Neoglycoproteins as potential biomarkers and vaccines for Chagas disease

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NEOGLYCOPROTEINS AS POTENTIAL BIOMARKERS AND VACCINES FOR CHAGAS DISEASE

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Dedication

I dedicate this dissertation to ME, my parents, Liliam Carvajal Montoya and Josue N. Garcia Acevedo, and my sister Camila Garcia Carvajal for their unconditional support during my Doctoral program.
NEOGLYCOPROTEINS AS POTENTIAL BIOMARKERS AND VACCINES FOR CHAGAS DISEASE

by

ELISA GARCIA CARVAJAL, M.Sc.

DISSERTATION

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Abstract

Trypanosoma cruzi, the causal agent of Chagas disease (CD), currently affects around 6 million people worldwide, predominantly in Latin America, where the parasite is endemic. The treatment of CD is based on two drugs, benznidazole (BZN) and nifurtimox (NFX). These drugs cause adverse effects and are more effective in the acute phase of the disease. Conventional serology assays can be used for diagnosis, but they are not suitable for determining chemotherapy effectiveness because patients take approximately 10-20 years to exhibit negative seroconversion. Although CD has become a global health problem, specific biomarkers are still needed for the follow-up of chemotherapy and vaccine development. T. cruzi has a complex glycocalyx in which glycosylphosphatidylinositol (GPI)-anchored glycoproteins (tGPI-MUC) are prominent. These GPI-MUCs are heavily O-glycosylated with oligosaccharides that are mostly branched. Many of them have α-Gal moieties at the non-reducing end and are highly immunogenic to humans. α-Gal residues are the principal targets of protective T. cruzi-specific anti-α-Gal antibodies present in the sera of acute and chronic CD (CCD) patients. Notably, these anti-α-Gal antibodies are known to seroconvert relatively quickly, i.e., within approximately 3-5 years. Therefore, these antibodies are attractive not only for serological diagnosis of CD but also for determining treatment efficacy. The use of tGPI-MUCs as biomarkers (BMKs) to target anti-α-Gal antibodies is less suitable due to the laborious cultivation of parasites, extraction and purification of entire tGPI-MUCs, and inhomogeneity of the material. Instead, utilizing small and homogeneous epitopes (including multiple copies thereof on an inert platform) to which anti-α-Gal antibodies of CD patients respond specifically may be a better approach. Our group has broadly used neoglycoproteins (NGPs) as an alternative for discovering BMKs for diagnosis of CD, early assessment of chemotherapeutic efficacy, and vaccine development. This approach eventually can replace purified T. cruzi lysates as antigens in conventional serological assays. Here we present the synthesis of novel immunogenic oligosaccharides believed to exist on the tGPI-MUC. We synthesized partial structures of potential T. cruzi cell-surface glycans and evaluated their antigenicity using CCD patient sera according to structural information from glycomics data. Chapter 1 briefly introduces Chagas disease and its causal agent, the protozoan parasite T. cruzi. Chapter 2 describes the modular synthesis
of three complexes α-Gal-containing oligosaccharides believed to exist in the tGPI-MUC of *T. cruzi*. This includes two tetrasaccharides \((G36s)_2\) and \((G37s)_2\) and one pentasaccharide \((G35s)_2\). It will also address their conjugation to BSA, preliminary data about the antibody response using sera from CCD patients, and the effect of glycan loading on the antibody reactivity. Chapter 3 discusses the optimized synthesis of α-Gal containing trisaccharide \((G24s)_2\) and the development of the conjugation procedure to synthesize neoglycoproteins (NGPs) using different carrier proteins (i.e., human serum albumin (HSA), ovalbumin (OVA), and tetanus toxoid (TT) proteins) and linkers of different lengths (i.e., SIA and SMCC linkers). Finally, chapter 4 features the discovery of a Galα(1,6)[Galα(1,2)]Galβ-containing NGP as a synthetic biomarker for CD. It will show the suitability of NGP11b as a biomarker for CCD diagnosis and chemotherapy follow-up by analyzing its reactivity with pooled sera from CCD and healthy individuals.
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1. Introduction

1.1. Chagas Disease generalities

Chagas Disease (CD), also known as American Trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). It was discovered by Carlos Justiniano Chagas, a Brazilian physician, in 1909. Since then, CD has remained a major social and public health problem in Latin America, principally in the seven southernmost American countries. The World Health Organization (WHO) has estimated 5.7 million infected people live in Latin America and 350,000 infected people live in the US.\(^1\)\(^2\) Even though CD is endemic in Latin American countries, CD cases have increased dramatically in non-endemic countries due to the migration from Latin America to Europe. For example, in Spain, 55367 migrants were estimated to have Chagas Disease in 2018.\(^3\) The migratory flow and non-vector transmission routes have turned CD into a public health problem worldwide.

*T. cruzi*, the causal agent of CD, is transmitted to humans and domestic animals by blood-sucking triatomine bugs, commonly known as “kissing bugs.” *Triatoma infestans, Rhodnius prolixus, and Triatoma dimidiate* are the principal species in parasite transmission. *T. infestans* is mainly found in sub-Amazonian endemic regions, *R. prolixus* is prevalent in northern South America and Central America, and *T. dimidiate* extends further North into Mexico. In the United States, eleven triatomine species have been reported, including *T. sanguisuga* and *T. protraca*, the widest distributed species.\(^4\) Although vector-borne transmission is still the main route for new human infections in endemic regions, *T. cruzi* can also be transmitted by congenital transmission, blood transfusion, organ transplantation, and contaminated food. These paths are the main route for new human infections in non-endemic regions.\(^1\)

CD is manifested in two phases, an acute phase, and a chronic phase. The acute phase begins 1-2 weeks after inoculation and lasts approximately four weeks. In most cases, the acute phase is asymptomatic, and less than 1-5% of patients experience severe acute disease.\(^5\) Although high levels of parasitemia are characteristic during this phase; the immune system controls the circulating parasite.\(^4\) After two months of infection, the parasitemia drops to levels that are not detectable by microscopy, and the patient evolves to
the chronic phase of *T. cruzi* infection. Asymptomatic chronic *T. cruzi* infection is the indeterminate form and is presented in around 70% of patients with chronic infection. In 20-30% of patients with chronic Chagas disease (CCD), a determinate form is developed after decades: cardiac, digestive (megaesophagus and megacolon), or cardio digestive complications.\(^1\)

The method of diagnosis of CD depends on the clinical manifestation and possible phase of infection. Direct detection of trypomastigotes by light microscopy in smear from whole blood or buffy coat is commonly used in the acute phase. Microscopy is helpful due to its wide availability and high specificity. However, its sensitivity depends on the detection of circulating parasites. Indirect detection of parasites using molecular methods like PCR presents higher sensitivity than direct methods. PCR has been proven helpful in diagnosing the acute phase and in the diagnosis of mother-to-child transmission; nonetheless, due to its dependence on a relative high concentration of parasites in the blood, it is unreliable in diagnosing the chronic phase. CD can also be indirectly detected by serological testing by determining IgG antibodies against *T. cruzi*. The whole parasite antigen (conventional serology), purified extracts, recombinant antigens, and synthetic peptides can detect IgG antibodies using enzyme-linked immunosorbent assays (ELISA), indirect haemagglutination (IHA) and immunofluorescence assays (IFAs). Generally, CD diagnosis must be made by more than one method to identify CD accurately.\(^6\)

Only two drugs, benznidazole (BZN) and nifurtimox (NFX), are available for CD treatment. NFX is a nitrofuran derivative. It is administrated orally in four doses of 10-20 mg/kg per day for 60-90 days. 43-97%\(^5\) of patients under NFX treatment develop side effects, including anorexia, weight loss, neurological disorders, digestive manifestations, fever, and rash. Due to the severe adverse effects, treatment is discontinued in 14-75% of cases. On the other hand, BZN is a nitroimidazole derivative. It is administrated orally at 5.0-7.5 mg/kg per day for 60 days. Most of the time it is preferred over NFX due to its better tolerability profile. The treatment is discontinued in 9-29% of cases, mainly due to side effects like hypersensitivity, digestive intolerance, and general symptoms such as anorexia, asthenia, headache, and sleeping disorders.\(^4,5,7\) Overall, the efficacy of both drugs is variable and depends on the drug dose, disease phase, patient age, medical history, and geographical
origin of the infection. Although CD belongs to the Neglected Tropical Disease (NTDs) group, there has been a tendency to study the reduction of antiparasitic dosage in the past years. The pilot studies include the BENEDITA\(^8\) trial, the EQUITY\(^9\) study, the MULTIBENZ (clinical trial NCT03191162) study, and, more recently, the TESEO\(^10\) study.

The assessment of CD treatment efficacy has been done by indirect detection of parasites through serological testing and polymerase chain reaction (PCR). Conventional serology is problematic due to the long time to observe negative seroconversion in patients (10-20 years).\(^{11,12}\) On the other hand, as the number of circulating parasites decreases due to chemotherapy, PCR reaches its detection limit and narrows its utility for drug efficacy assessments. In the last years, 25 biomarkers (BMK) have been used to measure the therapeutic efficacy of CD chemotherapy, including antibodies for host antigens, antibodies generated against parasite antigens, cytokine level, cytokine pattern in serum from patients, and markers to quantify cellular immune response populations.\(^{11}\) However, only a few have been evaluated using properly designed studies for therapeutic response in humans.\(^{11,13,14}\) Due to the difficulties in determining the response-to-treatment of patients after chemotherapy, there is a need for specific BMK for the early follow-up of Chagas disease chemotherapy efficacy.\(^7,11\)

### 1.2. Trypanosoma cruzi

There are four developmental phases in the life cycle of \textit{T. cruzi}, i.e., epimastigote, metacyclic trypomastigote, amastigote, and blood trypomastigote forms. Amastigotes and epimastigotes are replicative forms in mammalian cells and the insect midgut, respectively. Metacyclic trypomastigotes and bloodstream trypomastigotes are non-proliferative phases and are the parasite's infective stage. These morphological stages allow the parasite to adapt and survive the environment in each host.\(^{15}\)

\textit{T. cruzi} is also genetically diverse and classified into different strains or subtypes. Genetic variability of \textit{T. cruzi} was first discovered in Bahia, Brazil, using a panel of isoenzyme markers.\(^{16}\) This discovery opened the door to characterizing \textit{T. cruzi} strains using the molecular methods available at the time. As a result, high diversity was found, and the names of subtypes in the literature became confusing. It was not until 2009, during the Second
Satellite Meeting held in Buzios, Brazil, that the scientific community formally classified *T. cruzi* strains into six genotypes (TcI to TcVI) discrete typing units (DTUs). All six *T. cruzi* DTUs can infect mammals, overlap geographically, and coexist in the same population. TcI and TcII are the oldest and purest lineages, TcV and TcVI have a hybrid origin with TcII and TcIII as putative parentals, and TcIII and TcIV origins are not clear. Still, some believe they result from ancient hybridization between TcI and TcII.

TcI is the most frequent and broadly spread DTU; it has been found from the South of the United States to the North of Chile and Argentina and can be transmitted both in sylvatic and domestic cycles. It has been found in human infections in the US, Mexico, Central America, and the Andean and Amazon regions and is associated with severe cardiomyopathies. TcII is not common in North and Central America; nevertheless, it is prevalent in some regions of the Southern Cone of South America, particularly in Brazil and Chile. TcII is frequently found in Triatoma species and infected patients with megaesophagus and megacolon. TcIII and TcIV show similarities. They are believed to be the result of ancient hybridization between TcI and TcII and are mostly associated with sylvatic transmission cycles. They have been related as a cause of acute outbreaks in the Amazon basin. Lastly, TcV and TcVI are more common in domestic transmission cycles in the Southern Cone countries. TcV has been found in Argentina, Bolivia, Paraguay, Uruguay, Peru, Ecuador, and Louisiana. TcVI is more restricted to Southern Cone, representing more than 50% of the DTUs isolated in Argentina, and it is mostly associated with the cardiac and digestive forms of CD.

All *T. cruzi* DTUs have a thick coat of glycoconjugates covering their cell surface called the glycocalyx. This surface coat is composed of GPI-anchored glycoproteins, including major glycoprotein families of mucins, i.e., mucin-associated surface proteins (MASP), trans-sialidases (TS), and complex GPI-related glycolipids like glycoinositolphospholipids (GIPLs), and glycosylphosphatidylinositol-anchored mucins (GPI-MUC). GPI-MUC from the trypomastigote stage (tGPI-MUC) are heavily O-glycosylated through threonine or serine residues via α-N-acetylglucosamine units. The glycan portion of tGPI-MUC represents approximately 60% of the glycoprotein mass. The trisaccharide Galα1-3Galβ1-4GlcNAcα is the only O-linked glycan fully elucidated, representing about 10% of all tGPI-MUC.
The exact structures of the remaining 90% are still elusive, but notable structural features, including the presence of 4,6-di-O-substituted GlcNAcα and α-Galp residue at the non-reducing end, have been found.\textsuperscript{33-36} These α-Gal-containing glycans are absent in human cells due to the inactivation of the α1,3-galactosyltransferase (α1,3-GalT) gene in ancestral primates, thus becoming highly immunogenic glycotopes to humans.\textsuperscript{33,37} On account of that, protective \textit{T. cruzi}-specific anti-α-Gal antibodies present in the sera of chronic CD patients (CD anti-α-Gal Abs) strongly recognize tGPI-MUC O-glycans, specifically the α-Gal residues at the nonreducing end.\textsuperscript{31,33,38} CCD anti-α-Galp Abs and purified glycoproteins from \textit{T. cruzi} have been used to measure the efficacy of BZN treatment in children with chronic infection. After a 3-year follow-up, 58% of children exhibit negative seroconversion with chemiluminescent-ELISA (CL-ELISA).\textsuperscript{39,40} In contrast, only 9% of adults with CCD treated with BZN were negatively seroconverted by the antigen trypomastigote CL-ELISA (AT CL-ELISA) after a 1-year follow-up.\textsuperscript{41,42}

There is no prophylactic or therapeutic vaccine for CD. The approach for developing vaccines against CD has been focused on parasite lysates, purified proteins, peptides, DNA, and glycoproteins from the epimastigote stage.\textsuperscript{43,44} One of the difficulties among these approaches is the poor conservation among \textit{T. cruzi} DTUs. Therefore, poor protection has been observed.\textsuperscript{45,46,46} Besides, the purification and characterization of glycoproteins from the parasite present some challenges regarding the amount of extracted material and characterization. Although glycoconjugates are the significant epitopes of tGPI-MUC, they remain poorly explored as vaccine targets.\textsuperscript{47} The Galili epitope (Galα1-3Galβ1-4GlcNAcβ) is the only glycotope evaluated as a prophylactic α-Gal-based glycovaccine, showing efficacy against experimental acute Chagas disease.\textsuperscript{48}

While tGPI-MUCs are attractive as candidates for diagnostic and prognostic BMK, these results are far from perfect. Purifying tGPI-MUC requires the cultivation of large amounts of parasites, extensive mammalian cell tissue, prolonged extraction, and purification process, the glycoproteins exhibit structural inhomogeneity, and the results after each extraction are inconsistent. An alternative approach is the use of synthetic neoglycoproteins (NGPs). NGPs comprise a carrier protein (e.g., bovine serum albumin) decorated with containing oligosaccharides, based on structural information of O-glycans found in tGPI-mucins.\textsuperscript{31,36,49,50}
Our group has broadly used different NGPs, including NGP29b, a linear disaccharide from the β-galactofuranosylated GPI anchor of the tGPI-MUC, and NGP24b, a linear trisaccharide found in the protein portion of tGPI-MUC. Our evidence shows that NGP29b and NGP24b exhibit differential immunoreactivity similar to purified tGPI-MUC. The use of NGPs as BMKs for diagnosis of CD, early assessment of chemotherapeutic efficacy, and vaccine development can replace purified T. cruzi lysates as antigens in conventional serological assays.

Here we present the synthesis of novel immunogenic oligosaccharides believed to exist on the tGPI-MUC. Based on mass spectrometric glycomics data, we synthesized partial structures of potential T. cruzi cell-surface glycans and evaluated their antigenicity by ELISA using CCD patient sera. The serology was done in collaboration with Professor Igor Almeida in the Department of Biological Sciences at UTEP.

This dissertation is divided into four chapters. Chapter 2 will describe the modular synthesis of two tetrasaccharides \((G36)s_2\) and \((G37)s_2\) and one pentasaccharide \((G35)s_2\). It also will address their conjugation to BSA, the antibody response using sera from CD patients, and the effect of glycan loading on the antibody reactivity. We believe these glycotopes are present on tGPI-MUC and could be potential BMKs for diagnosing CD and early assessing chemotherapeutic efficacy. Chapter 3 will describe the optimized synthesis of \(\alpha\)-Gal containing trisaccharide \((G24)s_2\) previously developed in our lab and its conjugation to human serum albumin (HSA), ovalbumin (OVA), and tetanus toxoid (TT) proteins. We believe NGP vaccines induced protection via recognizing both peptides and glycopeptides presented by MHCII on the activated B cells and engagement of CD4+ T cells. The immunization experiments will be done in collaboration with Professor Igor C. Almeida in the Department of Biological Sciences at UTEP and Fikri Avci in the Department of Biochemistry at Emory University. Finally, chapter 4 will discuss the discovery of a Galα(1,6)[Galα(1,2)]Galβ-containing NGP as a synthetic biomarker for CCD. Here, the synthesis of a branched trisaccharide \((G11)s_2\) and its conjugation to bovine serum albumin (BSA) is described. We have investigated its suitability as a BMK by analyzing its sensitivity and selectivity with individual sera from CCD patients and healthy individuals. Finally, NGP11b was also assessed for chemotherapy follow-up after two years of treatment.
2. Novel branched α-Gal-containing oligosaccharides derived from Trypanosoma cruzi tGPI-mucins as a potential biomarker.

2.1. Introduction

Modification of proteins by glycans is one of the most common posttranslational modifications. These glycans play important roles in biological processes such as cell-extracellular matrix interactions, tissue developments, immune response, cell-cell interaction, and host-pathogen recognition, among others. Mucins are proteins heavily glycosylated with O-linked oligosaccharides and are the main component of the thick coat cell surfaces of T. cruzi.

The oligosaccharides from trypomastigotes GPI-mucins (tGPI-MUC) provide protection from the immune system of the mammalian host. The core protein tGPI-MUC is rich in Ser and Thr residues. These residues work as acceptor sites for the addition of N-acetylglucosamine through an O-linkage. The structure of the O-glycans from T. cruzi epimastigotes mucins (eGPI-MUC) has been determined. The 20% of the O-linked GlcNAc remains unsubstituted. The residual 80% of structures are elongated at the GlcNAc moiety in the reducing end to form complex and branched oligosaccharides. eGPI-MUC O-glycans differ by strain and genotype but can be categorized into two categories. The Core 1 (Galpβ1-4[Galpβ1-6]GlcNAcα) principally associated with the domestic transmission or Core 2 (Galfβ1-4[Galpβ1-6]GlcNAcα) expressed by strains isolated from T. cruzi sylvatic hosts. These branched trisaccharides are the skeleton for more complex higher-order O-glycans. Although eGPI-MUC O-glycans structures have been profoundly studied, the only O-linked glycan fully elucidated to date from tGPI-MUC is the linear trisaccharide Galα1-3Galβ1-4GlcNAcα, representing approximately 10% of all tGPI-mucin glycans. The structures of the remaining 90% have notable structural features, including the presence of 4,6-di-O-substituted GlcNAcα and α-Gal residue at the non-reducing.

Unpublished glycomics and reverse immunoglycomics data obtained by a former student from the Almeida’s Lab, Uriel Ortega-Rodriguez, elucidated O-linked oligosaccharides from T. cruzi tGPI-mucins. The oligosaccharides were released by reductive β-elimination followed by permethylation of the released glycans in their alditol forms. The glycans were analyzed by electrospray ionization tandem mass spectrometry, (ESI-MS/MSn)
and liquid chromatography-high resolution tandem mass spectrometry (LC-HR MS/MS). Collisional Activated Dissociation was used to get structural information through the generation of cross-ring fragments that can be further utilized to assign linkage. From these cross-ring fragments, it was found evidence of several glycoforms with the GlcNAc core substituted at the 4, and 6 position by Galfβ or Galpβ to generate the branched trisaccharide Galpβ1-46[Galp1-6]GlcNAcα.36 In combination with reverse immunoglycomics data collected by our group49,57,58, we suspected that this core is substitute by α-Gal residues at 3 or 2 position of the β-Gal residues.

Here we present the modular synthesis of three novels branched oligosaccharides with the common core Galβ(1→4)[Galβ(1→6)]GlcNAcα and α-Gal(1→3) residues at the non-reducing end (Figure 1). The oligosaccharides were assembled from three building blocks with orthogonal protecting groups to ensure regioselectivity and stereoselectivity. The oligosaccharides were conjugated to bovine serum albumin (BSA) and evaluated using sera from CCD patients and sera from healthy individuals as a negative control. Also, we will investigate the relationship between the payload and the antibody recognition by having four different NGP36b with different glycan loading.
Figure 1. Target oligosaccharides
2.2. Results and discussion

2.2.1. Synthesis of \((G35S)_2\), \((G36S)_2\), and \((G37S)_2\)

The synthetic strategy for the three complex oligosaccharides \(G35_{SH}\), \(G36_{SH}\), and \(G37_{SH}\) is based on a convergent approach using interchangeable building blocks of intermediate complexity. In this way, different analogs can be achieved by changing the combination of these building blocks. To accomplish these monosaccharides, we used orthogonal protecting groups based on acyl and silyl groups and stereoselective glycosylation using Kiso’s 4,6-di-\textit{tert}-butylsilylenegalactosyl trichloroacetimidate donor 14.\(^{59-61}\) In the final step, oligosaccharides were modified with a mercaptopropyl linker for the conjugation to maleimide-derivatized BSA.\(^{41,49,50,62}\)

The synthesis of the three target oligosaccharides \(G35_{SH}\), \(G36_{SH}\), and \(G37_{SH}\) was divided into three stages. 1) The synthesis of monosaccharides acceptors and donors 2, 4\(^3\), 14\(^59-61,64\), and 17\(^63\); 2) the synthesis of trisaccharide acceptor 3, disaccharide donor 1, and disaccharide acceptor 7; and 3) the assembly of fully protected oligosaccharides 5, 6 and 8.

Kiso’s 4,6-di-\textit{tert}-butylsilylenegalactosyl trichloroacetimidate donor 14 was synthesized according to the methodology previously reported by Kiso (Scheme 1).\(^{59-61}\) Thiogalactoside 11 was per benzoylated to donor 4 previously reported by Wong (Scheme 2).\(^63\) Although 4 was reported before, no spectroscopic data were reported. The \(^1\)H NMR spectrum in DMSO-\(d_6\) shows the anomeric proton overlap in a multiplet at 5.59 – 5.48 ppm, the methyl group from the \(p\)-thiotolyl group at 2.34 ppm, and the 24 anomeric protons in the region between 7.98 – 7.10 ppm. From the same galactoside 11, the naphthyl methyl (Nap) protecting group was installed in the hydroxyl at C-3 to give 15 in 67\% yield after two steps. The residual hydroxyl groups were benzoylated to furnish 16 in 91\% yield, following the selective removal of the Nap group using reducing conditions to yield acceptor 17 previously reported by Wong (Scheme 2).\(^63\)

The \(\alpha\)-N-acetyl-glucopyranoside (\(\alpha\)-GlcNAc) acceptor 2 was synthesized from unprotected GlcNAc. The allyl group was installed by a Fisher glycosylation in the anomeric carbon in a 1,2 cis configuration to give 20\(^65\) in 74 \% yield. Then, hydroxyl groups at C-4 and C-6 were protected with a benzylidene group to yield 21\(^66\) in 83\% yield, followed by
acetylation of hydroxyl at C-3 to furnish 22 in 89% yield. Finally, the benzylidene group was removed under acidic conditions to give 23 in 85% yield. Lastly, a tert-butyldimethylsilyl (TBDMS) group was installed in OH-6 to furnish acceptor 2 (Scheme 3) in 95% yield. $^1$H NMR spectroscopy confirmed the α-configuration of the anomeric proton in acceptor 2 ($\delta$ 4.82, d, $J_{1,2}$ = 3.6 Hz, 1H, H-1). The correlation between H-4 and -OH in the COSY-NMR confirmed the unprotected hydroxyl.

**Scheme 1.** Synthesis of 2,3-di-O-benzoyl-4,6-di-tert-butyldimethylsilyl-α-D-galactopyranosyl trichloroacetimidate (Galp $\alpha$) donor 14. a) 1. Cu(OTf)$_2$, Ac$_2$O, 0 °C to r.t; 2. TolSH, BF$_3$-Et$_2$O, solvent free, r.t, 70%. b) NaOMe, MeOH, rt, 95%. c) 1. DTBSOTf, pyr; 2. BzCl, pyr, 50% over two steps. d) NBS, Acetone, 62%. e) CCl$_3$CN, DBU DCM, 82%
Scheme 2. Synthesis of p-Tolyl 2,3,4,6-tetra-O-benzoyl-1-thio-β-D-galactopyranoside acceptor 17 and p-Tolyl 3-O-(2-methylnaphthyl)-2,4,6-tri-O-benzoyl-1-thio-β-D-galactopyranoside donor 4. a) BzCl, pyr, 91%. b) 1. Bu₂SnO, MeOH, reflux 2. Nap-Cl, Bu₄NBr, benzene, reflux, 67% over two steps. c) DDQ, DCM/H₂O (2:1), 80%.

Scheme 3. Synthesis of Allyl 2-Acetamido-3-O-acetyl-2-deoxy-6-O-tert-butylidydimethylsilyl-α-D-glucopyranoside (2). a) AllOH, BF₃·Et₂O, reflux, 2h, 74%. b) PhCH(OCH₃)₂, p-TsOH, CH₃CN, 83%. c) Ac₂O, pyr, 89%. d) AcOH 80%, 80 ºC, 85%. e) TBDMSOTf, DMAP, pyr, 95%.
The trisaccharide acceptor 3 was then synthesized by two consecutive glycosylations and one selective deprotection at C-6 of the GlcNAc at the reducing end. First, 17 was glycosylated with 14 to produce the fully protected disaccharide donor 1 in 88% yield (Scheme 4). $^1$H NMR confirmed the configuration of the new anomeric proton ($\delta$ 5.59, d, $J_{1':2'}$ = 3.8 Hz, 1H, H-1'). Then, this donor was used to synthesize fully protected trisaccharide 25 in 66% yield by glycosylation between 1 and 2 (Scheme 5). $^1$H NMR confirmed the configuration of the new anomeric proton ($\delta$ 5.00, d, $J_{1:2}$ = 8.0 Hz, 1H, H-1'), and $^1$H NMR confirmed the aromatic protons in acetone-d$_6$ to avoid interference with CDCl$_3$ signal at 7.26 ppm. The synthesis of trisaccharide 25 was improved compared with the previously reported by Schocker.$^{50}$ Subsequently, selective removal of the TBDMS group was accomplished using acetic acid (AcOH) to furnish trisaccharide acceptor 3 quantitatively. Similarly, disaccharide acceptor 7 was synthesized by the glycosylation of acceptor 2 with donor 4 in 55% yield, followed by selective deprotection of C-6 of the GlcNAc$\alpha$ at the reducing end under acid conditions to yield disaccharide acceptor 7 quantitatively (Scheme 6). $^1$H NMR spectroscopy confirmed the $\beta$-configuration of the new anomeric proton ($\delta$ 5.03, d, $J_{1:2}$ = 7.9 Hz, 1H, H-1').

Scheme 4. Synthesis of p-Tolyl 2,3-di-O-benzoyl-4,6-O-di-tert-butylsilylene-$\alpha$-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-1-thio-$\beta$-D-galactopyranoside (1). a) TMOSTf, DCM, 4Å MS, 88%
Scheme 5. Synthesis of Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethylsilylene-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Azetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (3) 

a) AgOTf, NIS, DCM, 4Å MS, 66%. b) AcOH:H₂O:THF,3:1:1, reflux, (quantitative)

The concept of regenerative glycosylation reaction, introduced by Demchenko,⁶⁸ was used in the construction of this disaccharide. Here, 3,3-difluoroxindole (HOFox) reacts with a thioglycoside to form a highly reactive OFox imidate. Then, the imidate will react with the glycosyl acceptor while regenerating HOFox aglycone. The released HOFox will react with another thioglycoside in the next cycle to form another HOFox donor, and so forth. Using 20-30 mol % of HOFox and a catalytic Lewis acid (TMSOTf), the reaction gives reasonable reactivities (1-2 h). The HOFox was synthesized as described elsewhere.⁶⁹
Scheme 6. Synthesis of Allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside(7). a) HOFox, NIS, TMSOTf, DCM, 4Å MS, 68%. b) AcOH:H₂O:THF, 3:1:1, reflux, quantitative

The tetrasaccharide G₃₆ˢʰ was synthesized by a glycosylation between acceptor 3 and donor 4 to generate a fully protected tetrasaccharide 5 in 69% yield (Scheme 7). ¹H NMR spectroscopy in DMSO-d₆ confirmed the protons in the aromatic region. Subsequently, the silylene protecting group was removed with HF·pyridine complex to afford 26 in 61% yield. The configuration of the new β-anomeric proton was confirmed in this deprotection step by ¹H NMR spectroscopy (δ 4.55, d, J = 7.9 Hz, 1H, H-1D). Radical anti-Markovnikov addition of thioacetic acid (AcSH) to the allyl glycoside produced 27 in 70% yield. Finally, the total deacylation under Zemplen conditions afforded the tetrasaccharide G₃₆ˢʰ in a 34% yield.

