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Synthesis of Galα(1,3)Galβ(1,4)GlcNAcα-, Galβ(1,4)GlcNAcα-, and GlcNAcα-containing neoglycoproteins and their immunological evaluation in the context of Chagas disease

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Abstract

The protozoan parasite, *Trypanosoma cruzi*, the etiologic agent of Chagas disease, has a cell surface covered by immunogenic glycoconjugates. One of the immunodominant glycotopes, the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα, is expressed on glycosylphosphatidylinositol-anchored mucins of the infective trypomastigote stage of *T. cruzi* and triggers high levels of protective anti-α-Gal antibodies in infected individuals. Here, we have efficiently synthesized the mercaptopropyl glycoside of that glycotope and conjugated it to maleimide-derivatized bovine serum albumin (BSA). Chemiluminescent-ELISA revealed that Galα(1,3)Galβ(1,4)GlcNAcα-BSA is recognized by purified anti-α-Gal antibodies from chronic Chagas disease patients ~230-fold more strongly than by anti-α-Gal antibodies from sera of healthy individuals (NHS anti-α-Gal). Similarly, the pooled sera of chronic Chagas disease patients (ChHSP) recognized Galα(1,3)Galβ(1,4)GlcNAcα ~20-fold more strongly than pooled NHS. In contrast, the underlying disaccharide Galβ(1,4)GlcNAcα, and the monosaccharide GlcNAcα or GlcNAcβ conjugated to BSA are poorly or not recognized by purified anti-α-Gal antibodies or sera from Chagasic patients or healthy individuals. Our results highlight the importance of the terminal Galα moiety for recognition by Ch anti-α-Gal antibodies and the lack of antibodies against nonself Galβ(1,4)GlcNAcα and GlcNAcα glycotopes. The substantial difference in binding of Ch vs. NHS anti-α-Gal antibodies to Galα(1,3)Galβ(1,4)GlcNAcα-BSA suggests that this neoglycoprotein might be suitable for experimental vaccination. To this end, the Galα(1,3)Galβ(1,4)GlcNAcα-BSA neoglycoprotein was then used to immunize α1,3-galactosyltransferase-knockout mice, which produced antibody titers 40-fold higher as compared to pre-immunization titers. Taken together, our results indicate that the synthetic

Galα(1,3)Galβ(1,4)GlcNAcα glycotope coupled to a carrier protein could be a potential diagnostic and vaccine candidate for Chagas disease.

Keywords: Biomarkers/Carbohydrates/Chagas disease/Immunization/Neoglycoprotein

Introduction

The surface of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease (ChD), is heavily coated by glycoproteins containing highly immunogenic glycans (Acosta-Serrano, A., Hutchinson, C., et al. 2007, Travassos, L.R. and Almeida, I.C. 1993). An immunodominant glycotope, Galα(1,3)Galβ(1,4)GlcNAcα, is abundantly expressed in the mammal-dwelling *T. cruzi* trypomastigote stage (Almeida, I.C., Ferguson, M.A., et al. 1994) and is not expressed on human cells, thus it is highly immunogenic to humans (Macher, B.A. and Galili, U. 2008, Travassos, L.R. and Almeida, I.C. 1993). The Galα(1,3)Galβ(1,4)GlcNAcα epitope contains a terminal, non-reducing αGal residue, which is highly conserved on trypomastigote-derived GPI-mucins (tGPI-mucins) of at least four major *T. cruzi* genotypes causing ChD in humans: TcI, TcII, TcV, and TcVI (Almeida, I.C., Krautz, G.M., et al. 1993, Izquierdo, L., Marques, A.F., et al. 2013, Soares, R.P., Torrecilhas, A.C., et al. 2012, Travassos, L.R. and Almeida, I.C. 1993). The Galα(1,3)Galβ(1,4)GlcNAcα glycotope contains the disaccharide Galα1,3Galβ, which is strongly recognized by Chagasic (Ch) anti-α-Gal Abs and to a much lesser extent by the natural anti-α-Gal Abs from healthy individuals (NHS anti-α-Gal) (Almeida, I.C., Ferguson, M.A., et al. 1994, Ashmus, R.A., Schocker, N.S., et al. 2013), which are produced mainly against Gram-negative enterobacteria of the human flora (Galili, U., Wang, L., et al. 1999). These enterobacteria (e.g., *E. coli*, *Enterobacter* spp., *Serratia* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., and *Citrobacter* spp.) have various types of non-reducing, terminal α-Gal-linked glycans, mostly Galα1,2-R, Galα1,4-R, and Galα1,6-R (where R is the remaining side chain or core glycan) on the lipopolysaccharide (LPS) core oligosaccharides or *O*-antigens (Wilkinson, S.G. 1996). The glycotope Galα(1,3)Galβ(1,4)GlcNAcα, so far not

reported in enterobacteria, and most likely other yet unidentified *T. cruzi*-specific cell surface saccharides with terminal αGal moieties, induce the major lytic, protective antibodies (Ch anti-α-Gal Abs) produced during both the acute and chronic stages of ChD (Almeida, I.C., Ferguson, M.A., et al. 1994, Almeida, I.C., Milani, S.R., et al. 1991, Avila, J.L., Rojas, M., et al. 1989, Gazzinelli, R.T., Pereira, M.E., et al. 1991, Milani, S.R. and Travassos, L.R. 1988, Travassos, L.R. and Almeida, I.C. 1993). These studies strongly indicate that lytic Ch anti-α-Gal Abs could be one of the main immunological mechanisms for controlling the parasitemia in both stages of the disease in humans. Thus, Galα(1,3)Galβ(1,4)GlcNAcα offers a potential route toward a carbohydrate-based vaccine against Chagas disease. Glycoconjugates are still unexplored as vaccine targets in *T. cruzi*, although these molecules are the most abundant and immunogenic antigens on the plasma membrane of all *T. cruzi* developmental stages (Acosta-Serrano, A., Hutchinson, C., et al. 2007, Buscaglia, C.A., Campo, V.A., et al. 2004, Frasch, A.C. 2000).

Here we describe the synthesis of glycosides of Galα(1,3)Galβ(1,4)GlcNAcα, and its truncated versions Galβ(1,4)GlcNAcα and GlcNAcα, as well as its diastereomer GlcNAcβ**,** all equipped with a thiol functionality (glycosides **1** – **4**, Figure 1) for their conjugation to the carrier protein bovine serum albumin (BSA). All neoglycoproteins (NGPs) were immunologically evaluated by chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) (Almeida, I.C., Covas, D.T., et al. 1997), using purified Ch anti-α-Gal Abs vs. NHS anti-α-Gal Abs, and Ch human serum pool (ChHSP) vs. normal human serum pool (NHSP). Lastly, the NGP Galα(1,3)Galβ(1,4)GlcNAcα-BSA was used to immunize α1,3-galactosyltransferase-knockout (α1,3GalT-KO) mice, which do not express terminal αGal epitopes in their cells (Tearle, R.G., Tange, M.J., et al. 1996, Thall, A.D., Murphy, H.S., et al. 1996). These animals are able to

produce lytic anti-α-Gal Abs, mimicking therefore the human humoral immune response against *T. cruzi* (Marques, Michael, Almeida et al., unpublished data).

The production of the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα and related analogs has been previously accomplished for a variety of uses, and mostly involves chemoenzymatic syntheses (Brinkmann, N., Malissard, M., et al. 2001, Fang, J., Li, J., et al. 1998, Qian, X., Sujino, K., et al. 1999, Vic, G., Hao Tran, C., et al. 1997), which are often efficient. However, some research groups prefer its chemical synthesis due to reagent availability, scalability, and derivatization options. For example, α-Gal trisaccharides have been chemically synthesized and coupled to Sepharose (Dahmén, J., Magnusson, G., et al. 2002), attached to a lipid for non-covalent association to target molecules (Litjens, R.E.J.N., Hoogerhout, P., et al. 2005), or attached to linkers such as p-nitrophenol esters (Plaza-Alexander, P. and Lowary, T.L. 2013) and 3-aminopropyl groups (Hanessian, S., Saavedra, O.M., et al. 2001, Wang, Y., Yan, Q., et al. 2005) to allow for further functionalization.

The four key features of our approach to an Galα(1,3)Galβ(1,4)GlcNAcα-containing NGP are: i) predominant use of acyl protecting groups that can be easily installed and cleanly removed; ii) utilization of Kiso's 4,6-di-*tert*butylsilyl protected galactosyl donor (Imamura, A., Kimura, A., et al. 2006) to ensure a stereoselective α-galactosylation; iii) utilization of easily accessible monosaccharide building blocks; and iv) use of an allyl glycoside at the non-reducing end of the trisaccharide allowing for the installation of a thiol functional group, via a thiol-ene reaction, for covalent conjugation to a carrier protein. Implementing these features, our strategy involves the synthesis of an acyl-protected disaccharide (Galα1,3Galβ), its conversion into a trichloroacetimidate donor, glycosylation of an appropriate allyl-glycoside GlcNAc acceptor to

produce a Galα(1,3)Galβ(1,4)GlcNAcα allyl glycoside, and further derivatization into a mercaptopropyl glycoside needed for protein conjugation.

BSA was chosen for the generation of NGPs because of its large number of conjugation sites per BSA molecule, its superior solubility properties, and its suitability as a carrier protein (Makela, O. and Seppala, I.J.T. 1986) and provider of T cell epitopes for the immunization of mice (Atassi, M.Z., Long, P.M., et al. 1982), as well as its capability to attach non-covalently to wells of microtiter plates. Previously, we discovered that Ch anti-α-Gal Abs recognize the disaccharide Galα(1,3)Galβ, which comprises the two terminal sugars of the glycotope trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα, much more strongly than Galα alone (Ashmus, R.A., Schocker, N.S., et al. 2013). In order to obtain information on the importance of Galβ(1,4)GlcNAcα or GlcNAc for antibody recognition, three additional BSA NPGs containing Galβ1,4GlcNAcα, GlcNAcα, or GlcNAcβ were synthesized and tested by CL-ELISA.

