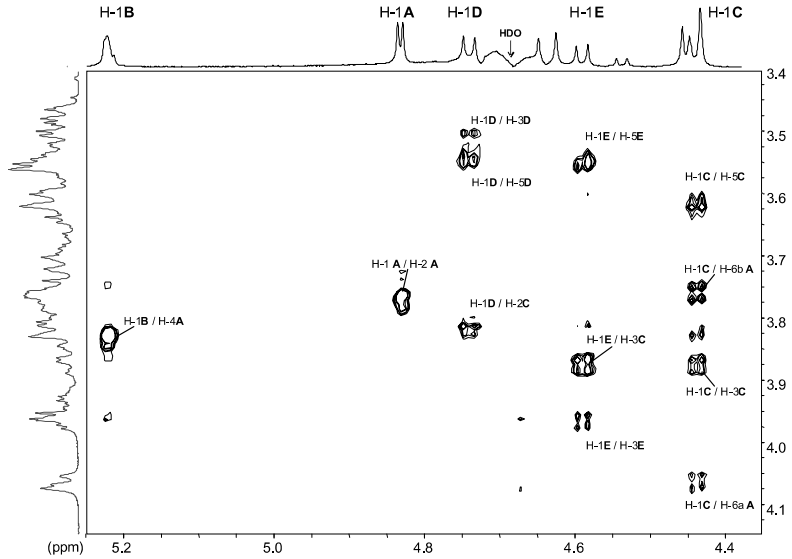


Figure 4. NOESY spectrum (500 MHz) of α-D-Neu*p*Ac-(2→3)-β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→2)]-β-D-Gal*p*-(1→6)-[β-D-Gal*f*-(1→4)]-α-D-Glc*p*NAc-OBn (**S-6**).

shifts for the Gal*p*-β-(1→2) unit were not affected by sialylation.

In conclusion, pentasaccharide **1** was chemically synthesized, for the first time, by a convergent approach employing the 'nitrile effect' concept. Analysis by NMR spectroscopy of alditol **7** confirmed the structure of the oligosaccharide present in the natural sources. Furthermore, the present study unequivocally established the sialylation site of the pentasaccharide at the less hindered (1→3)-linked galactopyranose.

3. Experimental

3.1. General methods

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 343 polarimeter. TLC was performed on 0.2 mm Silica Gel 60 F254 (Merck) aluminium supported plates. Column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Detection was effected by exposure to UV light or by spraying with 10% (v/v) sulfuric acid in EtOH and charring. NMR spectra were recorded with a Bruker AM 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 30 °C unless otherwise indicated. Chemical shifts are given relative to the signal of internal acetone standard at 2.16 and 30.8 ppm for ^{1}H NMR and ^{13}C NMR spectra, respectively, when recorded in D_2O. ^{1}H and ^{13}C assignments were supported by DEPT 135, homonuclear COSY, HETCOR and HMQC experiments. HMQC experiments were recorded in a Bruker Avance DPX-400. High resolution electrospray ionization mass spectra (ESIMS) were recorded on a Micromass Q-TOF Ultima Tandem Quadrupole/Time-of-Flight Instrument.

For the sialylation experiments, a recombinant TcTS expressed in *Escherichia coli* was kindly provided by A. C. C. Frasch (UNSAM, General San Martín, Buenos Aires Argentina). 3′-Sialyllactose was obtained from bovine colostrum by an adaptation of a reported method.[26] Analysis by HPAEC-PAD was performed using a Dionex DX-300 HPLC system with a pulse amperometric detector (PAD) and a CarboPac PA-100 ion exchange analytical column (4 × 250 mm), equipped with a guard column PA-100 (4 × 50 mm), with 70 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 mL/min at rt.

