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Selective sialylation of 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactose catalyzed by *Trypanosoma cruzi trans*-sialidase

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Abstract—*Trypanosoma cruzi*, the agent of Chagas' disease, expresses on its surface a *trans*-sialidase (TcTS) that transfers sialic acid from host glycoconjugates to terminal β-galactopyranosyl units of parasite mucins. This process is involved in infection and pathogenesis. The trisaccharide 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactose **1** is an external unit in the larger oligosaccharides of the mucins and a site for sialylation. The trisaccharide was previously synthesized in our laboratory. The last step of the synthesis was the hydrogenolysis of the crystalline benzyl trisaccharide. Herein we prove that the trisaccharide **1**, its alditol **3** and the benzyl glycoside **2** are good acceptors of sialic acid and effective inhibitors of the sialylation of *N*-acetyllactosamine catalyzed by TcTS. Furthermore, selective sialylation of the 1→3 linked galactopyranose in glycoside **2** was determined by one and two-dimensional NMR analysis. In contrast, the flexible 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol **3** is sialylated in either one of the two possible sites.
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1. Introduction

Trypanosoma cruzi is the agent of Chagas' disease, an American trypanosomiasis that affects about 18 million people in Latin America, and no effective treatment is available to date. Sialic acid in *T. cruzi* surface is involved in parasite–host cell interactions[1,2] and resistance to complement attack.[3]

Trypanosoma differ from other eukaryotes and prokaryotes in the mechanism by which sialic acid is incorporated into the glycoconjugates of the parasite. The transfer of sialic acid is catalyzed by a *trans*-sialidase (TcTS) anchored to the surface by glycosylphosphatidyl inositol[4–7] and does not require a nucleotide (i.e., CMP-sialic acid) as the monosaccharide donor. The transfer occurs from α-2,3-linked sialic acid in the host sialoglycoconjugates to terminal β-galactosyl units of the parasite mucins.[8,9] The *O*-chains are linked by α-GlcNAc and may be derived from two cores, Gal*p*(β1→4)GlcNAc or Gal*f*(β1→4)GlcNAc. The cores are further branched with various units of Gal*f* and/or Gal*p*.[10–13]

Variability in infectivity of different strains of *T. cruzi* may be the result of the presence of different oligosaccharides in the mucins and their ability to incorporate sialic acid from the host in the TcTS catalyzed reaction. It has been shown that this process is essential in infection and pathogenesis caused by *T. cruzi*.[14] Thus, the unique enzyme TcTS, absent in mammals is a good target for the development of chemotherapy against the parasite.

The 3D structure of TcTS was recently determined and the catalytic mechanism elucidated.[15–19] The enzyme has two subsites in the active centre: the sialic acid binding site and the galactose binding site that is absent in bacterial and viral sialidases. Binding of the sialic acid moiety of the donor triggers a conformational switch that creates the β-galactosyl acceptor-binding site.

In vitro, 3′-sialyllactose and lactose are widely used to assay for TcTS activity. In vivo, 3′-sialyl-*N*-acetyllactosamine structures are present in mammalian glycoconjugates. We have recently shown that compounds that interact with the lactose binding site might be good inhibitors of TcTS.[20] The acceptor substrate specificity of lactose derivatives was studied by high pH anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD). The lactose open chain derivatives

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lactitol and lactobionic acid were found to be good acceptors of sialic acid and lactitol effectively inhibited the transfer of sialic acid from sialyllactose to *N*-acetyllactosamine. Furthermore, the alditol inhibited parasite mucins re-sialylation when incubated with live trypanosomes and TcTS and diminished the *T. cruzi* infection in cultured Vero cells.

In connection with our project of correlating the structure of the parasite mucins oligosaccharides with the ability to act as substrates in the *trans*-sialidase reaction, we have undertaken their chemical synthesis, in particular of the Gal*f*-containing oligosaccharides.[21–23] The more complex chains in the mucins (Fig. 1) contain 2,3-di-*O*-(β-D-galactopyranosyl)-D-Gal*p* **1** as an external unit.[13] Trisaccharide **1,** previously synthesized,[24] presents two β-D-Gal*p* for possible sialylation. In the present work the acceptor substrate selectivity in the *trans*-sialidase reaction was studied for compound **1**, its benzyl glycoside **2** and the alditol **3** (Scheme 1). The ability of compounds **1**–**3** to act as competitive inhibitors for sialylation of *N*-acetyllactosamine was also investigated.

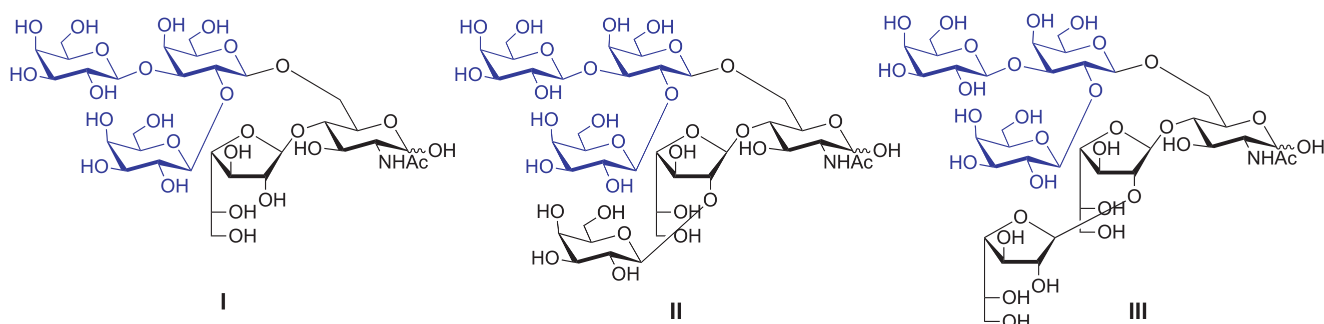
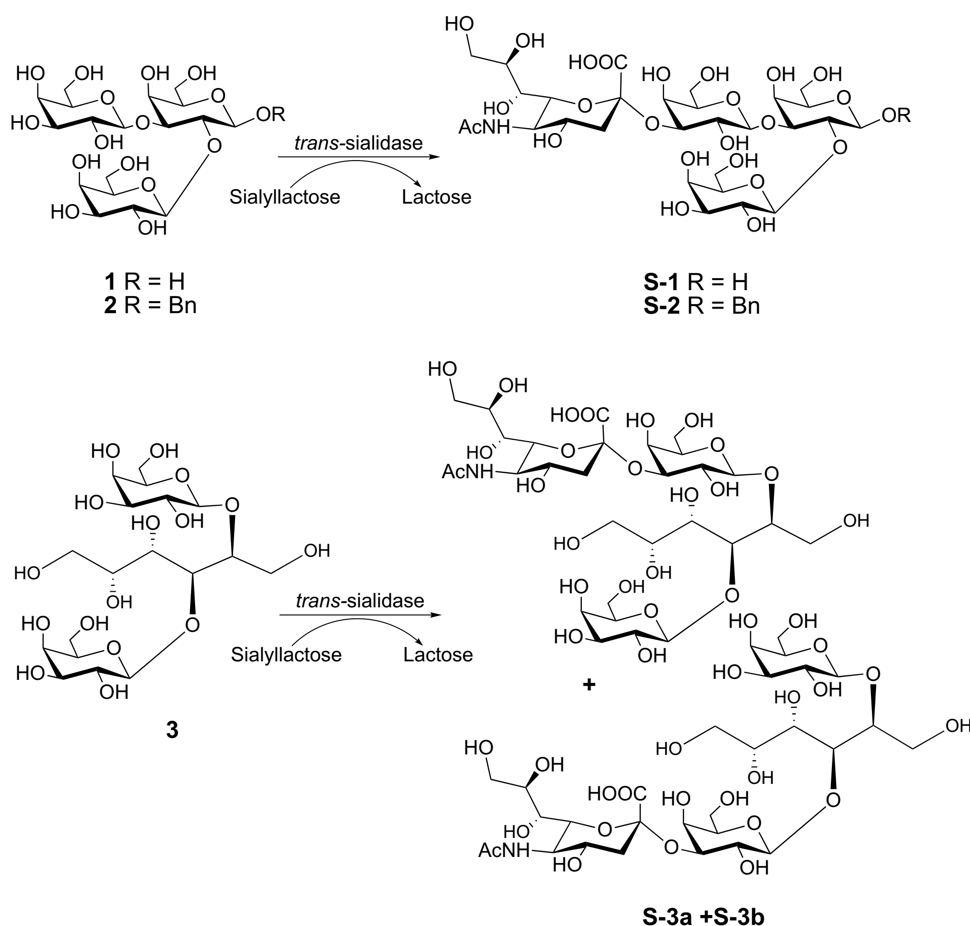