Results and Discussion

The α-Gal disaccharide **11** was synthesized from the known allyl β-galactoside **5** (Stevenson, D.E. and Furneaux, R.H. 1996), which was made from its peracetylated precursor following an optimized procedure (Khamsi, J., Ashmus, R.A., et al. 2012). Disaccharide **11** was synthesized in a 55% overall yield, starting with p-methoxybenzylation of allyl glycoside **5** at position 3 via its tin acetal to give **6**, followed by benzoylation of the three remaining hydroxyls to afford **7**. Oxidative cleavage of the p-methoxybenzyl group with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone furnished the β-Gal acceptor **8**. This acceptor was glycosylated with the known di-*tert*-butylsilylidene equipped α-Gal trichloroacetimidate donor **9** (Imamura, A., Kimura, A., et al.

2006), using trimethylsilyl trifluoromethanesulfonate catalysis to give disaccharide **10**. The di-*tert*-butylsilylidene group was cleaved with a large excess of 70% hydrogen fluoride in pyridine in THF, followed by acetylation of the two hydroxyls to give the peracylated allyl disaccharide **11** (Scheme 1).

The α-Gal disaccharide **11** was then treated with palladium (II) chloride in methanol to give the hemiacetal, which was filtered immediately after consumption of the starting material to avoid the formation of a polar by-product that we observed after two hours of reaction, and converted into the trichloroacetimidate **12** with trichloroacetonitrile in the presence of 1,8-diazabicycloundec-7-ene (Scheme 2). This donor was first used to glycosylate the allyl GlcNAc acceptor **13** with TMS-OTf, but produced a low-yielding mixture of anomers (1:4 α/β) likely due to the well-known poor nucleophilicity of the 4-OH of GlcNAc acceptors. The separation of the two diastereomeric trisaccharides proved to be difficult but could be accomplished by reversed phase FPLC. Replacing the acceptor **13** by the allyl 2-deoxy-2-azido-Glc acceptor **14** produced trisaccharide **15** in 46% yield, which could be purified by flash chromatography, and the azide was then reduced to an N-acetyl group with neat thioacetic acid to give the trisaccharide **16**. Radical addition of thioacetic acid with azobisisobutyronitrile in tetrahydrofuran under UV light gave the thioester **17**, followed by saponification under Zemplén conditions to afford the target trisaccharide **1** (Scheme 2).

The Galβ(1,4)GlcNAcα disaccharide **2** was synthesized as shown in Scheme 3 in a 70% overall yield from the allyl GlcNAc acceptor **13**. Through the use of a large excess of the known acetylated trichloroacetimidate β-Gal donor **18** (Schmidt, R.R. and Michel, J. 1980), and the use of boron trifluoride etherate at an unusual elevated temperature (Hendel, J.L., Wang, J.-W., et al. 2009), the Galβ(1,4)GlcNAcα disaccharide **19** was obtained in high yield (83%), followed by

radical addition of thioacetic acid to give the thioester **20**. Saponification under Zemplén conditions cleanly gave the target disaccharide **2**. The mercaptopropyl glycoside of GlcNAcα (**3**) was synthesized as previously described (Houseman, B.T., Gawalt, E.S., et al. 2003), while the mercaptopropyl glycoside of GlcNAcβ (**4**) was synthesized by radical addition of thioacetic acid to the known allyl glycoside **21** (Kiso, M. and Anderson, L. 1979) to give thioester **22**, followed by saponification to provide the target glycoside **4** (Scheme 3).

The mercaptopropyl glycosides oxidized to disulfides within hours-days of isolation, which could easily be reduced by tris(2-carboxyethyl)phosphine before their conjugation to BSA. The thiol groups on compounds **1-4** served as nucleophiles in the conjugate addition to commercially available maleimide-derivatized BSA in aqueous buffer at pH 7.2, as shown in Figure 2. This produced neoglycoproteins via thioether linkages, and the average number of saccharides conjugated per BSA molecule was estimated by matrix-assisted laser desorption ionization-time of flight mass spectrometry. The conjugation of 22-23 units of Galα(1,3)Galβ(1,4)GlcNAcα and 23-24 units of Galβ(1,4)GlcNAcα per molecule of BSA are shown in Figure 2. An average of 29 units of GlcNAcα and 25 units of GlcNAcβ were conjugated to BSA (see Supplemental Material, Page S3).

The four NPGs Galα(1,3)Galβ(1,4)GlcNAcα-BSA, Galβ(1,4)GlcNAcα-BSA, GlcNAcα-BSA, GlcNAcβ-BSA, and a BSA control conjugate in which the maleimide groups had been blocked with cysteine (Cys-BSA), were immobilized in 96-well polystyrene Nunc Maxisorp ELISA plates and antibody-binding responses were measured using CL-ELISA (Almeida, I.C., Covas, D.T., et al. 1997), with pooled Chagasic human sera (ChHSP) and normal human sera (NHSP), as well as Ch anti-α-Gal Abs and NHS anti-α-Gal Abs, purified as described (Almeida, I.C., Milani, S.R., et al. 1991). As shown in Figure 3A, Galα(1,3)Galβ(1,4)GlcNAcα-BSA

clearly displays a 20-fold differential between ChHSP and NHSP, whereas the NGPs Galβ(1,4)GlcNAcα-BSA, GlcNAcα-BSA, and GlcNAcβ-BSA all show minimal binding to either pooled sera. There was no significant difference between the weak antibody reactivity observed with GlcNAcα and GlcNAcβ. Cys-BSA proved to be an effective negative control. As shown in Figure 3B, Galα(1,3)Galβ(1,4)GlcNAcα-BSA displays a 230-fold differential between purified Ch and NHS anti-α-Gal antibodies, while neoglycoproteins Galβ(1,4)GlcNAcα-BSA, GlcNAcα-BSA, GlcNAcβ-BSA are practically not recognized by either antibodies. These results emphasize that the terminal Galα residue is crucial for Chagasic antibody binding, and demonstrates a convenient method to differentiate between *T. cruzi*-infected and non-infected sera. In addition, they show that, although Galβ(1,4)GlcNAcα and GlcNAcα are nonself glycotopes for humans, there is little or no antibody response against them in the sera of Chagasic patients (Fig. 3A).

Next, the in vivo response to Galα(1,3)Galβ(1,4)GlcNAcα-BSA was evaluated in C57Bl/6 α1,3galactosyltransferase-knockout (α1,3GalT-KO) mice. Akin to humans and in contrast to wild-type mice, these animals lack terminal Galα1,3-linked residues on glycoproteins, thus being able to produce high levels of anti-α-Gal antibodies (Tearle, R.G., Tange, M.J., et al. 1996, Thall, A.D., Murphy, H.S., et al. 1996). Sera collected from immunized and control animals were pooled separately and analyzed by CL-ELISA (Ashmus, R.A., Schocker, N.S., et al. 2013). As shown in Figure 4, sera from the Galα(1,3)Galβ(1,4)GlcNAcα-BSA-immunized mice displayed a 22-fold higher antibody response to Galα(1,3)Galβ(1,4)GlcNAcα-BSA after immunization as compared to pre-immunization levels, whereas mice immunized with BSA alone showed minimal antibody reactivity before and after immunization.

In conclusion, the mercaptopropyl glycoside of Galα(1,3)Galβ(1,4)GlcNAcα was efficiently synthesized in 12 steps from known monosaccharide building blocks. In contrast to the published chemical syntheses, an important feature of this synthesis is the ease of accessibility of the glycosyl acceptors, which are synthesized in only 2-3 steps from commercially available starting materials. In addition, our synthesis utilizes common and inexpensive glycosylation catalysts. The two key steps in this synthesis are the stereoselective installation of the terminal Galα unit into disaccharide **10** in 92% yield, and the challenging glycosylation of the 2-deoxy-2-azido acceptor **14** to give the correct stereoisomer (trisaccharide **15**) in 46% yield. With the exception of the p-methoxy group introduced into galactose derivative **6**, the di-*tert*-butylsilylidene protecting group of the galactosyl donor **9**, and the allyl group as a precursor of a hemiacetal in compound **11**, easily installable and removable acetyl and benzoyl protecting groups were used throughout the synthesis. Utilizing anomeric allyl groups allowed for the convenient conversion into mercaptopropyl glycosides that were needed for the conjugation to maleimide-derivatized BSA. The mercaptopropyl group of these glycosides is highly versatile as it is suitable for the conjugation to a large variety of other biomolecules and surfaces by conjugate addition to maleimides, nucleophilic substitution, and thiol-ene reaction. Finally, we showed that trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα, which is an immunodominant glycotope in infective *T. cruzi* trypomastigotes, is highly immunogenic in the context of *T. cruzi* infection in both mice and humans. We propose that the Galα(1,3)Galβ(1,4)GlcNAcα-BSA and its analogs containing different carrier proteins or peptides could be further explored as potential biomarkers or tools for the diagnosis and follow-up of chemotherapy of ChD, and as vaccine candidates.

