Probing for *Trypanosoma cruzi* Cell Surface Glycobiomarkers for the Diagnosis and Follow-Up of Chemotherapy of Chagas Disease

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Abstract *Trypanosoma cruzi* is a protozoan parasite that causes Chagas disease in humans. Linear and branched *O*-glycans with non-reducing, terminal α-galactosyl (α-Gal) glycotopes located on cell surface glycosylphosphatidylinositol (GPI)-anchored mucins of the infective trypomastigote form of the parasite are foreign to humans and elicit high levels of anti-α-Gal antibodies in Chagas disease patients (Ch anti-α-Gal antibodies). These antibodies have the capability to lyse the parasite in a complement-dependent or -independent manner. Ch anti-α-Gal antibodies have a considerably higher reactivity to the parasitic surface α-Gal glycotopes than the normal human serum (NHS) anti-α-Gal antibodies, which are present in every healthy human being. A series of ten mercaptopropyl saccharides with α-Gal moieties at the non-reducing end, all connected to another galactose unit, and five non-α-Gal-containing glycan controls were synthesized, and conjugated to maleimide-derivatized bovine serum albumin. This produced neoglycoproteins (NGPs), which were assembled into glycoarrays for the interrogation with sera of chronic Chagas disease patients and healthy individuals using chemiluminescent

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enzyme-linked immunosorbent assay (CL-ELISA). This study identified the terminal Galα(1,3)Galβ disaccharide as an immunodominant *T. cruzi* glycotope and biomarker, which shows a considerable binding differential between Ch and NHS anti-α-Gal antibodies. Therefore, this glycotope is suitable for the diagnosis of Chagas disease, and could also be potentially used for follow-up studies for the effectiveness of chemotherapy in Chagas disease patients.

1 Introduction

Chagas disease is an infectious disease caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted by blood-sucking insect vectors of the Reduviidae family, popularly known as “kissing bugs”. Transmission can also occur by blood transfusion, organ transplantation, ingestion of tainted foods and liquids, and by the congenital route. Currently, approximately 8–10 million people are infected, and approximately 12,000 die every year, mostly as a consequence of cardiomyopathy [1, 2]. Chagas disease is endemic to South and Central America; however, due to the migration of many thousands of chronically infected, asymptomatic individuals from endemic areas, Chagas disease now exists in non-endemic regions such as the U.S., Europe, Australia, New Zealand, and Japan [2–5].

The cell surface of *T. cruzi* has heavily *O*-glycosylated glycosylphosphatidylinositol (GPI)-anchored mucins, which are the major constituents of a particularly dense glycocalyx [6–8]. Unlike the *O*-glycans of GPI-mucins from the insect-derived developmental stages of the parasite, that have been characterized in several strains and genotypes, the structural details of the *O*-glycans of mucins from the mammal-dwelling trypomastigote form (tGPI-mucins) are mostly unknown [6, 9, 10]. However, through partial structural analysis and immunoassays it is known that the majority of these tGPI-mucin *O*-glycans contain highly immunogenic, non-reducing terminal α-galactopyranosyl residues [11], here abbreviated as α-Gal, αGal, or Galα, omitting the explicit designator “*p*” for pyranose. Interestingly, these terminal α-Gal residues are completely absent in GPI-mucins derived from the insect vector-dwelling epimastigote and metacyclic trypomastigote forms or stages [6–10]. The αGal residues are partial structures of most likely several immunodominant glycotopes and are recognized by the highly abundant, protective anti-α-Gal antibodies present in the sera of patients in the acute and chronic phases of Chagas disease [11, 12]. These antibodies are responsible for controlling the parasitemia in both stages of the infection [11–13]. The *O*-glycosylation of the *T. cruzi* mucins is a posttranslational modification in which α-*N*-acetylglucosamine (GlcNAcα) is added to a threonine side chain by the UDP-GlcNAc:polypeptide α-*N*-acetylglucosaminyltransferase in the Golgi apparatus [14]. This α-GlcNAc moiety is heavily 4,6-di-*O*-substituted, albeit 4-*O* monosubstitution also exists [11–16]. In addition, 2-*O*-substituted Gal, 3-*O*-substituted Gal, 4-*O*-substituted Gal, 6-*O*-substituted Gal, and 2,6-di-*O*-substituted Gal motives also exist, indicating that

tGPI-mucin *O*-glycans are galactose-rich and predominantly branched [11]. Nevertheless, the only tGPI-mucin *O*-glycan that has been fully characterized to date is the linear trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα. It is strongly recognized by Ch anti-α-Gal Abs, but only weakly by anti-α-Gal Abs from healthy individuals [normal human serum (NHS) anti-α-Gal Abs] [11], which are produced mainly against Gram-negative enterobacteria of the human flora [17]. These enterobacteria (e.g., *E. coli*, *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., *Salmonella* spp., *Citrobacter* spp., and *Shigella* spp.) have a number of different non-reducing, terminal αGal-linked glycans, mostly Galα(1,2)-R, Galα(1,4)-R and Galα(1,6)-R (where R is the remaining side chain or core glycan) on the lipopolysaccharide (LPS) core oligosaccharides or *O*-antigens [18]. The glycotope Galα(1,3)Galβ(1,4)GlcNAcα has so far not been reported in enterobacteria.