Similarly, the fully protected pentasaccharide 6 was synthesized by glycosylation between acceptor 3 and donor 1 in 40% yield (Scheme 8). The formation of by-product 35 affected the yield of this glycosylation. Similar to the synthesis of G₃₆ˢʰ, the silylene group was removed with HF·pyridine complex to afford 28 in 88% yield. Addition of AcSH by a radical anti-Markovnikov mechanism furnished propyl thioacetyl glycoside 29 in 70% yield. The aromatic protons and the configuration of the new β-anomeric proton were confirmed
by $^1$H NMR spectroscopy ($\delta$ 4.51, d, $J_{1-2}$ = 8.0 Hz, 1H, H-1D). The overall deprotection under Zemplen conditions afforded the pentasaccharide G35SH. Pentasaccharide was confirmed by MS.

Finally, fully protected tetrasaccharide 8 was synthesized by a glycosylation of acceptor 1 and donor 7 in a 69% yield (Scheme 9). Likewise, the silylene group was deprotected with HF-pyridine complex to afford 30 in 80% yield, followed by the radical addition of AcSH in an anti-Markovnikov fashion to give propyl thioacetyl glycoside 31 in 60% yield. Lastly, the complete deacylation under Zemplen conditions afforded the tetrasaccharide G37SH in a 34% yield.

Oligosaccharides G36SH and G37SH oxidized with air to form disulfides (G36S)$_2$ and (G37S)$_2$. After oxidation, disulfide compounds were purified by fast protein liquid chromatography (FPLC) using a Resource™ RPC column (polystyrene/divinyl benzene matrix). 2% ACN and 85% ACN were used as a solvent system with a flow rate of 2 mL/min. Compound (G36S)$_2$ was eluted at 10.63 min (Figure 2), and (G37S)$_2$ was eluted at 10.75 min (Figure 3). (G36S)$_2$ and (G37S)$_2$ could easily be located in the FPLC profiles by their absorption at 190 nm due to the disulfide chromophore.
Scheme 7. Synthesis of G36SH. a) HOFox, NIS, TMSOTf, DCM, 4Å MS, 72%. b) HF-pyr, THF, 61%. c) Thioacetic acid, DPAP, DCM, UV light (350 nm), 70%. d) NaOMe, MeOH
Scheme 8. Synthesis of $\text{G35}_{\text{SH}}$. a) HOFox, NIS, TMSOTf, DCM, 4Å MS, 40%. b) HF-pyr, THF, 88%. c) Thioacetic acid, DPAP, DCM, UV light (350 nm), 70%. d) NaOMe, MeOH
Scheme 9. Synthesis of G37SH. a) HOFox, NIS, TMSOTf, DCM, 4Å MS, 54%. b) HF-pyr, THF, 80%. c) Thioacetic acid, DPAP, DCM, UV light (350 nm), 60%. d) NaOMe, MeOH
Figure 2. FPLC chromatogram of (G36)$_2$
2.2.2. Synthesis of NGP36b

After reduction of (G36S)₂, with neutral TCEP, G36SH was conjugated to maleimide-derivatized BSA to yield NGP36b. The payload of the NGP was measured by comparative MALDI-TOF MS spectra (Scheme 10). In order to analyze the effects of the payload on antibody recognition, four NGP36b were synthesized with different numbers of glycan units (GU), i.e., 2, 6, 8, and 20 GU. Our first approach was to control the kinetics of the Michael-type addition of the thiol group to the maleimide. However, even at 5 min of reaction, the payload was the same as at 20 min (Figure 4). The payload achieved was 19 GU. Since the kinetic control was not successful, we decided to control the loading by changing the concentration...
of the linker and glycan. Low concentration (0.5 mM) gave low loading (NGP36b-2GU), and higher concentration (2 mM) gave medium loading (NGP36b-8GU) (Figure 5-Figure 8).

Scheme 10. Synthesis of NGP35b, NGP36b, and NGP37b. a) TCEP neutral 0.5 M, phosphate buffer pH 7.2, rt, 2h.
2.2.3. Synthesis of NGP37b and NGP35b

After reduction of \((G37)_2\) and \((G35)_2\) with neutral TCEP, \(G37_{SH}\) and \(G35_{SH}\) will be conjugated to maleimide-derivatized BSA to yield NGP37b and NGP35b. The payload of the NGP will be measured by comparative MALDI-TOF MS spectra.

**Figure 4.** G36_{SH} conjugation kinetics
Figure 5. MALDI-TOF NGP36b, 2 GU

Figure 6. MALDI-TOF NGP36b, 6 GU
Figure 7. MALDI-TOF NGP36b, 8 GU

Figure 8. MALDI-TOF NGP36b, 20 GU
2.2.4. Evaluation of NGP36b

With the NGP36b in hand, IgG antibody responses of pooled CCD patient sera with confirmed *T. cruzi* infection by PCR were studied using CL-ELISA. The patient sera were collected from individuals from Bolivia, where *T. cruzi* infection is endemic. Additionally, pooled sera from 27 healthy patients (NH) were interrogated as a negative control. As a positive control, we used NGP24b (payload 14 GU), the linear trisaccharide Galα1-3Galβ1-4GlcNAca conjugated to BSA. NGP24b was previously reported as a potential diagnostic and vaccine candidate for CCD due to its high immunogenicity in the context of *T. cruzi* infection in both mice and humans.50 As an antigen-negative control, 2-mercaptoethanol (2-ME) was conjugated to maleimide-derivatized BSA 34 (2-MEb).

CCD serum pools were prepared and cross-titrated against different concentrations [ng/well] of NGP24b, and NGP36b. Figure 9 shows that at different dilutions, CCD pooled sera exhibited strong IgG antibody reactivity with NGP36b and NGP24b. Contrary, NHSP pooled sera exhibited significantly lower antibody reactivity. The cross-titration revealed that serum dilution of 1:800 and 400 ng of NGP antigen per well offered the most significant differential IgG responses when comparing ChHSP vs. NHSP. At a sera dilution of 1/800, NGP24b has an antibody reactivity higher than NGP36b by a factor of 1.3. The difference in IgG reactivity between the ChHSP and the NHSP was a factor of 12 for NGP36b and a factor of 31 for NGP24b, similar to Galα1,3Galβ-containing NGPs synthesized in previous studies.49,50 When combining both NGPs in the same well at comparable well loading, no synergy in the antibody recognition was observed. This indicates that NGP36b behaves similar to NGP24b in the CL-ELISA, and that the extension with a Galβ moiety at C-6 of the GlcNAc does not seem to improve or disturb the recognition of the anti-a-Gal IgG antibodies.

Also, we wanted to investigate the effect of the NGP glycan density on antibody recognition. For this, we synthesized four NGPs with different glycan loading (2, 5, 8, and 20 glycans per molecule of BSA) and interrogated them with sera from CCD patients at two different dilutions (1/400 and 1/800). The cross titrations are presented in Figure 11-14. There is a clear effect of the glycan density with antibody recognition. In general, there is an increase in the reactivity of NGP36b-2GU to NGP36b-20GU. Specifically, at an antigen concentration of 100 ng/well and a sera dilution of 1/800, there is a 14-fold increase from
NGP36b-2GU to NGP36b-5GU and an 18-fold increase from NGP36b-2GU to NGP36b-8GU (Figure 10). We observed there is a saturation point after 8 GU due to the decrease by 0.3-fold from NGP36b-8GU to NGP36b-20GU. This suggested that higher glycan densities than 8 units per BSA do not result in higher antibody reactivities. The effect of glycan density in antibody recognition using CL-ELISA has not been studied in depth. Nevertheless, some reports have been found about how the glycan density contributes significantly to the variability of antibody recognition in NGP microarrays. To our knowledge, the effect of glycan density in biological activity was first observed by Krantz et al.\textsuperscript{70} Those authors showed that attaching increasing amounts of D-galactoside to BSA progressively increased its ability to bind to liver membranes even at low levels of incorporation (3 mol of D-galactoside per mol of BSA). When the glycan density was 25 D-galactosyl residues per BSA, the relative inhibitory power value increased 10\textsuperscript{6}-fold relative to unmodified BSA.\textsuperscript{70} Later in 2001, Cairo et al exhibited the effect of high binding epitope density by polymer results in greater numbers of receptors bounded per polymer.\textsuperscript{71} More recently, Bertozzi and Gildersleeve demonstrated that differences in glycan density of NGPs can affect the ability to form multivalent interaction in microarrays platforms.\textsuperscript{72,73}
Figure 9. Synergic effect on NGP24b-14GU and NGP36b-20GU. Titration with anti-human Ab at (A) 1/400 dilution. (B) 1/800 dilution.
**Figure 10.** Linear correlation between the glycan density and the antibody response at 100 ng/well

**Figure 11.** Antigen serum cross-titration of NGP36b with 2 glycan units (GU) per BSA.
Figure 12. Antigen serum cross-titration of NGP36b with 5 glycan units (GU) per BSA.

Figure 13. Antigen serum cross-titration of NGP36b with 8 glycan units (GU) per BSA.
Figure 14. Antigen serum cross-titration of NGP36b with 20 glycan units (GU) per BSA.

2.3. Conclusions

In this chapter, we developed a modular convergent synthetic route for the synthesis of complex oligosaccharides $G_{36}^{SH}$ (Gal$\alpha_1$3Gal$\beta_1$4Gal$\beta_1$6GlcNAc$\alpha$), $G_{35}^{SH}$ (Gal$\alpha_1$3Gal$\beta_1$4Gal$\alpha_1$3Gal$\beta_1$6GlcNAc$\alpha$), and $G_{37}^{SH}$ (Gal$\alpha_1$3Gal$\beta_1$4Gal$\alpha_1$3Gal$\beta_1$6GlcNAc$\alpha$), which are terminal glycans that could be present in the GPI-MUC of T. cruzi trypomastigotes. $G_{36}^{SH}$ was synthesized in seven steps from building blocks with an overall yield of 1.3%. $G_{37}^{SH}$ was synthesized in six steps from building blocks with an overall yield of 0.9%. Finally, $G_{35}^{SH}$ was also synthesized in 7 steps from building blocks. The overall yield before the final deprotection is 2%. This modular approach allowed the synthesis of a variety of tGPI-MUC-derived glycans. The acceptor 2, with C-4 unprotected grants the extension at this position. At the same time, the TBDMS group at C-6 can be removed selectively to allow the extension at position 6. Compound $G_{36}^{SH}$ was conjugated with different payloads to BSA controlling the concentration of the linker and glycan. Lower concentrations yielded lower payloads and vice versa.
The preliminary biological results from the screening of NGP36b with CCD sera suggested that it has a strong IgG Abs response, similar to the reactivity shown by NGP24b. From this data, we can also conclude that the substitution with a Galβ at C-6 of GlcNAc does not improve or inhibit antibody recognition. Also, we studied the effect of glycan density in antibody recognition. We have demonstrated that a glycan density higher than eight does not lead to higher antibody binding and reactivity.

2.4. Experimental section

2.4.1. General Information

The reactions were conducted under an inert atmosphere of argon and monitored by TLC on silica gel 60 F254 plates from EMD Millipore or Dynamic Adsorbents, Inc. All the glassware was oven dried at 120°C before use. All ACS-grade solvents were purchased from Thermo Fisher Scientific and were distilled using the proper drying agent. All the reagents were purchased from Acros Organic, SigmaAldrich, or Thermo Fisher Scientific and used without further purification unless otherwise stated. The 3Å and 4Å molecular sieves were purchased from Alfa Aesar and Thermo Fisher Scientific, respectively. Spots were identified under UV light (254 nm) and by staining with 4% sulfuric acid in ethanol. The compounds were purified by flash column chromatography on silica gel (40-60 μm). The ratio between silica and crude product ranged from 100:1 to 120:1 (dry w/w). FPLC purification was carried out with an AKTA Purifier 100 FPLC system from Cytiva (former GE Healthcare) using a Resource RPC column with a stationary phase of 15 μm polystyrene/divinylbenzene beads, solvent A: 2% ACN/H2O; solvent B: 85% ACN/H2O. Optical rotations were recorded on an ATAGO AP-300 Automatic Polarimeter. NMR experiments were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz. Chemical shifts were measured relative to tetramethylsilane (δ 0.00 ppm) as an internal standard or relative to the CDCl3 signal (δ 77.0 ppm) in 13C NMR spectra. In the case of NMR spectra measured in D2O, a solution of tetramethylsilane in CDCl3 in a sealed capillary was used as an external standard for calibration. MS analyses of the carbohydrate derivatives were performed on a high-resolution JEOL AccuTOF mass spectrometer using an electrospray ionization (ESI) source. Rayonet RPR200 photochemical reactor (USA) equipped with 16 UV lamps (350 nm) was used for the thiol-ene reactions. Glycoconjugates were characterized by matrix-assisted
laser/desorption/ionization mass spectrometer (MALDI)-TOF-MS (MALDI-8020, Shimadzu) using 10 mg/mL sinapinic acid, 0.1% trifluoroacetic acid, in 50% acetonitrile as a matrix. 96-well polystyrene Nunc MaxiSorp ELISA plates and CL-ELISA reagents were purchased from Thermo Scientific or Jackson ImmunoResearch, and luminescence was recorded on a Luminoskan Ascent, Thermo Scientific.

2.4.2. Synthetic procedures and spectroscopic characterization

2.4.2.1. Synthesis of α-Galactopyranoside donor 14

2.4.2.1.1. 2,3-Di-O-benzoyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl trichloroacetimidate (14).

A mixture of α,β-hydrolyzed sugar 13 (4.0 g, 7.5 mmol, 1 equiv) was dissolved in DCM (21.4 mL), CCl₃CN (15.19 mL, 150 mmol, 20 equiv) and DBU (1.4 mL, 9.0 mmol, 1.2 equiv) were consecutively added at 0 °C and stirred at rt for 15 min. Then, the residue was concentrated and purified by flash column chromatography on silica gel (EtOAc:hexanes = 3:1) to yield 14 (4.3 g, 86%), as a white powder. Rf 0.60 (EtOAc:hexanes = 3:1). ¹H NMR (400 MHz, CDCl₃) δ 8.56 (s, 1H, -NH); 8.05–7.90 (m, 4H, arom.); 7.57 – 7.46 (m, 2H, arom.); 7.44 – 7.32 (m, 4H, arom.); 6.79 (d, J₁₂ = 3.5 Hz, 1H, H-1); 6.04 (dd, J₂,₃ = 10.6, J₁,₂ = 3.6 Hz, 1H, H-2); 5.69 (dd, J₂,₃ = 10.6, J₃,₄ = 3.0 Hz, 1H, H-3); 5.01 (d, J₃,₄ = 3.1, Hz, 1H, H-4); 4.38 – 4.25 (m, 2H, H-6a,b); 4.17 (d, J = 2.6 Hz, 1H, H-5); 1.15 (s, 9H, tert-butyl), 0.98 (s, 9H, tert-butyl). ¹H NMR spectra matched the one previously described for this compound.

2.4.2.2. Synthesis of β-Galactopyranoside acceptor 17 and donor 4

2.4.2.2.1. p-Tolyl 3-O-(2-methylnaphthyl)-1-thio-β-D-galactopyranoside (15).

A solution of p-tolyl 1-thio-β-D-galactopyranoside (11) (1.0 g, 3.5 mmol, 1 equiv) was dissolved in anhydrous methanol (35 mL). Dibutyltin oxide was added (1.3 g, 5.2 mmol, 1.5 equiv), and the mixture was refluxed for 8 h. Methanol was evaporated, and the reaction mixture was resuspended in dry benzene (35 mL). Tetrabutylammonium bromide (563 mg, 1.7 mmol, 0.5 equiv) and 2-(Bromomethyl)naphthalene (1.16 g, 5.3 mmol, 1.5 equiv) were added, and the reaction mixture was refluxed for 12 h. Benzene was evaporated, and the crude mixture was dried under reduce pressure. The crude was purified by column chromatography (30:1 DCM:MeOH) to yield 15 (1.12 g, 76%) as a white powder. Rf 0.50 (4:1
EtOAc:Hexane). $^1$H NMR (400 MHz, DMSO) $\delta$ 8.04 – 7.75 (m, 4H), 7.67 – 7.30 (m, 5H), 7.12 (d, $J = 7.9$ Hz, 2H), 5.39 (d, $J = 6.2$ Hz, 1H, -OH), 4.96 – 4.62 (m, 4H, CH$_2$-Nap, 2-OH), 4.56 (d, $J = 9.7$ Hz, 1H, H-1), 4.04 (t, $J = 4.1$ Hz, 1H, H-4), 3.75 – 3.29 (m, 5H, H-2, H-6, H-5, H-3), 2.27 (s, 3H, CH$_3$). $^{13}$C NMR (101 MHz, DMSO) $\delta$ 136.6 (Cq), 135.8 (Cq), 132.7 (Cq), 132.3 (Cq), 131.2 (Cq), 130.2 (C-arom.), 129.3 (C-arom.), 127.5 (C-arom.), 127.4 (C-arom.), 125.9 (C-arom.), 125.9 (C-arom.), 125.6 (C-arom.), 125.6 (C-arom.), 88.1 (C-1), 82.5 (C-3), 78.9 (C-5), 70.2 (CH$_2$-Nap), 68.1 (C-2), 64.7 (C-4) 60.3 (C-6), 20.5 (CH$_3$). ESI-TOF HRMS: m/z [M+Na]$^+$ calcd for C$_{24}$H$_{26}$NaO$_5$S 449.1399, found 449.1331.

2.4.2.2. $p$-Tolyl 3-O-(2-methylnaphthyl)-2,4,6-tri-O-benzoyl-1-thio-$\beta$-D-galactopyranoside (16)

A solution of compound 15 (950 mg, 2.23 mmol, 1 equiv) in anhydrous pyridine (50 mL) was cooled down to 0 °C, and benzoyl chloride was added (1.5 mL, 13.4 mmol, 6 equiv). The resulting mixture was stirred at room temperature for 3 h under an argon atmosphere. The reaction mixture was quenched with methanol, stirred for 10 min, and evaporated under reduced pressure. The crude was re-dissolved in EtOAc and washed with saturated NaHCO$_3$ solution and brine. The organic layer was dried over anhydrous MgSO$_4$ and then concentrated under reduced pressure. The crude was purified by column chromatography (4:1 hexanes:EtOAc) to yield 16 (1.6 g, quantitative) as a white powder. R$_f$ 0.33 (4:1 EtOAc:Hexane). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.05 (t, $J = 7.0$ Hz, 4H, arom.), 7.97 (d, $J = 7.7$ Hz, 2H, arom.), 7.69 (d, $J = 7.8$ Hz, 1H, arom.), 7.66 – 7.56 (m, 4H, arom.), 7.53 – 7.35 (m, 12H, arom.), 7.18 (d, $J = 8.4$ Hz, 1H, arom.), 7.00 (d, $J = 7.8$ Hz, 2H, arom.), 5.98 (d, $J = 3.3$ Hz, 1H, H-4), 5.55 (t, $J = 9.7$ Hz, 1H, H-2), 4.84 (d, $J = 12.8$ Hz, 1H, CH$_2$), 4.77 (d, $J = 10.0$ Hz, 1H, H-1), 4.68 – 4.56 (m, 2H, CH$_2$-C-6), 4.48 (dd, $J = 11.5$, 5.1 Hz, 1H, H-6), 4.16-4.09 (m, 1H, H-5), 3.88 (dd, $J = 9.5$, 3.3 Hz, 1H, H-3), 2.32 (s, 3H, CH$_3$). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 166.16 (C=O), 165.81 (C=O), 165.15 (C=O), 138.21 (Cq-arom.), 134.5 (Cq-arom.), 133.9 (C-arom.), 133.4 (C-arom.), 133.3 (C-arom.), 133.1 (C-arom.), 133.0 (C-arom.), 132.9(C-arom.), 130.2 (C-arom.), 129.9 (C-arom.), 129.9 (C-arom.), 129.8 (C-arom.), 129.6 (Cq-arom.), 129.5 (C-arom.), 129.25 (Cq-arom.), 128.3 (C-arom.), 128.4 (C-arom.), 128.4 (C-arom.), 128.2 (C-arom.), 128.2 (C-arom.), 127.8 (C-arom.), 127.6 (C-arom.), 126.9 (C-arom.), 126.0 (C-arom.), 125.9 (C-arom.), 125.9 (C-arom.), 86.3 (C-1), 77.2 (C-3), 75.1 (C-5), 71.0 (CH$_2$-Nap), 69.4 (C-2), 66.8 (C-4), 63.1
ESI-TOF HRMS m/z calcd for C_{45}H_{38}NaO_{8}S [M+Na]^+: 761.2185, found: 761.2122.

2.4.2.2.3. p-Tolyl 2,4,6-tri-O-benzoyl-1-thio-β-D-galactopyranoside (17)

A solution of compound 16 (1.7 g, 2.3 mmol, 1 equiv) was dissolved in DCM:MeOH (2:1, 150 mL). 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (2.0 g, 9.2 mmol, 4 equiv) was added, and the solution was stirred at rt for 6 h. The reaction mixture was washed with saturated NaHCO$_3$ (aq) and brine. The organic phase was dried over anhydrous MgSO$_4$, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography (3:1 hexanes:EtOAc) to give 17 (0.9 g, 65%) as a white solid. R$_f$ 0.33 (3:1 hexanes:EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.12 – 8.07 (m, 2H, arom.), 8.06 – 8.02 (m, 2H, arom.), 7.99 – 7.95 (m, 2H, arom.), 7.64 – 7.55 (m, 3H, arom.), 7.51 – 7.39 (m, 8H, arom.), 7.02 (d, J = 7.9 Hz, 2H, arom.), 6.57 (d, J = 3.3 Hz, 1H, H-4), 5.26 (t, J = 9.6 Hz, 1H, H-2), 4.87 (d, J = 9.8 Hz, 1H, H-1), 4.57 (dd, J = 11.5, 7.1 Hz, 1H, H-6), 4.43 (dd, J = 11.6, 5.3 Hz, 1H, H-6), 4.15 (m, 2H, H-3, H-5), 2.96 (d, J = 6.6 Hz, 1H, OH), 2.34 (s, 3H, CH$_3$). ESI-TOF HRMS m/z calcd for C$_{34}$H$_{30}$NaO$_8$S [M+Na]^+: 621.1559, found: 621.1524. $^1$H NMR spectra matched the one previously described for this compound.

2.4.2.2.4. p-Tolyl 2,3,4,6-tetra-O-benzoyl-1-thio-β-D-galactopyranoside (4)

A solution of p-tolyl 1-thio-β-D-galactopyranoside (11)$^{74}$ (300 mg, 1 mmol, 1 equiv) in anhydrous pyridine (50 mL) was cooled down to 0 ºC, and benzoyl chloride was added (0.98 mL, 8 mmol, 8 equiv). The resulting mixture was stirred at rt overnight under an argon atmosphere. The reaction mixture was quenched with methanol, stirred for 10 min, and evaporated under reduced pressure. The crude was re-dissolved in EtOAc and washed with saturated NaHCO$_3$ solution and brine. The organic layer was dried over anhydrous MgSO$_4$ and concentrated under reduced pressure. The crude was purified by column chromatography (3:1 hexanes:EtOAc) to yield 4 (700 mg, 95%) as a white powder. R$_f$ 0.33 (3:1 EtOAc:Hexane). $^1$H NMR (400 MHz, DMSO) δ 7.98 – 7.89 (m, 4H, arom.), 7.86 – 7.47 (m, 15H, arom.), 7.45 – 7.38 (m, 2H, arom.), 7.38 – 7.29 (m, 2H, arom.), 7.17 – 7.10 (m, 2H, arom.), 5.93 (dd, J = 3.3, 1.0 Hz, 1H, H-4), 5.87 (ddd, J = 7.6, 3.3, 2.0 Hz, 1H, H-3), 5.59 – 5.48 (m, 2H, H-2, H-1), 4.78 (t, J = 6.1 Hz, 1H, H-5), 4.56 (dd, J = 11.5, 6.7 Hz, 1H, H-6), 4.48 (dd, J = 11.5,
5.3 Hz, 1H, H-6), 2.34 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO) δ 165.2 (C=O), 164.8 (C=O), 164.6 (C=O), 164.4 (arom.), 137.8 (C, arom.), 133.9 (arom.), 133.7 (arom.), 133.7 (arom.), 133.5 (arom.), 133.2 (arom.), 132.8 (C, arom.), 130.8 (arom.), 129.5 (arom.), 129.2 (arom.), 129.1 (arom.), 129.1 (C, arom.), 128.9 (arom.), 128.8 (arom.), 128.8 (arom.), 128.7 (C, arom.), 128.6 (arom.), 128.6 (arom.), 128.5 (arom.), 128.3 (C, arom.), 126.8 (C, arom.), 83.1 (C-1), 73.7 (C-5), 72.5 (C-3), 68.9 (C-4), 67.7 (C-2), 62.4 (C-6), 20.7 (CH₃). ESI-TOF HRMS m/z calcd for C₄₁H₃₄NaO₉ [M+Na]⁺: 725.1821, found: 725.1777.

2.4.2.3. Synthesis of α-N-acetyl-glucopyranoside acceptor 2

2.4.2.3.1. Allyl 2-Acetamido-4,6-benzylidine-2-deoxy-α-D-glucopyranoside (21)

Compound 20⁶⁵ (3.5 g, 13.4 mmol, 1 equiv) was dissolved in CH₃CN (300 mL). Benzaldehyde dimethyl acetal (3.1 mL, 20 mmol, 1.5 equiv) and p-Toluenesulfonic acid (350 mg, 1.3 mmol, 0.1 equiv) were added and the reaction mixture was stirred at rt for 2 h. The reaction mixture was neutralized with triethylamine, co-evaporated with toluene, and dried under reduce pressure. The crude was purified by column chromatography (9:0.5 DCM:MeOH) to yield 21 (3.9 g, 83%) as a white powder. Rf 0.26 (9:1 DCM:MeOH).¹H NMR (400 MHz, CDCl₃) δ 7.53 – 7.47 (m, 2H), 7.4 – 7.32 (m, 3H), 5.97 – 5.79 (m, 2H), 5.57 (s, 1H), 5.36 – 5.22 (m, 2H), 4.89 (d, J = 3.7 Hz, 1H, H-1), 4.31 – 4.18 (m, 3H), 4.04 – 3.73 (m, 5H), 3.60 (dd, J = 9.8, 8.5 Hz, 1H), 2.07 (s, 3H, CH₃). ESI-TOF HRMS m/z calcd for C₁₈H₂₃N₂O₆ [M+Na]⁺: 372.1423, found: 372.1404.

2.4.2.3.2. Allyl 2-Acetamido-3-O-acetyl-4,6-benzylidine-2-deoxy-α-D-glucopyranoside (22)

Compound 21 (719 mg, 2 mmol, 1 equiv) was dissolved in pyridine (100 mL). Acetic anhydride (0.78 mL, 8.0 mmol, 4 equiv) was added, and the reaction mixture was stirred at rt for 5 h. Pyridine was co-evaporated with toluene and the crude was dried under reduce pressure. The crude was purified by column chromatography (4:1 EtOAc:hexanes) to yield 22 (805 mg, quantitative) as a white powder. Rf 0.43 (4:1 EtOAc:Hexane).¹H NMR (400 MHz, CDCl₃) δ 7.48 – 7.41 (m, 2H, arom.), 7.39 – 7.33 (m, 3H, arom.), 5.89 (dddd, J = 16.9, 10.4, 6.4, 5.4 Hz, 1H, H-b), 5.80 (d, J = 9.5 Hz, 1H, -NH), 5.53 (s, 1H, CH₂Ph), 5.38 – 5.21 (m, 3H, H-c), 4.87 (d, J = 3.7 Hz, 1H, H-1), 4.35 (dddd, J = 10.5, 9.6, 3.8 Hz, 1H, H-2), 4.28 (dd, J = 10.3, 4.8 Hz, 1H, H-6), 4.20 (ddt, J = 12.8, 5.3, 1.4 Hz, 1H, H-a), 4.00 (ddt, J = 12.8, 6.4, 1.3 Hz, 1H, H-a),
3.93 (td, J = 9.9, 4.7 Hz, 1H, H-5), 3.82 – 3.68 (m, 2H, H-4, H-6), 2.06 (s, 3H, CH₃), 1.96 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.44 (C=O), 170.0 (C=O), 137.0 (Cq, arom.), 133.2 (C-b), 129.1 (C-arom.), 128.2 (C-arom.), 126.2 (C-arom.), 118.4 (C-c), 101.6 (C-1), 97.1 (C-1), 79.02 (C-4), 70.3 (C-3), 68.9 (C-6), 68.7 (C-a), 63.0 (C-5), 52.6 (C-2), 23.2 (CH₃), 20.9 (CH₃). ESI-TOF HRMS m/z calcld for C₂₀H₂₅NNaO₇ [M+Na]⁺: 414.1529, found: 414.1474.

2.4.2.3.3. Allyl 2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (23)

Compound 22 (805 mg, 2.3 mmol, 1 equiv) was dissolved in acetic acid 80% v/v (180 mL). The reaction mixture was stirred at 80°C for 5 hours and co-evaporated with EtOAc. The crude product was purified by column chromatography (9:1 DCM:MeOH) to yield 23 (592 mg, 85%) as a white solid. Rf 0.16 (9:0.5 DCM:MeOH). ¹H NMR (400 MHz, MeOD) δ 7.88 (d, J = 9.5 Hz, 1H, -NH), 5.98 (dddd, J = 16.9, 10.4, 6.4, 5.2 Hz, 1H, H-b), 5.35 (dq, J = 17.3, 1.7 Hz, 1H, H-c), 5.25 – 5.09 (m, 2H, H-c, H-3), 4.82 (d, J = 3.6 Hz, 1H, H-1), 4.24 (ddt, J = 13.0, 5.3, 1.5 Hz, 1H, H-a), 4.18 – 4.09 (m, 1H, H-2), 4.05 (ddt, J = 13.0, 6.3, 1.3 Hz, 1H, H-a), 3.83 (dd, J = 11.5, 1.9 Hz, 1H, H-6), 3.77 – 3.64 (m, 2H, H-6, H-5), 3.56 (t, J = 9.3 Hz, 1H, H-4), 2.04 (s, 3H, CH₃), 1.93 (s, 3H, CH₃). ¹³C NMR (101 MHz, MeOD) δ 173.5 (C=O), 172.8 (C=O), 135.3 (C-b), 118.1 (C-c), 97.7 (C-1), 75.1 (C-3), 73.8 (C-5), 69.8 (C-4), 69.3 (C-a), 62.3 (C-6), 53.3 (C-2), 22.5 (CH₃), 20.9 (CH₃). ESI-TOF HRMS m/z calcld for C₁₃H₂₁NNaO₇ [M+Na]⁺: 326.1216, found: 326.1147.

