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Synthesis of a model trisaccharide for studying the interplay between the anti α-Gal antibody and the trans-sialidase reactions in *Trypanosoma cruzi*

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ABSTRACT

Trypanosoma cruzi, the etiologic agent of Chagas disease, is covered by a dense glycocalix mainly composed by glycoproteins called mucins which are also the acceptors of sialic acid in a reaction catalyzed by a trans-sialidase (TcTS). Sialylation of trypomastigote mucins protects the parasite from lysis by the anti α-Gal*p* antibodies from serum. The TcTS is essential for the infection process since *T. cruzi* is unable to biosynthesize sialic acid. The enzyme specifically transfers it from a terminal β-D-Gal*p* unit in the host glycoconjugate to terminal β-D-Gal*p* units in the parasite mucins to construct the D-NeuNAc(α2→3)β-D-Gal*p* motif. On the other hand, although galactose is the most abundant sugar in mucins of both, the infective trypomastigotes and the insect stage epimastigotes, α-D-Gal*p* is only present in the infective stage whereas β-D-Gal*f* is characteristic of the epimastigote stage of the less virulent strains. Neither α-D-Gal*p* nor D-Gal*f* is acceptor of sialic acid. In the mucins, some of the oligosaccharides are branched with terminal β-D-Gal*p* units to be able to accept sialic acid in the TcTS reaction.

Based on previous reports showing that anti α-Gal*p* antibodies only partially colocalize with sialic acid, we have undertaken the synthesis of the trisaccharide α-D-Gal*p*(1→3)-[β-D-Gal*p*(1→6)]-D-Gal*p*, the smallest structure containing both, the antigenic D-Gal*p*(α1→3)-D-Gal*p* unit and the sialic acid-acceptor β-D-Gal*p* unit. The trisaccharide was obtained as the 6-aminohexyl glycoside to facilitate further conjugation for biochemical studies. The synthetic approach involved the α-galactosylation at O-4 of a suitable precursor of the reducing end, followed by β-galactosylation at O-6 of the same precursor and introduction of the 6-aminohexyl aglycone. The fully deprotected trisaccharide was successfully sialylated by TcTS using either 3′-sialyllactose or fetuin as donors. The product, 6-aminohexyl α-D-NeuNAc(2→3)-β-D-Gal*p*(1→6)-[α-D-Gal*p*(1→3)]-β-D-Gal*p*, was purified and characterized.

1. Introduction

Sialic acid and mucins are crucial constituents of the glycocalix that covers the surface of *Trypanosoma cruzi* [1–5]. Although the parasite lacks the necessary enzymes to biosynthesize the donor nucleotide CMPNeu5Ac [6], it manages to take sialic acid from the host by means of an efficient surface trans-sialidase (TcTS) [7,8]. The reaction is specific and involves the transfer of sialic acid (α2-3) linked to a β-D-Gal*p* unit in the donor to construct the same linkage in the acceptor [9,10]. The mucins, which are the most abundant surface glycoproteins in *T. cruzi* are the acceptors of sialic acid. It was shown that, in vitro, the enzyme is capable of transferring both, the common NeuNAc, and the less abundant glycolylneuraminic acid [11]. The oligosaccharides in *T. cruzi* mucins have distinctive features in comparison to vertebrate mucins [3]. In the first place, the linkage to the protein is through α-GlcNAc instead of α-GalNAc, which was not found in *T. cruzi* glycoconjugates. Secondly, galactose, the main monosaccharide in both, epimastigote and trypomastigote stages, is present in different configurations. Mucins from the insect epimastigote stage contain β-D-Gal*f* and β-D-Gal*p*. However, interstrain variations were observed and, depending on the lineage, only β-D-Gal*p* is incorporated. The oligosaccharides

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containing D-Gal*f* were characterized [12–14] and chemically synthesized [3,15,16]. The antigenic α-D-Gal*p*, on the other hand, is exposed only in mucins of trypomastigotes [17]. During the infection the host reacts by producing lytic antibodies which have higher affinity for the *T. cruzi* mucins than the natural anti-α-Gal human antibodies directed towards Gal(α1-3)-Gal(β1-4)GlcNAc [1,17]. The absence of this epitope in humans is the result of evolution since it is abundantly expressed in New World monkeys [18–20]. The anti α-Gal antibodies are the main cause of rejection in xenotransplantations [21,22]. The same α-Gal trisaccharide was characterized as the main oligosaccharide in *T. cruzi* trypomastigote mucins [17]. However, in order to be sialylated by the TcTS, the oligosaccharides must be branched with β-D-Gal*p* units since α-D-Gal*p* cannot function as acceptor. Since glycosylation of proteins is a result of the action of several glycosyl transferases and transglycosidases, microheterogeneity is usually observed. Accordingly, a mixture of higher oligosaccharides, some of them sialylated were also released from the mucins of *T. cruzi* by reductive β-elimination and were partially characterized. The fine structure of these branched oligosaccharides is still unknown. Sialylation of the mucins prevents lysis by anti-α-Gal antibodies. This effect is not due to interference of the reaction with the antibody, since sialylated and non-sialylated trypomastigotes bind the same amount of anti-α-Gal. Lysis was attributed to the inability of the anti-α-Gal to cause aggregation of the mucins and disrupt the surface of the parasite, in the presence of the sialic acid negative charge [1]. Sugar microheterogeneity of the mucins could explain the recent finding that sialic acid and α-Gal targets for antibody reaction only partially colocalize in the parasite surface [23]. Lineal α-Gal trisaccharide, which would not be sialylated, and higher branched oligosaccharides, acceptors of sialic acid, may be linked to the same mucin molecule or be part of different mucin populations.

In the present work we report the synthesis of α-D-Gal*p*(1→3)[β-D-Gal*p*(1→6)]-β-D-Gal*p*, functionalized as the 6-aminohexyl glycoside for further bioconjugation. This trisaccharide is the smallest structure which contains both, the antigenic unit D-Gal*p*(α1→3)-D-Gal*p* and a β-D-Gal*p* branch that may be acceptor of sialic acid. In fact, sialylation was achieved using recombinant trans-sialidase and the sialylated product was spectroscopically characterized.

2. Results and discussion

2.1. Synthesis of 6-aminohexyl α-D-Galp(1→3)[β-D-Galp(1→6)]-β-D-Galp(1)

A retrosynthetic analysis of target molecule **1** indicates that it can be obtained from synthons **2**–**4** (Fig. 1). Several groups have reported the synthesis of derivatives of the Gal*p*(α1→3)Gal*p* motif [24–33]. Most routes start from the benzylated trichloroacetimidate donor **2** which, by lacking a participating group at C-2, selectively affords the α1→3 linkage when reacting with acceptor **3** (Scheme 1).