Despite the existence of intraspecies polymorphism in the *O*-glycans of the GPI-mucins, the expression of highly immunogenic, non-reducing terminal αGal residues seems to be highly conserved in tGPI-mucins from at least four major *T. cruzi* genotypes or discrete typing units (DTUs) known to infect humans (i.e., TcI, TcII, TcV, and TcVI) [11, 19–21]. This is supported by numerous studies showing the abundant presence of high levels of protective Ch anti-α-Gal Abs in patients from different endemic and nonendemic regions [11, 12, 19, 22–30].

2 Results and Discussion

Here we present the identification of an immunodominant glycotope present on the *T. cruzi* cell surface that is strongly recognized by anti-α-Gal antibodies from chronic Chagas disease (CCD) patients. In order to identify potential *T. cruzi* αGal-containing glycotopes, we synthesized a biased library of ten glycans consisting of mono-, di-, and trisaccharides with terminal αGal moieties based on the partial structural information available for tGPI-mucin *O*-glycans [11]. The synthetic glycans were conjugated to bovine serum albumin (BSA) to produce neoglycoproteins (NGPs), which were assembled into glycoarrays and interrogated with pooled sera of *T. cruzi*-infected and healthy individuals using chemiluminescent enzyme-linked immunosorbent-assay (CL-ELISA) [24]. We reasoned that with a glycan library in hand, polyclonal anti-α-Gal Abs from CCD patients could be used as probes for the identification of immunodominant *T. cruzi* glycotopes. Antibody recognition of certain saccharides would suggest that these saccharides are immunogenic glycotopes or glycotope partial structures that exist on the cell surface of the infective trypomastigote form that lives in the human host. Due to the structural diversity of the trypomastigote cell surface *O*-glycans, several glycans with αGal moieties differently connected to another underlying sugar, most likely another galactose unit, could potentially be identified as immunogenic glycotopes.

For the synthesis of a potential αGal-containing library, three factors had to be considered: (a) the size and connectivity of the saccharide targets; (b) a suitable linker allowing for the conjugation to BSA; and (c) a versatile synthetic strategy

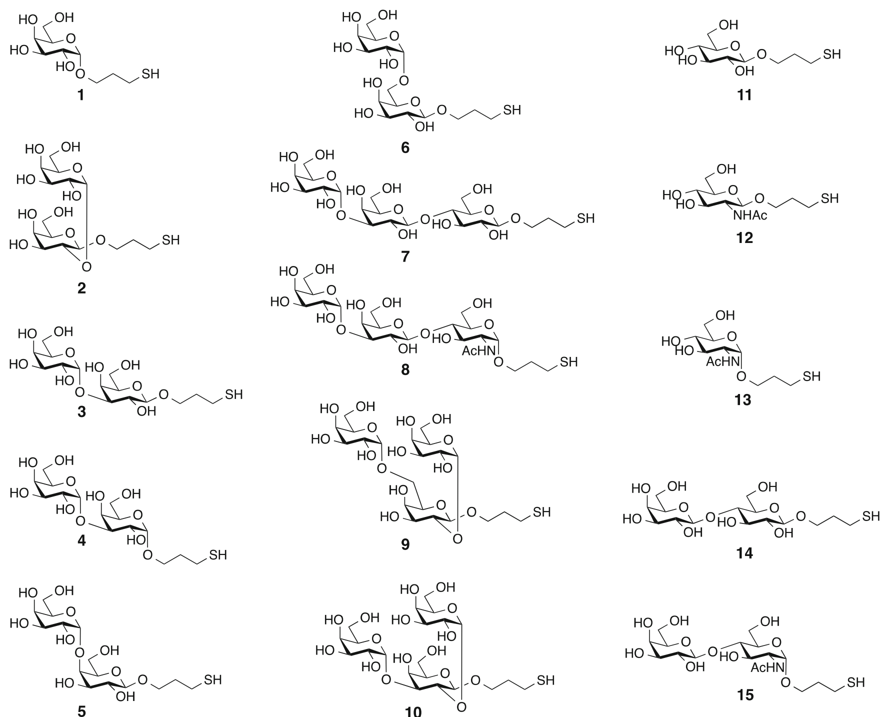


Fig. 1 A synthetic library of saccharides with mercaptopropyl linkers: ten saccharides with non-reducing αGal (**1–10**), and five saccharides that lack αGal (**11–15**) [32, 33]

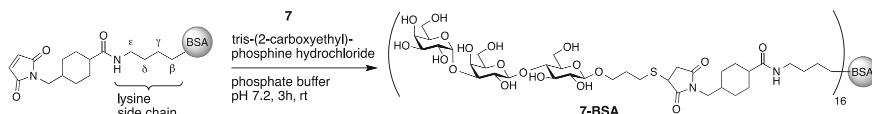
allowing for the use of common precursors. We synthesized a total of ten αGal-containing mercaptopropyl saccharides (**1–10**, Fig. 1) suitable for conjugation to commercially available maleimide-derivatized BSA. In order to shine light on whether Ch anti-α-Gal Abs can recognize a single monosaccharide, mercaptopropyl Galα (**1**) [31, 32] was included in the study. The mercaptopropyl glycosides of the following five αGal-containing disaccharides were also included: Galα(1,2)Galβ (**2**) [32]; Galα(1,3)Galβ (**3**) [32]; Galα(1,3)Galα (**4**); Galα(1,4)Galβ (**5**) [32]; and Galα (1,6)Galβ (**6**) [32]. In addition, the mercaptopropyl glycosides of Galα(1,3)Galβ (1,4)Glcα (**7**) [32], and the linear tGPI-mucin trisaccharide Galα(1,3)Galβ(1,4) GlcNAcα (**8**) [33] were selected as targets. Trisaccharide **8** serves as a positive control, and trisaccharide **7** may shine light on the importance of the third sugar at the reducing end for antibody recognition. In addition, the trisaccharides Galα(1,2) [Galα(1,6)]Galβ (**9**) and Galα(1,2)[Galα(1,3)]Galβ (**10**) were included because they represent branched *O*-glycans with two terminal αGal units. Lastly, six different putative negative controls that lack terminal αGal units were also included in the library: the mercaptopropyl glycosides of monosaccharides Galβ (**11**) [32], GlcNAcβ (**12**) [33], GlcNAcα (**13**) [33], the disaccharides Galβ(1,4)Glcβ (**14**) [32], and Galβ(1,4)GlcNAcα (**15**) [33], as well as cysteine [32, 33]. Including compounds **13** and **15** in the study will provide information on whether Ch anti-α-Gal

