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Expanding the knowledge of the chemical structure of glycoconjugates from *Trypanosoma cruzi* TcI genotype. Contribution to taxonomic studies

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ABSTRACT

One of the main obstacles to the treatment of Chagas disease is the genetic and phenotypical variance displayed by *T. cruzi* strains, resulting in differences in morphology, virulence, pathogenicity and drug susceptibility. To better understand the role of glycoconjungates in Chagas disease, we performed the molecular characterization of the *O*-linked chains from mucins and glycoinositolphospholipids (GIPLs) of the Silvio X10 clone 1 strain. We demonstrated the presence of a β-galactofuranose (β-Gal*f*) unity linked to the *O*-4 position of the α-*N*-acetylglucosamine (α-GlcNAc)*O*-4 in Tc-mucins. GIPLs analysis showed that the lipidic portion is exclusively composed of ceramide and the PI-oligossacharidic portion contains the Man4(AEP)GlcN-Ins-PO_4 core, substituted by ethanolamine-phosphate (EtNP) on the third distal mannose from inositol, which may or may not have a terminal β Gal*f* unity. These results confirm the classification of the Silvio X10/1 strain in group *T. cruzi* I. Again, it is noted that the study of *T. cruzi* surface glycoconjugates confirm the molecular results and the hypothesis that surface glycoconjugates may be interesting biomarker for the differentiation of trypanosomatid strains.

Key words: *T. cruzi*, glycans, mucins, glycoinositolphospholipids.

INTRODUCTION

Discovered in the early 20th century by the celebrated brazilian scientist Carlos Chagas, the parasitic disease bearing his name is caused by the protozoa *Trypanosoma cruzi* (Chagas 1909). Epidemiological data show that around nine million people are infected by *T. cruzi* in the world, with most cases in Latin America. Nonetheless, there is an increasing number of cases in non-endemic regions due to human migration (Rassi Jr et al. 2010, Bern 2015). Chagas disease is considered neglected, despite having the greatest socio-economic impact in Latin America among parasitic diseases, with productivity losses estimated at about 1.2 billion dollars a year (WHO 2012).

Chagas disease has different clinical manifestations, with most patients developing the asymptomatic indeterminate form in the chronic phase. However, around 45% of chronic patients present

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severe clinical manifestations, including cardiomyopathy or/and digestive dysfunctions. The variable degrees of severity for the chronic disease present substantial challenges, representing significant problems for potential drug trial candidate molecules, since there are no suitable determinants of endpoints of efficacy. The only two drugs currently available for treatment can have substantial side effects and variable efficacy (Le Loup et al. 2011, Zingales et al. 2014). The diversity observed for symptoms and severity shows significant variation correlating to endemic data. Whether these differences stem from characteristics derived from the host, environment, parasite strain or a sum of such components, it is still a matter of contention (Macedo et al. 2004, Coura and Borges-Pereira 2010).

T. cruzi strains present a large biochemical and genetic variability (Gomes et al. 2003, Macedo et al. 2004), leading to astounding differences in terms of morphology, tissue tropism, virulence and drug susceptibility (de Diego et al. 1998, Andrade and Magalhães 1997). Such different characteristics stimulated a search for new molecular markers that allow the correlation between protozoan genotype and clinical manifestations, leading to a more complete diagnosis and better treatment protocols. The most up to date classification splits *T. cruzi* strains into six major lineages or discrete typing units (DTU)s, named *T. cruzi* I to VI according to genetic and molecular markers (Zingales et al. 2009, Costales et al. 2015).

The Silvio X10 clone 1 strain is a member of the *T. cruzi* I group that finds frequent use in research models, both *in vivo* and *in vitro* (Marinho et al. 2009, Messenger et al. 2012). It was originally isolated from a *Rodnius prolixus* bug used in a xenodiagnosis test to a Chagas disease patient from the State of Pará in Brazil (Silveira et al. 1979). As TcI group member, it is related to human disease in Amazonia, the Andean countries, Central America, and Mexico, and clinical manifestations include cardiomyopathy. In these regions, chagasic megaoesophagus and megacolon are absent or very rare (Zingales et al. 2012, Miles et al. 2009). A recent study showed that this clone is resistant to traditional drug therapy due to the presence of an ABC transporter (Franco et al. 2015).

