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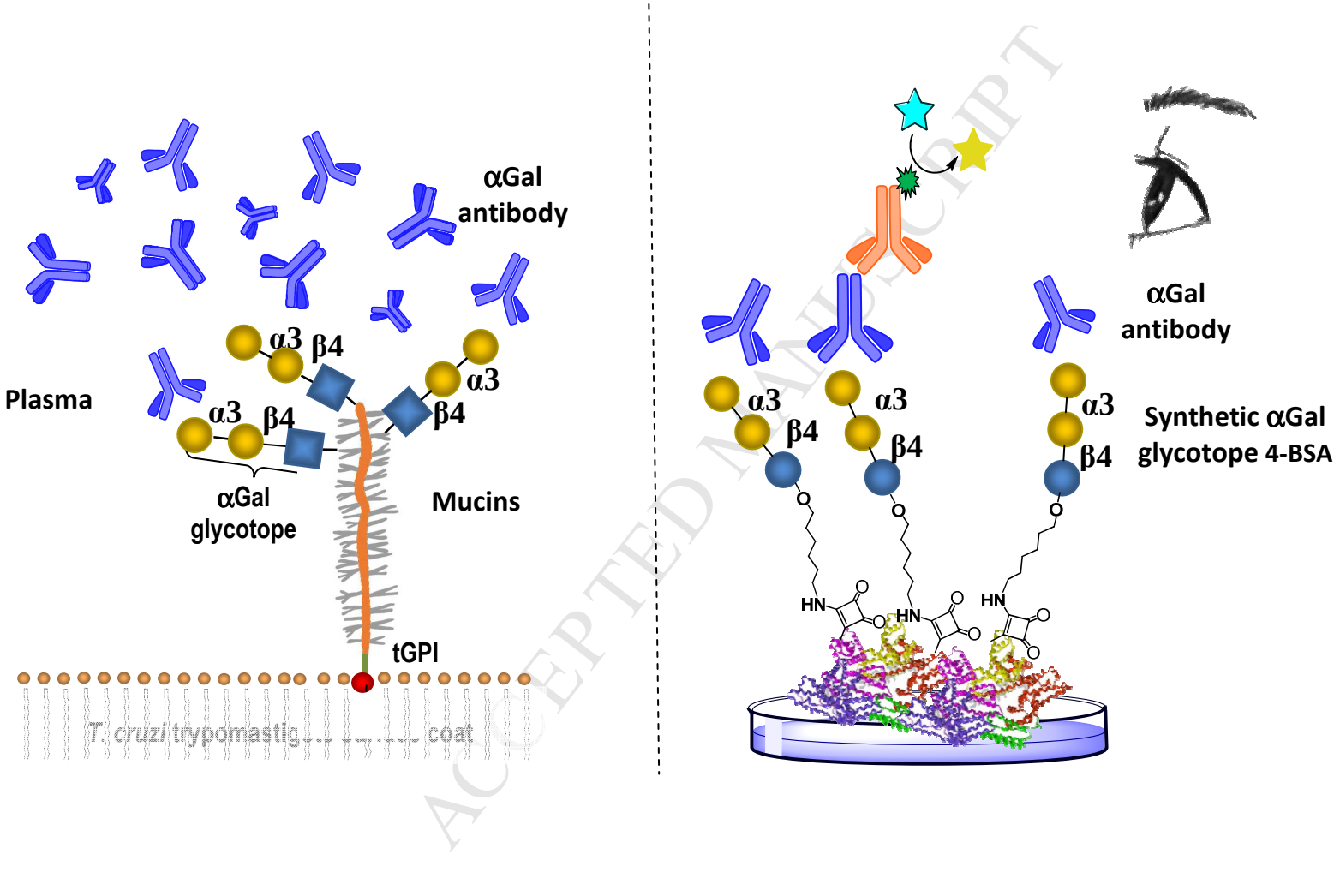

Synthesis and characterization of α-D-Gal*p*-(1→3)-β-D-Gal*p* epitope-containing neoglycoconjugates for Chagas disease serodiagnosis

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ABSTRACT

The immunodominant epitope α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc, expressed in the mucins of the infective trypomastigote stage of *Trypanosoma cruzi* has been proposed for multiple clinical applications, from serodiagnosis of protozoan caused diseases to xenotransplantation or cancer vaccinology. It was previously shown that the analogue trisaccharide, with glucose in the reducing end instead of GlcNAc, was as efficient as the natural trisaccharide for recognition of chagasic antibodies. Here we describe the synthesis of α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* functionalized as the 6-aminohexyl glycoside and its conjugation to BSA using the squarate method. The conjugate of 6-aminohexyl α-D-Gal*p*-(1→3)-β-D-Gal*p* was also prepared. Both neoglycoconjugates were recognized by serum samples of *Trypanosoma cruzi*-infected individuals and thus, are promising tools for the improvement of Chagas disease diagnostic applications.

Keywords: *Trypanosoma cruzi*, anti α-Gal, neoglycoconjugate, squarate conjugation method

1. Introduction

American trypanosomiasis, or Chagas disease, is a life-threatening infection caused by the protozoan parasite *Trypanosoma cruzi*, for which no vaccines or appropriate treatments are available. Current estimations indicate that ~6 million people are already infected and that ~100 million individuals are at risk of infection [1]. In endemic areas of Latin America, the parasite is primarily transmitted to humans through the bite of infected, blood-sucking triatomine bugs. Individuals may also be infected through blood transfusion, organ transplantation, congenital contamination or by the ingestion of tainted food and fluids [1].

The diagnosis of Chagas disease is challenging because it is often asymptomatic in its acute phase and evolves into a chronic stage in which it may present diverse clinical forms [1]. In addition, and due to the very low or null parasitemia during the chronic phase, the detection of parasites in blood samples by direct examination and/or by parasite-amplification methods such as hemoculture or xenodiagnoses is difficult and time-consuming. Several molecular methods, although highly specific, present suboptimal sensitivity and require technological expertise and expensive laboratory equipment (reviewed in [2]). Therefore, detection of anti-*T. cruzi* antibodies remains the most effective method for demonstrating direct exposure to the parasite. At present, the most widely used serologic methods are indirect hemagglutination assay (IHA), indirect immuno-fluorescence assay (IIF), and enzyme-linked immunosorbent assay (ELISA). Synthetic peptides spanning linear B-cell epitopes identified in parasite antigens may be used to increase serodiagnostic assay specificity [3][4][5].

The serodiagnostic potential of carbohydrate-rich molecules found on the surface of *T. cruzi* parasites has also been explored. In particular, the α-galactosyl-containing glycotopes that decorate the glycosylphosphatidylinositol (GPI)-anchored mucins (henceforth tGPI-mucins) of the bloodstream trypomastigote forms [6]. Since tGPI-mucins are expressed at staggering levels (i.e. ~10^6 molecules per parasite), and each may undergo multiple glycosylations, their attached α-galactosyl-containing glycotopes attached to them end up forming a quite dense and immunogenic ‘surface coat’ [7][8][9][10]. Structural data indicated that approximately 10% of the tGPI-mucins’ oligosaccharides consist of the linear

trisaccharide α-D-Gal*p*(1→3)-β-D-Gal*p*(1→4)-D-GlcNAc (**1**) known as "α-Gal" [11]. In the remaining tGPI-mucins glycans, this core trisaccharide is branched with β-D-Gal*p* residues, in as-yet undefined structures [12]. Once on the parasite surface, terminal β-D-Gal*p* units may be linked to sialic acid residues by means of the *trans*-sialidase reaction, thus leading to the formation of sialoglycomarkers crucial for parasite protection and virulence [8][9][10][11][12][13][14][15].

The α-Gal evolutionary distribution is unique, being abundantly expressed across the whole branch of mammals except for Old World monkeys, apes and humans [11]. Instead, these species produce large amounts of so-called anti α-Gal antibodies in response to cross-reactive glycotopes present in commensal enterobacteria [11]. Interestingly, the α-Gal glycotope is only weakly recognized by anti α-Gal antibodies from healthy individuals, whereas this structure and particularly the disaccharide α-D-Gal*p*-(1→3)-β-D-Gal*p* (**2**), elicit a strong humoral response in patients infected with *T. cruzi* [12][16]. Therefore, purified tGPI-mucins and/or synthetic neoglycoconjugates bearing glycotopes **1** and/or **2** were proven valuable tools for the improvement of serodiagnostic and therapy methods in Chagas disease [6][12][16] [17][18][19][20][21][22][23], and *Leishmania* and *Plasmodium infections* [11][24][25][26][27].

In addition to their diagnostic potential, antibodies to the αGal glycotope were proven to trigger the lysis of bloodstream trypomastigotes hence raising the possibility that they are involved in controlling parasitemia during *T. cruzi* infection [9]. Indeed, vaccination of α1,3GalT knockout mice with α-Gal-containing neoglycoconjugates was shown to protect against lethal challenge with *T. cruzi* parasites, chiefly by inducing a strong, cytolytic anti α-Gal antibody-mediated humoral response [28]. Again, quite similar results were obtained with *Leishmania* and *Plasmodium* parasites in α1,3GalT knockout mouse models [29][30][31] indicating that the αGal glycotope constitutes an appealing candidate for the development of a prophylactic vaccine to block main protozoan infections. In broader terms, artificial modulation of the αGal glycotope content was explored as a general strategy to increase/decrease the immunogenicity of xenotransplants and viral and/or cancer vaccines [11][32][33].

