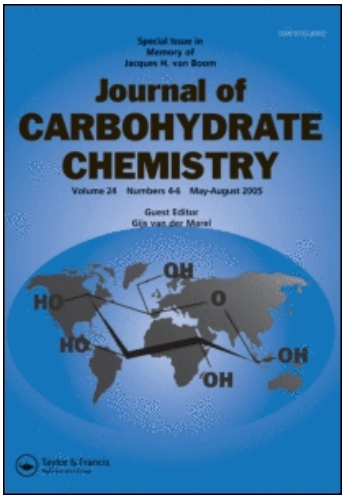

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Frontal Affinity Chromatography Coupled to Mass Spectrometry: An Effective Method for K_d Determination and Screening of α-Gal Derivatives Binding to Anti-Gal Antibodies (IgG)

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ABSTRACT

Frontal affinity chromatography with mass spectrometric detection (FAC/MS) was developed as an effective method for rapid determination of K_d values for α-Gal derivatives binding to human anti-Gal IgG antibodies. Using this method, K_d values for 23 α-Gal compounds were determined for the first time, including an α-Gal terminated *N*-linked oligosaccharide which mimics a single *N*-glycoform present on the surface of animal cells. A mixture of eight α-Gal derivatives, a model for an α-Gal compound library, was successfully screened against this anti-Gal IgG using FAC/MS. The analyte breakthrough sequence, indicated by the ion chromatogram, reflected the magnitude of the K_d values, confirming its potential application in the screening of new α-Gal derivatives and mimetics. Ten α-Gal derivatives were designed and synthesized chemically or enzymatically. Among the compounds analyzed, trivalent

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compound **26** demonstrated the strongest binding affinity to anti-Gal IgG with a K_d value of 3.1 μM. The α-Gal terminated *N*-linked oligosaccharide **28** had a K_d value of 8.6 μM.

Key Words: α-Gal; Anti-Gal antibody; K_d determination; Frontal affinity chromatography.

INTRODUCTION

Xenotransplantation,[1–3] the use of animals instead of humans as a source of organs, tissues and cells, presents a practical solution to the shortage of donated human organs for transplantation. However, xenotransplantation has been hampered by xenograft discordance mainly caused by hyperacute rejection (HAR). Recent studies indicate that HAR can be triggered by the recognition of naturally occurring human anti-Gal antibodies to carbohydrate epitopes bearing a Galα1–3Galβ terminus[4–10] (termed α-Gal or α-Gal epitopes) on the surface of xenograft cells. As a result, antibody-dependent cell-mediated cytotoxicity by human blood monocytes and macrophages along with the cascade of complement-mediated lysis of cells can destroy an organ in minutes to a few hours. Studies seeking to overcome this immunological barrier are concentrating on strategies that could eliminate or reduce the interaction between the α-Gal on tissues and the recipient anti-Gal antibodies. These include anti-Gal antibody immunoadsorption[11–17] and neutralization,[18–23] depletion or inhibition of complement in the recipient or expression of human complement-inhibiting proteins,[24–29] genetic engineering of an α-Gal 'knockout' donor animal or expression of other carbohydrate structures to mask the α-Gal epitopes,[30–35] and elimination of human anti-Gal B cells by α-Gal ricin,[36,37] etc. However, none of these strategies have so far solved the HAR problem found in xenotransplantation. The ultimate solution is likely to be a combination of some or all of these approaches.

Immunoadsorption and neutralization of anti-Gal antibodies are two strategies for vitiating the effects of α-Gal oligosaccharides in xenotransplantation. In immunoadsorption, a potential recipient is depleted of anti-Gal by passing their blood through an α-Gal epitope containing column.[11–17] A number of pig-to-primate organ xenotransplantations following anti-Gal immunoadsorption have been reported. This procedure was shown to extend the survival of pig organs from 1 to 23 days in baboon.[38] More recently, it was found to be difficult to deplete all of the anti-Gal antibody due to antibody heterogeneity. McKane and coworkers[39] found that immunoadsorbents derived from pentasaccharide **3** or trisaccharide **2** (Figure 1) were effective for IgG removal in all individuals. For IgM, no single immunoadsorbent achieves complete removal. For example, pentasaccharide **3** immunoadsorbent only removes anti-Gal IgM in 30% of individuals. Interestingly, the 3-deoxy-trisaccharide (Galα1-3Galβ1-4[3deoxy]GlcNAc) and 6-deoxy-trisaccharide (Galα1-3Galβ1-4[6deoxy]GlcNAc) were the best immunoadsorbents for IgM removal. These two deoxy derivatives were completely successful in 73% of individuals. The polymorphic nature of the anti-Gal antibody repertoire poses a considerable challenge when trying to develop an efficient antibody removal system. So far the best system is the combination of disaccharide **4** and

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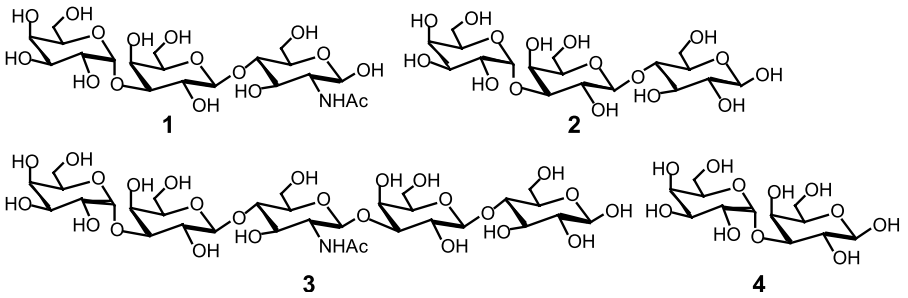

Figure 1. The structures of major α-galactosyl epitopes.

trisaccharide **2** attached to agarose which appeares to be efficient in removing anti-Gal IgG and IgM in all individuals.[39] Simpler, cheaper and more efficient structures would be desirable.

For anti-Gal antibody neutralization, synthetic α-Gal epitopes are infused into the recipient, so that the anti-Gal antibody in the recipient will be neutralized by these epitopes and is no longer free to attack the subsequently transplanted pig organ. Simon, Cooper and coworkers[18] reported the pharmacokinetic parameters for the intravenous administration of tri- and pentasaccharides **1** and **3** in the inhibition of hyperacute rejection of pig hearts transplanted into baboons. The circulatory pharmacokinetics of **1** and **3** were typical of small water-soluble molecules. Their half-lives in serum were 45–50 min, which indicated rapid renal clearance. In the period during which the blood oligosaccharide concentration was greater than 1 mM (initially maintained at 3–12 mM for more than 4 h), the serum cytotoxic activity against porcine cells was completely abolished. This work pointed out several obstacles that need to be overcome before this approach becomes practical. Firstly, large-scale production of α-Gal oligosaccharides[23,40–50] is needed to support more biological testing. Secondly, the affinity of anti-Gal antibodies for α-Gal oligosaccharides is not high. Thirdly, the α-Gal oligosaccharides are cleared from the blood too rapidly. Fourthly, the heterogeneity of anti-Gal antibodies leads to inefficient binding. Therefore, finding molecules that bind to anti-Gal antibody with high affinity is a priority.

Recently, we reported a series of neoglycopolymers with a polyacrylamide backbone bearing varying densities of α-Gal epitopes.[51] The binding affinity of the α-Gal epitope for the anti-Gal antibody was dramatically increased by the "multivalent effect". The IC_{50} values of some of these α-Gal polymers suggested nM binding to IgM and IgA. More progress has also been made by others with non-toxic α-Gal polymers such as α-Gal polylysine conjugate (GAS 914).[52] However, these are impractical because of their high molecular weights. Small α-Gal molecules, dendrimers[53–56] or well-defined α-Gal mimetics with high binding affinity may be a better choice.

Current assays for binding to anti-Gal involve traditional one-compound, one-assay formats. These are slow, thus limiting the number of compounds that can be tested and exclude the evaluation of compound libraries present as a mixture. Moreover, no reports are available on the absolute K_d values for interaction of α-Gal derivatives with anti-Gal subtype IgG, IgM or IgA. Only three compounds have been used to quantify the interaction of anti-Gal (mixture of IgG, IgA and IgM from α-Gal affinity column)

and α-Gal with K_d values using radioactive equilibrium dialysis.[57] This method is restricted to α-Gal derivatives. The need for a rapid and quantitative screening method is clear and we present such a method here.

Frontal affinity chromatography with mass spectrometric detection (FAC/MS) has been recently developed for the screening of compound mixtures.[58–65] In this method, a receptor (10 ∼ 1000 pmol) is immobilized on a suitable support material and packed into a micro-column (2 ∼ 40 μL). A mixture containing potential ligands is then continuously infused through the column. Active ligands will bind to the column until the capacity of the column becomes saturated, resulting in ligand breaking through at the infusion concentration. All non-retained compounds will break through earlier in the void volume of the system. The strongest binding component will appear last in the chromatogram. The components are identified with high sensitivity (down to 1 pmol/μL) and analyzed two-dimensionally using an electrospray ionization (ESI) mass spectrometer. An ion chromatogram is generated from which the breakthrough volume can be determined. The relationship between the breakthrough volume, $V - V_0$, and the concentration of ligand, [x], for the infusion of a single ligand is governed by Eq. 1, where B_t represents the dynamic binding capacity of the column and K_d is the dissociation constant of the ligand. This equation indicates that once B_t and [x] are known, the dissociation constant K_d of the ligand can be determined from a single measurement of its $V - V_0$.

$$V - V_0 = B_t/([x] + K_d) \quad (1)$$

V = breakthrough volume of bound ligand (μL)

V_0 = breakthrough volume of inactive compound (void marker) (μL)

B_t = binding capacity of the column (pmol)

[x] = concentration of the ligand (μM)

K_d = dissociation constant of the ligand [μM]

Herein, we report the application of FAC/MS to quantify the interaction between α-Gal derivatives and human anti-Gal IgG antibodies. Using this method, we were able to determine K_d values of 23 α-Gal derivatives binding to anti-Gal IgG for the first time. The compounds included chemically and enzymatically synthesized monovalent, di- to pentasaccharides, dimeric and trimeric ligands as well as a complex 14-mer α-Gal terminated *N*-linked oligosaccharides. A small α-Gal mixture was analyzed by this method to explore the potential application of FAC/MS for screening α-Gal libraries.

