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ARTICLE

The design and synthesis of an α-Gal trisaccharide epitope that provides a highly specific anti-Gal immune response

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Carbohydrate antigens displaying Galα(1,3)Gal epitopes are recognized by naturally occurring antibodies in humans. These anti-Gal antibodies comprise up to 1% of serum IgG and has been viewed as detrimental as they are responsible for hyperacute organ rejections. In order to model this condition, α(1,3)galactosyltransferase-knockout mice are innoculated agaisnt the Galα(1,3)Gal epitope. In our study, two α-Gal trisaccharide epitopes composed of either Galα(1,3)Galβ(1,4)GlcNAc or Galα(1,3)Galβ(1,4)Glc linked to a squaric acid ester moiety were examined for their ability to elicit immune response in KO mice. Both target epitopes were synthesized using a two-component enzymatic system using modified dissacharide substrates containing a linker moiety for coupling. While both glycoconjugate vaccines induced the required high anti-Gal IgG antibody titers, it was found that this response had exquisite specificity for the Galα(1,3)Galβ(1,4)GlcNAc hapten used, with little cross reactivity with the Galα(1,3)Galβ(1,4)Glc hapten. Our findings indicate that while homogenous glycoconjugate vaccines provide high IgG titers, the carrier and adjuvanting factors can deviate the specificty to an antigenic determinant outside the purview of interest.

Introduction

The α-Gal epitope (Galα1-3Gal) is a common type-2 terminus abundantly synthesized on lipids and proteins of non-primate mammals and New World monkeys through glycosylation via α1,3galactosyltransferase (α1,3GalT).[1,2] Interestingly, this epitope is absent in humans and Old World monkeys because the α1,3GalT gene was inactivated in ancestral Old World primates millions of years ago.[2,3,4] The lack of this epitope results in high levels of naturally-occurring anti-Gal antibodies (Abs), which constitute ~1% of circulating immunoglobulins.[5] The presence of large amounts of anti-Gal Abs is a key primary factor in the acute and hyperacute rejection of xenotransplants.[6,7,8,9]

Typically, in order to recapitulate the physiologic condition of α-Gal Abs in an animal model, α1,3GalT knockout (KO) mice[10] are inoculated with the α-Gal epitope via vaccination with rabbit red blood cells (RRBCs) or pig kidney membranes (PKM) homogenates having a high concentration of α-Gal epitopes. However, this method often results in a high production of anti-Gal IgM, with IgG production lower than in humans.[11,12] Our initial attempts to utilize RRBC ghosts to immunize KO mice also resulted in a primarily IgM response that failed to produce memory (Supp. Fig 1).

It has been established that the α-Gal dissacharide exhibits a high degree of conformational flexibility,[13] but the addition of even a single sugar unit severely impacts this flexibility.[14] Inhibition studies indicate that the preferred target antigen in the anti-Gal response is Galα(1,3)Galβ(1,4)GlcNAc.[15,16] However, structural insights suggest that there is no conformational difference between Galα(1-3)Galβ(1-4)Glc and Galα(1-3)Galβ(1-4)GlcNAc (Fig. 1),[14] and as a result are often used interchangeably when designing a hapten for a glycoconjugate vaccine.[13,14,17,18,19]

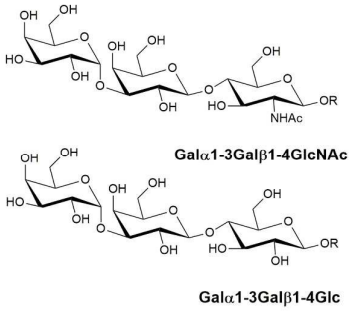

Figure 1. Structures of αGal trisaccharide epitope

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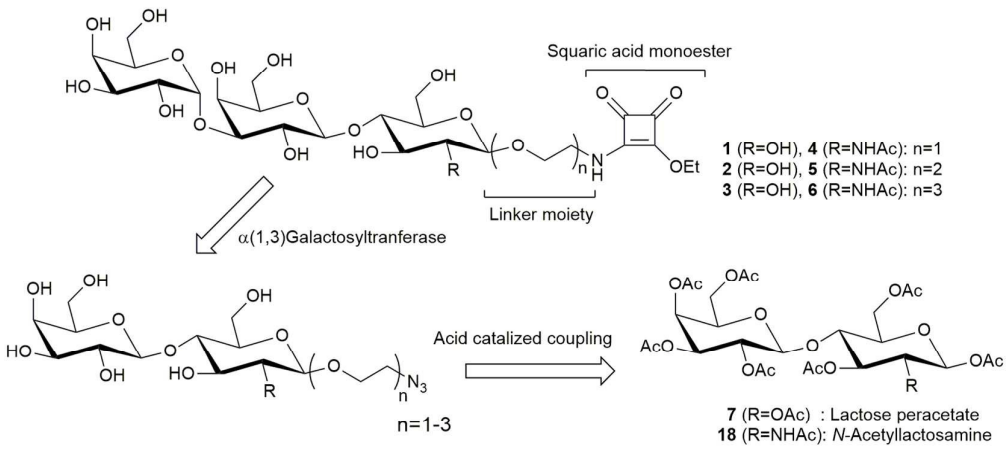

Figure 2. Synthetic strategy of the Galα(1,3)Galβ(1,4)Glc and Galα(1,3)Galβ(1,4)GlcNAc epitope.

During the course of our investigations using haptens based on both the Galα(1-3)Galβ(1-4)GlcNAc and Galα(1-3)Galβ(1-4)Glc epitopes, we discovered exquisite discrimination of the immune response between these two. Our findings demonstrated that while cross-reactivity is dominant with xenoreactive materials (RRBCs, PKM homogenate), the immune response can be tailored to a singular carbohydrate epitope if glycoside-pure materials are evaluated in the vaccination process, and that care must be taken in achieving a relevant approximation of the human condition of the anti-Gal response.

Results

Design of α-Gal epitopes

We designed α-Gal trisaccharide epitopes composed of either Galα(1,3)Galβ(1,4)Glc or Galα(1,3)Galβ(1,4)GlcNAc linked to a squaric acid ester moiety via 2-aminoethyl, 2-(2-aminoethoxy)ethyl, and 2-[2-(2-aminoethoxy)ethoxy]ethyl spacers (compounds **1**–**6**, Fig. 2). We varied the lengths of the spacer in an effort to determine its influence in inducing anti-Gal Abs. The squaric acid ester functionality serves as a handle that can be easily conjugated to carrier proteins via condensation with lysine residues of the protein.[20,21] We note that synthetic methods for the preparation of α-Gal trisaccharides have been reported, however, these involved a need to control selectivity during the glycosylation step.[22,23] We devised a strategy utilizing a chemoenzymatic reaction mediated by α(1,3)galactosyltransferase (α1,3GalT) for incorporating the terminal galactose to disaccharides, which resulted in the facile preparation of our targeted α-Gal epitopes.[24]

Syntheses of the Galα(1,3)Galβ(1,4)Glc epitopes

The syntheses of the Galα(1,3)Galβ(1,4)Glc epitopes were performed as shown in Scheme 1. Coupling of lactose peracetate **7** with 2-chloroethanol in the presence of BF_3-Et_2O to give **8** proceeded in 52% yield.[25] The reaction of peracetate **7** and 2-(2-chloroethoxy)ethanol was, however, inefficient (< 10% yield). Therefore, we utilized the chloroacetimidate strategy to append the 2-(2-chloroethoxy)ethyl and 2-[2-(2-chloroethoxy)ethoxy]ethyl linker moieties.[26] This was accomplished by treatment of **7** with hydrazine acetate to selectively remove the anomeric acetyl group, followed by nucleophilic addition of trichloroacetonitrile using DBU to give the α-isomer of compound **9**. The activated chloroacetimidate **9** readily reacted with 2-(2-chloroethoxy)ethanol or 2-[2-(2-chloroethoxy)ethoxy]ethanol in the presence of BF_3-Et_2O to give β-isomer of compounds **10** and **11** in 58% and 44% yield, respectively. Subsequent azidation and global deacetylation of compounds **8**, **10**, and **11** gave the azido-disaccharide intermediates, which were then used as substrates for the α1,3GalT-mediated incorporation of the terminal galactose. Enzymatic reactions were carried out in 50 mM Tris buffer (pH 7.0) containing 10 mM $MnCl_2$ at 37 °C (see Experimental Procedure) to give the α(1,3)-linked trisaccharides **15**–**17** in 34–54% yield. A unique ^{1}H-NMR peak was observed as a doublet at δ5.15–5.18 ppm (in D_2O solvent), which corresponds to the proton at the 1-position of an α(1,3)-linked galactose.[27] Reduction of azido compounds **15**–**17** to their corresponding primary amines followed by squarate coupling gave the target epitopes **1**–**3** in 39–66% yield.

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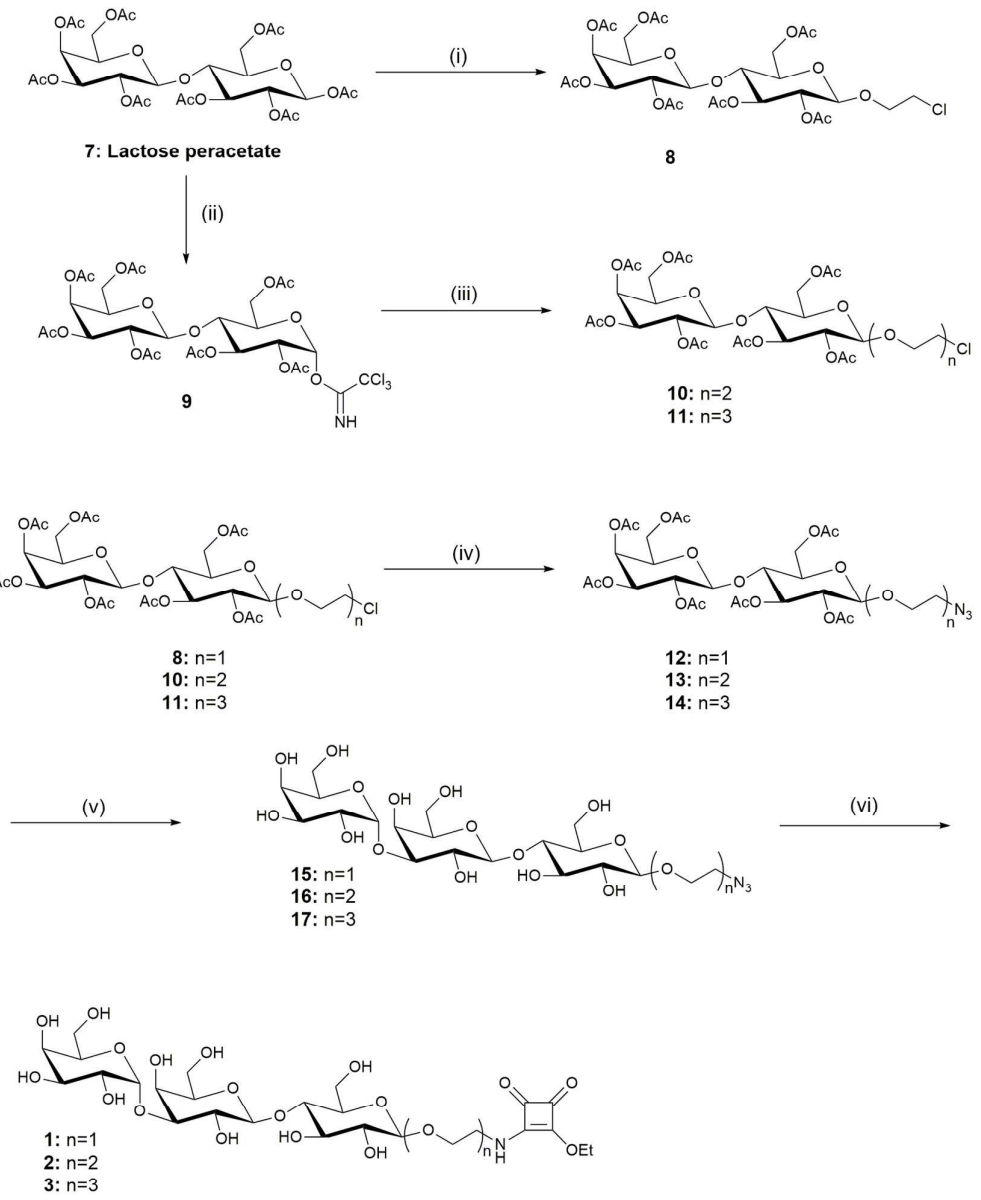

