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Glycoconjugate Journal
Official Journal of the International Glycoconjugate Organization

ISSN 0282-0080

Glycoconj J
DOI 10.1007/s10719-016-9676-0

Springer

Glycoconj J
DOI 10.1007/s10719-016-9676-0

ORIGINAL ARTICLE

Multivalent sialylation of β-thio-glycoclusters by *Trypanosoma cruzi* trans sialidase and analysis by high performance anion exchange chromatography

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Received: 29 December 2015 / Revised: 22 April 2016 / Accepted: 10 May 2016

Abstract The synthesis of multivalent sialylated glycoclusters is herein addressed by a chemoenzymatic approach using the trans-sialidase of *Trypanosoma cruzi* (TcTS). Multivalent β-thio-galactopyranosides and β-thio-lactosides were used as acceptor substrates and 3′-sialyllactose as the sialic acid donor. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was shown to be an excellent technique for the analysis of the reaction products. Different eluting conditions were optimized to allow the simultaneous resolution of the sialylated species, as well as their neutral precursors. The TcTS efficiently transferred sialyl residues to di, tri, tetra and octa β-thiogalactosides. In the case of an octavalent thiolactoside, up to six polysialylated compounds could be resolved. Preparative sialylation reactions were performed using the tetravalent and octavalent acceptor substrates. The main sialylated derivatives could be unequivocally assigned by MALDI mass spectrometry. Inhibition of the transfer to the natural substrate, *N*-acetyllactosamine, was also studied. The octalactoside caused 82 % inhibition of sialic acid transfer when we used equimolar concentrations of donor, acceptor and inhibitor.

Keywords *T. cruzi* trans-sialidase · β-galactopyranosides · Multivalent glycoclusters · Sialic acid · HPAEC · MALDI-TOF · Enzymatic sialylation

Introduction

The synthesis of multivalent glycoclusters has been undertaken by several groups with the aim to obtain high affinity ligands for specific binding to cellular proteins [1, 2]. In various approaches, multivalency is achieved by the simultaneous attachment of several protected sugar fragments to a suitably functionalized scaffold [3–8]. Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC), also known as the click reaction, has proved to be highly convenient for this purpose [9–13]. The analysis of these multivalent glycoclusters is frequently difficult as a consequence of their considerable molecular weight and highly branched structure, especially taking into consideration that most of the techniques used in polymer analysis, like size exclusion chromatography, are more suitable for linear molecules. Even Nuclear Magnetic Resonance (NMR) techniques are not always conclusive due to the complexity and the frequent lack of symmetry of these structures.

On the other hand, the classic methodologies for the analysis of small molecules (mainly adsorption and reversed phase chromatography), can be only partially adapted to the analysis of multivalent ligands, and are in general restricted to the less polar protected precursors. When dealing with the free, water-soluble final glycoclusters, limited methodologies are available. It is beyond discussion that the confirmation of their high

Electronic supplementary material The online version of this article (doi:10.1007/s10719-016-9676-0) contains supplementary material, which is available to authorized users.

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Published online: 15 June 2016

degree of purity is essential, as they are going to be used in biological tests. When multisialosides are obtained these issues become more important. The resulting glycoclusters are extremely polar and also labile to hydrolysis.

The critical role of sialic acid in a variety of biological processes including viral and bacterial adhesion is well known [14, 15]. Thus, the synthesis of glycoclusters exposing α-sialic acid residues remains an active area of research [16–18]. They have a potential for anti-adhesion therapies and would help to elucidate the features involved in carbohydrate-protein interactions.

Several methodologies have addressed the synthesis of multisialosides. 1-Thio-α-sialosyl residues have been attached to calixarene and dendrimeric scaffolds by S_N2 displacement of an activated leaving group [19, 20]. Click reaction has also been employed to link propargyl thiosialosides to azide-functionalized calixarene platforms, to obtain glycoclusters which were shown to be inhibitors of influenza A virus infection [21]. Recently, a fullerene derivative linked to a sialylthio-D-galactose disaccharide has also been synthesized [22]. In a different approach, a conveniently functionalized sialyllactose glycoside was connected to dendrimeric carbosilane cores, and the resulting glycoclusters showed potent inhibitory activity against hemolysis of human influenza virus [23]. It has also been reported that a trivalent glycopeptide mimetic binds to hemagglutinin H5 of avian influenza at the nanomolar level [24]. *T. cruzi* trans-sialidase (TcTS) allows the construction of terminal αSial(2–3)βGal motifs by transferring sialic acid from sialylglycans to β-galactopyranosides. *In vivo*, the reaction involves sialylglycoconjugates of the infected mammal and β-galactopyranosyl receptors of parasite mucin [25–27]. The enzyme has been cloned and successfully used to sialylate β-galactopyranoside units in diverse substrates [28]. The important role of TcTS in the pathogenesis of Chagas' disease has been extensively documented and explains the interest in the synthesis of inhibitors of this activity [29, 30]. One example is the synthesis of cyclic triazole bridged β-galactopyranosides [31]. These macromolecules were analyzed by reversed-phase HPLC with mixtures of water/acetonitrile and the inhibition assays were performed using a fluorometric method based on the hydrolysis of 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNANA) [32].