RESULTS AND DISCUSSION

Preparation of a Micro-column Containing an Immobilized Anti-Gal Antibody IgG

The preparation of the anti-Gal IgG FAC/MS column is illustrated in Figure 2. Human sera (type AB, Sigma) was diluted with PBS (pH 7.4), then passed through an

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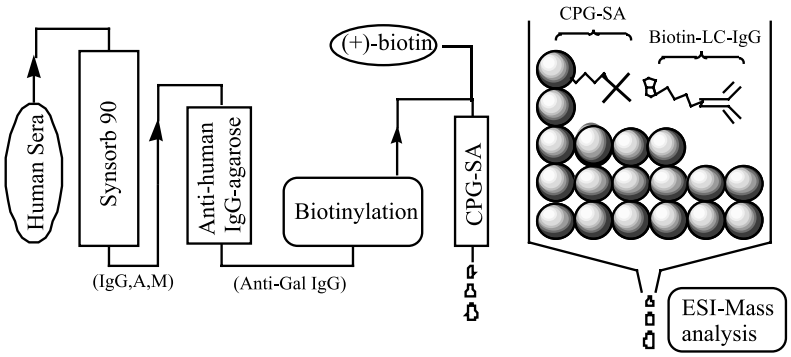

Figure 2. Preparation of the anti-Gal IgG FAC/MS column.

affinity column containing synthetic Galα1–3Galβ1–4Galβ-epitope linked to diatomaceous earth (Synsorb 90, Chembiomed Inc., Edmonton, Alberta, Canada).[66–68] Bound anti-Gal (IgG + IgM + IgA) were eluted with glycine buffer (pH 2.6) after extensive washing of the column. The eluate was immediately brought to pH 7 ∼ 8 with 1 M Na_2CO_3. The resulting anti-Gal antibodies were exhaustedly dialyzed against PBS (pH 7.4), then loaded on to a goat anti-human IgG agarose (Sigma) affinity column. After washing extensively with PBS (pH 7.4), the anti-Gal IgG was eluted with glycine buffer (100 mM, pH 2.8) and the eluate was immediately neutralized to pH 7 ∼ 8 with 1 M Na_2CO_3. The concentration of IgG was estimated by UV absorbance (280 nm).[a] Biotinylation of anti-Gal IgG was done by incubating it with NHS-LC-Biotin (Pierce) in the presence of Galα1–3Galβ1–4GlcNAc for 4 hours at room temperature. Galα1–3Galβ1–4GlcNHAc was added to protect the binding site of anti-Gal from biotinylation. The reaction mixture was diluted with PBS buffer (pH 7.4) and then concentrated by ultrafiltration (NMWL30,000). The process of dilution and ultrafiltration was repeated 5 times. Alternatively, the biotinylated anti-Gal IgG could be purified with a goat anti-Gal IgG immunoaffinity column or α-Gal affinity column. The biotinylated IgG was then infused through a pre-packed column containing controlled porous glass beads with streptavidin immobilized on the surface (CPG-SA) [8.5 cm long polyetheylene ether ketone (PEEK) column with an internal diameter of 750 μm] at slow rate (8 μL/min). The flow-through contained unbiotinylated anti-Gal IgG, which was re-biotinylated and re-infused through the column. The process was repeated 3 times until a preliminary FAC/MS analysis of selected α-Gal derivatives indicated that sufficient IgG had been immobilized to retard the ligands. Finally, the column was saturated with a solution of *d*-biotin in PBS buffer to block unoccupied streptavidin binding sites.

The column capacity, B_t, was determined by infusing compound **29** through the column at various concentrations and measuring the corresponding $V - V_0$ values. A plot of $1/\{[\mathbf{29}]\ (V - V_0)\}$ versus $1/[\mathbf{29}]$ was generated (Figure 3) correlating to

[a]The antibodies were purified by the method reported by Galili (see Refs. [66,67]), a method previously shown to yield only the IgG subtype.

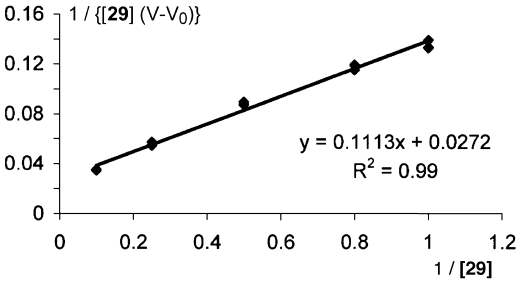

Figure 3. Determination of the column capacity by FAC/MS. Experiment was done with **29** and data points represent the mean of three replicates.

$y = 0.1113x + 0.0272$, where the reciprocal of the y-intercept indicates a B_t of 36.8 pmol ($1/B_t = 0.0272$). The negative reciprocal of the x-intercept indicated a K_d of 4.1 μM for this compound ($K_d/B_t = 0.1113$). The column was stable even after 300 runs.[b]

Chemo-enzymatic Syntheses of α-Gal Derivatives

A series of α-Gal trisaccharides with differing aglycons were synthesized as shown in Scheme 1. The synthesis of α-Gal trisaccharides **9a–e** started with the trisaccharide derivative **7** that was prepared from glycosylation of **5** and **6** according to a reported procedure.[41,51–56] Hydrogenation over PtO_2 in methanol, selectively reduced the azido group of **7** to the primary amine, which was reacted with the corresponding acyl chlorides to give compounds **8a–d**. Compound **8e** was obtained by coupling 2-(2-methoxyethoxy) acetic acid with **7** using 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroxyquinoline (IIDQ) as a promoter. The fully deprotected α-Gal trisaccharides **9a–e** were obtained from **8a–e** after deacetylation and hydrogenation.

The tetrasaccharide **11**, Galα1–3Galα1–3Galβ1–4GlcNAc–β–$(CH_2)_8$COOMe, was synthesized from **10** enzymatically using bovine α1,3-galactosyltransferase (α1,3-GalT). As shown in Scheme 2, tetrasaccharide **11** was obtained in 9.6% yield after the reaction mixture was incubated for 7 days in the presence of an excess of UDP-Gal. The low yield suggested that acceptor **10**, Galα1–3Galβ1–4GlcNAcβ$(CH_2)_8$COOMe, a product of the same enzyme reaction from Galβ1–4GlcNAcβ$(CH_2)_8$COOMe, is a poor acceptor for this enzyme. 1H, 2D-GCOSY, 2D-TOCSY and $^{13}C^1$H–HMQC NMR studies of **11** show H‴1 (5.17 ppm, J = 3.8 Hz), H″1 (5.19 ppm, J = 3.9 Hz), H″3 (4.09 ppm), C″3 (75 ppm). These data support the structure of **11**.

Three polyvalent, low molecular weight α-Gal clusters **22**, **24**, **26** were also synthesized as illustrated in Schemes 3–5. 1,4-di-{*N*-[*O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl)-(1 → 8)-3,6-di-oxo-octyl]carbamido}benzene **22** and 1,3,5-tri-{*N*-[*O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl)-(1 → 8)-3,6-di-oxo-

[b]The column should be stored in a refrigerator (4°C) in PBS (pH 7.4) buffer and warmed to room temperature before use. The B_t value of the column should be checked and calibrated periodically.

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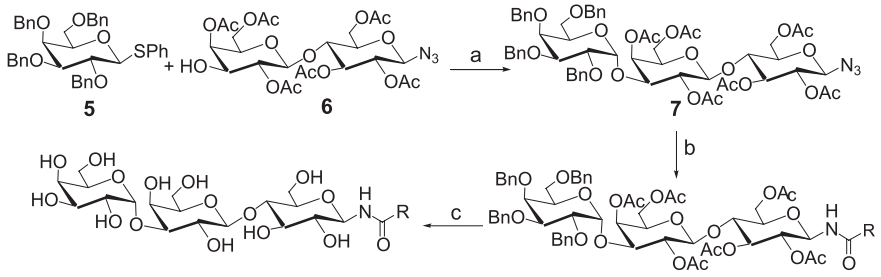


R: Ph **9a**; *o*-(OH)Ph, **9b**; *o*-(MeO)Ph, **9C**; *c*-C_6H_{11}, **9d**; $CH_3OCH_2CH_2OCH_2CO$, **9e.**

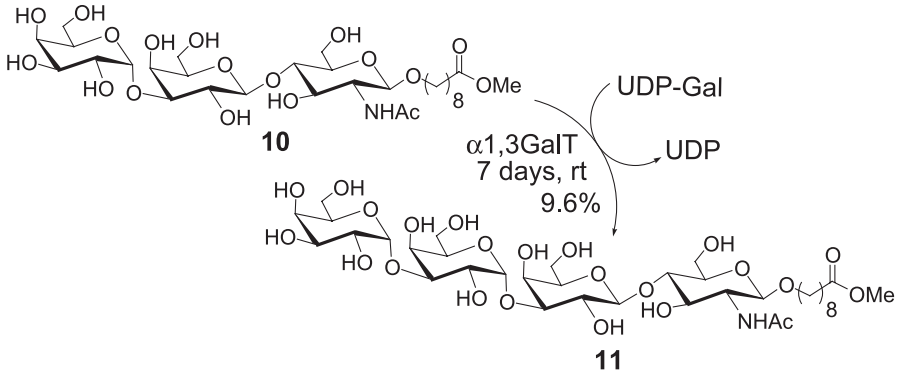
R: Ph **8a**; *o*-(OAc)Ph, **8b**; *o*-(MeO)Ph, **8C**; *c*-C_6H_{11}, **8d**; $CH_3OCH_2CH_2OCH_2CO$, **8e.**

Reagents: (a) NIS, TfOH, CH_2Cl_2, 4 A MS, -30 °C, 90%; (b) PtO_2, H_2, MeOH; then RCOCl, Et_3N, CH_2Cl_2, **8a**, 89%, **8b**, 81%, **8c**, 90%, **8d**, 89%; for **8e**, $CH_3OCH_2CH_2OCH_2COOH$, IIDQ, CH_2Cl_2, 54%; (c) Pd/C, H_2, MeOH; then NaOMe, MeOH, **9a**, 97%, **9b**, 91%, **9c**, 91%, **9d**, 96%, **9e**, 81%.

