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Comparative rates of sialylation by recombinant *trans*-sialidase and inhibitor properties of synthetic oligosaccharides from *Trypanosoma cruzi* mucins-containing galactofuranose and galactopyranose

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Abstract—The mucin-like glycoproteins of *Trypanosoma cruzi* have novel O-linked oligosaccharides that are acceptors of sialic acid in the *trans*-sialidase (TcTS) reaction. The transference of sialic acid from host glycoconjugates to the mucins is involved in infection and pathogenesis. The O-linked chains may contain galactofuranose in addition to the acceptor galactopyranose units. Thus far, the galactofuranose form was found in the mucins of strains belonging to the less infective lineage. The acceptor properties of the chemically synthesized oligosaccharides were now studied in order to correlate their structure with the ability to act as substrates. Recombinant TcTS and sialyllactose as donor were used. The reactions were followed by HPAEC-PAD. The K_m values were calculated for the free sugars, the sugar alditols and the benzyl glycosides. All the compounds showed to be good acceptors of sialic acid. Thus, the introduction of galactofuranose in the mucins of the strains of lineage 1 would not be responsible for the diminished virulence of the strains. The oligosaccharides and derivatives inhibited the transfer of sialic acid to the substrate *N*-acetyllactosamine with IC_{50} values between 0.6 and 4 mM.

1. Introduction

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

The surface of *T. cruzi* is dominated by glycoinositolphospholipids (GIPLs)[4,5] and mucin-like glycoproteins anchored to the membrane by a glycosylphosphatidylinositol moiety.[6,7]

The O-linked chains in these mucin-like, sialic acid acceptors, are linked to the protein by α-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*. Thus far, the galactofuranose form was found in the mucins of strains belonging to lineage 1,[8–11] whereas in the more infective Y[12] and CL[13–15] strains, galactose in the mucins is only present in the pyranose form. The mechanism by which the presence of Gal*f* correlates with the parasite lower virulence has not been fully elucidated. Gal*f* is an antigenic epitope[16] and an immunological reaction could influence the infection. On the other hand, as the Gal*p* units are the acceptors of sialic acid in the *trans*-sialidase reaction that involves the host glycoproteins, it is interesting to study the influence of the coexistence of Gal*f* on these properties. It was previously shown[9] that the oligosaccharitols released from the mucins are sialylated by native *trans*-sialidase, however no kinetic or comparative studies were reported. In our laboratory we have chemically synthesized the oligosaccharides-containing

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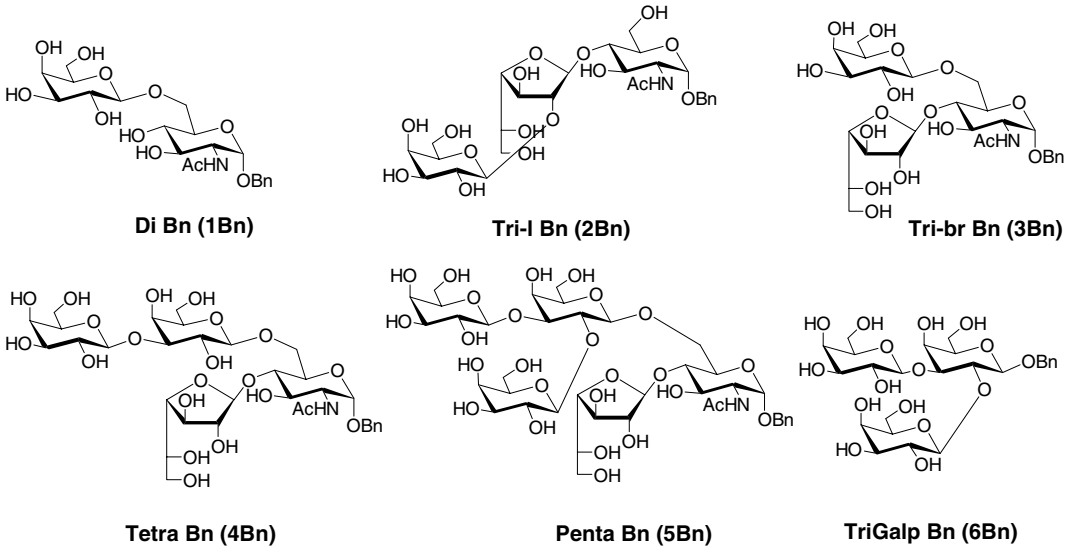
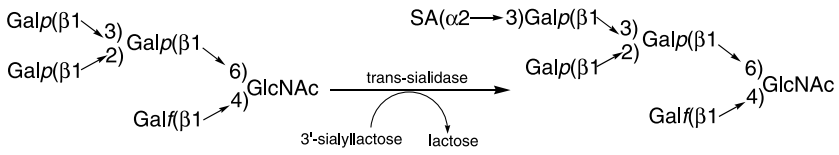
3–5 sugar units (Fig. 1).[17–20] The pentasaccharide is the major oligosaccharide in the mucins of the G strain[9] and presents two terminal β-D-Gal*p* for possible sialylation in the TcTS reaction (Scheme 1). We have recently demonstrated selective monosialylation of the terminal (1 → 3) linked galactopyranose.[20]