In accordance to the numerous clinical applications of αGal, a multiplicity of chemical and genetic engineering synthesis methods have been developed [6][11].

The purpose of our current project is develop and execute an efficient synthesis of an α-Gal glycotope and conjugate it to polypeptide carriers, to obtain diagnosis tools for Chagas disease. It was previously shown that the trisaccharide α-D-Gal*p*(1→3)-β-D-Gal*p*(1→4)-β-D-Glc*p* (**3**), with glucose in the reducing end instead of GlcNAc was as effective for recognition of chagasic antibodies as the natural trisaccharide **1** [16]. Thus, β-(1→4) glycosylation of D-GlcNAc derivatives being a difficult task [34], we decided to prepare trisaccharide **3** functionalized as the 6-aminohexyl glycoside **4** and conjugate it to BSA using the squaric acid chemistry [35], to afford **4-BSA** (Figure 1). The "squarate method" was previously used for the preparation of many glycoconjugates, including experimental vaccines [36]. A BSA conjugate of **3** was previously obtained by a conjugation chemistry which used the sugar glycoside with a thiol reactive linker and maleimide activated BSA [16]. The squarate method has the advantage of using a sugar derivative with a terminal amino group, which is more stable than the thiol group, and it does not require use of a derivatized peptide.

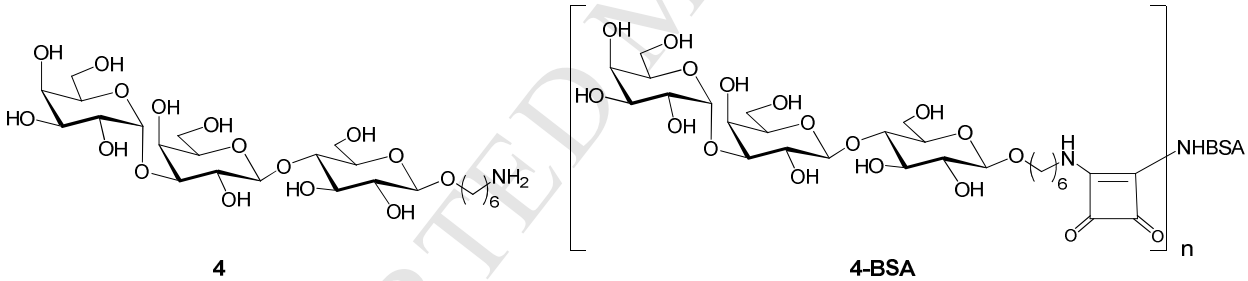


Figure 1. Target trisaccharide and neoglycoconjugate

2. Results and Discussion

*2.1 Synthesis of 6-aminohexyl α-D-Galp-(1→3)-β-D-Galp-(1→4)-β-D-Glcp (**4**)*

The synthesis of different derivatives of trisaccharide **3** has been previously accomplished. Wang et al. developed a downstream stepwise strategy [37]. Stocker and col. [38] and Almeida and col. [16] based their strategies on lactose glycosyl acceptors, lactose being an economical and easily available disaccharide, which already contains the β-D-

Gal*p*-(1→4)-β-D-Glc*p* motif. This fact greatly simplifies the total synthesis. In our case, we also used lactose as starting material for the synthesis of **4**. We planned that after a sequence of reactions leading to a selectively protect lactose, a glycosyl acceptor with the free OH-3' would be accessed, which could be then glycosylated with a α-D-Gal*p* donor. Subsequently, a spacer carrying a reactive amino group would be introduced [39]. Therefore, the retrosynthetic analysis of target molecule **4** indicated that it could be obtained from precursors **6** [40] and **7** (Figure 2).

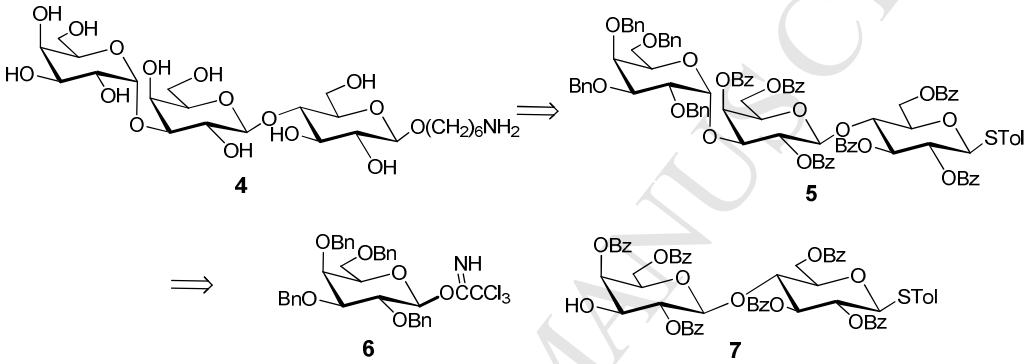
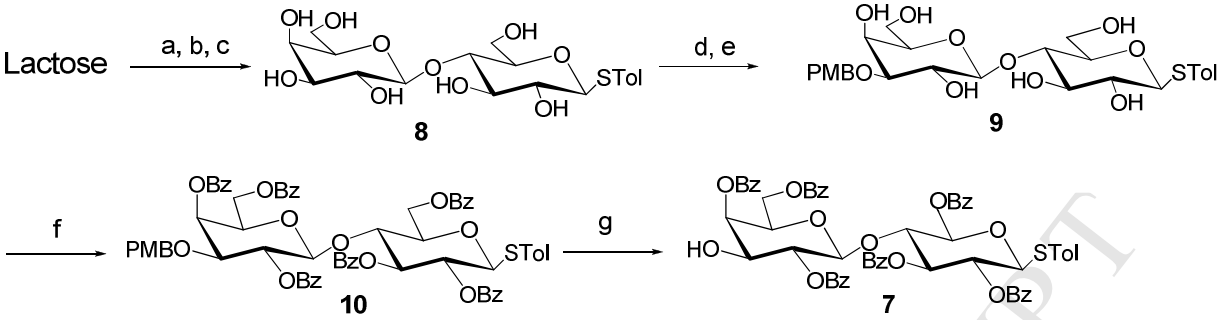


Figure 2. Target trisaccharide **4** and synthetic precursors **6** and **7**.

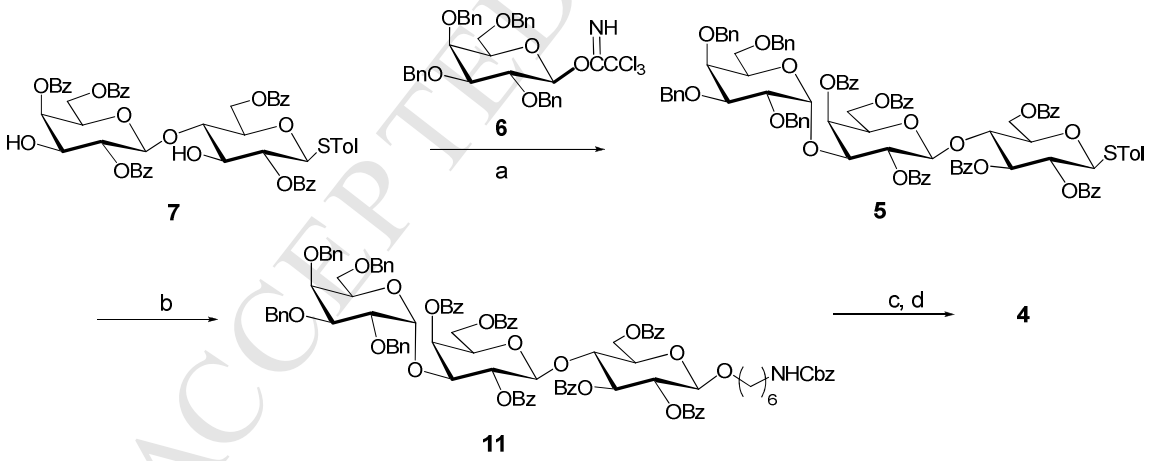
By treatment of lactose with Ac_2O/NaOAc, avoiding the use of pyridine or other solvents, per-*O*-acetylated lactose was obtained [41]. By glycosidation with thiocresol promoted by $BF_3.Et_2O$, followed by Zemplén deacetylation, we obtained **8** [42] (Scheme 1) with the anomeric S-tolyl function which could be later activated for further glycosylation with 6-aminohexanol. The differentiation of lactose OH-3' was performed by selective dibutyltinoxide-promoted alkylation with 4-methoxybenzyl chloride (PMBCl) [43] to yield compound **9** [44]. This strategy was also applied by Almeida et al. [16]. The pronounced downfield shift exhibited by C-3' (80.7 ppm) after 4-methoxybenzylation of **8**, compared with lactose (72.6 ppm) [45], confirmed the site of substitution in compound **9**.