T. cruzi surface is coated by a layer of glycoconjugates that play a role in many biological processes like survival, infectivity and parasite permanence in the host (Mendonça-Previato et al. 2013, 2008). Most glycoproteins and glycolipids are attached to the bilayer through glycophosphatidylinositol (GPI) anchors (Previato et al. 2004, Ferguson 1999) and are organized into large groups: glycoinositolphospholipids (De Lederkremer et al. 1991, Previato et al. 1990a), *T. cruzi* mucins (Tc-mucins) (Previato et al. 1994, 1995) and *trans*-sialidases (Previato et al. 1985, Schenkman et al. 1991).

Tc-mucins were described for the first time in 1975 as glycoproteins A, B and C from the epimastigote form of the γ strain (Alves and Colli 1975). Several years later, our group showed that those molecules bear resemblance to mammal mucins (Previato et al. 1994). Tc-mucins protect the parasite against the attack from proteases present in the intestinal tract of triatomines (Mortara et al. 1992). They also play pivotal roles in adhesion and invasion of mammal host cells, being the only possible acceptors of sialic acid in the parasite surface and thus *trans*-sialidase substrates (Ruiz et al. 1993, Previato et al. 1994).

Here, we describe for the first time, the structure of the main oligosaccharide molecules present in the surface of the epimastigote form of the *T. cruzi* Silvio X10/1 strain.

MATERIALS AND METHODS

Reagents

All solvents were purchased from Tedia (Fairfield, OH, USA). Resins and columns were acquired from BioRad (Richmond, CA, USA), and Restek (Bellefonte, PA, USA). Other chemical reagents

were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gas chromatography coupled with mass spectrometry (GC-MS) experiments were run in a Shimadzu GC-17A GC coupled with a Shimadzu GCMS-QP5050A mass spectrometer.

NMR experiments for the analysis of Tc-mucins and GIPL structures were carried out on a Bruker Ascend 500 MHz spectrometer equipped with a 5 mm BBI gradient probe at the Centro Nacional de Ressonância Magnética Nuclear, UFRJ, Brazil.

Parasite Culture

Epimastigote forms of the Silvio X10 clone 1 strain of *T. cruzi* (kindly provided by Dr. Bianca Zingales, Instituto de Química, USP, SP, Brasil) were kept in LIT (Liver Broth Infusion) medium (Camargo 1964), supplemented with hemin (10 µg/mL), folic acid (20 µg/mL), 10% fetal bovine serum (FBS) and gentamycin (25 µg/mL) at 28 ºC for seven days.

A pre-inoculum of 100 mL of LIT medium containing 10 mL of the *T. cruzi* culture described above was cultivated for 5 days at 28 °C and inoculated into 1L of the same medium (with 5% SFB) and kept under the same conditions for 5 more days. The parasites were then centrifuged for 10 minutes at 6000 *g*, washed thrice with 0.9% NaCl and the pellet, frozen. The wet weight obtained was approximately 90 g.

Total Carbohydrate Analysis

Approximately 2 x 10^7 parasites were washed with phosphate-buffered saline (PBS) and lyophilized. The material was subsequently submitted to a metanolisys reaction (18 hours at 80 °C), extracted with heptane and derivatized with 1:1 (v/v) mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1hour at room temperature). For the GC-MS analysis a DB-1 (30 m x 0.25 mm) column was used with Helium as carrier at a temperature range from 120 to 240 °C (2 °C/ min).

Glycoconjugates Purification

The defrosted cell mass was submitted to extraction according to Mendonça-Previato (Mendonça-Previato et al. 1983). After the extraction, the phenolic phase was discarded along with the interface and the aqueous phase was collected and dialyzed (Spectra 45 mm x 29 mm) for 48 hours in running water. The obtained material was lyophilized, solubilized in distilled water and applied to a Biogel P-10 (Bio-Rad, USA) column, being eluted with distilled water at a constant flow of 0.5 mL/min. Carbohydrate presence was detected through phenol/sulphuric acid assay (Dubois et al. 1956). The fractions containing GIPLs and Tc-mucins were collected, lyophilized and submitted to an extraction with a mix of chloroform, methanol and water (10:10:3) during 48 hours under heat and agitation. The insoluble TC-mucin rich part was filtered out, solubilized in distilled water and lyophilized. The soluble fraction containing GIPLs was concentrated in rotatory evaporator, washed with distilled water and lyophilized.