Herein we report the results of a comparative study on the rates of sialylation of the mucin oligosaccharides with the aim of relating the presence of Gal*f* with the ability of the oligosaccharides to act as substrates in the *trans*-sialidase reaction. In the trisaccharide **3**, Gal*f* is branching the acceptor unit Gal*p*β(1 → 6)GlcNAc (**1**). The K_m for the disaccharide **1** was also studied for comparison. In a previous work we have found that lactitol is a better acceptor than *N*-acetyllactosamine and is a good inhibitor of the *trans*-sialidase reaction.[21] Thus, the apparent K_m values were also calculated for the sugar alditols and the benzyl glycosides. The oligosaccharides were also tested as competitive inhibitors for the



Di	**1**	Gal*p*(β1 →6)GlcNAc
	1ol	Gal*p*(β1→6)GlcNAcol
	1Bn	Gal*p*(β1→6)GlcNAcOBn
Tri-l	**2**	Gal*p*(β1→2)Gal*f*(β1→4)GlcNAc
	2ol	Gal*p*(β1→2)Gal*f*(β1→4)GlcNAcol
	2Bn	Gal*p*(β1→2)Gal*f*(β1→4)GlcNAcOBn
Tri-br	**3**	Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAc
	3ol	Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAcol
	3Bn	Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAcOBn
Tetra	**4**	Gal*p*(β1→3)Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAc
	4ol	Gal*p*(β1→3)Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAcol
	4Bn	Gal*p*(β1→3)Gal*p*(β1→6)[Gal*f*(β1→ 4)]GlcNAcOBn
Penta	**5**	Gal*p*(β1→2)[Gal*p*(β1→3)]Gal*p*(β1→6)[Gal*f*(β1→4)]GlcNAc
	5ol	Gal*p*(β1→2)[Gal*p*(β1→3)]Gal*p*(β1→6)[Gal*f*(β1→4)]GlcNAcol
	5Bn	Gal*p*(β1→2)[Gal*p*(β1→3)]Gal*p*(β1→6)[Gal*f*(β1→4)]GlcNAcOBn
TriGalp	**6**	Gal*p*(β1→2)[Gal*p*(β1→3)]Gal
	6ol	Gal*p*(β1→2)[Gal*p*(β1→3)]Galol
	6Bn	Gal*p*(β1→2)[Gal*p*(β1→3)]GalOBn

Figure 1. Oligosaccharides from mucins of *Trypanosoma cruzi*. Structures of the chemically synthesized benzyl glycosides are shown. The abbreviations used for all the compounds analyzed are explained above.