2.4.2.3.4. Allyl 2-Acetamido-3-O-acetyl-2-deoxy-6-O-tert-butyldimethylsilyl-α-D-glucopyranoside (2)

A solution of compound 23 (300 mg, 1.0 mmol, 1 equiv) and N,N-dimethyl-4-aminopyrididine (24 mg, 0.2 mmol, 0.2 equiv) in anhydrous pyridine (20 mL) was cool down to 0°C. tert-Butyldimethylsilyl trifluoromethanesulfonate (340.5 μL, 1.5 mmol, 1.5 equiv) was slowly added to a solution. The mixture was warmed up to rt. After 15 min, the reaction was quenched with methanol, diluted with ethyl acetate, and sequentially washed with 1 M HCl(aq), saturated NaHCO₃(aq), and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude, which was purified by column chromatography (9:1 DCM:MeOH) to give 6 (392 mg, 95%) as a white syrup. Rf 0.43 (9:1 DCM:MeOH). [α]_D^29 = +63.23 (c = 0.9 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 5.99 – 5.74 (m,
2H, H-b, -NH), 5.29 (dq, J = 17.4, 1.6 Hz, 1H, H-c), 5.22 (dt, J = 10.5, 1.4 Hz, 1H, H-c), 5.13 (ddd, J = 10.6, 8.5, 1.8 Hz, 1H, H-3), 4.82 (d, J = 3.6 Hz, 1H, H-1), 4.26 – 4.15 (m, 2H, H-2, H-a), 3.98 (ddt, J = 12.9, 6.3, 1.3 Hz, 1H, H-a), 3.90 (dd, J = 10.7, 4.5, 1.7 Hz, 1H, H-6), 3.83 (dd, J = 10.6, 4.8 Hz, 1H, H-6), 3.79 – 3.66 (m, 2H, H-4, H-5), 3.34 (s, 1H, -OH), 2.09 (s, 3H, CH3), 1.95 (s, 3H, CH3), 0.91 (s, 9H, tert-butyl), 0.10 (s, 6H, 2 CH3). 13C NMR (101 MHz, CDCl3) δ 172.2 (C=O), 170.1 (C=O), 133.3 (C-b), 118.0 (C-c), 96.4 (C-1), 74.0 (C-3), 70.8 (C-4, C-5), 68.3 (C-a), 64.2 (C-6), 51.8 (C-2), 25.9 (tert-butyl), 23.2 (CH3), 21.0 (CH3), 18.3 (Cq), -5.4 (CH3), -5.5 (CH3). ESI-TOF HRMS m/z calcd for C19H35NNaO7Si [M+Na]+: 440.2080, found: 440.2014.

2.4.2.4. Synthesis of α-Gal Disaccharide Donor (1)

2.4.2.4.1. p-Tolyl 2,3-di-O-benzoyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-1-thio-β-D-galactopyranoside (1)

To a solution of β-Gal acceptor 17 (500 mg, 0.84 mmol, 1 equiv) and α-Gal donor 14 (841 mg, 1.3 mmol, 1.5 equiv) in anhydrous DCM (100 mL), freshly activated MS 4Å was added and stirred at rt for 1 h under Ar. Then, the mixture was cooled down to -10 °C and TMSOTf (45 µL, 0.25 mmol, 0.3 equiv) was added. The mixture was stirred for 45 min at -10 °C. the reaction was quenched with Et3N, filtered, and was washed with water, and brine. The organic phase was dried over anhydrous MgSO4, filtered, concentrated, and purified by column chromatography on silica gel (5:1 hexanes:EtOAc) to yield 1 (693 mg, 74%) as a white solid. Rf 0.60 (3:1 hexanes:EtOAc). [α]b29 = +120.71 (c = 0.67 in CHCl3). 1H NMR (400 MHz, CDCl3) δ 8.14 (d, J = 7.1 Hz, 2H, arom.), 7.99 (d, J = 7.0 Hz, 2H, arom.), 7.84 (d, J = 7.0 Hz, 2H, arom.), 7.71 (d, J = 7.1 Hz, 2H, arom.), 7.65 – 7.34 (m, 13H, arom.), 7.31 – 7.23 (m, 3H, arom.), 7.12 – 6.96 (m, 6H, arom.), 5.81 (d, J = 3.4 Hz, 1H, H-4), 5.72 (dd, J = 10.6, 3.5 Hz, 1H, H-2’), 5.64 (t, J = 9.7 Hz, 1H, H-2), 5.59 (d, J = 3.8 Hz, 1H, H-1’), 5.15 (dd, J = 10.7, 3.1 Hz, 1H, H-3’), 4.88 (d, J = 10.0 Hz, 1H, H-1), 4.51 (dd, J = 11.4, 6.8 Hz, 1H, H-6’), 4.33 – 4.23 (m, 2H, H-6’, H-3), 4.19 – 4.09 (m, 2H, H-4’, H-5’), 3.99 (dd, J = 13.1, 2.1 Hz, 1H, H-6), 3.60 (dd, J = 11.0, 2.2 Hz, 2H, H-5, H-6), 2.36 (s, 3H, CH3), 1.02 (s, 9H, tert-butyl), 0.79 (s, 9H, tert-butyl). 13C NMR (101 MHz, CDCl3) δ 166.2 (C=O), 166.0 (C=O), 165.5 (C=O), 165.1 (C=O), 164.9 (C=O), 138.5 (Cq), 134.3 (C arom.), 133.4 (C arom.), 133.2 (C arom.), 132.8 (C arom.), 132.7 (C arom.), 129.8 (C arom.), 129.8 (C arom.), 129.7 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.4 (Cq), 129.1 (Cq), 128.7 (C arom.), 128.4 (C arom.), 128.3 (Cq), 128.2 (C arom.), 128.07
2.4.2.5. Synthesis of Gal-β-(1→4)-GlcNAc disaccharide acceptor (7)

2.4.2.5.1. Allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-6-O-tert-butyldimethylsilyl-α-D-glucopyranoside (24)

Glycosyl acceptor 2 (150 mg, 0.36 mmol, 1 equiv) and glycosyl donor 4 (351 mg, 0.5 mmol, 1.4 equiv) were dissolved in dry CH₂Cl₂ (40 mL). Flame activated MS 4Å were added, and the reaction mixture was stirred at r.t for 1 h. The mixture was cooled down to -10 °C and NIS (186 mg, 0.83 mmol, 2.3 equiv), 3,3-difluoroxindole (45 mg, 0.27 mmol, 0.75 equiv), and TMSOTf (19.6 µL, 0.1 mmol, 0.3 equiv). The mixture was stirred for 90 min at -10 °C. Triethylamine (0.1 mmol) was added, and the reaction mixture was washed with saturated sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude, purified by column chromatography (3:2 EtOAc:hexanes) to give 24 (244 mg, 68%) as a yellow pale solid. Rf 0.40 (3:2 EtOAc:hexanes). [α]D²⁷ = +79.87 (c = 0.7 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.05 (ddd, J = 7.5, 1.4, 0.6 Hz, 4H, arom.), 7.98 – 7.91 (m, 2H, arom.), 7.79 – 7.72 (m, 2H, arom.), 7.66 – 7.35 (m, 10H, arom.), 7.25 – 7.19 (m, 2H, arom.), 5.96 (d, J = 3.4, 1H, H-4’), 5.87 – 5.67 (m, 3H, H-b, H-2’, -NH), 5.48 (dd, J = 10.3, 3.4 Hz, 1H, H-3’), 5.30 – 5.23 (m, 1H, H-3), 5.21 – 5.14 (m, 2H, H-c), 5.08 (d, J = 8.0 Hz, 1H, H-1’), 4.79 (d, J = 3.7 Hz, 1H, H-a), 4.50 (dd, J = 6.6, 1.1 Hz, 2H, H-6’), 4.27 – 3.95 (m, 5H, H-a, H-5’, H-2, H-4’), 3.87 (ddt, J = 12.9, 6.3, 1.3 Hz, 1H), 3.82 – 3.74 (m, 1H, H-6), 3.63 – 3.49 (m, 2H, H-6, H-5), 2.10 (s, 3H, CH₃), 1.96 (s, 3H, CH₃), 0.89 (s, 3H, tert-butyl), 0.08 (s, 3H, CH₃), 0.01 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.4 (C=O), 169.9 (C=O), 166.0 (C=O), 165.5 (C=O), 165.4 (C=O), 164.7 (C=O), 133.7 (C=O), 133.4 (C arom.), 133.4 (C-b), 133.3 (C arom.), 133.3 (C arom.), 130.0 (C arom.), 129.8 (C arom.), 129.7 (C arom.), 129.3 (Cq), 129.1 (Cq), 129.0 (Cq), 128.7 (C arom.), 128.6 (C arom.), 128.5 (C arom.), 128.3 (C arom.), 117.9 (C-c), 100.7 (C-1’), 96.2 (C-1), 74.8 (C-4), 72.2 (C3’), 71.5 (C-3), 71.2 (C-5’), 71.0 (C-5), 70.1 (C-2’), 68.2 (C-a), 67.9 (C-4’), 61.8 (C-6’), 60.8 (C-6), 52.3 (C-2), 25.8 (tert-butyl), 23.3 (CH₃), 21.0 (CH₃), 18.2 (Cq), -5.1 (CH₃), -
5.3 (CH₃). ESI-TOF HRMS m/z calcd for C₅₃H₆₁NNaO₁₆Si [M+Na]⁺: 1018.3657, found: 1018.3638.

2.4.2.5.2. Allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (7)

Compound 24 (199 mg, 0.2 mmol) was dissolved in a solution of AcOH:THF:H₂O 3:1:1 (40 mL). The solution was refluxed for 2 h and co-evaporated with EtOAc. The crude product was purified by column chromatography (20:1 DCM:MeOH) to yield 7 (170 mg, quantitative) as a yellow pale solid. R_	ext{f} 0.16 (20:1 DCM:MeOH). [α]₀²⁹ = +84.36 (c = 0.1 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.05 (dd, J = 14.1, 7.6 Hz, 4H, arom.), 8.01 – 7.94 (m, 2H, arom.), 7.75 (d, J = 8.6 Hz, 2H, arom.), 7.65 – 7.36 (m, 10H, arom.), 7.22 (t, J = 7.9 Hz, 2H, arom.), 5.97 (d, J = 4.6 Hz, 1H, H-4’), 5.87 – 5.71 (m, 3H, H-2’, H-b, -NH), 5.59 (dd, J = 10.4, 3.4 Hz, 1H, H-3’), 5.35 – 5.26 (m, 1H, H-3), 5.26 – 5.14 (m, 2H, H-c), 5.03 (d, J = 7.9 Hz, 1H, H-1’), 4.84 (d, J = 3.7 Hz, 1H, H-1), 4.59 (dd, J = 11.1, 6.5 Hz, 1H, H-6’), 4.46 – 4.38 (m, 1H, H-6’), 4.33 (t, J = 7.4 Hz, 1H, H-5’), 4.28 – 4.20 (m, 1H, H-2), 4.11 – 4.00 (m, 2H, H-4, H-a), 3.93 – 3.85 (m, 1H, H-a) 3.75 (dd, J = 12.5, 2.9 Hz, 1H, H-6), 3.67 – 3.56 (m, 2H, H-6, H-5), 2.75 (s, 1H, -OH), 2.11 (s, 3H, CH₃), 1.97 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.5 (C=O), 170.2 (C=O), 166.1 (C=O), 165.5 (C=O), 165.4 (C=O), 164.9 (C=O), 133.6 (C arom.), 133.4 (C arom.), 133.3 (C arom.), 133.2 (C-b), 130.0 (C arom.), 129.8 (C arom.), 129.8 (C arom.), 129.7 (C arom.), 129.3 (Cq), 129.0 (Cq), 129.0 (Cq), 128.7 (Cq), 128.7 (Cq), 128.6 (C arom.), 128.5 (C arom.), 128.3 (C arom.), 118.2 (C-c), 101.2 (C-1’), 96.3 (C-1), 75.1 (C-4), 72.0 (C-3’), 71.3 (C-3), 71.1 (C-5’), 70.8 (C-5), 70.2 (C-2’), 68.5 (C-a), 67.9 (C-4’), 61.6 (C-6’), 60.2 (C-6), 52.3 (C-2), 23.2 (CH₃), 21.0 (CH₃). ESI-TOF HRMS m/z calcd for C₄₇H₄₇NNaO₁₆ [M+Na]⁺: 904.2793, found: 904.2744.

2.4.2.6. Synthesis of Gal-α-(1→3)-Gal-β-(1→4)-GlcNAc trisaccharide acceptor (3)

2.4.2.6.1. Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethylsilylene-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-6-O-tert-butyldimethylsilyl-α-D-glucopyranoside (25)

Allyl glycosyl acceptor 2 (208 mg, 0.5 mmol, 2 equiv) and thioglycosyl donor 1 (277 mg, 0.25 mmol, 1 equiv) were dissolved in dry DCM (40 mL). Flame-activated MS 4Å were added, and the reaction mixture was stirred at rt for 1 h. Then, the mixture was cooled down
to -10 °C and NIS (84.4 mg, 0.37 mmol, 1.5 equiv) and AgOTf (19.2 mg, 0.08 mmol, 0.3 equiv) were added. The mixture was stirred for 40 min at -10 °C, then warmed up to rt and stirred for 40 more min. Triethylamine (0.08 mmol, 0.3 equiv) was added, and the reaction mixture was washed with saturated Sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude which was purified by column chromatography (3:2 hexanes:EtOAc) to give 25 (229 mg, 66%) as a yellow pale solid. [α]_D^20 = +215.91 (c = 0.1 in CHCl₃). Rf 0.27 (1:1 hexanes:EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 8.16 – 8.07 (m, 2H), 8.05 – 7.98 (m, 2H, arom.), 7.86 – 7.79 (m, 2H, arom.), 7.73 – 7.67 (m, 2H, arom.), 7.65 – 7.45 (m, 8H, arom.), 7.45 – 7.33 (m, 2H, arom.), 7.30 – 7.24 (m, 5H, arom.), 7.17 – 7.10 (m, 2H, arom.), 7.04 – 6.96 (m, 2H, arom.), 5.88 – 5.78 (m, 1H, H-b), 5.78 – 5.70 (m, 3H, H-4', H-2", -NH), 5.66 – 5.58 (m, 2H, H-1", H-2"), 5.31 – 5.16 (m, 3H, H-c, H-3), 5.06 (dd, J = 10.6, 3.2 Hz, 1H, H-3"), 5.00 (d, J = 8.0 Hz, 1H, H-1"), 4.79 (d, J = 3.7 Hz, 1H, H-1), 4.35 (dq, J = 11.3, 5.3 Hz, 2H, H-a), 4.22 – 4.12 (m, 2H, H-2, H-3"), 4.12 – 3.94 (m, 6H, H-a, H-6', H-6"), 3.93 – 3.84 (m, 2H, H-6, H-6"), 3.71 – 3.53 (m, 5H, H-6, H-6"), 2.02 (s, 3H, CH₃), 1.95 (s, 3H, CH₃), 1.02 (s, 9H, tert-butyl), 0.90 (s, 9H, tert-butyl), 0.77 (s, 9H, tert-butyl), 0.10 (s, 3H, CH₃), 0.03 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.3 (C=O), 169.9 (C=O), 166.03 (C=O), 166.01 (C=O), 165.4 (C=O), 165.1 (C=O), 164.3 (C=O), 133.5 (C arom.), 133.4 (C arom.), 133.3 (C-b), 132.9 (C arom.), 132.8 (C arom.), 132.7 (C arom.), 129.8 (Cq), 129.8 (C arom.), 129.6 (C arom.), 129.55 (C arom.), 129.52 (C arom.), 129.3 (Cq), 129.0 (Cq), 128.8 (C arom.), 128.6 (C arom.), 128.4 (Cq), 128.3 (C arom.), 128.2 (C arom.), 128.0 (C arom.), 117.9 (C-c), 100.8 (C-1"), 96.3 (C-1), 93.4 (C-1"), 74.8 (CH), 73.4 (C-3"), 71.5 (C-3), 71.2 (C-2"), 70.9(CH), 70.5 (H-3"), 70.2, 68.3 (C-6"), 67.1 (C-4"), 66.8, 66.3 (C-6"), 65.2 (C-2"), 61.9 (C-a), 61.0 (C-6), 52.3 (C-2), 29.7 (grease), 27.3 (tert-butyl), 27.1 (tert-butyl), 25.8 (tert-butyl), 23.3 (CH₃), 23.2 (Cq), 20.9 (CH₃), 20.5 (Cq), 18.2 (Cq), -5.1 (CH₃), -5.3 (CH₃). ESI-TOF HRMS m/z calcd for C₇₄H₉₁NNaO₂₂Si₂ [M+Na]^+: 1424.5469, found: 1424.5440.

2.4.2.6.2. **Allyl 2,3-di-O-benzoyl-4,6-di-tert-butyldiyline-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (3)**

Compound 25 (150 mg, 0.1 mmol) was dissolved in a solution of AcOH:THF:H₂O 3:1:1 (30 mL). The solution was refluxed for 2 h and co-evaporated with EtOAc. The crude product
was purified by column chromatography (20:1 DCM:MeOH) to yield 3 (137 mg, quantitative) as a white solid. Rf 0.30 (20:1 DCM:MeOH). [α]_D^20 = +160.15 (c = 0.1 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.17 – 8.11 (m, 2H, arom.), 8.03 – 7.95 (m, 2H, arom.), 7.88 – 7.80 (m, 2H, arom.), 7.74 – 7.69 (m, 2H, arom.), 7.64 – 7.35 (m, 11H, arom.), 7.29 – 7.23 (m, 4H, arom. and CDCl₃), 7.18 – 7.11 (m, 2H, arom.), 7.04 – 6.95 (m, 2H, arom.), 5.88 – 5.76 (m, 3H, H-b, H-4′, -NH), 5.73 (dd, J = 10.6, 3.6 Hz, 1H, H-2″), 5.64 (dd, J = 10.1, 7.9 Hz, 1H, H-2′), 5.59 (d, J = 3.6 Hz, 1H, H-1″), 5.34 – 5.17 (m, 3H, H-c, H-3), 5.08 (dd, J = 10.6, 3.2 Hz, 1H, H-3″), 4.93 (d, J = 7.9 Hz, 1H, H-1′), 4.84 (d, J = 3.6 Hz, 1H, H-1), 4.48 (dd, J = 11.2, 6.3 Hz, 1H, H-a), 4.27 – 4.18 (m, 3H, H-3′, H-5′), 4.14 – 3.99 (m, 5H, H-4, H-a, H-6″, H-6′), 3.91 (ddt, J = 12.8, 6.5, 1.3 Hz, 1H, H-6″), 3.81 (dd, J = 12.4, 2.5 Hz, 1H, H-6), 3.74 – 3.61 (m, 4H, H-6, H-6′, H-5), 3.52 (s, 1H, -OH), 2.03 (s, 3H, CH₃), 1.95 (s, 3H, CH₃), 1.00 (s, 9H, tert-butyl), 0.77 (s, 9H, tert-butyl). ¹³C NMR (101 MHz, CDCl₃) δ 171.3 (C=O), 170.1 (C=O), 166.04 (C=O), 166.03 (C=O), 165.5 (C=O), 165.1 (C=O), 164.6 (C=O), 133.5 (C arom.), 133.4 (C arom.), 133.1 (C-b), 132.9 (C arom.), 132.8 (C arom.), 132.7 (C arom.), 129.85 (Cq), 129.82 (C arom.), 129.79 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.2 (Cq), 129.1 (Cq), 128.8 (C arom.), 128.6 (C arom.), 128.4 (Cq), 128.25 (C arom.), 128.16 (C arom.), 128.0 (C arom.), 118.3 (C-c), 101.1 (C-1′), 96.3 (C-1), 93.7 (C-1″), 74.9, 73.5, 71.4 (C-3), 71.4 (C-2′), 71.0, 70.7, 70.5 (C-3″), 70.3, 68.6 (C-6″), 67.2 (C-2″), 66.9, 66.3 (C-6′), 65.2 (C-4′), 61.6 (C-a), 60.3 (C-6), 58.1, 52.3, 29.7, 27.3 (tert-butyl), 27.1 (tert-butyl), 23.2 (CH₃), 23.1 (Cq), 20.9 (CH₃), 20.5 (C). ESI-TOF HRMS m/z calcd for C₆₈H₇₇NNaO₂₂Si[M+Na]^+: 1310.4604, found: 1310.4569.

2.4.2.7. Synthesis of tetrascaccharide G₃₆SH

2.4.2.7.1. Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl-(1→3)-
2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3,4,6-tetra-O-benzoyl-1-thio-β-
D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside

(G36SH)

Glycosyl trisaccharide acceptor 3 (80 mg, 0.062 mmol, 1 equiv) and glycosyl donor 4 (130 mg, 0.186 mmol, 3.0 equiv) were dissolved in dry CH₂Cl₂ (15 mL). Flame activated MS 4Å were added, and the reaction mixture was stirred at rt for 1 h. The mixture was cooled down to -10 °C and NIS (64 mg, 0.28 mmol, 4.6 equiv), 3,3-difluoroxindole (6.3 mg, 0.037 mmol, 0.6 equiv), and TMSOTf (3.4 µL, 0.019 mmol, 0.3 equiv). The mixture was stirred for 30 min at -
10 °C. Triethylamine (0.3 mmol, 0.019 mmol) was added, and the reaction mixture was washed with saturated sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude, purified by preparative thin-layer chromatography (40:1 DCM:MeOH) to give 5 (80 mg, 69%) as a white solid. $R_f$ 0.26 (40:1 DCM:MeOH). [α]$_{D}^{29}$ = +123.55 (c = 0.1 in CHCl$_3$). $^{1}$H NMR (400 MHz, CDCl$_3$) δ 8.18 – 8.12 (m, 2H, arom.), 8.08 – 7.96 (m, 9H, arom.), 7.86 – 7.74 (m, 6H, arom.), 7.70 – 7.53 (m, 9H, arom.), 7.53 – 7.31 (m, 14H, arom.), 7.31 – 7.17 (m, 9H, arom.), 7.04 – 6.96 (m, 2H, arom.), 5.91 (dd, $J$ = 3.5, 1.4 Hz, 1H, H-4$_b$), 5.78 – 5.66 (m, 3H, H-b, H-2), 5.63 (d, $J$ = 9.5 Hz, 1H, -NH), 5.58 – 5.44 (m, 4H, H-2, H-1c, H-3d), 5.23 (dd, $J$ = 10.7, 8.8 Hz, 1H, H-3$\alpha$), 5.20 – 5.11 (m, 2H, H-c), 5.08 (dd, $J$ = 10.6, 3.1 Hz, 1H, H-3c), 4.70 (d, $J$ = 3.6 Hz, 1H, H-1$\alpha$), 4.62 (dd, $J$ = 11.4, 6.8 Hz, 1H, H-6), 4.51 – 4.45 (m, 2H, H-1$\beta$, H-1$\beta$), 4.44 – 4.31 (m, 2H, H-6, H-6), 4.23 – 3.98 (m, 7H, H-2$\alpha$, H-a, H-a, H-6, H-6), 3.97 – 3.92 (m, 1H, H-5), 3.91 – 3.84 (m, 2H), 3.78 – 3.67 (m, 3H, H-4$\alpha$, H-6, H-6), 3.64 (dd, $J$ = 10.9, 5.1 Hz, 1H, H-6), 3.56 – 3.48 (m, 2H, H-5), 1.98 (s, 3H, CH$_3$), 1.90 (s, 3H, CH$_3$), 0.96 (s, 9H, tert-butyl), 0.78 (s, 9H, tert-butyl). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.1 (C=O), 169.8 (C=O), 166.2 (C=O), 166.0 (C=O), 165.9 (C=O), 165.6 (C=O), 165.54 (C=O), 165.52 (C=O), 165.0 (C=O), 164.9 (C=O), 164.6 (C=O), 133.9 (C arom.), 133.6 (C arom.), 133.5 (C arom.), 133.45 (C arom.), 133.36 (C arom.), 133.33 (C arom.), 133.1 (C-b), 133.07 (C arom.), 132.9 (C arom.), 132.8 (C arom.), 130.0 (C arom.), 129.9 (C arom.), 129.83 (C arom.), 129.81 (C arom.), 129.79 (C arom.), 129.76 (C arom.), 129.7 (C arom.), 129.66 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.4 (Cq), 129.2 (Cq), 129.15 (C arom.), 129.11 (Cq), 129.0 (Cq), 128.69 (C arom.), 128.67 (C arom.), 128.57 (C arom.), 128.56 (C arom.), 128.3 (C arom.), 128.2 (C arom.), 128.1 (C arom.), 117.9 (CH$_2$-c), 102.0 (C-1), 101.0 (C-1), 95.9 (C-1$\alpha$), 94.8 (C-1$\alpha$), 76.2 (CH), 74.6 (CH), 72.0 (CH), 71.9 (CH), 71.4 (CH), 71.3 (CH), 70.6 (CH), 70.5 (CH), 70.4 (CH), 70.1 (CH), 69.4 (CH), 68.7 (CH$_2$), 68.2 (CH$_2$), 68.0 (C-4$\beta$), 67.6 (CH), 67.2 (CH), 66.2 (CH$_2$), 65.7 (CH), 62.1 (CH$_2$), 61.4 (CH$_2$), 52.1 (CH), 27.3 (3xCH$_3$, tert-butyl), 27.1 (3xCH$_3$, tert-butyl), 23.2 (CH$_3$), 23.1 (Cq), 21.0 (CH$_3$), 20.5 (Cq). ESI-TOF HRMS m/z calcd for C$_{102}$H$_{103}$NNaO$_3$Si[M+2Na]$^{2+}$: 955.8039, found: 955.8024.
2.4.2.7.2. *Allyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3,4,6-tetra-O-benzoyl-1-thio-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (26)*

The fully protected tetrasaccharide 5 (70 mg, 0.04 mmol, 1 equiv) was dissolved in 10.4 mL of anhydrous THF in a plastic conical tube and cooled to 0 °C. Then, 19.25 µL of HF-pyr (70%) was added and stirred for 2 h at rt under Ar. The reaction mixture was cooled again to 0 °C and quenched with a solution of saturated NaHCO₃. Finally, the mixture was extracted with EtOAc, washed with water and brine, dried over MgSO₄, concentrated, and purified by preparative thin-layer chromatography on silica gel (30:1 DCM:MeOH, two runs) to furnish the tetrasaccharide 26 (53 mg, 82%) as a white powder. $R_f$ 0.30 (0:1 DCM:MeOH). $[\alpha]_D^{29} = +126.78$ (c = 0.1 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.38 – 8.30 (m, 2H, arom.), 8.15 – 8.08 (m, 2H, arom.), 8.04 – 7.93 (m, 6H, arom.), 7.81 – 7.75 (m, 2H, arom.), 7.74 – 7.30 (m, 25H, arom.), 7.26 – 7.17 (m, 5H, arom.), 7.12 – 7.05 (m, 2H, arom.), 7.01 – 6.91 (m, 2H, arom.), 5.99 (dd, $J$ = 3.3, 1.3 Hz, 1H, H-4D), 5.86 – 5.58 (m, 7H, H-1C, H-2D, H-3D, H-2B), 5.46 (d, $J$ = 3.1 Hz, 1H), 5.24 – 5.10 (m, 4H), 4.93 (d, $J$ = 7.4 Hz, 1H, H-1B), 4.77 – 4.66 (m, 2H, H-1A, H-6), 4.55 (d, $J$ = 7.9 Hz, 1H, H-1B), 4.43 (dd, $J$ = 11.3, 6.6 Hz, 1H, H-6), 4.30 – 4.18 (m, 3H, H-3B, H-2A, H-5), 4.17 – 3.89 (m, 7H), 3.84 – 3.65 (m, 8H), 3.10 (t, $J$ = 6.7 Hz, 1H), 1.90 (s, 3H, CH₃), 1.89 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 171.2 (C=O), 169.9 (C=O), 166.2 (C=O), 165.94 (C=O), 165.89 (C=O), 165.5 (C=O), 165.1 (2xC=O), 165.0 (C=O), 164.6 (C=O), 134.1 (C arom.), 133.7 (Cq), 133.49 (C arom.), 133.47 (C arom.), 133.2 (C-b), 132.9 (C arom.), 132.7 (C arom.), 130.2 (C arom.), 130.0 (C arom.), 129.9 (C arom.), 129.8 (C arom.), 129.74 (C arom.), 129.69 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.3 (Cq), 129.2 (Cq), 128.9 (C arom.), 128.8 (Cq), 128.7 (C arom.), 128.6 (C arom.), 128.4 (C arom.), 128.33 (C arom.), 128.27 (C arom.), 128.223 (C arom.), 128.0 (C arom.), 117.9 (CH₂), 101.7 (C-1D), 101.5 (C-1B), 96.1 (C-1A), 93.2 (C-1C), 76.3 (CH), 72.7 (CH), 71.6 (CH), 71.4 (CH), 71.0 (CH), 70.7 (2xCH), 70.2 (CH), 70.1 (CH), 69.3 (CH), 68.8 (CH), 68.4 (CH), 68.3 (CH₂), 67.7 (CH₂), 67.3 (CH), 65.1 (CH), 63.0 (CH₂), 62.1 (CH₂), 61.7 (CH₂), 60.4 (CH), 52.1 (CH), 23.2 (CH₃), 20.8 (CH₃). ESI-TOF HRMS m/z calcd for C₉₅H₇₂N₂Na₂O₃₁ [M+2Na]²⁺: 885.7529, found: 885.7512.
2.4.2.7.3. 3-(Acetylthio)propyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-
benzoyl-β-D-galactopyranosyl-(1→4)-[2,3,4,6-tetra-O-benzoyl-1-thio-β-D-
galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside

(27)

Allyl tetrasaccharide 26 (52 mg, 0.03 mmol, 1 equiv) was dissolved in dry DCM (6 mL). DPAP (4 mg, 0.015 mmol, 0.5 equiv) and AcSH (6.4 μL, 0.09 mmol, 3 equiv) were added under Ar. The mixture was stirred under water cooling (~25 °C) for 15 min in a Rayonet UV reactor equipped with 350 nm lamps. The solution was co-evaporated with toluene and concentrated. The crude product was purified by flash column chromatography on silica gel (20:1 DCM:MeOH) to yield the acyl-protected tetrasaccharide 27 (38.4 mg, 70%) as a white solid. Rf 0.26 (20:1 DCM:MeOH). [α]D29 = +27.43 (c = 0.17 in CHCl3). 1H NMR (400 MHz, CDCl3) δ 8.39 – 8.29 (m, 2H, arom.), 8.16 – 8.08 (m, 2H, arom.), 8.05 – 7.94 (m, 6H, arom.), 7.83 – 7.75 (m, 2H, arom.), 7.72 – 7.30 (m, 27H, arom.), 7.24 – 7.17 (m, 4H, arom.), 7.09 (t, J = 7.7 Hz, 2H, arom.), 6.96 (t, J = 7.7 Hz, 2H, arom.), 6.04 – 5.92 (m, 2H, H-4'0), 5.85 – 5.69 (m, 3H, H-2D, H-3D), 5.66 – 5.58 (m, 2H, H-1c), 5.47 (s, 1H), 5.23 – 5.13 (m, 2H), 4.95 (d, J = 7.1 Hz, 1H, H-1d), 4.73 (dd, J = 11.2, 6.5 Hz, 1H), 4.63 (d, J = 3.7 Hz, 1H, H-1a), 4.56 (d, J = 7.9 Hz, 1H, H-1b), 4.42 (dd, J = 11.2, 6.8 Hz, 1H), 4.30 (t, J = 6.7 Hz, 1H), 4.26 – 4.06 (m, 5H), 4.04-3.97 (m, 1H), 3.97 – 3.88 (m, 2H), 3.81 – 3.63 (m, 5H), 3.53 (dt, J = 10.7, 5.6 Hz, 1H, -OCH2CH2CH2SAc), 3.18 – 3.04 (m, 2H, H-5, -OCH2CH2CH2SAc), 2.93 (dt, J = 14.1, 7.2 Hz, 1H, -OCH2CH2CH2SAc), 2.83 (dt, J = 13.5, 6.6 Hz, 1H, -OCH2CH2CH2SAc), 2.29 (s, 3H, CH3), 1.93 (s, 3H, CH3), 1.90 (s, 3H, CH3), 1.75 – 1.68 (m, 2H, -OCH2CH2CH2SAc). 13C NMR (101 MHz, CDCl3) δ 195.6 (C=O), 171.1 (C=O), 170.1 (C=O), 166.2 (C=O), 166.0 (C=O), 165.9 (C=O), 165.8 (C=O), 165.7 (C=O), 165.1 (C=O), 165.4 (C=O), 165.0 (C=O), 164.6 (C=O), 134.0 (C arom.), 133.7 (C arom.), 133.5 (C arom.), 133.1 (C arom.), 132.9 (C arom.), 132.7 (C arom.), 130.2 (C arom.), 130.0 (C arom.), 129.9 (C arom.), 129.8 (C arom.), 129.74 (C arom.), 129.7 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.3 (Cq), 129.2 (Cq), 129.0 (Cq), 128.91 (C arom.), 128.86 (Cq), 128.73 (C arom.), 128.7 (C arom.), 128.6 (C arom.), 128.4 (Cq), 128.34 (C arom.), 128.27 (C arom.), 128.2 (C arom.), 128.0 (C arom.), 101.7 (C-1b), 101.5 (C-1b), 96.9 (C-1a), 93.2 (C-1c), 76.4 (CH), 72.7 (CH), 71.6 (CH), 71.5 (CH), 70.9 (CH), 70.7 (3xCH), 70.2 (CH), 70.1 (CH), 69.2 (CH), 68.8 (CH), 68.3 (CH), 67.7 (CH2), 67.3 (CH), 65.6 (CH2), 65.1 (CH), 63.02 (CH2), 62.03 (CH2), 61.7 (CH2),
was stirred for 50 min at 3,1 mg, 0.0186 mmol, 0.3 equiv), and TMSOTf (3.4 µL, 0.0186 mmol, 0.3 equiv). The mixture was cooled down to 10ºC. Triethylamine (0.0186 mmol, 0.3 equiv) was added, and the

2.4.2.7.4. 3-Thiopropyl α-D-galactosyl-(1→3)-β-D-galactosyl-(1→4)-[β-D-galactosyl-(1→6)]-2-acetamido-2-deoxy-α-D-glucopyranoside (G36s)_2

The acyl-protected tetrasaccharide 27 (19 mg, 0.01 mmol) was dissolved in 6 mL of a solution of NaOMe 43 mM and stirred for 6 hours under Ar. The solution was then neutralized with Amberlyst-15, filtered through Celite, concentrated, re-dissolved in water, and lyophilized. The mercaptopropyl trisaccharide G36sh is produced, which oxidizes by handling in the water to yield the disulfide (G36s)_2 (3 mg, 34%). ^1H NMR (400 MHz, D_2O) δ 5.03 (d, J = 3.9 Hz, 2H, H-1), 4.78 (d, J = 3.5 Hz, 2H, H-1), 4.48 (d, J = 7.8 Hz, 2H, H-1), 4.34 (d, J = 7.7 Hz, 2H, H-1), 4.18 - 4.04 (m, 6H), 3.93 - 3.39 (m, 47H), 2.74 (t, J = 7.0 Hz, 4H, -OCH₂CH₂CH₂S⁻), 2.02 - 1.83 (m, 10H, CH₃, -OCH₂CH₂CH₂S⁻). ^13C NMR (101 MHz, D_2O) δ 174.2 (C=O), 103.1 (C-1), 102.6 (C-1), 96.7 (C-1), 95.4 (C-1), 78.1 (CH), 77.2 (CH), 75.1 (CH), 74.9 (CH), 72.7 (CH), 70.8 (CH), 70.6 (CH), 69.6 (2xCH), 69.32 (CH), 69.27 (CH), 69.1 (CH), 68.7 (CH), 68.1 (CH), 67.2 (CH₂), 66.3 (CH₂), 64.8 (CH), 61.0 (3xCH₂), 53.3 (CH), 34.7 (CH₂), 27.8 (CH₂), 21.9 (CH₃). ESI-TOF HRMS m/z calcd for C_{29}H_{51}N_{4}N₂O_{2}S [M+Na]+: 804.2572, found: 804.2514.

2.4.2.8. Synthesis of tetrasaccharide G35sh

2.4.2.8.1. Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3-di-O-benzoyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (6)

Glycosyl trisaccharide acceptor 3 (80 mg, 0.062 mmol, 1 equiv) and glycosyl disaccharide donor 1 (137 mg, 0.124 mmol, 2.0 equiv) were dissolved in dry CH₂Cl₂ (15 mL). Flame activated MS 4Å were added, and the reaction mixture was stirred at r.t for 1h. The mixture was cooled down to -10 ºC and NIS (41 mg, 0.186 mmol, 3 equiv), 3,3-difluoroxindole (3.1 mg, 0.0186 mmol, 0.3 equiv), and TMSOTf (3.4 µL, 0.0186 mmol, 0.3 equiv). The mixture was stirred for 50 min at -10 ºC. Triethylamine (0.0186 mmol, 0.3 equiv) was added, and the
reaction mixture was washed with saturated sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude, purified by preparative thin-layer chromatography (2:1:0.2 hexanes:EtOAc:MeOH, two runs) to give 6 (54 mg, 40%) as a white solid. Rf 0.16 (2:1:0.2 hexanes:EtOAc:MeOH, two runs). [α]b^29 = +92.94 (c = 0.1 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.14 – 8.06 (m, 4H), 8.05 – 7.97 (m, 4H), 7.85 – 7.80 (m, 4H), 7.79 – 7.71 (m, 4H), 7.68 – 7.35 (m, 23H), 7.31 – 7.23 (m, 8H), 7.19 – 7.12 (m, 4H), 7.07 – 6.97 (m, 4H), 5.79 (d, J = 3.3 Hz, 1H), 5.76 – 5.47 (m, 10H), 5.23 (dd, J = 10.8, 8.9 Hz, 1H), 5.16 – 5.04 (m, 5H), 4.65 – 4.59 (m, 3H), 4.52 (dd, J = 11.4, 6.6 Hz, 1H), 4.35 (dd, J = 11.3, 6.4 Hz, 1H), 4.31 – 3.95 (m, 14H), 3.94 – 3.83 (m, 2H), 3.80 – 3.70 (m, 21H), 3.69 – 3.54 (m, 7H), 3.52 (s, 1H), 1.96 (s, 3H), 1.90 (s, 3H), 1.00 (s, 18H), 0.79 (s, 9H), 0.77 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 171.1, 169.9, 166.2, 166.0, 165.9, 165.54, 165.52, 165.3, 165.0, 164.8, 164.5, 133.7, 133.5, 133.5, 133.1, 133.0, 132.82, 129.84, 129.82, 129.8, 129.74, 129.71, 129.6, 129.5, 129.4, 129.2, 129.1, 129.0, 128.9, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 117.8, 102.3, 101.0, 95.9, 94.3, 76.3, 73.9, 73.7, 71.8, 71.5, 70.8, 70.54, 70.46, 70.41, 69.39, 68.8, 68.1, 67.6, 67.4, 67.1, 66.4, 66.2, 65.9, 65.4, 62.1, 61.5, 52.0, 29.7, 27.32, 27.30, 27.11, 27.10, 23.21, 23.17, 23.1, 20.9, 20.5. ESI-TOF HRMS m/z calcd for C₁₂₃H₁₃₂NO₃⁰Si₂ [M+2Na]^2+: 1158.8945, found: 1158.8906.

2.4.2.8.2. Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethyldimethylsilylene-α-D-galactopyranosyl-(1→3)

2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-6-O-trimethylsilyl-α-D-glucopyranoside (35)

¹H NMR (400 MHz, CDCl₃) δ 8.16 – 8.07 (m, 2H, arom.), 8.04 – 7.96 (m, 2H, arom.), 7.85 – 7.79 (m, 2H, arom.), 7.74 – 7.68 (m, 2H, arom.), 7.64 – 7.34 (m, 11H, arom.), 7.18 – 7.10 (m, 2H, arom.), 7.04 – 6.96 (m, 2H, arom.), 5.89 – 5.69 (m, 4H), 5.68 – 5.56 (m, 2H), 5.32 – 5.23 (m, 2H), 5.23 – 5.16 (m, 2H), 5.07 (dd, J = 10.7, 3.2 Hz, 1H), 4.91 (d, J = 8.0 Hz, 1H, H-1’), 4.80 (d, J = 3.7 Hz, 1H, H-1), 4.47 (dd, J = 11.2, 6.4 Hz, 1H), 4.29 – 4.15 (m, 3H), 4.13 – 3.95 (m, 5H), 3.89 (ddt, J = 12.8, 6.4, 1.3 Hz, 1H), 3.81 (dd, J = 11.7, 3.3 Hz, 1H), 3.71 – 3.53 (m, 5H), 2.03 (s, 3H), 1.94 (s, 3H), 1.01 (s, 9H), 0.78 (s, 8H), 0.10 (s, 9H). ESI-TOF HRMS m/z calcd for C₇₁H₆₅NNaO₂₂Si₂ [M+Na]^+: 1383.4999, found: 1382.2265
2.4.2.8.3. **Allyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (28)**

Partially protected pentasaccharide 6 (53.9 mg, 0.024 mmol, 1 equiv) was dissolved in 7 mL of anhydrous THF in a plastic conical tube and cooled to 0 °C. Then, 40 μL of HF-Pyr (70%) was added and stirred for 2 h at rt under Ar. The reaction mixture was cooled again to 0°C and quenched with a solution of saturated NaHCO₃. Finally, the mixture was extracted with EtOAc, washed with water and brine, dried over MgSO₄, concentrated, and purified by preparative thin-layer chromatography on silica gel (20:1 DCM:MeOH, two runs) to furnish the pentasaccharide 28 (37 mg, 78%) as a white powder. $R_f$ 0.40 (9:1 DCM:MeOH). $[\alpha]_D^{29} = +7.94$ (c = 0.04 in CHCl₃). $^1$H NMR (400 MHz, CDCl₃) δ 8.48 – 8.28 (m, 4H), 8.03 – 7.83 (m, 4H), 7.81 - 7.40 (m, 22H), 7.40 – 7.15 (m, 10H), 7.16 – 6.86 (m, 12H), 5.95 (d, $J = 3.0$ Hz, 1H), 5.86 – 5.66 (m, 7H), 5.62 (dd, $J = 10.2, 7.9$ Hz, 1H), 5.42 (s, 1H), 5.25 – 4.99 (m, 6H), 4.87 (d, $J = 3.7$ Hz, 1H), 4.67 – 4.51 (m, 3H), 4.44 – 4.19 (m, 5H), 4.17 – 3.77 (m, 13H), 3.76 – 3.51 (m, 8H), 1.90 (s, 3H), 1.82 (s, 3H). $^{13}$C NMR (101 MHz, CDCl₃) δ 171.4, 169.8, 166.20, 166.17, 166.1, 165.8, 165.7, 165.1, 165.0, 164.9, 164.43, 164.38, 134.1, 133.5, 133.3, 133.2, 133.0, 132.9, 132.8, 132.7, 130.5, 130.3, 129.8, 129.7, 129.5, 129.42, 129.37, 129.2, 129.9, 128.8, 128.7, 128.5, 128.4, 128.3, 128.24, 128.17, 128.1, 127.9, 117.8, 101.8, 100.9, 96.3, 92.6, 76.1, 72.2, 71.9, 71.6, 71.4, 71.1, 70.9, 70.6, 70.5, 70.2, 69.2, 68.5, 68.2, 67.5, 67.4, 65.8, 65.5, 65.1, 62.9, 62.6, 62.5, 62.0, 52.1, 29.7, 23.2, 20.8. ESI-TOF HRMS m/z calcd for C₁₀₇H₁₀₁NNaO₃⁷ [M+2Na]⁺: 1018.7924, found: 1018.7913.

2.4.2.8.4. **3-(Acetylthio)propyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (29)**

Allyl pentasaccharide 28 (36.2 mg, 0.018 mmol, 1 equiv) was dissolved in dry DCM (3 mL). DPAP (2.3 mg, 0.009 mmol, 0.5 equiv) and AcSH (3.8 μL, 0.054 mmol, 3 equiv) were added under Ar. The mixture was stirred under water cooling (~25 °C) for 15 min in a Rayonet UV reactor equipped with 350 nm lamps. The solution was co-evaporated with
toluene and concentrated. The crude product was purified by preparative thin-layer chromatography on silica gel (20:1 DCM:MeOH, two runs) to yield the pentasaccharide 29 (24.4 mg, 63%) as a white powder. $R_f$ 0.60 (10:1 DCM:MeOH). $^1$H NMR (400 MHz, CD$_3$CN) δ 8.37 – 8.20 (m, 4H), 8.01 – 7.87 (m, 4H), 7.82 – 7.71 (m, 4H), 7.70 – 7.23 (m, 30H), 7.20 – 6.98 (m, 8H), 6.29 (d, $J = 9.6$ Hz, 1H), 5.88 (d, $J = 3.1$ Hz, 1H), 5.65 – 5.55 (m, 3H), 5.55 – 5.46 (m, 3H), 5.43 (dd, $J = 10.5$, 7.9 Hz, 1H), 5.09 – 5.01 (m, 3H), 4.83 (d, $J = 7.9$ Hz, 1H), 4.65 (d, $J = 3.5$ Hz, 1H), 4.60 (dd, $J = 10.4$, 3.1 Hz, 1H), 4.51 (d, $J = 8.0$ Hz, 1H), 4.44 – 4.28 (m, 3H), 4.26 (s, 1H), 4.18 (t, $J = 6.7$ Hz, 1H), 4.09 – 3.98 (m, 4H), 3.94 (dd, $J = 11.2$, 7.0 Hz, 1H), 3.92 – 3.78 (m, 3H), 3.73 – 3.41 (m, 13H), 3.29 – 3.18 (m, 2H), 3.15 (t, $J = 6.8$ Hz, 1H), 2.94 – 2.77 (m, 2H), 2.20 (s, 3H), 1.80 (s, 3H), 1.76 (s, 3H), 1.69 (q, $J = 6.5$ Hz, 2H). $^{13}$C NMR (101 MHz, CD$_3$CN) δ 195.6, 170.07 (2x), 165.8, 165.64, 165.58, 165.4, 165.3, 165.1, 165.0, 164.92, 164.90, 164.6, 134.2, 134.1, 133.5, 133.4, 133.3, 133.2, 133.15, 133.10, 130.2, 129.6, 129.5, 129.3, 129.2, 129.0, 128.74, 128.68, 128.6, 128.5, 128.45, 128.42, 128.34, 128.29, 117.3, 101.5, 101.1, 97.1, 92.4, 92.3, 76.9, 71.5, 71.2, 71.1, 70.9, 70.7, 70.6, 70.5, 70.4, 69.2, 68.0, 67.9, 67.6, 67.5, 66.5, 65.9, 65.7, 65.2, 61.9, 61.4, 51.4, 31.6, 29.8, 29.4, 28.9, 25.4, 22.4, 21.9, 20.3, 0.99, 0.96, 0.82, 0.79, 0.7, 0.61, 0.58, 0.5, 0.4, 0.3, 0.2, 0.1. ESI-TOF HRMS m/z calcd for C$_{109}$H$_{105}$N$_3$O$_{38}$S [M+2Na]$^+$: 1056.7915, found: 1056.7884.

2.4.2.8.5. 3-Thiopropyl $\alpha$-$D$-galactopyranosyl-(1$\rightarrow$3)-$\beta$-$D$-galactopyranosyl-(1$\rightarrow$4)-[$\alpha$-$D$-galactopyranosyl-(1$\rightarrow$3)-$\beta$-$D$-galactopyranosyl-(1$\rightarrow$6)]-2-Acetamido-3-O-acetyl-2-deoxy-$\alpha$-$D$-glucopyranoside (G35)$_2$

The acyl-protected tetrasaccharide 29 (24.4 mg, 0.01 mmol) was dissolved in 6 mL of a solution of NaOMe 0.1 M and stirred for 13 h under Ar. The solution was then neutralized with Amberlyst-15, filtered through Celite, concentrated, re-dissolved in water, and lyophilized. The mercaptopropyl trisaccharide G35$_{SH}$ is produced, which oxidizes by handling in the water to yield the disulfide (G35)$_2$ (2 mg). ESI-TOF HRMS m/z calcd for C$_{35}$H$_{61}$N$_3$O$_{26}$S [M+Na]$^+$: 966.3100, found: 966.3021.
2.4.2.9. Synthesis of tetrasaccharide G37SH

2.4.2.9.1. Allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-(2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethylsilyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (8)

Glycosyl disaccharide acceptor 7 (80 mg, 0.09 mmol, 0.5 equiv) and glycosyl disaccharide donor 1 (200 mg, 0.18 mmol, 1 equiv) were dissolved in dry DCM (30 mL). Flame activated MS 4Å were added, and the reaction mixture was stirred at r.t for 1h. The mixture was cooled down to -10 °C and NIS (60 mg, 0.27 mmol, 1.5 equiv), 3,3-difluoroxindole (15.2 mg, 0.09 mmol, 1.0 equiv), and TMSOTf (6.5 µL, 0.036 mmol, 0.2 equiv). The mixture was stirred for 30 min at -10 °C. Triethylamine (0.036 mmol, 0.2 equiv) was added, and the reaction mixture was washed with saturated sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo to give a crude, purified by preparative thin-layer chromatography (2:1:0.5 hexanes:EtOAc:MeOH) to give 8 (54 mg, 50%) as a white solid. [α]D²⁹ = +71.49 (c = 0.08 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.25 – 8.16 (m, 2H), 8.06 – 7.89 (m, 8H), 7.88 – 7.66 (m, 8H), 7.65 – 7.55 (m, 6H), 7.52 – 7.38 (m, 10H), 7.37 – 7.15 (m, 11H), 7.08 – 7.00 (m, 2H), 5.80 – 5.54 (m, 8H), 5.48 (dd, J = 10.5, 3.4 Hz, 1H), 5.25 (dd, J = 10.7, 8.4 Hz, 1H), 5.19 – 5.06 (m, 3H), 4.68 (d, J = 3.7 Hz, 1H), 4.59 (d, J = 7.8 Hz, 1H), 4.54 – 4.39 (m, 2H), 4.32 – 4.15 (m, 5H), 4.13 – 4.07 (m, 1H), 4.07 – 3.97 (m, 3H), 3.84 – 3.63 (m, 7H), 3.56 (dd, J = 10.8, 4.6 Hz, 1H), 2.06 (s, 3H), 1.92 (s, 3H), 0.98 (s, 9H), 0.81 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 171.2 (C=O), 169.9 (C=O), 166.3 (C=O), 165.95 (C=O), 165.88 (C=O), 165.5 (C=O), 165.4 (C=O), 165.35 (C=O), 165.33 (C=O), 164.9 (C=O), 164.6 (C=O), 133.7 (C arom.), 133.63 (C arom.), 133.57 (C arom.), 133.5 (C arom.), 133.3 (C arom.), 133.1 (C arom.), 133.0 (C arom.), 132.8 (C arom.), 129.93 (C arom.), 129.89 (C arom.), 129.81 (C arom.), 129.76 (C arom.), 129.6 (C arom.), 129.5 (C arom.), 129.4 (Cq), 129.3 (Cq), 129.2 (Cq), 129.1 (C arom.), 129.08 (C arom.), 128.98 (Cq), 128.8 (C arom.), 128.7 (C arom.), 128.64 (C arom.), 128.6 (Cq), 128.57 (C arom.), 128.5 (Cq), 128.3 (C arom.), 128.2 (C arom.), 128.1 (C arom.), 117.8 (CH₂), 102.2 (C-1), 101.2 (C-1), 96.0 (C-1), 94.8 (C-1), 76.6 (CH), 74.3 (CH), 72.0 (CH), 71.5 (CH), 71.4 (CH), 71.3 (CH), 70.7 (CH), 70.53 (CH), 70.48 (CH), 69.2 (CH), 68.4 (CH₂), 68.2
(CH₂), 67.8 (CH), 67.6 (CH), 67.2 (CH), 66.4 (CH₂), 66.1 (CH), 62.1 (CH₂), 61.1 (CH₂), 52.1 (CH), 27.3 (CH₃, t-butyl), 27.1 (CH₃, t-butyl), 23.2 (CH₃), 23.1 (Cq), 21.1 (CH₃), 20.5 (Cq). ESI-TOF HRMS m/z calcd for C₁₀₂H₁₀₃NNₐ₂O₃₁Si [M+2Na]²⁺: 955.8039, found: 955.8065.

2.4.2.9.2. **Allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→4)-[2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (30)**

Partially protected tetrasaccharide 8 (50 mg, 0.03 mmol, 1 equiv) was dissolved in 6 mL of anhydrous THF in a plastic conical tube and cooled to 0 °C. Then, 27.5 μL of HF-pyr (70%) was added and stirred for 2 h at rt under Ar. The reaction mixture was cooled again to 0°C and quenched with a solution of saturated NaHCO₃. Finally, the mixture was extracted with EtOAc, washed with water and brine, dried over MgSO₄, concentrated, and purified by preparative thin-layer chromatography on silica gel (30:1 DCM:MeOH,) to furnish the pentasaccharide 30 (38 mg, 82%) as a white powder. $R_f$ 0.26 (4:1 EtOAc:hexanes). [α]₀²⁹ = +76.63 (c = 0.33 in CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 8.38 – 8.30 (m, 2H), 8.08 – 8.00 (m, 4H), 7.99 – 7.89 (m, 4H), 7.83 – 7.65 (m, 8H), 7.64 – 7.52 (m, 6H), 7.52 – 7.32 (m, 12H), 7.27 – 7.17 (m, 6H), 7.16 – 7.06 (m, 2H), 7.05 – 6.97 (m, 2H), 5.82 – 5.61 (m, 8H), 5.58 (dd, $J$ = 10.4, 3.3 Hz, 1H), 5.30 – 5.20 (m, 2H), 5.19 – 5.07 (m, 2H), 4.74 (d, $J$ = 3.7 Hz, 1H), 4.64 (d, $J$ = 7.7 Hz, 1H), 4.54 – 4.39 (m, 3H), 4.34 – 4.17 (m, 4H), 4.10 – 3.92 (m, 4H), 3.90 – 3.53 (m, 10H), 2.05 (s, 3H), 1.91 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.2 (C=O), 169.9 (C=O), 166.0 (C=O), 165.9 (2xC=O), 165.7 (C=O), 165.5 (C=O), 165.4 (C=O), 165.1 (C=O), 165.0 (C=O), 164.9 (C=O), 134.0 (C arom.), 133.7 (C arom.), 133.5 (C arom.), 133.4 (C arom.), 133.3 (C arom.), 133.2 (C arom.), 133.1 (C arom.), 133.0 (C arom.), 132.9 (C arom.), 130.2 (C arom.), 130.0 (C arom.), 129.82 (C arom.), 129.76 (C arom.), 129.72 (C arom.), 129.67 (C arom.), 129.61 (C arom.), 129.56 (Cq), 129.5 (Cq), 129.3 (C arom.), 129.2 (Cq), 129.1 (C arom.), 129.0 (Cq), 128.9 (Cq), 128.8 (C arom.), 128.71 (C arom.), 128.67 (C arom.), 128.5 (C arom.), 128.4 (Cq), 128.34 (C arom.), 128.28 (C arom.), 128.2 (C arom.), 128.1 (C arom.), 117.8 (CH₂), 101.8 (C-1), 101.5 (C-1), 96.1 (C-1), 94.4 (C-1), 73.6 (CH), 72.0 (CH), 71.6 (CH), 71.5 (CH), 71.2 (CH), 70.72 (CH), 70.66 (CH), 70.5 (CH), 70.0 (CH), 69.2 (CH), 69.1 (CH), 68.3 (CH₂), 67.9 (CH₂), 67.8 (CH), 67.7 (CH), 65.9 (CH), 63.0 (CH₂), 62.4 (CH₂), 61.3 (CH₂), 52.1 (CH), 23.2 (CH₃), 21.1 (CH₃). ESI-TOF HRMS m/z calcd for C₉₄H₇₇N₂Na₂O₃₁ [M+2Na]²⁺: 885.7529, found: 885.7509.
2.4.2.9.3. 3-(Acetylthio)propyl 2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)[2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (31)

Allyl tetrasaccharide 30 (16.4 mg, 0.0095 mmol, 1 equiv) was dissolved in dry DCM (2 mL). DPAP (1.2 mg, 0.005 mmol, 0.5 equiv) and AcSH (2 µL, 0.028 mmol, 3 equiv) were added under Ar. The mixture was stirred under water cooling (~25 °C) for 15 min in a Rayonet UV reactor equipped with 350 nm lamps. The solution was co-evaporated with toluene and concentrated. The crude product was purified by preparative thin-layer chromatography on silica gel (20:1 DCM:MeOH, two runs) to yield the tetrasaccharide 31 (24.4 mg, 63%) as a white powder. $R_f$ 0.50 (20:1 DCM:MeOH). $^1$H NMR (400 MHz, MeOD) δ 8.40 – 8.29 (m, 2H), 8.09 – 7.99 (m, 4H), 7.99 – 7.93 (m, 2H), 7.93 – 7.87 (m, 2H), 7.86 – 7.20 (m, 31H), 7.11 – 7.04 (m, 2H), 7.05 – 6.93 (m, 2H), 5.89 – 5.82 (m, 2H), 5.74 – 5.53 (m, 5H), 5.25 – 5.15 (m, 2H), 4.98 (d, $J$ = 7.8 Hz, 1H), 4.76 (d, $J$ = 7.6 Hz, 1H), 4.58 – 4.51 (m, 2H), 4.49 – 4.40 (m, 2H), 4.42 – 4.31 (m, 2H), 4.21 – 3.98 (m, 6H), 3.91 – 3.75 (m, 4H), 3.68 – 3.58 (m, 3H), 3.51 (dd, $J$ = 11.5, 4.0 Hz, 2H), 3.08 (dt, $J$ = 10.7, 5.8 Hz, 1H), 2.86 (dt, $J$ = 14.0, 7.1 Hz, 1H), 2.77 (dt, $J$ = 13.7, 6.9 Hz, 1H), 2.19 (s, 3H), 2.00 (s, 3H), 1.89 (s, 3H), 1.60 (q, $J$ = 6.4 Hz, 2H). $^{13}$C NMR (101 MHz, MeOD) δ 197.4 (C=O), 173.4 (C=O), 172.3 (C=O), 167.5 (C=O), 167.4 (C=O), 167.3 (C=O), 167.1 (C=O), 166.93 (C=O), 166.89 (2xC=O), 166.7 (C=O), 166.5 (C=O), 135.1 (C arom.), 135.0 (C arom.), 134.9 (C arom.), 134.6 (C arom.), 134.5 (C arom.), 134.3 (C arom.), 134.24 (C arom.), 134.17 (C arom.), 131.6 (C arom.), 130.93 (C arom.), 130.89 (C arom.), 130.86 (C arom.), 130.82 (C arom.), 130.8 (C arom.), 130.6 (C arom.), 130.54 (C arom.), 130.52 (C arom.), 130.44 (C arom.), 130.43 (C arom.), 130.25 (C arom.), 130.18 (C arom.), 130.1 (C arom.), 130.05 (C arom.), 130.01 (C arom.), 129.96 (C arom.), 129.84 (C arom.), 129.82 (C arom.), 129.5 (C arom.), 129.4 (C arom.), 129.3 (C arom.), 129.3 (C arom.), 103.6 (C-1), 102.1 (C-1), 98.2 (C-1), 92.7 (C-1), 78.2 (CH$_2$), 73.6 (CH$_2$), 72.7 (3xCH), 72.5 (CH), 72.3 (CH), 72.1 (CH), 71.8 (CH), 71.5 (CH), 71.0 (CH), 70.4 (CH), 69.6 (CH), 69.5 (CH), 68.8 (CH), 67.3 (CH$_2$), 67.0 (CH), 63.1 (CH$_2$), 63.0 (CH$_2$), 53.2 (CH), 30.8 (CH), 30.6 (CH$_3$), 30.1 (CH$_2$), 26.6 (CH$_2$), 22.5 (CH$_3$), 21.4 (CH$_3$). ESI-TOF HRMS m/z calcd for C$_{96}$H$_{91}$NNa$_2$O$_{32}$S [M+2Na]$^{2+}$: 923.7520, found: 923.7497.
2.4.2.9.4. **3-Thiopropyl β-D-galactopyranosyl-(1→4)-[α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→6)]-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (G37)s₂**

The acyl-protected tetrasaccharide 30 (12.3 mg, 0.007 mmol) was dissolved in 8 mL of a solution of NaOMe 43 mM and stirred for 7 h under Ar. The solution was then neutralized with Amberlyst-15, filtered through Celite, concentrated, re-dissolved in water, and lyophilized. The mercaptopropyl trisaccharide G37SH is produced, which oxidizes by handling in the water to yield the disulfide (G37s)₂ (1.6 mg, 34%). ¹H NMR (400 MHz, D₂O) δ 5.06 (d, J = 3.9 Hz, 2H, H-1), 4.80 (d, J = 3.2 Hz, 2H, H-1), 4.47 (d, J = 7.8 Hz, 2H, H1), 4.42 (d, J = 7.9 Hz, 2H, H-1), 4.19 – 4.07 (m, 7H), 3.95 – 3.42 (m, 46H), 2.77 (t, J = 6.9 Hz, 4H, -OCH₂CH₂S⁻), 1.96 (s, 10H, 2xCH₃, -OCH₂CH₂S⁻). ESI-TOF HRMS m/z calcd for C₂₉H₅₁NNa₂O₂₁S [M+2Na]²⁺: 803.2494, found: 803.2484.