Precursor **3** was synthesized using a strategy similar to that used by Hanessian et al. [34] for the preparation of the analogue 3-methoxy-2-pyridyl glycoside (Scheme 1). We decided to use the tolyl thio-glycoside because it may be activated for further glycosylation with 6-aminohexanol or other aglycones. Thus, starting from tolyl 1-thio-β-D-galactopyranoside [35], the primary position was protected with a TBDPS group [36] and then, by successive treatment with triethylorthoacetate, benzoyl chloride and acid, to open the orthoacetate function, the glycosyl acceptor **3** was obtained in 92% yield from tolyl 1-thio-β-D-galactopyranoside. Reaction of trichloacetimidate **2** [37] with acceptor **3** at −68 °C using TMSOTf as activator and Et_2O as solvent, furnished thiodisaccharide **6** with the Gal(1→3) linkage only in the α-configuration, as confirmed by the chemical shift of C-1' (93.04 ppm) and the $J_{1',2'}$ value (3.4 Hz) in the ^{13}C and ^{1}H NMR spectra, respectively. Removal of the TBDPS group of **6** afforded the acceptor **7** (Scheme 2) with a total yield of 69% from the monosaccharide synthons **2** and **3**.

For the introduction of the β-D-Gal*p* unit, a derivative with a participating group at C-2 should be used. However, when the acetylated trichloroacetamidate **4a** and TMSOTf were used, the yield of trisaccharide **8a** was low (40%) due to the undesired migration of an acetate group from the donor **4a** to the primary hydroxyl of C-6 in the acceptor **7**. In agreement with a disaccharide structure, the NMR spectrum of this by-product showed only two anomeric signals at 93.2 and 87.0 ppm in the ^{13}C NMR spectrum corresponding to the α-D-Gal*p* and the β-thio-D-Gal*p*, respectively, and two acetate groups. Similar transesterifications have been observed for silver trifluoromethanesulfonate-promoted glycosylations of glycosyl halides [38]. Instead, no migration was observed when the benzoylated trichloroacetimidate **4b** was glycosylated with acceptor **7** (Scheme 2). Thus, after 18 h of reaction at −30 °C and purification by column chromatography, protected trisaccharide **8b** was obtained in 72% yield. The β-configuration of the newly formed linkage was confirmed by the large $J_{1,2}$ value (8.0 Hz) observed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum showed the three anomeric signals at 100.7 (C-1″), 93.4 (C-1′) and 87.2 ppm (C-1), consistent with the **8b** structure.

In order to use the non-protected thioglycoside as a substrate for TcTS, we attempted to remove the benzyl groups of **8b** by catalytic hydrogenolysis. However, very poor yields were obtained even after several days of reaction. Considering that the presence of sulfur could be poisoning the catalyst, we moved forward to the replacement of the thiotolyl anomeric group by the 6-aminohexyl aglycone planned out for the target product. This aglycone has the advantage of carrying a functional group for further conjugation. Thus, by activation of **8b** with NIS/HOTf and reaction with 6-benzyloxycarbonylamino-1-hexanol at −40 °C, glycosyl trisaccharide **9** was smoothly afforded in 71% yield. The ^{13}C NMR spectrum showed anomeric signals at δ 101.4 (C-1), and signals at 101.1 and 93.6 ppm, corresponding to C-1″ and C-1′, respectively, confirming the β-O-glycosidic character of the newly formed bond.

After *O*-deacylation of **9** with sodium methoxide, benzyl groups were easily removed by catalytic hydrogenolysis over 10% palladium on charcoal, using methanol containing 5% of formic acid as solvent. Free trisaccharide **1** was obtained in 59% yield. Anomeric ^{1}H signals were observed as doublets at δ 5.14 ($J_{1',2'}$ = 4 Hz, H-1′), 4.47 ($J_{1,2}$ = 7.3 Hz, H-1) and 4.45 ($J_{1'',2''}$ = 7.8 Hz, H-1″) and the ^{13}C NMR spectrum showed anomeric signals at 103.9 (C-1″), 103.1 (C-1), 95.8(C-1′) ppm. The structure of **1** was further confirmed by its HR ESIMS at m/z 1098.5067 (M+H), coincident with the calculated exact mass of the molecule.

2.2. Selective sialylation of 6-aminohexyl α-D-Galp(1→3) [β-D-Galp(1→6)]-β-D-Galp

Compound **1** was assayed as acceptor substrate for TcTS. Sialylation was addressed using 3′-sialyllactose as the sialic acid donor and incubation conditions as previously described (Scheme 3) [39].

The reaction was monitored by HPAEC-PAD using a Carbopac PA-100 column and, as expected, only one monosialylated product was obtained (Fig. 2). The earlier elution of the sialylated trisaccharide **S1** in comparison with 3′-sialyllactose could be explained by its branched structure, as reported for other oligosaccharides [40]. Accordingly, trisaccharide **1** was less retained than lactose and appeared included in the injection peak.

For the K_M and V_{max} determination of trisaccharide **1**, TcTS and 3′-sialyllactose were incubated with different amounts of **1**. The