Scheme 1. Sialylation of the major oligosaccharide in the mucins of the G strain.

transfer of sialic acid from sialyllactose to the conventional substrate *N*-acetyllactosamine.

2. Results

2.1. Separation of alditol oligosaccharides of *T. cruzi* mucins by HPAEC-PAD

Analysis of all the synthesized alditol oligosaccharides (compounds **2ol**–**5ol**), previously obtained from the mucins of *T. cruzi*, including Gal*p*(β1 → 6)GlcNAcol (**1ol**) and Gal*p*(β1 → 2)[Gal*p*(β1 → 3)]Galol (**6ol**) for comparison, was performed on a CarboPac PA-20 column under isocratic conditions (Fig. 2). All compounds could be perfectly resolved in less than 20 min and some unexpected retention times were obtained. The pentasaccharide **5ol** differs from the tetrasaccharide **4ol** in the presence of another external galactopyranose, however it eluted earlier under the conditions used. This could be the result of the combination of two factors: the more branched structure of the pentasaccharide and the minor proportion of the furanose form in the pentasaccharide than in the tetrasaccharide. It is known that the presence of a galactofuranose unit retards the elution in an anion exchange system.[13] Comparing the elution positions of the two different branched trisaccharides (**3ol** and **6ol**) it is evident the effect of the galactofuranose. Comparison of the two galactofuranose-containing disaccharides (**2ol** and **3ol**) showed that **3ol**, having a branched structure with an external Gal*f*, eluted later than the linear analogue with an internal one. The same results were obtained for the sialylated derivatives (Table 1) under different column and elution conditions.

2.2. Testing of the synthetic oligosaccharides as sialic acid acceptors in the *trans*-sialidase reaction

The transfer reaction of sialic acid from 3′-sialyllactose catalyzed by the *trans*-sialidase of *T. cruzi* (TcTS) was performed with the oligosaccharides **1**–**6** and the corresponding benzyl glycosides and alditols (Fig. 1). The

Table 1. Relative retention times in HPAEC-PAD of sialylated synthetic oligosaccharides from the mucins of *T. cruzi*, under two different conditions

Compound		Retention time[a]	
		Condition 1	Condition 2
Di	**1**	0.95	0.94
	1ol	0.45	0.50
	1Bn	0.55	0.58
Tri-l	**2**	2.94	1.39
	2ol	1.26	0.92
	2Bn	1.13	0.90
Tri-br	**3**	3.71	1.55
	3ol	0.88	0.94
	3Bn	1.55	1.07
Tetra	**4**	3.91	1.65
	4ol	2.29	1.15
	4Bn	2.46	1.20
Penta	**5**	2.95	1.39
	5ol	0.81	0.97
	5Bn	0.76	0.95
TriGalp	**6**	1.34	1.10
	6ol	0.54	0.60
	6Bn	0.72	0.75

[a] Relative to 3′-sialyllactose, t_R = 7.8–8.3 min under Condition 1 and 14.1–14.3 min under Condition 2.