3.2. *O*-[2,3-Di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-4,6-di-*O*-acetyl-α-D-galactopyranosyl] trichloroacetimidate (3)

To a stirred solution of 2,3-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-4,6-di-*O*-acetyl-α,β-D-galactopyranose[22] (**2**, 342 mg, 0.37 mmol) in anhydrous CH_2Cl_2 (3 mL) cooled to 0 °C, trichloroacetonitrile (1.71 mmol, 0.17 mL) and DBU (0.17 mmol, 26 μL) were added. After 1 h at rt, the solution was carefully concentrated under reduced pressure and the residue was immediately purified by column chromatography (7:3:0.03, toluene/EtOAc/TEA) to give 368 mg of foamy trichloroacetimidate **3** (92%, 0.34 mmol) as the only product: $R_f = 0.49$ (1:2, toluene/EtOAc), $[\alpha]_D$ +32.4 (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 8.59 (br s, 1H, N*H*), 6.55 (d, 1H, $J = 3.8$ Hz, H-1), 5.49 (dd, 1H, $J = 3.6$, 1.2 Hz, H-4), 5.35 (dd, 1H, $J = 3.5$, 1.1 Hz, H-4′ or H-4″), 5.33 (dd, 1H, $J = 3.4$, 1.0 Hz, H-4′ or H-4″), 5.13 (dd, 1H, $J = 7.7$, 10.5 Hz, H-2′ or H-2″), 5.08 (dd, 1H, $J = 7.7$, 10.6 Hz, H-2′ or H-2″), 4.99 (dd, 1H, $J = 10.6$, 3.6 Hz, H-3′ or H-3″), 4.97 (dd, 1H, $J = 10.5$, 3.4 Hz, H-3′ or H-3″), 4.64 (2d, 2H, $J = 7.7$ Hz, H-1′ and H-1″), 4.26 (m, 1H, H-5), 4.23 (dd, 1H, $J = 10.0$, 3.6 Hz, H-3), 4.20–4.13 (m, 3H), 4.10 (dd, 1H, $J = 3.8$, 10.0 Hz, H-2), 4.10 (dd, 1H, $J = 11.3$, 6.5 Hz), 4.01 (dd, 1H, $J = 11.1$, 6.6 Hz); 3.99 (dd, 1H, $J = 11.4$, 7.3 Hz), 3.91 (dt, 1H, $J = 6.7$, 1.2 Hz, H-5′ or H-5″), 3.89 (dt, 1H, $J = 6.6$, 1.2 Hz, H-5′ or H-5″), 2.17, 2.15, 2.11, 2.10, 2.08 (5s, 15H, C*H*$_3$CO), 2.07 (s, 6H, C*H*$_3$CO), 1.97 (s, 3H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$): δ 170.5, 170.3, 170.0, 169.9, 169.6, 169.4, 168.9 (CH_3CO), 160.7 (CCl_3CNH), 101.3, 100.4 (C-1′, C-1″), 95.6 (C-1); 75.7, 72.1 (C-2, C-3), 70.9 (×2), 70.6, 70.2, 69.8 (×2), 69.7, 69.5, 69.1, 67.0, 66.7 (C-4′, C-4″), 62.1 (C-6), 61.3 (C-6′, C-6″), 21.0, 20.9, 20.7 (×3), 20.6 (×3), 20.5 (×2) (CH_3CO).

3.3. Benzyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→2)-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)]-4,6-di-*O*-acetyl-β-D-galactopyranosyl-(1→6)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→4)]-2-acetamido-3-*O*-benzoyl-2-deoxy-α-D-glucopyranoside (5)

To a flask containing recently purified and dried benzyl 2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→4)-2-acetamido-3-*O*-benzoyl-2-deoxy-α-D-glucopyranoside[19] (**4**, 285 mg, 0.29 mmol), and activated 4 Å powdered molecular sieves, a solution of trichloroacetimidate (**3**, 368 mg, 0.34 mmol) in freshly distilled anhydrous CH_3CN (10 mL) was added, and the suspension was cooled to −40 °C under an argon atmosphere. After 15 min of vigorous stirring, TMSOTf (0.115 mmol, 21 μL) was added and the stirring continued for 4 h at −40 °C, and then for 16 h at −20 °C. The reaction was quenched by the addition of Et_3N (0.12 mmol, 16 μL), the mixture was allowed to reach rt, filtered and the molecular sieves were washed with CH_3CN. The filtrate was concentrated under reduced pressure and the residue was purified by column chromatography (1:1.7, *n*-hexane/EtOAc). A first fraction gave 100 mg of unreacted acceptor **4** ($R_f = 0.60$; 1:2, toluene/EtOAc). The next fraction afforded 314 mg of a foamy solid which