The purification was evaluated by electrophoresis in 15% polyacrylamide gel (SDS-PAGE) with a voltage of 90 V. 50 µg of the material were diluted in sample buffer (100 mM Tris-HCl pH 6.8; 2% SDS; 10% 2-β-mercaptoethanol; 0.012% glycerol and bromophenol blue), heated in boiling water for 5 minutes and applied into the gel. The presence of carbohydrates was revealed by Schiff staining (Fairbanks et al. 1971).

Tc-mucin Analysis

The material containing Tc-mucins was submitted to methanolysis with methanol-HCl at 80 °C for 18 hours. After the reaction, the fatty acids were extracted with n-heptane and derivatized with BSTFA/pyridine (1:1 v/v) for 1 h at room temperature. The products were analyzed by gas-liquid chromatography (GC) on a fused silica column of DB-1 (30 m × 0.25 mm.) using hydrogen as carrier gas.

The column temperature was programed from 120 to 240 °C at 2 °C/min.

The release of *O*-linked carbohydrate chains from Tc-mucins was performed through β-elimination (Previato et al. 1994). After the reaction, the material obtained was eluted through a Dowex 50WX8 ionic exchange column in hydrogen form of 100 mesh size. The material was thoroughly washed with methanol to remove boric acid, completely evaporated at 40 °C, solubilized in distilled water and applied through a Biogel P4 column along with $_{14}$C-labeled glucose. Samples of 300 µL were collected during the elution and monitored with orcinol/sulphuric acid in silica plates (Humbel and Collart 1975) and liquid scintillation (Beckman 6000LL, Beckman, Brea, CA, USA).

Appropriate samples were re-fractioned by HPLC (Shimadzu LC-20AD) in a porous graphitized carbon (PGC) column according to a gradient of 30% acetonitrile in 45 minutes and a total flow of 1 mM/min. Detection was performed with an UV detector module at 220 and 260 nm (Shimadzu SPD-20A).

After permethylation of the *O*-linked oligosaccharide alditols (Previato et al. 1990a), the samples were subjected to methanolysis (as described before). The obtained methyl glycosides were acetylated with acetic anhydride/pyridine (9:1 v/v) for 24 hours at room temperature. The monosaccharides were analyzed by gas chromatography (as described before) and identified by retention time.

GIPL Analysis

In order to separate PI-oligosaccharides from the lipid portion, 25 mg of intact purified GIPLs were submitted to alkaline degradation (Smith and Lester 1974). After adding chloroform and centrifuging the resulting mixture for 5 minutes at 2800 *g*, the organic phase was collected in a new tube. The extraction was repeated three times for an efficient separation. The aqueous phase containing oligosaccharides was neutralized with acetic acid and applied into a Dowex 50WX8 ionic exchange column in hydrogen form of 100 mesh size. The unbound material was lyophilized, re-solubilized in ultrapure water and eluted through a Biogel P4 column and 1 mL fractions were collected every 30 minutes. Fractions were monitored by the orcinol test in silica plates for carbohydrate detection and the positive fractions were grouped and lyophilized.

Fatty Acids and Long-Chain Base Analysis by GC-MS

The chloroformic fraction, gathered after the alkaline degradation described in the previous section, was washed with ultrapure water to remove salt and resuspended in chloroform. 100 µL of this material was evaporated under N_2 flow and submitted to methanolysis. The obtained methyl esthers were *N*-acetylated with acetic anhydride and the fatty acids extracted with heptane for separate analysis. After derivatization with BSTFA and pyridine as described previously, the samples were analyzed by GC-MS in a DB-1 column (30 m x 0.25 mm) with an oven temperature from 180 to 240 °C (3 °C/ min). In order to confirm our findings, the samples were also analyzed by MALDI-TOF in a Voyager DE-PRO MALDI-TOF spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada), equipped with 337 nm nitrogen laser. The instrument was operated in the negative ion reflectron mode at 20 kV accelerating voltage with time-lag focusing enabled in the University of Lille, France. The samples were resuspended in 500 µL of methanol, mixed with DHB (10 mg/mL in methanol) in a 1:1 ratio and 1 µL was spotted on the stainless steel plates.

O-Oligosaccharide Alditols and PI-Oligosaccharides Analysis by NMR Spectroscopy

The purified *O*-linked oligosaccharide alditols and the PI-oligosaccharides were subjected to D_2O exchange three times and finally resuspended in 500 µL of D_2O. Acetone was added as an internal standard.