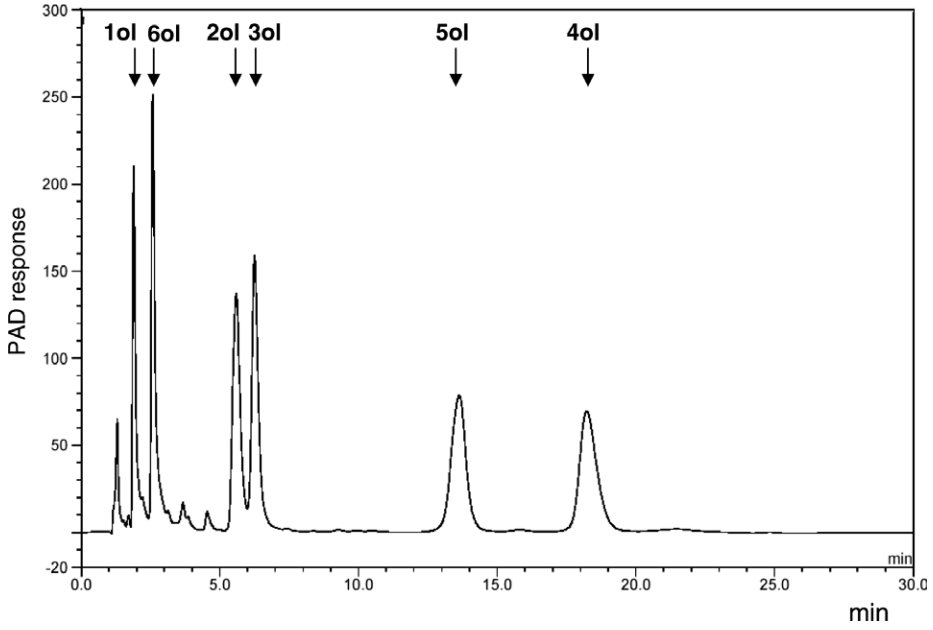


Figure 2. HPAEC-PAD on a CarboPac PA-20 column eluted with 80 mM NaOH of the chemically synthesized oligosaccharitols from *T. cruzi* mucins. The numbers correspond to: **1ol**, Gal*p*(β1 → 6)GlcNAcol; **2ol**, Gal*p*(β1 → 2)Gal*f*(β1 → 4)GlcNAcol; **3ol**, Gal*p*(β1 → 6)[Gal*f*(β1 → 4)]GlcNAcol; **4ol**, Gal*p*(β1 → 3)Gal*p*(β1 → 6) [Gal*f*(β1 → 4)]GlcNAcol; **5ol**, Gal*p*(β1 → 2)[Gal*p*(β1 → 3)]Gal *p*(β1 → 6)[Gal*f*(β1 → 4)]GlcNAcol; **6ol**, Gal*p*(β1 → 2)[Gal*p*(β1 → 3)]Galol.

Table 2. Kinetic parameters for the mucin oligosaccharides and derivatives in the TcTS reaction

Compound		K_m (mM)	V_{max} (nmoles/mg min)	Relative V_{max}[a]	Relative V_{max}/K_m	IC_{50} (mM)
Di	**1**	0.17 ± 0.03	0.409 ± 0.031	1	5.88	2.22 ± 0.17
	1ol	0.08 ± 0.02	0.077 ± 0.005	0.19	2.35	0.98 ± 0.03
	1Bn	0.05 ± 0.01	0.132 ± 0.047	0.32	6.46	1.11 ± 0.01
Tri-l	**2**	0.27 ± 0.01	0.217 ± 0.006	0.53	1.96	4.43 ± 0.15
	2ol	0.09 ± 0.01	0.260 ± 0.024	0.64	7.07	1.67 ± 0.09
	2Bn	0.31 ± 0.03	0.225 ± 0.001	0.55	4.23	0.85 ± 0.01
Tri-br	**3**	0.15 ± 0.01	0.091 ± 0.003	0.22	1.48	1.33 ± 0.12
	3ol	0.08 ± 0.01	0.476 ± 0.020	1.16	14.56	0.94 ± 0.10
	3Bn	0.15 ± 0.02	0.009 ± 0.001	0.02	0.15	0.86 ± 0.08
Tetra	**4**	2.35 ± 0.50	0.223 ± 0.017	0.55	0.23	0.76 ± 0.08
	4ol	0.18 ± 0.01	0.084 ± 0.007	0.21	1.14	1.26 ± 0.12
	4Bn	1.07 ± 0.05	0.055 ± 0.003	0.13	0.13	1.14 ± 0.10
Penta	**5**	0.17 ± 0.01	0.070 ± 0.003	0.17	1.01	0.85 ± 0.07
	5ol	0.82 ± 0.05	0.146 ± 0.001	0.36	0.44	0.61 ± 0.02
	5Bn	0.10 ± 0.01	0.071 ± 0.003	0.17	1.73	0.70 ± 0.03
TriGalp	**6**	0.08 ± 0.01	0.121 ± 0.001	0.30	3.71	0.86 ± 0.03
	6ol	0.25 ± 0.03	0.129 ± 0.008	0.31	1.26	0.93 ± 0.01
	6Bn	0.17 ± 0.01	0.089 ± 0.07	0.22	1.28	1.13 ± 0.05