antibodies have the ability to recognize the reducing end mono and disaccharide partial structures of the known *T. cruzi* tGPI mucin glycan Galα(1,3)Galβ(1,4)GlcNAcα. Lastly, including both anomers of GlcNAc (compounds **12** and **13**) may provide information on the importance of the configuration at the reducing end of Galα(1,3)Galβ(1,4)GlcNAcα.

The assembly of the glycans into a glycoarray requires their immobilization in microtiter plate wells. This can be accomplished by conjugation of the glycan to a protein, which adheres to Nunc MaxiSorp® microtiter plate wells made from polystyrene by non-covalent interactions. Initially, we chose maleimide-derivatized keyhole limpet hemocyanin (KLH) for the conjugation due to its high-loading capacity, but this protein showed poor water solubility and made accurate microplate-well loading impossible. Therefore, we decided to conjugate all glycosides (**1–15**) to BSA instead, which has superior solubility properties. The mercaptopropyl glycosides were conjugated to commercially available maleimide-activated BSA by 1,4-addition in aqueous solution at pH 7.2, which produced NGPs **1-BSA–15-BSA**. The conjugation was carried out in the presence of a water-soluble phosphine to reduce any sugar disulfides. The NGPs prepared in this manner have the tendency to aggregate over a time period of several months when kept in solution at 4 °C, which is in accordance with a recent study on the stability of BSA and lysozyme that had been exposed to reducing conditions [34]. Therefore we recommend storing the NGPs frozen at −20 to −80 °C, in small working aliquots to avoid repeated freeze-thaw cycles. Scheme 1 shows the conjugation of trisaccharide **7** to BSA producing the NGP **7-BSA** as an example. The average glycan load per BSA molecule can be determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Figure 2 illustrates the glycan load determination of **2-BSA** by MALDI-TOF-MS as a representative example.

Schemes 2, 3, and 4 illustrate the syntheses of mercaptopropyl glycosides **2**, **7**, and **8** as examples of synthetic strategies applied for the generation of the αGal-containing glycan library. The most important synthetic feature that we applied for the synthesis of all αGal-containing disaccharides and trisaccharides [32, 33], is the use of Kiso's di-*tert*-butylsilylidene galactosyl donor **18** (Scheme 2) allowing for the stereoselective α-galactosylation despite the presence of a benzoyl protecting group at position 2 [35, 36]. For the synthesis of mercaptopropyl disaccharide **2**, unprotected allyl β-galactoside **16** [37], synthesized from a peracetylated allyl galactoside precursor [38], was protected as its isopropylidene ketal [39] followed by silylation with *tert*-butyl-diphenylsilylchloride to give acceptor **17**



Scheme 1 Conjugate addition of mercaptopropyl saccharide **7** to Imject^{TM} maleimide-activated BSA from Thermo Fisher Scientific produced NGP **7-BSA** with a glycan load of 16 glycans per BSA molecule

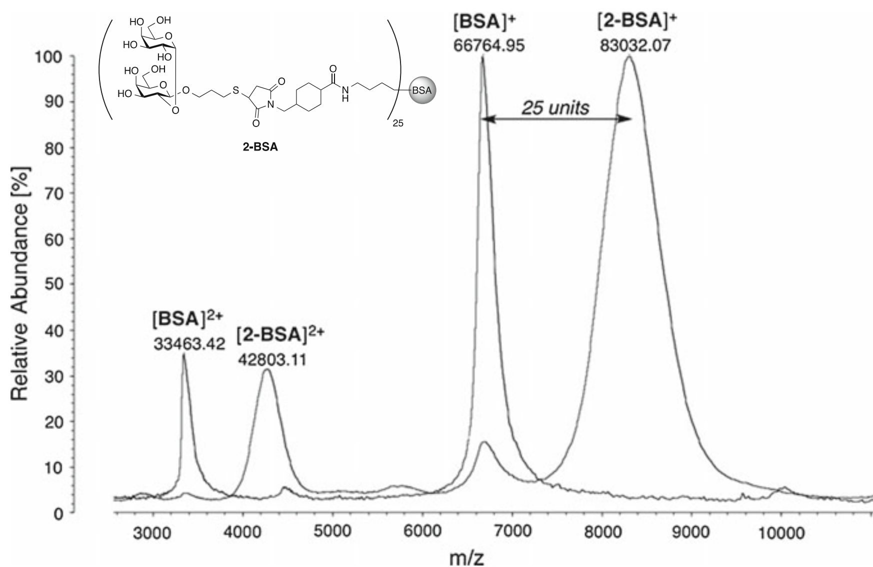
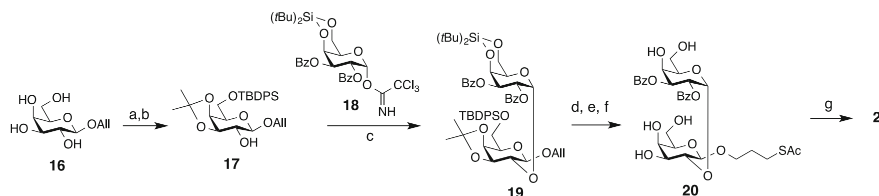
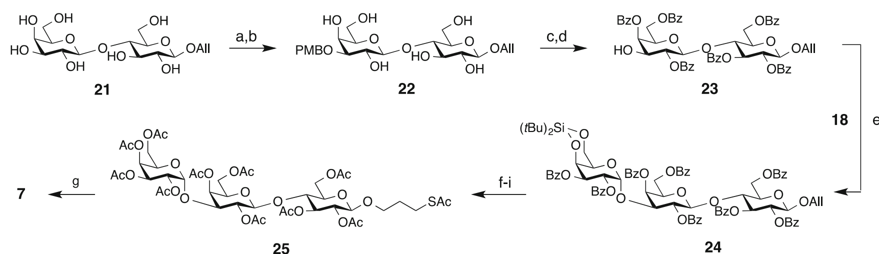