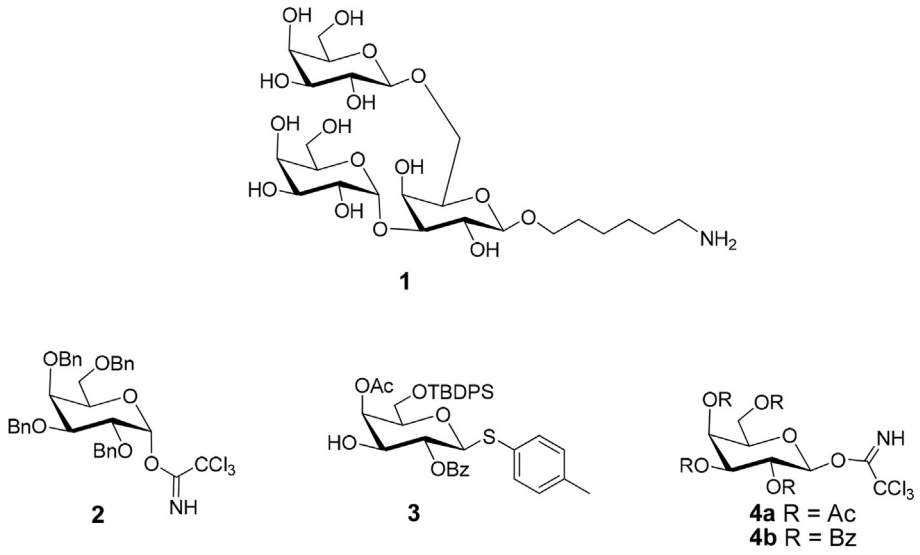
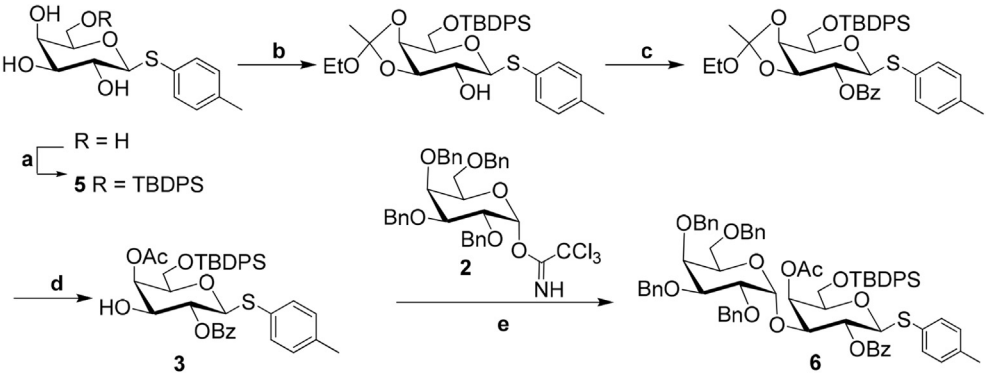
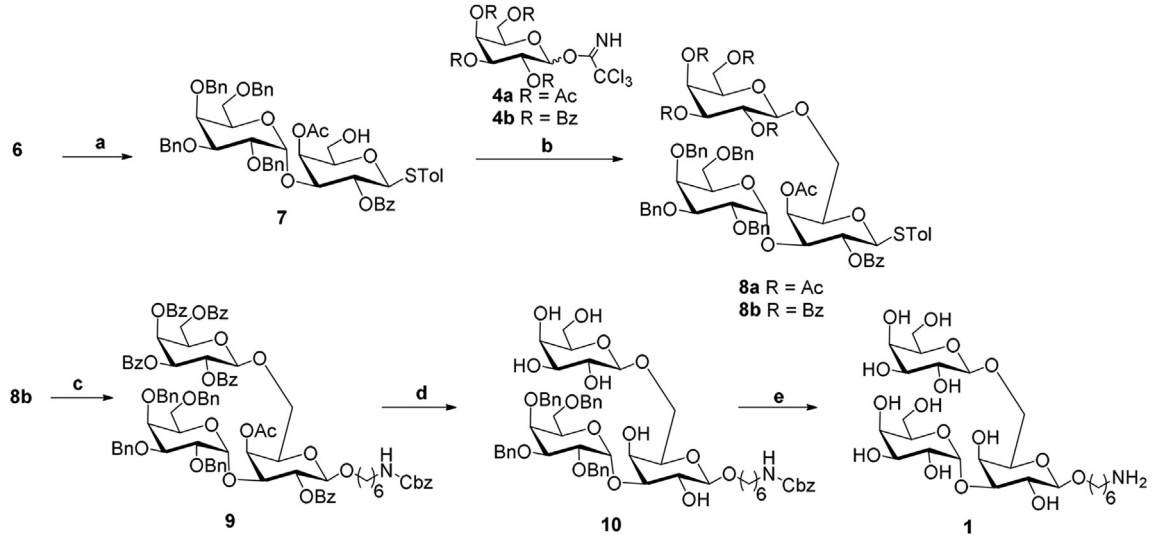


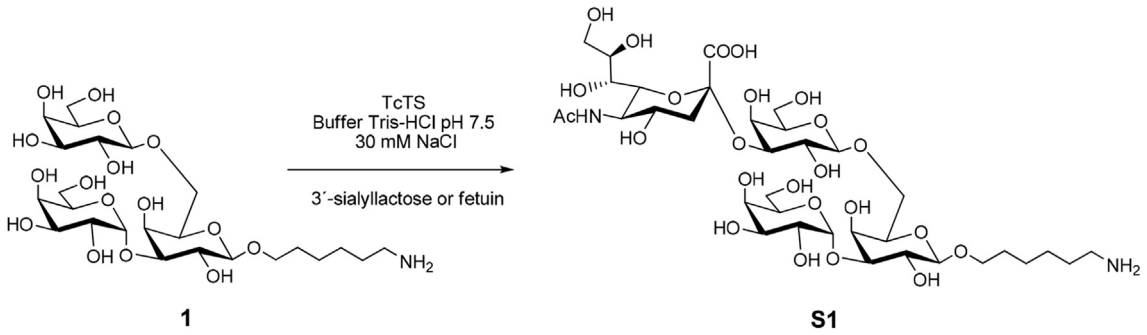
Fig. 1. Target trisaccharide **1** and synthetic precursors **2**–**4**.



Scheme 1. Synthesis of the D-Gal*p*(α1→3)-D-Gal*p* synthon. Reagents and conditions: (a) TBDPSCl, imidazole, DMF; (b) $MeC(OEt)_3$, pyridinium triflate, CH_2Cl_2; (c) BzCl, DMAP, CH_2Cl_2; (d) AcOH 90%, rt; (e) TMSOTf, Et_2O, 4 Å MS, −68 °C.



Scheme 2. Synthesis of trisaccharide **1**. Reagents and conditions: (a) TBAF, AcOH, THF, DMF; (b) TMSOTf, CH_2Cl_2, 4 Å MS, −30 °C; (c) 6-Benzyloxycarbonylamino-1-hexanol, NIS, HOTf, CH_2Cl_2, 4 Å MS, −40 °C; (d) NaOMe, MeOH, rt; (e) 10% Pd/C, H_2, 50 psi, 5% formic acid, MeOH.



Scheme 3. Sialylation of trisaccharide **1** by TcTS.