Scheme 1. Syntheses of trisaccharides **9a–e**.

octyl]carbamido}benzene **24** were synthesized chemically starting from galactose pentaacetate **12**. The glycosylation reaction of **12** and **13** was promoted by $BF_3 \cdot Et20$ in a solution of dichloromethane with 4 Å molecular sieves as a water scavenger (Scheme 3). The reaction gave **14** in 72% yield. Deacetylation of **14** by potassium carbonate in water and methanol gave **15**, which was treated with dibutyltin oxide and *p*-methoxybenzyl chloride[69] successively to selectively protect the 3-position of galactose **15** which was per-*O*-acetylated. Oxidation with ceric ammonium nitrate (CAN)[70] removed the methoxybenzyl group to afford acceptor **17**.

The coupling reaction of **17** and **18** was promoted by methyl triflate[71] in ether in the presence of 4 Å molecular sieves. The reaction gave the α1,3-linked disaccharide in



Scheme 2. Enzymatic synthesis of tetrasaccharide **11**.

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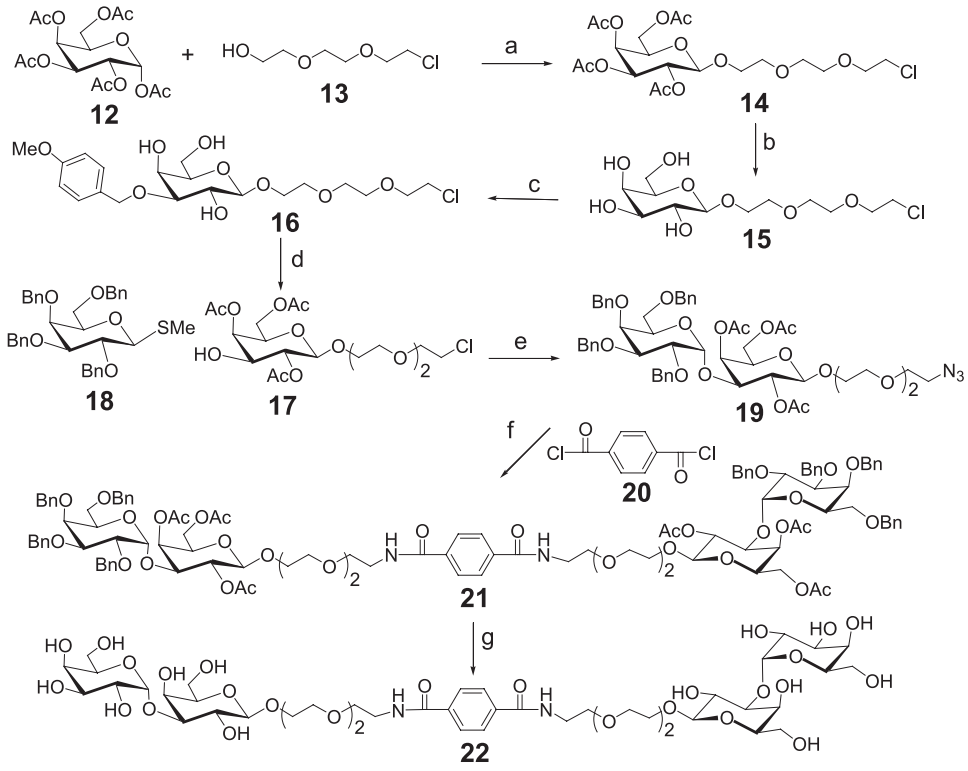

Reagents: (a) BF_3OEt_2, 72%; (b) K_2CO_3, MeOH/H_2O, 92%; (c) Bu_2SnO, MeOH, then MeOBnCl, Bu_4NBr, Benzene, 46%; (d) Ac_2O, Py, 90%, then CAN, CH_3CN/H_2O, 81%; (e) MeOTf, 84%, then NaN_3, DMF, 95%; (f) (i)PtO_2, H_2, MeOH, (ii) **20**, Et_3N, CH_2Cl_2, 66% from **19**; (g) Pd/C, MeOH/AcOH, H_2, then NaOMe, MeOH, 83%.

Scheme 3. Synthesis of the polyvalent α-Gal derivative **22**.

84% yield with an 8/1 (α/β) selectivity, which reacted with NaN_3 to form **19**. The azido group of **19** was then selectively reduced by hydrogenation (PtO_2 in MeOH) to give the amine. This amine was immediately used for coupling with terephthaloyl chloride (Scheme 3) and benzenetricarbonyl chloride (Scheme 4). The coupling reactions afforded protected dimer **21** (66%) and trimer **23** (62%). Debenzylation and deacetylation of **21** and **23** gave the desired products **22** and **24**, respectively. The trivalent α-Gal trisaccharide **26** was obtained in 64% yield from an enzymatic reaction of the trivalent lactoside **25**[72] with UDP-Gal using calf thymus α1, 3-galactosyltransferase (Scheme 5).

In order to investigate the interaction of a single complex-*N*-linked oligosaccharide[4–10,73–75] commonly found on the animal cell surface with the human anti-Gal antibody, an α-Gal triantennary *N*-glycan derivative **28** was also synthesized (Scheme 6).

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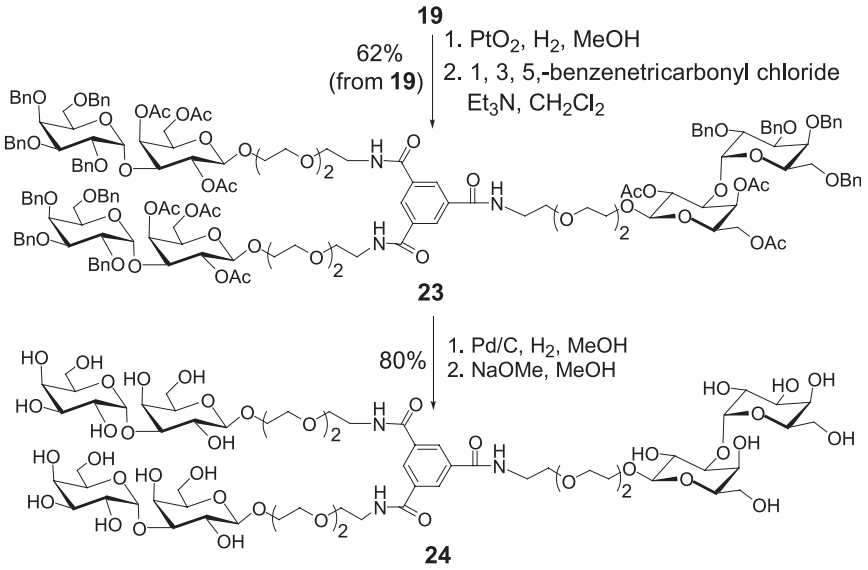
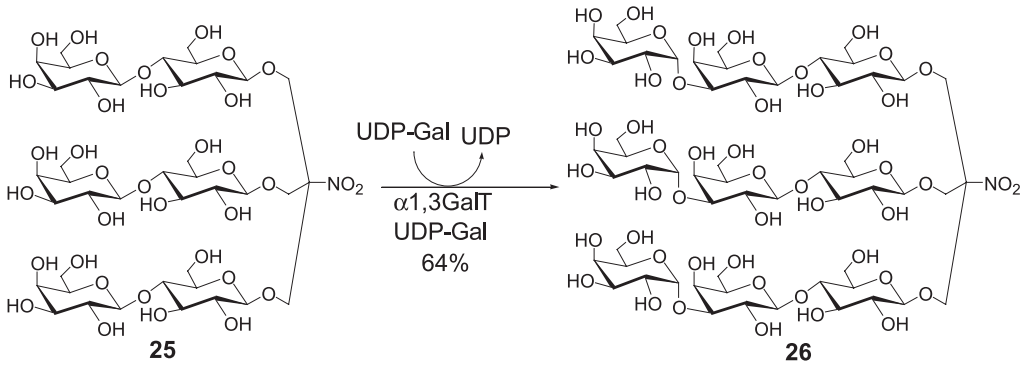


Scheme 4. Synthesis of the polyvalent α-Gal derivative **24**.

The tyrosinamide *N*-glycan **27**[76–79] was incubated with an excess of UDP-Gal and bovine α1,3-GalT[41] for one week at room temperature. The reaction mixture was filtered through glass wool and then ultrafiltered (NMWL 10,000) to remove the protein. The filtrate was lyophilized and purified on a Bio-Gel P-4 column. Further purification was carried out using semi-preparative HPLC. The sample was dissolved in water and applied to a 1 × 25 cm semi-preparative C18 reverse phase (RP) HPLC column, and eluted isocratically at 1 mL/min with 12% acetonitrile. The eluting peaks detected by UV absorbance at 280 nm were pooled and freeze dried (Figure 4). The major peak (1) corresponded to the desired α-Gal terminated oligosaccharide **28** with *m/z* of 2777 and 1400 representing the $M + Na^+$ and $M + 2Na^+$ peaks, respectively.