During the last years, we showed the potential of high performance anion exchange chromatography for the analysis of a variety of sialylated oligosaccharides, which were obtained by chemoenzymatic synthesis using the particular activity of TcTS. The usefulness of this enzyme for the preparation of sialylated ligands was assessed by the isolation and characterization of the sialylated products [33–36]. The acceptor and inhibitor properties of lactose derivatives and other chemically synthesized β-galactopyranosides in the TcTS reaction have been studied [28, 35]. The TcTS also transfers *N*-glycolylneuraminic acid to βGal units [37].

Our previous results prompted us to use the enzyme for multisialylation of β-galactosyl glycoclusters and analysis of the reaction by HPAEC. Chromatographic conditions were optimized to allow the simultaneous resolution of the acceptor substrates and their sialylated products in a single chromatographic run, which is of particular interest when performing kinetic studies.

Materials and methods

Enzyme catalysis β-Thiogalactosides and β-thiolactosides were prepared as previously reported [38, 39]. Then, they were incubated with recombinant TcTS (300 ng) in 20 mM Tris–HCl, pH 7.6 buffer, 30 mM NaCl, containing 1 mM 3′-sialyllactose (3′-SL, purchased from Elicityl, France) as a sialic acid donor, as described before [28]. For comparison of their capacity to act as acceptors, 1 mM of each, 3′-SL and polyvalent substrates were used. Experiments using 2, 3, 4 or 8 mM of 3′-SL were performed for di, tri, tetra and octavalent glycoclusters, respectively. In the case of incubations with equimolar concentrations of donor and acceptor substrates, the percentage of sialylation was calculated by integration of all the sialylated species present. When higher proportions of 3′-SL were used, the percentage of transfer was calculated by integration of all sialylated glycoclusters and non sialylated remaining species, if not otherwise indicated.

Inhibition of sialylation of *N*-acetyllactosamine (LN) The inhibition experiments were performed as described before [28]. Briefly, β-thiogalactosides and β-thiolactosides (1 mM) were incubated in 20 mM Tris–HCl, 30 mM NaCl, pH 7 buffer (20 μl), containing 1 mM 3′-SL as donor, 1 mM LN (purchased from Elicityl, France), and recombinant TcTS (300 ng) for 15 min at room temperature. After dilution with deionized water, analysis by HPAEC-PAD was performed. Inhibition was calculated considering the decrease in the percentage of 3′-sialyl-*N*-acetyllactosamine (calculated on the total amount of sialylated compounds) in the presence of the inhibitor.

Analysis by HPAEC Analysis by HPAEC-PAD was performed using a Dionex ICS 3000 HPLC system equipped with a pulsed amperometric detector. The following programs were used: *Condition 1*, isocratic elution with 100 mM NaOH at 25 °C; *Condition 2*, isocratic elution with 100 mM NaOH and 20 mM NaAcO at 25 °C; *Condition 3*, a lineal gradient over 60 min from 0 to 500 mM NaAcO in 100 mM NaOH at 25 °C; *Condition 4*, a lineal gradient over 45 min from 20 to 500 mM NaAcO in 100 mM NaOH at 25 °C. The same conditions were