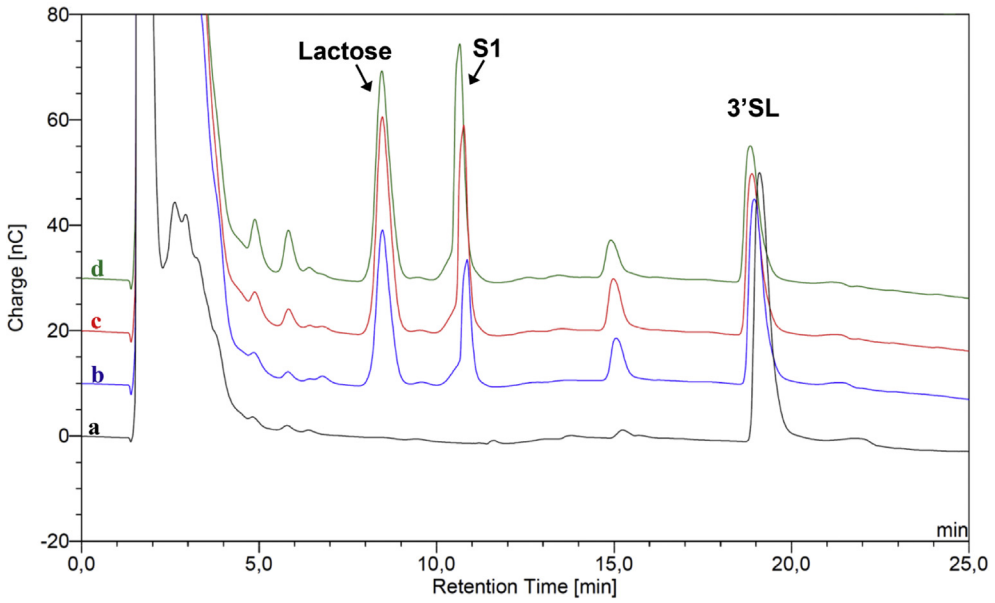


Fig. 2. HPAEC-PAD analysis of trisaccharide **1** sialylation by TcTS for K_M and V_{max} calculations. 3′-Sialyllactose (1 mM) and different concentrations of **1** were incubated in absence (a) or presence (b–d) of TcTS. The reaction mixtures were monitored by HPAEC-PAD using the elution conditions indicated in the Experimental section. Concentration of **1**: (a) 1 mM (without enzyme); (b) 0.25 mM; (c) 0.5 mM and (d) 0.75 mM.

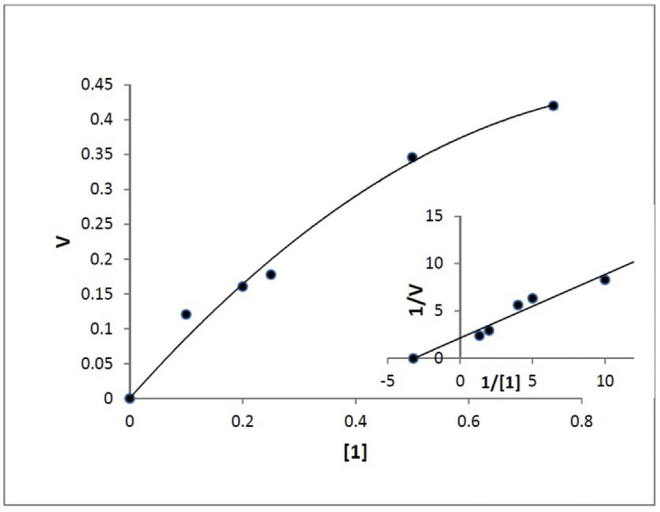


Fig. 3. Kinetic analysis of **1** as TcTS substrate. Plot of V (mmol x h^{-1} x mg^{-1}) vs. [**1**] (mM). Each point is the mean obtained from three replicate experiments. Inset: Lineweaver–Burk reciprocal plot.

obtained values, 0.35 mM and 0.42 nmol/mg. min respectively (Fig. 3), are similar to those observed for lactitol (K_M 0.26 mM) [39], the α-benzyl glycoside of the linear trisaccharide Gal*p*(β1→2) Gal*f*(β1→4)GlcNAc(K_M 0.31 mM) [41], and the α-benzyl glycosides of branched trisaccharides Galf (β1→4) [Gal*p*(β1→6)]GlcNAc and Gal*p*(β 1→3)[Gal*p*(β 1→6)]Gal*p*(K_M 0.72 mM) [41].

The ability to compete for the sialic acid with the conventional substrate *N*-acetyllactosamine was investigated by incubating trisaccharide **1** in concentrations ranging from 0 to 1.5 mM with *N*-acetyllactosamine, 3′-sialyllactose and TcTS. An IC_{50} value of 1.35 mM was calculated considering the decrease in the percentage of 3′-sialyllactosamine.

In order to characterize the sialylated product **S1** and with the aim to facilitate its purification, a preparative sialylation reaction was performed using fetuin as the sialic acid donor. The higher molecular weight of fetuin, compared with that of 3′-sialyllactose, simplified its separation from the sialylated trisaccharide. Purification was achieved by chromatography on a graphitized carbon column using a step gradient elution of acetonitrile/water (0→25% in acetonitrile). Unreacted trisaccharide **1** was eluted with 4–8% acetonitrile and the sialylated derivative with 20% acetonitrile. After evaporation of the solvent, a white powder was obtained with 36% yield. The structure of **S1**, with a sialic acid linked to the 3-O of

the β-D-Gal*p* unit, was confirmed by NMR spectroscopy. The ^{1}H NMR spectrum showed the diagnostic signals for the deoxy group of sialic acid, at δ 2.76 (H-3eq) and δ 1.8 (H-3ax) and a singlet corresponding to the NAc group at 2.04 ppm. The three anomeric protons were easily distinguished, with that of the α-D-Gal*p* unit at lower field (5.15 ppm). The sialylated β-D-Gal*p* showed the anomeric proton (4.55 ppm) and the characteristic H-3 signal (4.09 ppm) shifted to lower field than the analogue signals of trisaccharide **1** ($\delta_{H3''}$ 3.80–3.60), ascertaining the regiochemistry of the sialylation.

3. Conclusions

The trisaccharide α-D-Gal*p*(1→3)[β-D-Gal*p*(1→6)]-β-D-Gal*p* and its sialylated derivative **S1** were obtained, properly functionalized for further conjugation with a protein, activated gel matrices or polyethyleneglycol (PEG), with the aim of using them in immunological studies.

Trisaccharide **1** was synthesized starting from a tolyl β-D-1-*S*-Gal*p* derivative, precursor of the reducing end, bearing orthogonal protecting groups. Two galactosyl trichloroacetimidates, one benzylated and the other one benzoylated were consecutively used, in order to achieve the desire configurations. By the thioglycoside method using NIS/HOTf as activator, the 6-aminohexyl linker was introduced bearing a Cbz protective group that could be released together with the benzyl groups in the α-Gal*p* residue. For the introduction of the sialyl unit to afford derivative **S1**, a chemoenzymatic glycosylation methodology was used, taking advantage of the exceptional specificity of TcTS.

It was reported that sialylation of parasites prevents their lysis by the human anti α-Gal antibodies [1], however, the sugar epitopes can be recognized in mucins form the trypomastigote stage [23].

The tri- and tetrasaccharide here described could be useful as probes to study, in vitro, the interplay between sialylation and recognition by anti α-Gal antibodies, as a contribution to the knowledge of sialic acid glycobiology in *T. cruzi*.