Materials and Methods

Thin-layer chromatography was performed with silica gel on aluminum support, 8.0-12.0 µm, Sigma-Aldrich, and visualized by UV light or with 2% H_2SO_4 in ethanol, followed by heating. Flash chromatography was performed with silica gel, grade A, 32–63 µm, Dynamic Adsorbents. ^{1}H NMR spectra were recorded on a JEOL 600 MHz NMR spectrometer using tetramethylsilane or chloroform as an internal standard. ^{13}C NMR spectra were recorded on the same JEOL NMR spectrometer at 150 MHz. Optical rotations were recorded on an Atago AP300 automatic polarimeter. Mass spectra were recorded on a JEOL Accu TOF mass spectrometer using electrospray ionization, or on a Shimadzu Axima MALDI-TOF MS. Dichloromethane and pyridine were refluxed over calcium hydride and distilled, methanol was refluxed over magnesium and distilled. Reagents were purchased from Sigma-Aldrich, Acros Organics, Fisher Scientific, and Alfa Aesar. 96-well polystyrene Nunc MaxiSorp ELISA plates and CL-ELISA reagents were purchased from Thermo Scientific or Jackson ImmunoResearch, luminescence was recorded on a Luminoskan Ascent, Thermo Scientific.

3-thiopropyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-α-D-glucopyranoside (1)

To a flask containing **17** (0.027 g, 0.018 mmol), 3 mL of 0.5M NaOMe was added under argon, and stirred at room temperature for 30 minutes. HRMS showed full removal of acyl protecting groups, and all material was present as a mixture of thiol and disulfide. Amberlyst-15 ion-exchange resin was added and stirred until the solution was neutral, followed by filtration through Celite and evaporation of the solvent. The remainder was dissolved in water and lyophilized to give **1** as a white powder (0.011 g, quant.). ESI-TOF HRMS $[C_{23}H_{41}NO_{16}S + Na]^+$ calc. *m/z* = 642.2044, found 642.1980.

3-thiopropyl β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-α-D-glucopyranoside (2)

To a flask containing **20** (0.045 g, 0.051 mmol), 4 mL of 0.5M NaOMe was added under argon, and stirred at room temperature for 30 minutes. HRMS showed full removal of acyl protecting groups, and all the material was present as a disulfide. Amberlyst-15 ion-exchange resin was added and stirred until the solution was neutral, followed by filtration through Celite and evaporation of the solvent. The remainder was dissolved in water and lyophilized to give **2** as a white powder (0.024 g, quant.). ESI-TOF HRMS $[C_{34}H_{60}N_2O_{22}S_2 + Na]^+$ calc. *m/z* = 935.2977, found 935.2836.

3-thiopropyl 2-deoxy-2-acetamido-β-D-glucopyranoside (4)

To a flask containing **22** (0.059 g, 0.127 mmol), 4 mL of 0.5M NaOMe was added under argon, and stirred at room temperature for 2 hours. HRMS showed full removal of acyl protecting groups, and most of the material was present as a disulfide. Amberlyst-15 ion-exchange resin was added and stirred until the solution was neutral, followed by filtration through Celite and evaporation of the solvent. The remainder was dissolved in water and lyophilized to give **4** as a white powder (0.037 g, quant.). ESI-TOF HRMS $[C_{22}H_{40}N_2O_{12}S_2 + Na]^+$ calc. *m/z* = 611.1920, found 611.1707.

Allyl 3-*O*-(4-methoxybenzyl)-β-D-galactopyranoside (6)

A solution of **5** (Stevenson, D.E. and Furneaux, R.H. 1996) (0.409 g, 1.86 mmol) and Bu_2SnO (0.693 g, 2.79 mmol) in 18 mL anhydrous MeOH was stirred and refluxed under argon for 8 h. The solution was then quickly concentrated, and resuspended in 18 mL benzene. Bu_4NBr (0.30 g, 0.93 mmol) was added, followed by 4-methoxybenzyl chloride (0.378 mL, 2.79 mmol), and stirred at 80°C for 12 h. The solution was concentrated, and purified by column chromatography on silica gel ($CHCl_3$/MeOH 9:1) to give **6** as a white powder (0.430 g, 75%). Its ^1H and ^{13}C NMR spectra matched the ones previously described for this compound (Yoshida, T., Chiba, T., et al. 2001).

Allyl 3-*O*-(4-methoxybenzyl)-2,4,6-tri-O-benzoyl-β-D-galactopyranoside (7)

A solution of **6** (0.380g, 1.22 mmol) in 5 mL anhydrous pyridine was cooled to 0°C under argon. BzCl (0.854 mL, 7.35 mmol) was added dropwise, and stirred for 3h. The solution was diluted with EtOAc, washed once with 1M HCl, once with a sat. $NaHCO_3$ solution and once with brine, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (hexanes/EtOAc 2:1) to give **7** as a white powder (0.658 g, 90%). $[\alpha]_D^{28}$ 72.4 (c=1 in $CHCl_3$); R_f=0.38 (MeOH/$CHCl_3$ 1:9); ^1H NMR (600 MHz, $CDCl_3$, 300K) δ 8.18; 8.05; 7.97; 7.56-7.61; 7.43-7.50 (5m, 15H, 3 x Bz); 7.05; 6.59 (2m, 4H, 4-OMe-benzyl); 5.90 (m, 1H, H-4); 5.77 (m, 1H, OCH_2CHCH_2); 5.55 (dd, 1H, $^3J_{H1/H2}$=8.9 Hz, $^3J_{H2/H3}$=8.9 Hz, H-2); 5.18 (m, 1H, OCH_2CHCH_2); 5.07 (m, 1H, $OCH_2'CHCH_2$); 4.60-4.67 (m, 3H, H-1, H-6, CH_2PhOMe); 4.41-4.47 (m, 2H, H-6', $CH_2'PhOMe$); 4.35 (m, 1H, OCH_2CHCH_2); 4.13 (m, 1H, OCH_2CHCH_2'); 4.08 (m, 1H, H-5); 3.79 (dd, 1H, $^3J_{H3/H4}$=3.4 Hz, H-3); 3.70 (s, 3H, OCH_3) ppm. ^{13}C NMR

(150MHz, $CDCl_3$, 300K): δ 166.3; 166.0; 165.3; 159.3; 133.7; 133.5; 133.4; 133.1; 130.3; 129.6-130.1; 129.5; 129.4; 128.5-128.7; 128.4; 117.7; 113.7; 100.2 (C-1); 75.8; 71.5; 71.3; 70.7; 70.1; 66.8; 62.8; 55.2 ppm. ESI-TOF HRMS $[C_{38}H_{36}O_{10} + Na]^+$ calc. *m/z* = 675.2206, found 675.2001; $[C_{38}H_{36}O_{10} + K]^+$ calc. *m/z* = 691.1946, found 691.2022.

Allyl 2,4,6-tri-*O*-benzoyl-β-D-galactopyranoside (8)

To a solution of **7** (0.633 g, 0.97 mmol) in 20 mL CH_2Cl_2 and 1.1 mL H_2O, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (0.440 g, 1.94 mmol) was added in two portions, 30 minutes apart, and stirred vigorously for 12 h. The red and green solution was filtered through Celite, diluted with dichloromethane, and extracted with water (25 mL) and brine solution (25 mL), dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (EtOAc/hexanes 2:1) to give **8** as a white powder (0.504 g, 98%). Its ^1H and ^{13}C NMR spectra matched the ones previously described for this compound (Sherman, A.A., Yudina, O.N., et al. 2001).

Allyl 4,6-di-*O*-*tert*-butylsilylene-2,3-di-*O*-benzoyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranoside (10)

A solution of acceptor **8** (0.175 g, 0.329 mmol) and 4,6-di-*O*-tertbutylsilyl-2,3-di-*O*-benzoyl-α-D-galactopyranosyl trichloroacetimidate donor **9** (Imamura, A., Kimura, A., et al. 2006) (0.266 g, 0.395 mmol) in anhydrous dichloromethane (6 mL) was added to a 10 mL round bottomed flask with freshly activated, crushed 4Å molecular sieves and stirred under argon for 15 min. at 0°C.

TMSOTf (0.010 mL, 0.059 mmol) was added dropwise, and the mixture was gradually brought to room temperature and stirred for 2 h. To quench the reaction, Et_3N (0.010 mL, 0.072 mmol) was added and stirred. The solution was diluted with dichloromethane (50 mL) and extracted with water (2 × 25 mL) and brine solution (25 mL), dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (hexanes/EtOAc 3:1) to give **10** as a white powder (0.315 g, 92%). $[\alpha]^{28}_D$ 160.7 (c=1 in $CHCl_3$); R_f=0.55 (EtOAc/hexanes 1:2); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.09, 7.99, 7.84, 7.74, 7.60, 7.51, 7.41, 7.26, 7.13, 7.01 (10m, 25H, 5xBz); 5.79-5.87 (m, 2H, OCH_2CHCH_2, βGalH-4); 5.70-5.76 (m, 2H, αGalH-2, βGalH-2); 5.62 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGalH-1); 5.26 (m, 1H, OCH_2CHCH_2); 5.16 (m, 2H, $OCH_2'CHCH_2$, αGalH-3); 4.77 (d, 1H, $^3J_{H1/H2}$=8.3Hz, βGalH-1); 4.55 (dd, 1H, $^3J_{H5/H6}$=11.7Hz, $^2J_{H6/H6'}$= 6.9Hz, βGalH-6); 4.40 (m, 1H, OCH_2CHCH_2); 4.22-4.30 (m, 3H, βGalH-3, βGalH-5, αGalH-4); 4.19 (m, 1H, OCH_2CHCH_2'); 4.03-4.14 (m, 2H, αGalH-5, βGalH-6'); 3.64-3.71 (m, 2H, αGalH-6, αGalH-6'); 1.02 (s, 9H, *t*-butyl); 0.79 (s, 9H, *t*-butyl) ppm. ^{13}C NMR (150MHz, $CDCl_3$, 300K): δ 166.3, 166.1, 165.6, 165.4, 165.0, 133.6, 133.4; 133.4; 133.0; 132.9; 132.8; 129.5-129.9; 129.2; 128.8; 128.6; 128.3; 128.1; 118.0; 100.3 (βC-1); 94.2 (αC-1); 73.8; 71.5; 70.9; 70.7; 70.5; 70.2; 67.6; 67.1; 66.5; 65.9; 62.3; 27.4; 27.2; 25.4; 23.2; 20.7 ppm. ESI - TOF HRMS $[C_{58}H_{62}O_{16}Si + Na]^+$ calc. *m/z* = 1065.3705, found 1065.3587; $[C_{58}H_{62}O_{16}Si + K]^+$ calc. *m/z* = 1081.3444, found 1081.2728.