Figure 1. Structure of oligosaccharide alditols obtained from mucins of *Trypanosoma cruzi*.[13] The acceptor substrate studied is shown in blue.



Scheme 1.

2. Results and discussion

2.1. Testing of compounds 1–3 as sialic acid acceptors in the *trans*-sialidase reaction

Compound **1** is the external unit of the larger oligosaccharides of the mucins of *T. cruzi* and a site for sialylation. Trisaccharide **1** was previously synthesized in our laboratory.[24] The last step in the synthesis was the hydrogenolysis of crystalline benzyl trisaccharide **2**. Taking into account that the open chain derivative lactitol was a better acceptor than lactose and inhibited parasite mucins re-sialylation,[20] we now prepared the trisaccharide alditol **3** by reduction of trisaccharide **1** with $NaBH_4$.

Compounds **1**–**3** were analyzed as acceptor substrates for TcTS using the conditions previously described for incubation with the donor 3′-sialyllactose (Fig. 2). In all cases the reaction was fast and reached the equilibrium in about 30 min. Progress of the transference of sialic acid was followed by HPAEC. Sialylated trisaccharide **1** (**S-1**) is eluted later than sialyllactose and as a broad peak, since both anomers at the reducing end would be sialylated. As expected, the sialylated benzyl glycoside **2** (**S-2**) and the sialylated trisaccharide alditol **3** (**S-3**) were less retained in the column than sialyllactose. Only in the case of the alditol (Fig. 2C) a double peak in the region of the monosialylated compounds was detected, suggesting that both terminal galactoses could be sialylated. A very minor component with a larger retention time was also observed (not shown) but the area of this peak did not increase when excess of sialyllactose or longer incubation times were used, precluding the possibility that disialylation could be significative.

The three compounds showed to be good acceptors of sialic acid with similar values of transference. The benzyl trisaccharide **2** was the best acceptor with 75% of the sialic acid transferred from sialyllactose (Fig. 2B). The free trisaccharide showed 60% transference (Fig. 2A) and the alditol 61% (Fig. 2C). A minor amount of free sialic acid was detected indicating that TcTS has a poor neuraminidase activity.

2.2. Inhibition of sialylation of *N*-acetyllactosamine by compounds 1–3

It was interesting to find out if these acceptors of sialic acid were capable of inhibiting sialylation of *N*-acetyllactosamine. Different concentrations (0.5, 1.0 and 1.5 mM) of each trisaccharide derivative were tested in transfer reactions containing 3′-sialyllactose as donor, 1 mM of *N*-acetyllactosamine and TcTS. In all the reactions, the percentages of sialic acid transferred to *N*-acetyllactosamine decreased significantly while the ratio between sialyl *N*-acetyllactosamine and sialyllactose was slightly affected (Table 1, Fig. 3). These results indicated that in the presence of sialyllactose, most of the sialic acid is transferred to the trisaccharide diminishing the absolute amount of sialyl *N*-acetyllactosamine formed.

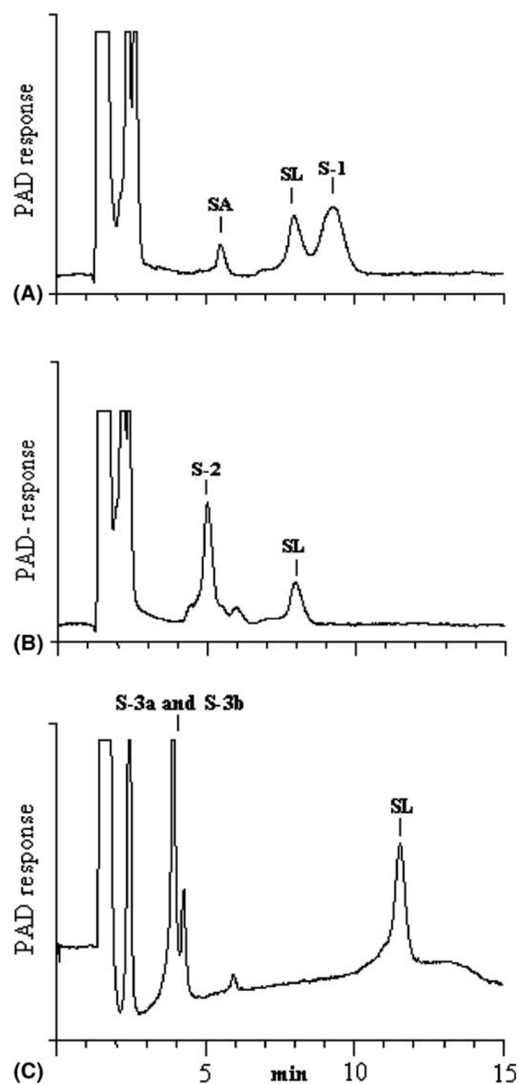


Figure 2. Analysis of compounds **1**–**3** as substrates for the *trans*-sialidase from *Trypanosoma cruzi*. Compounds **1**–**3** at 1 mM concentrations were incubated with 1 mM 3′-sialyllactose and *trans*-sialidase (40 ng) for 15 min and the reaction mixtures were analyzed by HPAEC-PAD in a PA-100 column as described under Experimental. (A) Substrate **1** analyzed under condition 1. (B) Substrate **2**; analyzed under condition 1. (C), Substrate **3**, analyzed under condition 2. **SA**, sialic acid; **S-1**, sialyl β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranose; **S-2**, benzyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranoside; **S-3a** and **S-3b**, 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-D-galactitol and 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-D-galactitol.