By *O*-benzoylation of **9**, followed by oxidative *O*-demethoxybenzylation, glycosyl acceptor **8** was obtained with 37 % yield, from lactose.



Scheme 1. Synthesis of the lactosyl acceptor **7**. Reagents and conditions: (a) Ac_2O/NaOAc; (b) TolSH, $BF_3.OEt_2$, CH_2Cl_2; (c) NaOMe/MeOH, rt; (d) Bu_2SnO, toluene, reflux; (e) NBu_4I, PMBCl, toluene, reflux; (f) BzCl, pyridine, rt; (g) DDQ, CH_2Cl_2-H_2O, rt.

With derivative **7** in hand, the next step was to glycosylate it with donor **6** [40]. Thus, by treatment of a solution of these compounds with TMSOTf in Et_2O at –55 ºC, the α-linked S-Tol trisaccharide **5** was obtained, as confirmed by the chemical shift of C-1'' (94.6 ppm) and the $J_{1'',2''}$ value (3.4 Hz) observed in the ^{13}C and ^{1}H NMR spectra, respectively (Scheme 2).



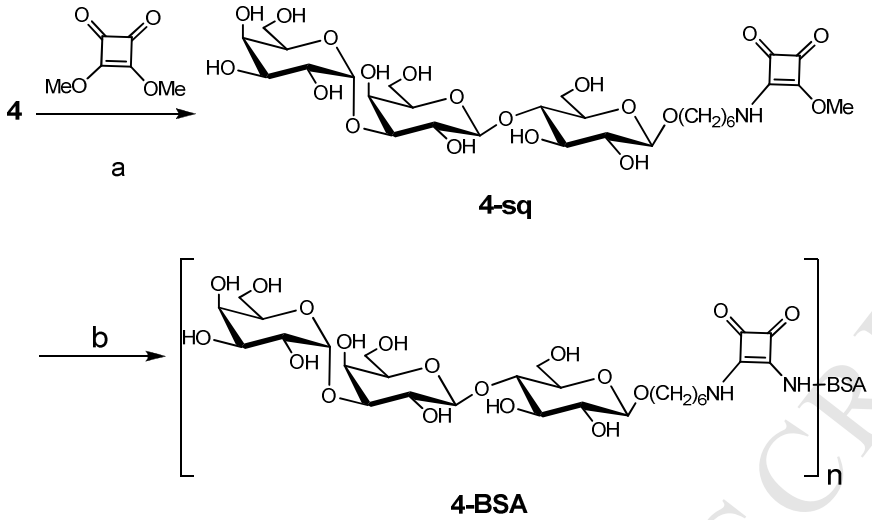
Scheme 2. Synthesis of **4**. Reagents and conditions: (a) TMSOTf, Et_2O, 4Å MS, –55 ºC; (b) 6-Benzyloxycarbonylamino-1-hexanol, NIS, HOTf, CH_2Cl_2, 4 Å MS, rt; (c) NaOMe, MeOH, rt; (d) 10 % Pd/C, H_2, 50 psi, 5% formic acid, MeOH.

The installation of the spacer was performed by activation of **5** with NIS/HOTf and reaction with 6-benzyloxycarbonylamino-1-hexanol, affording trisaccharide **11** in 74 % yield (Scheme 2). The ^{13}C NMR spectrum of **11** showed in the anomeric region signals at δ 101.18 and 101.12 corresponding to C-1' and C-1, respectively, confirming the β-*O*-glycosidic character of the newly formed bond, and a signal at 94.7 ppm corresponding to C-1''.

Deprotection of **11** was done by consecutive *O*-deacylation with sodium methoxide and catalytic hydrogenolysis over 10% palladium on charcoal, in methanol containing 5% of formic acid. Free trisaccharide **4** was obtained in 93 % yield for two steps (Scheme 2). In the anomeric region of the ^{1}H NMR spectrum doublets at δ 5.15 ($J_{1'',2''}$ 3.9 Hz, H-1''), 4.53 ($J_{1',2'}$ 7.8 Hz, H-1') and 4.49 ($J_{1,2}$ 8.0 Hz, H-1) were observed, and the ^{13}C NMR spectrum showed anomeric signals at 102.8 (C-1'), 101.9 (C-1) and 95.4 (C-1'') ppm. The structure of **4** was further confirmed by the m/z observed in the HR ESI mass spectrum, corresponding to the calculated exact mass of the molecule.

*2.2 Conjugation of 6-aminohexyl α-D-Galp-(1→3)-β-D-Galp-(1→4)-β-D-Glcp (**4**) to BSA*

As part of our project, we planned the conjugation of several oligosaccharides both to BSA and to some peptides specifically chosen on the basis of their antigenic activity against α-Gal antibodies [46][47]. We intended to optimize a conjugation protocol for trisaccharide **4** to BSA, to establish an easy procedure to monitor the reaction and extend the conditions to the conjugation with other peptides. Several methods have been described for the conjugation of oligosaccharides to peptides and proteins [48][49][50][51]. However, many of them use oxidative or reducing conditions that can modify the structure of the oligosaccharide to be conjugated. On the other hand, monitoring the progress of the conjugation reaction is usually difficult. We decided to use here the "squarate method" [52] that has the advantage that it is not necessary to derivatize the polypeptide.



Scheme 3. Conjugation of trisaccharide **4** to BSA. Regents and conditions: (a) 3,4-dimethoxy-3-cyclobutene-1,2-dione, KH_2PO_4-NaOH buffer (pH 7), rt; (b) BSA, borax -0.1 M KH_2PO_4 buffer (pH 9), rt.

Linker-equipped trisaccharide **4** was submitted to conjugation by the squarate method using conditions (hapten/peptide ratio, hapten and buffer concentrations, etc) optimized by Kovac and coworkers [53][54]. Dimethyl squarate was used as reagent, since it was described as the most convenient for conjugation [55]. Treatment of **4** with two equiv of dimethyl squarate at pH 7 afforded monomethyl monoamide squarate **4-sq** (Scheme 3). Complete conversion was confirmed by TLC analysis (see Experimental). Purification of **4-sq** from the buffer salts, the excess of dimethyl squarate and the monomethyl squarate formed as by product was performed by filtration through graphitized carbon SPE column. In the NMR spectra of **4-sq** some of the signals were split, as it is usually observed for squarate derivatives due to the double bond nature of the vinilogous C-N linkage [35]. Thus, in the ^{1}H NMR spectrum the signal corresponding to the O*C*H_3 group was observed as two singlets integrating for three hydrogens at 4.38 and 4.36 ppm. The splitting was also observed in the signal for the CH_2NH hydrogens of the linker, which as result of the substitution by the squarate were shifted downfield, from 3.00 ppm (triplet) in **4** to 3.48 and 3.62 ppm (two triplets) in **4-sq.** Diagnostic signals were also observed in the ^{13}C NMR spectrum: split signals corresponding to the O*C*H_3 group at 60.8 and 60.9 ppm and to the cyclobutene at 172-188 ppm.

Subsequently, **4-sq** was treated at pH 9 with BSA, in a 50:1 ratio, to afford the glycoconjugate. Also at this stage TLC was useful to monitor the reaction (see experimental). The conjugation mixture was subjected to dialysis which allowed the separation of the excess of **4-sq** and the salts. Lyophilization then afforded the neoglycoconjugate **4-BSA**, whose MALDI spectrum showed high glycosylation and absence of the unconjugated BSA (Figure 3).

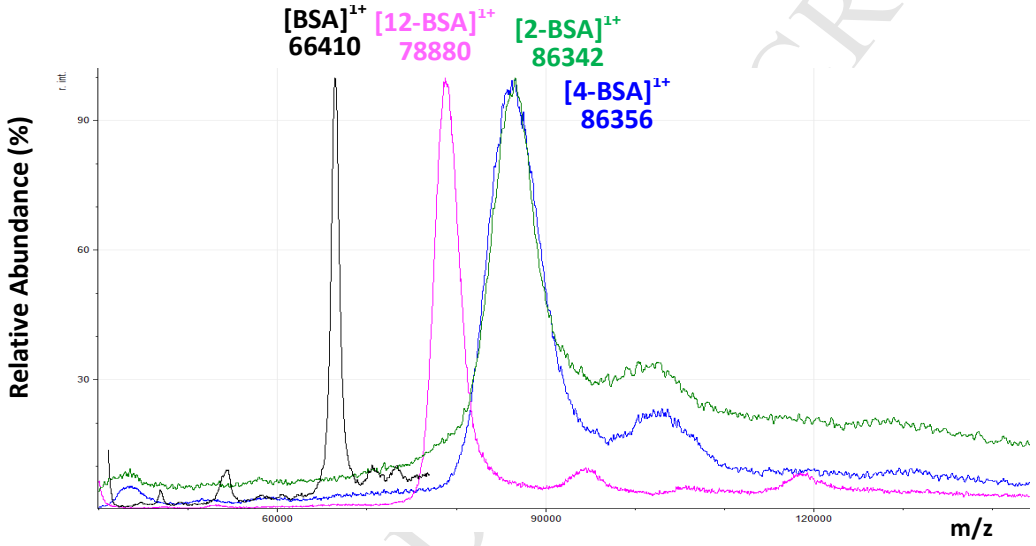


Figure 3. MALDI-TOF-MS spectra of free BSA and conjugates **2-BSA**, **4-BSA** and **12-BSA**.