Scheme 1. *Reagents and conditions*: (i) 2-chloroethanol, BF_3-Et_2O, CH_2Cl_2, 0°C then rt, overnight, 52%; (ii) (a)N_2H_4-OAc, DMF, rt, 4 h; (b) DBU, Cl_3CCN, CH_2Cl_2, 0°C then rt, 2h, 58% for 2 steps; (iii) 2-(2-chlroethoxy)ethanol or 2-[2-(2-chloroethoxy)ethoxy]ethanol, BF_3-Et_2O, CH_2Cl_2, 0°C then rt, overnight, 58% (n=2), 44% (n=3); (iv) NaN_3, DMF, 80°C, 48 h, 52-84%. (v) (a) MeONa, MeOH, rt, 5 h; (b) 4mM substrates, 20 μg/mL α1,3GalT, 5 mM UDP-galactose, 10 mM $MnCl_2$, 10 mM Tris-HCl (pH 7.0), 37°C for 72-168 h, 34-54%; (vi) (a) H_2/Pd-C, MeOH, rt, 24 h; (b) Squaric acid ethylester, Et_3N, 50% aq. EtOH, rt, 24h, 39-54%.

Syntheses of the Galα(1,3)Galβ(1,4)GlcNAc epitopes

The starting material *N*-acetyllactosamine **18** was prepared using the method of Wrodnigg et al.[28,29] Peracetylation of **18** followed by reaction with TMSOTf gave the oxazoline intermediate **20**, which was then coupled to azido-ethanol, 2-(2-azidoethoxy)ethanol or 2-[2-(2-azidoethoxy)ethoxy]ethanol under acidic conditions to yield compounds **21**–**23** (Scheme 2).[30] Subsequent deacetylation followed by α1,3GalT-mediated appendage of galactose gave the α(1,3)-linked trisaccharides **24** and **25**. An alternative strategy to access α(1,3)-linked trisaccharides is by exploiting two enzymatic systems [galactose 4-epimerase (GalE) and α1,3GalT] using the inexpensive UDP-glucose as the substrate. In this case, UDP-glucose is first catalytically converted *in situ* by GalE to UDP-galactose, which is then utilized as the substrate of α1,3GalT (Fig. 3). Such two-component enzyme system worked to afford compounds **25** and **26** in 52 and 57% yield, respectively. Hydrogenation of **24**–**26** to their corresponding primary amines followed by condensation with squaric acid ethyl ester furnished the target epitopes **4**–**6**.

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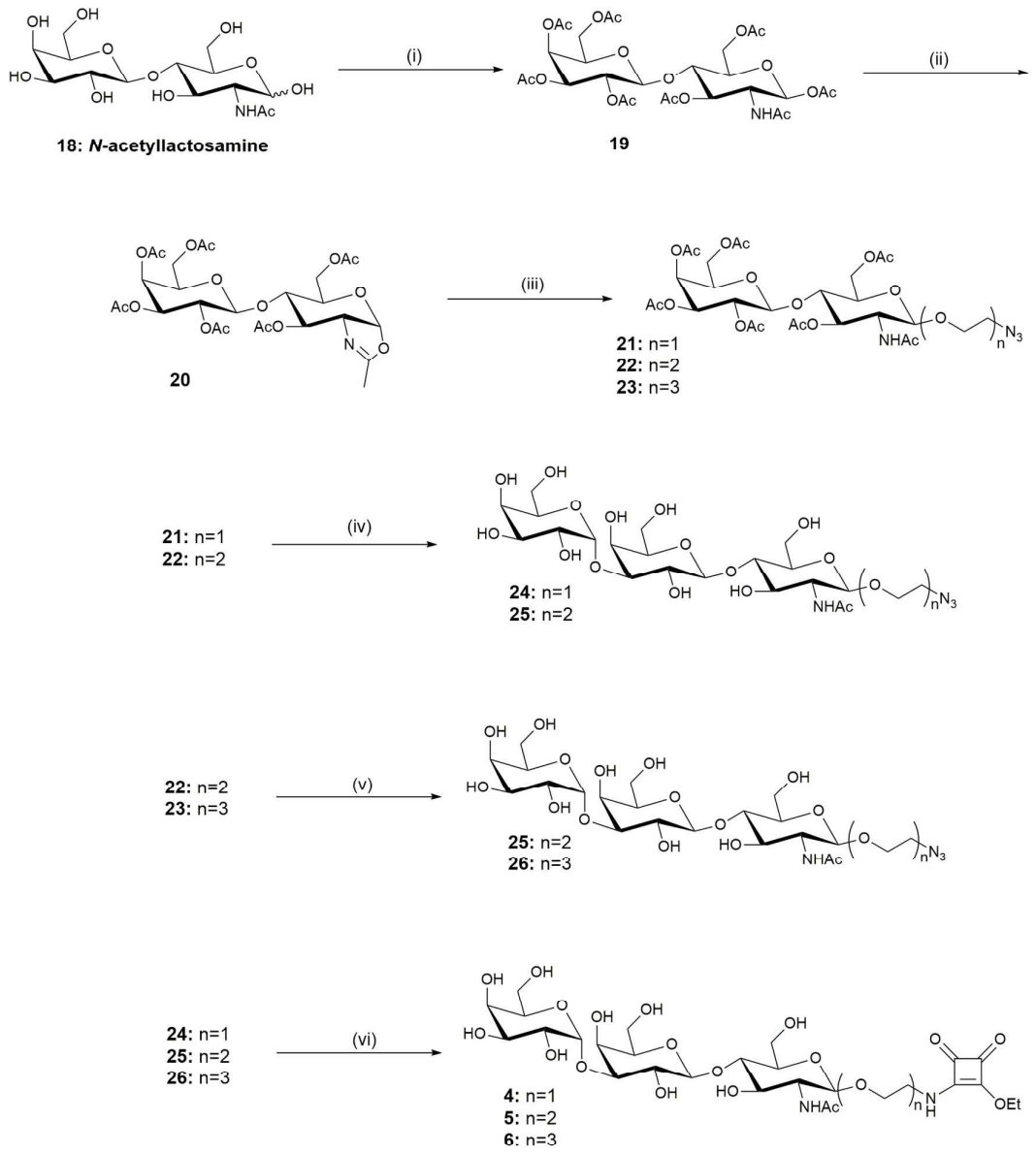

Scheme 2. *Reagents and conditions*: (i) Ac_2O, DMAP, pyridine, rt, overnight, 83%; (ii) TMSOTf, $ClCH_2CH_2Cl$, 50°C, 24h, 68%; (iii) 2-azidoethanol, 2-(2-azidoethoxy)ethanol or 2-[2-(2-azidoethoxy)ethoxy]ethanol, PPTS, $ClCH_2CH_2Cl$, 70°C, 24h, 40-74%; (iv) (a) MeONa, MeOH, rt, 5 h; (b) 4 mM substrates, 20 μg/mL α1,3GalT, 5 mM UDP-galactose, $MnCl_2$, 10 mM Tris-HCl (pH 7.0), 37°C for 72-168 h, 32-62%; (v) (a) MeONa, MeOH, rt, 5 h; (b) 4 mM substrates, 20 μg/mL α1,3GalT, 20 μg/mL GalE, 5 mM UDP-glucose, $MnCl_2$, 10 mM Tris-HCl (pH 7.0), 37°C for 72-168 h, 52-57% (vi) (a) H_2/Pd-C, MeOH, rt, 24 h; (b) Squaric acid ethylester, Et_3N, 50% aq. EtOH, rt, 24h, 47-74%.

Hapten conjugation, vaccination and α-Gal titer determination Targeted α-Gal epitopes **1**–**6** were conjugated to either immunogenic carrier protein keyhole limpet hemocyanin (KLH) or tetanus toxoid (TT) via amidation of the squaric acid moieties under basic borate buffer conditions.[21] In addition to alum,[31] preliminary results utilizing a Galα(1-3)Galβ(1-4)GlcNAc glycoconjugate as well as RRBC ghosts indicated that the formulation benefited from the use of the glycolipid adjuvant C34 (Supp. Fig 1). C34 has been shown to generally induce a class switch from IgM to IgG in mice.[32] The efficacies of the synthetic α-Gal conjugate vaccines were determined by vaccination of α1,3GalT-knockout mice (50 μg α-Gal conjugate/mouse).[10,33] The vaccine schedule of **1**-, **2**-, and **3**-conjugated KLH were 2-week interval injections for a total of 3 administrations (see experimental procedure). Anti-Gal antibody titers of mouse serum samples were determined by ELISA using compound **3**-BSA as the coating antigen (Fig. 4). Titers at 42 days were similar over the course of vaccination schedule in IgG (Midpoint IC_{50}; **1**:30,866, **2**:30,556, **3**:28,753) but

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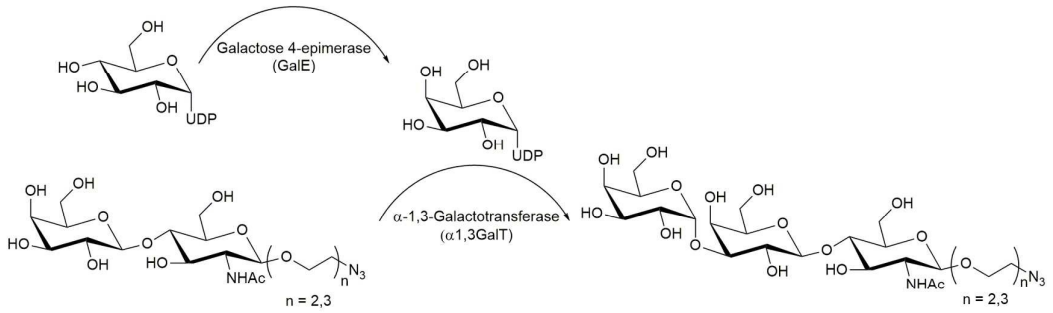