All experiments were recorded for 2 - 5 mg of *T. cruzi* polysaccharides in 0.5 mL of D_2O at 25 °C using Bruker AVANCE II 600 and 800 MHz and Varian Inova UNITY 500 MHz spectrometers in the NMR facilities of Centro Nacional de Ressonância Magnética Nuclear, Rio de Janeiro, Brazil, with standard pulse sequences for 1D proton, COSY, TOCSY (with mixing times of 60, 100 and 160 ms), ROESY (mixing time 300 ms) and HSQC. Spectra analysis was performed on Topspin software (Bruker Biospin) according to chemical shifts previously described (Previato et al. 1994, Carreira et al. 1996, Todeschini et al. 2001, Jones et al. 2004).

RESULTS AND DISCUSSION

The purification of Tc-mucins was verified by HPLC and Schiff coloration (data not shown). After β-elimination, the *O*-linked glycans present in the Tc-mucins were purified and fractioned by HPLC. The carbohydrates fractions were analyzed by NMR spectroscopy. Figure 1 shows the 1D-^{1}H spectra for those fractions. We are able to discern five different oligosaccharides with progressively higher molecular weight through the addition of β-Gal units from the presence of anomeric peaks. From top to bottom, we can observe a monosaccharide alditol with characteristic signals for the anomeric proton and β-galactofuranose (β-Gal*f*) at 5.10 ppm and for the acetyl group of N-acetylglucosaminitol (2.01 ppm); a disaccharide alditol with the additional anomeric signal of a β-galactopyranose (β-gal*p*) at 4.36 ppm and the subsequent additions of β-gal*p* residues (4.49; 4.84; 4.60 ppm) compounding the structures of tri, tetra and pentasacharide alditols.

Given the results provided by the 1D spectra, the next step was performing 2D experiments in order to further identify the oligosaccharides. The sequence of the carbohydrate residues was established through TOCSY, ROESY and HSQC.

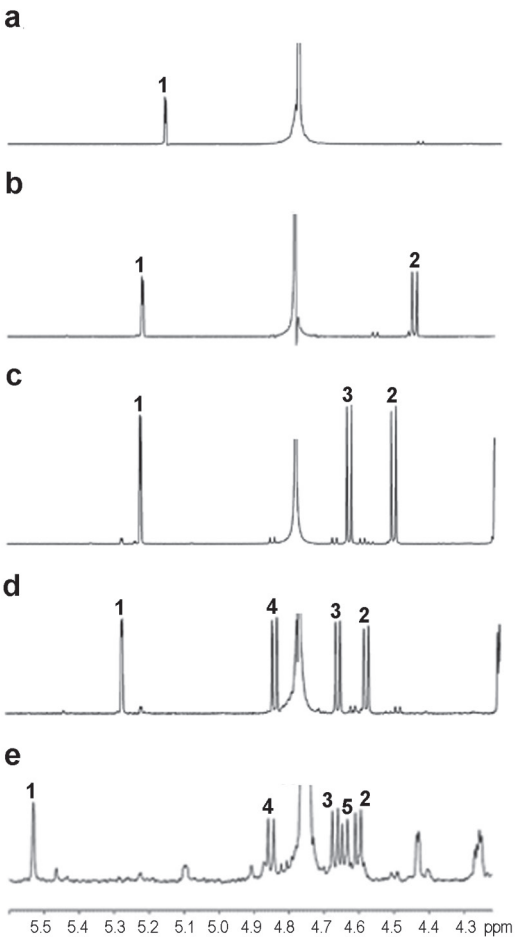

Figure 1 - ^{1}H NMR spectra of *O*-glycans released from mucins of *T. cruzi* strain Silvio X10/1. **a**- monosaccharide alditol; **b**- disaccharide alditol; **c**- trisaccharide alditol; **d**- tetrasaccharide alditol and **e**- pentasaccharide alditol. 1. Gal*f*-β-1-4-GlcNAc-ol; 2. Gal*p*-β-1-6-GlcNAc-ol; 3. Gal*p*-β-1-2-Gal*p*; 4 Gal*p*-β-1-3-Gal*p*; 5. Gal*p*-β-1-2-Gal*f*.