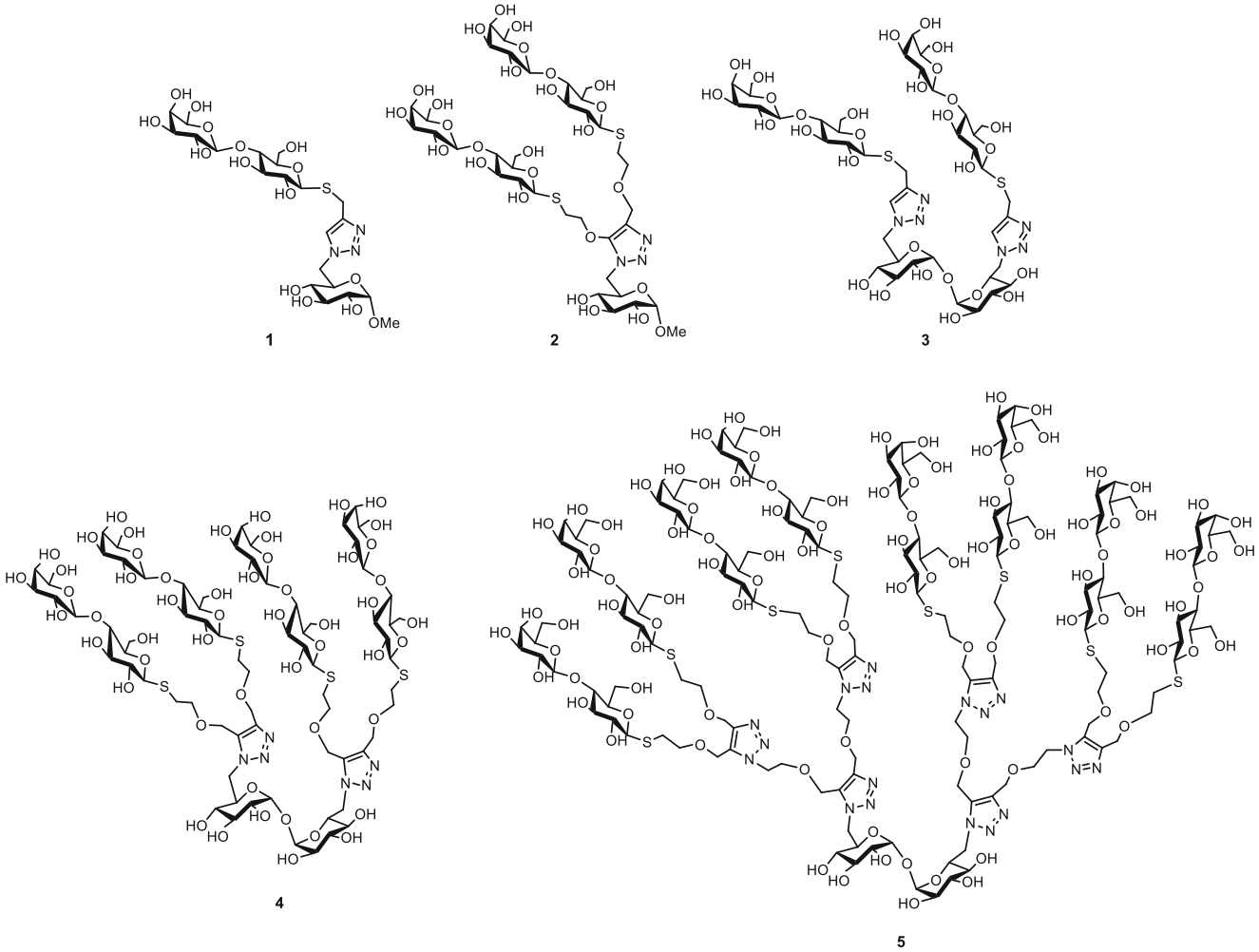


Fig. 1 Structures of multivalent thiolactosides **1**–**5**

used with two different columns: a CarboPac PA-20 ion exchange analytical column (3 × 150 mm) equipped with a guard column PA-20 (3 × 30 mm) and a CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm). The flow rates were 0.5 mL/min for the PA-20 column and 0.9 mL/min for the PA-100.

Table 1 Retention times (t_R, min) for compounds **1**–**7** under the different conditions used as described in Materials and Methods

Compound	PA-20		PA-100	
	Condition 3	Condition 4	Condition 3	Condition 4
1	3.9	1.9	7.4	2.5
2	7.9	4.3	13.5	13.1
3	11.8	7.9	16.5	17.1
4	9.6	10.9	16.9	22.8
5	13.8	25.4	17.8	73
6	16.3	n.d.	16.0	n.d.
7	16.9	n.d.	14.5	n.d.

Preparative sialylation of compound 4 Compound **4** (7.6 mg) and 3′-SL (5 mg) were incubated with 6.4 μg of recombinant TcTS in 0.4 mL of 20 mM Tris buffer pH 7.6 containing 30 mM NaCl for 90 min at 25 °C. The reaction was analyzed by HPAEC using a PA-100 column under condition 3. **$S_1$4** was purified by passing through an anion exchange resin (AG1X2, acetate form, BioRad, 1.2 × 10 cm). Neutral compounds, namely **4** and lactose, were eluted with water and the monosialylated **$S_1$4** with 50 mM pyridinium acetate buffer. The fractions containing a single peak with a retention time of ≈ 20 min were pooled, lyophilized and analyzed by MALDI-TOF MS. M/z found 2390.67, consistent with **$S_1$4**, $C_{87}H_{144}N_7O_{61}S_4$ $[M\text{-}H]^-$. The AG1X2 column was further eluted with a gradient from 50 to 500 mM pyridinium acetate buffer. The following fractions, which contained a peak at ≈ 25 min (**$S_2$4**), together with remaining 3′-SL, were pooled and analyzed by MALDI spectrometry. M/z found: 2682.55,