Figure 3. Two enzymatic system of synthesis for Galα(1,3)Galβ(1,4)GlcNAc type epitope

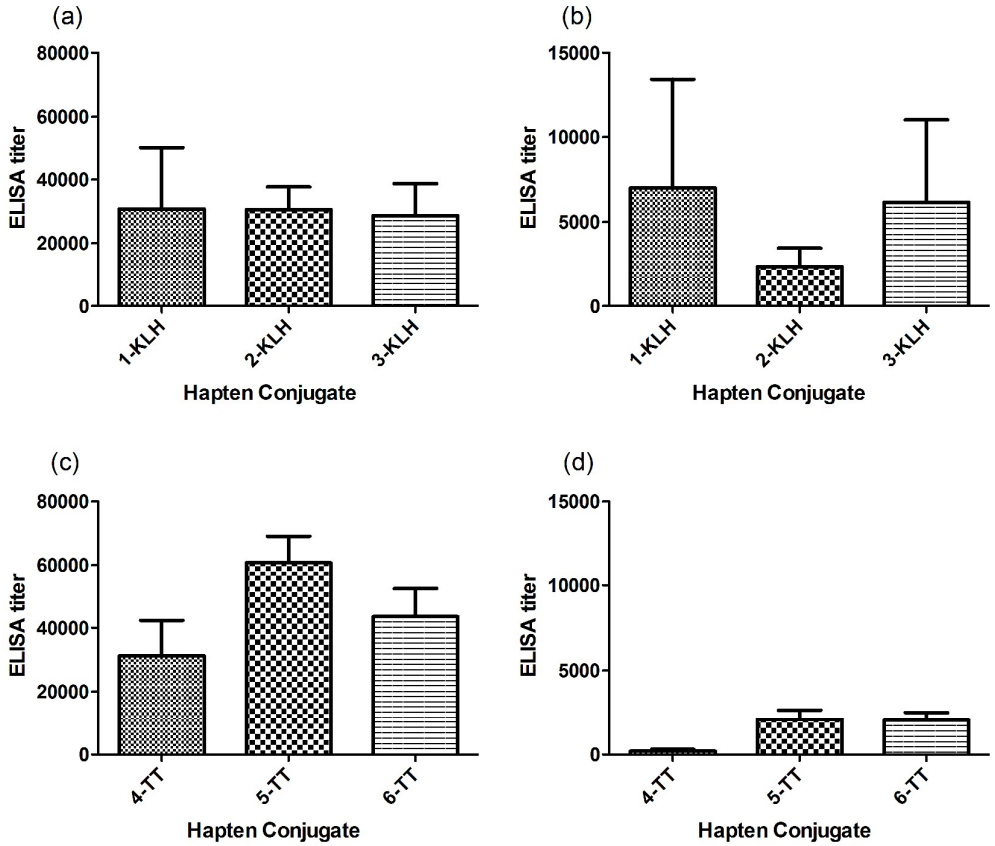

Figure 4. Midpoint anti-Gal antibody titers from compound **1**- (n=2), **2**- (n=4), and **3**-KLH (n=4) vaccinated mice, and compound **4**- (n=7), **5**- (n=7), and **6**-TT (n=7) vaccinated mice. (a) Anti-**3** IgG titers and (b) anti-**3** IgM (37 days) by ELISA using **3**-BSA as a coating antigen. (c) Anti-**6** IgG titers and (d) anti-**6** IgM (51 days) by ELISA using **6**-BSA as a coating antigen.

different for IgM (Midpoint IC_{50}; **1**:7,000, **2**:2,386, **3**:6,169). KLH, while an efficient T-cell epitope, presents difficulty in quantifying the conjugation efficiency. Thus, we changed to tetanus toxoid (TT) conjugated to a Galα(1,3)Galβ(1,4)GlcNAc type trisaccharide. In this series, **4**-, **5**-, and **6**-TT glycoconjugates were administered at 0, 4, and 8 weeks in an effort to increase the class-switching to IgG and stimulate a memory response (see experimental procedure). Antibody titers of mouse serum samples were determined by ELISA using compound **6**-BSA as the coating antigen (Fig. 4). Titers at 51 days were different over the course of vaccination schedule in IgG (Midpoint IC_{50}; **4**:31,203, **5**:60,748, **6**:43,568) and in IgM (Midpoint IC_{50}; **4**:193, **5**:2,084, **6**:2,000). The low IgM titers for these mice was preferable as it indicated an efficient memory IgG response was engendered. Furthermore, compound **5** having the 2-(2-aminoethoxy)ethyl linker trisaccharide results in the highest titer values of all six haptens, indicating this linker length is preferable to maximize the IgG titres than that of other linker lengths

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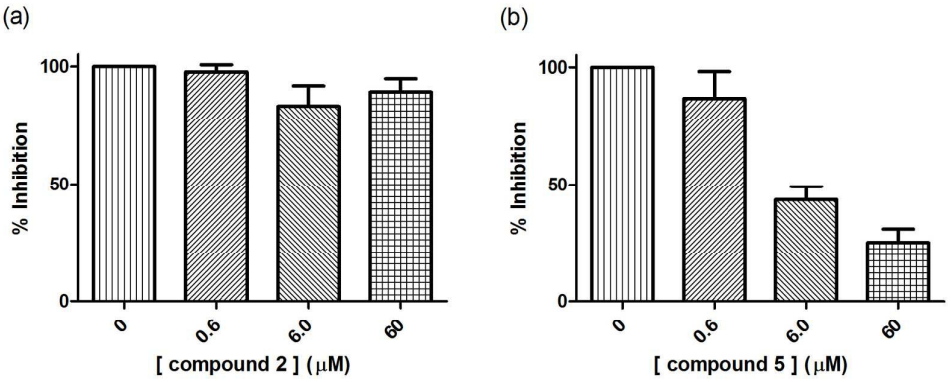

Figure 5. The bar graph for the competitive interaction between anti-**5** antibody sera and compound **6**-BSA conjugate with compound **2** (a) and compound **5** (b) as competitors, which are presented at concentrations of 0, 0.6, 6.0, and 60 μM.

(compounds **4** and **6**). Sham-vaccinated mice showed no appreciable titer.

α-Gal antibody specificity

Among the three TT-conjugates, **5**-TT which contains the 2-(2-minoethoxy)ethyl linker, elicited Abs with the highest titers as determined by ELISA. In order to assess the specificity of the α-Gal antibodies induced by **5**-TT vaccination, we performed competitive ELISA in the presence of varying concentrations (0, 0.6, 6.0, and 60 μM) of either the Galα(1,3)Galβ(1,4)Glu **2** or Galα(1,3)Galβ(1,4)GlcNAc **5** epitope. As depicted in Fig. 5, Galα(1,3)Galβ(1,4)Glc **2** showed no inhibitory effect up to 60 μM (Fig. 5a) whereas the Galα(1,3)Galβ(1,4)GlcNAc **5** epitope displayed a concentration-dependent inhibition (with approximate IC_{50} of ~6.0 μM; Fig. 5b) of the Ab-antigen binding. Moreover, the squaric acid ethylester attached to the 2-(2-minoethoxy)ethyl spacer (but devoid of the trisaccharide unit) also showed no inhibition of antigen binding, indicating that the Abs specifically recognize the α-Gal epitope but not the linker moiety (data not shown). Our results exemplify the exquisite specificity of **5**-TT-elicited Abs for the Galα(1,3)Galβ(1,4)GlcNAc-type epitope over Galα(1,3)Galβ(1,4)Glc.

Discussion

The role of carbohydrates as key biological ligands is well established. Yet, in stark comparison to peptides and proteins, carbohydrates have been notoriously difficult structures to develop effective vaccines against. This low immunogenicity has been attributed to the flexibility and high solvation of glycans.[34] In order to improve the response, it is often necessary to engender greater T-cell help via conjugation of the desired carbohydrate epitope to a T-cell epitope carrier.[34] We employed this strategy in order to achieve high levels of IgG titers in mice. Studies often use the Galα(1-3)Galβ(1-4)GlcNAc and Galα(1-3)Galβ(1-4)Glc epitopes interchangeably, as the natural antibody response is primarily directed against the α-Gal terminus.[13,14] However, evidence suggests that part of the natural Ab response to αGal is directed against the Galβ(1-4)GlcNAc portion of the trisaccharide.[14] This has been attributed to the relative inflexibility of the Galβ(1-4)GlcNAc glycosidic linkage.

We prepared two highly homologous α-Gal epitopes, Galα(1,3)Galβ(1,4)GlcNAc and Galα(1,3)Galβ(1,4)Glc, both predicated upon a chemoenzymatic reaction with α1,3GalT. Using dissacharide substrates functionalized with an ethylene glycol linker, a two-component enzymatic synthesis provided rapid access to these two haptens. In addition, this synthetic scheme was greatly enhanced by utilizing GalE.[22] This allowed the preparation of the very expensive but essential intermediate, UDP-galactose substrate, which was obtained from the inexpensive UDP-glucose.

With facile routes to Galα(1,3)Galβ(1,4)GlcNAc and Galα(1,3)Galβ(1,4)Glc epitopes, antibody selectivity could be evaluated and thus vaccination of these carbohydrate epitopes was initiated. To enhance the vaccination process, a glycosphingolipid bearing an α-galactosyl headgroup and acyl chain terminated with a phenyl moiety, termed C34, was also added to the vaccine cocktail.[32] C34 has been shown to induce a class switch from IgM to IgG in mice. Utilizing this vaccine, high levels of anti-Gal IgG were achieved (Fig. 4), but it is clear that this response has a specificity for the reducing end of the glycone.

α-Gal antibodies in humans readily cross-react with a variety of xenoantigens bearing the α-Gal terminus, the lack of antibody cross-reactivity pattern seen between the Galα(1,3)Galβ(1,4)GlcNAc and Galα(1,3)Galβ(1,4)Glc epitope-vaccines we have prepared (See Fig. 5) demonstrates an exquisite discrimination between these two epiopes. This result clearly indicates that the *N*-acetyl moiety is the key element in antigen recognition by these antibodies generated by both vaccines. We hypothesize this is due to the conformational stability imparted by the additional residue at the reducing end of the oligosaccharide. This stability may provide a higher stochastic exposure of this antigenic determinant during synapse formation, thus driving specificity to this end and outcompeting the response to the terminus. While this subtle change in a single functional group drastically alters antibody recognition, cases of antibody discrimination

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between these two functional groups has been documented and thus highlight the subtle importance of steric and electronic effects in determining antibody-carbohydrate epitope recognition. Similar discrimination has been reported in the isolation of anti-Gal monoclonal Abs,[35] but not in a polyclonal response. Our results demonstrate the ability of this glycoconjugate formulation to discriminate these epitopes almost entirely in the polyclonal response.