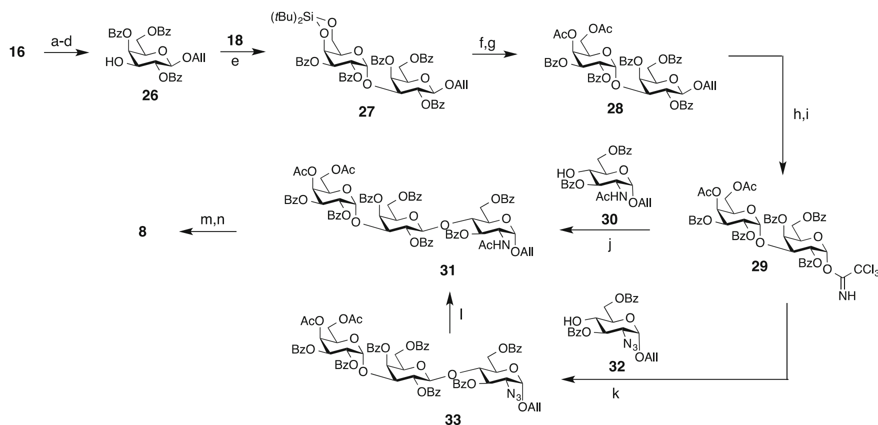
Fig. 2 MALDI-TOF mass spectra of BSA and **2-BSA**: in this case, the average loading is 25 disaccharide units per BSA molecule, based on the *m/z* difference of $[BSA]^+$ and $[\mathbf{2\text{-}BSA}]^+$



Scheme 2 Synthesis of mercaptopropyl disaccharide **2**: **a** 2,2-dimethoxypropane, pTsOH, DMF (85%); **b** TBDPSCl, DMAP, DCM (95%); **c** TMSOTf, DCM, 4 Å MS (82%); **d** HF-pyr, THF, 0 °C (91%); **e** DCM, H_2O, TFA (98%); **f** AcSH, AIBN, THF, 350 nm (83%); **g** NaOMe, MeOH (quant.)



Scheme 3 Synthesis of the αGal-containing mercaptopropyl trisaccharide **7**: **a** Bu_2SnO, MeOH, 65°C; **b** PMBCl, Bu_4NBr, benzene, 65°C (54% two steps); **c** BzCl, pyr (quant.); **d** DDQ, CH_2Cl_2, H_2O (72%); **e** TMSOTf, DCM, 4 Å MS (85%); **f** HF-pyr, THF (90%); **g** NaOMe, MeOH (quant.); **h** Ac_2O, pyr; **i** AcSH, AIBN, THF, 350 nm (83% over two steps)



Scheme 4 Synthesis of Galα1,3Galβ1,4GlcNAcα-$(CH_2)_3$SH (**8**): **a** Bu_2SnO, MeOH; **b** PMBCl, Bu_4NCl, benzene (75% two steps); **c** BzCl, pyr (91%); **d** DDQ, DCM, H_2O (98%); **e** TMSOTf, DCM, 4 Å MS (92%); **f** HF-pyr, THF (90%); **g** Ac_2O, pyr (89%, two steps); **h** $PdCl_2$, MeOH (87%); **i** CCl_3CN, DCM, DBU (84%); **j** TMSOTf, DCM, 4 Å MS (30% α/β 1:4, separable by FPLC); **k** TMSOTf, DCM, 4 Å MS (46%); **l** AcSH (77%); **m** AcSH, AIBN, THF, 350 nm (89%); **n** NaOMe, MeOH (quant.)

[40]. α-Galactosylation with the Kiso donor **18** [35, 36] afforded the fully protected allyl disaccharide **19**. Removal of the di-*tert*-butylsilylidene group with tetrabutylammonium fluoride or potassium fluoride in the presence of 18-crown-6 failed, but its removal was accomplished with hydrofluoric acid-pyridine complex, which simultaneously also removed the *tert*-butyldiphenylsilyl group to furnish the dibenzoylated allyl disaccharide. Radical addition of thioacetic acid to the double bond [31, 41, 42] of the allyl glycoside gave the thioester **20**, which was deprotected under Zemplén conditions to afford mercaptopropyl disaccharide **2** (Scheme 2).