The synthesis and conjugation to BSA of the 6-aminohexyl glycosides of α-D-Gal*p*-(1→3)-β-D-Gal*p* (**2**) and β-D-Gal*p* (**12**) was performed in a similar way, affording glycoconjugates **2-BSA** and **12-BSA** (Figure 4, synthesis not shown). Aliquots of the conjugation mixtures were taken at different times, and analyzed by SDS-PAGE under denaturing conditions, followed by Coomasie Brilliant blue staining (Fig 5A). Results allowed to conclude that after 24 h the reaction was complete, since no unconjugated BSA was observed (Fig 5A).

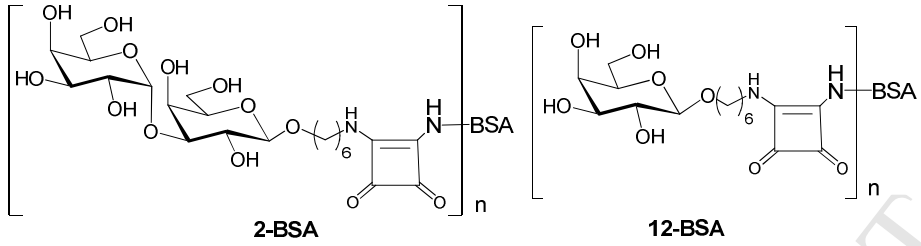


Figure 4. Conjugates **2-BSA** and **12-BSA** prepared by the squarate method.

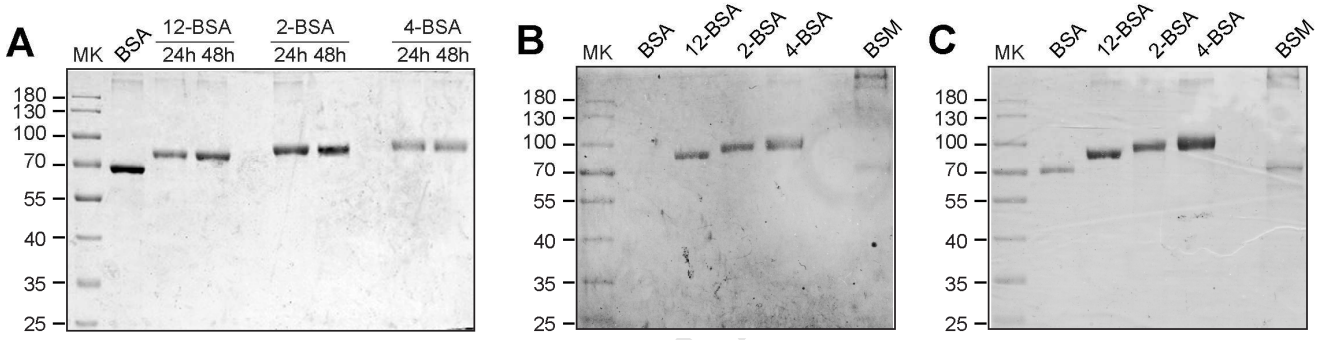


Figure 5. SDS-PAGE (10 %) under denaturing conditions of the neoglycoconjugates. **A)** Samples containing ~1 µg of **12-BSA**, **2-BSA** or **4-BSA** collected at either 24 or 48 h of the conjugation reaction. The gel was stained with Coomassie Brilliant blue. BSA was used as control. **B)** and **C)** Samples containing ~1 µg of **12-BSA**, **2-BSA** or **4-BSA** collected at 48 h of the conjugation reaction. The gel was stained with Schiff reagent **(B),** followed by Coomassie Brilliant blue **(C)**. BSA and bovine submaxillary mucin (BSM) were used as negative and positive controls for Schiff reagent staining, respectively. Molecular markers (in kDa) are indicated.

The neoglycoconjugates were characterized by MALDI-TOF-MS (Figure 3). The apparent molecular mass observed for these neoglycoconjugates (~80 kDa for **12-BSA,** and ~85 kDa for both **2-BSA** and **4-BSA**, Figure 5A) in the SDS-PAGE, strongly correlate with MALDI-TOF-MS data. The sugar load of the conjugates was estimated on the basis of the shift in the molecular mass of the BSA (Table 1). This allowed us to calculate the carbohydrate content per protein molecule, and to estimate that 35, 38 and 29 units of **12**, **2**

and **4**, respectively, were attached to BSA. Accordingly, **12-BSA**, **2-BSA** and **4-BSA** but not BSA yielded a coloured band upon periodate-Schiff staining for carbohydrate (Figure 5B). Gels were next stained with Coomasie Brilliant blue to reveal total proteins (Fig 5C). Densitometric analyses indicated an apparent lower carbohydrate/protein ratio of **4-BSA** (0.321) as compared to **12-BSA** (0.884) and **2-BSA** (0.972), hence further supporting its reduced content (on molar basis) of attached carbohydrates. BSA contains 59 units of lysine per molecule [56]. The degree of glycosylation achieved (Table 1) although not complete, was similar to that obtained by other authors [53].

Table 1. Sugar load of the neoglycoconjugates **2-BSA**, **4-BSA** and **12-BSA**

	Molecular weight (Da)		Glycotope /BSA ratio	Conjugation efficiency (%)
	Calculated	Experimental		
BSA	66500	66410		
12	389.1686			
2	551.2214			
4	713.2742			
12-BSA		78880	34.8	70
2-BSA		86342	38.3	77
4-BSA		86356	29.3	59

The immunological validation of the synthesized neoglycoconjugates was performed using an ELISA format and a panel of human serum samples. As shown in Fig 6A, **2-BSA** and **4-BSA** were recognized in a dose-dependent manner by anti α-Gal antibodies affinity-purified from Chagas positive sera but not by antibodies purified from Chagas negative sera. Conversely, and in accordance with previous results [16], **12-BSA** was not recognized by anti α-Gal antibodies purified from Chagas positive sera (Figure 6A).

We next assessed the recognition of the neoglycoconjugates by chronic Chagasic sera. In this case, an additional conjugate, from BSA coupled to a synthetic peptide spanning an immunodominant sequence from *T. cruzi* Antigen 2 (**BSA-TcAg2**) was included in the analysis for comparison purposes. Paired comparisons between signals obtained for Chagas disease positive and negative sera indicated that **4-BSA, 2-BSA** and **BSA-TcAg2**, but neither **12-BSA** nor **BSA,** display statistically significant differences in their recognition by either population and, hence display positive predictive value for

Chagas disease serodiagnosis (Figure 6B). **4-BSA** and **2-BSA** yielded highly variable and dispersed (i.e. not normally distributed) reactivity profiles, with few positive sera displaying high signals and most of them exhibiting moderate or negative signals (Figure 6B). Most interestingly, **4-BSA** and **2-BSA** displayed quite similar reactivity towards individual serum samples (Figure 6C), strongly suggesting they are exposing similar glycotope(s) on their attached carbohydrates. In this context, it is worth noting that previous glycoarray-based data [16] mapped the binding of anti-α-Gal antibodies from Chagasic patients mainly to the disaccharide α-D-Gal*p*-(1→3)-β-D-Gal*p* (**2**), which is conserved between **4-BSA** and **2-BSA.**

To further address this issue, we performed competitive ELISA assays. Plates were coated with **4-BSA** and assayed with 3 chronic Chagasic sera (selected on the basis of their reactivity to this molecule as shown in Fig 6B). For competition studies, and before being added to the plate, serum samples were incubated for 30 min with 10 µg of compound **12, 2** or **4** diluted in PBS (phosphate buffered saline). As shown in Fig 6D, pre-incubation with compounds **2** or **4** yielded inhibition of **4-BSA** recognition by Chagasic sera whereas pre-incubation with compound **12** or PBS had a negligible effect on this phenomenon. Moreover, competition with compound **4** did not yield an improved inhibition as compared to compound **2**, strongly suggesting that **2** is the main target of anti-α-Gal antibodies elicited during *T. cruzi* infection. Neither compound **2** nor compound **4** were able to inhibit **BSA-TcAg2** recognition by the same set of Chagas positive sera, further supporting the specificity of competition results (Figure 6D).

Taken together, these results indicate i) that anti α-Gal obtained from Chagas positive sera recognize neoglycoconjugates **2-BSA** and **4-BSA**; ii) that this recognition can be explained by or ascribed to their attached carbohydrates; and iii) that, as previously proposed [16], the disaccharide **2** bears the main glycotope recognized by anti-α-Gal antibodies elicited during *T. cruzi* infections. Most importantly, our data support **2-BSA** and **4-BSA** neoglycoconjugates as suitable reagents to improve Chagas disease diagnostic applications, a major need in this field of research.