Scheme 5. Enzymatic synthesis of the polyvalent α-Gal derivative **26**.

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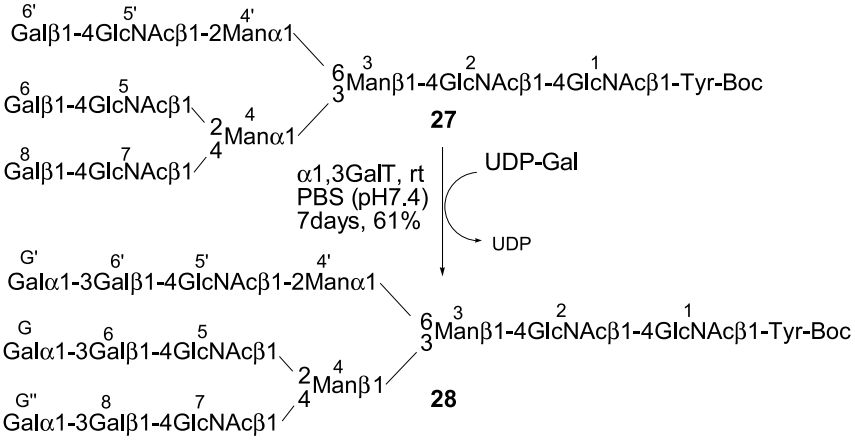

Scheme 6. Enzymatic synthesis of α-Gal oligosaccharide **28**.

The proton NMR spectrum of **28** possessed readily identifiable Galα, Galβ, GlcNAc and Man anomeric protons as shown in Figure 5. Three anomeric protons between 5.145–5.151 ppm were assigned to Galα residues G, G′, G″. The resonance frequencies of the Galβ (6′, 8, 6) anomeric protons were significantly influenced by the attachment of Galα1 residues (G, G′, G″) which resulted in a downfield shift to 4.533–4.568 ppm from the original 4.458–4.482 ppm in **27**.

Determination of K_d Values of α-Gal Derivatives by FAC/MS Analysis and the Screening of an Eight Compound Mixture Model Library

The dissociation constants (K_d) of α-Gal derivatives binding to anti-Gal IgG were calculated from Eq. 1 after their corresponding breakthrough volumes, $V - V_o$, were measured by FAC/MS analysis at a fixed concentration ([x]). For operation in the frontal analysis chromatography mode, the anti-Gal IgG column was washed with

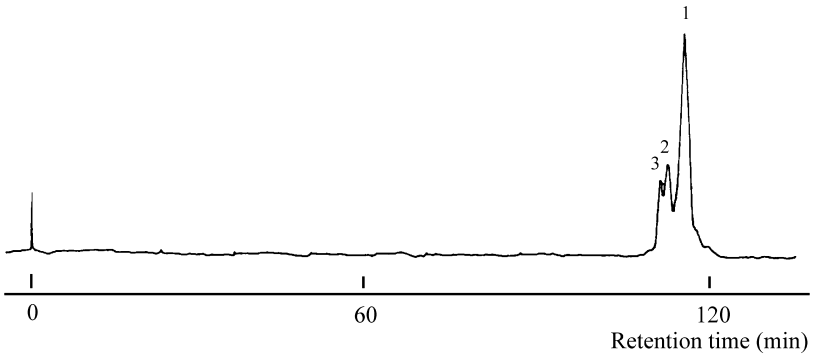

Figure 4. Semipreparative RP–HPLC purification of α-Gal oligosaccharide **28**.

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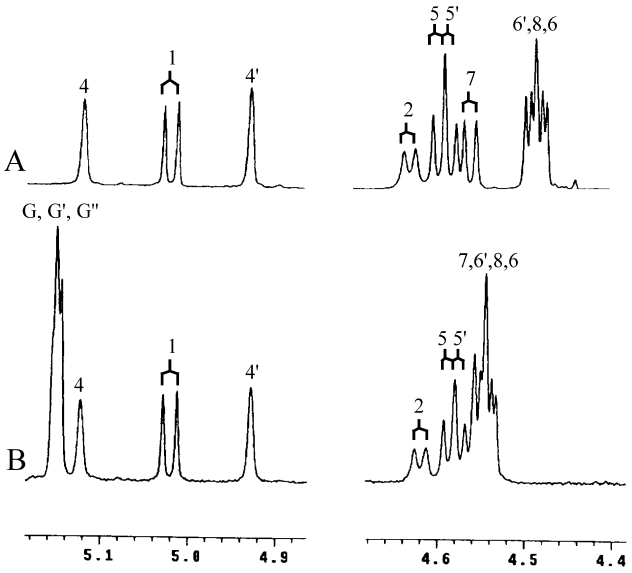

Figure 5. Anomeric proton signals (600 MHz, D_2O) for *N*-linked oligosaccharide **27** (A) and α-Gal-*N*-linked oligosaccharide **28** (B). G, G′, G″ (5.145–5.151 ppm); 6′, 8, 6 (4.458–4.482 ppm, A; 4.533–4.568 ppm, B).

ammonium acetate solution (NH_4OAc, 2 mM, pH 7.4) to remove the PBS buffer[b], and then directly connected to an electrospray mass spectrometer. The column was flushed with ammonium acetate buffer continuously until the flow was switched to a second solution (NH_4OAc, 2 mM, pH 7.4) containing α-Gal derivatives (2 μM) and a void

Table 1. Molecular weight and dissociation constants of synthetic α-gal derivatives.

α-Gal	*m/z* (MNa^+)[a]	$K_d \pm s$[b] [μM]
9a	630.2	8.5 ± 0.7
9b	646.3	9.3 ± 1.1
9c	660.1	11.6 ± 0.3
9d	636.2	13.0 ± 0.6
9e	642.3	15.5 ± 0.6
11	900.5	8.9 ± 0.2
22	1099.5	8.5 ± 0.2
24	1598.7	8.3 ± 0.5
26	1632.7	3.1 ± 0.7
28	2777.0	8.6 ± 0.7

All values are determined from triplicate experiments.
[a]Monoisotopic molecular weight of the singly charged sodium adduct.
[b]Dissociation constant with their corresponding standard deviations.

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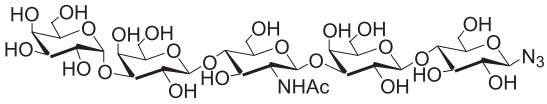
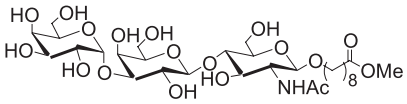
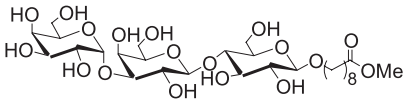
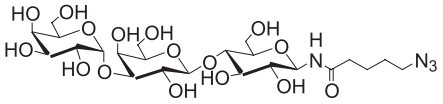
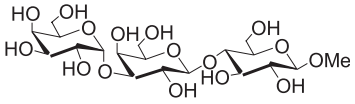
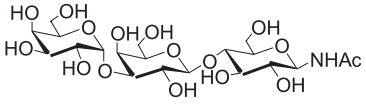
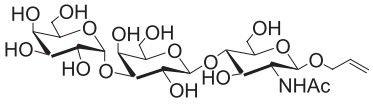
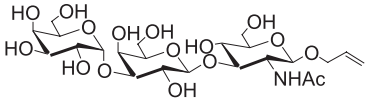
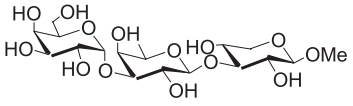
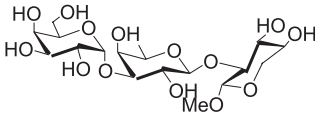
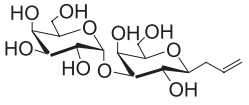
Table 2. Dissociation constants of other α-Gal derivatives.

Structure	Compound	*m/z* (MNa^+)[a]	$K_d \pm s$[b] [μM]
	**29**	917.3	4.1 ± 0.4
	**30**	738.4	8.3 ± 0.4
	**31**	697.3	7.5 ± 0.2
	**32**	651.2	9.8 ± 0.2
	**33**	541.2	6.5 ± 0.5
	**34**	568.2	8.8 ± 0.4
	**35**	608.3	15.7 ± 0.6
	**36**	608.3	19.7 ± 2.5
	**37**	495.1	15.1 ± 2.4
	**38**	495.1	17.6 ± 2.8
	**39**	389.1	12.6 ± 0.5

(*continued*)

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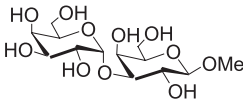
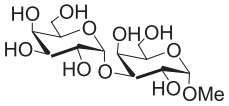
Table 2. Continued.

Structure	Compound	*m/z* (MNa^+)[a]	$K_d \pm s$[b] [μM]
	**40**	379.1	15.1 ± 2.5
	**41**	379.1	8.5 ± 0.4

All values are determined from triplicate experiments with the exception of compound **29** which was determined from Figure 3.

[a]Monoisotopic molecular weight of the singly charged sodium adduct.

volume marker (methoxycarbonyloctyl D-Manα1-3-[D-Manα(1–6)]-β-D-mannopyranoside, **42**, M + Na^+, 697.3, 1 μM). The retention volume, $(V - V_{void\ marker})_{anti\text{-}Gal}$, was obtained by mass spectrometric analysis of the elution front.[58–65]

Compound that binds non-specifically to column elements (walls, beads or protein) would also demonstrate breakthrough volumes greater than the void volume of the system, which can potentially introduce errors to K_d values of a given compound. To control for such errors, a control experiment using the same analyte was carried out in a parallel blank column. This blank column of CPG-SA had the same size as the anti-Gal IgG column but contained no immoblized anti-Gal IgG. The control experiment measured the non-specific retention volume $(V - V_{void\ marker})_{blank}$. Then $V - V_o$ was obtained by substracting of the non-specific retention volume $(V - V_{void\ marker})_{blank}$ from the retention volume of the ligand in the anti-Gal column $(V - V_{void\ marker})_{anti\text{-}Gal}$ as shown in Eq. 2.

$$V - V_o = (V - V_{void\ marker})_{anti-Gal} - (V - V_{void\ marker})_{blank} \quad (2)$$

The dissociation constant (K_d) was generated from Eq. 1 by inputting the observed $V - V_o$ volume and chosen [x]. Compound **42** was used as the void marker for all of the K_d determination experiments except for compound **31** which has the same M + Na^+ value as **42**. In this case, *n*-octyl β-D-glucopyanoside (M + Na^+, 315.0) was selected as the void marker. The K_d values of the synthetic α-Gal derivatives are summarized in Table 1, while Table 2 shows other α-Gal derivatives.