4. Experimental

4.1. General methods

The solvents used were distilled, dried and stored according to standard procedures. Analytical thin layer chromatography (TLC) was performed on Silica Gel 60 F254 (Merck) aluminium supported plates (layer thickness 0.2 mm). Visualization of the spots was effected by exposure to UV light and charring with a solution of 5% (v/v) sulfuric acid in EtOH, containing 0.5% *p*-anisaldehyde. Column chromatography was carried out with Silica Gel 60 (230–400 mesh, Merck). Optical rotations were measured with a Perkin-Elmer 343 digital polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AMX 500 instrument. Chemical shifts (δ) are reported in ppm, with residual chloroform (δ 7.27 for ^{1}H and δ 77.16 for ^{13}C) or acetone (δ 2.16 for ^{1}H and δ 29.84 for ^{13}C) as internal references. Assignments of ^{1}H and ^{13}C NMR spectra were assisted by 2D 1H COSY and HSQC experiments. High resolution mass spectra (HRMS) were obtained by Electrospray Ionization (ESI) and Q-TOF detection. Analysis by HPAEC-PAD was performed using a Dionex ICS 3000 HPLC system equipped with a pulse amperometric detector. A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a PA-100 guard column (4 × 50 mm) was used with a linear gradient program from 0 to 500 mM NaOAc in 100 mM NaOH over 60 min at a flow rate of 0.9 mL/min at 25 °C.

For the sialylation experiments a recombinant TcTS expressed in *Escherichia coli* was kindly provided by the group of O. Campetella from Universidad Nacional General San Martin (Buenos Aires, Argentina).

4.2. 4-Methylphenyl 6-O-tert-butyldiphenylsilyl-1-thio-β-D-galactopyranoside (**5**)

To a solution of 4-methylphenyl 1-thio-β-D-galactopyranoside [35] (0.62 g, 2.15 mmol) and imidazole (0.3 g, 4.3 mmol) in dry DMF (8 mL), TBDPSCl was added (728 μL, 2.8 mmol) and the reaction mixture was stirred for 3 h at rt. The solution was diluted with EtOAc (100 mL), extracted with H_2O (2 × 150 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (2:1 hexane-EtOAc) to give **5** (1.0 g, 96%) as a foamy solid. The NMR data were coincident with those reported [36].

4.3. 4-Methylphenyl 4-O-acetyl-2-O-benzoyl-6-O-tert-butyldiphenylsilyl-1-thio-β-D-galactopyranoside (**3**)

To a solution of **5** (0.43 g, 0.82 mmol) in anhydrous CH_2Cl_2 (8 mL), triethylorthoacetate (302 μL, 1.64 mmol) and pyridiniumtriflate (2 mg) were added and the solution was stirred during 3 h at rt. Then, DMAP (0.20 g, 1.64 mmol) was added and the solvent was removed under reduced pressure. The syrup obtained was dried under vacuum. The residue was redissolved in CH_2Cl_2 (8 mL) and BzCl (145 μL, 1.26 mmol, 1.5 eq) was added. After stirring the solution during 2 h at rt, the excess BzCl was quenched by addition of MeOH (3 mL) and the solution was concentrated under reduced pressure. Then, the residue was dissolved in AcOH (90%), stirred for 15 min at rt, diluted with CH_2Cl_2 (100 mL) and washed successively with water (100 mL), saturated $NaHCO_3$ (ss) and water (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure to afford **3** (0.53 g, 96%) as a white foam. $[\alpha]_D$ +5.1 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35–6.90 (m, 19H, aromatic), 5.51 (d, 1H, $J_{3,4}$ = 3.4 Hz, H-4), 5.18 (dd, 1H, $J_{1,2}$ = 9.9 Hz, $J_{2,3}$ = 9.8 Hz, H-2), 4.78 (d, 1H, H-1), 4.02 (dd, 1H, H-3), 3.84–3.72 (m, 2H, H-5, H-6a), 3.71 (dd, 1H, $J_{5,6b}$ = 5.2 Hz, $J_{6a,6b}$ = 8.4, H-6b), 2.29 (s, 3H, SArCH_3), 2.03 (s, 3H, COCH_3), 1.04 (s, 9H, SiC(CH_3)$_3$); ^{13}C NMR (126 MHz, $CDCl_3$): δ 171.0 and 166.5 (CO), 138.0, 135.6, 135.5, 135.5, 133.3, 133.1, 132.9, 132.9, 132.7, 129.9, 129.8, 129.8, 129.7, 129.5, 129.5, 129.4, 128.4, 128.2, 127.8, 127.8, 127.7 and 127.7 (aromatic), 86.8 (C-1), 77.6 (C-5), 73.0 (C-3), 71.8 (C-2), 70.0 (C-4), 61.9 (C-6), 21.2 (SArCH$_3$), 20.8 (COCH$_3$), 26.8 and 19.1 (SiC(CH$_3$)$_3$). ESIMS: *m/z* calcd for $C_{38}H_{42}O_7SSiNa$ $[M+Na]^+$ 693.23182. Found: 693.23100.

4.4. 4-Methylphenyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-4-O-acetyl-2-O-benzoyl-6-O-tert-butyldiphenylsilyl-1-thio-β-D-galactopyranoside (**6**)

A solution of thioglycoside **3** (1 g, 1.6 mmol) in dry Et_2O (10 mL) was added to a stirred suspension of trichloroacetimidate **2** (1.36 g, 2 mmol) [37] in dry Et_2O (8 mL), cooled at −68 °C, containing 4 Å activated MS under argon atmosphere. TMSOTf (100 μL, 0.54 mmol, 0.3 eq) was added and the stirring was continued for 20 h, when TLC analysis showed consumption of compound **3** and the formation of a main product with R_f 0.6 (4:1 hexane-EtOAc).