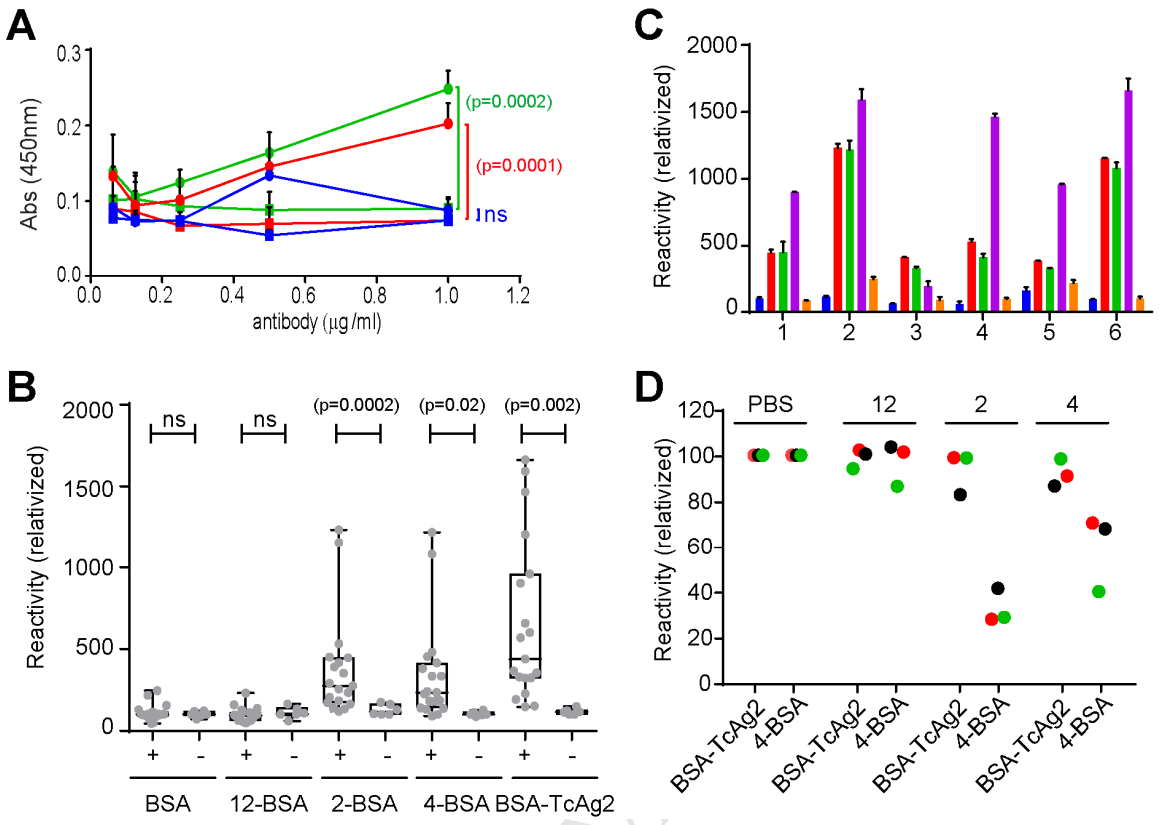


Figure 6. Immunological validation of neoglycoconjugates. A) Reactivity of anti α-Gal antibodies purified from Chagas positive sera (circles) or antibodies purified from Chagas negative sera (squares) against **4-BSA** (red), **2-BSA** (green) and **12-BSA** (blue) neoglycoconjugates. Each point was tested in duplicate and mean ± SD OD_{450nm} values are plotted against antibody concentration. Significant differences between the indicated populations' medians were evaluated by the ANOVA and Tukey post-test, and the p values are informed. Non-significant differences are denoted as ns. B) Dot plot analysis of reactivity values (expressed as the % of reactivity of a control Chagas negative serum assayed in parallel) using unconjugated **BSA**, **12-BSA**, **2-BSA**, **4-BSA** and **BSA-TcAg2**. The ELISA plates were coated with the indicated antigen and incubated with 19 serum samples from chronic Chagas-positive individuals (+) or 6 Chagas negative individuals (-). The median and SD for each group are indicated by box and whiskers. Significant differences between the indicated populations' medians were evaluated by the Mann-Whitney test, and the p values are informed. Non-significant differences are denoted as ns. C) Reactivity (expressed as the % of reactivity of a control Chagas negative serum assayed

in parallel) of 6 selected serum samples from Chagas-positive individuals towards **12-BSA** (blue), **4-BSA** (red), **2-BSA** (green), **BSA-TcAg2** (purple) or BSA (orange). Each point was tested in duplicate and mean ± SD OD_{450nm} values were indicated. D) Reactivity values (expressed as the % of reactivity of PBS-added sera) towards **4-BSA** or **BSA-TcAg2** of 3 Chagasic serum samples treated with 10 μg of the indicated glycan before being added to the plate. Each serum sample is indicated by a different color.

3. Conclusions

The synthesis of trisaccharide **4** was achieved using a synthetic path somewhat different from those reported for similar glycosides**.** Conjugation to BSA of **4** and the 6-aminohexyl α-D-Gal*p*-(1→3)-β-D-Gal*p* was performed by the squarate method which allowed the direct derivatization of the lysines amino groups by the carbohydrate moieties. The synthesized neoglycoconjugates were successfully characterized both structurally and functionally. They were specifically recognized by serum samples of *T. cruzi*-infected patients, indicating they constitute suitable, and much needed tools for improve diagnostic of Chagas disease.

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, charring with 5% (v/v) sulfuric acid in EtOH containing 0.5% *p*-anisaldehyde, or with 0.25% ninhydrin in acetone with traces of pyridine. Column chromatography was carried out with Silica Gel 60 (230–400 mesh, Merck) and for reverse phase with RP18/ graphitized carbon Strata™ catridges (500 mg/6ml) from Phenomenex. 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, relative to chloroform (δ 7.27 for ^{1}H and δ 77.16 for ^{13}C). Assignments of ^{1}H and ^{13}C NMR spectra were assisted by 2D ^{1}H COSY and HSQC experiments. High resolution mass spectra (HRMS) were obtained by Electrospray Ionization (ESI) and Q-TOF detection. UV-MALDI-TOF analysis of the conjugates was performed using a 4800 Plus Maldi TOF-TOF AB-Sciex spectrometer equipped with a NdYAG laser.

4.2 Synthesis

4.2.1 *4-Methylphenyl 3-O-(4-methoxybenzyl)-β-D-galactopyranosyl-1-thio-β-D-glucopyranoside (**9**)*

To a suspension of 4-methylphenyl 1-thio-β-lactoside (**8**, 1.5 g, 3.34 mmol) [42] in dry toluene (80 mL), Bu_2SnO (1.72 g, 2 equiv) was added and the mixture was stirred under reflux using a Dean-Stark trap to remove the formed water. After 4 h complete dissolution of the starting material was observed. The solution was brought to room temperature and after addition of NBu_4I (0.14 g, 0.45 equiv) and 4-methoxybenzyl chloride (0.13 mL, 4.0 mmol, 1,2 equiv) the mixture was refluxed overnight using a conventional reflux condenser. Then, TLC analysis showed the formation of a product of R_f 0.33 (9:1 CH_2Cl_2-MeOH) and some unreacted **8** (R_f 0.08). The mixture was filtrated, the filtrate was concentrated under reduce pressure and column chromatography (9:1 CH_2Cl_2-MeOH). afforded compound **9** as a syrup (0.80 g; 43 %), $[\alpha]_D$ –14 (*c* 1; MeOH). ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.38-6.87 (m, 8H, aromatic), 4.60 (d, 1H, *J* 11.9 Hz, OCH_{2a}Ar), 4.58 (d, 1H, *J* 10.1 Hz, H-1), 4.46 (d, 1H, *J* 11.7 Hz, OCH_{2b}Ar), 4.25 (d, 1H, *J* 7.8 Hz, H-1´), 3.88 (d, 1H, *J* 3.4 Hz, H-4´), 3.74-3.53 (m, 4H, H-6a, H-6b, H-6´a, H-6´b), 3.73 (s, 3H, CH_3O), 3.43-3.30 (m, 4H, H-3, H-4, H-5 and H-5´), 3.23 (dd, 1H, *J* 9.6, 3.1 Hz, H-3´), 3.08 (td, 1H, *J* 8.8, 6.0 Hz, H-2´), 2.27 (s, 3H, CH_3Ar) ppm. ^{13}C NMR (126 MHz, DMSO-d_6): δ 158.7, 136.5, 131.1, 130.9, 130.3, 129.6, 129.4, 113.6 (aromatics), 103.7(C-1´), 87.1(C-1), 80.8 (C-3'), 80.1, 78.8, 76.4, 75.5 (C-3, C-4, C-5 and C-5´), 72.2 (C-2), 70.0 (*C*H_2OAr), 69.7 (C-2´), 64.7 (C-4´), 60.5, 60.4 (C-6 and C-6´), 55.2 (CH_3O), 20.7 (*C*H_3Ar). ESIMS: *m*/*z* calcd for $C_{27}H_{36}O_{11}SNa$ $[M+Na]^+$ 591.1875. Found: 591.1882.