Conclusion

In summary a highly efficient, cost-effective chemoenzymatic route has been developed to achieve glycoconjugate vaccines for two common α-Gal haptens. Anti-Gal Abs represent a significant portion of the natural antibody repertoire in humans, and are contentious agents in xenotransplantation. The naturally occurring α-Gal antibody repertoire binds with several modes to α-Gal termini. However, utilizing two chemically pure Galα(1,3)Gal epitopes, we were able to generate a highly honed immune response able to discriminate the Galα(1,3)Galβ(1,4)GlcNAc and Galα(1,3)Galβ(1,4)Glc epitopes. The importance of an N-acetyl for a hydroxyl group substitution between these two epitopes was critical in polyclonal selectivity seen. The information and materials garnered from this campaign are expected to facilitate future vaccine and monoclonal antibody development for both cancer-related as well as drugs of abuse treatments.

Materials and methods

General Methods

^{1}H and ^{13}C NMR spectra were obtained using a Bruker 500 or 600 MHz instrument unless otherwise noted. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, 7.26 ppm) or (CD_3OD, 3.31 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.0 ppm) or (CD_3OD, 49.0 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet). Electrospray Ionization (ESI) mass were obtained on a ThermoFinnigan LTQ Ion Trap. Analytical thin layer chromatography (TLC) was performed on Merck precoated analytical plates, 0.25 mm thick, silica gel 60 F254. Preparative TLC (PTLC) separations were performed on Merck analytical plates (0.50 mm thick) precoated with silica gel 60 F254. Flash chromatography separations were performed on Aldrich silica gel (60 Å pore size, 40-63 μm particle size, 230-400 mesh). The LC/MS analysis was performed using an Agilent G-1956D single quadrupole mass spectrometer equipped with an 1100 series LC system from Agilent Technologies. HPLC separations were performed on a Vydac 218TP C_{18} reversed phase preparative (10–15 μm) HPLC column using a gradient of acetonitrile and water. High resolution mass spectra were obtained in the Scripps Center for Mass Spectrometry. Protein concentration was determined by BCA assay (Pierce BCA Protein Assay Kit) with analysis on a plate reader (Molecular Devices SpectraMax 250) at 562 nm[36], and determination of copy number of protein conjugates was carried out using comparative MALDI-MS, using sinapinic acid as the matrix on an Applied Biosystems VoyagerDE STR MS[37].

Enzyme Expression

The vectors pET15b-GalE and pET15b-α1,3galT encoding the enzymes Galactose-4-epimerase (GalE), and α1,3-Galactosyltransferase (α1,3GalT), respectively, were a generous gift from Prof. Peng George Wang. Proteins were overexpressed in the *E. coli* expression strain BL21(DE3) (Invitrogen) and purified using TALON® metal affinity resin (Clontech). For GalE expression, 50 mL LB/Ampicillin (100 ug/mL) culture was inoculated with an overnight culture and grown at 37°C with shaking (250 rpm) to an OD_{600} ~ 0.6 followed by the addition of isopropyl-1-thio-β-D-galactoside at a final concentration of 0.5 mM. Cells were grown for an additional 3 h at 37°C with shaking and subsequently, harvested by centrifugation at 5837 rpm (~4000 rcf) and 4°. The cell pellet yield was ~ 0.22g per 50 mL culture. For α1,3GalT expression, cell growth was conducted at 20°C and continued for an additional 18 h after IPTG induction. Approximately 0.30 g of cell paste was obtained for α1,3GalT. Cell pellets were stored at -80°C until further processing

Purification of Enzymes

Cell pellets were thawed on ice and then lysed with 5 mL of 0.2 g/L lysozyme (Sigma) in 20 mM Sodium phosphate buffer (pH 7.0) containing 1% Triton X-100. Cells were disrupted by gentle pipetting followed by incubation at 37°C on rotator for 20 min. Subsequently, a 5 uL aliquot of a 2,000 unit/mL DNase I stock (New England Biolab) was added to the solution, followed by further incubation for 20 min at 37°C on rotator. The soluble fraction of the cell lysate was obtained by centrifugation at 13,200 rpm (~16,000 rcf) and 4°C for 20 min and was added to 2 mL of TALON resin (Clonetech) pre-equilibrated with 20 mM Sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl. The column was washed with 10 mL of 20 mM Sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl and the protein was eluted using a step gradient with increasing concentrations of imidazole (20, 50, 100, and 200 mM) in 20 mM Sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl. Protein purity in each 5mL fraction was visualized by SDS-PAGE (supplementary information 2). Fractions containing pure enzyme were pooled and concentrated to ~0.3-0.5 mL using an Amicon 4 centrifugal filter (MWCO 10-kDa) followed by dialysis against PBS. The final enzyme solution was added to equal volume of glycerol to make a 50% glycerol solution for storage at -20°C. Protein concentration was determined by BCA method. Overall protein yield from a 50 mL culture was ~1.5 mg for GalE and ~1.4 mg for α1,3GalT.

Synthesis of 1-position modified β-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside)-2-ethoxycyclobutene-3,4-dione derivatives

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2-Chloroethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (8)

To a mixture of Lactose peracetate **7** (5.0 g, 7.36 mmol) and 2-chlroethanol (0.494 ml, 7.36 mmol) in CH_2Cl_2 (50 ml) was added by dropwise addition of BF_3-Et_2O (1.20 ml, 9.58 mmol) at 0 ^oC over a time course of 20 min. The mixture was diluted with CH_2Cl_2 and washed with ice H_2O and saturated aqueous $NaHCO_3$ (two times), dried over Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Pentane:AcOEt=1:1) to afford **8** (2.69 g, 52%) as a yellow oil.
^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.33 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.19 (t, *J* = 9.3 Hz, 1H, H-3), 5.09 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2'), 4.94 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.90 (dd, *J* = 9.5, 7.9 Hz, 1H, H-2), 4.53 (d, *J* = 7.9 Hz, 1H, H-1'), 4.51 – 4.45 (m, 2H, H-1, H-6a), 4.21 – 3.97 (m, 4H, H-6b, H-6a', H-6b', OCH*H*), 3.88 – 3.83 (m, 1H, H-5'), 3.82 – 3.68 (m, 2H, H-4, OC*H*H), 3.67 – 3.52 (m, 3H, H-5, CH_2Cl), 2.14, 2.11, 2.05, 2.04, 2.03, 2.03, 1.95 (7 x s, 21H, 7 x $COCH_3$). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 170.70, 170.49, 170.41, 170.08, 170.07, 169.42, 101.44, 101.24, 76.54, 73.14, 72.97, 71.82, 71.35, 71.09, 70.31, 69.50, 66.99, 62.24, 61.19, 42.79, 21.22, 21.17, 21.08, 21.01, 20.88. MS (ESI), m/z 699.1900 $(M + H)^+$ ($C_{28}H_{39}ClO_{18}$ requires 699.1898). TLC; R_f 0.29 (Pentane:AcOEt=1:1).

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranose trichloracetimidate (9)

To a mixture of Lactose peracetate **7** (0.5 g, 0.74 mmol) in DMF (5 ml) was added hydrazine acetate (0.73 g, 0.81 mmol). Upon completion of anomeric deacetylation by TLC, 1 ml of water was added to the mixture and concentrated in vacuo. The residue was diluted with AcOEt (20 ml) and washed with 1% HCl (20 ml), saturated $NaHCO_3$ (20 ml), and Brine (20 ml), and the organic phase was dried over Na_2SO_4. The organic phase was filtered and concentrated in vacuo to give the crude glycosyl hemiacetal. The dried crude glycosyl hemiacetal compound in CH_2Cl_2 (10 ml) was treated with DBU (0.044 ml, 0.30 mmol) and to the solution was added trichloroacetonitrile (0.22 ml, 2.2 mmol) at 0^{o}C and stirred at room temperature for 3 hrs. The mixture was concentrated and purified by silica gel column chromatography (Hexane:AcOEt=1:1) to give compound **9** as yellow oil (0.337 g, 58%).
The spectrum for compound **9** was the same as reported previously for the α isomer [24]. ^{1}H NMR (500 MHz, Chloroform-*d*) δ8.65 (s, 1H, NH), 6.47 (d, *J* = 3.8 Hz, 1H, H-1), 5.54 (t, *J* = 9.7 Hz, 1H, H-3), 5.34 (d, *J* = 3.4 Hz, 1H, H-4'), 5.19 – 4.99 (m, 2H, H-2, H-2'), 4.94 (dd, *J* = 10.4, 3.4 Hz, 2H, H-3'), 4.61 – 4.36 (m, 2H, H-6a, H-1'), 4.20 – 4.01 (m, 4H, H-5, H-6b, H-6a', H-6b'), 3.97 – 3.75 (m, 2H, H-4, H-5'), 2.14, 2.09, 2.08 – 2.01, 1.99, 1.95 (s, 21H, 7 x $COCH_3$). TLC; R_f 0.34 (Pentane:AcOEt=1:1).

2-(2-Chloroethoxy)ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (10)

To a mixture of chloroimidate **8** (0.244 g, 0.312 mmol) and 2-(2-chlroethoxy)ethanol (0.037 ml, 0.343 mmol) in CH_2Cl_2 (50 ml) was added by dropwise addition of BF_3-Et_2O (1.20 ml, 9.58 mmol) at -20 ^oC over a time course of 30 min and stirred at room temperature for 3 hrs. The mixture was neutralized with Et_3N (0.056 ml, 0.4 mmol) and concentrated in vacuo. The residue was purified by preparative Thin layer chromatography (CH_2Cl_2:AcOEt=1:1) to afford **10** (0.134 g, 58%) as a yellow oil.
^{1}H NMR (500 MHz, Chloroform-*d*) δ5.34 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.19 (t, *J* = 9.3 Hz, 1H, H-3), 5.10 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2'), 4.95 (dd, *J* = 10.4, 3.5 Hz, 1H, H-3'), 4.89 (dd, *J* = 9.5, 7.9 Hz, 1H, H-2), 4.56 (d, *J* = 7.8 Hz, 1H, H-1'), 4.52 – 4.46 (m, 2H, H-1, H-6a), 4.17 – 4.04 (m, 3H, H-6b, H-6a', H-6b'), 3.91 (ddd, *J* = 11.0, 4.8, 3.6 Hz, 1H, OCH*H*), 3.86 (ddd, J = 7.4, 6.3, 1.2 Hz, 1H, H-5'), 3.79-3.56 (m, 8H, OC*H*H, OCH_2, CH_2O, CH_2Cl, H-5), 2.14, 2.11, 2.07-2.00, 1.96 (7 x s, 21H, $COCH_3$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.49, 170.48, 170.27, 170.19, 169.89, 169.80, 169.20 (7 x *C*OCH_3), 101.20, 100.71, 76.37, 72.94, 72.81, 71.80, 71.55, 71.13, 70.83, 70.46, 69.26, 69.19, 66.75, 62.10, 60.93, 42.9, 21.01, 20.95, 20.87, 20.77, 20.64. MS (ESI), m/z 765.1978 $(M + Na)^+$ ($C_{30}H_{43}ClO_{19}$ requires 765.1979). TLC; R_f 0.71 (CH_2Cl_2:AcOEt=1:1).