Comparing the K_d values of all of the monovalent trisaccharides in Tables 1 and 2, compound **33** showed the strongest binding to anti-Gal IgG with a K_d value of 6.5 μM. Pentasaccharide **29** (K_d 4.1 μM) has the highest binding affinity of all of the monovalent α-Gal derivatives analyzed. A modest ''multivalent effect'' was reflected by compound **26** with a K_d of 3.1 μM, better than other monovalent trisaccharides. The α-Gal dimer disaccharide **22** (K_d 8.5 μM) and trimer disaccharide **24** (K_d 8.3 μM)

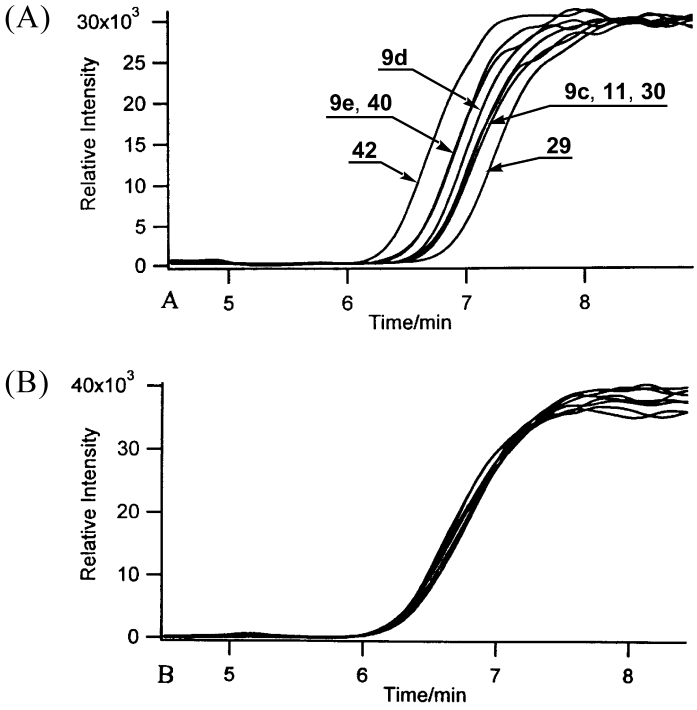


Figure 6. Frontal chromatogram of the eight compound mixture (A) and the corresponding blank frontal chromatogram (B).

showed two times higher affinity than the monomer disaccharide **40** (K_d 15.1 μM). The single α-Gal-*N*-linked oligosaccharide **28** had a K_d value of 8.6 μM. The K_d values of these multivalent compounds, i.e. **22**, **24**, **26**, **28** are corrected for valency (i.e. they are based on the number of galactosyl residues).

To illustrate the potential of FAC/MS screening of α-Gal derivatives and mimetics, a mixture of eight oligosaccharides, (**29**, **30**, **11**, **9c**, **9d**, **40**, **9e** and **42**, in 2 mM NH_4OAc, pH 7.4), was analyzed. Each of the eight compounds was present at a concentration of 2 μM, except for compound **42** that was present at 1 μM. The analysis gave the ion chromatograms shown in Figure 6A, which shows compound **42** breaking through the column first (left curve), followed by **9e**, **40**, **9d**, **9c**, **11**, **30** and finally **29**. Compounds **9e** and **40** had virtually the same breakthrough volume. The most retarded compound was the pentasaccharide **29**.

To avoid the false indication of activity caused by non-specific binding to column elements (walls, beads or protein), a control experiment using a blank column was also carried out. Figure 6B shows the blank column FAC/MS analysis of the eight compound mixture. It indicates that these eight compounds broke through the column together with void volume marker **42**. Therefore, the breakthrough sequence in Figure 6A is compatible with the magnitude of their K_d values.

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CONCLUSION

In summary, FAC/MS has been shown to be an efficient method to estimate the K_d values of individual oligosaccharides binding to isolated human anti-α-Gal IgG. The method is also useful in screening mixtures of oligosaccharides suggesting its application in higher-throughput situations such as the screening of small libraries. The oligosaccharides (di- to pentasaccharides) tested all had K_d values in the range 4.1 – 20 μM, indicating that only modest added interaction takes place beyond the Gal (α1 → 3)Gal sequence. Modification of the aglycon in the α-Gal trisaccharide has little or no effect on binding. Additionally, polyvalency of the ligands had little if any effect on the bivalent IgG binding. It will be interesting to see if similar behavior will be observed for IgA and IgM counter parts.

EXPERIMENTAL

General. ^1H and ^{13}C spectra were recorded on Varian Mercury 400 NMR, Varian Unity 500 and Varian Inova 300 and 600 spectrometers. For solutions in D_2O, the residual DOH peak at 300, 400 and 500 MHz was set at 4.67 ppm (ambient temperature, ca 20–23°C) while at 600 MHz the DOH signal was set at 4.76 ppm at 30°C. FAB high resolution mass spectra were run at the mass spectrometry facility at University of California, Riverside. ESI high resolution mass spectra were recorded on a Micro-mass ZabSep Hydroid Sector-TOF at University of Alberta. Baker silica gel (40μm) was used for column chromatography and E. Merck precoated TLC plates were used for thin-layer chromatography. Size-exclusion chromatography was performed on Bio-Gel P2 or P4 supports using distilled water as the eluent. Dialysis was performed using Spectra/Por molecularporous membrane (16 mm cylinder diameter, molecular weight cutoff 14 kD). C18 Sep-Pak sample preparation cartridges were from Waters (Missisauga, ON, Canada). The C18 Reverse phase HPLC column (1 × 25 cm, dp 5 μ) was from Beckman.

FAC/MS Analysis. All solutions were infused simultaneously using a multi-syringe pump (PHD 2000, Harvard Apparatus) at a flow rate of 8 μL/min per syringe (1-mL syringes). A Rheodyne valve (model 9725) was used for flow switching. The column eluent was combined with the make-up flow (acetonitrile) in a mixing tee,[58–64] to give a total flow rate of 16 μL/min directed into the Hewlett-Packard series 1100 MSD electrospray single quadrupole mass spectrometer, operating in the positive-ion mode. A chamber voltage of − 4000 V with a grounded electrospray needle, N_2 drying gas flow rate of 4 L/min, and N_2 nebulizer pressure of 480 mba were used. Breakthrough volumns were measured as midpoints in the selected ion chromatograms for each *m/z* value.

General procedure for the syntheses of trisaccharides 8a–d.[41] Trisaccharide **7** (0.2 mmol) in ethanol (10 mL) was stirred under hydrogen (40 lb/in^2) in the presence of PtO_2 (cat.) for 1 h. After the reaction, PtO_2 was removed by filtration and the filtrate was concentrated. To the residue in dry dichloromethane (10 mL) was added TEA (0.6

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mmol) and acetyl chloride (0.4 mmol) at −78°C. The mixture was allowed to warm to room temperature and stirred for 2 h. The solution was washed with 0.2 N HCl, $NaHCO_3$, brine and dried over Na_2SO_4. Solvent removal followed by chromatographic purification on silica gel column afforded **8a–d**.

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-β-D-glucopyranosyl] benzamide (8a).** 89%, R_f = 0.20, 2/1 hexane/ethyl acetate, white foamy solid.[1]H NMR (400 MHz, $CDCl_3$) δ 1.87 (s, 3H, OAc), 1.99 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.14 (s, 3H, OAc), 3.55 (d, J = 6.4 Hz, 2H, H-6a′, H-6a″), 3.74 (t, J = 6.4 Hz, 1H, H-6b′), 3.88 (m, 6H), 4.13 (m, 4H), 4.37 (d, J = 7.6 Hz, 1H), 4.46 (t, J = 9.6 Hz, 2H), 4.54 (d, J = 11.6 Hz, 2H, PhCH_2 –), 4.75 (m, 3H, H-1, H-1′, H-6a), 4.86 (d, J = 11.6 Hz, 1H, PhCH_2 –), 4.96 (d, J = 11.2 Hz, 1H, PhCH_2 –), 5.02 (t, J = 9.2 Hz, 1H, H-4), 5.11 (brs, 1H, H-1″), 5.17 (dd, J_1 = J_2 = 8.4 Hz, 1H, H-2), 5.44 (m, 2H, H-2′, H-3), 5.50 (brs, 1H, H-4′), 7.09 (d, J = 9.2 Hz, 1H, CON*H*-), 7.30–7.55 (m, 23H, ArH), 7.80 (d, J = 7.6 Hz, 1H, ArH). [13]C NMR (100.6 MHz, $CDCl_3$) δ 172.9, 171.6, 171.5, 171.4, 170.5, 169.9, 168.2, 139.8, 139.7, 139.1, 133.9, 133.5, 129.9, 129.5(2C), 129.4, 129.3(m), 129.1, 129.0, 128.8 (m), 128.7, 128.6 (m), 128.4, 102.0, 96.1, 79.9, 79.5, 76.7, 76.6, 76.4, 76.0, 75.7, 74.8, 74.4, 74.3, 74.1, 73.3, 72.2, 71.6, 70.9, 69.5, 65.9, 63.3, 62.6, 21.9 (m), 21.6. HR-FABMS Calcd for $C_{65}H_{73}NO_{22}Na^+$ 1242.4522, Found 1242.4511