*4.2.2 4-Methylphenyl 2,4,6-tri-O-benzoyl-3-O-(4-methoxybenzyl)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzoyl-1-thio-β-D-glucopyranoside (**10**)*

To a solution of **9** (0.75 g, 1.31 mmol) in anhydrous pyridine (9 mL) stirred at 0 °C, BzCl was added (3 ml, 4.8 equiv), and the stirring was continued overnight at room temperature. TLC analysis showed complete conversion of the starting material to a faster moving product (R_f 0.72, 1:1 hexane-AcOEt). The excess BzCl was quenched by addition of water (1 mL) and after 0.5 h of stirring the solution was diluted with CH_2Cl_2 (100 mL) and washed successively with water (100 mL), saturated $NaHCO_3$ (ss) and water (100 mL), and dried (Na_2SO_4). The solution was concentrated under reduced pressure and co-evaporated several times with toluene. Crude product (**10**, 1.39 g, 89 %) was used for the following reaction without further purification. A fraction of the crude product was purified by column chromatography (3:1 hexane-AcOEt), $[\alpha]_D$ +13 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, CDCl3): δ 8.13-6.51 (m, 38 H, arom.), 5.74 (t, 1H, *J* 9.3 Hz, H-3), 5.61 (d, 1H, *J* 3.3 Hz, H-4'), 5.41 (dd, 1H, *J* 9.7, 7.7 Hz, H-2'), 5.38 (dd, 1H, J 9.7 Hz, H-2), 4.82 (d, 1H, *J* 10.0 Hz, H-1), 4.61 (d, 1H, *J* 8.0 Hz, H-1'), 4.56 (dd, 1H, *J* 11.9, 1.9 Hz, H-6a), 4.52 (d, 1H, *J* 12.5 Hz, OCH_{2a}Ph), 4.41 (dd, 1H, *J* 11.9, 5.4 Hz, H-6b), 4.29 (d, 1H, *J* 12.5 Hz, OCH_{2b}Ph), 4.04 (t, 1H, *J* 9.5 Hz, H-4), 3.82 (ddd, 1H, *J* 9.9, 5.3, 2.0 Hz, H-5), 3.71 (dd, 1H, *J* 11.2, 5.8 Hz, H-6'a), 3.68 (m, 1H, H-5'), 3.67 (s, 3H, CH_3O), 3.59 (dd, 1H, *J* 10.0, 3.3 Hz, H-3'), 3.51 (dd, 1H, *J* 10.8, 6.5 Hz, H-6'b), 2.23 (s, 3H, CH_3Ar) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.8, 165.7, 165.5, 165.4, 165.2, 164.7 (*C*OBz), 159.1, 138.3, 133.7, 133.6, 133.3, 133.25, 133.20, 133.1, 133.0, 130.2, 130.1, 129.9, 129.8, 129.79, 129.71, 129.6, 129.5, 129.4, 129.3, 129.2, 129.1, 129.0, 128.6, 128.4, 128.1, 127.8, 113.6 (aromatics), 101.1 (C-1'), 86.0 (C-1), 77.2 (C-5), 76.0 (C-4), 75.7 (C-3'), 74.1 (C-3), 71.4 (C-5'), 71.3 (C-2'), 70.4 (O*C*H_2Ph), 70.3 (C-2), 66.0 (C-4'), 62.7 (C-6), 61.5 (C-6'), 55.1 (CH_3O), 21.1 (*C*H_3Ar) ppm. ESIMS: *m*/*z* calcd for $C_{69}H_{60}O_{16}SNa$ $[M+Na]^+$ 1215.3448. Found: 1215.3402.

*4.2.3 4-Methylphenyl 2,3,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→3)-2,3,6-tri-O-benzoyl -1-thio-β-D-glucopyranoside (**7**)*

To a solution of **10** (1.64 g, 1.37 mmol) in CH_2Cl_2-H_2O (22:1, 23 mL) DDQ (0.77 g, 3.4 mmol) was added and the solution was stirred in dark during 16 h. TLC analysis

showed the presence of a product with R_f 0.41 (2:1 hexane-EtOAc) and remaining starting material (R_f 0.58). After adding 0.02 g more of DDQ the reaction was completed in 2 h.

The solution was diluted with CH_2Cl_2 (100 mL) and the organic phase was washed successively with saturated $NaHCO_3$ (ss) (2 x 50 ml), water (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (3:1 hexane-EtOAc) to afford **7** as an amorphous solid (1.05 g, 72 %), $[\alpha]_D$ +27 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11- 6.91 (m, 34 H, H-arom.), 5.72 (t, 1H, *J* 9.4 Hz, H-3), 5.49 (d, 1H, *J* 3.4 Hz, H-4'), 5.41 (t, 1H, *J* 9.8 Hz, H-2), 5.29 (dd, 1H, *J* 10.0, 7.8 Hz, H-2'), 4.83 (d, 1H, *J* 10.0 Hz, H-1), 4.72 (d, 1H, *J* 7.9 Hz, H-1'), 4.70 (d, 1H, *J* 1.9, 12.0 Hz, , H-6a), 4.57 (dd, 1H, J 12.0, 5.2 Hz, H-6b), 4.11 (t, 1H, *J* 9.6 Hz, H-4), 3.95 (dd, 1H, *J* 10.0, 3.5 Hz, H-3'), 3.88 (ddd, 1H, *J* 10.0, 5.1, 1.9 Hz, H-5), 3.77-3.65 (m, 2H, H-5'and H-6'a), 3.52-3.43 (m, 1H, H-6'b), 2.25 (s, 3H, C*H*$_3$Ar) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 166.5, 165.9, 165.8, 165.6, 165.5, 165.1 (CO), 138.4, 133.8, 133.5, 133.4, 133.3, 133.0, 130.15, 130.1, 129.9, 129.62, 129.56, 128.8, 128.62, 128.57, 128.5, 128.45, 128.37, 128.1, 127.7 (aromatics), 100.6 (C-1`), 86.1 (C-1), 77.1 (C-5), 75.9 (C-4), 74.0 (C-3), 73.7 (C-2'), 71.9 (C-3'), 71.5 (C-5'), 70.2 (C-2), 70.0 (C-4'), 62.8 (C-6), 61.3 (C-6'), 21.2 (*C*H$_3$Ar) ppm. ESIMS: *m/z* calcd for $C_{61}H_{52}O_{16}SNa$ $[M+Na]^+$ 1095.2874. Found: 1095.2871.

*4.2.4 4-Methylphenyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzoyl-1-thio-β-D-glucopyranoside (**5**)*

To a stirred suspension of trichloroacetimidate **6** (0.45 g, 0.65 mmol) [40] in anhydrous Et_2O (10 mL) containing 4 Å activated MS under argon atmosphere cooled at −55 ºC, a solution of disaccharide acceptor **7** (0.56 g, 0.52 mmol) in anhydrous Et_2O (7 mL) and TMSOTf (38 µL, 0.21 mmol) were added and the stirring was continued for 18 h. The reaction was quenched with Et_3N, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (7:2 hexane-EtOAc). Fractiond of R_f 0.29 gave compound **5** (0.68 g, 65 %) as a foamy solid, $[\alpha]_D$ +27 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.05-6.88 (m, 54 H, H-arom.), 5.75 (t, 1H, *J* 9.3 Hz, H-3), 5.61 (d, 1H, *J* 3.3 Hz, H-4'), 5.58 (dd, 1H, *J* 10.1, 7.9 Hz, H-2'), 5.39 (t, 1H, *J* 9.7 Hz, H-2), 5.06 (d, 1H, *J* 3.4 Hz, H-1''), 4.83 (d, 1H, *J* 9.9 Hz, H-1), 4.65 (d, 1H, *J* 11.5 Hz, OC*H*$_2$Ph), 4.60

(d, 2H, *J* 8.4 Hz, H-1' and H-6a), 4.46 (dd, 1H, *J* 11.9, 5.2 Hz, H-6b), 4.41 (dd, 2H, *J* 12.0, 4.4 Hz, OC*H*$_2$Ph), 4.36 - 4.33 (m, 2H, OC*H*$_2$Ph), 4.23 (d, 3H, *J* 11.7 Hz, OC*H*$_2$Ph), 4.04 (t, 1H, *J* 9.5 Hz, H-4), 3.98 (dd, 1H, *J* 10.1, 3.3 Hz, H-3'), 3.85 (dd, 1H, *J* 10.3, 5.0 Hz, H-5), 3.80 (dd, 1H, *J* 10.1, 3.4 Hz, H-2''), 3.74- 3.68 (m, 2H, H-5'' and H-6'a), 3.59 (t, 1H, *J* 6.5 Hz, H-5'), 3.44 (dd, 1H, *J* 10.1, 2.8 Hz, H-3''), 3.38 (dd, 1H, *J* 11.3, 6.9 Hz, H-6'b), 3.22 (dd, 1H, *J* 9.2, 6.5 Hz, H-6''a), 3.19 (d, 1H, *J* 2.9 Hz, H-4''), 3.11 (dd, 1H, *J* 9.3, 6.1 Hz, H-6''b), 2.23 (s, 3H, C*H*$_3$Ar) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.7, 165.6, 165.5, 165.4, 165.1, 164.4 (CO), 138.7, 138.4, 138.3, 138.2, 133.7, 133.29, 133.22, 133.18, 132.8, 130.1, 129.8, 129.7, 129.63, 129.57, 129.5, 129.3, 129.1, 128.9, 128.6, 128.5, 128.41, 128.38, 128.35, 128.31, 128.2, 128.1, 128.05, 128.02, 128.0, 127.9, 127.8, 128.7, 127.67, 127.60, 127.4, 127.3, 127.2, 127.1, 127.0 (aromatics), 101.2 (C-1'), 94.6 (C-1''), 85.9 (C-1), 78.5 (C-3''), 77.2 (C-5), 76.0 (C-4), 75.1 (C-2''), 74.8 (C-4''), 74.4 (O*C*H$_2$Ph), 74.1 (C-3), 73.2 (O*C*H$_2$Ph), 73.1 (O*C*H$_2$Ph), 73.0 (C-3'), 72.3 (O*C*H$_2$Ph), 71.6 (C-5'), 71.1 (C-2'), 70.2 (C-2), 69.8 (C-5''), 68.8 (C-6''), 65.7 (C-4'), 62.6 (C-6), 61.3 (C-6'), 21.1 (*C*H$_3$Ar) ppm. ESIMS: m/z calcd for $C_{95}H_{86}O_{21}SNa$ [M+Na]+ 1617.5280. Found: 1617.5201.