The reaction mixture was quenched with trimethylamine, filtered, diluted with CH_2Cl_2 (100 mL) and successively washed with $NaHCO_3$ (ss) and water (100 mL). After drying with Na_2SO_4, the solvent was evaporated under reduced pressure and the residue was purified by column chromatography (60:1 toluene-AcOEt) to afford **6** (0.82 g, 46%) as a foamy solid. $[\alpha]_D$ +78 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.02–7.00 (m, 39 H, aromatic), 5.55 (br s, 1H, H-4), 5.43 (dd, 1H, $J_{1,2}$ = 10.3 Hz, $J_{2,3}$ = 10.3 Hz, H-2), 5.18 (br s, 1H, H-

1′), 4.64 (m, 2H, H-1 and CH_2Ph), 4.60–4.15 (m, 7H, CH_2Ph), 4.01 (d, 1H, H-3), 3.83 (d, 1H, $J_{2',3'}$ = 10.3 Hz, H-2′), 3.79 (br s, 1H, H-5′), 3.69 (m, 1H, H-6a), 3.58 (m, 2H, H-5, H-6b), 3.43 (d, 1H, H-3′), 3.38 (t, 1H, $J_{6'a,6'b}$ = 8.8 Hz, H-6'a), 3.08 (m, 2H, H-4'and H-6'b), 2.21 (s, 3H, SArCH_3), 1.66 (s, 3H, COCH_3), 0.97 (s, 9H, SiC(CH_3)$_3$); ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.0 and 164.8 (CO), 138.7, 138.7, 138.4, 138.3, 135.6, 133.1, 133.0, 132.9, 132.4, 129.9, 129.8, 129.8, 129.7, 129.6, 129.5, 129.0, 128.3, 128.2, 128.1, 128.1, 128.0, 127.8, 127.7, 127.7, 127.4, 127.3 and 125.2 (aromatic), 93.0 (C-1′), 87.5 (C-1), 78.8 (C-3′), 77.8 (C-5), 75.7 (C-2′), 74.9 (C-4′), 74.4, 73.3, 73.2 and 73.1 (CH_2Ph), 72.8 (C-3), 69.8 (C-5′), 69.6 (C-6′), 69.3 (C-2), 64.9 (C-4), 62.2 (C-6), 21.1 (SArCH_3), 20.4 (COCH_3), 26.7 and 19.1 (SiC(CH_3)$_3$). ESIMS: *m/z* calcd for $C_{72}H_{77}O_{12}SSi$ $[M+H]^+$ 1193.4905. Found: 1193.4897.

4.5. 4-Methylphenyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-[2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→6)]-4-O-acetyl-2-O-benzoyl-1-thio-β-D-galactopyranoside (**8b**)

To a solution of **6** (0.44 g, 0.37 mmol) in anhydrous THF (5 mL), TBAF (0.19 g, 0.73 mmol) and AcOH (44 μL, 0.77 mmol) were added and the solution was stirred for 2 h at rt. The mixture was diluted with CH_2Cl_2 (100 mL), washed with water (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure to afford **7** (0.35 g) as a white solid.

To a stirred suspension of trichloroacetimidate **4b** (0.27 g, 0.36 mmol) [37] in anhydrous CH_2Cl_2 (4 mL) containing 4 Å activated MS under argon atmosphere cooled at −30 °C, a solution of disaccharide acceptor **7** (0.25 g, 0.25 mmol) in anhydrous CH_2Cl_2 (3 mL) was added. TMSOTf (18 μL, 0.08 mmol) was added and the stirring was continued for 18 h. After addition of trimethylamine, the mixture was filtered and diluted with CH_2Cl_2 (100 mL), extracted with $NaHCO_3$ (ss), washed with water (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (3:1 hexane-EtOAc) to give **8b** (0.28 g, 72%) as a foamy solid. $[\alpha]_D$ +98 (*c* 1, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.11–7.07 (m, 49 H, aromatic), 5.97 (d, 1H, $J_{3'',4''}$ = 3.5 Hz, H-4″), 5.78 (dd, 1H, $J_{1'',2''}$ = 7.9 Hz, $J_{2'',3''}$ = 10.4, H-2″), 5.55 (dd, 1H, H-3″), 5.53 (d, 1H, $J_{3,4}$ = 3.3 Hz, H-4), 5.49 (t, 1H, J $J_{1,2}$ = 10 Hz, $J_{2,3}$ = 10 Hz, H-2), 5.08 (d, 1H, $J_{1',2'}$ = 3.4 Hz, H-1′), 4.93 (d, 1H, H-1″), 4.73 (d, 1H, *J* = 11.5 Hz, CH_2Ph), 4.68–4.56 (m, 5H, H-6″a and CH_2Ph), 4.45–4.36 (m, 3H, CH_2Ph), 4.28 (d, 2H, *J* = 11.8 Hz, CH_2Ph), 4.24 (m, 1H, H-5″), 4.00 (dd, 1H, H-3), 3.94 (dd, 1H, $J_{6a,6b}$ = 11.0, $J_{5,6a}$ = 4.3 Hz, H-6a), 3.90–3.84 (m, 2H, H-2′ and H-6b), 3.84–3.73 (m, 2H, H-5′ and H-5), 3.52 (dd, 1H, $J_{2',3'}$ = 10.1, $J_{3',4'}$ = 2.8 Hz, H-3′), 3.36 (dd, 1H, $J_{6'a,6'b}$ = 9.5, $J_{5',6'a}$ = 6.8 Hz, H-6'a), 3.20 (d, 1H, H-4′), 3.15 (dd, 1H, $J_{5',6'b}$ = 5.5 Hz, H-6'b), 2.28 (s, 3H, SArCH_3), 1.79 (s, 3H, COCH_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.1 (CO), 166.0, 165.5, 165.5, 165.2, 164.9 (CO), 138.7, 138.6, 138.5, 138.25, 138.1, 133.5, 133.3, 133.2, 133.2, 133.1, 132.6, 130.0, 129.8, 129.8, 129.8, 129.7, 129.7, 129.5, 129.4, 129.4, 129.3, 129.0, 128.7, 128.6, 128.5, 128.4, 128.3, 128.3, 128.3, 128.1, 128.1, 127.9, 127.6, 127.5, 127.4, 127.3 (aromatic), 100.7 (C-1″), 93.4 (C-1′), 87.2 (C-1), 78.7 (C-3′), 76.8 (C-5), 75.6 (C-2′), 74.9 (C-4′), 74.5, 73.3, 73.3 and 73.2 (CH_2Ph), 72.9 (C-3), 71.8 (C-3″), 71.3 (C-5″), 69.8 (C-5′), 69.7 (C-2″), 69.3 (C-6′), 69.1 (C-2), 68.0 (C-4″), 67.5 (C-6), 65.6 (C-4), 61.9 (C-6″), 21.1 (SArCH_3), 20.4 (COCH_3). ESIMS: *m/z* calcd for $C_{90}H_{85}O_{21}S$ $[M+H]^+$ 1533.5304. Found: 1533.5305.