Allyl 2,3-di-*O*-benzoyl-4,6-di-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranoside (11)

A solution of **10** (0.464 g, 0.444 mmol) in anhydrous THF (7 mL) was added to a 50 mL plastic conical tube and stirred under argon at rt. A solution of HF-pyridine (70% HF, 30% pyridine) (0.223 mL, 8.88 mmol) was added to the reaction mixture and stirred for 3 h, then quenched with 0.5 mL saturated $NaHCO_3$. The solution was diluted with EtOAc and extracted with water and brine, dried over $MgSO_4$, and concentrated. The compound was then added to a 25 mL round bottom flask in 5 mL anhydrous pyridine, and Ac_2O was added (0.252 mL; 2.66 mmol) and stirred for 12 h. The solvent was then co-evaporated with toluene, and the remainder was purified by column chromatography on silica gel (hexanes/EtOAc 2:1) to give **11** as a white powder (0.389 g, 89% in 2 steps). $[\alpha]^{28}_D$ 163.0 (c=1 in $CHCl_3$); R_f=0.20 (EtOAc/hexanes 1:2); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.15; 7.99; 7.70; 7.61; 7.51; 7.44; 7.39; 7.28; 7.24; 7.10; 7.01 (11m, 25H, 5 x Bz); 5.80-5.86 (m, 2H, OCH_2CHCH_2, βGalH-4); 5.76 (dd, 1H, $^3J_{H2/H3}$=9.6 Hz, βGalH-2); 5.67 (d, 1H, $^3J_{H1/H2}$=4.1 Hz, αGalH-1); 5.61 (dd, 1H, $^3J_{H2/H3}$=11.0 Hz, αGalH-2); 5.41 (dd, 1H, $^3J_{H3/H4}$=3.4 Hz, αGalH-3); 5.26 (m, 1H, OCH_2CHCH_2); 5.09-5.17 (m, 2H, $OCH_2'CHCH_2$, αGalH-4); 4.78 (d, 1H, $^3J_{H1/H2}$= 7.6 Hz, βGalH-1); 4.57 (dd, 1H, $^3J_{H5/H6}$=11.0 Hz, $^2J_{H6/H6'}$=6.2 Hz, βGalH-6); 4.41 (m, 1H, OCH_2CHCH_2); 4.27-4.32 (m, 2H, βGalH-3, βGalH-5); 4.19 (m, 2H, OCH_2CHCH_2', αGalH-5); 4.11 (dd, 1H, $^3J_{H5/H6}$=6.6 Hz, βGalH-6'); 3.96 (m, 2H, αGalH6, αGalH6'); 1.97-2.03 (m, 6H, 2x Ac) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 170.1; 169.8; 166.2; 166.0; 165.4; 165.1; 164.9; 133.6; 133.5; 133.4; 133.1; 133.0; 129.8; 129.8; 129.7; 129.5; 129.5; 129.3; 128.8; 128.6; 128.4; 128.3; 128.3; 128.2; 118.1; 100.3 (βC-1); 93.4 (αC-1); 73.4; 71.5; 70.6; 70.2; 67.9; 67.6; 66.8; 65.5; 62.3; 61.5; 20.8; 20.6 ppm. ESI-TOF HRMS $[C_{54}H_{50}O_{18} + NH_4]^+$ calc. *m/z* = 1004.3341, found 1004.3070.

Trichloroacetimidate 2,3-di-O-benzoyl-4,6-di-O-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-O-benzoyl-β-D-galactopyranoside (12)

To a solution of **11** (0.369 g, 0.374 mmol) in MeOH (6 mL), $PdCl_2$ (0.0398 g, 0.225 mmol) was added and stirred for 2 h at room temperature until consumption of most of the starting material. After 2 h, a degradation product can be observed. The solution was filtered through Celite, concentrated, and purified by column chromatography on silica gel (EtOAc/hexanes 2:3) to give the α and β anomers (0.308 g, 87%). A recovered compound assumed to be remaining starting material was actually the vinyl glycoside. The anomeric product mixture was then placed into a round-bottomed flask, 10 mL anhydrous CH_2Cl_2 was added under argon, and the solution was cooled to 0°C. CCl_3CN (0.325 mL, 3.24 mmol) was added, followed by dropwise addition of DBU (0.015 mL, 0.097 mmol) and the mixture was brought to rt over 3 h. The solution was concentrated and purified by column chromatography on silica gel (EtOAc/hexanes 1:2) to give **12** as a white powder (0.295 g, 84%). $[\alpha]^{27}_D$ 186.2 (c=1 in $CHCl_3$); R_f=0.65 (acetone/hexanes 1:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.64 (s, 1H, NH); 8.10; 7.94; 7.67-7.71; 7.53-7.63; 7.47; 7.36-7.41; 7.23-7.30; 7.12; 7.02 (9m, 25H, 5 x Bz); 6.90 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGalH-1) 5.99 (d, 1H, $^3J_{H4/H5}$=2.8 Hz,αGalH-4); 5.94 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, αGalH-2); 5.76 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGal'H-1); 5.65 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, αGal'H-2); 5.49 (dd, 1H, $^3J_{H3/H4}$=3.4 Hz,αGal'H-3); 5.28 (m, 1H, αGal'H-4); 4.76 (dd, 1H, $^3J_{H3/H4}$=3.4 Hz, αGalH-3); 4.65 (m, 1H, αGalH-5); 4.44 (dd, 1H, 7.6 Hz, 11.7 Hz, αGalH-6); 4.41 (dd, 1H, $^3J_{H5/H6}$=11.7 Hz, αGal'H-5); 4.31 (dd, 1H, 5.5 Hz, 11.7 Hz, αGalH-6'); 4.09-4.14 (m, 1H, αGal'H-6); 4.02 (dd, 1H, $^2J_{H6/H6'}$=6.2 Hz, αGal'H-6'); 1.98-2.06 (m, 6H, 2x Ac) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 170.2; 169.8; 166.0; 165.6; 165.3; 165.0; 160.5; 133.9; 133.0-133.3; 129.6-129.9;

129.4; 128.8; 128.5; 128.3; 128.2; 93.8 (αGalC-1); 93.2 (αGal'C-1); 90.9 (CCl_3); 70.1; 69.5; 68.7; 67.8; 66.7; 66.0; 62.5; 60.9; 20.9; 20.6 ppm. ESI-TOF HRMS did not show a molecular ion peak for $[C_{53}H_{46}Cl_3NO_{18}]^+$.

Allyl 2-deoxy-2-acetamido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (13)

To a solution of allyl 2-deoxy-2-acetamido-α-D-glucopyranoside (Gavard, O., Hersant, Y., et al. 2003) (3.98 g, 15.24 mmol) in 80 mL anhydrous AcCN, 1-benzoylimidazole (5.46 mL, 36.56 mmol) was added via a plastic syringe, and was heated at 80°C for 12 h. After evaporation of the solvent the remainder was dissolved in EtOAc and extracted twice with water and once with brine solution, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (toluene/EtOAc 2:1) to give **13** as a white powder (4.79 g, 67%). Its ^1H and ^{13}C NMR spectra matched the ones previously described for this compound (Danac, R., Ball, L., et al. 2007). A minor byproduct with a higher R_f value was identified as the tri-*O*-benzoylated compound.

Allyl 2-deoxy-2-azido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (14)

Compound **14** was prepared similarly to a published synthesis with slight variations in the solvent and the time period over which BzCl was added (Danac, R., Ball, L., et al. 2007): A solution of allyl 2-deoxy-2-azido-α-D-glucopyranoside (Gavard, O., Hersant, Y., et al. 2003) (0.30 g, 1.223 mmol) in 10 mL anhydrous pyridine was cooled to -20°C, and BzCl (0.350 mL, 3.01 mmol) was added dropwise in 3 portions of 0.117 mL each over 1 h, and stirred for an

additional 1 h. The solution was diluted with EtOAc and extracted twice with water and once with brine solution, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (hexanes/EtOAc 5:2) to give **14** as a white powder (0.364 g, 66%). $[\alpha]^{25}_D$ 161.0 (c=1 in $CHCl_3$) R_f=0.48 (EtOAc/hexanes 1:2); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.05-8.11; 7.58; 7.43-7.48 (m, 10H, 2 x Bz); 5.95 (m, 1H, $OCH_2C\mathit{H}CH_2$); 5.64 (dd, 1H, $^3J_{H3/H4}$=9.6 Hz, H-3); 5.36 (m, 1H, $OC\mathit{H}_2CHCH_2$); 5.25 (m, 1H, $OC\mathit{H}_2$'$CHCH_2$); 5.09 (d, 1H, $^3J_{H1/H2}$=4.1 Hz, H-1); 4.73 (dd, 1H, $^3J_{H5/H6}$=4.8 Hz, $^2J_{H6/H6'}$=12.4 Hz, H-6); 4.60 (dd, 1H, $^3J_{H5/H6'}$=2.1 Hz, H-6'); 4.29 (m, 1H, $CH_2CHC\mathit{H}_2$); 4.12 (m, 2H, H-5, $CH_2CHC\mathit{H}_2$'); 3.77 (dd, 1H, $^3J_{H4/H5}$=9.6 Hz, H-4); 3.47-3.54 (broad, 1H, 4-OH); 3.44 (dd, 1H, $^3J_{H2/H3}$=11.0 Hz, H-2) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 167.2; 167.0; 133.7; 133.4; 133.0; 130.1; 129.7-130.0; 129.2; 128.6; 128.6; 118.5; 97.0 (C-1); 74.0; 70.7; 70.0; 69.0; 63.5; 61.2 ppm. ESI-TOF HRMS $[C_{23}H_{23}N_3O_7 + H]^+$ calc. m/z= 454.1614, found 454.1912. A minor byproduct of this reaction was identified as the tri-*O*-benzoylated compound.