2.4.3. **Conjugation procedure of NGP36b**

The conjugation of the thiol-containing (G36s)₂ to BSA was performed using the Imject™ Maleimide-Activated BSA kit (Thermo Fisher Scientific, Waltham, MA, catalog #77116). The conjugation procedure was similar to the one described by the manufacturer and previously published. The disulfide containing (G36s)₂ (756 µg, 0.97 µmol) was reduced with 0.8-1.0 µL of a 5 mM Bond-Breaker TCEP Solution (0.9 equiv per disulfide bond). The final glycan concentration was 3 mM, and the final concentration of TCEP was 0.13 mM. The solution was agitated on a shaker for 30 min to furnish thiol G36SH. Then, Maleimide derivatized BSA (2 mg) was dissolved in 200 µL of conjugation buffer (83 mM sodium phosphate, 0.1M EDTA, 0.9M NaCl, 0.1M sorbitol, and 0.02% sodium azide; pH 7.2) to give a 10 mg/mL solution. The thiol solution was added to the reconstituted BSA and agitated at rt for 2 h. The final glycan concentration was 2 mM. The conjugation mixture was diluted with ultrapure water to a volume of 1 mL and desalted using an Amicon Ultra 10K centrifugal filter (10 min at 4,000 × g, rt). The mixture was washed with 1 mL of ultrapure water three times following the same procedure. The filtrate was removed, and 500 µL of ultrapure water was added to the conjugate solution. The solution was transferred onto a 2 mL ZebaTM spin desalting column (7K MWCO), provided in the kit, previously washed with 1 mL of ultrapure pure water 4 times, and centrifuged at 1,000 × g for 2 min at 10 ºC. The
filtrate was lyophilized and stored at -50 ºC. The payload (average number of G36SH per BSA molecule) was calculated by comparative MALDI-TOF MS spectra.

**NGP36b (Galpa1,3Galpβ1,4[Galpβ1,6]GlcNAc – linker – BSA):** MALDI-TOF MS: m/z for BSA [M+H]+ = 66431; for NGP36b [M+H]+ = 86127.

![MALDI-TOF NGP36b](image)

**Figure 15. MALDI-TOF NGP36b**

2.4.3.1. Conjugation of NGP36b with different payloads

BSA and Sulfo-SMCC linker were dissolved in conjugation buffer pH 7.2 (see Table 1 for concentrations). The mixture was agitated on a shaker for 30 min to yield the linker-BSA conjugate. The conjugation mixture was diluted with conjugation buffer pH 7.2 to a volume of 0.4 mL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of conjugation buffer pH 7.2 three times following the same procedure. The disulfide-containing glycan (G36S)2 was dissolved in conjugation buffer pH 7.2 and reduced with 10-26 µL of a 0.5 mM neutral Bond-Breaker TCEP Solution (0.9 equiv per disulfide bond). The solution was agitated on a shaker for 30 min to furnish thiol G36SH. The thiol solution was added to the linker-BSA 34 and stirred at rt for 2 h. The purification was accomplished as same as described above. The conjugation mixture was diluted with ultrapure water to a volume of 0.4 mL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of water three times following the same procedure. The filtrate was removed,
and 500 µL of water was added to the conjugate solution. The solution was transferred onto a 2 mL Zeba™ spin desalting column (7K MWCO), provided in the kit, previously washed with 1 mL of ultrapure water 4 times, and centrifuged at 1,000 × g for 2 min at 10 ºC. The filtrate was lyophilized and stored at -50 ºC.

Table 1. Glycan and linker concentration for conjugation

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<th>NGP36b payload</th>
<th>BSA concentration (mg/mL)</th>
<th>Sulfo-SMCC concentration (mM)</th>
<th>G36SH concentration (mM)</th>
</tr>
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<td>0.5</td>
</tr>
<tr>
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<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>3.3</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

2.4.4. CL-ELISA of tGPI-MUC and NGP11b

The antigens were immobilized to Nunc MaxiSorp polystyrene microplates for 16 h at 4 ºC in Carbonate-Bicarbonate Buffer (CBB), 200 mM, pH 9.6. After antigen loading, the plates were blocked with 200 µL per well Phosphate-Buffered Saline (PBS) containing 1% BSA (PBS-B) and incubated for 1 h at 37 ºC. The plates were then washed three times with 200 µL of PBS containing 0.05% Tween 20 detergent (PBS-T). Pools of human sera or individual patient sera were analyzed in duplicate at a dilution of 1:400 and 1:800, in PBS-B and 0.05% PBS-TB. The plates were incubated for 1 h at 37 ºC, followed by washing with 200 µL of PBS-T. 50 µL of Biotinylated goat Anti-human IgG antibodies, diluted at 1:5000 in PBS-TB, was added to each well and incubated for 1 h at 37 ºC. After washing three times with 200 µL of PBS-T, 50 µL of High sensitivity Neutravidin-Horseradish Peroxidase (NA-HRP) diluted in PBS-TB was added at 1:5000 dilution. The plates were incubated again for 1 h at 37 ºC. After a final round of washing with PBS-T, the plates were developed with 50 µL of the substrate Super Signal ELISA Pico Stable Peroxide Solution and Super Signal ELISA Pico Luminol Enhancer and CBB/0.1% BSA in a 1:1:8 ratio (v/v/v), and relative luminescent units (RLU)
were measured using a Luminoskan Accent Luminometer. The titers were calculated by dividing the average of patient RLU values by the cutoff of each plate, which was determined by utilizing the standard deviation multiplier at 99.5%.

2.4.5. **Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)**

To determine the mass of BSA and NGPs, 1 µL of BSA (0.4 mg/mL) was combined into a 1.5 mL microcentrifuge tube with 2.5 µL NGPs (5 µg/mL) and 2.5 µL of sinapic acid (20 mg/mL, 50% acetonitrile, 0.1% TFA). Two microliters of the combined sample were spotted onto a 48-well steel MALDI plate and allowed to crystallize at room temperature for approximately 20 minutes. The mass spectra were acquired using a SHIMADZU MALDI-8020 mass spectrometer set to linear mode with a scan range of 20,000 to 100,000 \( m/z \). Data acquisition included a laser power of 115 and profiles at 200. Spectra were processed by Threshold Apex set at constant Threshold, smoothing filter width 400. BSA was utilized as a calibration mass before NGP data acquisition. \([\text{BSA}+\text{H}]^+ = 66431\) and \([\text{BSA}+2\text{H}]^{2+} = 33216\).

2.5. **Additional information**

Another structural feature in tGPI-MUC is the disaccharide Galα(1→2)Galβ.\(^{33-35}\) The Galα(1→2), donor \(\text{41}\) was synthesized with a Fmoc group at position 2. Fmoc group can be removed selectively using triethylamine in the presence of the pivaloyl and isopropylidene. Using \(\text{11}\) as starting material, isopropylidene was installed at C-3 and C-4 to give \(\text{43}\) in 60% yield. Then, the pivaloyl group was installed selectively at C-6 in 60% yield. The \(^1\text{H NMR}\) of \(\text{43}\) and \(\text{44}\) agree with the previously reported.\(^{75,76}\) Furthermore, Fmoc protecting group was installed at C-2 in 86% yield. \(^1\text{H NMR}\) and HR-MS confirmed the installation of the Fmoc group (Scheme 11). Afterward, disaccharide \(\text{42}\) was synthesized by the glycosylation of \(\text{2}\) with \(\text{41}\), using regenerative glycosylation conditions. Compound \(\text{42}\) was furnished in 53% yield (Scheme 12). Selective removal of the Fmoc group needs to be more explored. Removal of Fmoc with TEA in dry DCM general by-products including the hydrolysis products.
Scheme 11. Synthesis of donor 45. a) 2,2-DMP, p-TsOH, acetone, 60%. b) PicCl, pyr, 60%. c) FmocCl, pyr, DCM, 86%

Scheme 12. Synthesis of disaccharide 42. a) HOFox, NIS, TMSOTf, DCM, 4Å MS, 53%.

2.5.1. Synthetic procedures

2.5.1.1. Synthesis of Galβ(1→3)GlcNAcα with Fmoc protecting group

2.5.1.1.1. p-Tolyl-3,4-O-isopropylidene-1-thio-β-D-galactopyranoside (43)

Compound 11 (500 mg, 1.7 mmol, 1 equiv) was dissolved in acetone (100 mL). 2,2-DMP (4.3 mL, 35 mmol, 20 equiv) and p-toluenesulfonic acid (33 mg, 0.17 mmol, 0.1 equiv) were added and the reaction mixture was stirred at rt for 3 h. The reaction mixture was neutralized with triethylamine, evaporated, and dried under reduce pressure. The crude was purified by column chromatography (1:1 hexanes:EtOAc) to yield 43 (343 mg, 60%) as a white powder. Rf 0.26 (1:1 hexanes:EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.49 – 7.40 (m, 2H), 7.16 – 7.09 (m, 2H), 4.42 (dd, $J = 10.2, 1.9$ Hz, 1H), 4.21 – 4.12 (m, 1H), 4.16 – 4.06 (m, 2H), 3.97 (dd, $J = 11.3, 7.1$ Hz, 1H), 3.90 – 3.75 (m, 2H), 3.55 (ddt, $J = 9.2, 6.9, 2.1$ Hz, 1H), 2.75 (t, $J$
2.3 Hz, 1H), 2.39 (d, \( J = 8.8 \) Hz, 1H), 2.33 (d, \( J = 1.9 \) Hz, 3H), 1.42 (d, \( J = 1.8 \) Hz, 3H), 1.33 (d, \( J = 1.8 \) Hz, 3H). Signals corresponded with reference. 

2.5.1.1.2. **p-Tolyl-3,4-O-isopropylidene-6-O-pivaloyl-1-thio-\( \beta \)-D-galactopyranoside (44)**

A solution of compound 43 (100 mg, 0.3 mmol, 1 equiv) in anhydrous pyridine (50 mL) was cooled down to 0 °C, and pivaloyl chloride was added (188 µL, 1.5 mmol, 5 equiv). The resulting mixture was stirred at room temperature for 30 min under an argon atmosphere. The reaction mixture was quenched with methanol, stirred for 10 min, and evaporated under reduced pressure. The crude was re-dissolved in EtOAc and washed with saturated NaHCO₃ solution and brine. The organic layer was dried over anhydrous MgSO₄ and then concentrated under reduced pressure. The crude was purified by column chromatography (hexanes:EtOAc = 2:1) to yield 44 (75.4 mg, 60%) as a white powder. Rf 0.4 (1:2 EtOAc:Hexane). \(^1\)H NMR (400 MHz, CDCl₃) δ 7.50 – 7.39 (m, 2H, arom.), 7.10 (d, \( J = 7.9 \) Hz, 2H, arom.), 4.41 (d, \( J = 10.3 \) Hz, 1H, H-1), 4.38 – 4.28 (m, 2H, H-6), 4.16 (dd, \( J = 5.4, 2.3 \) Hz, 1H, H-4), 4.09 (dd, \( J = 6.9, 5.4 \) Hz, 1H, H-3), 3.98 (ddd, \( J = 7.4, 5.2, 2.3 \) Hz, 1H, H-5), 3.57 (dd, \( J = 10.2, 6.9 \) Hz, 1H, H-2), 2.60 (s, 1H, -OH), 2.33 (s, 3H, CH₃), 1.45 (s, 3H, CH₃), 1.33 (s, 3H, CH₃), 1.21 (s, 9H, 3 CH₃ piv). Signals corresponded with reference.

2.5.1.1.3. **p-Tolyl-2-O-fluorenymethyloxycarbonyl-3,4-O-isopropylidene-6-O-pivaloyl-1-thio-\( \beta \)-D-galactopyranoside (41)**

Compound 44 (520 mg, 1.27 mmol, 1 equiv) was dissolved in 55 mL of DCM:pyr 10:1 the solution was cooled down to 0 °C, and FmocCl was added (657 mg, 2.54 mmol, 2 equiv). The resulting mixture was stirred at room temperature for 45 min under an argon atmosphere. The reaction mixture was quenched with methanol, stirred for 10 min, and evaporated under reduced pressure. The crude was re-dissolved in EtOAc and washed with saturated NaHCO₃ solution and brine. The organic layer was dried over anhydrous MgSO₄ and then concentrated under reduced pressure. The crude was purified by column chromatography (5:1 hexanes: EtOAc) to yield 41 (691 mg, 86%) as a white powder. Rf 0.26 (1:5 EtOAc:Hexane). \(^1\)H NMR (400 MHz, CDCl₃) δ 7.77 (ddt, \( J = 7.6, 2.2, 1.0 \) Hz, 2H), 7.67 (dddd, \( J = 7.4, 5.7, 1.9, 0.9 \) Hz, 2H), 7.44 – 7.38 (m, 4H), 7.31 (tdd, \( J = 7.5, 2.9, 1.2 \) Hz, 2H), 7.11 – 7.06 (m, 2H), 4.87 (dd, \( J = 10.3, 7.2 \) Hz, 1H), 4.65 – 4.58 (m, 2H), 4.41 – 4.31 (m, 4H), 4.31 – 4.25
(m, 1H), 4.21 (dd, $J = 5.3, 2.1$ Hz, 1H), 3.98 (ddd, $J = 7.2, 4.8, 2.2$ Hz, 1H), 2.33 (s, 3H), 1.55 (s, 3H), 1.35 (s, 3H), 1.21 (s, 9H).

2.5.1.1.4. Allyl 2-O-fluorenlymethyloxycarbonyl-3,4-O-isopropylidene-6-O-pivaloyl-1-thio-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-6-O-tert-butyldimethylsilyl-α-D-glucopyranoside (42)

Glycosyl acceptor 2 (150 mg, 0.36 mmol, 1 equiv) and glycosyl donor 41 (341 mg, 0.54 mmol, 1.5 equiv) were dissolved in dry CH$_2$Cl$_2$ (45 mL). Flame-activated MS 4Å were added, and the reaction mixture was stirred at rt for 1 h. The mixture was cooled down to -10 °C and NIS (186 mg, 0.83 mmol, 2.3 equiv), 3,3-difluoroxindole (45 mg, 0.27 mmol, 0.75 equiv), and TMSOTf (19.6 µL, 0.1 mmol, 0.3 equiv). The mixture was stirred for 30 min at -10 °C and then at rt for 3 more h. Triethylamine (0.1 mmol) was added, and the reaction mixture was washed with saturated sodium thiosulfate anhydrous (aq) and brine. The organic phase was dried over anhydrous MgSO$_4$, filtered, and concentrated in vacuo to give a crude, purified by column chromatography (3:2 EtOAc:hexanes) to give 42 (175 mg, 53%) as a yellow pale solid. $R_f$ 0.26 (3:2 EtOAc:hexanes). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.74 (dd, $J = 7.6, 4.1$ Hz, 2H, arom.), 7.60 (dd, $J = 7.6, 3.9$ Hz, 2H, arom.), 7.45 – 7.27 (m, 4H, arom.), 5.88 (ddt, $J = 16.6, 11.1, 5.8$ Hz, 1H, H-b), 5.68 (dd, $J = 10.6, 7.7$ Hz, 2H, H-1’, ), 5.32 – 5.12 (m, 3H), 4.79 (d, $J = 3.8$ Hz, 1H), 4.54 (dd, $J = 5.7, 2.0$ Hz, 1H), 4.43 (dd, $J = 7.7, 2.1$ Hz, 1H), 4.27 – 3.91 (m, 10H), 3.85 (d, $J = 11.2$ Hz, 1H), 3.70 – 3.55 (m, 3H), 2.04 (s, 3H), 1.95 (s, 3H), 1.40 (s, 3H), 1.29 (s, 3H), 1.06 (s, 9H), 0.86 (s, 9H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 178.1, 177.2, 171.3, 170.0, 144.1, 144.0, 141.4, 141.2, 133.4, 127.7, 127.6, 127.0, 126.9, 125.2, 125.1, 119.9, 119.9, 118.0, 109.6, 96.7, 95.7, 72.1, 71.6, 71.5, 70.9, 70.5, 70.2, 68.1, 67.2, 66.8, 63.0, 62.3, 60.4, 51.7, 47.4, 38.6, 29.6, 27.0, 26.0, 24.3, 23.3, 21.09, 21.06, 18.4, 14.2, -5.13, -5.24. ESI-TOF HRMS m/z calcd for C$_{48}$H$_{67}$NNaO$_{15}$Si [M+Na]$^+$: 948.4178, found: 948.4132.
3. Synthetic neoglycoprotein of Gal\(\alpha1\)-3Gal\(\beta1\)-4GlcNA\(\alpha\) glycoside as potential vaccines for Chagas disease

3.1. Introduction

CD is a neglected tropical disease caused by the parasite *T. cruzi*. An estimated 8 million infected people live in Latin America, and 350,000 infected people reside in the US.\(^1\) CD was initially considered endemic to Latin America; however, due to flux migration from endemic countries to the US and Europe, it has spread worldwide, becoming a global public health problem.\(^3\) Approximately 20-30% of patients with chronic Chagas disease (CCD) develop cardiomyopathy and digestive megas Syndromes, leading to not only disability and death but also to social and economic burden.\(^1,5\) Treatment of CD is centered on two drugs (i.e., benznidazole and nifurtimox) that are very effective in the acute phase. Unfortunately, most cases are diagnosed in the chronic stage, where the treatment is partially effective with serious side effects, reason why around 10–20% of patients with CCD undergo premature termination of treatment.

There is no preventive or therapeutic vaccine for *T. cruzi* infection. The vaccine development approach has focused on parasite lysates, purified proteins, peptides, DNA, and glycoproteins from the epimastigote stage.\(^43,44,47,77\) The use of these molecules as a glycotopes do not protect completely against *T. cruzi* infection because they are not conserved among the different parasite DTUs.\(^78–80\) Additionally, the material after extraction is inconsistent, and the purification of lysates, DNA, and glycoproteins from the parasite requires extensive mammalian cell tissue, prolonged extraction, and purification.

tGPI-MUCs are heavily \(O\)-glycosylated with 4,6-di-\(O\)-substituted GlcNA\(\alpha\) and \(\alpha\)-Galp residue at the non-reducing end.\(^33–36\) These \(\alpha\)-Gal glycotopes induce high levels of *T. cruzi*-specific anti-\(\alpha\)-Gal antibodies in CCD patients.\(^31,33,38\) Our group has broadly used different NGPs, including NGP29b, a linear disaccharide from the \(\beta\)-galactofuranosylated GPI anchor of the tGPI-MUC,\(^41\) and NGP24b, a linear trisaccharide found in the protein portion of tGPI-MUC.\(^50\) Our evidence shows these NGPs show differential immunoreactivity, yet they have been poorly investigated for vaccine development.
Glycoconjugate vaccines have played an important role in preventing infectious diseases such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*. There is strong evidence that glycovaccine activate adaptive immune responses. When carbohydrates epitopes are conjugated to a carrier protein and used to immunize mammals, they activate T cells that help B cells to produce IgG antibodies against the polysaccharide component. These glycoproteins prompt IgM-to-IgG switching, memory B cell development, and long-lived T cell memory. The glycan portion of the glycoconjugate is recognized and internalized by the B cell receptor (BCR) and internalized. Then, a peptide-glycan is generated in the endolysosomes and binds to the MHCII, which presents it on the B cell's surface. αβ TCR from T cells recognize the glycan and produce cytokines (IL-4 and IL-2). These induce the maturation of B cells to become memory B cell and promote the production of carbohydrate-specific IgG antibodies.

A recent study exhibited an effective prophylactic α-Gal-based glycoconjugate vaccine, able to protect against acute murine CD. Portillo et al. demonstrated the efficacy of two α-Gal-based vaccine formulations using as an epitope the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcβ (Galili epitope) conjugated to HSA and tested in the α1,3GalT-KO mouse model, which closely resembles the major protective human anti-glycan response observed in CD infection. This study has shown that immunogenic glycoconjugate vaccines induce protection via recognition of both peptides and glycopeptides presented by MHCII on the activated B cells and engagement of CD4+ T cells.

We considered that T cells could recognize carbohydrates if they are covalently linked to a peptide that allows MHCII to present the hydrophilic carbohydrate on the surface of the antigen-presenting cell (APC). We have evidence that the distance between the glycan and the peptide affects the recognition of the αβ TCR (Almeida group, unpublished data). Based on these preliminary data, we hypothesize that if the distance is short (~4 Å), the recognition is stronger, resulting in long-term protection against *T. cruzi*.

Here we describe the synthesis of the α-Gal containing trisaccharide NGPs using three carrier proteins and two different linkers, i.e., Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate), and Sulfo-SIA (sulfosuccinimidyl iodoacetate). The trisaccharide was synthesized by two consecutive glycosylation and
modified at the reducing end by a thiopropyl group for conjugation. Our synthetic approach synthesized the trisaccharide in 6 steps from known monosaccharides. We report the spectroscopic data of (G24s)2 for the first time. The oligosaccharide (G24s)2 was conjugated to human serum albumin (HSA), ovalbumin (OVA), and tetanus toxoid (TT) using SMCC and SIA as likers. The NGPs will be evaluated in the α1,3GalT-KO mouse model to give us insights into the induction of lytic antibodies resulting in long-term protection against T. cruzi.

3.2. Results and discussion

3.2.1. Retrosynthetic analysis

The retrosynthetic analysis of G24SH is shown in Figure 16. This would be prepared from the disaccharide donor 1 and GlcNAc acceptor 2. Disaccharide donor 1 would be synthesized through chemical glycosylation of acceptor 14 with donor 17. The synthesis of donor 17 and acceptor 14 followed a conventional synthetic route described in Chapter 2.

Figure 16. Retrosynthetic analysis of G24SH
3.2.2. Synthesis of trisaccharide (G24$_3$)$_2$

Compound (G24$_3$)$_2$ was synthesized by two consecutive glycosylations and the overall deprotection. The synthesis of fully protected trisaccharide 25 is described in detail in Chapter 2. Briefly, compound 25 was synthesized by two consecutive glycosylation. First, compound 17 was glycosylated with 14 to produce 1 in 88% yield. Then, compound 2 was glycosylated with 1 to furnish 25 in 66% yield (Scheme 13). $^1$H NMR spectroscopy confirmed the configuration of the new $\beta$-anomeric proton ($\delta$ 5.00, d, $J_{1-2}$ = 8.0 Hz, 1H, H-1'), and $^1$H NMR spectroscopy confirmed the correct number of aromatic protons in acetone-d$_6$ to avoid interference with CDCl$_3$. Subsequently, the tert-butyldimethylsilyl group was removed using AcOH to furnish 3 quantitatively. Then, the silylene group was removed with HF-pyridine complex to afford 32 (88% yield), followed by the radical anti-Markovnikov addition of AcSH to give 29 in 80% yield. Finally, general deacylation provided the target glycoside (G24$_3$)$_2$ in 45% yield. Although (G24$_3$)$_2$ was synthesized by Scheckter, et al.,$^{57}$ no spectroscopic data was provided. Here, we fully characterized (G24$_3$)$_2$ by $^1$H, $^{13}$C, COSY, and HSQC NMR spectroscopy. According to the $^1$H NMR in D$_2$O, three anomeric protons were found at 5.12, 4.87, and 5.42 ppm ($J_{1-2}$ = 3.9, 3.1, and 7.7 Hz, respectively). The singlet identified at 1.99 ppm corresponds to the protons of the methyl group in the acetyl group. According to $^{13}$C NMR spectroscopy the carbon of the carbonyl group was identified at 174.40 ppm. $^{13}$C DEPT-135 NMR identified six methylene at 66.19, 61.04, 60.98, 60.01, 34.84, and 28.01 ppm. Finally, fifteen methines were identified corresponding to the carbon backbone of the trisaccharide.
Scheme 13. Synthesis of mercaptopropyl glycosides G24SH. a) AgOTf, NIS, DCM, 4Å MS, 66%. b) AcOH:H₂O:THF,3:1:1, reflux, quantitative. c) HF-pyr, THF, 70%. d) AcSH, DPAP, DCM, UV light, 80%. e) NaOMe, MeOH.
Compound (G24S)_2 was previously synthesized in 12 synthetic steps from known monosaccharides. Here, we synthesized (G24S)_2 in 6 steps from known building blocks. The two strategic steps in the synthetic route were installing the terminal Galα unit at the non-reducing end to form disaccharide 1 in 88% yield and the challenging glycosylation of the 2-deoxy-2-N-acetyl acceptor 2 to give the correct stereoisomer (25) in 66% yield. Fukase and coworkers did a similar glycosylation with similar yield.93,94

Schocker, et al. synthetic route for (G24S)_2 is illustrated in Scheme 14. Three notable improvements to this synthetic strategy were made. First, Schocker, et al. removed the silylene in 46 and acetylated the two hydroxyls to afford 47. In our approach, we avoid the removal of the silylene group, the presence of electro-donating groups increases the electrophilicity of the disaccharide donor 1. Second, Schocker, et al. used an allyl group in compound to protect the anomeric carbon. The allyl group is further removed and a trichloroacetimidate group is installed as an activated group. This strategy increases the synthetic route by two steps. In our synthesis, this problem is avoided by changing the allyl group by a thioglycoside (compound 1, Scheme 13). The thiotolyl group at the anomeric center acts as a protecting group as well as an activating group. Lastly, they used unarmed GlcNAc acceptors (compound 49 and 50, Scheme 14). The presence of electro-withdrawing groups decreases the reactivity of hydroxyl group at position 4. Our approach changed the benzoyl group at C-6 for a TBDMS group without modifying the N-Acetyl group. This change increased the yield of the glycosylation step from 46% to 66%.

Similar to Schocker, et al., we functionalized the (G24S)_2 with a mercaptopropyl linker needed for conjugation to carrier proteins. The mercaptopropyl linker provides versatility for conjugation with a large variety of bifunctional linkers.
Scheme 14. Synthesis of *G24*$_{SH}$ took from reference$^{57,95}$, a) Bu₂SnO, MeOH; b) PMBCl, Bu₄NCl, benzene (75% two steps); c) BzCl, pyr (91%); d) DDQ, DCM, H₂O (98%); e) TMSOTf, DCM, 4 Å MS (92%); f) HF-pyr, THF (90%); g) Ac₂O, pyr (89%, two steps); h) PdCl₂, MeOH (87%); i) CCl₃CN, DCM, DBU (84%); j) TMSOTf, DCM, 4 Å MS (30% a/b 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.).

After oxidation, disulfide compounds were purified by fast protein liquid chromatography (FPLC) using a Resource™ RPC column (polystyrene/divinyl benzene matrix). 2% ACN and 85% ACN were used as a solvent system with a flow rate of 2 mL/min. Compound \( \text{[G24s]}_2 \) was eluted at 11.2 min (Figure 20). UV–vis spectroscopy was used to detect the disulfide bond at 190 nm.