crystallized from 1:1 hexane/ether and was characterized as pentasaccharide **5** (57%), $R_f = 0.40$ (1:2, toluene/EtOAc), mp 118–120 °C, $[\alpha]_D$ −19.2 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$, Table 1): only the protecting groups are listed δ 8.01–7.85 (m, 10H, arom.), 7.61–7.23 (m, 20 H, arom), 2.17, 2.07, 2.04, 2.02, 2.01, 2.00, 1.97, 1.96 (8s, 30H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$, Table 2): only the protecting groups are listed δ 171.0, 170.3, 170.2, 169.9, 169.8, 169.7, 169.3, 168.9 (CH$_3$*C*O), 167.1 (CH$_3$*C*ONH), 165.8, 165.6, 165.4, 165.3 (Ph*C*O); 136.8, 133.5, 133.3, 133.0, 132.9, 129.9, 129.8, 129.6, 129.5, 129.4, 128.9, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1 (arom.), 20.9, 20.6, 20.5, 20.4, 20.3 (*C*H$_3$CO). Anal. Calcd for $C_{94}H_{101}NO_{41}$: C, 59.40; H, 5.36; N, 0.74. Found: C, 58.92; H, 5.29; N, 0.74.

3.4. Benzyl β-D-galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-α-D-glucopyranoside (6)

To a flask containing benzyl derivative **5** (176 mg, 0.093 mmol) was added 2 mL of a cooled 0.5 M sodium methoxide solution. After 1 h of stirring at 0 °C, TLC examination showed that the reaction was completed giving only one more polar compound. H_2O (0.2 mL) was added and the resulting solution was decationized by elution with CH_3OH/H_2O (9:1) through a column of Amberlite IR 120 cationic resin. Evaporation of the solvent, and co-evaporation with H_2O three times, gave 82.2 mg (0.086 mmol) of **6** as a glassy solid (92% yield) which was crystallized from EtOH/Et_2O (1:2) to give very hygroscopic crystals: mp 205–208 °C; $R_f = 0.43$ (7:1:2, *n*-PrOH/EtOH/H_2O); $[\alpha]_D$ +43.2 (*c* 0.8, H_2O); ^1H NMR (D_2O, Table 1): only the protecting groups are listed δ 7.41–7.33 (m, 5H, arom.); ^{13}C NMR (D_2O, Table 2): only the protecting groups are listed δ 174.8 (CH$_3$*C*ONH), 137.5, 129.3, 129.1, 128.9 (arom). Anal. Calcd for $C_{39}H_{61}NO_{26}\cdot H_2O$: C, 47.60; H, 6.49; N, 1.43. Found: C, 47.71; H, 6.45; N, 1.34.

3.5. β-D-Galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-α,β-D-glucopyranose (1)

To a solution of benzyl glycoside **6** (50 mg, 0.052 mmol) in 9:1 CH_3OH/H_2O (4 mL), was added a catalytic amount of 10% Pd/C Deguzza type E101 NE/W and the suspension was hydrogenated for 5 h at 2 atm. The catalyst was filtered and the solution was passed through a C-8 reverse phase cartridge and eluted with H_2O. Evaporation of the solvent afforded compound **1** (41.7 mg, 92%) as a glassy solid: $R_f = 0.25$ (7:1:2, *n*-PrOH/EtOH/H_2O); $[\alpha]_D$ −11.1 (*c* 1.0, H_2O); ^1H NMR (D_2O): δ anomeric region 5.29 (d, 0.8H, $J = 2.8$ Hz, Gal*f* for α anomer), 5.27 (d, 0.2H, $J = 2.4$ Hz, Gal*f* for β anomer), 5.15 (d, 0.8H, $J = 3.5$ Hz, α-Glc*p*NAc), 4.64 (d, 0.2H, $J = 8.3$ Hz, Gal*p* β-(1→2) or Gal*p* β-(1→3) for β anomer), 4.59 (d, 0.8H, $J = 7.6$ Hz, Gal*p* β-(1→2) or Gal*p* β-(1→3) for α anomer), 4.50 (d, 0.2H, $J = 7.4$ Hz, Gal*p* β-(1→6) for β anomer), 4.49 (d, 0.8H, $J = 7.7$ Hz, Gal*p* β-(1→6) for α anomer). β-GlcNAc, Gal*p* β-(1→2) or Gal*p* β-(1→3) for β anomer and Gal*p* β-(1→2) or Gal*p* β-(1→3) for α anomer signals are overlapped with DHO signal. ^{13}C NMR (D_2O): δ anomeric region 108.4 (Gal*f*), 104.7 (Gal*p* β-(1→3)), 103.6 (Gal*p* β-(1→2)), 102.4 (Gal*p* β-(1→6)), 95.7 (β-Glc*p*NAc), 91.3 (α-Glc*p*NAc). ESIMS *m*/*z* calcd for $C_{32}H_{55}NO_{26}Na$ $[M+Na]^+$: 892.2910. Found: 892.2916.