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-β-D-glucopyranosyl] acetylsalicylamide (8b).** 81%, R_f = 0.15, 1:1 hexane/ethyl acetate, white foamy solid.[1]H NMR (400 MHz, $CDCl_3$) δ 1.86 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.38 (s, 3H, OAc), 3.54 (d, J = 6.4 Hz, 2H, H-6a″, H-6a′), 3.72 (t, J = 6.4 Hz, 1H, H-6b′), 3.84 (m, 6H), 4.11 (m, 4H), 4.37 (d, J = 8.0 Hz, 1H), 4.45 (m, 2H), 4.53 (d, J = 11.2 Hz, 2H, PhCH_2 –), 4.67 (d, J = 11.6 Hz, 1H, PhCH_2 –), 4.75 (m, 2H, H-1′, H-6a), 4.79 (d, J = 11.6 Hz, 1H, PhCH_2 –), 4.95 (m, 2H, H-1, H-6b), 5.09 (d, J = 3.6 Hz, 1H, H-1″), 5.15 (dd, J_1 = 10.0 Hz, J_2 = 18.0 Hz, 1H, H-2′), 5.37 (t, J = 9.0 Hz, 1H, H-2), 5.44 (t, J = 9.0 Hz, 1H, H-3), 5.47 (d, J = 3.2 Hz, 1H, H-4′), 7.08 (d, J = 9.2 Hz, 1H, CON*H*-), 7.14 (d, J = 8.0 Hz, 1H, ArH), 7.27–7.42 (m, 20H, ArH), 7.52 (t, J = 8.0 Hz, 1H, ArH), 7.77 (d, J = 8.0 Hz, 1H).[13]C NMR (100.6 MHz, $CDCl_3$) δ 172.4, 171.6, 171.5, 171.4, 170.5, 170.1, 169.9, 166.5, 149.6, 139.8, 139.7, 139.1, 133.9, 130.8, 129.5 (m), 129.4, 129.3 (m), 129.1, 128.9 (m), 128.8, 128.7 (m), 128.6, 127.6, 127.4, 124.6, 101.9, 96.1, 79.5, 79.3, 76.8, 76.7, 76.4, 75.9, 75.8, 74.6, 74.4, 74.3, 74.0, 73.6, 72.2, 72.1, 71.6, 70.9, 69.6, 65.9, 63.3, 62.6, 22.2, 21.9 (m), 21.8 (m), 21.6. HR-FABMS Calcd for $C_{67}H_{75}NO_{24}Na^+$ 1300.4576, Found 1300.4535.

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-β-D-glucopyranosyl]–*o*-anisoylamide (8c).** 90%, R_f = 0.20, 1:1 hexane/ethyl acetate, white foamy solid. [1]H NMR (500 MHz, $CDCl_3$) δ 1.84 (s, 3H, OAc), 1.96 (s, 3H, OAc), 2.00 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.11 (s, 3H, OAc), 3.52 (d, J = 6.5 Hz, 2H, H-6a″, H-6a′), 3.69 (t, J = 9.0 Hz, 1H, H-6b′), 3.83 (m, 6H), 3.95 (s, 3H, OCH_3), 4.00 (dd, J_1 = 9.0 Hz, J_2 = 3.5 Hz, 1H, H-3″), 4.07 (t, J = 6.0 Hz, 2H), 4.19 (m, 1H), 4.34 (d, J = 8.0 Hz, 1H), 4.41 (d, J = 11.5 Hz, 2H, PhCH_2 –), 4.51 (d, J = 11.5 Hz, 2H,

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$PhCH_2$ –), 4.66 (d, J = 11.5 Hz, 1H, $PhCH_2$ –), 4.72 (m, 2H), 4.83 (d, J = 12.0 Hz, 1H), 4.92 (d, J = 11.0 Hz, 1H, 6a), 5.08–5.13 (m, 3H, H-2, H-1″, H-1), 5.34 (m, 1H, H-2′), 5.48 (m, 2H, H-3, H-4′), 6.95 (d, J = 8.5 Hz, 1H, CONH-), 7.06 (t, J = 7.5 Hz, 1H, ArH), 7.27–7.39 (m, 15H, ArH), 7.48 (dd, J_1 = 7.5 Hz, J_2 = 9.0 Hz, 1H, ArH), 8.20 (dd, J_1 = 2.0 Hz, J_2 = 7.5 Hz, 1H, ArH), 8.61 (d, J = 9.0 Hz, 1H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.3, 171.1, 170.9, 170.0, 169.4, 166.3, 158.6, 139.4, 138.8, 134.4, 133.3, 129.1, 128.9 (m), 128.8 (m), 128.6, 128.4, 128.3 (m), 128.2, 128.1, 121.9, 120.9, 112.1, 101.6, 95.8, 79.2, 79.0, 76.5, 76.4, 76.1, 75.5, 75.2, 74.3, 74.0, 73.9, 73.8, 73.5, 71.9, 71.3, 71.2, 70.6, 69.2, 65.7, 63.1, 62.2, 56.4, 21.5, 21.4 (m), 21.3, 21.1. HR-FABMS Calcd for $C_{66}H_{75}NO_{23}Na^+$ 1272.4627, Found 1272.4678.

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-β-D-glucopyranosyl] cyclohexylamide (8d).** 89% (diaisomer 2:1), R_f = 0.25, 2:1 hexane/ethyl acetate, white foamy solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.25 (m, 6H, c-Hex), 1.75 (m, 4H, c-Hex), 1.81 (s, 3H, OAc), 1.91 (s, 3H, OAc), 2.00 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.28 and 2.42 (t, J = 11.0 Hz, 1H, diaisomer 2:1, c-Hex), 3.21 and 3.09 (m, 1H, diaisomer 2:1), 3.48 (d, J = 6.5 Hz, 2H), 3.61–3.81 (m, 7H), 4.03 (m, 3H), 4.29 (d, J = 7.5 Hz, 1H), 4.37 (m, 2H), 4.48 (d, J = 11.0 Hz, 2H), 4.63 (d, J = 11.5 Hz, 1H, PhCH_2 –), 4.69 (m, 2H, $PhCH_2$ –), 4.80 (m, 2H, PhCH_2, H-4), 4.89 (d, J = 11.5 Hz, 1H, H-6a), 5.04 (d, J = 3.0 Hz, 1HH-1″), 5.08 (t, J = 8.0 Hz, 1H, H-2), 5.20 (t, J = 9.5 Hz, 1H, H-2′), 5.27 (m, 1H, H-3), 5.43 (m, 1H, H-4′), 6.26 (d, J = 9.5 Hz, 1H, CONH-), 7.24–7.35 (m, 20H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 181.1, 176.9 & 175.6 (diaisomer), 172.0, 171.1, 170.9, 170.1, 169.5, 139.2, 138.7, 129.0 (m), 128.8 (m), 128.7, 128.5, 128.4 (m), 128.2, 128.1, 101.5, 95.6, 79.1, 78.6, 76.3, 76.1, 75.9, 75.5, 75.2, 74.3, 73.9(2C), 73.6, 73.0, 71.7, 71.1, 70.5, 69.1, 67.7, 67.6, 67.4, 65.5, 62.9, 62.2, 50.8, 48.3, 46.6 and 45.8, 43.5 and 42.7, 40.9, 37.2, 30.4, 30.2, 29.9, 29.5, 29.4, 26.5, 26.4, 26.3, 26.1. HR-FABMS Calcd for $C_{65}H_{79}NO_{22}Na^+$ 1248.4991, Found 1248.5012.

***N*-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-β-D-glucopyranosyl]–2-(2-methoxyethoxy) acetamide (8e).** Trisaccharide **7** (250 mg, 0.21 mmol) in ethanol (10 mL) was stirred under hydrogen (40 lb/in^2) for 1 h. After the reaction, PtO_2 was removed by filtration and the filtrate was evaporated. To the residue in dry dichloromethane (10 mL) was added 2-(2-methoxyethoxy) acetic acid (72 uL, 0.63 mmol) and 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquonoline (IIDQ, 0.38 ml, 1.26 mmol). The mixture was stirred overnight at room temperature. Solvent removal followed by chromatographic purification on silica gel column (eluent: 1/2 hexane/ethyl acetate) afforded **8e** (0.15 g, 54 %, R_f = 0.45, 1:3 hexane/ethyl acetate) as a white foamy solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.82 (s, 3H, OAc), 1.93 (s, 3H, OAc), 2.01 (s, 6H, OAc), 2.07 (s, 3H, OAc), 2.08 (s, 3H, OAc), 3.40 (s, 3H, OMe), 3.51 (m, 2H, H-6a″, H-6a′), 3.54 (dd, J_1 = 6.5 Hz, J_2 = 8.5 Hz, 2H), 3.61 (m, 2H), 3.66 (t, J = 7.0 Hz, 1H), 3.74–3.84 (m, 6H), 3.95–4.12 (m, 6H), 4.31 (d, J = 8.0 Hz, 1H), 4.39 (m, 2H), 4.49 (d, J = 12.0 Hz, 1H, $PhCH_2$ –), 4.64 (d, J = 11.5 Hz, 1H, $PhCH_2$ –), 4.69 (m, 2H, $PhCH_2$ –), 4.81 (d, J = 12.0 Hz, 1H, H-6a), 4.91 (m, 2H, H-1′, H-1), 5.04 (d, J = 3.0 Hz, 1H, H-1″), 5.09 (dd, J_1 = 8.0 Hz, J_2 = 10.0 Hz, 1H, H-2), 5.24 (m, 2H, H-2′, H-3), 5.43 (d, J = 3.0 Hz, 1H, H-4′), 7.24–7.37 (m, 20H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ171.6, 171.2 (2C), 171.6, 170.9, 170.2, 169.5, 139.4, 139.3, 138.7,

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129.1 (m), 129.0 (m), 128.9 (m), 128.8 (m), 128.7, 128.4 (m), 128.3, 128.2, 128.1, 79.1, 76.4, 76.1, 75.9, 75.5, 75.3, 74.3, 74.0, 73.9, 73.6, 73.5, 72.2, 71.7, 71.5, 71.1, 71.0, 70.5, 69.2, 65.5, 62.9, 62.1, 59.7, 21.5, 21.4 92C), 21.3 (2C), 21.1. HR-FABMS Calcd for $C_{65}H_{77}NO_{24}Na^+$ 1254.4733, Found 1254.4786.