Scheme 3 illustrates the synthesis of mercaptopropyl trisaccharide **7** from allyl lactoside **21** [43], which was p-methoxybenzylated at position 3 in the galactose ring via its tin-acetal to give compound **22**. It was converted into glycosyl acceptor **23** by perbenzoylation, followed by oxidative removal of the p-methoxybenzyl group using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). α-Galactosylation of acceptor **23** with the Kiso donor **18** furnished the fully protected allyl trisaccharide **24**, which was treated with hydrofluoric acid-pyridine complex to remove the di-*tert*-butylsilylidene group. Debenzoylation followed by acetylation, followed by radical addition of thioacetic acid by a thiol-ene reaction afforded trisaccharide **25**. The reason for the debenzoylation-acetylation strategy was a more straightforward purification of the thiol-ene reaction product by silica gel column chromatography. Upon deesterification of the fully esterified trisaccharide **25** using Zemplén conditions the target mercaptopropyl trisaccharide **7** was obtained.

The synthesis of mercaptopropyl trisaccharide **8** is much more challenging than the synthesis of its analog **7** (Scheme 3) due to the presence of *N*-acetylglucosamine

instead of a glucose moiety at the reducing end. The glycan moiety Galα(1,3)Galβ(1,4)GlcNAcα present in the tGPI mucins of *T. cruzi* [11] is an anomer of the well-known Galili epitope Galα(1,3)Galβ(1,4)GlcNAcβ [44] present on all mammalian cell surfaces with the exception of humans and Old World monkeys. For the Galili epitope and its glycosides, several chemical and chemo-enzymatic syntheses have been reported [45–52], but to the best of our knowledge there are no reports on the synthesis of mercaptopropyl trisaccharide **8** and analogs thereof with its unusual α-configuration at the reducing end. In order to develop a synthesis for **8** we envisioned to utilize the Kiso donor **18**, and a strategy that makes use of predominantly acyl protecting groups that can be easily installed and cleanly removed (Scheme 4). Allyl β-galactoside **16** was converted into its tin acetal followed by p-methoxybenzylation at position 3. Benzoylation of the remaining hydroxyl groups and oxidative removal of the p-methoxybenzyl group with DDQ afforded acceptor **26**. This acceptor was glycosylated with the donor **18**, using trimethylsilyl trifluoromethanesulfonate (TMSOTf) catalysis to give disaccharide **27**. The di-*tert*-butylsilylidene group was cleaved with hydrofluoric acid-pyridine complex in tetrahydrofuran (THF), followed by acetylation of the two hydroxyls to afford the peracylated allyl disaccharide **28**. Treatment with palladium(II) chloride in methanol gave the hemiacetal, which was filtered immediately after consumption of the starting material to avoid the formation of a polar by-product that is observable after 2 h of reaction, and was converted into the α-trichloroacetimidate **29** with trichloroacetonitrile in the presence of 1,8-diazabicycloundec-7-ene (DBU). This donor was at first used to glycosylate the allyl GlcNAc acceptor **30** [33, 53], obtained from allyl 2-deoxy-2-acetamido-α-D-glucopyranoside [54], by selective benzoylation with *N*-benzoylimidazole [55, 56]. The glycosylation was accomplished with TMSOTf, but trisaccharide **31** was obtained as an anomeric mixture (1:4 α/β) of low yield, most likely due to the well-known poor nucleophilicity of the 4-OH of GlcNAc acceptors [57]. The separation of the two diastereomeric trisaccharides by column chromatography proved to be difficult, but can be accomplished by FPLC. Replacement of the acceptor **30** with the allyl 2-deoxy-2-azido-Glc acceptor **32** [33], produced from allyl 2-deoxy-2-azido-α-D-glucopyranoside [54] by selective benzoylation [53], furnished trisaccharide **33** in 46% yield, which was purified by flash chromatography. Reduction of the azide and installation of an *N*-acetyl group with neat thioacetic acid (AcSH) gave the trisaccharide **31**. Radical addition of AcSH in the presence of azobisisobutyronitrile (AIBN) in THF under UV light gave the thioester (not shown). All ester-protecting groups were removed under Zemplén conditions to afford the target trisaccharide **8**.

For glycoarray interrogation, pooled sera from ten CCD patients (ChSP) and pooled sera from ten healthy individuals (NHSP), obtained from the ISGlobal, Hospital Clinic, Universitat de Barcelona, were used. CCD patients had been diagnosed by two conventional ELISA tests, one with a lysate of *T. cruzi* parasites (Ortho-Clinical Diagnostics, Raritan, NJ, USA), and the other one with recombinant antigens (BioELISA Chagas, Biokit S.A., Barcelona, Spain). Healthy individuals tested negative in these two ELISAs. The synthetic αGal-containing NGPs **1-BSA–10-BSA**, cysteine conjugated to BSA (Cysteine-BSA), as well as the five

non-αGal-containing NGPs (**11-BSA–15-BSA**), were immobilized in microtiter plate wells, and the resulting glycoarrays were subjected to CL-ELISA [24] in two glycoarray sets. Unlike our previously published results, in which 125 ng of NGP was immobilized per well, and sera dilutions of 1:100 and 1:300 were used [32], here we decreased the quantity of NGP per well to 24 ng (Fig. 3) and 12 ng (Fig. 4), and increased the serum dilution to 1:800, because under these conditions most NGPs show higher reactivity differentials (ratios) between ChSP and NHSP.