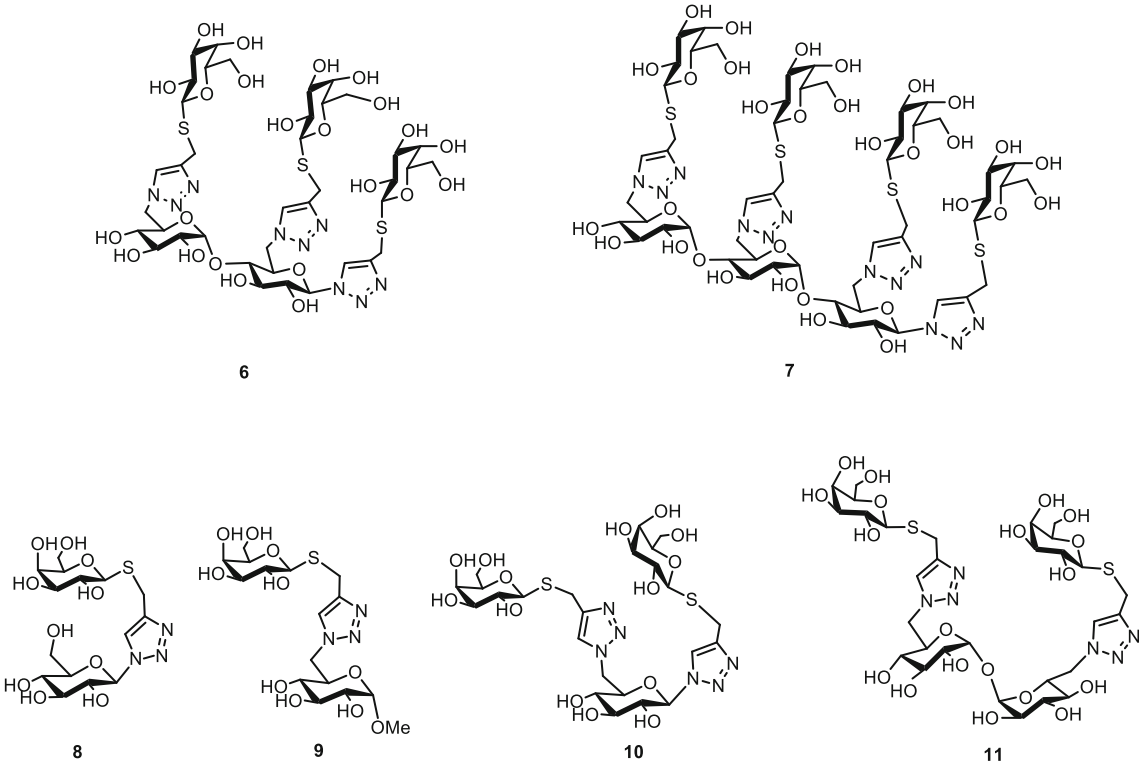
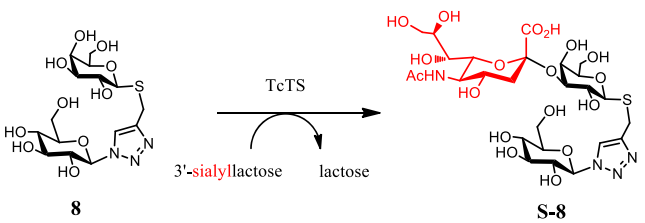


Fig. 2 Structures of the β-thiogalactosides **6–11**

consistent with **$S_2$4**, $C_{98}H_{161}N_8O_{69}S_4$ $[M\text{-}H]^-$. The fractions eluted with 500 mM pyridinium acetate buffer showed a single peak at ≈ 30 min, consistent with the trisialylated derivative. M/z found: 2973.40, compatible with **$S_3$4**, $C_{109}H_{178}N_9O_{77}S_4$ $[M\text{-}H]^-$.

Preparative sialylation of compound 5 Compond **5** (4.7 mg) was incubated as described above with 3′-SL (1 mg) and recombinant TcTS (3.2 μg) for 60 min at 25 °C. The monosialylated compound **$S_1$5** was purified by passing through an anion exchange resin (AG1X2, acetate form, BioRad, 1.2 × 10 cm). The analysis of the column fractions by HPAEC using a PA-100 column under condition 3 showed that **$S_1$5** was eluted with 50 mM pyridinium acetate buffer. The fractions containing a single peak with a retention time of ≈ 21 min were pooled, lyophilized and analyzed by MALDI-TOF. M/z found 4549.75, consistent with **$S_1$5**, $C_{167}H_{276}N_{19}O_{109}S_8$ $[M\text{-}H]^-$.



Scheme 1 Sialylation of compound **8** by *T. cruzi* trans-sialidase

Matrix-assisted laser desortion/ionization time-of-flight mass spectrometry (MALDI-TOF MS) MS spectra were acquired in an Ultraflex II (Bruker Daltonics) MALDI-TOF-TOF, using α-cyano-4-hydroxyhydroxycinnamic acid (CHCA) or 6-aza-2-thiothymine (ATT) as matrix. The ions were detected in negative linear or reflectron modes.

Results and discussion

Multivalent thiogalactopyranosides and thiolactosides can be analyzed by HPAEC

The chromatographic resolution of multivalent lactosides built on methyl α-D-glucopyranoside or trehalose (Fig. 1), was studied using lactoside **1** as a reference. The retention times

Table 2 Evaluation of compounds **1**, **8** and **9** as substrates and inhibitors of TcTS

	t_R X (min)[a]	t_R SX (min)[a]	% SX[b]	% Inhibition
1	7.4	16	48	18
8	11	21	46	48
9	3.5	15	83	68

[a] t_R , retention times of the compounds (X) and their sialylated derivatives (SX) using condition 3 and the PA-100 column

[b] Calculated by integration of the peaks of all sialylated compounds observed in the chromatogram

Table 3 Evaluation of divalent compounds **2**, **3**, **10** and **11** as substrates and inhibitors of TcTS

	t_R X[a]	t_R SX[a]	t_R S_2X	% SX[b]	% S_2X[b]	% total transfer	% Inhibition
2	13.5	19.6	26.1	54	12	66	57
3	16.5	22.5	29.3	65	5	70	56
10	16.2	24.7/25.2	37.9	47	6	53	39
11	14.5	21.7	32.4	51	8	59	34

[a] $\mathbf{t_R}$, retention times of the compounds (X) and their sialylated derivatives (SX and S_2X) using condition 3 and the PA-100 column

of di- (**2** and **3**), tetra- (**4**) and octavalent (**5**) glycoclusters [39] were determined using two HPAEC columns and solvent systems (Table 1). Even the largest glycocluster **5**, could be efficiently analysed with this methodology.