4.6. 6-Benzyloxycarbonylaminohexyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-[2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→6)]-4-O-acetyl-2-O-benzoyl-β-D-galactopyranoside (**9**)

Thioglycoside **8b** (0.24 g, 0.16 mmol), 6-benzyloxycarbonylamino-1-hexanol (0.06 g, 0.22 mmol) [42] and NIS (0.04 g, 0.17 mmol, 1.1eq) were dissolved in anhydrous CH_2Cl_2 (6 mL) containing 4 Å activated molecular sieves. The solution was cooled at −40 °C and then TfOH (2.5 μL, 0.016 mmol) was added. After 2 h of stirring the mixture was filtered and diluted with CH_2Cl_2 (100 mL), extracted with $NaHCO_3$ (ss) and $Na_2S_2O_4$ (100 mL, 10% v/v), washed with water (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by column chromatography (15:1 toluene-EtOAc) to give **9** (0.19 g, 71%) as white foam. $[\alpha]_D$ +81 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11–7.11 (m, 50H, aromatic), 5.99 (d, 1H, $J_{3'',4''}$ = 3.5 Hz, H-4″), 5.80 (dd, 1H, $J_{1'',2''}$ = 7.9, $J_{2'',3''}$ = 10.4 Hz, H-2″), 5.60 (dd, 1H, H-3″), 5.48 (d, 1H, $J_{3,4}$ = 3.3 Hz, H-4), 5.41 (dd, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 10.3 Hz, H-2), 5.08 (m, 3H, H-1′ and CH_2Ph), 4.90 (d, 1H, H-1″), 4.75 (d, 1H, *J* = 11.5 Hz, CH_2Ph), 4.69–4.58 (m, 5H, H-6″a and CH_2Ph), 4.43 (m, 3H, H-6″b and CH_2Ph), 4.35–4.25 (m, 4H, H-1, H-5″ and CH_2Ph), 4.03 (m, 2H, H-6a and H-3), 3.90 (dd, 1H, $J_{1',2'}$ = 3.4, $J_{2',3'}$ = 10 Hz, H-2′), 3.86 (t, 1H, $J_{5',6'a}$ = 6.8 Hz, $J_{5',6'b}$ = 5.6 Hz H-5′), 3.79–3.70 (m, 2H, H-5 and H-6b), 3.59 (dd, 1H, $J_{3',4'}$ = 2.8 Hz, H-3′), 3.40 (dd, 1H, $J_{6'a,6'b}$ = 9.5 Hz, H-6'a), 3.29 (d, 1H, H-4′), 3.22 (dd, 1H, H-6'b), 3.49 and 3.06 (m, 2H, OCH_2), 3.00–2.87 (m, 2H, CH_2N), 1.80 (s, 3H, COCH_3), 1.25–0.91 (m, 8H, CH_2); ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.2, 166.0, 165.6, 165.5, 165.4, 164.7 and 156.2 (CO), 138.7, 138.6, 138.5, 138.3, 133.5, 133.2, 133.2, 133.0, 130.0, 129.7, 129.7, 129.6, 129.6, 129.3, 129.2, 129.0, 128.7, 128.6, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.6, 127.5, 127.5, 127.4, 127.3 and 127.3 (aromatic), 101.4 (C-1), 101.1 (C-1″), 93.6 (C-1′), 78.7 (C-3′), 75.5 (C-2′), 74.9 (C-4′), 74.4, 73.3, 73.2, 73.1 (CH_2Ph), 72.9 (C-5), 71.8 (C-3), 71.6 (C-3″), 71.2 (C-5″), 70.7 (C-2), 69.7 (C-5′), 69.7 (C-2″), 69.5 (OCH_2), 69.4 (C-6′), 68.2 (C-6), 68.0 (C-4″), 66.5 (CH_2Cbz), 65.6 (C-4), 61.9 (C-6″), 40.8 (CH_2N), 29.5, 29.0, 26.1, 25.4 (CH_2), 20.4 (COCH_3). ESIMS: *m/z* calcd for $C_{97}H_{98}O_{24}N$ $[M+H]^+$ 1661.6557. Found: 1661.6513.

4.7. 6-Aminohexyl α-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→6)]-β-D-galactopyranoside (**1**)

Trisaccharide **9** (0.16 g, 0.1 mmol) was treated with a cooled solution of 0.3 M MeONa in MeOH (6 mL) and the mixture was stirred at rt during 1.5 h. The solution was deionized by elution with MeOH trough a column loaded with Ambrelite IR-120 plus resin (200 mesh, H^+ form). The eluate was concentrated under reduced pressure to afford **10** as a white solid (0.1 g, 93%). $[\alpha]_D$ +28 (*c* 1, $CHCl_3$); ^{1}H NMR (500 MHz, D_2O): anomeric signals δ 4.86 (m, 2H, H-1′ and CH_2Ph), 4.32 (d, 1H, $J_{1'',2''}$ = 7.7 Hz, H-1″), 4.17 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1); ^{13}C NMR (126 MHz, D_2O): anomeric region δ (ppm) 103.9 (C-1″), 103.1 (C-1), 96.0 (C-1′). ESIMS calcd for $C_{60}H_{75}NO_{18}$ [M+H] 1098.50624; found: 1098.50671.

Trisaccharide **10** (0.1 g, 0.09 mmol) was dissolved in MeOH/H_2O (1:1, 2 mL) and further purified with a RP-18 column (500 mg) in order avoid the presence of sulfur impurities. After evaporation of the solvent, compound **10** was redissolved in methanol (5 mL) containing 5% of formic acid and 10% Pd/C (15 mg). The mixture was hydrogenated at 55 psi during 16 h. Then, the solution was filtered through Celite and the filtrate was concentrated under reduced pressure, to afford **1** (32.6 mg, 59%). $[\alpha]_D$ +60 (*c* 1, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 5.14 (d, 1H, $J_{1',2'}$ = 4 Hz, H-1′), 4.47 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1), 4.45 (d, 1H, $J_{1'',2''}$ = 7.8 Hz, H-1″), 4.22 (d, 1H, $J_{3,4}$ = 3.2 Hz, H-4), 4.18 (dd, 1H, $J_{5'',6''a}$ = 6.3 Hz, $J_{5'',6''b}$ = 6.3 Hz, H-5″), 4.06 (dd, 1H, $J_{5,6a}$ = 4.1 Hz, $J_{6a,6b}$ = 10.8, H-6a), 4.01 (d, 1H, $J_{3',4'}$ = 3.4 Hz, H-4′), 3.95–3.86 (m, 6H, H-3′, OCH_2a, H-4″, H-6b, H-5 and H-2′), 3.80–3.60 (m, 9H, H-3, H-6'a, H-6'b, H-6″a, H-6″b, H-5′, OCH_2b, H-3″ and H-2), 3.52 (dd, 1H, $J_{2'',3''}$ = 9.9, H-2″), 3.01 (m, 2H, CH_2N), 1.70–1.60 (m, 4H, CH_2), 1,45–1.35 (m, 4H, CH_2); ^{13}C NMR (126 MHz, D_2O): δ 103.9 (C-1″), 103.1 (C-1), 95.8 (C-1′), 77.7 (C-3), 75.8 (C-5′), 74.2 (C-5), 73.3 (C-3″), 71.5 (C-5″), 71.4 (C-2″), 71.1

(OCH_2), 69.9 (C-3′), 69.8 (C-2 and C-4′), 69.5 (C-6), 69.2 (C-4″), 68.8 (C-2′), 65.4 (C-4), 61.6 (C-6′ and C-6″), 40.0 (CH_2N), 29.1, 27.2, 25.9, 25.2 (CH_2). ESIMS calcd for $C_{24}H_{46}NO_{16}$ [M+H] 604.2817; found: 604.2804.