The ROESY spectra revealed inter-residue cross peaks between anomeric protons and linkage carbons (data not shown). The HSQC spectra of all five alditols (Figures 2-6) showed the expected pattern for a furanose ring with C_4 being strongly deshielded and also confirmed the substitution positions hinted at by the ROESY experiments. The chemical shifts observed for these samples were compared with the ones found for the Colombian, Dm28 and G strains (Previato et al. 1994, Todeschini et al. 2009, Agrellos et al. 2003).

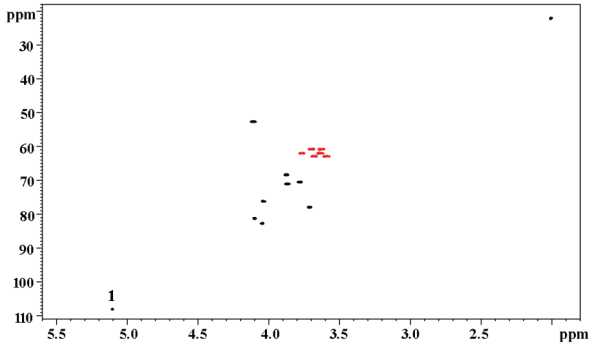

Figure 2 - ^{1}H-^{13}C HSQC spectrum of the monosaccharide alditol from epimatigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red). 1. Gal*f*-β-1-4-GlcNAc-ol.

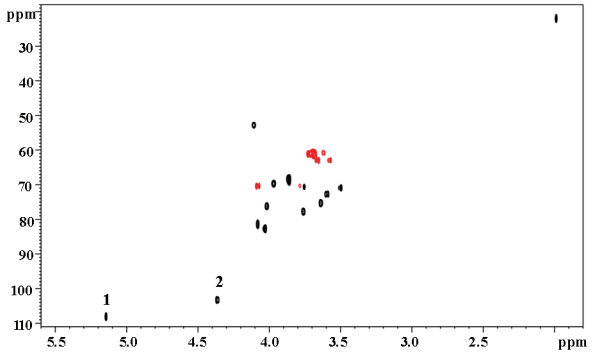

Figure 3 - ^{1}H-^{13}C HSQC spectrum of the disaccharide alditol from epimatigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red). 1. Gal*f*-β-1-4-GlcNAc-ol; 2. Gal*p*-β-1-6-GlcNAc-ol.

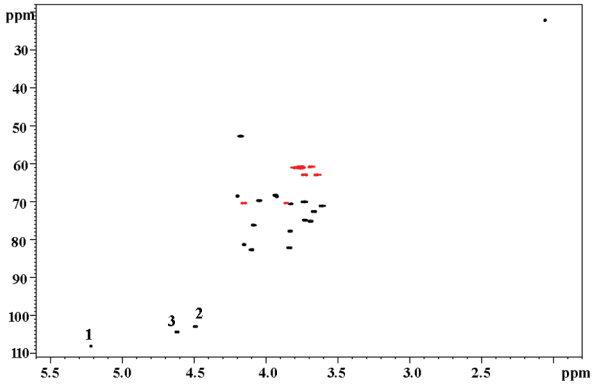

Figure 4 - ^{1}H-^{13}C HSQC spectrum of the trisaccharide alditol from epimatigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red). 1. Gal*f*-β-1-4-GlcNAc-ol; 2. Gal*p*-β-1-6-GlcNAc-ol; 3. Gal*p*-β-1-2-Gal*p*.

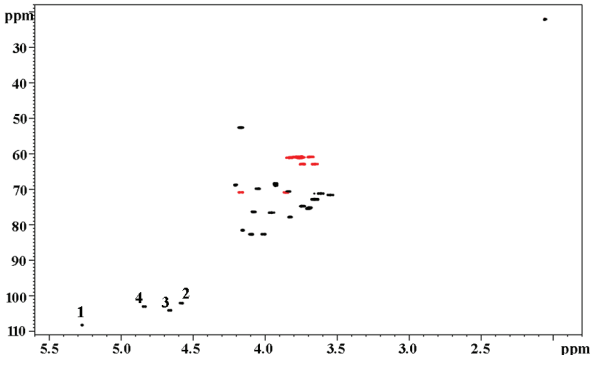

Figure 5 - ^{1}H-^{13}C HSQC spectrum of the tetrasaccharide alditol from epimatigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red). 1. Gal*f*-β-1-4-GlcNAc-ol; 2. Gal*p*-β-1-6-GlcNAc-ol; 3. Gal*p*-β-1-2-Gal*p*; 4 Gal*p*-β-1-3-Gal*p*.