Allyl 2,3-di-*O*-benzoyl-4,6-di-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-azido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (15)

A solution of acceptor **14** (0.126 g, 0.279 mmol) and donor **12** (0.304 g, 0.279 mmol) in anhydrous CH_2Cl_2 (6 mL) was placed in a 10 mL round bottomed flask with freshly activated, crushed 4Å molecular sieves and stirred under argon for 15 min at 0°C. TMS-OTf (0.015 mL, 0.0835 mmol) was added dropwise to the reaction mixture, which was gradually brought to room temperature and stirred for 2 h. The reaction was quenched with Et_3N (0.02 mL, 0.143 mmol),

filtered through Celite, concentrated and purified by column chromatography on silica gel (hexanes/EtOAc 2:1) to give **15** as a slightly yellow powder (0.175 g, 46%). $[\alpha]^{26}_{D}$ 106.1 (c=1 in $CHCl_3$) R_f=0.53 (EtOAc/hexanes 1:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.20; 8.02; 7.97; 7.68; 7.55-7.64; 7.36-7.52; 7.31; 7.23; 7.11; 6.97 (10m, 35H, 7 x Bz); 5.93 (m, 1H, OCH_2CHCH_2); 5.87 (dd, 1H, $^3J_{H2/H3}$=9.5 Hz, $^3J_{H3/H4}$=9.5 Hz, αGlcH-3); 5.66 (dd, 1H, $^3J_{H1/H2}$=9.5 Hz,βGalH-2); 5.56 (m, 1H, βGalH-4); 5.53 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGalH-1); 5.34 (m, 1H, OCH_2CHCH_2); 5.30 (dd, 1H, $^3J_{H2/H3}$= 10.3 Hz, αGalH-2); 5.24 (m, 1H, OCH_2'$CHCH_2$); 5.06 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGlcH-1); 4.95 (m, 1H, αGalH-3); 4.80 (d, 1H, $^3J_{H1/H2}$=7.9 Hz, βGalH-1); 4.53-4.60 (m, 2H, βGalH-6, βGalH-6'); 4.26 (m, 1H, OCH_2CHCH_2); 4.05-4.18 (m, 5H, OCH_2CHCH_2, βGalH-3, βGalH-5, αGlcH-4, αGalH-4); 4.01 (m, 1H, αGalH-5); 3.87 (dd, 1H, $^3J_{H5/H6}$=11.0 Hz, $^2J_{H6/H6'}$=6.9 Hz, αGalH-6); 3.82 (dd, 1H, $^3J_{H5/H6'}$=11.7 Hz, αGalH-6'); 3.73-3.78 (m, 2H, αGlcH-5, αGlcH-6); 3.40-3.49 (m, 2H,αGlcH-2, αGlcH-6'); 1.95-1.99 (m, 6H, 2 x Ac) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 170.3; 169.7; 166.0; 165.9; 165.7; 165.2; 164.8; 164.5; 133.8; 133.5; 133.2; 133.0-133.1; 132.9; 129.2-130.0; 128.5-128.9; 128.0-128.4; 118.7; 101.3 (βGalC-1); 96.9 (αGlcC-1); 92.9 (αGalC-1); 76.4; 73.2; 71.3; 70.8; 70.6; 69.1; 69.0; 67.9; 67.8; 67.3; 66.8; 64.6; 62.5; 61.8; 61.4; 61.1; 20.7; 20.5 ppm. ESI-TOF HRMS $[C_{74}H_{67}N_3O_{24} + Na]^+$ calc. *m/z* = 1399.4458, found 1399.4391; $[C_{74}H_{67}N_3O_{24} + K]^+$ calc. *m/z* = 1420.3752, found 1420.3016.

Allyl 2,3-di-*O*-benzoyl-4,6-di-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (16)

To a flask containing **15** (0.125 g, 0.0904 mmol), was added 8 mL of thioacetic acid, and was stirred for 24 h at 40°C. The solution was concentrated by two co-evaporations with toluene, and purified by column chromatography on silica gel (EtOAc/hexanes 1:1 → 3:1) to give **16** as a white powder (0.097g, 77%). $[\alpha]^{26}_{D}$ 94.3 (c=1 in $CHCl_3$); R_f=0.15 (hexanes/EtOAc 1:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.20; 8.02; 7.96; 7.68; 7.55-7.63; 7.47-7.53; 7.36-7.45; 7.28-7.35; 7.22; 7.15; 7.03; 6.95 (10m, 35H, 7 x Bz); 5.84-5.92 (m, 2H, $OCH_2C\mathit{H}CH_2$, NH); 5.52-5.67 (m, 5H, βGalH-2, βGalH-4, αGalH-1, αGlcNAcH-3); 5.26-5.31 (m, 2H, αGalH-2, $OC\mathit{H}_2CHCH_2$); 5.23 (m, 1H,$OC\mathit{H}_2CHCH_2$); 4.90-4.94 (m, 2H, αGlcNAcH-1, αGalH-3); 4.80 (d, 1H, $^3J_{H1/H2}$= 7.6 Hz, βGalH-1); 4.50-4.59 (m, 3H, αGlcNAcH-2); 4.06-4.22 (m, 4H, βGalH-3, $OCH_2CHC\mathit{H}_2$); 4.00 (m, 2H, $OCH_2CHC\mathit{H}_2$); 3.88 (dd, 1H); 3.78-3.84 (m, 2H); 3.66-3.72 (m, 2H); 1.96-2.00 (m, 6H, 2 x Ac); 1.86 (s, 3H, NH*Ac*) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 170.3; 170.2; 169.7; 166.6; 166.1; 165.9; 165.7; 165.2; 164.8; 164.6; 133.9; 133.5; 133.3; 133.1; 133.1; 132.9; 130.0; 129.5-129.8; 129.4; 129.2; 128.9; 128.6-128.8; 128.0-128.4; 118.6; 101.3 (βGalC-1); 96.4 (αGlcNAcC-1); 92.9 (αGalC-1); 75.9; 73.2; 71.7; 71.4; 70.8; 69.0; 68.8; 67.9; 67.8; 67.3; 66.8; 64.6; 62.5; 61.8; 61.1; 52.1; 29.8; 23.3; 20.8; 20.5 ppm. ESI-TOF HRMS $[C_{76}H_{71}NO_{25} + H]^+$ calc. *m/z* =1398.4393, found 1398.4308; $[C_{76}H_{71}NO_{25} + Na]^+$ calc. *m/z* = 1420.4213, found 1420.4487; $[C_{76}H_{71}NO_{25} + K]^+$ calc. *m/z* = 1436.3935, found 1436.3893.

3-(acetylthio)propyl 2,3-di-*O*-benzoyl-4,6-di-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-3,6-di-*O*-benzoyl-α-*D*-glucopyranoside (17)

To a solution of **16** (0.030 g, 0.022 mmol) and AIBN (0.004 g, 0.022 mmol) in anhydrous THF (3 mL), thioacetic acid (0.016 mL, 0.222 mmol) was added and stirred under argon for 5 min. The solution was then placed in a Rayonet UV reactor (350 nm) and stirred for 12 h under water cooling (~ rt). The solution was concentrated by two co-evaporations with toluene, and purified by column chromatography on silica gel (EtOAc/Hex 2:1) to give **17** as a white powder (0.028g, 89%). $[\alpha]^{26}_D$ 94.2 (c=0.5 in $CHCl_3$); R_f=0.48 (EtOAc/hexanes 2:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.20; 8.01; 7.96; 7.68; 7.55-7.63; 7.47-7.53; 7.20-7.45; 7.13; 7.06; 6.95 (10m, 35H, 7 x Bz); 6.19 (d, 1H, $^3J_{NH/H2}$=9.3 Hz, NH); 5.52-5.67 (m, 4H, βGalH-2, αGalH-2); 5.52 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGalH-1); 5.28 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, $^3J_{H3/H4}$=10.3 Hz, αGalH-3); 4.92 (m, 1H, αGalH-4); 4.83 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGlcNAcH-1); 4.79 (d, 1H, $^3J_{H1/H2}$=8.3 Hz, βGalH-1); 4.52-4.59 (m, 3H, αGlcNAcH-2); 4.09-4.16 (m, 2H); 4.04 (m, 1H); 4.00 (m, 1H, αGalH-5); 3.87 (dd, 1H, $^3J_{H5/H6}$=11.7 Hz, $^2J_{H6/H6'}$=6.9 Hz, αGalH-6); 3.81 (dd, 1H, $^3J_{H5/H6'}$=11.7 Hz, αGalH-6'); 3.68-3.78 (m, 3H, OC*H*$_2$CH$_2$CH$_2$); 3.62 (dd, 1H); 3.44 (m, 1H, OC*H*$_2$CH$_2$CH$_2$); 3.09 (m, 1H, OCH$_2$CH$_2$C*H*$_2$); 2.95 (m, 1H, OCH$_2$CH$_2$C*H*$_2$'); 2.32 (s, 3H, S*Ac*); 1.97-1.99 (m, 6H, 2 x Ac); 1.85-1.92 (m, 5H, NH*Ac*, OCH$_2$C*H*$_2$, OCH$_2$C*H*$_2$') ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 195.8; 170.5; 170.3; 169.7; 166.5; 165.9; 165.7; 165.1; 164.6; 133.9; 133.5; 133.2; 133.1; 133.1; 133.0; 132.8; 129.3-130.0; 128.6-129.2; 128.0-128.4; 101.3 (βGalC-1); 97.4 (αGlcNAcC-1); 92.9 (αGalC-1); 76.1; 73.2; 71.8; 71.4; 70.8; 69.1; 67.9; 67.8; 67.3; 66.8; 66.0; 64.6; 62.5; 61.8; 61.1; 51.9; 30.7; 29.8; 29.3; 25.5; 23.2; 20.8; 20.5 ppm. ESI-TOF HRMS $[C_{78}H_{75}NO_{26}S + H]^+$ calc. *m/z* = 1474.4376, found 1474.4222.

Allyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (19)

To a solution of acceptor **13** (Danac, R., Ball, L., et al. 2007) (3.60 g, 7.63 mmol) and donor **18** (Schmidt, R.R. and Michel, J. 1980) (14.0g, 28.42 mmol) in 60 mL anhydrous CH_2Cl_2, BF_3-OEt_2 (1.93 mL, 15.25 mmol) was added and immediately brought to 35-40°C. After 3 h, Et_3N (2.35 mL, 16.87 mmol) was added. The solution was washed one time with a saturated $NaHCO_3$ solution, and the aqueous layer was extracted with CH_2Cl_2. The organic phases were combined, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography on silica gel (EtOAc/Hex 2.3:1) to give **19** as a white powder (5.10 g, 83%). $[\alpha]^{22}_D$ 56.6 (c=1 in $CHCl_3$); R_f=0.30 (EtOAc/hexanes 2:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.07; 7.61; 7.52; 7.47 (4m, 10H, 2 x Bz); 5.91 (m, 1H, $OCH_2C\mathit{H}CH_2$); 5.85 (d, 1H, $^3J_{NH/H2}$=9.6 Hz, NH); 5.62 (dd, 1H, $^3J_{H2/H3}$=11.0 Hz, $^3J_{H3/H4}$=8.3 Hz, αGlcNAcH-3); 5.30 (m, 1H, $OC\mathit{H}_2CHCH_2$); 5.25 (m, 1H, $OC\mathit{H}_2$'$CHCH_2$); 5.13 (m, 1H, βGalH-4); 5.10 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, βGalH-2); 4.91 (d, 1H, $^3J_{H1/H2}$=3.4 Hz, αGlcNAcH-1); 4.82 (dd, 1H, $^3J_{H3/H4}$=3.4 Hz, βGalH-3); 4.69 (m, 1H, αGlcNAcH-6); 4.61 (d, 1H, $^3J_{H1/H2}$=8.3 Hz, βGalH-1); 4.47 (m, 1H, αGlcNAcH-2); 4.41 (dd, 1H, $^2J_{H6/H6'}$=4.1 Hz, $^3J_{H5/H6'}$=11.7 Hz, αGlcNAcH-6'); 4.22 (m, 1H, $OCH_2CHC\mathit{H}_2$); 4.07-4.15 (m, 2H, αGlcNAcH-4, αGlcNAcH-5); 4.03 (m, 1H, $OCH_2CHC\mathit{H}_2$'); 3.64 (dd, 1H, $^3J_{H5/H6}$=8.3 Hz, $^2J_{H6/H6'}$=11.0 Hz, βGalH-6); 3.48 (dd, 1H, $^3J_{H5/H6'}$=5.5 Hz, βGalH-6'); 3.36 (dd, 1H, 6.2 Hz, 8.3 Hz, βGalH-5); 1.80-2.10 (m, 15H, 4 x Ac, NH*Ac*) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 170.2; 169.9; 169.4; 166.4; 166.1; 133.6; 133.5; 133.2; 129.8; 129.7; 128.7; 128.6; 118.6; 101.1 (βGalC-1); 96.5 (αGlcNAcC-1); 76.4; 72.2; 71.0; 70.6; 69.4; 68.9; 68.8; 66.3; 62.6; 60.0; 52.2; 23.2; 20.5-20.8 ppm. ESI-TOF HRMS $[C_{39}H_{45}NO_{17} + H]^+$ calc. *m/z* = 800.2766,

found 800.2864; $[C_{39}H_{45}NO_{17} + Na]^+$ calc. *m/z* = 822.2585, found 822.2022; $[C_{39}H_{45}NO_{17} + K]^+$ calc. *m/z* = 838.2325, found 838.1110.

3-(acetylthio)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-3,6-di-*O*-benzoyl-α-D-glucopyranoside (20)

To a solution of **19** (0.050 g, 0.063 mmol) and AIBN (0.010 g, 0.063 mmol) in anhydrous THF (3 mL), thioacetic acid (0.045 mL, 0.63 mmol) was added and stirred under argon for 5 min. The solution was then placed in a Rayonet UV reactor (350 nm) stirred for 12 h under water cooling (~ rt). The solution was concentrated by two co-evaporations with toluene, and purified by column chromatography on silica gel (EtOAc/Hex 2:1) to give **20** as a white powder (0.046g, 84%). $[\alpha]^{22}_D$ 45.6 (c=0.9 in $CHCl_3$); R_f=0.25 (EtOAc/hexanes 2:1); ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 8.08; 7.61; 7.52; 7.47 (4m, 10H, 2 x Bz); 6.15 (d, 1H, $^3J_{NH/H2}$=9.6 Hz, NH); 5.58 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, $^3J_{H3/H4}$=8.3 Hz, αGlcNAcH-3); 5.13 (m, 1H, βGalH-4); 5.10 (dd, 1H, $^3J_{H2/H3}$=10.3 Hz, βGalH-2); 4.80-4.84 (m, 2H, αGlcNAcH-1, βGalH-3); 4.70 (m, 1H, αGlcNAcH-6); 4.60 (d, 1H, $^3J_{H1/H2}$=8.3 Hz, βGalH-1); 4.48 (m, 1H, αGlcNAcH-2); 4.40 (dd, 1H, $^2J_{H6/H6'}$=4.1 Hz, $^3J_{H5/H6'}$=11.7 Hz, αGlcNAcH-6'); 4.09 (m, 2H, αGlcNAcH-4, αGlcNAcH-5); 3.80 (m, 1H, OC*H*$_2$CH$_2$CH$_2$); 3.61 (dd, 1H, $^3J_{H5/H6}$= 8.3 Hz, $^2J_{H6/H6'}$=11.0 Hz, βGalH-6); 3.47 (m, 2H, OC*H*$_2$'CH$_2$CH$_2$, βGalH-6'); 3.37 (m, 1H, βGalH-5); 3.10 (m, 1H, OCH$_2$CH$_2$C*H*$_2$); 2.96 (m, 1H, OCH$_2$CH$_2$C*H*$_2$'); 2.35 (s, 3H, S*Ac*); 1.85-2.05 (m, 17H, NH*Ac*, 4 x Ac, OCH$_2$C*H*$_2$CH$_2$) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 195.7; 170.5; 170.1; 169.9; 169.4; 166.3; 166.1; 133.5; 129.8; 129.7; 128.7; 128.6; 101.1 (βGalC-1); 97.4 (αGlcNAcC-1); 76.4; 72.2; 71.0; 70.6; 69.4; 68.9; 66.3; 66.1; 62.6; 60.0; 52.1; 30.7; 29.8; 29.3; 25.6; 23.1; 20.5-20.8 ppm. ESI-

TOF HRMS $[C_{41}H_{49}NO_{18}S + H]^+$ calc. *m/z* = 876.2749, found 876.3192; $[C_{41}H_{49}NO_{18}S + Na]^+$ calc. *m/z* = 898.2568, found 898.2413.