2.3. Selective sialylation of benzyl 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside

Acosta Serrano et al.[25] showed that oligosaccharitol **I** (Fig. 1) obtained by reductive β-elimination from mucins of *T. cruzi*, accepted only one sialic acid when incubated with *trans*-sialidase and 3′-sialyllactose as donor. With the natural product it could not be determined if only one of the Gal*p* units was selectively sialylated or if sialylation of either one of the residues precluded sialylation of the other. In order to solve this question,

Table 1. Inhibition of sialic acid transfer to *N*-acetyllactosamine by compounds **1**–**3**[a]

Substrate	Concentration (mM)	S-compound[b] (%)	SLN (%)	Inhibition (%)	SL (%)	SLN/SL
Compound **1**	0	0	53		47	1.1
	0.5	36	32	40	32	1.0
	1.0	52	23	57	25	0.9
	1.5	70	11	79	19	0.6
Compound **2**	0	0	62		38	1.6
	0.5	17	50	19	33	1.5
	1.0	32	37	40	31	1.2
	1.5	46	25	60	29	0.9
Compound **3**	0	0	51		49	1.0
	0.5	33	34	33	33	1.0
	1.0	48	27	47	25	1.1
	1.5	79	9	82	12	0.8

[a] Reaction conditions: 1 mM 3′-sialyllactose (SL), 1 mM *N*-acetyllactosamine (LN), *trans*-sialidase and different concentrations of inhibitors.
[b] Sialylated substrate. Percentages were calculated over the total amount of sialic acid present (1 mM).

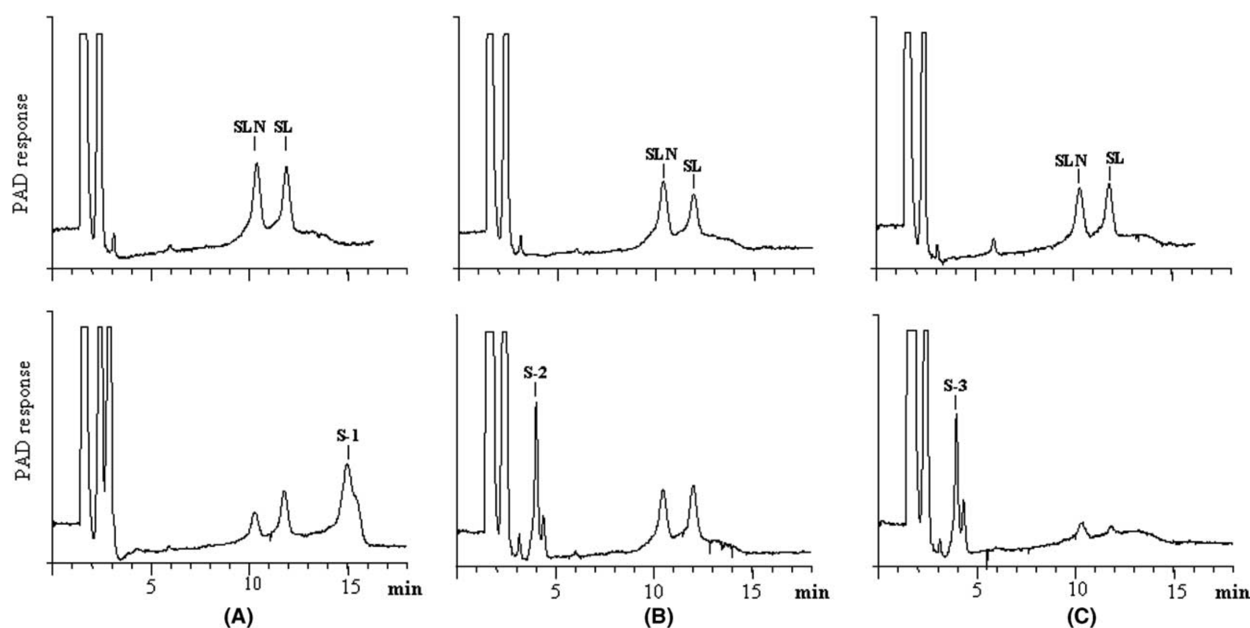


Figure 3. Inhibition of sialylation of *N*-acetyllactosamine by trisaccharides **1**–**3**. Reaction mixtures in 20 mM Tris pH 7 buffer containing 1 mM 3′-sialyllactose (SL), 1 mM *N*-acetyllactosamine (LN) and 40 ng *trans*-sialidase were incubated with 1 mM of compounds **1**–**3** (panels A–C) for 30 min at 25 °C and analyzed by HPAEC-PAD. A CarboPac PA-100 column was used under condition 2. For all panels the upper chromatogram represents the *N*-acetyllactosamine sialylation (SLN) in the absence of inhibitor. For abbreviations see Figure 2.

we used as substrate of the TcTS reaction a glycoside of the terminal trisaccharide of **I** (Fig. 1). The selectivity of the transfer reaction with respect to the two terminal β-D-Gal*p* units was studied by NMR spectroscopy of the purified sialylated oligosaccharide. We selected the benzyl glycoside **2** for this study because interpretation of the results was facilitated by the absence of anomeric mixtures, otherwise obtained by sialylation of the free trisaccharide **1**. On the other hand, the hydrophobic aglycon helped for purification of the product by anionic chromatography from the remaining sialyllactose used as donor. The reaction was checked by HPAEC and after 3 h it was stopped by heating at 65 °C for 2 min. Under these conditions, 68% sialylation of compound **2** was obtained, no evidence of the presence of the other monosialylated derivative or of the disialylated compound was found, even though the reaction was analyzed by HPAEC at different times of incubation. The reaction product was purified on a 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 sialylated derivative of the benzyl trisaccharide **1** was more retained by de anion exchange resin than the sialyllactose remaining in the reaction and could be successfully purified by this method.

2.4. Characterization of benzyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranoside S-2

The expected molecular mass of the *trans*-sialidation product was confirmed by Q-TOF positive ion spray mass spectrometry. Peaks at 886.32, 908.30 and 924.27

were assigned to $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively (Fig. 4, **S-2**). The corresponding fragments at 595.22 and 617.21 due to the loss of sialic acid from $[M+H]^+$ and $[M+Na]^+$ were also detected. The fragment due to the loss of galactose from $[M+H]^+$ was observed at *m/z* 724.27. The spectrum also showed the peak corresponding to the loss of OBn at 778.27 and fragments due to glycosidic cleavage. An abundant ion (70%) at *m/z* 454.16 corresponded to the non-reducing terminal fragment sialic acid–Gal (B-type carbenium ion).[26] Once established the monosialylation of compound **2**, the next step was the assignment of the linkage position, which was achieved by mono- and bidimensional NMR spectroscopy.