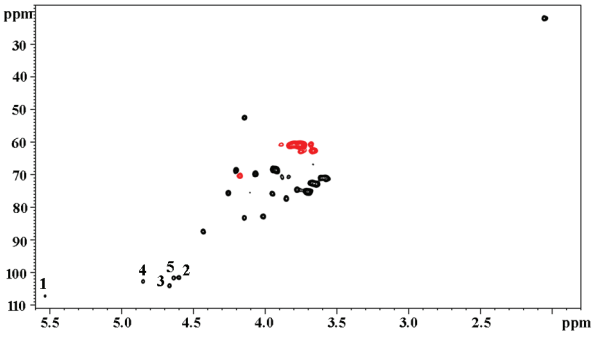

Figure 6 - ^{1}H-^{13}C HSQC spectrum of the pentasaccharide alditol from epimatigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red). 1. Gal*f*-β-1-4-GlcNAc-ol; 2. Gal*p*-β-1-6-GlcNAc-ol; 3. Gal*p*-β-1-2-Gal*p*; 4 Gal*p*-β-1-3-Gal*p*; 5. Gal*p*-β-1-2-Gal*f*.

Confirmation of the structure predicted by NMR spectroscopy was provided through methylation analysis. Oligosaccharides were permethylated, methanolyzed and finally acetylated with acetic anhydride and analyzed by GC-MS. The results shown in Table I corroborate the structure of the oligosaccharides, since they identify the linkage positions for galactose and *N*-glucosamine residues.

Taking into account the results shown thus far, Figure 7 shows the expected structure for the oligosaccharides present in the *O*-linked glycan that make up the carbohydrate portion of the mucin molecules of the epimastigote form of the Silvio X10/1 *T. cruzi* strain.

TABLE I

	Ratio to GlcNAc-ol				
Partially acetylated and methylated glicosides	**Oligo 1**	**Oligo 2**	**Oligo 3**	**Oligo 4**	**Oligo 5**
2,3,5,6-Gal*f*	0.5	0.5	0.5	0.4	0.0
2,3,4,6-Gal*p*		0.5	0.5	1.0	1.6
3,5,6-Gal*f*					0.7
2,4,6-Gal*p*			0.7	0.0	
4,6-Gal*p*				0.7	1.0
1,2,3,5-GlcNAc-ol		1.0	1.0	1.0	1.0
1,2,3,5,6-GlcNAc-ol	1.0				