Although the PA-100 column is the usually recommended column for the resolution of oligosaccharides differing in their molecular weights [40, 41], we obtained a better resolution using the PA-20 column. This can be ascribed to the smaller particle size of the latter and/or to the dendrimeric shape of the triazole-containing compounds tested. Even under isocratic eluting conditions, we observed a better performance for the PA-20 column, resolving the five compounds in less than 30 min. On the other hand, in the PA-100 column, the retention time for the octavalent compound **5** was too large. The use of a gradient solvent system also afforded a much better resolution for the PA-20 column, resulting in a well resolved 15 min chromatographic run.

Compound **2**, built on a methyl α-glucoside platform, had a shorter retention time than **3**, having a trehalose scaffold. This behavior could be attributed to the presence of the disaccharide platform in **3** that provides the molecule with more OH groups and one extra triazole ring. Also, the more extended conformation of **3** with respect to **2** would allow a stronger interaction with the stationary phase.

Two structural features present in the glycoclusters had to be taken into consideration when analyzing the retention times. An increase in the multivalency, and so, in the number of OH groups, inforced the interactions with the stationary phase, but, on the other hand, the aromatic triazole rings seemed to have the opposite effect. Thus, retention times for the multivalent compounds were shorter than those expected if only the number of sugar residues were considered. As an example, the retention time in the PA-100 column (condition 3) for the octavalent lactoside **5** (with 6 triazole rings) is only 1 min higher than that for the structurally related tetralactoside **4** (with 2 triazole rings).

The same tendency was observed for tri- and tetrathiogalactosides **6** and **7**, belonging to the thiogalactoside family (Fig. 2), that share a similar linear platform and differ in two sugar residues and one triazole ring. Using the PA-100 column under condition 3, trivalent galactoside **6** had a retention time of 16.0 min, while the tetravalent **7** eluted earlier at 14.5 min.

Table 4 Transfer of sialic acid to divalent compounds **2**, **3**, **10** and **11** using different proportions of 3'-SL

X	% X		% SX		% S_2X	
	(1:1)[a]	(1:2)[b]	(1:1)[a]	(1:2)[b]	(1:1)[a]	(1:2)[b]
2	52	43	39	45	9	12
3	53	29	43	58	4	13
10	45	32	47	53	8	15
11	61	52	34	38	5	10

Calculated by integration of all sialylated glycoclusters (SX and S_2X) and non sialylated remaining species (X)

[a] Incubations were performed using 1 mM X and 1 mM 3′-SL

[b] Incubations were performed using 1 mM X and 2 mM 3′-SL

S-galactopyranosides as acceptors and inhibitors in the TcTS reaction. Analysis by HPAEC-PAD

Comparison of monovalent glycoclusters Two different monovalent galactopyranosides (compounds **8** and **9**, Fig. 2) were analyzed as acceptor substrates and inhibitors of TcTS. The reaction is shown for compound **8** (Scheme 1). Conditions for incubations with the enzyme were as previously described [28] using 1 mM 3′-SL as donor and 1 mM of the substrate if not otherwise indicated. As HPAE chromatography was also suited for the analysis of the smallest members of the β-thiogalactoside family, the reaction mixtures could be easily followed by this technique (PA100, condition 3, Table 2).

Table 5 Evaluation of compounds **4**–**7** as substrates and inhibitors of TcTS

	Valency	% SX	% S_2X	% Total transfer	% Inhibition
4	4	40	11	51	35
5	8	38	10	48	82
6	3	48	12	60	66
7	4	51	12	63	63

Calculated by integration of the peaks of all sialylated compounds observed in the chromatogram. Incubations were performed using 1 mM X and 1 mM 3′-SL

Table 6 Retention times (t_R) for compounds **4**–**7**, and their polysialylated derivatives using condition 3 and the PA-100 column

		Retention times, t_R (min)						
	Valency	X	SX	S_2X	S_3X	S_4X	S_5X	S_6X
4	4	16.0	20.2	25.1	30.1			
5	8	17.6	20.8	24.1	27.5	31.1	34.9	38.5
6	3	16.6	23.7	33.1/33.8	46.6			
7	4	14.5	19.9	25.4	28.4			

Retention time of compound **8**, with one more free hydroxyl group (namely the Glc 6-OH group) was longer than that of **9**, and their corresponding sialylated derivatives followed the same order. Compound **9** was a better substrate for TcTS reaching 83 % sialylation and suggesting that the linkage to the primary carbon of the glucose, being more flexible, would facilitate the interaction with the enzyme. In accordance **9** caused a higher inhibition (68 %) of sialylation of the natural substrate *N*-acetyllactosamine (LN). We had already reported an enhanced inhibitory activity of **9** with respect to **8** towards the β-galactosidase from *E. coli* [38].