[a] Relative V_{max} are expressed with respect to the disaccharide **1**.

reactions were followed by high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) under two different conditions.

In all cases, the reaction was fast and reached the equilibrium in about 15 min. In Table 1, the elution positions relative to 3′-sialyllactose (3SL) of all the sialylated oligosaccharides obtained in the *trans*-sialidase reaction are recorded. As expected, all the alditols and benzyl glycosides are less retained than the free oligosaccharides. From the three trisaccharides, the one containing only galactopyranose (TriGalp: Gal*p*(β1 → 2)[Gal*p*(β1 → 3)] Gal*p*) is eluted well ahead than the other two (Tri-l: Gal*p*(β1 → 2)Gal*f*(β1 → 4)GlcNAc and Tri-br: Gal*p* (β1 → 6)[Gal*f*(β1 → 4)]GlcNAc). All the derivatives of the branched trisaccharide (Tri-br) eluted later than those of the linear trisaccharide (Tri-l) with the internal Gal*f*, with the exception of the alditol **3ol** under condition 1.

Interestingly further branching of the tetrasaccharide (Tetra) to afford the pentasaccharide (Penta) caused a lower retention time under both conditions. We estimated the apparent K_m for each acceptor to quantify the relative affinity of TcTS for the substrates (Table 2). Gal*p*(β1 → 6)GlcNAcol (**1ol**), Gal*p*(β1 → 6)[Gal*f*(β1 → 4)] GlcNAcol (**3ol**), and Gal*p*(β1 → 2)Gal*f*(β1 → 4) GlcNAcol (**2ol**) had similar K_m values in the range of that for lactitol (0.05 mM). These results show that the presence of galactofuranose as internal or branching unit does not interfere with the reaction. They also showed high catalytic efficiency as measured by the relative V_{max}/K_m values. The tetra- and pentasaccharide alditols, both containing a terminal non-reducing galactofuranose in addition to the acceptor galactopyranose, are poorer acceptors than the smaller alditols.

2.3. Testing of the synthetic oligosaccharides as inhibitors of sialylation of *N*-acetyllactosamine in the *trans*-sialidase reaction

Inhibition of sialylation of the substrate *N*-acetyllactosamine by the synthetic mucin oligosaccharides and derivatives was studied (Table 2). The IC_{50} values ranged between 0.6 and 4.4 mM being the best competitive inhibitor the alditol of the pentasaccharide (**5ol**). The pentasaccharide is the most abundant oligosaccharide in the mucins of the epimastigote and metacyclic forms of *T. cruzi*.[9] Accordingly, also the free pentasaccharide and its benzyl glycoside showed low values of IC_{50}. Again, no correlation between the presence of galactofuranose and the inhibition values could be inferred.

3. Discussion

The O-linked oligosaccharides in the mucins of *T. cruzi* may be modified with galactofuranose.[8–11] This modification was only found in strains belonging to the less infective lineage 1. Since the *trans*-sialidase was related to the infection, it was important to study if the Gal*f* interferes with the *trans*-sialidase reaction. In this article, we show that the presence of galactofuranose is not related with the acceptor properties of the galactopyranose in the oligosaccharides. Although it was previously shown that the oligosaccharide alditols obtained from the mucins were acceptors of sialic acid, no quantitative data were reported. The present results showed that the introduction of an external galactofuranose in going from Gal*p*(β1 → 6)GlcNAcol (**1ol**) to Gal*p*(β1 → 6)[Gal*f*(β1 → 4)] GlcNAcol (**3ol**) does not affect the sialylation of the Gal*p*. Neither the presence of an internal Gal*f* in Gal*p*(β1 → 2)Gal*f*(β1 → 4)GlcNAcol (**2ol**)