3.6. β-D-Galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-D-glucitol (7)

To a 0 °C solution of pentasaccharide **1** (20 mg, 0.023 mmol) in CH_3OH/H_2O (12:1), $NaBH_4$ (30 mg, 0.79 mmol, 34 equiv) was added in four portions with stirring. The reaction was allowed to stand for 16 h at 5 °C, until TLC analysis showed that the reaction was complete. The resulting mixture was purified by elution with CH_3OH/H_2O (9:1) through a column of Amberlite IR 120 cationic resin. Evaporation of the solvent, and co-evaporation with CH_3OH (×3), gave 15.9 mg (0.081 mmol, 80% yield) of the desired alditol **7**, which was recrystallized from absolute EtOH to give a very hygroscopic crystalline product: 158–160 °C; $R_f = 0.18$ (7:1:2, *n*-PrOH/EtOH/H_2O); $[\alpha]_D$ +1.1 (*c* 0.4, H_2O); ^1H NMR (D_2O): δ 5.26 (d, 1H, $J = 2.2$ Hz, H-1 Gal*f*), 4.83 (d, 1H, $J = 8.0$ Hz, H-1 Gal*p* β-(1→2) or β-(1→3)), 4.66 (d, 1H, $J = 7.6$ Hz, H-1 Gal*p* β-(1→2) or β-(1→3)), 4.58 (d, 1H, $J = 7.7$ Hz, H-1 Gal*p* β-(1→6)), 4.20 (d, 1H, $J = 3.3$ Hz, H-4 Gal*p* β-(1→6)), 4.18–4.15 (m, 2H), 4.10 (dd, 1H, $J = 6.8$, 3.5 Hz), 4.07 (dd, 1H, $J = 6.8$, 4.0 Hz), 4.08–4.03 (m, 1H), 4.00 (dd, 1H, $J = 9.7$, 3.3 Hz, H-3 Gal*p* β-(1→6)), 3.95 (dd, 1H, $J = 9.7$, 7.7 Hz, H-2 Gal*p* β-(1→6)), 3.93–3.91 (m, 3H), 3.87–3.63 (m, 19H), 3.61 (dd, 1H, $J = 9.9$, 7.6 Hz, H-2 Gal*p* β-(1→2) or β-(1→3)), 3.54 (dd, 1H, $J = 10.0$, 7.9 Hz, H-2 β-(1→2) or β-(1→3)), 2.05 (s, 3H, C*H*$_3$CO); ^{13}C NMR (D_2O): δ 175.7 (CH$_3$*C*ONH), 109.4 (C-1 Gal*f*); 105.3, 104.2 (C-1 Gal*p* β-(1→2) and β-(1→2)), 103.2 (C-1 Gal*p* β-(1→6)), 83.9, 83.8, 82.7, 79.1, 77.8, 77.6, 76.6, 76.4, 75.9, 74.1, 74.0, 72.4, 72.1 (C-6 Glc*p*NAc), 71.9, 71.1, 70.1, 70.0, 69.9, 69.6, 64.1 (C-6 Gal*f*), 62.3, 62.2, 62.1 (×2) (C-1 Glc*p*NAc, C-6 Gal*p* β-(1→6), β-(1→2) and β-(1→3)), 52.3 (C-2 Glc*p*NAc), 23.4 (*C*H$_3$CO). ESIMS *m*/*z* calcd for $C_{32}H_{57}NO_{26}Na$ $[M+Na]^+$: 894.3067. Found: 894.3073. Calcd for $C_{32}H_{58}NO_{26}$ $[M+H]^+$: 872.3247. Found: 872.3275.