Full ^{13}C and ^{1}H NMR assignments of **2** were performed based on COSY, HETCOR and NOESY experiments (Tables 2 and 3).[27–30] The resonances of the H-2 and H-3 appeared deshielded at 3.89 and 3.92 ppm, respectively, because of the glycosidic linkages. An interresidue NOE between Gal*p* H-1′ and Gal*p* H-2 was observed in the NOESY spectrum that allowed the straightforward assignment of the Gal*p* (1→2) linked unit. Therefore, the assignments for the benzylated Gal*p* and for the other terminal Gal*p* residue could also be deduced. Confirmation due to other NOE correlations was not possible because of the superposition of Gal*p* H-1 and Gal*p* H-1″ signals (δ = 4.59 ppm). On the other hand, the linkage assignment is in accordance with the assumption that the most crowded C-1′ of the β-D-Gal*p*-(1→2) unit would appear more shielded (103.7 ppm) than the C-1″ of the β-D-Gal*p*-(1 → 3) unit (104.3 ppm). The resonances of C-2 and C-3 appeared at low field, 77.5 and 82.9 ppm, respectively.

Compound **2** presents two non-reducing terminal Gal*p* that could be sites for sialylation. However, treatment of benzyl glycoside **2** with TcTS and sialyllactose as sialic acid donor, gave only one sialylated product, **S-2**.

The presence of a sialyl residue in **S-2** was evident by the reporter signals at 1.77 ppm (H-3a) and 2.71 ppm (H-3e) in the ^{1}H NMR spectrum[28,29] (Fig. 5). Integration of these signals also showed that only one sialic acid unit has been introduced.

The anomeric protons of **S-2** appeared at 4.60 ppm ($J_{1,2}$ = 7.6 Hz), 4.74 ppm ($J_{1,2}$ = 8.0 Hz) and 4.66 ppm ($J_{1,2}$ = 7.8 Hz), the latter was partially overlapped with the HDO broad signal. In a spectrum performed at 45 °C, overlapping with HDO was avoided and the three anomeric signals appeared at 4.74 ppm ($J_{1,2}$ = 9.0 Hz),

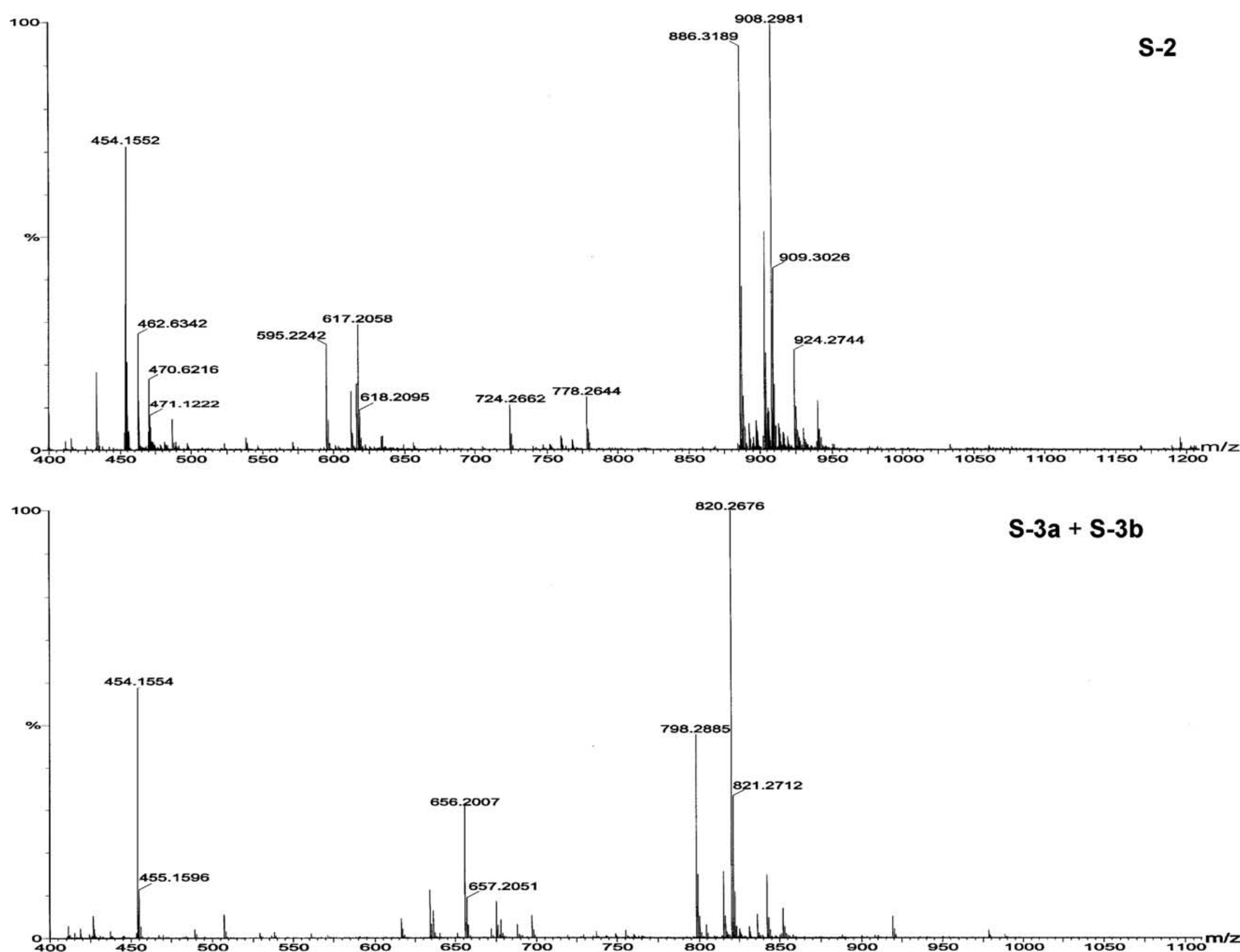


Figure 4. Positive ion mode ESI Q-TOF Tandem Quadrupole/Time-of-Flight for compound **S-2**, and the mixture **S-3a** + **S-3b**.