*4.2.5 6-Benzyloxycarbonylaminohexyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzoyl-β-D-glucopyranoside (**11**)*

Thioglycoside **5** (0.26 g, 0.16 mmol), 6-benzyloxycarbonylamino-1-hexanol (0.058 g, 0.23 mmol, 1.4 equiv) and NIS (0.04 g, 0.18 mmol, 1.1 equiv) were dissolved in anhydrous CH_2Cl_2 (5 mL) containing 4 Å activated molecular sieves. The solution was cooled at −10 ºC and then TfOH (2 µL, 0.02 mmol) was added. After 12 h of stirring at rt the mixture was filtered and diluted with CH_2Cl_2 (100 mL), extracted with $NaHCO_3$ (ss) and $Na_2S_2O_4$ (50 mL, 10 % v/v), washed with water (2 x 25 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by column chromatography (15:2 toluene-EtOAc) to give **11** (0.21 g, 74%) as white foam, $[\alpha]_D$ +32 (*c* 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.07-6.94 (55 H, H-arom.), 5.76 (t, 1H, *J* 9.5 Hz, H-3), 5.61 (d, 1H, *J* 2.8 Hz, H-4'), 5.59 (dd, 1H, *J* 8.0, 10 Hz, H-2'), 5.42 (dd, 1H, *J* 9.9, 7.9 Hz, H-2), 5.07 (s, 2H, CH_2Cbz), 5.05 (d, 1H, *J* 3.4 Hz, H-1''), 4.66-4.62 (m, 3H, H-1, H-1' and OC*H*$_2$Ph), 4.58-4.54 (m, 2H, H-6a and N*H*Cbz), 4.45 (dd, 1H, *J* 12.1, 4.7 Hz, H-6b), 4.41 (dd, 2H, *J*

11.9, 4.9 Hz, OC*H*$_2$Ph), 4.34 (d, 2H, *J* 11.7 Hz, OC*H*$_2$Ph), 4.22 (dd, 3H, *J* 11.7, 6.2 Hz, OC*H*$_2$Ph), 4.14 (t, 1H, *J* 9.5 Hz, H-4), 3.96 (dd, 1H, *J* 10.1, 3.3 Hz, H-3'), 3.82-3.70 (m, 5H, H-2'', H-5, H-5'', H-6'a and OCH$_2$a), 3.59 (t, 1H, *J* 6.5 Hz, H-5'), 3.44 (d, 1H, *J* 2.8, 10 Hz, H-3''), 3.43-3.39 (m, 2H, OCH$_2$b and H-6'b), 3.19 (m, 2H, H-6''a and H-4''), 3.11 (dd, 1H, *J* 9.3, 6.2 Hz, H-6''b), 2.98 (q, 2H, *J* 6.6 Hz, C*H*$_2$N), 1.53 (m, 2H, C*H*$_2$), 1.20-1.07 (m, 2 C*H*$_2$) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.8, 165.6, 165.5, 165.4, 165.1, 164.5 (CO), 138.8, 138.5, 138.2, 133.4, 133.23, 133.20, 130.1, 129.8, 129.7, 129.6, 129.5, 129.4, 128.6, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.6, 127.3, 127.0 (aromatics), 101.2 (C-1'), 101.1 (C-1), 94.7 (C-1''), 78.6 (C-3''), 76.0 (C-4), 75.1 (C-2''), 74.9 (C-4''), 74.4 (O*C*H$_2$Ph), 73.3, 73.1, 73.0 (C-5, C-3' and 2 O*C*H$_2$Ph), 72.8 (C-3), 72.3 (O*C*H$_2$Ph), 71.7 (C-2), 71.6 (C-5'), 71.1 (C-2'), 70.0 (*C*H$_2$O), 69.8 (C-5''), 68.7 (C-6''), 66.5 (*C*H$_2$Cbz), 65.8 (C-4'), 62.5 (C-6), 61.4 (C-6'), 40.8 (*C*H$_2$N), 29.6, 29.1, 26.1 and 25.4 (4 CH$_2$) ppm. ESIMS: *m/z* calcd for $C_{102}H_{99}O_{24}NaN$ $[M+H]^+$ 1722.6635. Found: 1722.6646.

*4.2.6 6-Aminohexyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (**4**)*

Trisaccharide **11** (0.17 g, 0.1 mmol) was dissolved in CH_2Cl_2 (1 mL) and treated with a cooled solution of 0.1 M NaOMe in MeOH (8 mL). After 3 h of stirring at rt TLC showed total conversion of **11** (R_f 0.46, 9:0.3 CH_2Cl_2-MeOH) into a compound of R_f 0.15. The solution was concentrated under reduced pressure in order to evaporate the CH_2Cl_2 and then deionized by elution with MeOH trough a column loaded with Amberlite IR-120 plus resin (200 mesh, H^+ form). The eluate was concentrated under reduced pressure and coevaporated several times with water to afford a white solid (0.10 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 C*H*$_2$Ph), 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').

The solid was dissolved in methanol (9.5 mL) containing 5% of formic acid and 10 % Pd/C (20 mg). The mixture was hydrogenated at 55 psi until all the starting material was converted into a lower running component (R_f 0.23, 3:2:0.1 CH_2Cl_2-MeOH-NH_4OH) (48 h). After filtration, the solution was concentrated under reduced pressure, redissolved in water and purified on a RP-18 column (2 g) eluting with a step gradient from 0 % → 100 % of

MeOH. Fractions of R_f 0.23 were concentrated under reduced pressure, to afford **4** (0.48 g, 98 %). $[\alpha]_D$ +29 (*c* 0.5, CH_3OH); ^1H NMR (500 MHz, D_2O): δ 5.15 (d, 1H, *J* 3.9 Hz, H-1''), 4.52 (d, 1H, *J* 7.8 Hz, H-1'), 4.49 (d, 1H, *J* 8.0 Hz, H-1), 4.21-4.18 (d, 2H, H-5'' and H-4'), 4.03- 3.90 (m, 4H, H-4'', H-6a, H-3'' and OCH_2a), 3.87 (dd, 1H, *J* 10.4, 3.8 Hz, H-2''), 3.83-3.57 (m, 12H, H-6b, H-6'a, H-6'b, H-6''a, H-6''b, H-5, H-5', H-4, H-3, H-3', H-2' and OCH_2b), 3.31 (td, 1H, *J* 8.0, 2.4 Hz, H-2), 3.00 (t, 2H, *J* 7.6 Hz, NCH_2), 1.66 (dp, 4H, 2 CH_2), 1.41 (p, 4H, 2 CH_2) ppm. ^{13}C NMR (126 MHz, D_2O): δ 102.8 (C-1'), 102.0(C-1), 95.4 (C-1''), 78.7, 77.2, 75.0, 74.7, 74.5, 69.6 (C-4, C-3', C-5, C-3, C-2' and C-5'), 72.80 (C-2), 70.8 (C-5''), 70.4 (*C*H_2), 69.3 (C-3''), 69.1 (C-4''), 68.2 (C-2''), 64.8 (C-4'), 61.0, 60.9, 60.2 (C-6, C-6' and C-6''), 39.4 (*C*H_2N), 28.4, 26.6, 25.2, 24.5 (*C*H_2) ppm. ESIMS calcd for $C_{24}H_{46}NO_{16}$ [M+H] 604.2816; found: 604.2803.