4.8. 6-Aminohexyl α-D-galactopyranosyl-(1→3)-[5-N-acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→6)]-β-D-galactopyranoside (**S1**)

To a solution of 6-aminohexyl α-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→6)]-β-D-galactopyranoside (**1**, 0.01 g) and fetuin (0.2 g) in 1 mL of 20 mM Tris-HCl buffer (pH 7.4), containing 30 mM NaCl, recombinant TcTS (0.022 mg) was added and the solution was incubated for 5 h at 25 °C. The synthesis was performed in triplicate and each reaction was analyzed by HPAEC. The three incubation mixtures were combined and purified with a graphitized carbon SPE column (500 mg). The column was eluted with water (10 mL) followed by a step gradient from 0 to 25% of CH_3CN in water. Fractions were collected and analyzed by HPAEC. Neutral trisaccharide **1** was eluted with 4–8% of CH_3CN and **S1** with 20% of CH_3CN. The pooled fraction were concentrated under vacuum at rt, redissolved in water and lyophilized to afford 5.4 mg of **S1** as an amorphous white powder. ^{1}H NMR (500 MHz, D_2O): δ 5.15 (s, 1H, $J_{1',2'} = 3.9$ Hz, H-1′), 4.55 (d, 1H, $J_{1'',2''} = 7.9$ Hz, H-1″), 4.48 (d, 1H, $J_{1,2} = 8.0$ Hz, H-1), 4.24 (d, 1H, $J_{3,4} = 3.2$ Hz, H-4), 4.19 (t, 1H, $J = 6.3$ Hz, H-5″), 4.09 (dd, 1H, $J_{2'',3''} = 10.0$, $J_{3'',4'''} = 3.5$ Hz, H-3″), 4.08 (dd, 1H, $J_{5,6a} = 3.5$ Hz, $J_{6a,6b} = 10.0$ Hz, H-6a), 4.02 (d, 1H, $J_{3',4'} = 3.4$ Hz, H-4′), 3.97–3.80 (m, 9H, H-3′, OCH_2a, H-4″, H-6b, H-5_{Neu5Ac}, H-5, H-2′, H-$9a_{Neu5Ac}$ and H-7_{Neu5Ac}), 3.80–3.55 (m, 13 H, H-6″a, H-6″b, H-6'a, H-6'b, H-3, OCH_2b, H-5′, H-2, H-$9b_{Neu5Ac}$, H-8_{Neu5Ac}, H-2″, H-4_{Neu5Ac} and H-6_{Neu5Ac}), 3.01 (m, 2H, CH_2N), 2.76 (dd, 1H, $J = 12.5$, 4.6 Hz, H-$3eq_{Neu5Ac}$), 2.04 (s, 3H, CH_3Ac), 1.80 (t, 1H, $J = 12.5$ Hz, H-$3ax_{Neu5Ac}$), 1.67 (m, 4H, CH_2) and 1.43 (m, 4H, CH_2) ppm; ^{13}C NMR (126 MHz, D_2O): δ 175.8 and 174.6 (CO and CO_{Neu5Ac}), 103.6 (C-1″), 103.3 (C-1), 100.7 (C-2_{Neu5Ac}), 96.0 (C-1′), 77.9 (C-3), 76.6 (C-3″), 75.7 (C-5′), 74.3 (C-5), 72.6 (C-7_{Neu5Ac}), 71.6 (C-5″), 71.2 (OCH_2), 70.1, 69.9 × 3 (C-4′, C-2, C-2″ and C-3′), 69.6 (C-6), 73.6 and 69.1 (C-4_{Neu5Ac} and C-6_{Neu5Ac}), 69.0 (C-2′), 68.8 (C-8_{Neu5Ac}), 68.3 (C-4″), 65.6 (C-4), 63.3 (C-9_{Neu5Ac}), 61.7 × 2 (C-6′ and C-6″), 52.4 (C-5_{Neu5Ac}), 40.4 (C-3_{Neu5Ac}), 40.2 (CH_2N), 29.2, 27.3, 26.0 and 25.3 (CH_2), 22.8 ($COCH_3$). ESIMS: m/z calcd for $C_{34}H_{61}O_{24}N_2$ $[M+H]^+$ 895.37708. Found: 895.37665.

4.9. Enzyme kinetics

Reactions mixtures of 20 μL of 20 mM Tris-HCl pH 7 buffer, containing 30 mM NaCl, 1 mM 3′-sialyllactose as donor and different concentrations of trisaccharide **1** as acceptor substrate, were incubated with 304 ng of purified TcTS for 15 min at rt. For K_M calculations a set of 6 concentrations between 0 and 1 mM of **1** was used. Each incubation was performed at least in duplicate. Samples were then diluted 3 times with deionized water and analyzed by HPAEC using D-galacturonic acid as internal standard. The extent of sialylation was calculated from the decrease in concentration of 3′-sialyllactose. K_M and V_{max} were determined using the Lineweaver-Burk method [43].

4.10. Inhibition of sialylation of N-acetyllactosamine

Reaction mixtures of 20 μL containing 20 mM Tris-HCl pH 7 buffer, 30 mM NaCl, 1 mM 3′-sialyllactose as donor, 1 mM *N*-acetyllactosamine, and different concentrations of compound **1** (0–1.5 mM) were incubated with 304 ng of purified TcTS for 15 min at rt. Samples were then diluted 3 times with deionized water and analyzed by HPAEC. Inhibition was calculated from the amount of 3′-sialyl-*N*-acetyllactosamine with respect to the total amount of sialylated compounds obtained with or without inhibitor. The IC_{50} values indicate the concentration of inhibitor required to give 50% inhibition under the assay conditions.

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Appendix A. Supplementary data

Supplementary data related to this article (NMR spectra for compounds **3**, **6**, **8b**, **9**, **1** and **S1**) can be found at http://dx.doi.org/10.1016/j.carres.2017.08.007.

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