Table 2. ^{1}H NMR chemical shifts for compounds **2** and **S-2** (500 MHz, D_2O)

Oligosaccharide	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 ($J_{6a,6b}$)	H-6b ($J_{5,6b}$)	H-7	H-8	H-9	CO*C*H$_3$	Ph*C*H$_2$
2												
β-D-Gal*p*-*O*-Bn	4.59	3.89	3.92	4.14	3.66	3.74	3.69					4.93, 4.72
	(7.6)	(9.8)	(3.0)	(1.1)	(7.7)	(11.5)	(4.5)					(11.5)
β-D-Gal*p*-(1→2)	4.71	3.46	3.55	3.84	3.50	3.60	3.56					
	(7.8)	(10.2)	(3.6)	(1.1)	(5.4)	(11.5)	(6.8)					
β-D-Gal*p*-(1→3)	4.59	3.55	3.59	3.86	3.62	3.71	3.67					
	(7.6)	(10.0)	(3.2)	(1.1)	(7.8)	(11.7)	(4.4)					
S-2												
β-D-Gal*p*-*O*-Bn	4.60	3.89	3.93	4.15	3.66[a]	3.75–3.64[b]						4.94, 4.73
	(7.6)	(9.7)	(3.2)	(0.9)	n.d.	n.d.						(11.6)
β-D-Gal*p*-(1→2)	4.74	3.45	3.56	3.84	3.52[a]	3.75–3.64[b]						
	(8.0)	(10.1)	(3.4)	n.d.	n.d	n.d.						
β-D-Gal*p*-(1→3)	4.66	3.60	4.05	3.90	3.62[a]	3.62–3.52[b]						
	(7.8)	(9.8)	(3.2)	n.d.	n.d.	n.d.						
Neu5Acα (2→3)			1.77 ax	3.64	3.79	3.52		3.53	3.84	3.84 a	1.98	
			(12.4)							n.d.		
			2.71 ec	n.d.	n.d.	n.d.		n.d.	n.d.	3.56 b		
			(12.0, 4.6)							n.d.		

[a,b]Signals could be interchanged.

Table 3. ^{13}C NMR chemical shifts for compounds **2** and **S-2** (500 MHz, D_2O)

Oligosaccharide	*C*-1	*C*-2	*C*-3	*C*-4	*C*-5	*C*-6	*C*-7	*C*-8	*C*-9	C*OC*H$_3$	CO*C*H$_3$	*Ph*CH$_2$
2												
β-D-Gal*p*-*O*-Bn	101.3	77.5	82.9	69.3	75.2	61.4						71.8
β-D-Gal*p*-(1→2)	103.7	72.2	73.3	69.1	75.6	61.2						
β-D-Gal*p*-(1→3)	104.3	71.7	73.4	69.2	75.7	61.5						
S-2												
β-D-Gal*p*-*O*-Bn	101.3	77.5	83.0	69.3	75.5[d]	61.4[a]						71.9
β-D-Gal*p*-(1→2)	103.7	72.3[e]	73.2[c]	69.1[b]	75.3[d]	61.3[a]						
β-D-Gal*p*-(1→3)	104.5	70.1	76.3	68.1[b]	75.2[d]	61.1[a]						
Neu5Acα (2→3)	173.8	100.5	40.1	68.8	52.2	73.4[c]	68.8	72.4[e]	63.3	175.6	22.6	70.9

[a–e]Signals could be interchanged.

4.64 ppm ($J_{1,2}$ = 7.9 Hz) and 4.58 ppm ($J_{1,2}$ = 6.6 Hz). Sialylation of compound **2** caused a downfield shift of the characteristic double doublet assigned to H-3 (δ 4.05 ppm, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 3.2 Hz) of one of the Gal*p* units. The COSY and TOCSY (Fig. 6) experiments allowed the assignment of the signals of the sialylated Gal*p*. In fact, the H-3″ (δ 4.05 ppm) correlated to the H-1″ (δ = 4.66 ppm) that was partially overlapped by HDO. Both H-3″ and H-1″ resonances were shifted downfield by 0.46 and 0.07 ppm each one in compound **S-2** when compared to those in compound **2**. The corresponding H-2″ appeared also shifted downfield at 3.60 ppm. The resonances for the protons of the terminal non-sialylated Gal*p* unit were also assigned.

To identify further, which Gal*p* has been glycosylated, a NOESY experiment was performed. In this case, an interresidue NOE between Gal*p* H-1″ and H-3 (δ = 3.93 ppm) could not be established because H-1″ was overlapped by HDO. However, the interresidue NOE between non-sialylated Gal*p* H-1′ (δ = 4.74 ppm) and Gal*p* H-2 (δ = 3.89 ppm) was detected, allowing the glycosidic linkage assignment of the Galp-(1→2)-β-D-Gal*p*-OBn. The observation that the chemical shift of H-3′ in the Gal*p*-(1→2) linked unit was not affected confirmed that sialylation occurred on the H-3″ of the β-D-Gal*p*-(1→3) linked unit.

In addition, the TOCSY experiment showed the characteristic three intrarresidue correlations between H-1/H-2, H-1/H-3 and H-1/H-4 for each Gal*p* unit (Fig. 6). The HMQC allowed the assignment of the anomeric carbons and glycosidation sites.

2.5. Preparation of 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol 3

Reduction of trisaccharide **1** with sodium borohydride afforded 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol **3**. The NMR analysis of trisaccharide alditol **3** allowed partial assignment of the signals. In the ^{1}H NMR spectrum, resonances for the hydrogens of both Gal*p* units were superimposed, in particular, those for H-3′ and H-3″, precluding assignment of each Gal*p* spin system. However, the alditol moiety of **3** was unequivocally assigned. The large value for $J_{3,4}$ (9.3 ppm) indicated a *trans* periplanar relationship between H-3 and H-4, and the small magnitude for $J_{2,3}$ and $J_{4,5}$ (1.5 and 1.4 Hz, respectively), indicated a *gauche* disposition between H-2 and H-3, and H-4 and H-5, respectively.