On the other hand, the structurally related lactoside **1**, was shown to be a moderate acceptor of sialic acid, similar to compound **8** (48 % and 46 % of sialic acid transfer, respectively). However, the inhibition of **1** was barely 18 %, in contrast to the general behavior expected for competitive inhibitors, which is usually in agreement with the sialic acid acceptor properties as mentioned above. It must be taken into consideration that other factors, like the reversibility of the reaction, solubility and aggregation, may also play a role in the global process.

Comparison of divalent glycoclusters Four different divalent thioglycoclusters (compounds **2**, **3**, **10** and **11**, Figs. 1 and 2) were analyzed as substrates and inhibitors of TcTS by HPAEC under condition 3 (PA100). Two of them were built on a glucopyranoside platform, **10** substituted with thiogalactoses and **2** with thiolactoses, whereas **11** and **3** were built on trehalose platforms and substituted with thiogalactoses and thiolactoses, respectively.

The products of mono- and disialylation of divalent conjugates **2**, **3**, **10** and **11** could be detected under the conditions described (Table 3). The incorporation of the first sialyl residue did not hamper the introduction of a second one as confirmed by increasing the amount of the donor 3′-SL (see below). It is worthy to point out that **3** and **11** are symmetric and thus, only one monosialylated product can be obtained. On the other hand, two possible monosialylated derivatives are possible for **2** and **10**. Whereas two peaks were detected in the chromatogram consistent with the two possible monosialylated products of **10**, only one peak was observed in the case of **2**, most probably because of the similarities of both sialylated products.

Considering the mono and di-sialylated species, an average of 60 % of sialic acid transfer was obtained, being the lactose-containing derivatives **2** and **3**, slightly better acceptors and moderately better inhibitors than the galactosides **10** and **11**.

In an attempt to determine if the degree of sialylation was dependent on the amount of the donor, another set of experiments using two equivalents of 3′-SL was performed and compared with the incubations in equimolar concentrations described above. The results are summarized in Table 4. As our interest was focused on the distribution of sialic acid over each compound X, the ratio between X and the total X-containing species (X + SX + S_2X), was calculated. When 2 equivalents of 3′-SL were used, the increase in disialylation was significant, although the availability of sialic acid did not linearly correlate with the total sialylation.

Multivalent glycoclusters Four different multivalent glycoclusters were analyzed as acceptors and inhibitors of

Table 7 Transfer of sialic acid to compounds **4, 5, 6** and **7** (1 mM)

	Valency	% X	% SX	% S_2X	% S_3X	% S_4X	% S_5X	% S_6X
4 (1:1)[a]	4	54	33	12	1.4			
4 (1:4)[c]	4	24	23	29	21	3.6		
5 (1:1)[a]	8	-	81	17	2	-		
5 (1:8)[d,f]	8	-	74	20	4	1	-	-
5 (1:8) 60'[e,f]	8	-	27	30	32	6	4	1
6 (1:1)[a]	3	53	38	8.0	0.3			
6 (1:3)[b]	3	18	40	35	3.8			
7 (1:1)[a]	4	64	32	4.0	-	-		
7 (1:4)[b]	4	20	45	29	6.3	0.4		

[a] using 1 mM 3′-SL; [b] 3 mM 3′-SL; [c] 4 mM 3′-SL; [d] 8 mM 3′-SL; [e] 8 mM 3′-SL, 60 min incubation. Calculated by integration of all sialylated glycoclusters and non sialylated remaining species (X)

[f] Compound **5** was not included as it overlaps with 3′-SL under the conditions used. The values represent the relative percentages of the tabulated species

TcTS and the results are shown in Table 5. The similarites in the structures seem to be reflected in their behavior as sialic acid acceptors, being the β-thiogalactosides **6** and **7**, built on maltoside scaffolds, slightly better acceptors than the β-thiolactosides **4** and **5**, which were grafted on trehalose platforms. However, the octalactoside **5** was the better inhibitor for the competitive sialylation of LN. The significant inhibition obtained (82 %) when equimolar concentrations of the substrate and the inhibitor were used, encourages further *in vivo* studies with this ligand.

With the aim to verify that the reaction is useful for the preparation of multisialylated compounds, another set of