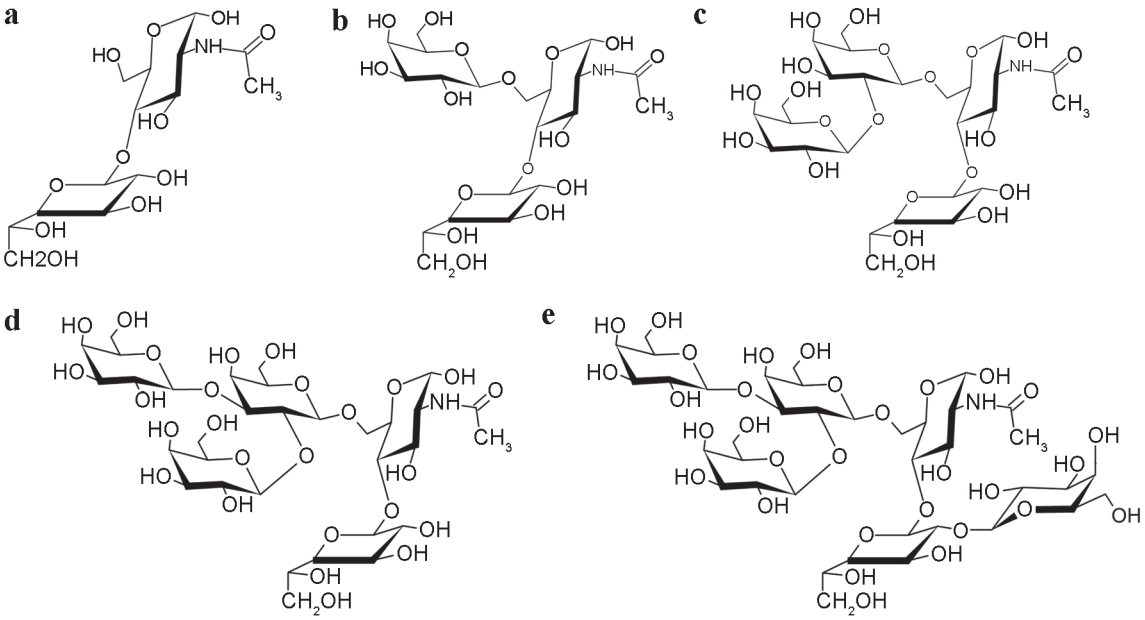

Figure 7 - Representation of *O*-glycan structures from mucins of epimastigotes from *T. cruzi* strain Silvio X10/1. **a-** monosaccharide alditol; **b-** disaccharide alditol; **c-** trisaccharide alditol; **d-** tetrasaccharide alditol and **e-** pentasaccharide alditol.

Next, we analyzed the structure of the GIPLs from the epimastigote surface. The glycan portion was analyzed by GC, after permethylation, methanolyzation and acetylation, showing the presence of mannose and galactose (in pyranose and furanose rings) in a 2:1 ratio (data not shown). The samples were also subjected to NMR analysis (Figure 8), showing the presence of two different structures: one of them containing a terminal residue of Man (1→2), while the other portrays a Gal*f* (1→3) linked to this residue as shown in Figure 9.

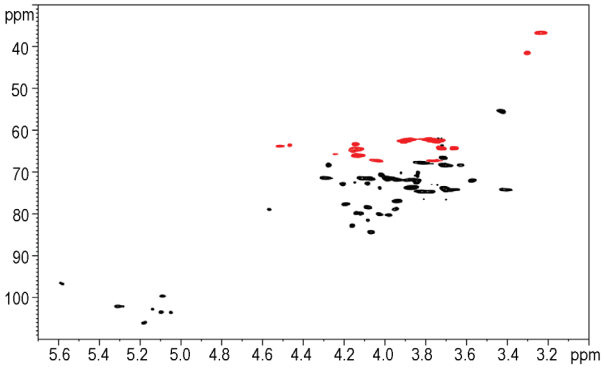

Figure 8 - ^{1}H-^{13}C HSQC spectrum of the PI-oligosaccharide isolated of GIPLs from epimastigotes of *T. cruzi* strain Silvio X10/1 (CH black, CH_2 red).

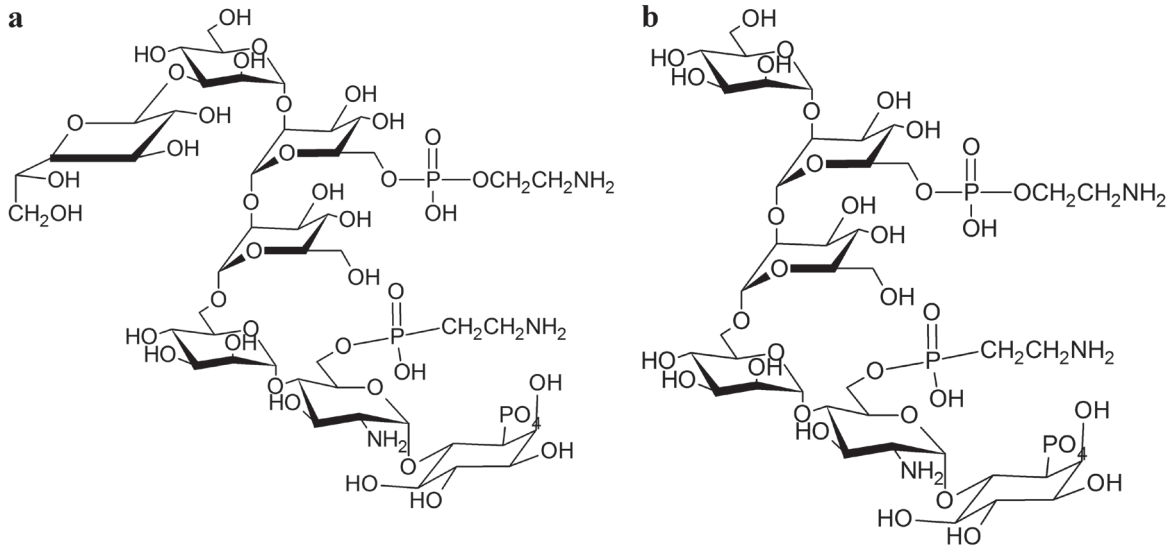

Figure 9 - Representation of PI-oligosaccharide structures isolated from GIPLs of epimastigotes from *T. cruzi* strain Silvio X10/1.