impaired the reaction. Thus, the diminished virulence of the strains belonging to lineage 1 is not caused by interference of sialylation by galactofuranose. Probably, the antigenicity of galactofuranose-containing glycoconjugates accounts for parasite inactivation by the mammalian immune system.[16] In addition, in the present paper we report for the first time the elution time values with respect to 3′-sialyllactose of the mucin sialylated oligosaccharides. Two different conditions were used for the HPAEC-PAD analysis. We also report the elution times for the chemically synthesized neutral alditols previously obtained by reductive elimination of the mucins. This sensitive method could be advantageous for the characterization of strains-containing galactofuranose.

Few inhibitors were reported for TcTS and they were mostly directed to the sialic acid binding site. The known sialidase inhibitor, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), has to be used ~100 times more concentrated to inhibit TcTS.[22] The 2,3-difluor derivative of sialic acid was also required in high concentrations (20 mM) to inactivate the enzyme completely.[23] Cylclohexene phosphonate derivatives were also reported as weak inhibitors.[24] Compounds directed to the lactose binding site were also inhibitors for the sialylation of *N*-acetyllactosamine.[21] In particular lactitol also prevented the apoptosis caused by the *trans*-sialidase of *T. cruzi*.[25] The alditols analyzed in the present work showed IC_{50} values in the same order of lactitol. Derivatization of these oligosaccharitols could lead to better inhibitors of the TcTS reaction.

4. Experimental

4.1. Oligosaccharide substrates

The mucin oligosaccharides, their alditols, and benzyl glycosides were prepared as described.[17,18,20,26] The synthesis of Gal*p*(β1 → 2)Gal*f*(β1 → 4)GlcNAcol will be described elsewhere.

4.2. General procedures

For the sialylation experiments a recombinant TcTS expressed in *Escherichia coli* was kindly provided by A. C. C. Frasch (UNSAM, General San Martin, Buenos Aires, Argentina). 3′-Sialyllactose was obtained from bovine colostrum by an adaptation of a reported method.[27]

Analysis by HPAEC-PAD was performed using a Dionex DX-3000 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 eluted with 60 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 ml/min at room temperature.

Condition 2. A CarboPac PA-20 ion exchange analytical column (3 × 150 mm) equipped with a guard column PA-20 (3 × 30 mm) was used with the following program: a linear gradient over 30 min from 20 to 200 mM NaAcO in 100 mM NaOH at a flow rate of 0.5 ml/min at room temperature.

Condition 3. A CarboPac PA-20 ion exchange analytical column (3 × 150 mm) equipped with a guard column PA-20 (3 × 30 mm) was eluted with 20 mM NaOH at a flow rate of 0.4 ml/min at room temperature.

4.3. Enzyme kinetics

Reaction mixtures of 20 μl containing 20 mM Tris–HCl, pH 7 buffer, 30 mM NaCl, 1 mM 3′-sialyllactose as donor, and compounds **1**–**6** as acceptor substrates were incubated with 300 ng purified TcTS for 15 min at room temperature. Samples were then diluted 12 times with deionized water and analyzed by HPAEC. For K_m calculations a set of 6 points corresponding to 0–1 mM of each compound was used. Each incubation was performed at least in duplicate. D-Galacturonic acid was used as internal standard. The extent of sialylation was calculated from the decrease in concentration of 3′-sialyllactose. The K_m and V_{max} were determined using the Lineweaver–Burk method.

4.4. 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 compounds **1**–**6** were incubated with 300 ng purified TcTS for 15 min at room temperature. Samples were then diluted 12 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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