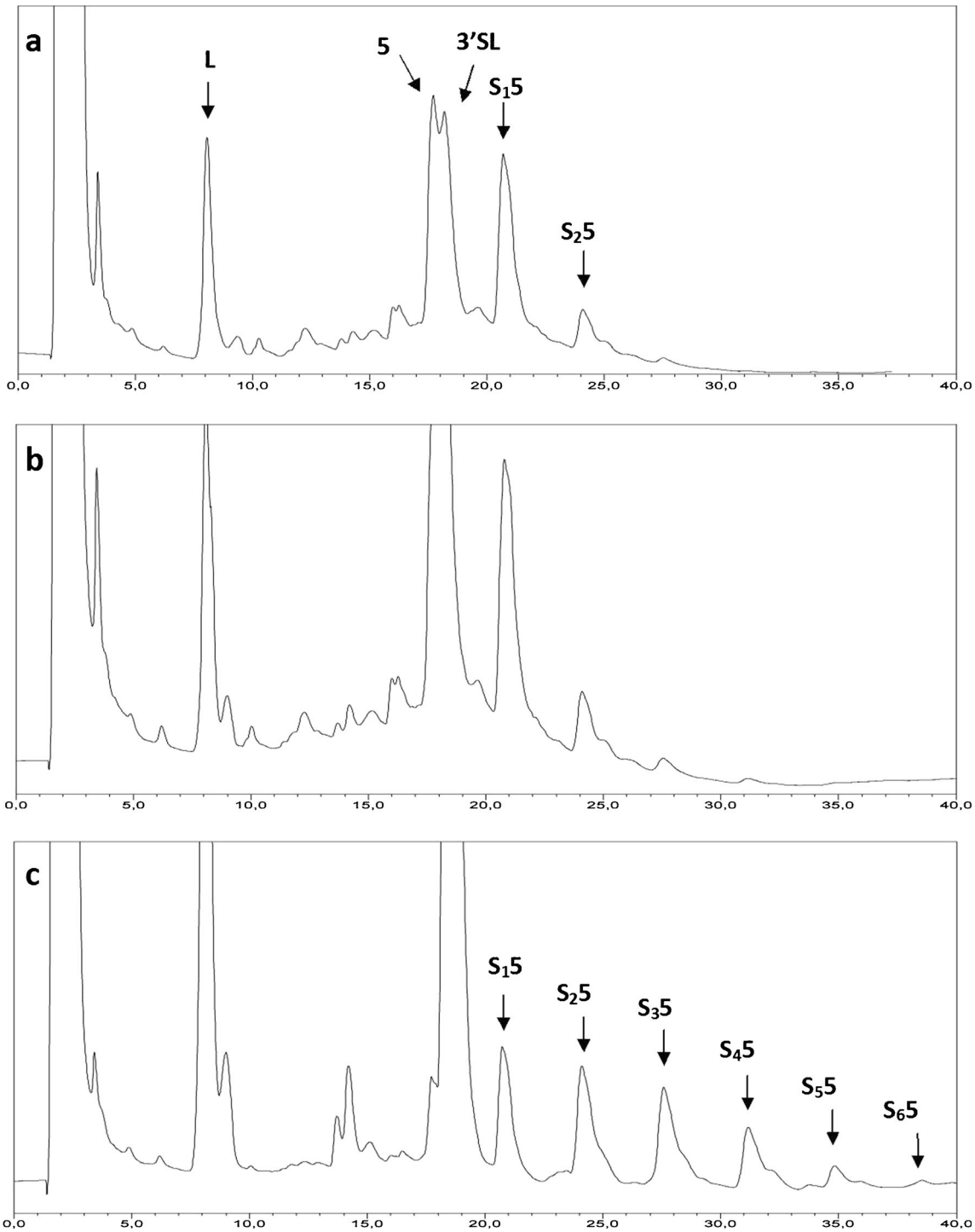


Fig. 3 Polysialylation of **5**. Compound **5** (1 mM) was incubated with (**a**) 1 mM 3′-SL for 15 min; (**b**) 8 mM 3′-SL for 15 min; (**c**) 8 mM 3′-SL for 60 min. Analysis by HPAEC was optimized using condition 3 (CarboPac PA-100 column, linear gradient from 0 to 500 mM NaAcO over 60 min in 100 mM NaOH, flow 0.9 mL/min)

experiments was carried out using different proportions of 3′-SL. The incubations were followed by HPAEC. The retention times of the different sialylated species obtained from glycoclusters **4**, **5**, **6** and **7** are listed in Table 6.

The increase in the concentration of 3′-SL resulted in a substantial rise in the sialylated species (Table 7). For the analysis of the sialylation products of compound **5**, a better resolution of $\mathbf{S_1}$**5** from the remaining 3′-SL was achieved using a PA-100 column (Fig. 3). Using equimolar quantities of the glycocluster **5** and 3′-SL (1 mM) the monosialylated compound $\mathbf{S_1}$**5** could be detected together with lesser amounts of disialylated $\mathbf{S_2}$**5** and trisialylated species $\mathbf{S_3}$**5** (Fig. 3a). The confirmation that the peak at ≈ 21 min (Fig. 3a) corresponded to $\mathbf{S_1}$**5** was achieved by its isolation and characterization by MALDI spectrometry as described below. The retention times observed for the following peaks were consistent with the presence of polysialylated species obtained by successive addition of sialic acid residues, as was later verified for the preparative sialylation of compound **4** (see below, Fig. 4). In the case of multisialylation, the product distribution changed with the incubation time. After 60 min of reaction (Fig. 3c) there was an increase in species with a higher degree of sialylation. The scope of HPAEC was evident for the simultaneous detection of multisialosides carrying up to 6 sialyl residues. Peak shoulders in the chromatograms are ascribed to minor structural differences due to the many possible regioisomers with the same number of sialic acid residues.