*4.2.7 1-[6-Aminohexyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-1-thio-β-D-glucopyranosyl]-2-methoxycyclobutene-3,4-dione (**4-sq**)*

To a solution of **4** (0.025 g, 0.046 mmol) in 0.1 M KH_2PO_4-NaOH buffer (pH 7, 3.57 ml) 3,4-dimethoxy-3-cyclobutene-1,2-dione (13 mg, 0.09 mmol) was added and the solution was stirred at room temperature. The pH was maintained at 7 by addition of small amounts of Et_3N (10 μL). TLC examination after 24 h of reaction showed total conversion of **4** (R_f 0.23, 3:2:0.1 CH_2Cl_2-MeOH-NH_4OH) into a faster moving compound (R_f 0.43). The solution was concentrated under reduced pressure and purified by passing through a graphitized carbon SPE column (500 mg). The column was eluted with water (10 mL) followed by a step gradient from 0 to 40 % of CH_3CN in water. By concentration of fractions eluted with 40 % CH_3CN compound **4-sq** was obtained (15 mg, 46 %) as a syrup. ^1H NMR (500 MHz, D_2O): δ 5.15 (d, 1H, *J* 3.9 Hz, H-1''), 4.53 (d, 1H, *J* 7.8 Hz, H-1'), 4.48 (d, 1H, *J* 8.0 Hz, H-1), 4.38-4.36 (m, 3H, CH_3O-squarate$_a$ and CH_3O-Ssquarate$_b$), 4.23-4.18 (m, 2H, H-5'' and H-4'), 4.03-3.89 (m, 4H, H-4'', H-6a, H-3'' and OCH_2a), 3.87 (dd, 1H, *J* 10.4, 3.8 Hz, H-2''), 3.83-3.57 (m, 7H, H-6'a, H-6b, H-6'b, H-6''a, H-6''b, H-4, H-3', H-5, H-2', H-3, H-5' and OCH_{2b}, NHCH_2-squarate$_a$), 3.48 (t, 1H, *J* 6.8 Hz, NHCH_2-squarate$_b$), 3.33-3.28 (m, 1H, H-2), 1.64 (d, 4H, *J* 6.9 Hz, 2 CH_2), 1.41-1.36 (m, 4H, 2 CH_2) ppm. ^{13}C NMR (126 MHz, D_2O): δ 189.2, 183.2, 183.0, 177.6, 177.1, 172.9 (cyclobutene), 102.8 (C-1'), 102.0 (C-1), 95.4 (C-1''), 78.6, 77.2, 75.0, 74.7,

74.5, 69.6 (C-2', C-3, C-3', C-4, C-5 and C-5'), 72.8 (C-2), 70.8 (C-5''), 70.5 (O*C*H_2), 69.3 (C-3''), 69.1 (C-4''), 68.2 (C-2''), 64.8 (C-4'), 61.0, 60.9, 60.8, 60.7, 60.2 (C-6, C-6', C-6'', *C*H_3O_a and *C*H_3O_b), 44.5, 44.3 (*C*H_2N), 29.7, 29.4, 28.5 x 2, 25.2, 25.1, 24.6, 24.5 ppm (4 CH_2, duplicated signals [35]) ppm.

*4.2.8 1-[6-Aminohexyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-1-thio-β-D-glucopyranosyl]-2-[BSA]-methoxycyclobutene-3,4-dione (**4-BSA**)*

A solution of **12** (7 mg, 0.0098 mmol) and BSA (0.013 g, $1.96x10^{-4}$ mmol) in a molar ratio 50:1, in 0.05 M borax -0.1 M KH_2PO_4 buffer (pH 9, 0.98 ml) was stirred at room temperature. The pH was maintained at 9 by addition of small amounts of Et_3N (10 µL were necessary). By TLC the gradual conversion of **12** (R_f 0,43 CH_2Cl_2-MeOH-NH_4OH 3:2:0.1) into components of R_f 0 was observed. The solution dialyzed against deionized water using a MWCO 12000-14000 membrane and the retained solution was lyophilized to afford conjugate **4-BSA** (13.4 mg, 79 %) as an amorphous white powder.

4.3 MALDI-TOF MS

The MALDI-TOF spectra were recorded in positive ion mode. Each sample was dissolved in 2 % acetic acid and 2 % acetonitrile aqueous solution, to give a final 300 µM sample solution. Pre-loading mix was prepared by adding 5 µL of sample solution to 5 µL of matrix solution (sinapinic acid 10 mg/mL in 7:3 acetonitrile-water containing 0.1 % TFA). The mixture (1 µL) was applied to the MALDI-plate spot and allowed to air dry. Once dried, 1000 adquisitions shots were fired per spot and the accumulated final spectra were saved.

4.4 SDS-PAGE

Aliquots containing ~1 µg of protein taken at the indicated conjugation times were analyzed by SDS-PAGE (10 % gels). Commercial bovine submaxillary mucin (BSM, Sigma) was used as positive control. The gels were stained with Coomassie brilliant blue or subjected to the periodate-Schiff staining technique [57] and, following image acquisition, counterstained with Coomassie brilliant blue. Densitometric analyses were carried out with ImageJ 1.45s Software (NIH, USA).

4.5 Immunological assays

4.5.1 Serum samples

Serum samples from *T. cruzi*-infected subjects have been described [4][46][56] and were obtained from the Laboratorio de Enfermedad de Chagas, Hospital de Niños "Dr. Ricardo Gutierrez". All procedures were approved by the research and teaching committee and the bioethics committee of this institution, and followed the Declaration of Helsinki Principles. Written informed consent was obtained from all individuals (or from their legal representatives), and all samples were decoded and de-identified before they were provided for research purposes. Chagasic patients were coursing the chronic stage of the disease without cardiac or gastrointestinal compromise. Serum samples were analyzed for *T. cruzi*-specific antibodies with the following commercially available kits: ELISA using total parasite homogenate (Wiener lab, Argentina) and indirect hemmaglutination assay (IHA, Polychaco, Argentina). Serum samples from healthy individuals that gave negative results in the aforementioned tests were obtained from different blood banks: Fundación Hemocentro Buenos Aires (Buenos Aires, Argentina), Hospital de Enfermedades Infecciosas 'Dr. Francisco Javier Muñiz' (Buenos Aires, Argentina), Hospital Italiano de Buenos Aires (Buenos Aires, Argentina) and Hospital Municipal 'Dr. Diego E. Thompson' (San Martín, Buenos Aires, Argentina). Anti-α-Gal antibodies were obtained by affinity chromatography of serum samples from chronic Chagasic patients on silica particles linked to trisaccharide **1** as described [58], whereas antibodies from Chagas-negative individuals were obtained by protein-A affinity chromatography [4].

4.5.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were performed using flat-bottomed 96-well Nunc-Immuno plates (Nunc, Roskilde, Denmark), as described previously [59]. Briefly, BSA-based glycoconjugates were dissolved in carbonate buffer (pH 9.6) as a coating buffer at 10 µg/ml and incubated overnight at 4°C. The plates were blocked for 1 h with PBS supplemented with 4% skim milk and 0.05% Tween 20 (blocking buffer) at 37°C. Serum samples were prepared in blocking buffer (at 1:500 dilution) were then added to the plate. Anti α-Gal or control immunoglobulins were also appropriately diluted in blocking buffer

before being added to the plate. Following incubation for 1 h at 37°C and washings with PBS/T, peroxidase-conjugated goat IgG to human IgG (Sigma) diluted 1:5,000 in 4% skim milk in PBS/T was added to the plates and incubated at 37°C for 1 h. The plates were washed and incubated with 100 µl of freshly prepared citrate-phosphate buffer (pH 4.2) containing 0.2% hydrogen peroxide and 0.5 mM 3,3',5,5'-tetramethylbenzidine (Sigma). The reaction was stopped with 50 µl of 2 M sulfuric acid, and the absorbance at 450 nm was read. Each sample was assayed in triplicate, unless otherwise indicated. *T. cruzi* Antigen 2 (TcAg2) was identified by means of early immunological screenings [60], and its immunodominant sequences mapped by high-density peptide microarrays [47]. A TcAg2-derived peptide (sequence: NH_2-KKKQKTAPFGQAAAGDKPSPFGQAC) was custom synthesized (GenScript) and coupled through its *C*-terminal cysteine residue to maleimide-activated BSA (Sigma) as described [61]. ELISA plates coated with 100 µl of a solution containing 5 µg/ml of this conjugate were prepared and evaluated as described above.

For competitive ELISA**,** the serum samples were diluted up to 10 µl in PBS containing 10 µg of the indicated synthetic carbohydrate. After 30 min of incubation at room temperature, the serum-carbohydrate mixtures were diluted up to 1:500 in 4 % skim milk PBS/T buffer, added to **4-BSA** or **BSA-TcAg2** coated plates, and processed by ELISA, as described above. Absorbance at 450 nm in the control wells in which the serum samples were incubated for 30 min with 10 µl of PBS without carbohydrate was taken as 100% reactivity [59].

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Appendix: Supplementary material

Supplementary data to this article (NMR spectra for compounds **4, 5, 7, 9, 10** and **11**) can be found online at doi:….

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- ✓ Trisaccharide α-D-Galp(1→3)-β-D-Galp(1→4)-D-GlcNAc is a main component of *Trypanosoma cruzi* trypomastigote mucins with immunological properties.
- ✓ The analogue α-D-Gal*p*(1→3)-β-D-Gal*p*(1→4)-β-D-Glc*p* derivatized as the 6-aminohexyl glycoside was synthesized. This trisaccharide and the analogue glycoside of α-D-Gal*p*(1-3)-β-D-Gal*p*, were conjugated to BSA by the squarate method.
- ✓ ELISA assays revealed that these neoglycocojugates were recognized by sera from chagasic patients, indicating its potential as diagnostic tools.