3.2.3. Optimization of conjugation with Succinimidyl 4-\((\text{N-maleimidomethyl})\text{cyclohexane-1-carboxylate} \) (sulfo-SMCC) and \( \text{N-Sulfosuccinimidyl iodoacetate} \) (Sulfo-SIA) linker

The conjugation procedure with the SMCC linker has been very well established in our group. The results are reproducible, giving, on average, 20 units of glycans attached to the protein. First, the accessible lysine side chains of the protein are amidated with the succinimide functionality of the sulfo-SMCC linker. In this step, the concentration of the linker must be 4 mM; a lower concentration will yield a lower glycan payload. Then, the sugar is conjugated to the maleimide functionality by a thiol-Michael addition producing an NGP. Here, the sugar concentration must be 4 mM as well (Scheme 15).

![Scheme 15. General conjugation with SMCC linker](image)

Scheme 15. General conjugation with SMCC linker

![Scheme 16. General conjugation with SIA linker](image)

Scheme 16. General conjugation with SIA linker
The synthesis of NGP using the sulfo-SIA linker had not been performed before in our lab, the conjugation procedure with this linker needed to be established. \( \text{G3}_{\text{SH}} (\alpha \text{Gal}) \) and cysteamine were used as a model (Scheme 16). Sulfo-SIA is a heterobifunctional crosslinker with an \( N \)-hydroxysuccinimide (NHS) and an iodo acetyl as reactive groups.

**Table 2.** Conjugation procedure of SIA link

<table>
<thead>
<tr>
<th>Entry</th>
<th>Linker</th>
<th>Equivalents/concentration (mM)</th>
<th>Coupling time</th>
<th>Glycan (mM)</th>
<th>Payload(^\text{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SIA</td>
<td>50/5</td>
<td>30 min(^a)/1h(^b)</td>
<td>5</td>
<td>9(^d) (aggre.)</td>
</tr>
<tr>
<td>2</td>
<td>Sulfo-SIA</td>
<td>50/15</td>
<td>30 min(^a)/1h(^b)</td>
<td>10</td>
<td>24(^f)/14(^e)</td>
</tr>
<tr>
<td>3</td>
<td>Sulfo-SIA</td>
<td>50/15</td>
<td>30 min(^a)/1h(^b)</td>
<td>15</td>
<td>20(^f)/13(^e)</td>
</tr>
<tr>
<td>4</td>
<td>Sulfo-SIA</td>
<td>50/15</td>
<td>30 min(^a)/1h(^b)</td>
<td>15</td>
<td>24(^f)/14(^e)</td>
</tr>
<tr>
<td>5</td>
<td>Sulfo-SIA</td>
<td>50/50</td>
<td>30 min(^a)/1h(^b)</td>
<td>15</td>
<td>34(^f)/25(^e)</td>
</tr>
</tbody>
</table>

\(^a\)succinimidyl iodoacetate, TCEP (tris(2-carboxyethyl)phosphine). conc. concentration
\(^b\)Linker-protein conjugation
\(^c\)Linker-protein-glycan conjugation
\(^d\)glycans attached per protein determined by MALDI-TOF
\(^e\)Units of KM9 (Gal\(\alpha\)1-3Gal\(\beta\))
\(^f\)Units of KM3 (Gal\(\alpha\))
\(^g\)Units of cysteine

Initially, the conditions developed for the conjugation with the SMCC linker were tried with the SIA linker (entry 1, **Table 2**). Even though the conjugation was successful, only nine units of \( \text{G3}_{\text{SH}} \) were attached to the protein, and aggregation was observed. The addition of DMSO helps with the linker solubility but promotes aggregation. To avoid using organic solvents, a water-soluble version of the SIA linker was purchased. The carbonyl substitution of NHS with lysins side chain was performed at pH 7.2, and the nucleophilic substitution was performed at pH 8.3. **Table 2** lists all the conditions explored. In entry 2, linker and glycan concentrations tripled and duplicated, respectively. Increasing the concentration of both linker, and glycan increases the payload of the NGP 1.5-fold. Then, the concentration of
glycan was increased to 15 mM (entry 3); however, no change in the NGP payload was observed. We suspected that using TCEP-HCl could be too acidic and may hydrolyze the glycan resulting in a low conjugation yield. For this reason, we decided to use a neutral TCEP solution for the reduction reaction. Following the same concentration as in entry 3 but using neutral TCEP solution instead of acidic TCEP, the conjugation payload was the same; thus, the low payload is not caused by hydrolysis of the glycan (entry 4). Finally, the linker concentration was increased by 3.3-fold, resulting in an increase in NGP payload of 1.7-fold. These results are in accordance with the nearly 20 lysine residues in HSA accessible for covalent attachment. Conditions listed in entry 5 were tried with different glycans (Table 2). As mentioned before, 34 units of cysteine were attached to the protein and 26 units of monosaccharide \( \text{G3SH} \). For bigger molecules, like disaccharide \( \text{G9SH} \) and branched trisaccharide \( \text{G11SH} \), 17 units of each compound were attached to the protein. For the linear trisaccharide \( \text{G24SH} \), only seven units of the compound were attached because the amount of compound needed for the conjugation was not available (Figure 17).
Figure 17. Conjugation of different glycans using SIA as a linker and HSA as a carrier protein.
3.2.4. Synthesis of NGPs

With the trisaccharide in hand, we performed the conjugations of \((G24S)_2\) to HSA and OVA. Using \((G24S)_2\) and the SMCC linker and yielded the **NGP24h-smcc** with a payload of 24 GU (**Figure 18**). On the other hand, the same trisaccharide was conjugated to OVA using the SIA linker to yield the **NGP24ova-sia** with a payload of 7 GU (**Figure 19**). The loading of **NGP24h-smcc** is in accordance with the almost 20 lysine residues available for modification in HSA. The payload of the **NGP24ova-sia** reaches half of the payload reported in literature when OVA is used as a carrier protein.

**Figure 18.** MALDI-TOF of **NGP24h-smcc**.
3.3. Conclusions

In this chapter, we developed a synthetic route for the synthesis of trisaccharide (Gal\(\alpha_1,3\)Gal\(\beta_1,4\)GlcNAc\(\alpha\)), one of the principal immunoglycotopes of GPI-MUC from \textit{T. cruzi} trypomastigotes. Compound \textbf{G24\textsubscript{SH}} was synthesized in 6 synthetic steps from building blocks with an overall yield of 2.2%. This route was improved from the previously reported synthesis by Schocker, who achieved the synthesis of \textbf{G24\textsubscript{SH}} in 12 synthetic steps.\textsuperscript{50,95} The spectroscopic characterization of \textbf{G24\textsubscript{SH}} was reported for the first time. The conjugation procedure using sulfo-SMCC linker was optimized, and a conjugation procedure using sulfo-SIA was developed. The conjugation procedure was successfully achieved from small molecules such as cysteine to trisaccharides such as \textbf{G11\textsubscript{SH}}. \textbf{G24\textsubscript{SH}} was successfully conjugated to HSA using the sulfo-SMCC linker to yield \textbf{NGP24h-smcc} with a payload of 24 GU. Equally, \textbf{G24\textsubscript{SH}} was conjugated to OVA with sulfo-SIA linker to furnish \textbf{NGP24ova-sia} with a payload of 7 GU. These NGPs will be evaluated in the \(\alpha_1,3\)GalT-KO mouse model to give insights into the induction of lytic antibodies resulting in long-term protection against \textit{T. cruzi}.

\textbf{Figure 19.} MALDI-TOF of \textbf{NGP24ova-sia}.
3.4. Experimental section

3.4.1. General Information

The reactions were conducted under an inert atmosphere of argon and monitored by TLC on silica gel 60 F254 plates. All solvents were distilled by standard procedures. All the reagents were purchased from commercial sources and used as received unless otherwise stated. The compounds were purified by flash column chromatography on silica gel (40-60 μm). NMR experiments were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz. Chemical shifts were measured relative to tetramethylsilane (δ 0.00 ppm) or the solvent peak. The signal assignments were made by COSY, HSQC, and HMBC experiments, and the coupling constants were measured from the 1D experiments. Electrospray mass spectra were recorded on a JEOL Accu Time Of Flight (TOF) Rayonet RPR200 photochemical reactor (USA) equipped with 16 UV lamps (350 nm) was used for the thiol-ene reactions. Glycoconjugates were characterized using a Matrix-Assisted Laser Desorption Ionization (MALDI)-TOF MS (sinapic acid was used as a matrix).

3.4.2. Synthetic procedures and spectroscopic characterization

3.4.2.1. Deprotection of trisaccharide acceptor 3

3.4.2.1.1. **Allyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (32)**

Trisaccharide acceptor 3 (52 mg, 0.04 mmol) was dissolved in 5 mL of anhydrous THF in a plastic conical tube and cooled to 0 ºC. Then, 30 μL of HF-Pyr (70%) was added and stirred for 3 h at rt under Ar. The reaction mixture was cooled again to 0ºC and quenched with a solution of saturated NaHCO3. Finally, the mixture was extracted with EtOAc, washed with water and brine, dried over MgSO4, concentrated, and purified by preparative thin-layer chromatography on silica gel (20:1 DCM:MeOH, two runs) to furnish the pentasaccharide 32 (38 mg, 82%) as a white powder. $R_f$ 0.33 (10:1 DCM:MeOH). $[\alpha]_D^{29} = +234.91$ (c = 0.04 in CHCl3). $^1$H NMR (400 MHz, CDCl3) δ 8.29 – 8.19 (m, 2H, arom.), 7.91 (d, $J = 7.7$ Hz, 2H, arom.), 7.79 (d, $J = 7.7$ Hz, 2H, arom.), 7.65 (d, $J = 7.8$ Hz, 2H, arom.), 7.62 – 7.47 (m, 6H, arom.), 7.44 – 7.30 (m, 4H, arom.), 7.26 – 7.16 (m, 3H, arom.), 7.10 (t, $J = 7.7$ Hz, 2H, arom.), 6.95 (t, $J = 7.7$ Hz,
Hz, 2H, arom.), 5.93 – 5.61 (m, 6H, H-b, H-4', -NH, H-2", H-2', H-1"), 5.34 – 5.13 (m, 4H, H-3", H-3, H-c), 5.01 (d, J = 8.0 Hz, 1H, H-1'), 4.84 (d, J = 3.6 Hz, 1H, H-1), 4.48 – 4.35 (m, 2H), 4.27 (dt, J = 9.9, 4.9 Hz, 2H), 4.19 – 4.03 (m, 4H), 3.97 – 3.80 (m, 3H), 3.79 – 3.55 (m, 6H), 3.42 (d, J = 31.4 Hz, 3H), 2.02 (s, 3H, CH3). 13C NMR (101 MHz, CDCl3) δ 171.4 (C=O), 170.4 (C=O), 166.2 (C=O), 165.0 (C=O), 165.2 (C=O), 165.1 (C=O), 164.7 (C=O), 133.6 (C arom.), 133.4 (C arom.), 133.2 (C arom.), 133.0 (C-b), 132.9 (C arom.), 132.8 (C arom.), 130.0 (C arom.), 129.7, 129.6 (C arom.), 129.3 (Cq), 129.2 (Cq), 128.8 (C arom.), 128.6 (C arom.), 128.4 (Cq), 128.2 (C arom.), 128.0 (C arom.), 118.1 (CH2), 101.4 (C-1'), 96.3 (C-1), 92.9 (C-1"), 74.9 (CH), 72.9 (CH), 71.4 (CH), 71.0 (CH), 70.9 (CH), 70.7 (CH), 69.7 (CH), 69.0 (CH), 68.5 (CH2), 67.4 (CH), 65.3 (CH), 62.8 (CH2), 62.4 (CH2), 60.4 (CH), 60.0 (CH2), 52.3 (CH), 23.2 (CH3), 20.9 (CH3). ESI-TOF HRMS m/z calcd for C50H61NNaO22 [M+Na]+: 1070.3583, found: 1070.3562

3.4.2.1.2. 3-(Acetyltia)propyl 2,3-di-O-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (33)

Allyl pentasaccharide 32 (37.9 mg, 0.03 mmol, 1 equiv) was dissolved in dry DCM (3 mL). DPAP (4.2 mg, 0.016 mmol, 0.5 equiv) and thioacetic acid (7 µL, 0.1 mmol, 3 equiv) were added under Ar. The mixture was stirred under water cooling (~25 °C) for 15 min in a Rayonet UV reactor equipped with 350 nm lamps. The solution was co-evaporated with toluene and concentrated. The crude product was purified by preparative thin-layer chromatography on silica gel (20:1 DCM:MeOH, two runs) to yield the tetrascarbohydrate 33 (28.1 mg, 70%) as a white powder. Rf 0.33 (10:1 DCM:MeOH) 1H NMR (400 MHz, CDCl3) δ 8.22 (d, J = 7.7 Hz, 2H), 7.97 (d, J = 7.6 Hz, 2H), 7.83 – 7.78 (m, 2H), 7.69 (d, J = 7.7 Hz, 2H), 7.66 – 7.33 (m, 11H), 7.24 (d, J = 8.4 Hz, 6H), 7.13 (t, J = 7.6 Hz, 2H), 6.98 (t, J = 7.7 Hz, 2H), 6.04 (d, J = 9.4 Hz, 1H), 5.79 (d, J = 3.1 Hz, 1H), 5.73 – 5.62 (m, 3H), 5.34 – 5.26 (m, 1H), 5.19 (dd, J = 10.6, 3.1 Hz, 1H), 4.95 (d, J = 8.0 Hz, 1H), 4.75 (d, J = 3.6 Hz, 1H), 4.47 (dd, J = 11.2, 6.4 Hz, 1H), 4.33 – 4.19 (m, 3H), 4.11 (t, J = 6.7 Hz, 1H), 4.03 (t, J = 9.6 Hz, 1H), 3.89 – 3.54 (m, 10H), 3.38 – 3.30 (m, 1H), 3.01 (dt, J = 14.0, 7.1 Hz, 1H), 2.89 (dt, J = 13.6, 6.6 Hz, 1H), 2.64 (s, 1H), 2.31 (s, 3H), 2.04 (s, 3H), 1.97 (s, 4H), 1.83 (dq, J = 18.2, 6.5 Hz, 2H). 13C NMR (101 MHz, CDCl3) δ 195.8 (C=O), 171.3 (C=O), 170.7 (C=O), 166.2 (C=O), 166.1 (C=O), 165.2 (C=O),
165.1 (C=O), 164.7 (C=O), 133.3 (Carom.), 133.5 (Carom.), 133.0 (C arom.), 132.9 (C arom.),
132.8 (C arom.), 130.0 (C arom.), 129.7 (C arom.), 129.59 (C arom.), 129.56 (C arom.), 129.3
(Cq), 129.2 (Cq), 128.8 (C arom.), 128.6 (C arom.), 128.4 (Cq), 128.2 (C arom.), 128.0 (C
arom.), 101.2 (C-1'), 97.2 (C-1), 92.9 (C-1"), 75.0 (CH), 72.9 (CH), 71.5 (CH), 71.0 (CH), 70.9
(CH), 70.7 (CH), 69.7 (CH), 69.0 (CH), 67.4 (CH), 65.9 (CH2), 65.3 (CH), 62.9 (CH2), 62.9 (CH2),
60.1 (CH2), 52.2 (CH), 30.6 (CH3), 29.2 (CH2), 25.5 (CH2), 23.1 (CH3), 20.9 (CH3). ESI-TOF
HRMS m/z calcld for C_{62}H_{65}NNaO_{23}S [M+Na]^+: 1246.3566, found: 1246.3512
3.4.2.1.3. 3 Thiopropyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-
Acetamido-3-O-acetyl-2-deoxy-α-D-glucopyranoside (G_{24S})_{2}

The acyl-protected tetrasaccharide 33 (13.3 mg, 0.007 mmol) was dissolved in 17 mL
of a solution of NaOMe 43 mM and stirred for 6 h under Ar. The solution was then neutralized
with Amberlyst-15, filtered through Celite, concentrated, re-dissolved in water, and
lyophilized. The mercaptopropyl trisaccharide G_{24SH} is produced, which oxidizes by
handling in the water to yield the disulfide (G_{24S})_{2} (6.4 mg, 46%). 1H NMR (400 MHz, D_{2}O)
δ 5.02 (d, J = 4.2 Hz, 1H), 4.77 (d, J = 3.1 Hz, 1H), 4.42 (d, J = 7.8 Hz, 1H), 4.07 (dd, J = 8.2, 4.8
Hz, 2H), 3.92 – 3.87 (m, 1H), 3.86 – 3.50 (m, 18H), 3.44 (dq, J = 12.6, 6.2 Hz, 1H), 2.74 (t, J =
6.9 Hz, 1H), 1.96-1.84 (m, 5H). 13C NMR (101 MHz, D_{2}O) δ 102.9 (CH), 96.6 (CH), 95.4 (CH),
79.0 (CH), 77.2 (CH), 75.1 (CH), 70.9 (CH), 70.6 (CH), 69.70 (CH), 69.66 (CH), 69.3 (CH), 69.2
(CH), 68.3 (CH), 66.1 (CH2), 64.8 (CH), 61.0 (CH2), 61.0 (CH2), 60.0 (CH2), 53.4 (CH), 34.8
(CH2), 30.9 (CH), 28.0 (CH2), 22.0 (CH3). ESI-TOF HRMS m/z calcld for C_{46}H_{80}N_{2}NaO_{32}S_{2}
Figure 20. FPLC chromatogram of (G24s)_2

3.4.3. Conjugation procedure

3.4.3.1. Succinimidyl iodoacetate (SIA) linker

The carrier protein was reconstituted in conjugation buffer (0.083M sodium phosphate, 0.1M EDTA, 0.9M NaCl, 0.1M sorbitol and 0.02% sodium azide; pH 7.2) to get the concentration listed in Table 3. Separately, the SIA linker was dissolved in 20 μL of ultrapure water. This solution was added to the carrier protein solution, and the volume was adjusted to give the concentrations in Table 3. The mixture was agitated in a shaker for 30 min to give the protein–linker conjugate. The conjugation mixture was diluted with ultrapure water to a volume of 0.3 μL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of borate buffer (50mM sodium borate, pH 8.3, 5mM EDTA) two times following the same procedure. The disulfide-containing glycan (G24s)_2 was dissolved in borate buffer pH 8.2 and reduced with 10-26 μL of a 0.5 mM
neutral Bond-Breaker TCEP Solution (0.9 equiv per disulfide bond). The solution was agitated on a shaker for 30 min to furnish thiol G24SH. The thiol solution was added to the linker-protein conjugate. The glycan volume was adjusted to yield the concentration in Table 3 and agitated at rt for 1.5 h. The purification was accomplished as same as described above. The conjugation mixture was diluted with ultrapure water to a volume of 0.4 mL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of water four times following the same procedure. The filtrate was removed, and 500 µL of water was added to the conjugate solution. The solution was transferred onto a 2 mL ZebaTM spin desalting column (7K MWCO), previously washed with 1 mL of ultrapure water 4 times, and centrifuged at 1,000 × g for 2 min at 10 ºC. The filtrate was lyophilized and stored at -50 ºC.

3.4.3.2. Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker

The carrier protein was reconstituted in conjugation buffer (0.083M sodium phosphate, 0.1M EDTA, 0.9M NaCl, 0.1M sorbitol and 0.02% sodium azide; pH 7.2) to get the concentration listed in Table 3. Separately, the SMCC linker was dissolved in 20 µL of ultrapure. This solution was added to the carrier protein solution, and the volume was adjusted to give the concentration in Table 3. The mixture was agitated in a shaker for 30 min to give the protein–linker conjugate. The conjugation mixture was diluted with ultrapure water to a volume of 0.3 µL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of conjugation buffer two times following the same procedure. The disulfide-containing glycan (G24s)2 was dissolved in conjugation buffer and reduced with 10-26 µL of a 0.5 mM neutral Bond-Breaker TCEP Solution (0.9 equiv per disulfide bond). The solution was agitated on a shaker for 30 min to furnish thiol G24SH. The thiol solution was added to the linker-protein conjugate; the glycan volume was adjusted to yield the concentration in Table 3 and agitated at rt for 1.5 h. The purification was accomplished as same as described above. The conjugation mixture was diluted with ultrapure water to a volume of 0.4 mL and desalted using an 0.5 mL Amicon Ultra 10K centrifugal filter (15 min at 14,000 × g, rt). The mixture was washed with 0.4 mL of water four times following the same procedure. The filtrate was removed, and 500 µL of water was added to the conjugate solution. The solution was
transferred onto a 2 mL Zeba™ spin desalting column (7K MWCO), previously washed with 1 mL of ultrapure water 4 times and centrifuged at 1,000 × g for 2 min at 10 °C. The filtrate was lyophilized and stored at -50 °C.

**Table 3.** HSA, OVA, and TT conjugation conditions

<table>
<thead>
<tr>
<th>Entry</th>
<th>Linker</th>
<th>Protein</th>
<th>Protein conc. (mg/mL)</th>
<th>Linker conc. (mM)</th>
<th>Glycan conc. (mM)</th>
<th>Payload&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sulfo-SIA</td>
<td>HSA</td>
<td>40</td>
<td>50</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>OVA</td>
<td>20</td>
<td>50</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Sulfo-SM CC</td>
<td>HSA</td>
<td>3.3</td>
<td>5</td>
<td>5.8</td>
<td>24</td>
</tr>
</tbody>
</table>

<sup>a</sup>Glycans attached per protein determined by MALDI-TOF

**3.4.4. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)**

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) was used to determine the mass of the glycoconjugates. The glycoconjugate (125 µg/mL) was combined with 2 µL of matrix (10 mg/mL sinapinic acid, 50% acetonitrile, 0.1% TFA). One microliter of the solution was spotted in a 48-well steel MALDI plate and allowed to crystallize at r.t for 10 minutes. The mass spectra were acquired using a SHIMADZU MALDI-8020 mass spectrometer, and BSA was used as an external standard.
4. Discovery of a Galα(1,6)[Galα(1,2)]Galβ-containing neoglycoprotein as a synthetic biomarker for Chagas Disease

4.1. Introduction

Chagas Disease (CD) is a neglected tropical disease (NTD) caused by the protozoan parasite *T. cruzi*. CD is endemic to Latin America but has spread worldwide due to migration and transmission routes. Initially, CD was considered endemic to Latin America; however, due to migration events and other transmission routes (congenital route, blood transfusion, organ transplantation, and contaminated food), cases have been identified in nonendemic countries in Europe and North America. In the United States, cases transmitted by local vectors have been identified. Even though CD has been recognized as a risk to global public health, its treatment relies on two drugs with variable efficacy and severe secondary effects. Although understanding the etiology and epidemiology of CD has been under intense investigation during the last years, it is still difficult to completely define its epidemiology. This complexity is attributable to several factors, including a variety of widely distributed mammals that serve as reservoirs for *T. cruzi*, the variety of vectors involved in the transmission, and the genetic diversity of the parasite. Genetically, *T. cruzi* is diverse and classified into different strains or subtypes. *T. cruzi* DTUs are distinctively distributed through endemic and non-endemic regions. All six *T. cruzi* DTUs can infect mammals and geographically overlap and coexist in the same population. TcI and TcII are the oldest and purest lineages, TcV and TcVI have a hybrid origin with TcII and TcIII as putative parentals, and TcIII and TcV origin is not clear. Still, some believe they result from ancient hybridization between TcI and TcII. TcI is the most frequent and broadly spread DTU; it has been found from the south of the United States to the north of Chile and Argentina and can be transmitted both in sylvatic and domestic cycles. It has been found in human infections in the US, Mexico, Central America, and the Andean and Amazon regions and is associated with severe cardiomyopathies. On the other hand, TcII is not common in North and Central America; nevertheless, it is prevalent in some regions of the Southern Cone of South America, particularly in Brazil and Chile. TcII is frequently found in Triatoma species and infected patients with megaesophagus and megacolon.
All *T. cruzi* DTUs have a complex cell surface containing different glycoconjugates linked to the plasma membrane through glycosylphosphatidylinositol (GPI)-anchors, including the significant families of GPI-anchored mucins (GPI-MUC). Many O-glycans found on the GPI-anchored mucins of *T. cruzi* trypomastigotes (tGPI-MUC) contain terminal nonreducing α-galactopyranosyl residues (α-Gal). These α-Gal-containing glycans are highly immunogenic to humans due to their absence on human cells. Patients with CD produce substantial amounts of lytic, protective anti-α-Gal antibodies against α-Gal epitopes found on tGPI-MUC. However, there is evidence that the levels of O-linked α-galactosylation may vary among the DTUs. Therefore, the ability of the host to clear the parasite using these antibodies might depend on the expression of those sugars on tGPI-MUC. Nevertheless, α-Gal-containing glycotopes of tGPI-MUC may be highly specific biomarkers for accurate diagnosis between DTUs. They could be used for the early assessment of post-chemotherapeutic outcomes as antigens in chemiluminescent enzyme-linked immunosorbent assays (CL-ELISA). Here, we describe the synthesis of neoglycoprotein NGP11b, a decorated bovine serum albumin (BSA) with copies of the tGPI-MUC-derived glycotope Galα(1,6)[Galα(1,2)]Galβ and its evaluation as an appropriate BMK to differentiate among *T. cruzi* DTUs and its potential for monitoring disease clearance after chemotherapy. The reactivity of NGP11b was assessed by CL-ELISA using sera of CD patients from Mexico and Venezuela. The antibody reactivities of *T. cruzi*-positive and *T. cruzi*-negative sera to NGP11b, TcI-tGPI-MUC, and TcII-tGPI-MUC were compared. Furthermore, we present the first preliminary assessment of NGP11b as a BMK in the monitoring of anti-α-Gal antibody levels in patients who have undergone treatment with BNZ.

### 4.2. Results and discussion

#### 4.2.1. Synthesis of (G11s)_2

The branched mercaptopropyl glycoside of α-D-galactopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→6)]-β-galactopyranoside G11\textsubscript{SH} was synthesized according to the synthetic route in Scheme 17. Fully protected trisaccharide 37 was synthesized by double glycosylation of the partially protected acceptor 36 using Kiso’s α-Gal donor 14 to introduce selectively α-Gal moieties. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) was used as
an activator to yield the glycoside 33 in 72% yield. Removal of silylene protecting group using hydrofluoric acid-pyridine (HF-Py) complex followed by the hydrolysis of the isopropylidene acetal with TFA:H₂O:DCM furnishes partially protected glycoside 39. Subsequently, installing a mercaptopropyl acetyl group at the reducing end by radical anti-Markovnikov addition of AcSH afforded the thioester 40 in 76% yield. Finally, complete diacylation of thioester 40 using Zemplén conditions furnished trisaccharide G11SH, which oxidized to the disulfide (G11S)₂ after exposure to oxygen.

4.2.2. Synthesis of NGP11b

After reduction of (G11)₅ with neutral TCEP, G11SH was conjugated to maleimide-derivatized BSA to yield NGP11b (Figure 21). The average number of conjugated glycans per BSA molecule was determined by matrix-assisted laser desorption ionization-time of flight (MALDI – TOF) mass spectrometry. The average number of glycans per BSA molecule was calculated by subtracting the average mass of the commercial maleimide-derivatized BSA from the average mass of NGP11b (Figure 21).
Scheme 17. Synthesis of mercaptopropyl glycosides G11\textsubscript{SH}. a) TMSOTf, DCM, 4Å MS (72%); b) HF-pyr, THF (90%); c) TFA/H\textsubscript{2}O/DCM (96%); d) Thioacetic acid, DPAP, DCM, UV light (350 nm) (76%); e) NaOMe, MeOH (quantitative).
Figure 21. (A) Conjugation to maleimide derivatized BSA to afford NGP11b. a) TCEP neutro 0.5M, phosphate buffer pH 7.2, rt, 2h. (B) Overlaid MALDI-TOF MS spectra of BSA, BSA-maleimide, and NGP11b.

4.2.3. Evaluation of NGP11b as a BMK for Chagas Disease by CL-ELISA

The suitability of NGP11b as a BMK was examined and compared to Tcl-MUC and TcII-MUC extracted from *T. cruzi* trypomastigotes. The antibody-binding response was measured by CL-ELISA using pooled sera from 42 CCD patients (ChHSP) from Venezuela, 16 CCD patients (ChHSP) from Mexico and 27 healthy donors (NHSP) from El Paso. Figure 22 shows the CL-ELISA cross-titrations changing the antigen loading (ng/well) and the serum dilution. Both Tcl-MUC and TcII-MUC exhibit significative differential immunoreactivities between CCDSP and NHSP, even at low antigen concentration (1 ng/well). The lower reactivity of
NHSP indicates that anti-α-Gal antibodies from healthy individuals have a weakly binding with tGPI-MUCs and NGP11b. Differently, anti-α-Gal antibodies from CCD patients strongly bind with tGPI-MUC and NGP11b in a dose-dependent fashion. A 15-fold differential antibody response was observed at a sera dilution of 1:400 and 50 ng/well of NGP11b between CCDSP and NHSP. At the same sera dilution and antigen concentration, the differential antibody response between CCDSP and NHSP was a factor of 30 and 35 for Tcl-MUC and TclI-MUC, respectively. The large gap between NGP11b and tGPI-MUCs is due to the abundant O-linked oligosaccharides and other epitopes that elicit not only lytic anti-α-Gal antibody but other IgG antibodies. Three other NGPs have been reported to exhibit a significant difference between CCDSP and NHSP.41,117

![Graph](image)

**Figure 22.** Cross-titration of varying quantities of immobilized NGP11b, Tcl tGPI-MUC, and TcII tGPI- at different serum dilutions. Chronic Chagas Human Sera Pool (CCDSP) (n=58) was obtained from patients from Venezuela and Mexico with CCD, and Normal Human Sera Pool (NHSP) (n=27) was obtained from healthy donors.

Although the cross-titrations shown in Figure 22 indicate the potential of NGP11b as a BMK, sensitivity and specificity parameters needed to be established. Knowledge of CCD patients’ “true” disease state is needed to calculate sensitivity and specificity based on NGP11b as a BMK. Antibody-binding response of individual sera from 58 patients with
confirmed CCD and 27 healthy donors were measured using NGP11b, TcI-MUC, and TcII-MUC by CL-ELISA (Figure 23).