2-[2-(2-Chloroethoxy)ethoxy]ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (11)

Chloroimidate **9** (0.276 g, 0.353 mmol) and 2-[2-(2-chloroethoxy)ethoxy]ethanol (0.041 ml, 0.282 mmol) were allowed to react under the same condition as described for the preparation of **10** to give **11** (0.096 g, 44%) as a colorless oil.
^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.30 (dd, *J* = 3.4, 1.1 Hz, 1H, H-4'), 5.15 (t, *J* = 9.3 Hz, 1H, H-3), 5.06 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2'), 4.91 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.85 (dd, *J* = 9.6, 7.9 Hz, 1H, H-2), 4.53 (d, *J* = 7.9 Hz, 1H, H-1'), 4.49 – 4.37 (m, 2H, H-1, H-6a), 4.13 – 4.00 (m, 3H, H-6b, H-6a', H-6b'), 3.92 – 3.52 (m, 15H, H-4, H-5', 3 x OCH_2, 2 x CH_2O, CH_2Cl, H-5), 2.11, 2.08, 2.02, 2.00, 1.92 (7 x s, 21H, $COCH_3$).^{13}C NMR (101 MHz, $CDCl_3$) δ 170.39, 170.37, 170.17, 170.08, 169.79, 169.68, 169.09, 101.11, 100.65, 76.31, 72.85, 72.68, 71.70, 71.41, 71.03, 70.71, 70.70, 70.42, 69.15, 69.10, 66.67, 62.06, 60.86, 42.84, 20.92, 20.86, 20.77, 20.69, 20.68, 20.67, 20.55. MS (ESI), m/z 787.2419 $(M + H)^+$ ($C_{32}H_{47}ClO_{20}$ requires 787.2422). TLC; R_f 0.55 (CH_2Cl_2:AcOEt=1:1).

2-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (12)

To a solution of **8** (0.0841 g, 0.12 mmol) in DMF (4 ml) was added sodium azide (78.2 mg, 1.20 mmol). The mixture was stirred at 60 ^oC for 48 h. The solvent was removed under reduced pressure, and the residue was diluted with AcOEt and washed with H_2O, dried over Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by preparative thin layer chromatography (Pentane:AcOEt=1:1) to afford **12** (0.0652 g, 77%) as a colorless oil.
^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.34 (dd, *J* = 3.4, 1.2 Hz, 1H, H-4'), 5.19 (t, *J* = 9.2 Hz, 1H, H-3), 5.10 (dd, *J* = 10.4, 7.9 Hz,

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1H, H-2'), 4.97 – 4.88 (m, 2H, H-3', H-2), 4.55 (d, *J* = 7.9 Hz, 1H, H-1'), 4.52 (dd, *J* = 12.0, 2.2 Hz, 1H, H-6a), 4.49 (d, *J* = 7.8 Hz, 1H, H-1), 4.17 – 4.03 (m, 3H, H-6b, H-6a', H-6b'), 3.98 (ddd, *J* = 10.6, 5.0, 3.4 Hz, 1H, OCH*H*), 3.86 (ddd, *J* = 7.5, 6.3, 1.3 Hz, 1H, H-5'), 3.84 – 3.77 (m, 1H, H-4), 3.67 (ddd, *J* = 10.6, 8.2, 3.3 Hz, 1H, OC*H*H), 3.61 (ddd, *J* = 9.9, 5.0, 2.2 Hz, 1H, H-5), 3.46 (ddd, *J* = 13.3, 8.2, 3.4 Hz, 1H, CH*H*N_3), 3.26 (ddd, *J* = 13.4, 5.0, 3.3 Hz, 1H, C*H*H N_3), 2.14, 2.11, 2.05, 2.04, 1.95 (s, 21H, 7 x COCH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ170.45, 170.25, 170.17, 169.86, 169.79, 169.18, 101.22, 100.58, 76.28, 72.95, 72.87, 71.63, 71.10, 70.83, 69.26, 68.77, 66.74, 61.92, 60.92, 50.65, 20.97, 20.92, 20.84, 20.76, 20.63. MS (ESI), m/z 728.2121 $(M + H)^+$ ($C_{28}H_{39}N_3O_{18}$ requires 728.2102). TLC; R_f 0.34 (Pentane:AcOEt=1:1).

2-(2-Azidoethoxy)ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (13)

Compound **10** (0.061 g, 0.0775 mmol) was allowed to react under the same condition as described for the preparation of **12** to give **13** (0.030 g, 52%) as a colorless oil.
^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.33 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.19 (t, *J* = 9.3 Hz, 1H, H-3), 5.09 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2'), 4.94 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.89 (dd, *J* = 9.5, 7.9 Hz, 1H, H-2), 4.56 (d, *J* = 7.9 Hz, 1H, H-1'), 4.52 – 4.45 (m, 2H, H-1, H-6a), 4.15 – 4.03 (m, 3H, H-6b, H-6a', H-6b'), 3.91 (dt, *J* = 11.1, 4.4 Hz, 1H, OCH*H*), 3.86 (ddd, *J* = 7.5, 6.3, 1.2 Hz, 1H, H-5'), 3.79 (t, *J* = 9.4 Hz, 1H, H-4), 3.72 (ddd, *J* = 10.9, 6.6, 4.1 Hz, 1H, OC*H*H), 3.66 – 3.56 (m, 5H, OCH_2, CH_2O, H-5), 3.38 – 3.32 (m, 2H, CH_2N_3), 2.14, 2.11, 2.05, 2.03, 1.95 (s, 21H, $COCH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.49, 170.47, 170.27, 170.19, 169.88, 169.81, 169.19, 101.18, 100.74, 76.37, 72.91, 72.77, 71.81, 71.12, 70.80, 70.49, 70.31, 69.23, 69.20, 66.73, 62.09, 60.92, 50.88, 21.00, 20.95, 20.84, 20.78, 20.77, 20.76, 20.65. MS (ESI), m/z 750.2563 $(M + H)^+$ ($C_{30}H_{43}N_3O_{19}$ requires 750.2562). TLC; R_f 0.19 (Pentane:AcOEt=1:1).

2-[2-(2-azidoethoxy)ethoxy]ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (14)

Compound **11** (0.3 g, 0.381 mmol) was allowed to react under the same condition as described for the preparation of **13** to give **14** (0.241 g, 84%) as a colorless oil.
^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.34 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.19 (t, *J* = 9.3 Hz, 1H, H-3), 5.10 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2'), 4.94 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.89 (dd, *J* = 9.6, 7.9 Hz, 1H, H-2), 4.56 (d, *J* = 7.9 Hz, 1H, H-1'), 4.52 – 4.41 (m, 2H, H-1, H-6a), 4.18 – 4.02 (m, 3H, H-6b, H-6a', H-6b), 3.95 – 3.84 (m, 2H, OCH*H*, H-5'), 3.78 (t, *J* = 9.5 Hz, 1H, H-4), 3.75 – 3.54 (m, 10H, H-5, OC*H*H, 2 x OCH_2, 2 x CH_2O), 3.39 (t, *J* = 5.0 Hz, 2H, CH_2N_3), 2.14, 2.11, 2.05, 2.05 – 2.00 , 1.96 (s, 21H, $COCH_3$) . ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.72, 170.69, 170.49, 170.40, 170.12, 170.00, 169.42, 101.45, 100.99, 76.65, 73.19, 73.00, 72.04, 71.36, 71.08, 71.06, 70.76, 70.42, 69.49, 69.41, 66.98, 62.39, 61.17, 51.05, 21.23, 21.18, 21.07, 21.00, 20.87. MS (ESI), m/z 794.2828 $(M + H)^+$ ($C_{32}H_{47}N_3O_{20}$ requires 794.2826). TLC; R_f 0.51 (CH_2Cl_2:AcOEt=1:1).

2-Azidoethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside (15)

To a solution of **12** (0.044 g, 0.0569 mmol) in dry MeOH (10 ml) was added 0.5 M NaOMe (0.224 ml, 0.112 mmol). The mixture was stirred at room temperature for 5 h, and then neutralized with CH_3COOH (0.0064 ml, 0.112 mmol). The mixture was concentrated in vacuo, and the residue was partially purified by silica gel column chromatography (CH_2Cl_2:MeOH=3:1 to 1:1) to afford deacetylate crude compound, and an aliquot of this compound was used for enzyme reaction without further purification. To a solution of 5 mMUDP-Galactose and 4mM deacylated compound (0.0123 g, 0.03 mmol) in 50 mM Tris-HCl (pH 7.0) supplemented with 10 mM $MnCl_2$ was added 1,3αGalT (20 μg/mL) to a final volume of 7.5 ml. The solution was incubated at 37°C with agitation and reaction progress was monitored by TLC. When the deacetylated compound was consumed, α1,3GalT was removed from reaction mixture using an Amicon 4 filter (MWCO 10-kDa) by centrifugation at 7,830 rpm and room temperature for 15 min. Filtrates were concentrated in vacuo, and the residue was dissolved in H_2O and then passed through a chloride anion-exchange column [Dowex 1 (Cl^-)]. The eluate was concentrated and the residue was purified on a VYDAC C_{18} reversed phase semipreparative (250 mm × 22 mm, 10–15 μm) HPLC column using H_2O and 0.1% TFA in CH_3CN mobile phases. A linear gradient was employed (95:5 H_2O/0.1%TFA in CH_3CN → 5:95 H_2O/0.1%TFA in CH_3CN) over a period of 40 min at a flow rate of 10 mL/min. Fractions containing the desired product were collected, frozen, and lyophilized to give compound **15** (5.9 mg, 34%) as a white solid.
^{1}H NMR (500 MHz, Deuterium Oxide) δ 5.14 (d, *J* = 3.7 Hz, 1H, H-1''), 4.53 (t, *J* = 7.4 Hz, 2H, H-1', H-1), 4.19 (d, *J* = 6.0 Hz, 2H, H-4', H-5''), 4.08 – 3.91 (m, 3H, H-6a, H-4'', H-3''), 3.88 – 3.51 (m, 16H), 3.40 – 3.30 (m, 1H, H-2).^{13}C NMR (500 MHz, Deuterium Oxide) δ 102.33, 101.66, 94.92, 78.05, 76.68, 74.54, 74.27, 73.89, 72.23, 70.32, 69.06, 68.77, 68.61, 68.03, 67.69, 64.29, 60.48, 60.41 59.61, 50.02. MS (ESI), m/z 574.2090 $(M + H)^+$ ($C_{20}H_{35}N_3O_{16}$ requires 574.2090). TLC; R_f 0.56 (2-propanol:AcOEt:H_2O=2:2:1).