General procedure for the synthesis of trisaccharides 9a–e. Pd/C (10%, 100 mg) was added to a solution of **8a–e** (500 mg) in methanol (30 mL). The black suspension was charged with compressed hydrogen (50 lb/in^2) after degassing. The reaction took 5 h until no more hydrogen was consumed. The mixture was filtered and the filtrate was evaporated to give a residue that was dissolved in anhydrous methanol (500 mL) followed by the addition of NaOMe to adjust the pH value of the solution to 9.0. The mixture was stirred for 2 h. Dowex (H^+ form) was added to neutralize the solution. The resin was filtered and the filtrate was concentrated to afford **9a–e** as a white solid after lyophilization. Further purification on Bio-Gel P2 used water as eluent.

***N*-[*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-β-D-glucopyranosyl] benzamide (9a).** 97%, R_f = 0.55, 8/2/2 2-methylpropanol/H_2O/ethyl acetate. ^{1}H NMR (500 MHz, D_2O) δ 3.49 (t, J = 8.5 Hz, 1H, H-2), 3.54–3.88 (m, 15H), 4.06 (m, 2H, H–5″ & H-4′), 4.41 (d, J = 8.0 Hz, 1H, H-1′), 5.01 (d, J = 4.0 Hz, 1H, H-1″), 5.07 (d, J = 9.0 Hz, 1H, H-1), 7.40 (t, J = 7.5 Hz, 2H, ArH), 7.51 (t, J = 7.5 Hz, 1H, ArH), 7.69 (d, J = 8.5 Hz, 2H, ArH). ^{13}C NMR (125.7 MHz, D_2O) δ 172.3, 133.4, 129.5, 128.1, 103.4, 96.1, 81.4, 78.6, 77.8, 77.1, 75.9, 75.7, 75.2, 72.0, 71.5, 70.2, 69.9, 69.7, 68.8, 65.5, 61.7, 61.6, 60.6. HR-FABMS Calcd for $C_{25}H_{37}NO_{16}Na^+$ 630.2010, Found 630.1987.

***N*-[*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-β-D-glucopyranosyl] salicylamide (9b).** 91%, R_f = 0.60, 10/2/2 2-methylpropanol/H_2O/ethyl acetate. ^{1}H NMR (400 MHz, D_2O) δ 3.53 (t, J = 9.2 Hz, 1H, H-2), 3.60–3.95 (m, 15H), 4.06 (m, 2H, H-4″ & H-3″), 4.11 (m, 2H, H-5″ & H-4′), 4.47 (d, J = 7.6 Hz, 1H, H-1′), 5.07 (d, J = 3.6 Hz, 1H, H-1″), 5.16 (d, J = 9.2 Hz, 1H, H-1), 6.83 (m, 2H, ArH), 7.36 (m, 1H, ArH), 7.75 (dd, J_1 = 1.6 Hz, J_2 = 8.0 Hz, 1H, ArH).^{13}C NMR (100.6 MHz, D_2O) δ 171.4, 162.6, 134.8, 129.4, 119.4, 117.8, 116.6, 102.9, 95.5, 79.5, 78.2, 77.2, 76.4, 75.2, 75.1, 71.8, 70.9, 69.6, 69.3, 69.2, 68.3, 64.9, 61.1, 60.9, 60.1. HR-FABMS Calcd for $C_{25}H_{37}NO_{17}Na^+$ 646.1959, Found 646.1934.

***N*-[*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-β-D-glucopyranosyl]-*o*-anisoylamide (9c).** 91%, R_f = 0.50, 8/2/2 2-methylpropanol/H_2O/ethyl acetate. ^{1}H NMR (400 MHz, D_2O) δ 3.57 (t, J = 9.2 Hz, 1H, H-2), 3.65–4.01 (m, 18H), 4.18 (m, 2H, H-5″ & H-4′), 4.52 (d, J = 7.6 Hz, 1H, H-1′), 5.12 (d, J = 4.0 Hz, 1H, H-1″), 5.19 (d, J = 9.2 Hz, 1H, H-1), 7.11 (m, 2H, ArH), 7.56 (m, 1H, ArH), 7.75 (dd, J_1 = 1.6 Hz, J_2 = 7.6 Hz, 1H, ArH).^{13}C NMR (100.6 MHz, D_2O) δ 170.1, 157.6, 134.3, 130.5, 121.2, 120.9, 112.5, 103.0, 95.6, 79.7, 78.4, 77.4, 76.6, 75.4, 75.2, 71.8, 71.0, 69.7, 69.4, 69.3, 68.4, 65.0, 61.2, 61.1, 60.2, 56.1. HR-FABMS Calcd for $C_{26}H_{39}NO_{17}Na^+$ 660.2116, Found 660.2101.

***N*-[*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-β-D-glucopyranosyl] cyclohexylamide (9d).** 96%, R_f = 0.70, 8/2/2 2-methylpropanol/H_2O/ethyl acetate. ^{1}H NMR (500 MHz, D_2O) δ 1.06–1.25 (m, 6H, c-Hex), 1.52–1.69 (m, 4H, c-Hex), 2.16 & 2.55 (t, J = 11.5 Hz, diaisomer, 2/1, 1H, c-Hex), 3.31 (t,

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J = 9.0 Hz, 1H, H-2), 3.45 (t, J = 5.5 Hz, 1H), 3.54–3.82 (m, 13H), 3.88 (d, J = 3.0 Hz, 1H, H-4″), 4.05 (m, 2H, H-5″ & H-4′), 4.38 (d, J = 7.5 Hz, 1H, H-1′), 4.84 (d, J = 9.0 Hz, 1H, H-1), 5.00 (d, J = 4.0 Hz, 1H, H-1″).^{13}C NMR (125.7 MHz, D_2O) δ 182.2 & 178.6, 103.4, 96.1, 79.7, 78.7, 77.8, 77.0, 75.9, 75.7, 72.1, 71.5, 70.2, 69.9, 69.8, 68.8, 67.2, 67.1, 65.5, 61.7, 61.6, 60.6, 46.5, 45.6, 42.9, 40.5, 29.6 (m), 29.5, 28.8 (m). HR-FABMS Calcd for $C_{25}H_{43}NO_{16}Na^+$ 636.2479, Found 636.2477.

***N*-[*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-β-D-glucopyranosyl]–2-(2-methoxyethoxy)acetamide (9e).** 81%, R_f = 0.20, 8/2/2 2-methylpropanol/H_2O/ethyl acetate. ^{1}H NMR (400 MHz, D_2O) δ 3.34 (s, 3H, OMe), 3.47 (t, J = 9.2 Hz, 1H, H-2), 3.59–3.91 (m, 19H), 3.96 (d, J = 3.2 Hz, 1H, H-4″), 4.12 (m, 3H, H-5″, H-4′, H-3″), 4.47 (d, J = 7.6 Hz, 1H, H-1′), 5.00 (d, J = 9.2 Hz, 1H, H-1), 5.08 (d, J = 3.6 Hz, 1H, H-1″).^{13}C NMR (100.6 MHz, D_2O) δ 174.0, 102.8, 95.5, 78.9, 77.9, 77.2, 76.5, 75.5, 75.1, 71.4, 70.9, 70.8, 70.2, 69.6, 69.5, 69.3, 69.1, 68.3, 64.9, 61.1, 60.9, 59.9, 58.1. HR-FABMS Calcd for $C_{23}H_{41}NO_{18}Na^+$ 642.2221, Found 642.2240.

8-Methoxycarbonyloctyl α-D-galactopyranosyl-(1 → 3)-α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-2-deoxy-2-acetamidoglucopyranoside (11). Trisaccharide **10** (6 mg, 8.4 μmol), UDP-Gal (76.8 mg, 15 × 8.4 μmol) and 10 μL of alkaline phosphatase (1U/μL) were added to a 2.5-mL solution of bovine α1,3-Gal T (2.5 U in PBS), $MnCl_2$ (10 mM) and BSA (0.1%). The mixture was incubated with agitation at room temperature for one week. The reaction mixture was then filtered through glass wool and loaded onto two sequential C18 Sep-Pak cartridges which were prewashed successively with 20 mL HPLC MeOH and 20 mL H_2O. The cartridges were washed with water (80 mL) and eluted with MeOH (15 mL). The eluate was concentrated, redissolved in water, filtered through a Millex-GV 0.22-μM filter, and lyophilized to yield crude product (5.3 mg) as a white solid. The crude product was dissolved in a small amount of CH_2Cl_2/MeOH/H_2O (60/40/1) and loaded onto an IATROBEAD (IATRON Laboratories INC.) column. The product was purified by chromatography (eluent: 60/40/1 CH_2Cl_2/MeOH/H_2O) to give tetrasaccharide **11** (0.7 mg, 9.6%, R_f = 0.45, 65/35/8 CH_2Cl_2/MeOH/H_2O) as a white solid. Unreacted starting material (4.0 mg) was recovered after chromatography. ^{1}H NMR (600 MHz, D_2O) δ 1.30 (br., 8H), 1.54 (t, J = 6.0 Hz, 2H), 1.60 (t, J = 7.2 Hz, 2H), 2.03 (s, 3H), 2.39 (t, J = 7.8 Hz, 2H), 3.57–4.03 (m, 24H), 4.09 (dd, J_1 = 3.0 Hz, J_2 = 10.2 Hz, H-3″), 4.20 (m, H-4′, H-5″, H-5‴)J = 2.4 Hz, H-4″), 4.52 (d, J = 7.8 Hz, H-1), 4.55 (d, J = 7.8 Hz, H-1′), 5.17 (d, J = 3.6 Hz, H-1‴), 5.19 (d, J = 3.6 Hz, H-1″).^{1}H-^{13}C HMQC (600 MHz, D_2O) C_3″ (75.0 ppm), C_4″ (66.3 ppm), C_4‴ (70.0 ppm). HR-ESMS Calcd for $C_{36}H_{63}NO_{23}Na^+$ 900.3688, Found 900.3693.