Figure 3 represents the first of two glycoarray studies and shows the CL-ELISA responses for **1-BSA–11-BSA**, **14-BSA** and **Cysteine-BSA**, most of which had been previously studied under different conditions [32], using ChSP and NHSP at sera dilutions of 1:800. It is noticeable that the NGPs **3-BSA**, **7-BSA**, and **8-BSA** show strong CL-ELISA reactivities with ChSP, and the greatest differentials between ChSP and NHSP of ~11 fold. All three NGPs share a common non-reducing terminal Galα(1,3)Galβ moiety, which is highly indicative for the disaccharide Galα(1,3)Galβ being an immunodominant *T. cruzi* trypomastigote cell surface glycotope.

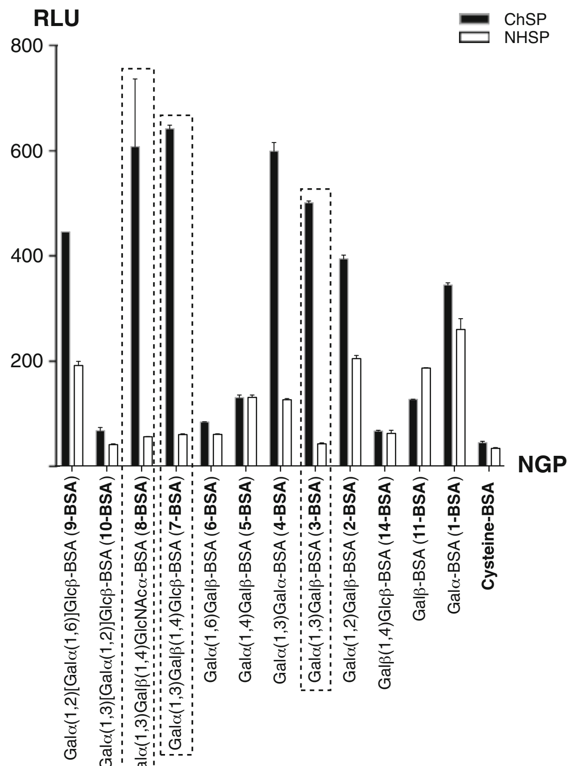


Fig. 3 CL-ELISA reactivities of ChSP and NHSP to αGal- and βGal-containing NGPs. Each NGP was tested with ChSP and NHSP in duplicate. **Cysteine-BSA** was used as a negative control. The three NGPs with strong reactivities and large differentials between ChSP and NHSP are highlighted (dashed boxes). The amount of NGP and Cysteine-BSA loading in each well was 24 ng; sera were diluted at 1:800. RLU, relative luminescence units

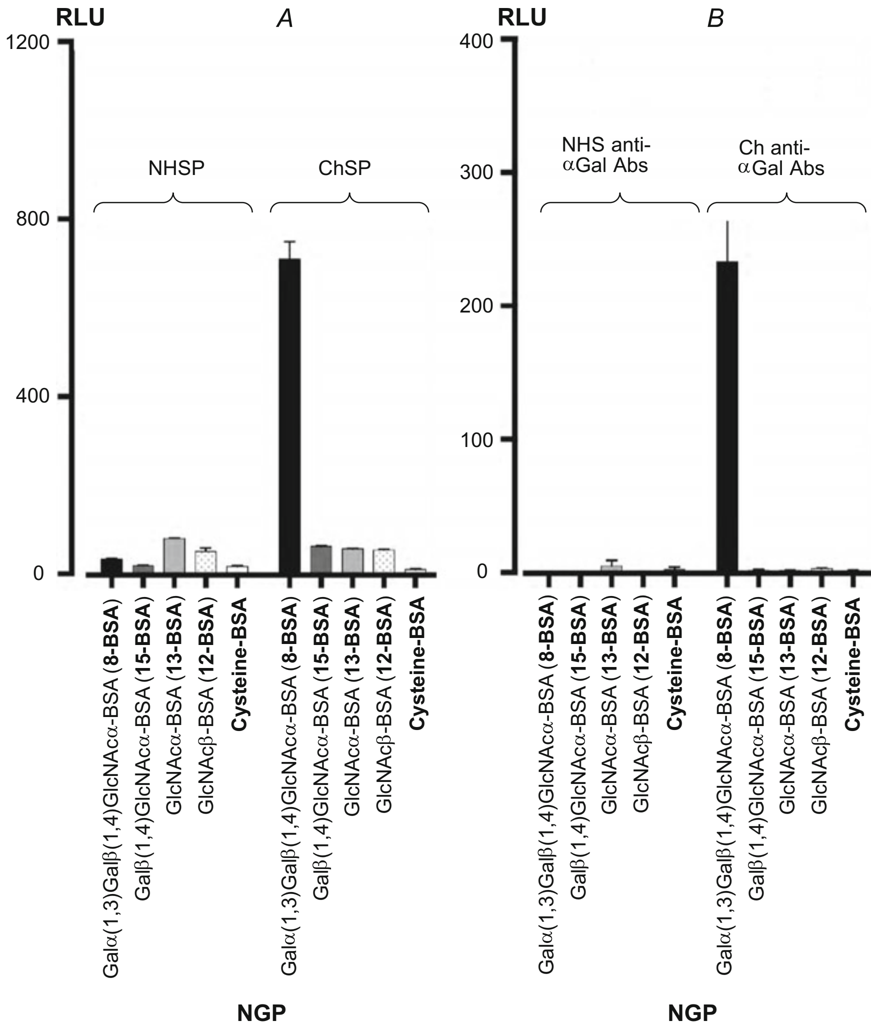


Fig. 4 **A** CL-ELISA reactivity of NHSP versus ChSP to NGPs and Cysteine-BSA at 12 ng/well; sera dilution 1:800. **B** CL-ELISA reactivity of purified NHS anti-α-Gal Abs versus Ch anti-α-Gal to NGPs and Cysteine-BSA at 12 ng/well; antibody concentration: 1 μg/mL. RLU, relative luminescence units. Figure modified from Ref. [33]