2-(2-Azidoethoxy)ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside (16)

Compound **13** (0.030 g, 0.0400 mmol) was allowed to react under the same condition as described for the preparation of deacetylated compound from compound **12** and the aliquot (0.0137 g, 0.03 mmol) of this compound was used for enzyme reaction to give compound **16** (6.7 mg, 36%) as a white solid.
^{1}H NMR (500 MHz, Deuterium Oxide) δ 5.16 (d, *J* = 3.9 Hz, 1H, H-1''), 4.54 (dd, *J* = 7.9, 5.1 Hz, 2H, H-1', H-1), 4.27 – 4.16 (m, 2H, H-4', H-5''), 4.15 – 3.92 (m, 4H), 3.92 – 3.57 (m, 17H), 3.57 – 3.48 (m, 2H, CH_2N_3), 3.41 – 3.31 (m, 1H, H-2). ^{13}C NMR (151 MHz, D_2O) δ 102.36, 101.61, 94.93, 78.10, 76.69, 74.55, 74.23, 73.88, 72.28, 70.33, 69.17, 69.08, 68.79, 68.66, 68.62, 68.23,

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67.70, 64.30, 60.49, 60.42, 59.64, 49.67. MS (ESI), m/z 618.2350 $(M + H)^+$ ($C_{22}H_{39}N_3O_{17}$ requires 618.2352). TLC; R_f 0.50 (2-propanol:AcOEt:H_2O=2:2:1).

2-[2-(2-azidoethoxy)ethoxy]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside (17)

Compound **14** (0.059 g, 0.0743 mmol) was allowed to react under the same condition as described for the preparation of deacetylated compound from compound **12** and the aliquot (0.015 g, 0.03 mmol) of this compound was used for enzyme reaction to give compound **17** (14.3 mg, 68%) as a white solid.
^{1}H NMR (500 MHz, Deuterium Oxide) δ^1H NMR (500 MHz, Deuterium Oxide) δ 5.17 (d, *J* = 3.9 Hz, 1H, H-1''), 4.55 (dd, *J* = 7.9, 2.7 Hz, 2H, H-1', H-1), 4.29 – 4.15 (m, 2H, H-4', H-5''), 4.12– 3.93 (m, 4H), 3.93 – 3.58 (m, 21H), 3.56 – 3.49 (m, 2H, CH_2N_3), 3.37 (td, *J* = 7.7, 2.4 Hz, 1H, H-2). ^{13}C NMR (126 MHz, D_2O) δ 103.25, 102.49, 95.82, 79.01, 77.60, 75.44, 75.14, 74.77, 73.19, 71.23, 70.08, 69.98, 69.89, 69.68, 69.59, 69.52, 69.14, 68.60, 65.21, 61.37, 61.31, 60.55, 50.53. MS (ESI), m/z 662.2613 $(M + H)^+$ ($C_{24}H_{43}N_3O_{18}$ requires 662.2614). TLC; R_f 0.47 (2-propanol:AcOEt:H_2O=2:2:1).

1-(2-aminoethyl) α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside)-2-ethoxycyclobutene-3,4-dione (1)

To a solution of **15** (0.0074 g, 0.0129 mmol) in MeOH (5 ml) was added 5% Pd-C (5.0 mg). The mixture was stirred under hydrogen atmosphere at room temperature for 24 h. The solution was filtered through a pad of celite, and then concentrated under reduced pressure to afford azide reduced crude compound, and this compound was used for next reaction for further purification. To a solution of amine compound in 50% aqueous EtOH (2ml) was added Diethyl squarate (0.0094 ml, 0.0636 mmol) and Et_3N (0.0072 ml, 0.0518 mmol), and stirred at room temperature for 24 hrs. The elute was concentrated and the residue was purified on a VYDAC C_{18} reversed phase semipreparative (250 mm × 22 mm, 10−15 μm) HPLC column using water and CH_3CN mobile phases. A linear gradient was employed (95:5 H_2O/CH_3CN → 5:95 H_2O/CH_3CN) over a period of 40 min at a flow rate of 10 mL/min. Fractions containing the desired product were collected, frozen, and lyophilized to give compound **1** (0.0034 mg, 34% from compound **15**) as a colorless oil.
^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.15 (t, *J* = 3.0 Hz, 1H, H-1''), 4.75 (ddd, 2H, OCH_2CH_3), 4.52 (ddt, *J* = 6.6, 4.9, 1.8 Hz, 2H, H-1', H-1), 4.20 (q, *J* = 5.0, 3.2 Hz, 2H, H-4', H-5''), 4.08 – 3.55 (m, 19H), 3.33 (s, 1H, H-2), 1.55 – 1.34 (m, 3H, CH_2CH_3).
^{13}C NMR (600 MHz, Deuterium Oxide) δ 191.76, 190.35, 188.52, 188.46, 183.00, 182.92, 176.96, 176.76, 173.28, 102.36, 101.70, 94.93, 78.11, 76.70, 74.87, 74.57, 74.30, 74.27, 73.96, 73.91, 72.31, 72.03, 70.46, 70.35, 70.15, 70.11, 69.24, 69.09, 68.90, 68.79, 68.63, 68.38, 67.71, 64.31, 60.50, 60.43, 59.65, 43.71, 43.64, 14.57, 14.53, 14.48. MS (ESI), m/z 672.2343 $(M + H)^+$ ($C_{26}H_{41}NO_{19}$ requires 672.2345). TLC; R_f 0.13 (2-propanol:AcOEt:H_2O=2:2:1).

1-[2-(2-aminoethoxy)]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside)-2-ethoxycyclobutene-3,4-dione (2)

Compound **16** (0.0067 g, 0.0108 mmol) was allowed to react under the same condition as described for the preparation of **1** to give **2** (0.0051 g, 66% from compound **16**) as a colorless oil.
^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.16 (d, *J* = 3.7 Hz, 1H, H-1''), 4.77 – 4.70 (m, 2H, OCH_2CH_3), 4.52 (td, *J* = 9.1, 8.6, 4.1 Hz, 2H, H-1', H-1), 4.23 – 4.15 (m, 2H, H-4', H-5''), 4.11 – 3.52 (m, 23H), 3.34 (d, *J* = 8.3 Hz, 1H, H-2), 1.58 – 1.37 (m, 3H, OCH_2CH_3). ^{13}C NMR (151 MHz, D_2O) δ 188.43, 182.94, 182.85, 176.91, 176.63, 173.27, 102.38, 101.65, 94.93, 78.16, 76.69, 74.87, 74.56, 74.29, 73.96, 72.29, 72.03, 70.46, 70.34, 70.17, 69.54, 69.24, 69.09, 68.96, 68.80, 68.63, 68.31, 68.24, 67.72, 64.31, 60.50, 60.42, 59.64, 43.47, 42.72, 14.51. MS (ESI), m/z 716.2609 $(M + H)^+$ ($C_{28}H_{45}NO_{20}$ requires 716.2608). TLC; R_f 0.26 (2-propanol:AcOEt:H_2O=2:2:1).

1-[2-{2-(2-aminoethoxy)]ethoxy]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-β-D-glucopyranoside)-2-ethoxycyclobutene-3,4-dione (3)

Compound **17** (0.0089 g, 0.014 mmol) was allowed to react under the same condition as described for the preparation of **1** to give **3** (0.0058 g, 54% from compound **17**) as a colorless oil.
^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.15 (dd, *J* = 3.8, 1.7 Hz, 1H, H-1''), 4.78 – 4.68 (m, 2H, OCH_2CH_3), 4.57 – 4.47 (m, 2H, H-1', H-1), 4.26 – 4.14 (m, 2H, H-4', H-5''), 4.10 – 3.53 (m, 27H), 3.42 – 3.28 (m, 1H, H-2), 1.54 – 1.36 (m, 3H, OCH_2CH_3). ^{13}C NMR (151 MHz, D_2O) δ 191.76, 188.40, 182.90, 176.85, 176.61, 173.30, 102.36, 101.59, 94.92, 78.12, 76.70, 74.56, 74.26, 73.90, 72.29, 70.34, 70.16, 69.23, 69.18, 69.09, 68.97, 68.90, 68.79, 68.63, 68.27, 67.71, 64.31, 60.49, 60.42, 59.65, 43.47, 43.35, 14.52. MS (ESI), m/z 760.2867 $(M + H)^+$ ($C_{30}H_{49}NO_{21}$ requires 760.2870). TLC; R_f 0.33 (2-propanol:AcOEt:H_2O=2:2:1).

Synthesis of 1-position modified α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside derivatives.

2-Acetoamido-1,3,6-tri-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-glucopyranose (19)

To a solution of *N*-acetyllactosamine **18** (0.5 g, 1.3 mmol) in pyridine (5 ml) was added acetic anhydride (2.84 ml, 30 mmol) and 4-dimethylaminopyridine (0.061 g, 1.3 mmol), and the mixture was stirred at room temperature for overnight. The mixture was concentrated in vacuo and the resulting residuepurified by silica gel column chromatography (Toluene:AcOEt=1:9) to give compound 5 as yellow oil (0.73 g, 83%). The spectrum for compound **19** was the same as reported previously for the α isomer.[38]
^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.09 (d, *J* = 3.6 Hz, 1H, H-1), 5.74 (d, *J* = 9.2 Hz, 1H, NH), 5.36 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.23 (dd, *J* = 11.1, 8.4 Hz, 1H, H-3), 5.11 (dd, *J* = 10.5, 7.9 Hz, 1H, H-2'), 4.97 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.53 (d, *J* = 7.9 Hz, 1H, H-1'), 4.44 – 4.32 (m, 2H, H-2, H-6a), 4.19 – 4.05 (m,

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3H', H-6b, H-6a', H-6b'), 3.97 – 3.81 (m, 3H, H-5, H-4, H-5), 2.18, 2.15, 2.11, 2.09, 2.06, 1.96, 1.93 (7 x s, 21H, 7 x COCH_3). MS (ESI), m/z 678.2240 $(M + H)^+$ ($C_{28}H_{39}NO_{18}$ requires 678.224). TLC; R_f 0.20 (AcOEt:Toluene=6:1).

2-Methyl-{3,6-di-O-acetyl-1,2-dideoxy-4-O-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-glucopyrano}-[2,1-d]-oxazoline (20)

To a solution of peracetate **19** (0.49 g, 0.723 mmol) in 1,2-dichlroethane was added TMSOTf (0.161 ml, 0.889 mmol), and the resulting mixture was stirred at 50 °C for 5hrs. To the mixture was added Et_3N (0.145 ml, 1.04 mmol) and concentrated in vacuo. The residue was purified by silica gel column chromatography (Toluene:AcOEt:Et_3N=100:200:1) to afford **20** (0.304 g, 68%) as a yellow oil.
^{1}H NMR (600 MHz, Chloroform-*d*) δ 5.88 (d, *J* = 7.3 Hz, 1H, H-1), 5.61 (dd, *J* = 2.6, 1.1 Hz, 1H, H-3), 5.34 (dd, *J* = 3.6, 1.2 Hz, 1H, H-4'), 5.14 (dd, *J* = 10.4, 8.0 Hz, 1H, H-2'), 4.97 (dd, *J* = 10.4, 3.5 Hz, 1H, H-3'), 4.62 (d, *J* = 8.0 Hz, 1H, H-1'), 4.18 (dd, *J* = 12.0, 2.4 Hz, 1H, H-6a), 4.15 – 4.06 (m, 3H, H-2, H-6a', H-6b'), 4.04 (dd, *J* = 12.0, 5.9 Hz, 1H, H-6b), 3.92 (ddd, *J* = 7.6, 6.4, 1.2 Hz, 1H, H-5'), 3.63 (dt, *J* = 9.5, 1.4 Hz, 1H, H-5), 3.46 (ddd, *J* = 9.5, 5.7, 2.3 Hz, 1H, H-4), 2.13, 2.08, 2.07, 2.02, 2.01, 2.00, 1.94 (7 x s, 21H, 7 x COCH_3). MS (ESI), m/z 618.2031 $(M + H)^+$ ($C_{26}H_{35}NO_{16}$ requires 618.2029). TLC; R_f 0.32 ($CHCl_3$:MeOH=20:1).