3.7. Synthesis of benzyl 5-*N*-acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-α-D-glucopyranoside (S-6)

Benzyl pentasaccharide **6** (3.5 mg) and 3′-sialyllactose (3.0 mg) were incubated with 22.5 μg of recombinant TcTS in 1 mL of 20 mM Tris buffer pH 7.6 containing 30 mM NaCl for 14 h at 25 °C. The synthesis was performed in triplicate and each reaction analyzed by HPAEC. A typical chromatogram is shown in Figure 2. The three incubation mixtures were combined, and sialylated benzyl β-D-galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-α-D-glucopyranoside (**S-6**) was purified by passing through an anion exchange resin (AG1X2, acetate form, BioRad, 1.2 × 15 cm). Neutral compounds, namely benzyl β-D-galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-β-D-galactopyranosyl-(1→6)-[β-D-galactofuranosyl-(1→4)]-2-acetamido-2-deoxy-α-D-glucopyranoside (**6**) and lactose were eluted with H_2O and sialylated compounds with 200 mM pyridinium acetate buffer pH 5. Fractions (1.5 mL) were collected and analyzed by HPAEC. The remaining sialyllactose was eluted with the first 30 mL of 200 mM pyridinium acetate buffer, further elution with the same buffer afforded sialylated compound **6** (**S-6**). After neutralization with pyridine, the pooled fractions were concentrated under vacuum at rt. Compound **S-6** was further purified by passing through a SepPack C8 cartridge (Alltech) eluting with H_2O to obtain 3.6 mg of a colourless syrup: $[\alpha]_D$ +9.0 (*c* 0.4, H_2O); ^{1}H NMR (D_2O, Fig. 3): δ 7.40–7.32 (m, 5H, arom.), 5.29 (br s, 1H, H-1 B), 4.90 (d, 1H, $J = 3.4$ Hz, H-1 A), 4.80 (d, 1H, $J = 7.7$ Hz, H-1 D), 4.70, 4.69 (2d, 2H, $J = 12.0$ Hz, CH_2Ph), 4.65 (d, 1H, $J = 7.9$ Hz, H-1 E), 4.49 (d, 1H, $J = 7.1$ Hz, H-1 C), 4.15 (d, 1H, $J = 3.3$ Hz, H-4 C), 4.14 (d, 1H, $J = 11.2$, 1.4 Hz, H-6a A), 4.04 (dd, 1H, $J = 10.0$, 3.2 Hz, H-3 E), 4.04–3.98 (m, 3H, H-2,3,4 B), 3.94 (dd, 1H, $J = 9.7$, 3.3 Hz, H-3 C), 3.91–3.72 (m, 14H), 3.71–3.50 (m, 16H), 2.70 (dd, 1H, $J = 12.3$, 4.6 Hz, H-3e NeuAc), 1.97 (s, 3H, CH_3CO NeuAc), 1.89 (s, 3H, CH_3CO), 1.75 (t, 1H, $J = 12.3$ Hz, H-3a NeuAc); ^{13}C NMR (D_2O): δ 175.7, 174.8 (CH_3*C*ONH); 137.5, 129.3, 129.1, 128.9 (arom.); 108.3 (C-1 B), 104.6 (C-1 E), 103.4 (C-1 D), 102.5 (C-1 C), 96.4 (C-1 A), 83.8 (C-3 C); 82.6, 81.8 (C-2,4 B); 78.5 (C-4 A); 76.4, 76.3 (C-3 B, C-3 E); 76.2, 76.0, 75.5, 75.3, 74.4, 73.6, 73.4, 72.5, 72.2, 71.0, 70.5 (*C*H_2Ph), 70.2, 69.9, 69.8, 69.3 (C-4 C) 68.9, 68.8, 68.4, 68.1, 63.4, 63.2, 61.9, 61.6, 61.5, 54.2 (C-2 A), 52.2 (C-5 NeuAc), 40.6 (C-3 NeuAc); 22.6, 22.3 (*C*H_3CO). ESIMS *m/z* calcd for $C_{50}H_{79}NO_{34}$ $[M+H]^+$: 1251.4514. Found: 1251.4556. Calcd for $C_{50}H_{78}NO_{34}Na$ $[M+Na]^+$: 1273.4334. Found: 1273.4295.

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Supplementary data

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