Preparative sialyltation of tetravalent compound 4 and octavalent compound 5 To confirm the retention times assigned to monosialylated compounds **4** and **5**, preparative sialylations were performed using **4** or **5** as substrates and 3′-SL as the sialic acid donor. The reactions were monitored by HPAEC and, under the used conditions (PA-100 column, condition 3), 57 % and 63 % of monosilalylation with respect to the total sialylated compounds **4** (Fig. 4) and **5**, respectively, were obtained. The products were purified using an AG1X2 resin (acetate form) column. After elution of neutral sugars with water, the acidic components were eluted with a gradient of pyridinium acetate buffer. For compound **4**, three sialylated species, $\mathbf{S_1}$**4**, $\mathbf{S_2}$**4** and $\mathbf{S_3}$**4**, were isolated and their structures confirmed by MALDI MS spectrometry (Fig S1, see Supporting information). For example, for $\mathbf{S_1}$**4**, a major peak at 2390.67 was detected (negative mode), corresponding to the $[M\text{-}H]^-$ anion, while the diagnostic peaks for $\mathbf{S_2}$**4** and $\mathbf{S_3}$**4** appeared at 2682.55 and 2973.40 respectively (Fig. S3-S5).

For compound **5**, the sialylated derivative with retention time of ≈ 21 min was also isolated. The detection of a peak at m/z = 4549.75 in the MALDI-TOF MS spectra (negative mode), assigned to the $[M\text{-}H]^-$, confirmed the monosialylated structure of $\mathbf{S_1}$**5** (Fig. S5).

Conclusions

Multisialylation of polyvalent glycoclusters could be achieved using the TcTS. The reaction, as well as the inhibitory properties of the complex β-galactopyranosides for the transfer of sialic acid to the natural substrate *N*-acetyllactosamine, can be accurately followed by HPAEC. The eluting conditions can be optimized for the analysis of the neutral multivalent glycoclusters as well as for the simultaneous detection of neutral substrates and their sialylated derivatives, which is of particular interest for quantitative studies on the trans-sialidase reaction. Moreover, this technique is compatible with the analysis of sialosides, which are labile even under mild acid conditions. Usually, a colorimetric assay based on the hydrolysis of MuNANA is used for the analysis of potential inhibitors of TcTS [32]. However, this method does not discriminate between sialidase and trans-sialidase activity. A radioactive method has also been used [25].

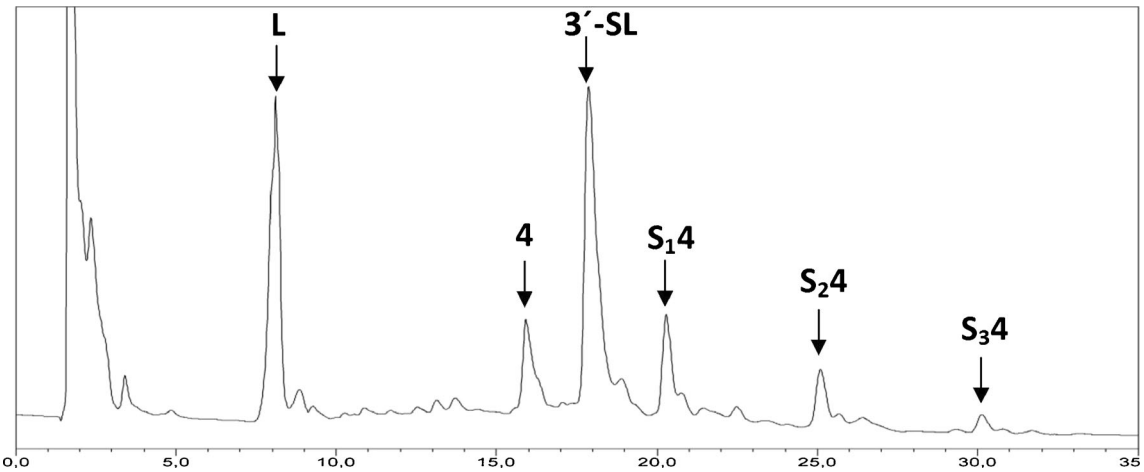


Fig. 4 Polysialylation of **4**. Compound **4** was incubated with 3′-SL for 25 min. Analysis by HPAEC was optimized using condition 3 (CarboPac PA-100 column, linear gradient from 0 to 500 mM NaAcO over 60 min in 100 mM NaOH, flow 0.9 mL/min)

The chemoenzymatic approach using the TcTS, together with HPAEC as an analytical resource, proved to be a convenient strategy for the preparation and analysis of polysialylated compounds with potential as anti-adhesion agents against microbial infections. MALDI-TOF mass spectrometry was chosen as a complementary methodology for the molecular weight determination of the polysialylated glycoclusters.

Acknowledgments We thank O. Campetella and his group from Universidad Nacional General San Martín (UNSAM) Argentina, for the kind gift of trans-sialidase from *T. cruzi*, and the personal of CEQUIBIEM (CONICET-UBA) for the MALDI experiments. Support for this work from the National Agency for Promotion of Science and Technology, ANPCyT, the National Research Council CONICET and the University of Buenos Aires is gratefully acknowledged. María E. Cano is a fellow from CONICET. Rosalía Agustí, María Laura Uhrig and Rosa M. de Lederkremer are research members of CONICET.

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