**Figure 23.** Group scatter plot analysis of sera from CCD patients of NH individuals with NGP11b, TcI tGPI-MUC, and TcII tGPI-MUC.

Based on this data, NGP11b diagnosed 55/58 positive CCD cases, while TcI and TcII diagnosed 51/58 and 32/58 positive CCD cases, respectively (Table 4). The specificity of all the antigens is 100%, which means that all of them can rule out the disease in patients in whom the disease is truly absent. On the other hand, the sensitivity is variable within the
three antigens. Using a titer cut-off value of 1.000, **NGP11b**, TcI and TcII exhibited a sensitivity of 95%, 88%, and 55%, respectively (Table 5).

**Table 4.** Immunoreactivity of sera of CCD patients and plasma of negative controls with TcI tGPI-MUC, TcII GPI-MUC, and NGP11b.

<table>
<thead>
<tr>
<th>Disease/Control</th>
<th>n</th>
<th>TcI tGPI-MUC</th>
<th>TcII tGPI-MUC</th>
<th>NGP11b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>CCD</td>
<td>58</td>
<td>51</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Negative control a</td>
<td>27</td>
<td>0</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

a Plasma from umbilical cord samples with negative real-time PC and non-conventional serology (with nine in-house T. cruzi antigens) for CCD

**Table 5.** Sensitivity, specificity, and other diagnostic parameters of TcI tGPI-MUC, TcII tGPI-MUC, and NGP11b, in the comparison of CCD vs. NC.

<table>
<thead>
<tr>
<th>Parameter a,b</th>
<th>TcI tGPI-MUC (%)</th>
<th>TcII tGPI-MUC (%)</th>
<th>NGP11b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>87.9</td>
<td>55.2</td>
<td>94.8</td>
</tr>
<tr>
<td>Specificity</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>FPR</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>PPV</td>
<td>100.0</td>
<td>100.0</td>
<td>100.p0</td>
</tr>
<tr>
<td>NPV</td>
<td>79.4</td>
<td>50.9</td>
<td>90.0</td>
</tr>
</tbody>
</table>

a Calculated based on CCD (n=58) and NC (n=27) immunoreactivity with TcI tGPI-MUC, TcII tGPI-MUC, and NGP11b figure 2B
Sensitivity = \frac{\text{true positive (TP)}}{\text{TP} + \text{false negative (FN)}} \times 100. \text{ Specificity} = \frac{\text{true negative (TN)}}{\text{TN} + \text{false positive (FP)}} \times 100. \text{ False-positive rate (FPR)} = 100 - \text{specificity}. \text{ Positive predictive value (PPV)} = \frac{\text{TP}}{\text{TP} + \text{FP}}. \text{ Negative predictive value (NPV)} = \frac{\text{TN}}{\text{TN} + \text{FN}}.

This result suggests that NGP11b is better for detecting T. cruzi in patients in whom the parasite is truly present. Receiver-operating characteristics (ROC) curves were plotted to understand the relationship between sensitivity and specificity of NGP11b, Tcl-MUC, and TcII-MUC (Figure 24). Area under the curve (AUC) values extracted from the ROC curves indicate that NGP11b (AUC=0.9974) exhibited higher sensitivity and specificity than Tcl-MUC (AUC=0.9891) and TcII-MUC (AUC=0.7998). According to these results, NGP11b promises a good BMK for CCD diagnosis.

![Figure 24](image.png)

**Figure 24.** ROC curves for NGP11b, Tcl tGPI-MUC, and TcII tGPI-MUCm comparing the reactivity of sera from CCD patients with healthy individuals. AUC, the area under the curve is indicated in gray. 95% confidence intervals are indicated in brackets.

Previous studies have shown that although the lipid moiety of the tGPI anchor is preserved among different DTUs, the diversity of the glycan part trigger differential invasion patterns and different reactivity with anti-α-Gal antibodies, suggesting that the glycans expressed in the cell surface of the parasite can vary in each DTU.\(^{115,118}\) These differences between DTUs glycoconjugates have also been observed in a small protein region of the trypomastigote small surface antigen (TSSA).\(^{119–121}\) The amino acid residues change according to DTUs. Thus, Tcl, TcII, and TcIV have specific TSSA epitopes; TcII, TcV, and TcVI share a common TSSA epitope, and TcV and TcVI share an additional epitope.\(^{119,120,122}\) In this study, we found an interesting similar antibody-binding response between NGP11b and Tcl-
MUC when individual sera from patients with confirmed CCD were used. This result shows that the glycoptope Galα1,6[Galα1,2]Galβ is most expressed in TcI-MUC than in TcII-MUC and could be used as a serological tool to differentiate between DTUs.

We also investigate the suitability of NGP11b for assessing chemotherapy efficacy in CCD patients. Six CCD patients were subjected to standard-of-care (SOC) chemotherapy with benznidazole (5 mg/Kg/day, 60 days). Patients were followed up for 24 months. RLU of sample average was divided by each plate cutoff, calculated as the mean value of 9 NHSP. Dotted horizontal red line: titer equal to 1.0. Green dashed line: titer equal to 0.9. Our data show that even though individual patients had different anti-α-Gal antibody levels before chemotherapy, all patients showed a significant reduction. Patient #6 revealed an antibody reactivity below the cut-off, suggesting negative seroconversion for the anti-α-Gal antibody after two years of chemotherapy. There are no studies investigating the evolution of antibody seroconversion using glycan-based biomarkers. Only a few reports have measured the efficacy of BZN treatment using tGPI-MUC. For example, after a 3-year follow-up, 58% of children with CCD exhibit negative seroconversion. Differently, only 9% of adults with CCD treated with BZN were negatively seroconverted by the antigen trypomastigote CL-ELISA (AT CL-ELISA) after a 1-year follow-up (Figure 25).
**Figure 25.** Anti-α-Gal antibody reactivity of the sera of patients with Chronic Chagas Disease (CCD) against NGP11b analyzed by CL-ELISA before and after chemotherapy for monitoring the efficacy of BZN in patients. Data interpretation: positive result, titer equal or greater than 1.0; inconclusive result, titer <1.0 and >0.9; negative result, titer equal or <0.9. [99.5% CI for all data collected].

Our data suggest that NGP11b can be utilized as a CCD-specific BMK for diagnosis and drug efficacy assessment. Due to the lack of sufficient specificity, batch-to-batch inconsistencies, and the consuming time purification processes of tGPI-MUC, NGP11b could eventually replace it.

**4.3. Conclusions**

Neoglycoprotein NGP11b was produced by the synthesis and conjugation of the branched trisaccharide with two terminal α-Gal units Galα(1,2)[Galα(1,6)]Galβ-(CH$_2$)$_3$/SH. NGP11b was serologically evaluated as a BMK for Chagas Disease diagnosis and early
assessments of drug efficacy in patients. Our results suggested that Galα(1,6)[Galα(1,2)]Galβ may be an immunodominant partial glycopeptide of terminal *T. cruzi* glycans according to the antibody response of sera from patients with CCD measured by CL-ELISA. Using a cohort of 58 individual sera from CCD patients and 27 healthy donors, we demonstrated that NGP11b can distinguish significantly between *T. cruzi* positive and *T. cruzi* negative infection into two distinct groups by CL-ELISA. Based on these results, NGP11b has a sensitivity of 95%. In contrast, when tGPI-MUC is used as a BMK with the same cohort, the accuracy was only 88%. This result shows that NGP11b can potentially replace tGPI-MUC as a BMK for CCD in serological assays. From our results, we also showed that a synthetic *T. cruzi* glycopeptide can be used for the early assessment of drug efficacy in CCD patients. In summary, our results support the antigenicity and the sensitivity of the α-Gal epitopes in NGP11b. Also, the significant differential antibody reactivities between sera from CCD patients and healthy individuals, placed the NGP11b as a suitable BMK for diagnosis and potential follow-up of CCD chemotherapy.

4.4. Experimental section

4.4.1. General Information

The reactions were conducted under an inert atmosphere of argon and monitored by TLC on silica gel 60 F254 plates. All solvents were distilled following standard procedures. All the reagents were purchased from commercial sources and used as received unless otherwise stated. The compounds were purified by flash column chromatography on silica gel (40-60 μm). NMR experiments were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer at 400 and 101 MHz. Chemical shifts were measured relative to tetramethysilane (δ 0.00 ppm) or the solvent peak. The signal assignments were made by COSY, HSQC, and HMBC experiments, and the coupling constants were measured from the 1D experiments. Electrospray mass spectra were recorded on a JEOL Accu Time Of Flight (TOF). Rayonet RPR200 photochemical reactor (USA) equipped with 16 UV lamps (350 nm) was used for the thiol-ene reactions. Glycoconjugates were characterized using a Matrix-Assisted Laser Desorption Ionization (MALDI)-TOF MS (sinapic acid was used as a matrix). 96-well polystyrene Nunc MaxiSorp ELISA plates and CL-ELISA reagents were purchased
from Thermo Scientific or Jackson ImmunoResearch, and luminescence was recorded on a Luminoskan Ascent, Thermo Scientific.

4.4.2. Synthetic procedures and spectroscopic characterization

4.4.2.1. Synthesis of (G11)$_2$

4.4.2.1.1. Allyl 2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethoxysilyl-α-D-galactosyl-(1→2)-[2,3-di-O-benzoyl-4,6-O-di-tert-butyldimethoxysilyl-α-D-galactosyl-(1→6)]-3,4-O-isopropylidene-β-D-galactoside (37)

To a solution of the known galactoside acceptor 36$^{123}$ (165 mg, 0.63 mmol, 1 equiv) and trichloroacetimidate donor 14$^{59-61,124}$ (896 mg, 1.34 mmol, 2 equiv) in anhydrous DCM (12 mL) freshly activated crushed molecular sieves (4 Å) were added and the mixture was stirred under Ar for 1 h at 0 °C. Then, TMS-OTf (12 µL, 66 µmol, 0.1 equiv) was added dropwise, the reaction mixture was continuously stirred for 45 min at 0 °C, and finally quenched with Et$_3$N (66 µmol, 0.1 equiv). The mixture was diluted with DCM, the molecular sieves was filtered off, and the mixture was washed with water and brine. The organic layer was dried over MgSO$_4$, concentrated, and purified by flash column chromatography on silica gel (EtOAc:hexanes = 1:6) to afford the fully protected trisaccharide 37 (584 mg, 72%) as a white powder. $R_f$ 0.28 (EtOAc:hexanes = 1:4). $[a]_{D}^{26}$ +65.9 (c = 0.08 in CH$_2$Cl$_2$). $^1$H NMR (400 MHz, CDCl$_3$, 300K) δ 8.04-7.93 (m, 8H, arom.), 7.60-7.46 (m, 4H, arom.), 7.45-7.31 (m, 8H, arom.), 5.79-5.70 (m, 2H), 5.67-5.56 (m, 3H), 5.49 (m, 1H, OCH$_2$CH=CH$_2$), 5.33 (d, $J$ = 3.2 Hz, 1H), 4.95 (d, $J$ = 17.1 Hz, 1H), 4.89-4.75 (m, 3H), 4.35-3.90 (m, 11H), 3.85-3.83 (m, 1H), 3.76-3.62 (m, 2H), 3.59-3.52 (m, 1H), 1.43 (s, 3H, CCH$_3$), 1.12 (s, 18H, 2 × tBu-Si), 1.07 (s, 3H, CCH$_3$), 0.96 (s, 18H, 2 × tBu-Si) ppm. $^{13}$C NMR (101 MHz, CDCl$_3$, 300K) δ 166.2 (2 × C=O), 166.0 (C=O), 165.7 (C=0), 133.3 (CH, arom., OCH$_2$CH=CH$_2$), 133.2 (CH, arom.), 133.1 (CH, arom.), 133.0 (CH, arom.), 130.0 (C, arom.), 129.8 (C, arom.), 129.8 (CH, arom.), 129.7 (CH, arom.), 129.6 (CH, arom.), 129.5 (C, arom.), 128.4 (CH, arom.), 128.3 (CH, arom.), 117.9 (C), 117.7 (CH$_2$), 110.0 (C), 101.3 (CH, C-1), 96.4 (CH, C-1), 96.1 (CH, C-1), 78.0 (CH), 75.7 (CH), 73.6 (CH), 71.5 (CH), 71.3 (2 × CH), 70.8 (CH), 70.7 (CH), 70.2 (CH$_2$), 68.7 (CH), 68.5 (CH), 67.0 (CH), 66.9 (2 × CH$_2$), 66.7 (CH$_2$), 66.6 (CH), 28.0 (CH$_3$), 27.5 (CH$_3$, tBu), 27.3 (CH$_3$, tBu), 27.2 (CH$_3$, tBu), 26.1
(CH₃), 23.3 (C), 23.2 (C), 20.8 (C), 20.7 (C) ppm. ESI-TOF HRMS m/z calcd for C₁₅₈H₁₇₂NaO₂₂Si₂ [M+Na]⁺: 1303.5305, found: 1303.5257.

4.4.2.1.2. **Allyl 2,3-di-O-benzoyl-α-D-galactosyl-(1→2)-[2,3-di-O-benzoyl-α-D-galactosyl-(1→6)]-3,4-O-isopropylidene-β-D-galactoside (38)**

The fully protected trisaccharide 37 (120 mg, 0.09 mmol) was dissolved in 12 mL of anhydrous THF in a plastic conical tube and cooled 0 °C. Then, 120 μL of HF-Pyr (70%) was added and stirred for 30 min. at 0 °C and 1 h at rt under Ar. The reaction mixture was cooled again to 0 °C and quenched with saturated a NaHCO₃ solution. Finally, the mixture was extracted with EtOAc, washed with water and brine, dried over MgSO₄, concentrated, and purified by flash column chromatography on silica gel (EtOAc:hexanes = 4:1) to furnish the desilylated trisaccharide 38 (85 mg, 90%) as a white powder. [α]D +162.7 (c = 0.14 in CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃, 300K) δ 8.03-7.93 (m, 8H, arom.), 7.55-7.43 (m, 4H, arom.), 7.41-7.29 (m, 8H, arom.), 5.77-5.60 (m, 5H), 5.48 (m, 1H, OCH₂CH=CH₂), 5.36 (s, 1H), 4.96 (dd, dJ = 17.2, 1.3 Hz, 1H), 4.88 (d, dJ = 10.4 Hz, 1H), 4.45 (d, dJ = 11.6 Hz, 2H), 4.31 (t, dJ = 3.6 Hz, 1H), 4.17 (d, dJ = 8.4 Hz, 1H), 4.15-4.04 (m, 3H), 4.01-3.89 (m, 6H), 3.89-3.83 (m, 1H), 3.74 (dd, dJ = 9.9, 5.8 Hz, 1H), 3.64-3.50 (m, 2H), 3.40-3.26 (m, 2H, -OH), 2.86-2.76 (m, 1H, -OH), 1.45 (s, 3H, CCH₃), 1.06 (s, 3H, CCH₃), 0.91-0.79 (m, 1H, -OH) ppm. ¹³C NMR (101 MHz, CDCl₃, 300K) δ 166.1 (C=O), 166.0, (2 × C=O), 165.8 (C=O), 133.5 (CH, arom.), 133.4 (CH, arom.), 133.3 (CH, arom., OCH₂CH=CH₂), 129.9 (CH, arom.), 129.8 (CH, arom.), 129.7 (CH, arom.), 129.6 (CH, arom.), 129.5 (CH, arom.), 129.4 (CH, arom.), 128.6 (CH, arom.), 128.5 (CH, arom.), 117.8 (CH₂), 110.3 (C), 101.3 (CH, C=1), 96.8 (CH, C=1), 96.7 (CH, C=1), 78.1 (CH), 77.4 (CH), 73.5 (CH), 71.4 (CH), 71.0 (CH), 70.9 (CH), 70.4 (CH), 70.2 (CH₂), 69.8 (CH), 69.3 (CH), 69.0 (2 × CH), 68.6 (CH), 66.6 (CH₂), 63.8 (CH₂), 63.2 (CH₂), 28.1 (CH₃), 26.1 (CH₃) ppm. ESI-TOF HRMS m/z calcd for C₅₂H₅₆NaO₂₀ [M+Na]⁺: 1023.3263, found: 1023.3252.

4.4.2.1.3. **Allyl 2,3-di-O-benzoyl-α-D-galactosyl-(1→2)-[2,3-di-O-benzoyl-α-D-galactosyl-(1→6)]-β-D-galactoside (39)**

To a solution of trisaccharide 38 (260 mg, 0.26 mmol) in DCM (8 mL), H₂O (1.0 mL) and TFA (1.0 mL) were consecutively added, and the mixture was vigorously stirred at rt for
30 min. After disappearance of starting material based on TLC, the resulting solution was twice co-evaporated with EtOH (10 mL). The residue was then further dried under vacuum and purified by flash column chromatography on silica gel (DCM/MeOH = 9:1) to afford the partially deprotected 39 (240 mg, 96%) as a colorless syrup. $\alpha_0^{26}$ +215.6 (c = 0.05 in CH$_2$Cl$_2$). $^1$H NMR (400 MHz, MeOD, 300K) $\delta$ 8.02-7.86 (m, 8H, arom.), 7.55-7.46 (m, 4H, arom.), 7.45-7.29 (m, 8H, arom.), 5.72-5.61 (m, 5H), 5.55 (m, 1H, OCH$_2$CH=CH$_2$), 5.31 (d, $J$ = 3.2 Hz, 1H), 4.94 (dd, $J$ = 17.4, 1.5 Hz, 1H), 4.86-4.79 (m, 1H), 4.61 (t, $J$ = 6.0 Hz, 1H), 4.37-4.27 (m, 3H), 4.10 (t, $J$ = 6.0 Hz, 1H), 4.02-3.91 (m, 2H), 3.85-3.69 (m, 9H), 3.52 (dd, $J$ = 12.5, 5.9 Hz, 1H) ppm. $^1$C NMR (101 MHz, MeOD, 300K) $\delta$ 166.1 (C=O), 166.0 (2 ´ C=O), 165.8 (C=O), 133.7 (CH, arom.), 133.2 (CH, arom.), 133.1 (CH, arom.), 133.0 (CH, arom., OCH$_2$CH=CH$_2$), 129.7 (C, arom.), 129.6 (C, arom.), 129.5 (C, arom.), 129.4 (CH, arom.), 129.3 (CH, arom.), 129.2 (CH, arom.), 128.2 (CH, arom.), 128.1 (CH, arom.), 116.1 (CH$_2$), 102.6 (CH, C-1), 96.2 (CH, C-1), 96.1 (CH, C-1), 75.6 (CH), 72.8 (CH), 72.2 (CH), 71.3 (2 ´ CH), 71.0 (CH), 70.32 (CH), 69.7 (CH$_2$), 69.5 (CH), 69.2 (CH), 69.0 (CH), 67.7 (CH), 67.5 (CH), 66.4 (CH$_2$), 61.1 (CH$_2$), 61.0 (CH$_2$) ppm. ESI-TOF HRMS m/z calcd for C$_{49}$H$_{52}$NaO$_{20}$ [M+Na]$^+$: 983.2950, found: 983.2975.

4.4.2.1.4. 3-(Acetylthio)propyl 2,3-di-O-benzoyl-$\alpha$-D-galactosyl-(1$\rightarrow$2)-[2,3-di-O-benzoyl-$\alpha$-D-galactosyl-(1$\rightarrow$6)]-$\beta$-D-galactoside (40)

To a solution of allyl trisaccharide 39 (230 mg, 0.24 mmol) and AIBN (20 mg, 0.12 mmol) in anhydrous THF (3 mL) under Ar, thioacetic acid (43 µL, 0.60 mmol) was added, and the mixture was stirred under water cooling (~ 25 ºC) for 6 h in a Rayonet UV reactor equipped with 350 nm lamps. The solution was then co-evaporated with toluene and concentrated to near dryness. The crude product was purified by flash column chromatography on silica gel (CHCl$_3$/MeOH = 14:1) to afford the acyl-protected trisaccharide 40 (188 mg, 76%) as a white solid. $\alpha_0^{26}$ +100.1 (c = 0.07 in MeOH). $^1$H NMR (400 MHz, MeOD, 300K) $\delta$ 8.02-7.85 (m, 8H, arom.), 7.62-7.29 (m, 12H, arom.), 5.72-5.57 (m, 5H), 5.49 (s, 1H), 5.31 (d, $J$ = 2.4 Hz, 1H), 4.65-4.58 (m, 1H), 4.37-4.28 (m, 2H), 4.23 (d, $J$ = 7.0 Hz, 1H), 4.13-3.90 (m, 3H), 3.86-3.64 (m, 9H), 3.59-3.50 (m, 1H), 2.95-2.83 (m, 1H), 2.72-2.55 (m, 2H), 2.23 (s, 3H) ppm. $^1$C NMR (101 MHz, MeOD, 300K) $\delta$ 195.9 (C=O), 166.1 (C=O), 166.0 (2 ´ C=O), 165.8 (C=O), 133.2 (CH, arom.), 133.1 (CH, arom.), 133.0 (CH, arom., OCH$_2$CH=CH$_2$), 129.7 (C, arom.), 129.6 (C, arom.), 129.5 (C, arom.), 129.4 (CH, arom.), 129.3 (CH, arom.), 129.2 (CH, arom.), 128.2 (CH, arom.), 128.1 (CH, arom.), 116.1 (CH$_2$), 102.6 (CH, C-1), 96.2 (CH, C-1), 96.1 (CH, C-1), 75.6 (CH), 72.8 (CH), 72.2 (CH), 71.3 (2 ´ CH), 71.0 (CH), 70.32 (CH), 69.7 (CH$_2$), 69.5 (CH), 69.2 (CH), 69.0 (CH), 67.7 (CH), 67.5 (CH), 66.4 (CH$_2$), 61.1 (CH$_2$), 61.0 (CH$_2$) ppm.
(CH, arom.), 129.7 (C, arom.), 129.6 (C, arom.), 129.5 (C, arom.), 129.3 (CH, arom.), 129.2 (CH, arom.), 128.3 (CH, arom.), 128.2 (CH, arom.), 128.1 (CH, arom.), 103.5 (CH, C-1), 96.1 (CH, C-1), 96.0 (CH, C-1), 75.6 (CH), 72.8 (CH), 72.1 (CH), 71.3 (2 × CH), 71.0 (CH), 70.2 (CH), 69.6 (CH), 69.3 (CH), 69.1 (CH), 67.7 (CH), 67.7 (CH2), 67.6 (CH), 66.3 (CH2), 61.0 (2 × CH2), 29.3 (CH2), 25.3 (CH2) ppm. ESI-TOF HRMS m/z calcd for C_{51}H_{56}NaO_{23}S [M+Na]^+: 1059.2932, found: 1059.2925.

4.4.2.1.5. 3-Thiopropyl α-D-galactosyl-(1→2)-[α-D-galactosyl-(1→6)]-β-D-galactoside
[\text{G11}_{\text{SH}}]

The acyl-protected trisaccharide 40 (188 mg, 0.18 mmol) was dissolved in 20 mL of anhydrous 0.25 M NaOMe, and stirred for 2 hours under Ar. The solution was then neutralized with Amberlyst-15, filtered through Celite, concentrated and finally dissolved in water and lyophilized. Initially, the unprotected mercaptopropyl trisaccharide 7 is produced, which oxidizes by handling on air within hours to the disulfide G11_{SH} (118 mg, quant.) as an off-white solid. Rf 0.25 (iPrOH/H₂O = 5:1 w/ 3 drops AcOH). [α]_D^{26} 61.6 (c = 0.03 in H₂O). ¹H NMR (400 MHz, D₂O, 300 K) δ 5.34 (d, 2H, J = 3.8 Hz, H-1), 4.93 (d, 2H, J = 2.7 Hz, H-1), 4.50 (d, 2H, J = 7.9 Hz, H-1), 4.14-4.29 (m, 2H), 3.53-4.06 (m, 42H), 3.31 (s, 4H), 2.71-2.88 (m, 4H), 1.95-2.11 (m, 4H), 1.87 (s, 12H) ppm. ¹³C NMR (101 MHz, D₂O, 300K) δ 103.3 (C-1), 98.1 (C-1), 97.9 (C-1), 73.0 (CH), 71.5 (CH), 70.9 (CH), 70.6 (CH), 69.5 (CH), 69.3 (CH), 69.2 (2 × CH), 69.1 (CH), 68.7 (CH₂), 68.3 (CH), 68.2 (CH), 66.3 (CH₂), 61.2 (CH₂), 60.9 (CH₂), 34.3 (CH₂), 28.4 (CH₂), 23.3 (CH) ppm. ESI-TOF HRMS m/z calcd for C_{42}H_{74}NaO_{32}S_{2} [M+Na]^+: 1177.3502, found: 1177.3310

4.4.3. Conjugation of the glycan to maleimide-derivatized bovine serum albumin (BSA)

The kit for conjugating the thiol-containing glycan 7 to BSA (Imject™ Maleimide-Activated BSA, 77116 cat. number) was purchased from Thermo Fisher Scientific, and the conjugation procedure followed was similar to the ones described by the manufacturer and previously published.¹¹⁶ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 0.8 mg, 2.8 μmol) was dissolved in 250 μL of conjugation buffer provided in the kit (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M sodium chloride, 0.02% sodium azide, pH 7.2). The
TCEP·HCl solution was added to a 1.5 mL microcentrifuge tube containing sugar-disulfide 8 (2.7 mg, 2.4 μmol). The mixture was agitated in a shaker for 30 min to furnish sugar-thiol 7. An aliquot of 10 μL (0.11 mg of 7) was set aside for the colorimetric determination of the thiol concentration. The maleimide-activated BSA (2 mg, 15-25 moles of maleimide/mole BSA) was reconstituted with 200 μL of ultrapure water to produce a 10 mg/mL solution. The trisaccharide solution was added to the reconstituted BSA and incubated at rt for 2-3 h in a shaker. After that time, 18.3 μL (corresponding to the same quantity of 7 if no conjugation had occurred) were removed from the mixture to determine the concentration of unreacted thiol. This aliquot was diluted to 2.75 mL with reaction buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA), combined with 50 μL of Ellman’s reagent [5,5’dithiobis-(2-nitrobenzoic acid) = DTNB] solution (4 mg DTNB in 1 mL of reaction buffer), and reacted for 15 min at rt. With a UV Vis spectrophotometer, the absorbance at 412 nm was measured. The thiol concentration was determined using the molar extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB, $\varepsilon = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$), and the amount of sugar conjugated, typically 2.0 μmol, was calculated.

The conjugation mixture was diluted with ultrapure water to a volume of 1 mL and desalted using an Amicon Ultra 3K centrifugal filter and was centrifuged for 20 min at 4,000 × g, rt. The mixture was washed with 1 mL of ultrapure water three times following the same procedure. The tube with the filtrate was removed, and 500 μL of ultrapure water was added to the NGP11b solution remaining in the filter. Since a small amount of aggregation can occur, the solution/suspension was transferred onto a 2 mL ZebaTM spin desalting column (7K MWCO) provided in the kit that was previously washed with 1 mL of ultrapure pure water four times by centrifuged at 1,000 × g for 2 min, rt. This procedure removed all salts and aggregated protein. The filtrate was lyophilized and can be stored at -50 ºC for at least six months. In our hands, this combination of filtration and size exclusion chromatography avoids or minimizes aggregation of the NGP. To determine the NGP11b quantity, a solution of 1-2 mg in 1-3 mL of ultrapure water was prepared. The concentration was determined with a Pierce BCA Protein Assay Reagent kit using a spectrophotometer at a detection wavelength of 562 nm.
4.4.4. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

To determine the mass of BSA and NGP11b, 1 µL of BSA (125 µg/mL) was combined into a 1.5 mL microcentrifuge tube with 1 µL NGP11b (670 µg/mL) and 2 µL of matrix (10 mg/mL sinapinic acid, 50% acetonitrile, 0.1% TFA). Two microliters of the combined sample: matrix were spotted onto a 48-well steel MALDI plate and allowed to crystallize at room temperature for approximately 20 minutes. The mass spectra were acquired using a SHIMADZU MALDI-8020 mass spectrometer set to linear mode with dithering at a scan range of 10,000 to 100,000 m/z. Data acquisition included a laser power of 110, laser rep. Rate (Hz) 50, accumulated shots 5, blast shots 2, profiles at 200, pulse extraction set to 66431, and a blanking mass of 15000. Spectra were processed by Threshold Apex set at constant Threshold, Gaussian smoothing, smoothing filter width 200, and peak width 2. BSA standard was used for calibration and internal references set at [BSA+H]^+ = 66431 and [BSA+2H]^2+ = 33216 with a 5ppm mass tolerance.

4.4.5. CL-ELISA of tGPI-MUC and NGP11b

tGPI-MUC were extracted and purified as described by the Almeida group. CL-ELISA was performed as described elsewhere to assess the specificity of IgG from patients infected with CD against NGP11b compared to purified tGPI-MUC. The antigens were immobilized to Nunc MaxiSorp polystyrene microplates for 16 h at 4 °C in Carbonate-Bicarbonate Buffer (CBB), 200 mM, pH 9.6. After antigen loading, the plates were blocked with 200 µL per well Phosphate-Buffered Saline (PBS) containing 1% BSA (PBS-B) and incubated for 1 h at 37 °C. The plates were then washed three times with 200 µL of PBS containing 0.05% Tween 20 detergent (PBS-T). Pools of human sera or individual patient sera were analyzed in triplicate at a dilution of 1:200-1:1600 and 1:800, respectively, in PBS-B and 0.05% PBS-TB. The plates were incubated for 1 h at 37 °C, followed by washing three times with 200 µL of PBS-T. 50 µL of Biotinylated goat Anti-human IgG antibodies, diluted at 1:10,000 in PBS-TB, was added to each well and incubated for 1 h at 37 °C. After washing three times with 200 µL of PBS-T, 50 µL of High sensitivity Neutravidin-Horseradish Peroxidase (NA-HRP) diluted in PBS-TB was added at 1:5,000 dilution. The plates were
incubated again for 1 h at 37 °C. After a final round of washing with PBS-T, the plates were developed with 50 µL of the substrate Super Signal ELISA Pico Stable Peroxide Solution and Super Signal ELISA Pico Luminol Enhancer and CBB/0.1% BSA in a 1:1:8 ratio (v/v/v), and relative luminescent units (RLU) were measured using a Luminoskan Accent Luminometer. The titers were calculated by dividing the average of patient RLU values by the cutoff of each plate, which was determined by utilizing the standard deviation multiplier at 99.5%. The Confidence Interval (CI) for nine true negative sera was calculated as described by Frey et al. (CI 99.5% 9 controls = 3.537)\(^3\).