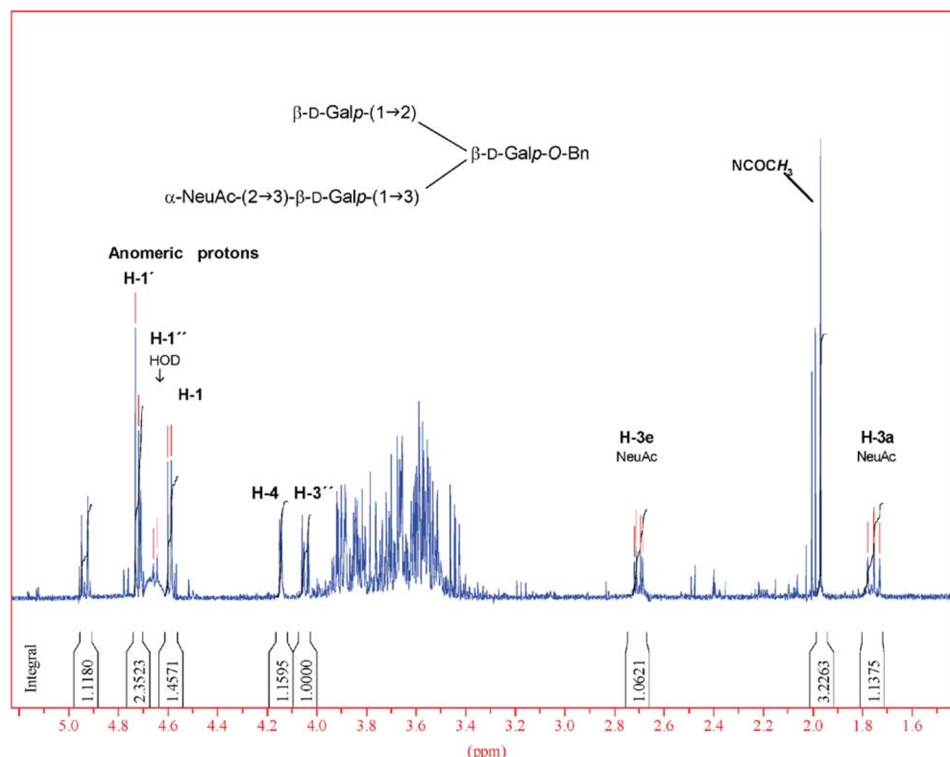


Figure 5. ^{1}H NMR spectrum (500 MHz, D_2O) of benzyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranoside **S-2**. Key resonances are labelled.

These values are in agreement with an extended planar zigzag conformation of the alditol backbone in solution.[31–33] Also in the ^{13}C NMR spectrum, only the alditol skeleton could be assigned (Experimental).

2.6. Sialylation of 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol

Sialylation of the trisaccharide alditol **3** gave two peaks in the region of the monosialylated oligosaccharides when analyzed by HPAEC (Fig. 2C). A preparative experiment of *trans*-sialylation of compound **3** was performed with sialyllactose as donor and TcTS. After 2 h of incubation at 25 °C the mixture was analyzed by HPAEC showing 94% of transference and an almost complete disappearance of sialyllactose. The two monosialylated alditols, **S-3a** and **S-3b**, could be separated from sialyllactose on the AG1X2 column, but not from each other. Integration of the peaks in the HPAEC gave a 1:0.8 ratio between the two monosialylated derivatives.

The molecular mass for **S-3a** and **S-3b** was confirmed by Q-TOF positive ion spray mass spectrometry (Fig. 4). Pseudomolecular ions at 798.29 and 820.27 corresponded to $[M+H]^+$ and $[M+Na]^+$. As in the case of **S-2**, also the peak at 454.16 corresponding to the non-reducing terminal fragment sialyl-Gal (B-type ion) was strong.

Analysis of the mixture by NMR spectroscopy confirmed that each one of the terminal Gal*p* was sialylated. The ^{1}H NMR spectrum (Fig. 7) showed in the anomeric region, two pairs of doublets (δ 4.53 ppm, $J = 8$ Hz; δ 4.43 ppm, $J = 7.8$ Hz and δ 4.50 ppm, $J = 7.8$ Hz, δ 4.45 ppm, $J = 7.8$ Hz) in the ratio 1:0.8. On the other hand, the diagnostic sialyl signals[28,29] of the more shielded H-3a appeared as two triplets centred at 1.76 and 1.75 ppm, respectively, with similar coupling constants ($J = 12.2$ Hz). The integration of these signals corroborated the proportion of sialylated products. Moreover, the two deshielded H-3e appeared superimposed (2.70 ppm, dd, $J = 12.6$, 4.6 Hz) and the integration value was as expected. The H-3′ and H-3″ signals of the sialylated Gal*p* units were downfield shifted to 4.06 and 4.07 ppm, in the ^{1}H NMR spectrum.

3. Conclusions

The 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside external unit in the mucins of *T. cruzi* is selectively monosialylated by a recombinant *trans*-sialidase. Analysis by NMR spectroscopy unequivocally proved that the less hindered 1→3 linked galactopyranose was sialylated. In the case of the disaccharide alditol **3** the open zig-zag conformation adopted by the alditol turned both Gal*p* units accessible for TcTs recognition. Compounds **1-3** inhibited the transfer of sialic acid from 3′-sialyllactose to *N*-acetyllactosamine.

4. Experimental

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Optical rotations were

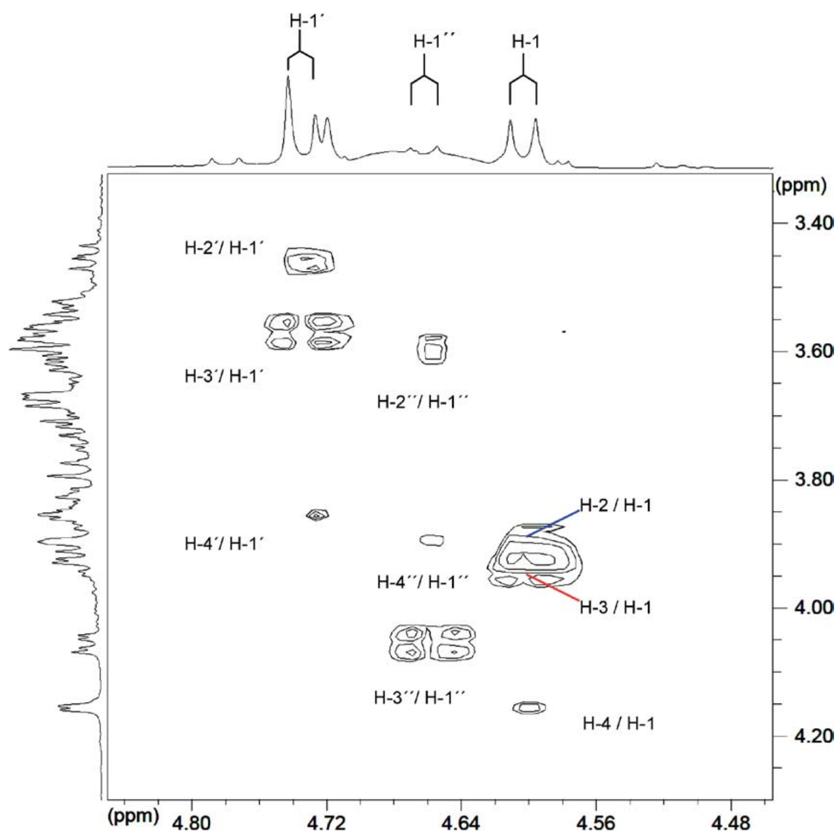


Figure 6. Partial 400 MHz TOCSY spectrum of benzyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranoside **S-2**. The correlations for the three β-D-Gal*p* units are assigned.

measured with a Perkin–Elmer 343 polarimeter, with a path length of 1 dm, concentrations are given in g/100 mL. TLC was performed on 0.2 mm silica gel 60 F254 (Merck) aluminium supported plates. 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 in D_2O 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 2.16s and 30.8 ppm for ^{1}H NMR and ^{13}C NMR spectra, respectively. ^{1}H and ^{13}C assignments were supported by DEPT 135, homonuclear COSY and HETCOR experiments. HMQC and 2D-TOCSY (mixing time of 80 ms) experiments were recorded in a Bruker Avance DPX-400. High resolution mass spectra (HRMS) were recorded on a Micromass Q-TOF Ultima Tandem Quadrupole/Time-of-Flight Instrument equipped with an electrospray ionization (ESI) source.