2-Azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1,4)-*O*-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (21)

To a solution of **20** (0.1 g, 0.162 mmol) and 2-azidoethanol (0.141 mg, 1.62 mmol) in 1,2-dichlroethane(10 ml) was added Pyridinium *p*-TsOH (0.005 g, 0.0199 mmol) and stirred at 70 °C for overnight. The mixture was cooled and neutralized with pyridine (0.25 ml, 3.1 mmol), and concentrated in vacuo. The residue was purified by silica gel column chromatography (n-hexanes:AcOEt=1:1) to afford **21** (0.067 g, 59%) as a colorless oil.
^{1}H NMR (600 MHz, Chloroform-*d*) δ 5.86 (d, *J* = 9.3 Hz, 1H, NH), 5.33 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.13 – 5.04 (m, 2H, H-2', H-3), 4.95 (dd, *J* = 10.5, 3.4 Hz, 1H, H-3'), 4.56 (d, *J* = 7.7 Hz, 1H, H-1'), 4.53 – 4.46 (m, 2H, H-1, H-6a), 4.13 – 3.93 (m, 5H, H-2, H-6b, H-6a', H-6b' OC*H*H), 3.86 (ddd, *J* = 7.5, 6.3, 1.3 Hz, 1H, H-5'), 3.77 (t, *J* = 8.6 Hz, 1H, H-4), 3.67 – 3.59 (m, 2H, OCH*H*, H-5), 3.45 (ddd, *J* = 13.4, 8.3, 3.3 Hz, 1H, CH*H*N_3), 3.24 (ddd, *J* = 13.4, 5.0, 3.2 Hz, 1H, C*H*HN_3), 2.12, 2.09, 2.05, 2.04 – 2.00, 1.94 (7 x s, 21H, 7 x COCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.68, 170.48, 170.47, 170.44, 170.20, 170.12, 169.39, 101.11, 100.84, 75.88, 72.79, 72.44, 70.90, 70.78, 69.15, 68.26, 66.68, 62.17, 60.85, 53.32, 50.67, 23.34, 20.96, 20.94, 20.73, 20.71, 20.60. MS (ESI), m/z 705.2462 $(M + H)^+$ ($C_{28}H_{40}N_4O_{17}$ requires 705.2461). TLC; R_f 0.35 (n-hexanes:acetone=1:1).

2-(2-Azidoethoxy)ethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1,4)-*O*-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (22)

Oxazoline **20** (0.12 g, 0.194 mmol) and 2-(2-azidoethoxy)ethanol (0.254 ml, 1.94 mmol) were allowed to react under the same condition as described for the preparation of **21** to give **22** (0.108 g, 74%) as a white solid.
^{1}H NMR (600 MHz, Chloroform-*d*) δ 5.99 (d, *J* = 9.5 Hz, 1H, NH), 5.35 (dd, *J* = 3.4, 1.2 Hz, 1H, H-4'), 5.08 (ddd, *J* = 26.6, 10.1, 8.1 Hz, 2H, H-2', H-3), 4.96 (dd, *J* = 10.5, 3.4 Hz, 1H, H-3'), 4.58 (d, *J* = 7.9 Hz, 1H, H-1'), 4.52 – 4.40 (m, 2H, H-1, H-6a), 4.21 – 3.99 (m, 3H, H-6b, H-6a', H-6b'), 3.95 – 3.83 (m, 2H, H-2, OC*H*H), 3.83 –3.63 (m, 8H, H-5', H-4, H-5, OCH*H*, 2 x OCH_2), 3.52 – 3.23 (m, 2H, CH_2N_3), 2.14, 2.11, 2.07, 2.05, 1.98 – 1.93 (7 x s, 21H, 7 x COCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.62, 170.50, 170.42, 170.27, 170.20, 170.11, 169.37, 101.38, 101.08, 75.95, 72.76, 72.75, 70.98, 70.76, 70.06, 69.17, 68.66, 66.70, 62.40, 60.87, 53.36, 51.02, 23.27, 20.96, 20.93, 20.72, 20.59. MS (ESI), m/z 749.2726 $(M + H)^+$ ($C_{30}H_4N_4O_{18}$ requires 749.2723). TLC; R_f 0.40 (n-hexanes:acetone=1:1).

2-[2-(2-Azidoethoxy)ethoxy]ethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1,4)-*O*-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (23)

Oxazoline **20** (0.079 g, 0.128 mmol) and 2-[2-(2-azidoethoxy)ethoxy]ethanol (0.224 mg, 1.28 mmol) were allowed to react under the same condition as described for the preparation of **21** to give **23** (0.040 g, 40%) as a colorless oil.
^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.28 (d, *J* = 9.6 Hz, 1H, NH), 5.33 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4'), 5.09 (dd, *J* = 10.5, 7.9 Hz, 1H, H-2'), 5.01 (dd, *J* = 10.2, 8.7 Hz, 1H, H-3), 4.94 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3'), 4.64 (d, *J* = 8.3 Hz, 1H, H-1), 4.52 – 4.41 (m, 2H, H-1', H-6a), 4.14 – 4.04 (m, 4H, H-2, H-6b, H-6a', H-6b'), 3.89 – 3.54 (m, 15H, H-5, H-4, H-5', OCH_2 x 3, CH_2O x 2), 3.50 (ddd, *J* = 5.3, 4.4, 0.9 Hz, 2H, CH_2N_3), 2.12, 2.10, 2.06 – 2.01, 1.95 (7 x s, 21H, 7 x COCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.60, 170.57, 170.52, 170.44, 170.23, 170.14, 169.35, 102.04, 101.23, 76.33, 73.51, 72.68, 71.79, 71.08, 70.75, 70.60, 69.95, 69.23, 68.78, 66.72, 62.51, 60.87, 53.58, 50.60, 23.11, 21.01, 21.00, 20.76, 20.74, 20.63. MS (ESI), m/z 793.2985 $(M + H)^+$ ($C_{32}H_{48}N_4O_{19}$ requires 793.2974). TLC; R_f 0.50 (n-hexane:acetonet=1:1).

2-Azidoethyl α-D-galactopyranosyl-(1,3)–β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (24)

To a solution of **21** (0.0187 g, 0.0265 mmol) in dry MeOH (10 ml) was added 0.5 M NaOMe (0.5 ml, 0.25 mmol). The mixture was stirred at room temperature for 5 h, and then neutralized with CH_3COOH (0.017 ml, 0.3 mmol). The mixture was concentrated in vacuo, and the residue was purified on a VYDAC C_{18} reversed phase semi preparative (250 mm × 22 mm, 10–15 μm) HPLC column using H_2O and 0.1% TFA in CH_3CN mobile phases. A linear gradient was employed (95:5 H_2O/0.1%TFA in CH_3CN → 5:95 H_2O/0.1%TFA in CH_3CN) over a period of 40 min at a flow rate of 10 mL/min. Fractions containing the desired product were collected, frozen, and lyophilized to give deacetylated compound quantitatively. To a solution of 5 mM UDP-Galactose and 4 mM deacetylated compound (0.0118 g, 0.0192 mmol) in 50 mM Tris-HCl (pH 7.0)

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supplemented with 10 mM $MnCl_2$ was added 1,3αGalT (20 μg/mL) to a final volume of 7.5 ml. The solution was incubated at 37°C with agitation and reaction progress was monitored by TLC. When the deacetylated compound was consumed, 1,3αGalT was removed using an Amicon 4 filter (MWCO 10-kDa) by centrifugation at 7,830 rpm and room temperature for 15 min. Filtrates were then concentrated in vacuo and the residue was dissolved in H_2O and passed through a chloride anion-exchange column [Dowex 1 (Cl^-)]. The eluate was concentrated and the residue was also purified on a VYDAC C_{18} reversed phase semi preparative (250 mm × 22 mm, 10–15 μm) HPLC column using H_2O and 0.1% TFA in CH_3CN mobile phases. A linear gradient was employed (95:5 H_2O/0.1%TFA in CH_3CN → 5:95 H_2O/0.1%TFA in CH_3CN) over a period of 40 min at a flow rate of 10 mL/min. Fractions containing the desired product were pooled, frozen, and lyophilized to afford compound **24** (5.1 mg, 32%) as a white solid.

^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.07 (d, *J* = 3.9 Hz, 1H, H-1''), 4.53 (d, *J* = 8.3 Hz, 1H, H-1'), 4.47 (d, *J* = 7.8 Hz, 1H, H-1), 4.16 – 4.06 (m, 2H, H-4', H-5''), 3.98 (ddd, *J* = 11.5, 5.6, 3.0 Hz, 1H), 3.94 (d, *J* = 3.6 Hz, 1H), 3.92 (d, *J* = 2.1 Hz, 1H), 3.87 (dd, *J* = 10.4, 3.3 Hz, 1H), 3.81 – 3.50 (m, 14H), 3.41 (ddd, *J* = 13.9, 7.7, 3.0 Hz, 1H, C*H*HN$_3$), 3.34 (ddd, *J* = 13.8, 5.8, 3.0 Hz, 1H, CH*H*N$_3$), 1.97 (s, 3H, $COCH_3$). ^{13}C NMR (151 MHz, D_2O) δ 174.19, 102.32, 100.50, 94.94, 78.19, 76.70, 74.57, 74.30, 2.06, 71.56, 70.35, 69.11, 68.63, 68.27, 67.70, 64.32, 61.99, 60.50, 60.43, 59.62, 54.50, 49.87, 21.76. MS (ESI), m/z 615.2353 (M + H)$^+$ ($C_{22}H_{38}N_4O_{16}$ requires 615.2355). TLC; R_f 0.22 (2-propanol:AcOEt:H_2O=2:2:1).

2-(2-Azidoethoxy)ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (25)

Compound **22** (0.079 g, 0.105 mmol) was allowed to react under the same condition as described for the preparation of compound **24**. The aliquot (0.015 g, 0.03 mmol) of deacetylated compound from **22** gave compound **25** (12.2 mg, 62% from compound **22**) as a white solid.