None of these NGPs are significantly recognized by NHSP. Interestingly, the branched trisaccharide Galα(1,3)[Galα(1,2)]Galβ in **10-BSA** is practically not recognized by these antibodies. It resembles the blood group B antigen Galα(1,3)[Fucα(1,2)]GalβR, which is also not recognized by NHS anti-α-Gal Abs [58] or Ch anti-α-Gal Abs (Almeida I.C., unpublished data). The Galα(1,3)Galα containing

NGP **4-BSA**, which differs from the Galα(1,3)Galβ containing NGP **3-BSA** only in its anomeric configuration at the reducing end, also shows a strong reactivity with ChSP, but a somewhat smaller differential between ChSP and NHSP of only ~5:1 is observed. The strong reactivity with ChSP exhibited by **4-BSA** could be due to the fact that the Ch anti-α-Gal antibodies cannot distinguish between the two possible configurations of the galactose moiety at the reducing end. Another potential reason could be that *T. cruzi* trypomastigotes express both disaccharides, however, so far the Galα(1,3)Galα glycotope has not been identified by analysis of *T. cruzi*-derived lysates, and its existence remains unconfirmed. The Galα(1,2)Galβ-containing NGPs **2-BSA** and **9-BSA** also have strong reactivities with ChSP, which matches reports of high levels of anti-α-Gal Abs with specificity toward the Galα(1,2)Gal epitope in the sera of CCD patients [30]. However, the differentials between ChSP and NHSP reactivity to **2-BSA** and **9-BSA** are much lower (~2 fold) than those observed for **3-BSA**, **7-BSA**, and **8-BSA**. The NGP **1-BSA**, which contains only the monosaccharide αGal, gave a medium strong CL-ELISA response with a poor differential of ~1.3 fold between ChSP and NHSP. **5-BSA** [Galα(1,4)Galβ BSA] and **6-BSA** [Galα(1,6)Galβ BSA] show weak reactivities with ChSP, indicating that neither of them is a major immunogenic *T. cruzi* glycotope. Their reactivities are similar to the ones displayed by the βGal-containing NGP **11-BSA** and the lactose-containing NGP **14-BSA**, which only have slightly higher reactivities than the negative control **Cysteine-BSA** (Fig. 3).

The second glycoarray included the NGPs **8-BSA**, **12-BSA**, **13-BSA**, and **15-BSA**, as well as the negative control **Cysteine-BSA** [33]. Unlike the first glycoarray in which 24 ng of NGP was immobilized in each well, the second glycoarray was constructed immobilizing only 12 ng of NGP per well. Figure 4A shows CL-ELISA results of the glycoarray interrogation using ChSP and NHSP. Galα(1,3)Galβ(1,4)GlcNAcα-BSA (**8-BSA**) shows a 20-fold differential between ChSP and NHSP, whereas the NGPs GlcNAcβ-BSA (**12-BSA**), GlcNAcα-BSA (**13-BSA**) and Galβ(1,4)GlcNAcα-BSA (**15-BSA**) are only weakly recognized by antibodies of ChSP or NHSP. As expected, **Cysteine-BSA** showed practically no reactivity. In addition, the glycoarray was interrogated with Ch and NHS anti-α-Gal Abs (Fig. 4B), which had been purified by affinity chromatography using immobilized Galα(1,3)Galβ(1,4)GlcNAcβ [13]. Galα(1,3)Galβ(1,4)GlcNAcα-BSA (**8-BSA**) displays a 230-fold differential between Ch-αGal Abs and NHS-αGal Abs, while the other NGPs (**12-BSA**, **13-BSA**, and **15-BSA**) and **Cysteine-BSA** remain practically unrecognized by either Abs. Our results show that the terminal αGal moiety of Galα(1,3)Galβ(1,4)GlcNAcα is essential for Ch antibody recognition. Although GlcNAcα and Galβ(1,4)GlcNAcα, which are underlying partial structures of Galα(1,3)Galβ(1,4)GlcNAcα, are nonself glycotopes for humans, there are only very weak antibody responses against them in ChSP. The αGal-containing glycoarray/CL-ELISA method presented here, especially when carried out with only 12 ng or 24 ng of antigen/well under dilute conditions is highly suitable for the differentiation between *T. cruzi*-infected and non-infected sera.