### 4.5. Additional information

**Vaccination groups (n= 8 mice per group (40 total):**

1. **KM11h (20ug/dose/mouse) – 100ul – (4 doses)**
2. **KM11b (20ug/dose/mouse) – 100ul – (4 doses)**
3. **Me-HSA (20ug/dose/mouse) – 100ul – (4 doses)**
4. **Me-BSA (20ug/dose/mouse) – 100ul – (4 doses)**
5. **PBS – 100ul – (4 doses)**

![Immunizations Diagram](image)

**Prophylactic immunization of GalαT-KO mice with Galα3LN (KM11h and KM11b)**
Matrix-Assisted Laser Desorption Ionization-Time Of Flight mass spectra of NGP11h

Survival Rate
IgG levels by CL-ELISA after vaccination
5. References


(2) World Health Organization. *Chagas Disease in Latin America: An Epidemiological Update Based on the 2010 Estimates*; Wkly Epidemiol Rec; 6; 2015; pp 33–44.


(58) Montoya, A. L. Discovery Of Glycan-Based Biomarkers For Cutaneous Leishmaniasis And Chagas Disease By Reversed Immunoglycomics. 197.


6. Appendix

$^1$H NMR, 400 MHz, CDCl$_3$, compound (14)

$^1$H NMR, 400 MHz, CDCl$_3$, compound (15)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (15)

COSY NMR, 400 MHz, CDCl$_3$, compound (15)
HSQC NMR, 400 MHz, CDCl$_3$, compound (15)

ESI-TOF HR mass spectrum of compound (15)

$\text{C}_{24}\text{H}_{26}\text{O}_5\text{S} = 426.1501$

Calculated [M+Na$^+$] $\text{m/z} = 449.1399$

Experimental [M+Na$^{1+}$] $\text{m/z} = 449.1331$
ESI-TOF HR mass spectrum of compound (15) with isotopic simulation

\[
\text{C}_{28}\text{H}_{39}\text{O}_{3}\text{S} = 426.1501
\]
Calculated [M+Na\textsuperscript{+}] m/z = 449.1399
Experimental [M+Na\textsuperscript{+}] m/z = 449.1331

\[
^1\text{H NMR, 400 MHz, CDCl}_3, \text{ compound (16)}
\]
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (16)

COSY NMR, 400 MHz, CDCl$_3$, compound (16)
HSQC NMR, 400 MHz, CDCl3, compound (16)

ESI-TOF HR mass spectrum of compound (16)

Calculated [M+Na]+ m/z = 761.2185
Experimental [M+Na]+ m/z = 761.2122
ESI-TOF HR mass spectrum of compound (16) with isotopic simulation

16
C_{45}H_{38}O_{8}S = 738.2287
Calculated [M+Na^+] m/z = 761.2185
Experimental [M+Na^+] m/z = 761.2122

^1^H NMR, 400 MHz, CDCl3, compound (17)
ESI-TOF HR mass spectrum of compound (17)

C_{34}H_{30}O_{8}S = 598.1661
Calculated [M+Na\textsuperscript{+}] m/z = 621.1559
Experimental [M+Na\textsuperscript{+}] m/z = 621.1524

ESI-TOF HR mass spectrum of compound (17) with isotopic simulation

C_{34}H_{30}O_{8}S = 598.1661
Calculated [M+Na\textsuperscript{+}] m/z = 621.1559
Experimental [M+Na\textsuperscript{+}] m/z = 621.1524
$^1$H NMR, 400 MHz, CDCl$_3$, compound (4)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (4)
COSY NMR, 400 MHz, CDCl₃, compound (4)

HSQC NMR, 400 MHz, CDCl₃, compound (4)
ESI-TOF HR mass spectrum of compound (4)

C_{41}H_{34}O_{9}S = 598.1661
Calculated [M+Na]: m/z = 725.1821
Experimental [M+Na]: m/z = 725.1777

ESI-TOF HR mass spectrum of compound (4) with isotopic simulation
$^1$H NMR, 400 MHz, CDCl$_3$, compound (20)

ESI-TOF HR mass spectrum of compound (20)

C$_{17}$H$_{20}$O$_6$ = 261.12
Calculated [M+Na]$^+$: m/z = 284.1110
Experimental [M+Na]$^+$: m/z = 284.1107
$^1$H NMR, 400 MHz, CDCl$_3$, compound (21)

ESI-TOF HR mass spectrum of compound (21)
ESI-TOF HR mass spectrum of compound (21) with isotopic simulation

C_{10}H_{20}NO_6 = 349.1525
Calculated [M+Na]^+: m/z = 372.1423
Experimental [M+Na]^+: m/z = 372.1031

^1H NMR, 400 MHz, CDCl_3, compound (22)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (22)

COSY NMR, 400 MHz, CDCl$_3$, compound (22)
HSQC NMR, 400 MHz, CDCl₃, compound (22)

ESI-TOF HR mass spectrum of compound (22)

C₂₀H₂₅NO₇ = 391.1631
Calculated [M+Na]⁺: m/z = 414.1529
Experimental [M+Na]⁺: m/z = 414.1474
ESI-TOF HR mass spectrum of compound (22) with isotopic simulation

\[
\text{Ph} \quad \text{O} \quad \text{O} \\
\text{AcO} \quad \text{AcHN} \quad \text{OAl} \\
\text{22}
\]

\[
\text{C}_{20}\text{H}_{25}\text{NO}_{7} = 391.1631
\]

Calculated [M+Na]^+: m/z = 414.1529
Experimental [M+Na]^+: m/z = 414.1474

\[\text{^1H NMR, 400 MHz, CDCl}_3, \text{compound (23)}\]
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (23)

COSY NMR, 400 MHz, CDCl$_3$, compound (23)
HSQC NMR, 400 MHz, CDCl3, compound (23)

ESI-TOF HR mass spectrum of compound (23)

C_{13}H_{21}NO_7 = 303.1318
Calculated [M+Na]^+: m/z = 326.1216
Experimental [M+Na]^+: m/z = 326.1147
ESI-TOF HR mass spectrum of compound (23) with isotopic simulation

$C_{13}H_{21}NO_7 = 303.1318$

Calculated [M+Na]$^+ \text{: m/z } = 326.1216$

Experimental [M+Na]$^+ \text{: m/z } = 326.1147$

$^1$H NMR, 400 MHz, CDCl$_3$, compound (2)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (2)

COSY NMR, 400 MHz, CDCl$_3$, compound (2)
HSQC NMR, 400 MHz, CDCl3, compound (2)

ESI-TOF HR mass spectrum of compound (2)

C_{12}H_{16}NO_{3}Si = 417.2183
Calculated [M+Na]^+: m/z = 440.2080
Experimental [M+Na]^+: m/z = 440.2014
ESI-TOF HR mass spectrum of compound (2) with isotopic simulation

\[ \text{C}_{19} \text{H}_{35} \text{NO}_7 \text{Si} = 417.2183 \]

Calculated [M+Na]^+: m/z = 440.2080
Experimental [M+Na]^+: m/z = 440.2014

\[ ^1\text{H NMR, 400 MHz, CDCl}_3, \text{compound (1)} \]
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (1)

$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (1)
COSY NMR, 400 MHz, CDCl$_3$, compound (1)

HSQC NMR, 400 MHz, CDCl$_3$, compound (1)
ESI-TOF HR mass spectrum of compound (1)

C_{62}H_{64}O_{15}Si = 1108.3735
Calculated [M+Na]^+: m/z = 1131.3633
Experimental [M+Na]^+: m/z = 1131.3623

ESI-TOF HR mass spectrum of compound (1) with isotopic simulation
$^{1}H$ NMR, 400 MHz, CDCl$_3$, compound (25)

$^{1}H$ NMR, 400 MHz, Acetone-d$_6$, compound (25)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (25)

COSY NMR, 400 MHz, CDCl$_3$, compound (25)
HSQC NMR, 400 MHz, CDCl₃, compound (25)

ESI-TOF HR mass spectrum of compound (25)

C₉₆H₇₅NO₂₂Si₂ = 1401.5571
Calculated [M+Na]⁺: m/z = 1424.5469
Experimental [M+Na]⁺: m/z = 1424.5440
ESI-TOF HR mass spectrum of compound (25) with isotopic simulation

C_{25}H_{25}NO_{25}Si = 1401.5571
Calculated [M+Na]^+: m/z = 1424.5469
Experimental [M+Na]^+: m/z = 1424.5440

^1H NMR, 400 MHz, CDCl$_3$, compound (3)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (3)

COSY NMR, 400 MHz, CDCl$_3$, compound (3)
HSQC NMR, 400 MHz, CDCl₃, compound (3)

ESI-TOF HR mass spectrum of compound (3)

C₆₈H₇₇NO₂₂Si = 1287.4706
Calculated [M+Na]⁺: m/z = 1310.4604
Experimental [M+Na]⁺: m/z = 1310.4569
ESI-TOF HR mass spectrum of compound (3) with isotopic simulation

$C_{68}H_{64}NO_{28}Si = 1287.4706$
Calculated [M+Na]$^+$: m/z = 1310.4604
Experimental [M+Na]$^+$: m/z = 1310.4569

$^1$H NMR, 400 MHz, CDCl$_3$, compound (24)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (24)

$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (24)
COSY NMR, 400 MHz, CDCl$_3$, compound (24)

HSQC NMR, 400 MHz, CDCl$_3$, compound (24)
ESI-TOF HR mass spectrum of compound (24)

C_{53}H_{61}NO_{16}Si = 995.3760
Calculated [M+Na]⁺: m/z = 1018.3657
Experimental [M+Na]⁺: m/z = 1018.3382

ESI-TOF HR mass spectrum of compound (24) with isotopic simulation

C_{53}H_{61}NO_{16}Si = 995.3760
Calculated [M+Na]⁺: m/z = 1018.3657
Experimental [M+Na]⁺: m/z = 1018.3382
$^1$H NMR, 400 MHz, CDCl$_3$, compound (7)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (7)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (7)

COSY NMR, 400 MHz, CDCl$_3$, compound (7)
HSQC NMR, 400 MHz, CDCl₃, compound (7)

ESI-TOF HR mass spectrum of compound (7)

**Chemical Structure**

**Calculated** 
\[
\text{[M+Na]⁺: m/z = 904.2793}
\]

**Experimental** 
\[
\text{[M+Na]⁺: m/z = 904.2744}
\]
ESI-TOF HR mass spectrum of compound (7) with isotopic simulation

\[
\text{Calculation: } [M+Na]^+: m/z = 904.2793
\]

Experimental [M+Na]^+: m/z = 904.2744

\[\text{C}_{47}\text{H}_{47}\text{NO}_{16}=881.2895\]

\[\text{1H NMR, 400 MHz, CDCl}_3, \text{ compound (5)}\]

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$^1$H NMR, 400 MHz, DMSO-$d_6$, compound (5)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (5)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (5)

COSY NMR, 400 MHz, CDCl$_3$, compound (5)
HSQC NMR, 400 MHz, CDCl₃, compound (5)

ESI-TOF HR mass spectrum of compound (5)
ESI-TOF HR mass spectrum of compound (5) with isotopic simulation

C$_{100}$H$_{103}$NO$_8$Si = 1885.6283
Calculated [M+2Na]$^{2+}$: m/z = 955.8039
Experimental [M+2Na]$^{2+}$: m/z = 955.8024

$^1$H NMR, 400 MHz, CDCl$_3$, compound (26)
\(^{13}\text{C} \text{NMR, 100 MHz, } \text{CDCl}_3, \text{ compound (26)}\)

\[^{13}\text{C} \text{DEPT-135 NMR, 100 MHz, } \text{CDCl}_3, \text{ compound (26)}\]
COSY NMR, 400 MHz, CDCl₃, compound (26)

HSQC NMR, 400 MHz, CDCl₃, compound (26)
ESI-TOF HR mass spectrum of compound (26)

\[ C_{94}H_{87}NO_{31} = 1865.6283 \]
Calculated \([\text{M+2Na}]^{2+}: m/z = 885.7529 \]
Experimental \([\text{M+2Na}]^{2+}: m/z = 885.7512 \]

ESI-TOF HR mass spectrum of compound (26) with isotopic simulation

\[ C_{94}H_{87}NO_{31} = 1865.6283 \]
Calculated \([\text{M+2Na}]^{2+}: m/z = 885.7529 \]
Experimental \([\text{M+2Na}]^{2+}: m/z = 885.7512 \]
$^1$H NMR, 400 MHz, CDCl$_3$, compound (27)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (27)
$^{13}\text{C DEPT-135 NMR, 100 MHz, CDCl}_3$, compound (27)

$\text{COSY NMR, 400 MHz, CDCl}_3$, compound (27)
HSQC NMR, 400 MHz, CDCl₃, compound (27)

ESI-TOF HR mass spectrum of compound (27)

C₉₆H₉₁NO₃S = 1801.5245
Calculated [M+2Na]⁺: 923.7520
Experimental [M+2Na]⁺: 923.7485
ESI-TOF HR mass spectrum of compound (27) with isotopic simulation

$C_{96}H_{91}NO_{32}S = 1801.5245$
Calculated $[M+2Na]^{2+}: 923.7520$
Experimental $[M+2Na]^{2+}: 923.7485$

$^1H$ NMR, 400 MHz, CDCl$_3$, compound (G36)$_s$
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (G36)$_s$

$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (G36)$_s$
COSY NMR, 400 MHz, CDCl₃, compound (G36)ₙ

HSQC NMR, 400 MHz, CDCl₃, compound (G36)ₙ
ESI-TOF HR mass spectrum of compound (G36\textsubscript{SH})

![ESI-TOF HR mass spectrum of compound (G36\textsubscript{SH})](image)

ESI-TOF HR mass spectrum of compound (G36\textsubscript{SH}) with isotopic simulation

![ESI-TOF HR mass spectrum of compound (G36\textsubscript{SH}) with isotopic simulation](image)

C\textsubscript{29}H\textsubscript{51}NO\textsubscript{21}S = 781.2674
Calculated [M+Na]\textsuperscript{+}: m/z = 804.2572
Calculated [M+Na]\textsuperscript{2+}: m/z = 804.2514
$^1$H NMR, 400 MHz, CDCl$_3$, compound (6)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (6)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (6)

COSY NMR, 400 MHz, CDCl$_3$, compound (6)
HSQC NMR, 400 MHz, CDCl₃, compound (6)

ESI-TOF HR mass spectrum of compound (6)

ESI-TOF HR mass spectrum of compound (6) with isotopic simulation

Calculated [M+2Na]²⁺: m/z = 1158.8945
Experimental [M+2Na]²⁺: m/z = 1158.8906

C₁₇₂H₁₃₂NO₃₂Si₂ = 2271.8095

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\[ C_{123}H_{129}NO_{37}Si_2 = 2271.8095 \]

Calculated [M+2Na]\(^{2+}\): m/z = 1158.8945

Experimental [M+2Na]\(^{2+}\): m/z = 1158.8906

\[ \text{H NMR, 400 MHz, CDCl}_3, \text{compound (35)} \]
ESI-TOF HR mass spectrum of compound (35)

C_{17}H_{24}NO_2Si = 359.5102
Calculated [M+2Na]^2+: m/z = 1383.4999
Calculated [M+2Na]^3+: m/z = 1382.2250

^1H NMR, 400 MHz, CDCl₃, compound (28)
$^1$H NMR, 400 MHz, Acetonitrile-d$_3$, compound (28)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (28)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (28)

COSY NMR, 400 MHz, CDCl$_3$, compound (28)
HSQC NMR, 400 MHz, CDCl₃, compound (28)

ESI-TOF HR mass spectrum of compound (28)

C₉₀H₁₀₂NO₅ = 1991.6052
Calculated [M+2Na]²⁺: m/z = 1018.7924
Experimental [M+2Na]²⁺: m/z = 1018.7913
ESI-TOF HR mass spectrum of compound (28) with isotopic simulation

C_{10}H_{16}NO_{2} = 1991.6052
Calculated [M+2Na]^{2+}: m/z = 1018.7924
Experimental [M+2Na]^{2+}: m/z = 1018.7913

1H NMR, 400 MHz, Acetonitrile-d₃, compound (29)
$^{13}$C NMR, 100 MHz, Acetonitrile-d$_3$, compound (29)

$^{13}$C DEPT-135 NMR, 100 MHz, Acetonitrile-d$_3$, compound (29)
COSY NMR, 400 MHz, Acetonitrile-d$_3$, compound (29)

HSQC NMR, 400 MHz, Acetonitrile-d$_3$, compound (29)
ESI-TOF HR mass spectrum of compound (29)

C_{109}H_{105}NO_{38}S = 2067.6035
Calculated [M+2Na]^{2+}: m/z = 1056.7915
Experimental [M+2Na]^{2+}: m/z = 1056.7884

ESI-TOF HR mass spectrum of compound (29) with isotopic simulation

C_{109}H_{105}NO_{38}S = 2067.6035
Calculated [M+2Na]^{2+}: m/z = 1056.7915
Experimental [M+2Na]^{2+}: m/z = 1056.7884
ESI-TOF HR mass spectrum of compound (G35$_{SH}$)

$C_{35}H_{61}NO_{26}S = 947.3203$

Calculated [M+Na]$^+$: m/z = 966.3100
Experimental [M+Na]$^+$: m/z = 966.3119

ESI-TOF HR mass spectrum of compound (G35$_{SH}$) with isotopic simulation

$C_{35}H_{61}NO_{26}S = 947.3203$

Calculated [M+Na]$^+$: m/z = 966.3100
Experimental [M+Na]$^+$: m/z = 966.3119
\(^1\)H NMR, 400 MHz, CDCl\(_3\), compound (8)

\(^{13}\)C NMR, 100 MHz, CDCl\(_3\), compound (8)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (8)

COSY NMR, 400 MHz, CDCl$_3$, compound (8)
HSQC NMR, 400 MHz, CDCl₃, compound (8)

ESI-TOF HR mass spectrum of compound (8)

C₁₀₂H₁₀₃NO₃Si = 1865.6283
Calculated [M+2Na]⁺ : m/z = 955.8039
Experimental [M+2Na]⁺ : m/z = 955.8065
ESI-TOF HR mass spectrum of compound (8) with isotopic simulation

C_{100}H_{120}N_{20}O_{23}Si = 1865.6283
Calculated [M+2Na]^2+: m/z = 955.8039
Experimental [M+2Na]^2+: m/z = 955.8065

^1H NMR, 400 MHz, CDCl_3, compound (30)
$^{1}H$ NMR, 400 MHz, MeOD, compound (30)

$^{13}C$ NMR, 100 MHz, CDCl$_3$, compound (30)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (30)

COSY NMR, 400 MHz, CDCl$_3$, compound (30)
HSQC NMR, 400 MHz, CDCl$_3$, compound (30)

ESI-TOF HR mass spectrum of compound (30)

Calculated [M+2Na]$^{2+}$: m/z = 885.7529
Experimental [M+2Na]$^{2+}$: m/z = 885.7509
ESI-TOF HR mass spectrum of compound (30) with isotopic simulation

\[
\text{C}_{30}\text{H}_{43}\text{NO}_{13} = 1865.6283
\]
Calculated [M+2Na]^{2+}: m/z = 885.7529
Experimental [M+2Na]^{2+}: m/z = 885.7509

\(^1\)H NMR, 400 MHz, CDCl\(_3\), compound (31)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (31)

COSY NMR, 400 MHz, CDCl$_3$, compound (31)

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HSQC NMR, 400 MHz, CDCl$_3$, compound (31)

![HSQC NMR spectrum of compound (31)](image)

ESI-TOF HR mass spectrum of compound (31)

![ESI-TOF HR mass spectrum of compound (31)](image)

Calculated [M+2Na]$^{2+}$: 923.7520
Experimental [M+2Na]$^{2+}$: 923.7497

$C_{65}H_{21}NO_{15}S = 1801.5245$
ESI-TOF HR mass spectrum of compound (31) with isotopic simulation

C_{26}H_{20}NO_7S = 1801.5245
Calculated [M+2Na]^2+: 923.7520
Experimental [M+2Na]^2+: 923.7497

^1H NMR, 400 MHz, CDCl_3, compound (G37)s
COSY NMR, 400 MHz, CDCl₃, compound (G37)ₜ

ESI-TOF HR mass spectrum of compound (G37)ₜ
ESI-TOF HR mass spectrum of compound (G37)₅ with isotopic simulation

C₃₀H₃₈NO₄S = 781.2674
Calculated [M+2Na]⁺: m/z = 803.2494
Calculated [M+2Na]⁺: m/z = 803.2484

¹H NMR, 400 MHz, CDCl₃, compound (32)
$^1$H NMR, 400 MHz, Acetonitrile-$d_3$, compound (32)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (32)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (32)

COSY NMR, 400 MHz, CDCl$_3$, compound (32)
HSQC NMR, 400 MHz, CDCl$_3$, compound (32)

[Graph of HSQC NMR spectrum]

ESI-TOF HR mass spectrum of compound (32)

[Graph of ESI-TOF HR mass spectrum]

Calculated [M+Na]$^+$: m/z = 1170.3583
Experimental [M+Na]$^+$: m/z = 1170.3562
ESI-TOF HR mass spectrum of compound (32) with isotopic simulation

C_{60}H_{22}NO_{33} = 1147.3685
Calculated [M+Na]^+: m/z = 1170.3583
Experimental [M+Na]^+: m/z = 1170.3562

\(^1\text{H NMR, 400 MHz, CDCl}_3\), compound (33)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (33)

$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (33)
COSY NMR, 400 MHz, CDCl₃, compound (33)

HSQC NMR, 400 MHz, CDCl₃, compound (33)
ESI-TOF HR mass spectrum of compound (33)

\[
\text{C}_{62}\text{H}_{65}\text{NO}_{23}\text{S} = 1246.3566
\]
Calculated [M+Na]^+: m/z = 1246.3566
Experimental [M+Na]^+: m/z = 1246.3512

ESI-TOF HR mass spectrum of compound (33) with isotopic simulation

\[
\text{C}_{62}\text{H}_{65}\text{NO}_{23}\text{S} = 1246.3566
\]
Calculated [M+Na]^+: m/z = 1246.3566
Experimental [M+Na]^+: m/z = 1246.3512
$^1$H NMR, 400 MHz, CDCl$_3$, compound (G24)$_s$

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (G24)$_s$
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (G24)$_5$

COSY NMR, 400 MHz, CDCl$_3$, compound (G24)$_5$
HSQC NMR, 400 MHz, CDCl₃, compound (G24)$_S$

ESI-TOF HR mass spectrum of compound (G24)$_S$
ESI-TOF HR mass spectrum of compound (G24)_5 with isotopic simulation

\[ \text{C}_{46}\text{H}_{80}\text{N}_2\text{O}_{32}\text{S}_2 = 1236.4136} \]

Calculated [M+Na]^+ : m/z = 1259.3906
Experimental [M+Na]^+ : m/z = 1259.3906

\(^1\text{H NMR, 400 MHz, CDCl}_3\), compound (37)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (37)

COSY NMR, 400 MHz, CDCl$_3$, compound (37)
HSQC NMR, 400 MHz, CDCl₃, compound (37)

ESI-TOF HR mass spectrum of compound (37)

m/z [M+Na]⁺ calculated: 1303.5305
m/z [M+Na]⁺ experimental: 1303.5302.
ESI-TOF HR mass spectrum of compound (37) with isotopic simulation

\[ m/z \ [M+Na]^+ \text{ calculated: 1303.5305} \]
\[ m/z \ [M+Na]^+ \text{ experimental: 1303.5302}. \]

\[ ^1H \text{ NMR, } 400 \text{ MHz, CDCl}_3, \text{ compound (38)} \]
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (38)

COSY NMR, 400 MHz, CDCl$_3$, compound (38)
HSQC NMR, 400 MHz, CDCl₃, compound (38)

ESI-TOF HR mass spectrum of compound (38)
ESI-TOF HR mass spectrum of compound (38) with isotopic simulation

![ESI-TOF HR mass spectrum of compound (38) with isotopic simulation](image)

\[m/z \ [\text{M+Na}]^{-} \text{ calculated: 1023.3263}
\]
\[m/z \ [\text{M+Na}]^{-} \text{ experimental: 1023.3251}
\]

\[1^\text{H} \text{ NMR, 400 MHz, } \text{CDCl}_3, \text{ compound (39)}
\]

![1H NMR, 400 MHz, CDCl₃, compound (39)](image)
$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (39)

COSY NMR, 400 MHz, CDCl$_3$, compound (39)
HSQC NMR, 400 MHz, CDCl₃, compound (39)

ESI-TOF HR mass spectrum of compound (39)

m/z [M+Na]+ calculated: 983.2950
m/z [M+Na]+ experimental: 983.2974
ESI-TOF HR mass spectrum of compound (39) with isotopic simulation

\[
\text{m/z [M+Na]}^+ \text{ calculated: } 983.2950 \\
\text{m/z [M+Na]}^+ \text{ experimental: } 983.2974
\]

\[\begin{align*}
\text{39} \\
\text{m/z [M+Na]}^+ \text{ calculated: } 983.2950 \\
\text{m/z [M+Na]}^+ \text{ experimental: } 983.2974
\end{align*}\]

\[\begin{align*}
\text{1H NMR, 400 MHz, CDCl}_3, \text{ compound (40)}
\end{align*}\]
\(^{13}\text{C} \text{NMR, 100 MHz, CDCl}_3, \text{compound (40)}\)

ESI-TOF HR mass spectrum of compound (40)
ESI-TOF HR mass spectrum of compound (40) with isotopic simulation

m/z [M+Na]+ calculated: 1059.2932
m/z [M+Na]+ experimental: 1059.2935

1H NMR, 400 MHz, CDCl₃, compound (41)

(G11)₂
\[ ^{13}\text{C NMR, 100 MHz, CDCl}_3, \text{compound (41)} \]

\[ \text{COSY NMR, 400 MHz, CDCl}_3, \text{compound (41)} \]
HSQC NMR, 400 MHz, CDCl₃, compound (41)

ESI-TOF HR mass spectrum of compound (41)

m/z [M+Na]+ calculated: 1177.3502
m/z [M+Na]+ experimental: 1177.3331
ESI-TOF HR mass spectrum of compound (41) with isotopic simulation

\[\text{(G11S)}_2\]

m/z [M+Na]+ calculated: 1177.3502  
m/z [M+Na]+ experimental: 1177.3331

\[\text{\textsuperscript{1}H NMR, 400 MHz, CDCl}_3, \text{compound (43)}\]
$^1$H NMR, 400 MHz, CDCl$_3$, compound (44)

$^1$H NMR, 400 MHz, CDCl$_3$, compound (45)
$^1$H NMR, 400 MHz, CDCl$_3$, compound (42)

$^{13}$C NMR, 100 MHz, CDCl$_3$, compound (42)
$^{13}$C DEPT-135 NMR, 100 MHz, CDCl$_3$, compound (42)

COSY NMR, 400 MHz, CDCl$_3$, compound (42)
HSQC NMR, 400 MHz, CDCl$_3$, compound (42)

ESI-TOF HR mass spectrum of compound (42)

Calculated [M+Na]: m/z = 948.4178
Experimental [M+Na]: m/z = 948.4132
7. Vita

Elisa García Carvajal was born in Medellin, Colombia. In 2007 she graduated from i.e. Escuela Normal Superior María Auxiliadora high school, standing out in the pedagogy area. The same year she was admitted to the University of Antioquia, one of the best public universities in Colombia. She was awarded a tuition scholarship for three academic years during her bachelor's studies. In 2012, she joined the Interdisciplinary Group of Molecular Studies (GIEM). Under the direction of Professor Mario Vázquez, she completed all required coursework and her undergrad research work studying natural products from endemic fruits in Colombia. From this work, she published one article in a peer-reviewed journal. In 2015 she got her BS degree in chemistry. She continued her education in the Chemistry MS program conducting research in the Chemistry of Colombia Plant group under the guidance of Professor Wilson Cardona. Throughout her MS studies, she studied the synthesis of hybrids molecules for the discovery of a new drug for Chagas disease treatment, and she published six articles in a peer-reviewed journal. During her graduate studies at the University of Antioquia, she also served as a representative student council of the Chemical Science graduate program committee. In 2017, she was accepted into the Chemistry Doctoral Program at UTEP. Dr. Almeida’s and Dr. Michael’s R01 grant (NIH) supported her as a teaching and research assistant. During her Ph.D. studies, Elisa presented her research at multiple national and international conferences. She was awarded the best porter in the Carbohydrate Division at the ACS National Meeting in San Diego 2022. She was awarded the DAAD-RISE scholarship to do an international summer internship in Germany at Institut für Bioprozess- und Analysenmesstechnik, where she received specialized training on biosensors design. Elisa’s dissertation, “Synthesis of neoglycoproteins as biomarkers and potential vaccines for Chagas disease” was supervised by Dr. Katja Michael and Dr. Igor C. Almeida. Following graduation, Elisa will become a postdoctoral researcher at the University of Alberta in Canada.

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