The methyl esters obtained from the lipidic portion of the GIPL molecules were analyzed by GC-MS (Figure 10), revealing the presence of hexa and octadecanoate; as well as tetra, penta and hexacosanoate methyl esters with a predominance of the C24:0 structure corresponding to the ceramide formed by sphinganine and lignoceric acid. This result was further confirmed by MALDI-TOF mass spectrometry (Figure 11) and conforms to structures described by our group for other strains (Previato et al. 1990b).

The major *O*-glycan structures found in Tc-mucins contain Gal*f*, much like the ones displayed by the Dm28c and Colombian strains (Agrellos et al. 2003, Buscaglia et al. 2006).

The GIPL molecules exhibit two different saccharidic structures, one of them containing Gal*f*, while the lipidic portion is composed mainly by a sphinganine long chain and lignoceric acid.

The *T. cruzi* GIPLs find no counterpart in the mammal hosts and that is also valid for the Gal*f* present in the parasite mucins, making them, as well as their biosynthesis pathways, potential therapeutic targets. Unfortunately, there is currently no experimental data based on GIPL-deficient *T. cruzi* strains.

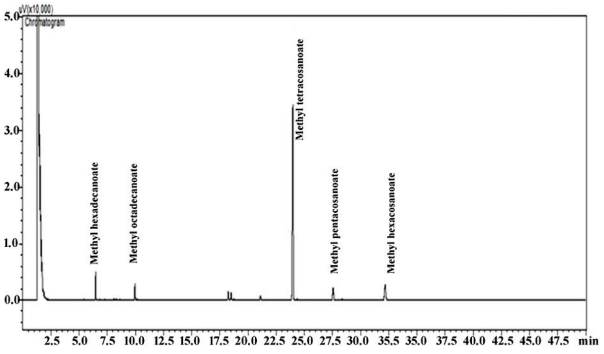

Figure 10 - GC analysis of methyl esthers from GIPLs fatty acids from epimastigotes of *T. cruzi* Silvio X10/1 strain.

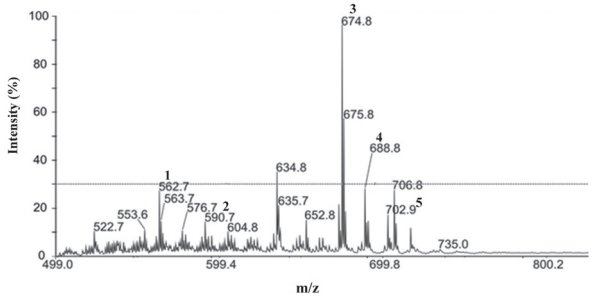

Figure 11 - Negative mode MALDI-TOF mass spectrometry analysis of GIPLs ceramide of epimastigotes of Silvio X10/1 *T. cruzi* strain. 1. N-palmitoylsphinganine; 2. N-stearylsphinganine; 3. N-lignoceroylsphinganine; 4. N-pentacosanoylsphinganine; 5. N-hexacosanoylsphinganine.

GIPLs and mucin-like molecules are abundant in the membrane of parasitic protozoa that are common etiologic agents of medical and veterinary diseases (Ferguson 1997, Mendonça-Previato et al. 2013, Giorgi and De Lederkremer 2011, Buscaglia et al. 2006, DosReis et al. 2002, Lederkremer and Bertello 2001). Although it has been known that structural differences exist in the composition of such molecules among different strains of *T. cruzi* (Mendonça-Previato et al. 2013, Acosta-Serrano et al. 2001, Frasch 2000, Lederkremer and Bertello 2001), there is scarce information regarding its immunobiological functions following the course of infection. In addition, so far, no one has described the relationship between the glycan composition vs. the biological effect of such parasitic glycoconjugates. Certainly, the identification of receptors and signaling pathways triggered by glycan structures expressed by specific *T. cruzi* strains might provide new insights for the development of therapies that inhibit detrimental immune responses or potentiate beneficial immune responses observed during infection. This kind of information, besides extending our knowledge about parasite molecules that stimulate/regulate the host immune system during *T. cruzi* infection, may also reveal interesting biomarkers for the differentiation of trypanosomatid strains. Further efforts are needed in this lively area to better understand the biology of *T. cruzi*.

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