^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.11 (d, *J* = 3.9 Hz, 1H, H-1''), 4.55 (dd, *J* = 8.3, 1.6 Hz, 1H, H-1'), 4.51 (d, *J* = 7.9 Hz, 1H, H-1), 4.19 – 4.11 (m, 2H, H-4', H-5''), 4.01 – 3.87 (m, 4H), 3.85 – 3.59 (m, 17H), 3.55 (d, *J* = 7.0 Hz, 1H), 3.49 – 3.43 (m, 2H, CH_2N_3), 2.01 (s, 3H, $COCH_3$). ^{13}C NMR (151 MHz, D_2O) δ 174.04, 102.30, 100.44, 94.93, 78.18, 76.69, 74.53, 74.21, 72.04, 70.32, 69.08, 68.80, 68.62, 67.69, 64.31, 60.48, 60.42, 59.62, 54.53, 49.71, 21.66. MS (ESI), m/z 659.2617 (M + H)$^+$ ($C_{24}H_{42}N_4O_{17}$ requires 659.2618). TLC; R_f 0.31 (2-propanol:AcOEt:H_2O=2:2:1).

2-(2-Azidoethoxy)ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (25)

In an alternative method for the generation of compound **25**, the deacylated compound from **22** was allowed to react in a coupled enzyme system containing both 1,3αGalT and GalE. To a solution of 5 mM UDP-Glucose, 4 mM deacetylated compound (0.015 g, 0.03 mmol) in 50 mM Tris-HCl (pH 7.0) supplemented with 10 mM $MnCl_2$ was added 1,3αGalT (20 μg/mL) and GalE (20 μg/mL)[22] to a final volume of 7.5 ml. The same work-up procedure as described for compound **24** afforded compound **25** (11.2 mg, 57%) as a white solid.

2-[2-(2-azidoethoxy)ethoxy]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (26)

The deacylated compound from **23** was allowed to react under the same conditions as described for the preparation of compound **25** to give compound **26** (11 mg, 54% from compound **23**) as a white solid.

^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.15 (d, *J* = 3.9 Hz, 1H, H-1''), 4.63 – 4.51 (m, 1H, H-1'), 4.48 (d, *J* = 7.9 Hz, 1H, H-1), 4.25 – 4.16 (m, 1H, H-4'), 4.08 – 3.89 (m, 4H), 3.90 – 3.48 (m, 25H, CH_2N_3), 2.05 (s, 3H, $COCH_3$). ^{13}C NMR (151 MHz, D_2O) δ 174.03, 102.32, 100.47, 94.94, 78.21, 76.70, 74.57, 74.24, 72.07, 70.48, 70.35, 69.15, 68.84, 68.64, 68.50, 67.71, 64.32, 60.50, 59.65, 54.55, 49.74, 21.69. MS (ESI), m/z 703.2879 (M + H)$^+$ ($C_{26}H_{46}N_4O_{18}$ requires 703.288).

1-(2-aminoethyl) α-D-galactopyranosyl-(1,3)-β-Dgalactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside-2-ethoxycyclobutene-3,4-dione (4)

To a solution of **24** (0.0038 g, 0.00647 mmol) in MeOH (5 ml) was added 5% Pd-C (5.0 mg). The mixture was stirred under hydrogen atmosphere at room temperature for 24 h. The solution was filtered through a pad of celite, and then concentrated under reduced pressure to afford azide reduced crude compound, and this compound was used for next reaction for further purification. To a solution of amine compound in 50% aqueous EtOH (2ml) was added Diethyl squarate (0.0047 ml, 0.032 mmol) and Et_3N (0.0035 ml, 0.023 mmol), and stirred at room temperature for 24 hrs. The elute was concentrated and the residue was purified on a VYDAC C_{18} reversed phase semi preparative (250 mm × 22 mm, 10–15 μm) HPLC column using water in CH_3CN mobile phases. A linear gradient was employed (95:5 H_2O/CH_3CN → 5:95 H_2O/CH_3CN) over a period of 40 min at a flow rate of 10 mL/min. Fractions containing the desired product were collected, frozen, and lyophilized to give compound **4** (2.5 mg, 54% from compound **24**) as a colorless oil.

^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.16 (d, *J* = 3.9 Hz, 1H, H-1''), 4.75 (ddd, 2H, OC*H*$_2$CH$_3$), 4.61 – 4.48 (m, 2H, H-1', H-1), 4.26 – 4.16 (m, 2H, H-4', H-5''), 4.10 – 3.92 (m, 4H), 3.91 – 3.53 (m, 16H), 2.00 (s, 3H, $COCH_3$), 1.46 (q, *J* = 7.5 Hz, 3H, CH$_2$C*H*$_3$). MS (ESI), m/z 713.2607 (M + H)$^+$ ($C_{28}H_{44}N_2O_{19}$ requires 713.2611). TLC; R_f 0.32 (2-propanol:AcOEt:H_2O=1:1:1).

1-[2-(2-aminoethoxy)]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside-2-ethoxycyclobutene-3,4-dione (5)

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Compound **25** (0.0112 g, 0.0177 mmol) was allowed to react under the same condition as described for the preparation of **4** to give **5** (0.0063 g, 47%) as a colorless oil.
^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.16 (d, *J* = 3.9 Hz, 1H, H-1''), 4.77 – 4.67 (m, 2H, OCH_2CH$_3$), 4.57 (dd, *J* = 13.2, 8.1 Hz, 2H, H-1', H-1), 4.27 – 4.15 (m, 2H, H-4', H-5''), 4.08 – 3.90 (m, 4H), 3.91 – 3.47 (m, 20H), 2.04 (s, 3H, COCH$_3$), 1.46 (q, *J* = 7.4 Hz, 3H, CH$_2$CH_3). ^{13}C NMR (151 MHz, D$_2$O) δ 188.46, 173.92, 173.17, 102.33, 100.53, 94.94, 78.23, 77.99, 76.69, 74.87, 74.56, 74.29, 72.04, 71.98, 70.35, 70.17, 69.23, 69.10, 68.79, 68.63, 68.53, 68.47, 68.06, 67.71, 64.31, 60.49, 60.42, 59.67, 54.51, 43.50, 21.67, 14.51. MS (ESI), m/z 757.2873 (M + H)$^+$ ($C_{30}H_{48}N_2O_{20}$ requires 757.2875). TLC; R_f 0.41 (2-propanol:AcOEt:H$_2$O=1:1:1).

1-[2-{2-(2-aminoethoxy)]ethoxy]ethyl α-D-galactopyranosyl-(1,3)-β-D-galactopyranosyl-(1,4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside-2-ethoxycyclobutene-3,4-dione (6)

Compound **26** (0.0114 g, 0.0168 mmol) was allowed to react under the same condition as described for the preparation of **4** to give **6** (0.01 g, 74%) as a colorless oil.
^{1}H NMR (600 MHz, Deuterium Oxide) δ 5.16 (d, *J* = 3.9 Hz, 1H, H-1''), 4.77 – 4.67 (m, 2H, OCH_2CH$_3$), 4.57 (dd, *J* = 13.2, 8.1 Hz, 2H, H-1', H-1), 4.27 – 4.15 (m, 2H, H-4', H-5''), 4.08 – 3.90 (m, 4H), 3.91 – 3.47 (m, 24H), 2.04 (s, 3H, COCH$_3$), 1.46 (q, *J* = 7.4 Hz, 3H, CH$_2$CH_3). ^{13}C NMR (151 MHz, D$_2$O) δ 188.38, 173.95, 102.41, 100.53, 94.94, 74.87, 74.57, 74.29, 72.07, 72.02, 70.48, 70.35, 70.16, 69.30, 69.21, 69.16, 68.98, 68.80, 68.64, 68.56, 68.07, 67.71, 64.32, 60.50, 60.43, 59.58, 54.53, 43.48, 21.69, 14.53. MS (ESI), m/z 801.3131 (M + H)$^+$ ($C_{32}H_{52}N_2O_{21}$requires 801.3135). TLC; R_f 0.13 (2-propanol:AcOEt:H$_2$O=1:1:1).

Animals

Mice used have disrupted (knockout) α1,3galactosyltransferase genes[16] and are referred to as knockout (KO) mice (6-10 week old). Mice were group-housed in an AAALAC-accredited vivarium containing temperature- and humidity-controlled rooms, with mice kept on a reverse light cycle (lights on: 9PM-9AM). All experiments were performed during the dark phase, generally between 1PM-4PM. General health was monitored by both the scientists and veterinary staff at The Scripps Research Institute. All studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conjugation of Haptens

Dialyzed KLH, TT or BSA in 0.5M Borate buffer (pH 9.0) was added to the hapten [ca. 447 (compound **1**), 420 (compound **2**), 393 (compound **3**) equiv. for KLH, 205 (compound **4**), 193 (compound **5**), 180 (compound **6**) equiv. for TT, and 92 (compound **3**), 87 (compound **6**) equiv. for BSA] to give a final concentration of 1.25 mg/mL in 0.5M Borate buffer (pH 9.0) and the solutions mixed (rt, 24 h). In order to quantify copy number (hapten density) for each **3**-BSA and **6**-BSA conjugates, samples were submitted for MALDI-TOF analysis and compared MW of **3**-BSA or **6**-BSA and unmodified BSA, respectively, as per the formula: copy number = (MW αGal BSA – MW BSA) / (MW αGal – MW EtOH); MW αGal = 759 (compound **3**), 800 (compound **6**) Da, MW EtOH = 46 Da; MW **3**-BSA = 76,514 Da, MW **6**-BSA = 78,102, MW BSA = 66,432 Da (see supplementary information 2). The resulting protein conjugates were dialyzed into PBS (pH 7.4) at 4 °C. KLH and TT conjugates were used for immunization; BSA conjugates were used for ELISA plate coating.

Vaccine formulation/administration and blood collection

On a per mouse basis, 50 μg of KLH-conjugate (or 50 μg of TT-conjugate) in ~50 μL PBS pH 7.4 was combined with 50 μL of Alhydrogel® (10 mg/mL, Invivogen). The resulting suspension was mixed and subsequently administered to mice intraperitoneally on days 0, 14, and 28 for KLH-conjugates (or 0, 28, 42 for TT-conjugate). Each mouse was bled (via retro-orbital sinus) on days 28 and 42 for KLH-conjugates (or 37 and 51 for TT-conjugates). Blood samples were then centrifuged at 10,000 rpm for 10 min to collect sera.

ELISA

Production of anti-αGal IgG antibody was evaluated by ELISA. Microtiter plates (Costar 3690) were incubated with coating antigen-BSA in PBS (4 μg/mL, 25 μL) (18 h, 37 °C). Then, 5% nonfat milk in PBS (30 min, 37 °C) was added to block nonspecific binding. Mouse sera in 2% BSA were serially diluted across the plate before incubation in a moist chamber (24 h, 4 °C). The plate was washed with dH$_2$O before incubation with peroxidase-conjugated donkey anti-mouse IgG (SouthernBiotech) in a moist chamber (2 h, 25 °C). The plates were further washed with dH$_2$O before being developed with using TMB (Thermo Pierce) and the absorbance at 450 nm measured on a microplate reader (SpectraMax M2e Molecular Devices). Titers were calculated as the dilution corresponding to 50% of the maximum absorbance from a plot of the absorbance versus log(dilution) using GraphPad Prism 6. Competitive ELISA was also performed in a similar manner but with an added step: serum was incubated with dilution of compounds 2 and 5 (0, 0.6, 6.0, 60 μM) in hapten-BSA coated plates for 24 h (4°C).

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