Based on the CL-ELISA results illustrated in Fig. 3, the question of specific glycotope recognition by Ch anti-α-Gal Abs arises: Does each of the three Galα(1,3)Galβ-containing NGPs **3-BSA**, **7-BSA**, and commercial Galα(1,3)Galβ(1,4)GlcNAcβ conjugated to BSA (purchased from V-Labs), which is also strongly recognized by ChSP and shows a favorable differential between ChSP and NHSP [32], recruit its own set of antibodies from the ChSP, or are the three glycotopes recognized by the same anti-α-Gal Abs? To address this question, we compared the CL-ELISAs of the individual NGPs immobilized in microtiter plate wells at different quantities, with that of the combined NGPs immobilized in the same microtiter plate wells at different quantities between 5 and 125 ng, and CL-ELISA reactivities were measured at three different sera dilutions (1:100, 1:200, and 1:400) (Fig. 5) [32]. We hypothesized if each NGP was recognized by a different set of antibodies, one would expect significantly higher RLU readings for the combined NGPs. However, the curves of the four experimental sets and the RLU readings resemble each other quite closely (Fig. 5). As expected, NHSP showed almost no binding to **3-BSA**, **7-BSA**, or Galα(1,3)Galβ(1,4)GlcNAcβ-BSA up to 125 ng. With ChSP, the titration curves for **3-BSA**, **7-BSA**, and Galα(1,3)Galβ(1,4)GlcNAcβ-BSA were similar. This indicates that antibodies from CCD patients recognize all three saccharides (Galα(1,3)Galβ, Galα(1,3)Galβ(1,4)Glcβ, and Galα(1,3)Galβ(1,4)GlcNAcβ) to a similar extent. As can be seen in the titration curves of Fig. 5, right panel, when the NGPs **3-BSA**, **7-BSA**, and Galα(1,3)Galβ(1,4)GlcNAcβ-BSA are combined in the same wells, no significant increase in the fluorescence signal was observed at a serum dilution of 1/100, showing that only a very small or no synergistic effect exists. This experiment suggests that for the most part the same antibodies recognize all three NGPs (**3-BSA**, **7-BSA**, and Galα(1,3)Galβ(1,4)GlcNAcβ-BSA) confirming that Galα(1,3)Galβ is the immunodominant disaccharide glycotope that is specifically recognized, regardless of whether it is linked to a short alkyl chain, or whether it has a 1,4 linkage to βGlc, or βGlcNAc.

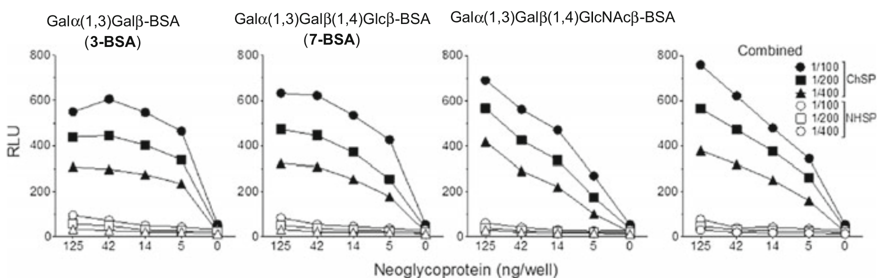


Fig. 5 CL-ELISA reactivity of ChSP and NHSP to the Galα(1,3)Galβ-containing NGPs **3-BSA**, **7-BSA**, and the commercial NGP Galα(1,3)Galβ(1,4)GlcNAcβ-BSA, alone or combined. Each NGP (alone or combined) was tested with ChSP and NHSP in duplicate. Figure modified from Ref. [32]

3 Conclusions

Synthetic strategies were developed for the synthesis of a biased library of ten *T. cruzi* glycans with terminal αGal moieties, which can be interrogated with sera of CCD patients to identify potentially immunodominant glycotopes. One of the glycans synthesized is the mercaptopropyl glycoside of Galα(1,3)Galβ(1,4) GlcNAcα, which is the only structurally defined *T. cruzi* tGPI mucin glycan. It was prepared in 12 steps from known monosaccharide building blocks. The two key features utilized throughout all glycan syntheses is the stereoselective installation of the terminal αGal moiety with the di-*tert*-butylsilylidene protected "Kiso donor" **18**, and the installation of allyl glycosides at the reducing ends. We have performed thiol-ene reactions by radical addition of thioacetic acid to the double bond of these allyl glycosides, and saponification of the resulting thioester afforded mercapto-propyl thioglycosides. However, allyl glycosides are very versatile, as they can be easily converted into hemiacetals, or into aldehydes by ozonolysis, which can then be further derivatized.

All glycans were conjugated to BSA, and the resulting NGPs were immobilized in wells of microtiter plates, thus generating a glycoarray that was subjected to CL-ELISA using pooled sera from CCD patients (ChSP), and healthy individuals (NHSP). ChSP strongly recognized the terminal disaccharide Galα(1,3)Galβ indicating that this glycotope is immunodominant. No matter if this disaccharide is connected to a short alkyl residue or to a glucose or *N*-acetylglucosamine moiety, the same set of Ch anti-α-Gal antibodies seem to recognize this disaccharide. The terminal disaccharide Galα(1,3)Galβ is specifically recognized by Ch anti-α-Gal antibodies with a large differential of 10–20 fold between CCD sera and the NHS when only 12 or 24 ng of the NGP is immobilized per well, and when the sera are diluted at 1:800. All other saccharides synthesized and conjugated to BSA gave poor reactivity differentials between ChSP and NHSP or had significantly weaker reactivity with ChSP. Interestingly, the nonself Galβ(1,4)GlcNAcα and GlcNAcα glycotopes are not recognized by ChSP, which stresses that the terminal αGal residue is essential for ChSP binding. Our data indicate that based on the large differential in reactivity, fully synthetic, structurally defined Galα(1,3) Galβ-containing NGPs could be used as biomarkers for the diagnosis of Chagas disease and could potentially be used for follow-up of chemotherapy, thus replacing purified and heterogeneous tGPI-mucins currently used for these purposes [19, 21, 23–25, 59]. In addition, synthetic Galα(1,3)Galβ-containing glycoconjugates could potentially be suitable for the development of glycan-based therapeutic and/or preventive vaccines for experimental vaccination against *T. cruzi* infection, as we recently proposed [33].

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