Lactose was from Calbiochem. Lactitol, *N*-acetyllactosamine and 3′-sialyllactose were purchased from Sigma. For preparative sialylations, 3′-sialyllactose was obtained from bovine colostrum by an adaptation of a reported method.[34] Benzyl 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside and 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactose were synthesized as previously described.[24]

Analysis by HPAEC-PAD was performed using a Dionex DX-300 HPLC system equipped with a pulse amperometric detector (PAD). The following columns and conditions were used:

Condition 1: A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was used with the following program: 50 mM NaAcO in 100 mM NaOH for 10 min followed by a linear gradient in 80 min from 50 to 500 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 mL/min at room temperature.

Condition 2: A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was used with the following program: a linear gradient over 80 min from 100 to 500 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 mL/min at room temperature.

4.1. Sialylation of compounds 1–3 with *trans*-sialidase of *T. cruzi*

A recombinant TcTS expressed in *E. coli* was kindly provided by A. C. C. Frasch (UNSAM, General San Martín, Buenos Aires, Argentina). Reaction mixtures of 20 μL containing 20 mM Tris pH 7 buffer, 30 mM NaCl, 1 mM 3′-sialyllactose as donor and 1 mM acceptor substrate were incubated with 40 ng of purified *trans*-sialidase for 15 min at 25 °C. After incubation, reaction mixtures were diluted with 100 μL of deionized water and frozen. Sialylation of acceptors was calculated as the percentage of the sialylated product obtained over

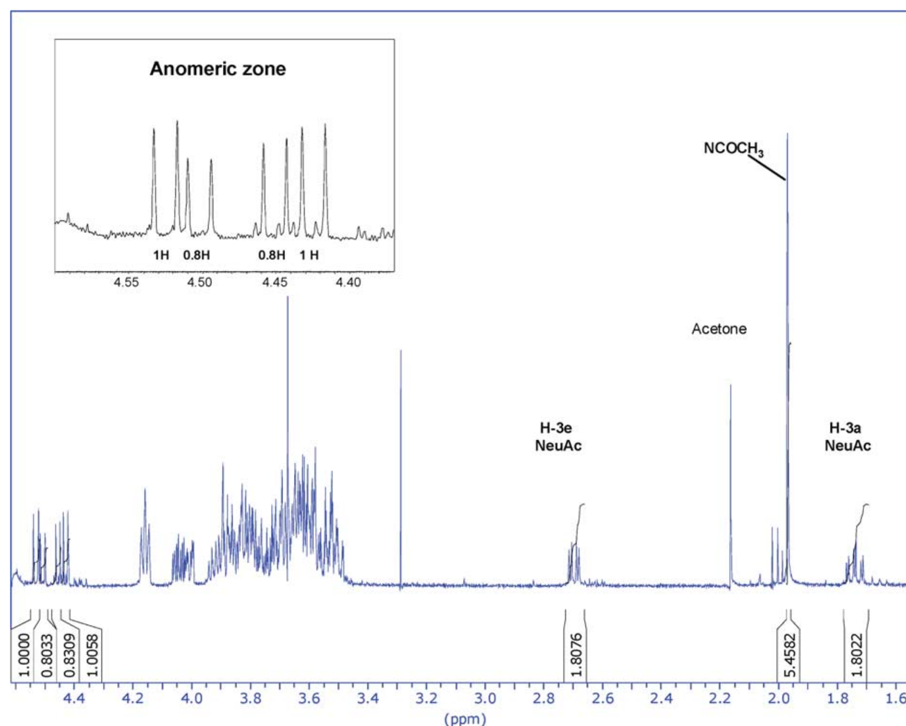


Figure 7. ^{1}H NMR spectrum (500 MHz, D_2O) of the mixture of **S-3a** and **S-3b**. Anomeric zone is shown enhanced.

the total amount of sialic acid (free or linked to a saccharide).

4.2. Inhibition of sialylation of *N*-acetyllactosamine by compounds 1–3

Reaction mixtures of 20 μL containing 20 mM Tris–HCl pH 7 buffer, 30 mM NaCl, 1 mM 3′-sialyllactose, 1 mM *N*-acetyllactosamine and different concentrations of inhibitors were incubated with purified TcTS for 30 min at room temperature. Samples were then diluted 36 times with deionized water and analyzed by HPAEC. Inhibition was calculated from the percentages of 3′-sialyl-*N*-acetyllactosamine obtained with or without inhibitor.

4.3. Synthesis of 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol 3

To a cooled solution (0 °C) of 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactose (25 mg, 0.05 mmol) in 3:7 methanol–water (3.5 mL), sodium borohydride (60.0 mg, 1.62 mmol) was added, and the mixture was stirred at room temperature for 4 h. Excess of reductant was destroyed by the addition of a solution of 50% HAcO, decationized by passing it through a column of Amberlite IR-120 plus, and concentrated to dryness. The residue was co-evaporated with water and methanol. Crystallization from 2-propanol gave 22 mg (87%) of alditol **3**: mp 158–160 °C, R_f 0.27 (7:1:2 *n*-propanol–ethanol–water), $[\alpha]_D = -21.6$ (c 0.5, H_2O); ^{1}H NMR (D_2O, 500 MHz): δ 4.43 (d, 1H, $J = 7.9$ Hz, H-1′ or H-1″); 4.40 (d, 1H, $J = 7.7$ Hz, H-1″ or H-1′); 4.14 (m, 2H, H-2, H-5), 3.97 (dd, 1H, $J = 9.3$, 1.5 Hz, H-3); 3.89 (dd, 1H, $J = 11.5$, 7.1 Hz, H-1a); 3.84 (dd, 1H, $J = 9.3$, 1.4 Hz, H-4); 3.83 (dd, 1H, $J = 3.4$, 0.8 Hz, H-4′ or 4″); 3.80 (dd, 1H, $J = 3.4$, 1.0 Hz, H-4′ or H-4″); 3.76 (dd, 1H, $J = 11.5$, 6.0 Hz, H-1b); 3.71 (dd, 1H, $J = 11.7$, 8.6 Hz, H-6a′ or H-6a″); 3.70 (dd, 1H, $J = 11.7$, 8.1 Hz, H-6a′ or H-6a″); 3.68 (dd, 1H, $J = 11.7$, 3.8 Hz, H-6b′ or H-6b″); 3.66 (dd, 1H, $J = 11.7$, 4.2 Hz, H-6b′ or H-6b″); 3.63 (m, 2H, H-5′ and H-5″); 3.59 (m, 2H, H-6a and H-6b); 3.57 (dd, 1H, $J = 9.9$, 3.4 Hz, H-3′ or H-3″); 3.56 (dd, 1H, $J = 9.9$, 3.4 Hz, H-3′ or H-3″); 3.47 (dd, 1H, $J = 9.9$, 7.7 Hz, H-2′ or H-2″); 3.46 (dd, 1H, $J = 9.9$, 7.9 Hz, H-2′ or H-2″); ^{13}C NMR (D_2O, 125.8 MHz): δ 104.5, 104.4 (C-1′, C-1″); 80.9 (C-2); 77.8 (C-3); 75.8, 75.6 (C-5′, C-5″); 73.3, 73.1 (C-3′, C-3″); 71.7, 71.6 (C-2′, C-2″); 69.9 (C-5); 69.2, 69.1 (C-4, C-4′, C-4″); 63.5 (C-6); 61.7 (C-1); 61.6, 61.5 (C-6′, C-6″). HRMS (ESI+) *m/z* calculated for $C_{18}H_{35}O_{16}$ ($M+H^+$) requires 507.1925; found: 507.1913. Calculated for $C_{18}H_{34}O_{16}Na$ ($M+Na^+$) requires 529.1745; found: 529.1732.