3-(acetylthio)propyl 2-deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (22)

To a solution of **21** (Kiso, M. and Anderson, L. 1979) (0.081 g, 0.209 mmol) and AIBN (0.034 g, 0.209 mmol) in anhydrous THF (5 mL), thioacetic acid (0.149 mL, 2.09 mmol) was added and stirred under argon for 5 min. The solution was then placed in a Rayonet UV reactor (350 nm) and stirred for 12 h under water cooling (~ rt). The solution was concentrated by two co-evaporations with toluene, and purified by column chromatography on silica gel ($CHCl_3$/MeOH 25:1) to give **22** as a white powder (0.082g, 85%). $[\alpha]^{26}_D$ 11.9 (c=1 in $CHCl_3$); R_f=0.30 (MeOH/$CHCl_3$ 1:9) ^{1}H NMR (600 MHz, $CDCl_3$, 300K) δ 6.20 (d, 1H, $^3J_{NH/H2}$=8.9 Hz, NH); 5.17 (dd, 1H, $^3J_{H2/H3}$=8.9 Hz, $^3J_{H3/H4}$=10.3 Hz, H-3); 5.02 (dd, 1H, $^3J_{H4/H5}$=9.62 Hz, H-4); 4.50 (d, 1H, $^3J_{H1/H2}$=8.3 Hz, H-1); 4.20 (dd, 1H, $^3J_{H5/H6}$=12.4 Hz, $^2J_{H6/H6'}$=4.8 Hz, H-6); 4.06 (dd, 1H, $^3J_{H5/H6'}$=12.4 Hz, H-6'); 3.92 (m, 1H, H-2); 3.85 (m, 1H, OC*H*$_2$CH$_2$CH$_2$); 3.64 (m, 1H, H-5); 3.42 (m, 1H, OC*H*$_2$'CH$_2$CH$_2$); 3.00 (m, 1H, OCH$_2$CH$_2$C*H*$_2$); 2.75 (m, 1H, OCH$_2$CH$_2$C*H*$_2$'); 2.28 (s, 3H, S*Ac*); 1.80-2.10 (m, 13H, NH*Ac*, 3 x Ac, OCH$_2$C*H*$_2$CH$_2$); 1.69 (m, 1H, OCH$_2$C*H*$_2$'CH$_2$) ppm. ^{13}C NMR (150 MHz, $CDCl_3$, 300K): δ 196.7; 171.0; 170.8; 170.6; 169.5; 100.8 (C-1); 72.9; 71.8; 68.7; 67.5; 62.2; 54.4; 30.7; 29.4; 25.4; 23.3; 20.6-20.9 ppm. ESI-TOF HRMS $[C_{19}H_{29}NO_{10}S + H]^+$ calc. *m/z* = 464.1590, found 464.1340; $[C_{19}H_{29}NO_{10}S + Na]^+$ calc. *m/z* = 486.1410, found 486.1100; $[C_{19}H_{29}NO_{10}S + K]^+$ calc. *m/z* = 502.1149, found 502.0768.

Immunization Protocol

Groups of five female C57Bl/6 α1,3GalT-KO mice (Tearle, R.G., Tange, M.J., et al. 1996, Thall, A.D., Murphy, H.S., et al. 1996) were immunized subcutaneously with 20 µg Galα(1,3)Galβ(1,4)GlcNAcα-BSA in 200 µl PBS/dose/immunization or 20 µg BSA alone in 200 µl PBS . All animals were immunized four times at 7-day intervals and sacrificed 14 days after the last immunization. Blood was collected by cardiac puncture and serum was separated through centrifugation for analysis by CL-ELISA. All animal procedures were performed according to the vertebrate animal protocols A-201211-1 and A-201411-1, approved by the University of Texas at El Paso's Institutional Animal Care and Use Committee.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

α1,3-GalT-KO, α1,3-galactosyltransferase-knockout; Abs, antibodies; AcSH, thioacetic acid; AIBN, azobisisobutyronitrile; BF_3-Et_2O, boron trifluoride etherate; BSA, bovine serum albumin; Ch anti-α-Gal, anti-α-Gal antibodies purified from sera of patients with chronic Chagas disease; ChD, Chagas disease; CL-ELISA, chemiluminescent-ELISA; ChHSP, pooled sera of chronic Chagas disease patients; DBU, 1,8-diazabicycloundec-7-ene; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DTBS, di-*tert*butylsilyl; $DTBS(OTf)_2$, di-*tert*butylsilyl

bis(trifluoromethanesulfonate); FPLC, fast protein liquid chromatography; HF-pyr, hydrogen fluoride in pyridine; MALDI-TOF, Matrix-assisted laser desorption ionization Time-of-Flight; NGP, neoglycoprotein; NHSP, normal human serum pool; NHS anti-α-Gal, anti-α-Gal antibodies from sera of healthy individuals; NMR, nuclear magnetic resonance; RLU, relative luminescence units; TCEP, tris(2-carboxyethyl)phosphine; tGPI-mucins, trypomastigote-derived GPI-mucins; TMSOTf, trimethylsilyl trifluoromethanesulfonate

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Legends to figures

Figure 1. Target mercaptopropyl saccharides of Galα(1,3)Galβ(1,4)GlcNAcα (1), Galβ(1,4)GlcNAcα (2), GlcNAcα (3), and GlcNAcβ (4).

Figure 2. (**A**) Scheme of conjugation of neoglycoproteins to BSA. a) tris-(2-carboxyethyl)phosphine, phosphate buffer pH 7.2 and maleimide-activated BSA. (**B** and **C**) Matrix-assisted laser desorption ionization-time of flight mass spectra of Galα(1,3)Galβ(1,4)GlcNAcα-BSA and Galβ(1,4)GlcNAcα-BSA, respectively.

Figure 3. (**A**) CL-ELISA reactivity of normal human sera pool (NHSP) vs Chagasic human sera pool (ChHSP) to neoglycoproteins (NGP). (**B**) CL-ELISA reactivity of purified normal human sera anti-α-Gal Abs (NHS anti-α-Gal) vs Chagasic anti-α-Gal Abs (Ch anti-α-Gal) to neoglycoproteins. RLU, relative luminescence units.

Figure 4. CL-ELISA reactivity of α1,3GalT-KO mouse serum to Galα(1,3)Galβ(1,4)GlcNAcα-BSA and BSA before (□) and after (■) immunization with the respective neoglycoproteins.

Scheme 1. Synthesis of disaccharide **11.** a) Bu_2SnO, MeOH, reflux; 4-OMe-benzyl-Cl, Bu_4NBr, benzene, reflux (75%); b) BzCl, pyr (91%); c) DDQ, CH_2Cl_2/H2O (98%); d) TMSOTf, DCM, 0°C, 4Å MS (92%); e) HF-pyr, THF; f) Ac_2O, pyr (89%, 2 steps).

Scheme 2. Synthesis of mercaptopropyl trisaccharide **1**. a) $PdCl_2$, MeOH (87%); b) CCl_3CN, DBU, CH_2Cl_2 (84%); c) TMSOTf, molecular sieves 4Å, CH_2Cl_2 (30% of 1:4 α/β anomers, FPLC separable); d) TMSOTf, 4Å MS, CH_2Cl_2 (46%); e) AcSH (77%); f) AcSH, AIBN, THF, UV light (350 nm) (89%); g) NaOMe, MeOH (quant.).

Scheme 3. Synthesis of mercaptopropyl glycosides **2** and **4**. a) BF_3-Et_2O, CH_2Cl_2, 35-40°C (83%); b) AcSH, AIBN, THF, UV light (350 nm) (84-85%); c) NaOMe, MeOH (quant.).

Figure 1

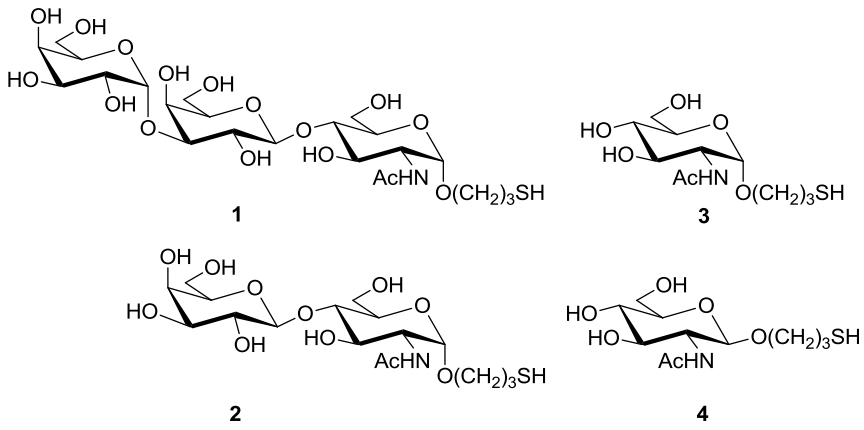
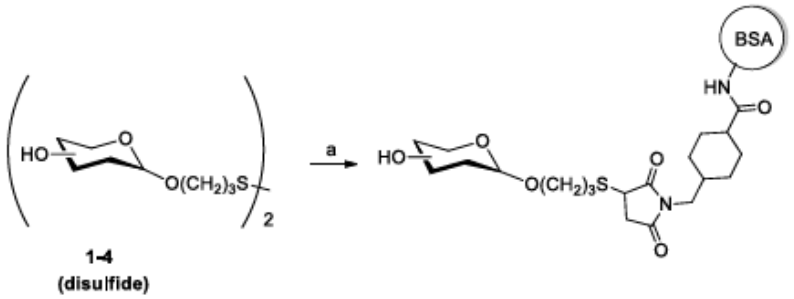


Figure 2



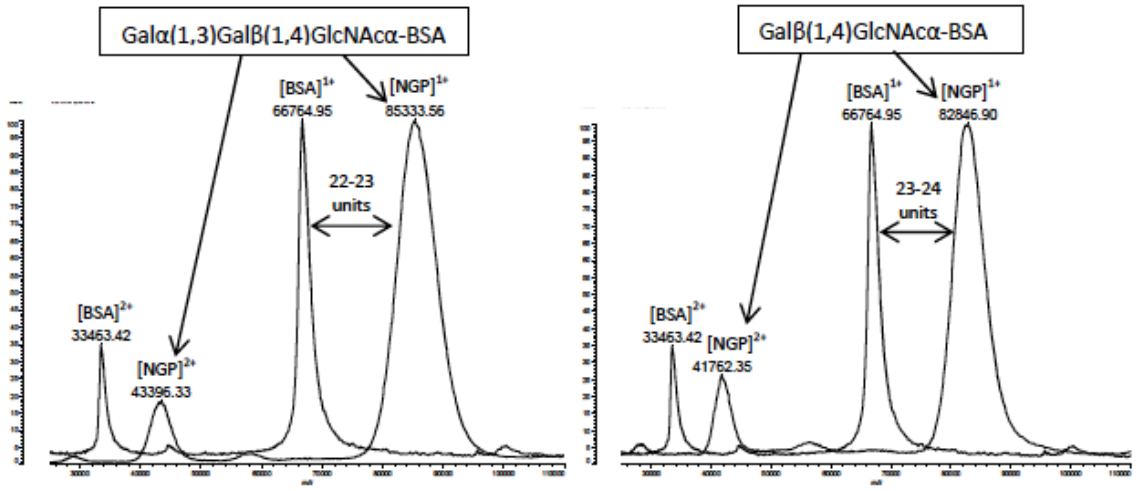


Figure 3

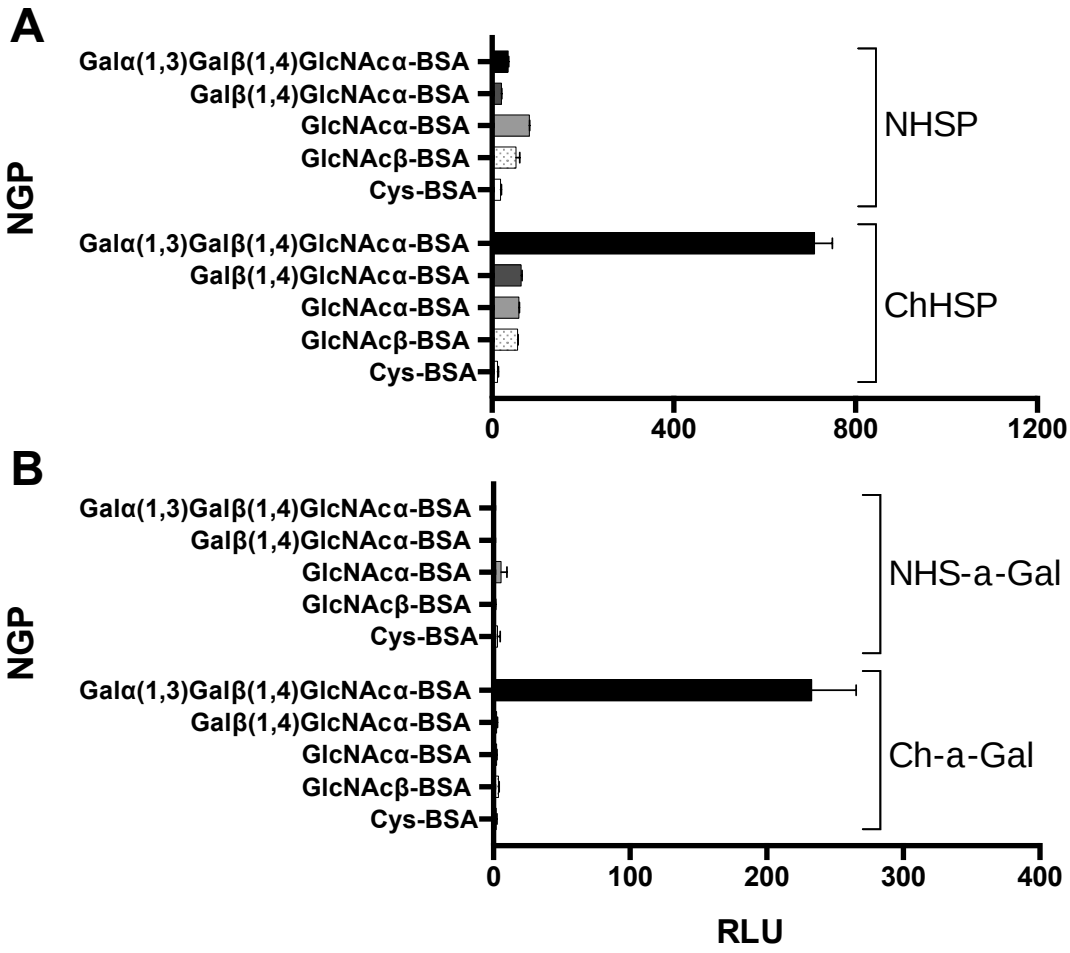
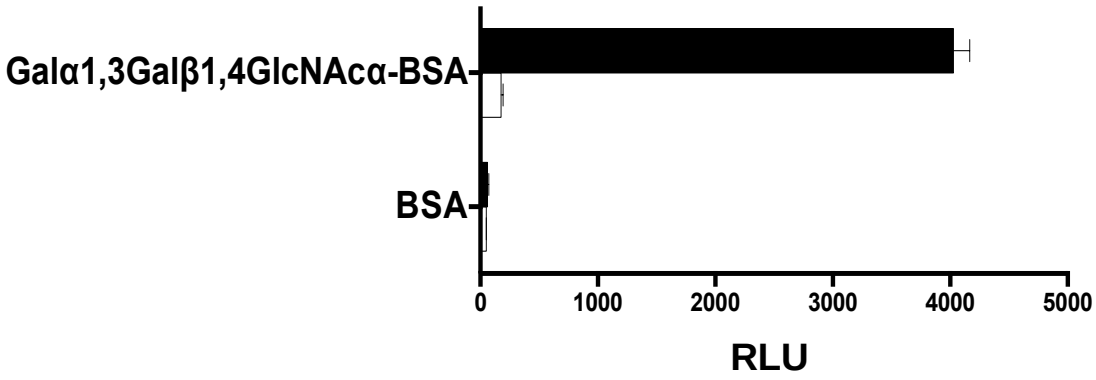
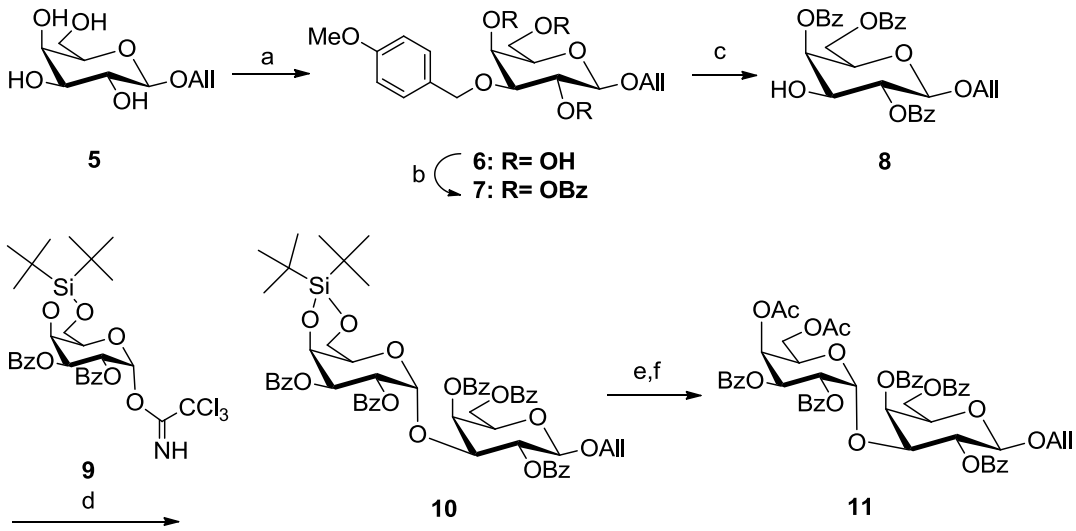


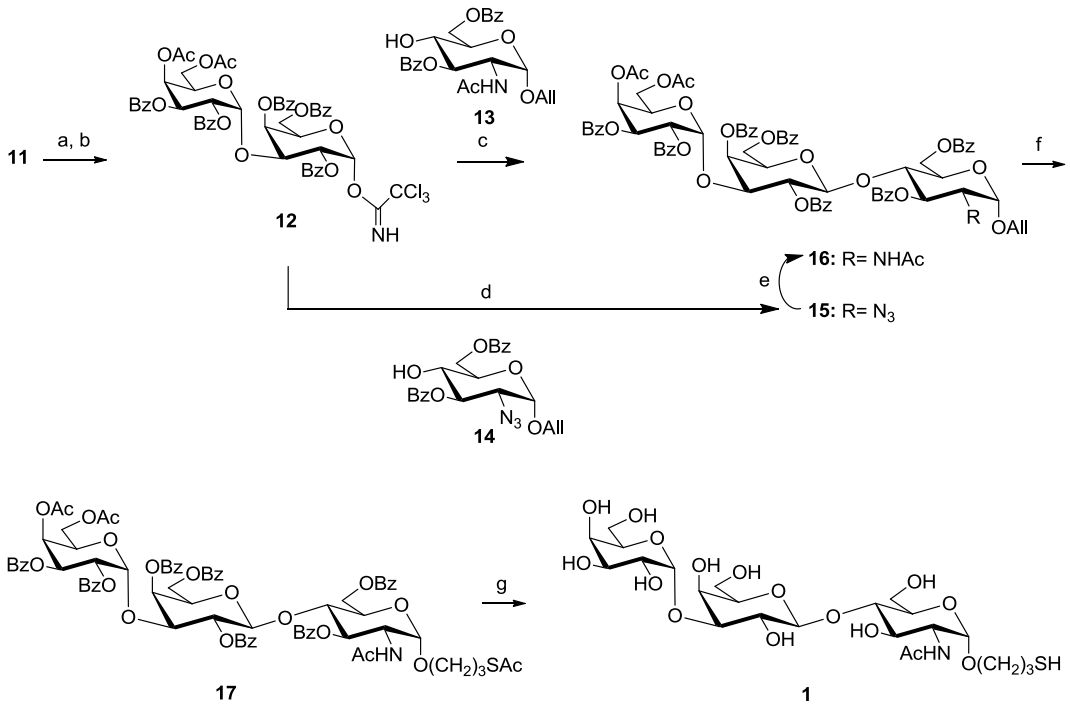
Figure 4



Scheme 1



Scheme 2



Scheme 3