4.4. Synthesis of benzyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-galactopyranoside S-2

Benzyl 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside (3.5 mg) and 3′-sialyllactose (3.5 mg) were incubated with 20 μg of TcTS in 500 μL of 200 mM Tris buffer pH 7.6 containing 30 mM NaCl for 3 h at 25 °C. The synthesis was performed in triplicate and each reaction analyzed by HPAEC. The three incubations mixtures were combined and sialylated benzyl 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside **S-2** was purified by passing through an anion exchange resin

(AG1X2, acetate form, BioRad, 1.2 × 15 cm). Neutral compounds, namely benzyl 2,3-di-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside and lactose were eluted with water and sialylated compounds with 200 mM pyridinium acetate buffer pH 5. Fractions (2 mL) were collected and analyzed by HPAEC. Remaining sialyllactose eluted with the first 35 mL of 200 mM pyridinium acetate buffer, fractions eluted after 60 mL of buffer was passed, contained sialylated compound **2 S-2**. After neutralization with pyridine, the pooled fractions were concentrated under vacuum at room temperature. Compound **S-2** was further purified by passing through a SepPack C8 cartridge (Alltech) eluting with water to obtain 7 mg of a colourless syrup: $[\alpha]_D = -1.4$ (*c* 0.3, H_2O); HRMS (ESI+) *m/z* calculated for $C_{36}H_{56}NO_{24}$ $(M+H^+)$ requires 886.3192; found: 886.3189. Calculated for $C_{36}H_{55}NO_{24}Na$ $(M+Na^+)$ requires 908.3012; found: 908.2981.

4.5. Synthesis of 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-D-galactitol and 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→3)]-D-galactitol S-3a and S-3b

2,3-Di-*O*-(β-D-galactopyranosyl)-D-galactitol (10 mg) and 3′-sialyllactose (10 mg) were incubated with 10 μg of TcTS in 1 mL of 200 mM Tris buffer pH 7.6 containing 30 mM NaCl for 3 h at 25 °C. The reaction was analyzed by HPAEC. Sialylated 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitols (**S-3a** and **S-3b**) were purified by passing through an anion exchange resin (AG1X2, acetate form, BioRad, 1.2 × 15 cm) and eluted as above. Compounds **S-3a** and **S-3b** were less retained in the anionic resin than sialyllactose and eluted after 10 mL of 200 mM pyridinium acetate buffer was passed. Fractions containing sialylated 2,3-di-*O*-(β-D-galactopyranosyl)-D-galactitol were pooled and evaporated as above. The sialylated alditols were further purified through a SepPack C8 cartridge eluting with water to obtain 5 mg of a white amorphous solid: HRMS (ESI+) *m/z* calculated for $C_{29}H_{52}NO_{24}$ $(M+H^+)$ requires 798.2879; found: 798.2885. Calculated for $C_{29}H_{51}NO_{24}Na$ $(M+Na^+)$ requires 820.2699; found: 820.2676. ^1H NMR (D_2O, 500 MHz): δ 4.53 (d, 1H, $J = 8.0$ Hz, H-1′ or H-1″ **S-3a**); 4.50 (d, 0.8H, $J = 7.8$ Hz, H-1′ or H-1″ **S-3b**); 4.45 (d, 0.8H, $J = 7.8$ Hz, H-1′ or H-1″ **S-3b**); 4.43 (d, 1H, $J = 7.8$ Hz, H-1′ or H-1″ **S-3a**); 4.17 (m, 3.6H, H-2 and H-5 **S-3a** and **S-3b**); 4.07 (dd, 1H, $J = 9.7$, 3.3 Hz, H-3″ **S-3a**); 4.06 (dd, 0.8H, $J = 9.9$, 3.5 Hz, H-3″ **S-3b**); 4.02 (dd, 0.8H, $J = 9.3$, 1.7 Hz, H-3 **S-3b**); 4.01 (dd, 1H, $J = 9.3$, 1.7 Hz, H-3 **S-3a**); 3.93 (dd, 0.8H, $J = 11.6$, 7.0 Hz, H-1a **S-3b**); 3.92 (dd, 1H, $J = 11.6$, 6.8 Hz, H-1a **S-3a**); 3.90–3.57 (m, 34H); 3.56–3.49 (m, 3.6H, H-2′ and H-2″ **S-3a** and **S-3b**); 2.70 (dd, 1.8H, $J = 12.6$, 4.6 Hz, H-3e **S-3a** and **S-3b**); 1.97 (s, 5.4H, CH_3CON); 1.76 (t, 0.8H, $J = 12.2$ Hz, H-3a **S-3b**); 1.75 (t, 1H, $J = 12.2$ Hz, H-3a **S-3a**). ^{13}C NMR (D_2O, 125.8 MHz): δ 175.6 ($NCOCH_3$); 174.4 (C-1 Neu5Ac), 104.5, 104.4, 104.3, 104.2 (C-1′, C-1″); 100.4, 100.3 (C-2 Neu5Ac); 80.9, 80.6 (C-2); 77.8, 77.7 (C-3); 76.2, 76.1 (C-3″); 75.8, 75.6, 75.5; 73.5, 73.4, 73.2, 73.1, 72.4, 72.3, 71.7, 71.6, 70.2, 70.1, 70.0, 69.2, 69.1, 68.9, 68.8, 68.7, 68.0, 67.9, 63.6, 63.5, 63.2 (×2), 61.7 (×2), 61.6 (×3), 61.6, 61.5, 61.4, 59.9, 52.3 (C-5 Neu5Ac); 40.3 (C-3 Neu5Ac); 22.6 ($NCOCH_3$).

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