1-Chloro-3,6-dioxa-8-octyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactoside (14). To dry dichloromethane (300 mL) in a 3-neck flask was added galactose pentaacetate **12** (48.0 g, 123 mmol), 2-[2-(2-chloroethoxy)ethoxy]ethanol (**13**) (31.2 g, 28 mL, 185 mmol) and 4 Å molecular sieves (10 g). After the reaction mixture was stirred for 1 h, $BF_3 \cdot Et_2O$ (52.0 g, 45 mL, 370 mmol) was added dropwise during 3 h and the resulting mixture was stirred for 48 h. The suspension was filtered through a pad of celite and the filtrate was poured into a pre-cooled $NaHCO_3$ solution. The organic layer

was separated, and the aqueous solution was extracted twice with dichloromethane. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo to give an oil. The crude product was purified by flash column chromatography eluting with a mixture of hexane and ethyl acetate (1/1), to give the product **14** (44.2 g, 72%) as a colorless oil.^{1}H NMR (500 MHz, $CDCl_3$) δ 1.93 (s, 3H, OAc), 1.99 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.10 (s, 3H, OAc), 3.57–3.62 (m, 8H), 3.70 (m, 3H), 3.87–3.91 (m, 2H), 4.06–4.13 (m, 2H), 4.53 (d, J = 8.0 Hz, 1H, H-1), 4.96 (dd, J_1 = 3.6 Hz, J_2 = 10.4 Hz, 1H, H-3), 5.14 (dd, J_1 = 8.4 Hz, J_2 = 10.4 Hz, 1H, H-2), 5.33 (d, J = 3.6 Hz, 1H, H-4). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.0, 170.9, 170.7, 170.1, 102.0, 72.0, 71.6, 71.3 (m), 71.0, 69.7, 69.5, 67.8, 62.0, 43.4, 21.4, 21.3 (2C), 21.2. HR-FABMS Calcd for $C_{20}H_{31}O_{12}ClNa^+$ 521.1402, Found 521.1426.

1-Chloro-3,6-dioxa-8-octyl β-D-galactoside (15). To a 1000 mL flask containing **14** (44.0 g, 88.3 mmol) was added a mixture of methanol (150 mL) and 1 M Na_2CO_3 aqueous solution (200 mL). The mixture was stirred at room temperature for 36 h. The reaction solution was poured into a 1000 mL Erlenmeyer flask cooled in an ice–water bath. Methanol (600 mL) was added and a white solid precipitated. The suspension was filtered and washed with methanol. The filtrate was neutralized with Dowex resin (H^+ form) until pH 6 was achieved. The resin was removed by filtration and washed with water and the filtrate was concentrated in vacuo. Toluene was added to further remove water via azeotropic distillation to give 1-chloro-3,6-dioxa-8-octyl β-D-galactoside (**15**) (26.8 g, 92%). ^{1}H NMR (500 MHz, D_2O) δ 3.35 (m, 1H, H-3), 3.47–3.70 (m, 15H), 3.76 (d, J = 3.6 Hz, 1H, H-4), 3.92 (m, 1H, H-5), 4.26 (d, J = 8.0 Hz, 1H, H-1). ^{13}C NMR (125.7 MHz, D_2O) δ 103.4, 75.8, 73.3, 71.4, 71.3, 70.3, 70.1, 70.0, 69.2 (m), 61.6, 43.7.

1-Chloro-3,6-dioxa-8-octyl 3-*O*-p-methoxybenzyl-β-D-galactoside (16). Compound **15** (26.6 g, 80.5 mmol) was dissolved in anhydrous methanol (150 mL) to which dibutyltin oxide (30.1 g, 120.8 mmol) was added. After the suspension was refluxed for 6 h under an atmosphere of nitrogen, the solution was cooled to room temperature followed by removal of methanol in vacuo. Then dry benzene (500 mL), *p*-methoxybenzyl chloride (35.6 mL, 161.0 mmol), TBAB (13.3 g, 40.2 mmol) and 4 Å MS (10 g) were added. The mixture was refluxed for 2 h under N_2, then cooled to 0°C when diethylamine (20 mL) was added to quench the reaction. The reaction mixture was diluted with methanol (300 mL), filtered through a pad of celite and concentrated. The residue was purified by column chromatography (silica gel, eluent: ethyl acetate then ethyl acetate/ethanol: 10/1, 5/1) to afford **16** (16.7 g, 46%) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.35 (dd, J_1 = 9.0 Hz, J_2 = 3.0 Hz, 1H, H-3), 3.41 (t, J = 5.5 Hz, 1H, H-5), 3.59–3.86 (m, 17H), 3.93 (d, J = 5.5 Hz, 1H, H-6a), 3.99 (m, 1H, H-4), 4.25 (d, J = 8.0 Hz, 1H, H-1), 4.63 (s, 2H, $PhCH_2O$ –), 6.84 (d, J = 9.6 Hz, 2H, ArH), 7.28 (d, J = 9.6 Hz, 2H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 160.0, 130.7, 130.3 (2C), 114.6 (2C), 104.3, 80.6, 75.2, 72.4, 72.0, 71.4, 71.1, 71.0 (m), 69.3, 67.6, 62.6, 56.0, 43.3. HR-FABMS Calcd for $C_{20}H_{31}O_9ClNa^+$ 473.1554, Found 473.1550.

1-Chloro-3,6-dioxa-8-octyl 2,4,6-tri-*O*-acetyl-β-D-galactoside (17). To compound **16** (14 g, 31 mmol) in a 500 mL flask was added a mixture of Ac_2O (100 mL) and pyridine (150 mL), followed by addition of DMAP (120 mg). The reaction was complete after 3 h. The solvents were removed in vacuo to give an oil, which

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was dissolved in chloroform (300 mL). The solution was washed sequentially with 0.5 N HCl, water, $NaHCO_3$ and brine. It was then dried over anhydrous Na_2SO_4 and evaporation of the solvent afforded an oil which was purified by column chromatography (silica gel, hexane/ethyl acetate: 1/3, 1/6, sequentially) to give 1-chloro-3,6-dioxa-8-octyl 2,4,6-tri-*O*-acetyl-3-*O*-*p*-methoxybenzyl-β-D-galactoside (16.1 g, 90%) as an colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.95 (s, 3H, OAc), 1.99 (s, 3H, OAc), 2.06 (s, 3H, OAc), 3.46 (dd, J_1 = 9.6 Hz, J_2 = 3.6 Hz, 2H, H-3), 3.53–3.66 (m, 12H), 3.71 (s, 3H), 3.86 (m, 1H, H-5), 4.08 (m, 2H, H-6a,b), 4.24 (d, J = 11.6 Hz, 1H, PhCH_2 –), 4.38 (d, J = 8.4 Hz, 1H, H-1), 4.54 (d, J = 11.6 Hz, 1H, PhCH_2 –), 5.00 (dd, J_1 = 9.6 Hz, J_2 = 8.0 Hz, 1H, H-2), 5.41 (d, J = 2.8 Hz, 1H, H-4), 6.78 (d, J = 8.6 Hz, 2H, ArH), 7.10 (d, J = 8.6 Hz, 2H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.0, 170.9, 170.0, 160.0, 130.2, 130.1 (2C), 114.4 (2C), 101.9, 76.8, 71.9, 71.6, 71.2 (m), 71.1, 71.0, 69.4, 66.7, 62.6, 55.9, 43.5, 21.5, 21.4, 21.3. HR-FABMS Calcd for $C_{26}H_{37}O_{12}ClNa^+$ 599.1871, Found 599.1884. 1-Chloro-3,6-dioxa-8-octyl 2,4,6-tri-*O*-acetyl-3-*O*-*p*-methoxybenzyl-β-D-galactoside (16.0 g, 27.7 mmol) was dissolved in a mixture (300 mL) of CH_3CN and water (9/1). With ice water bath cooling, CAN (38.0 g, 69.4 mmol) was added to the reaction vessel portionwise during 2 h. After the reaction was kept for two more hours, the solvents were partially removed in vacuo and water was added followed by extraction with chloroform three times. The combined organic phase was washed with $NaHCO_3$ and brine. The solution was dried over anhydrous Na_2SO_4 and concentrated in vacuo to give an oil, which was purified by flash chromatography to give **17** (10.3 g, 81%) yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.03 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.14 (s, 3H, OAc), 3.59–3.73 (m, 12H), 3.83 (m, 1H, H-3), 3.93 (m, 1H, H-5), 4.12 (m, 2H, H-6a.b), 4.48 (d, J = 7.8 Hz, 1H, H-1), 4.95 (dd, J_1 = 10.0 Hz, J_2 = 7.8 Hz, 1H, H-2), 5.29 (d, J = 3.6 Hz, 1H, H-4). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.7, 171.6, 171.2, 101.7, 73.2, 72.0, 71.8, 71.7, 71.3 (m), 71.1, 70.4, 69.6, 62.6, 43.4, 21.6, 21.4, 21.3. HR-FABMS Calcd for $C_{18}H_{29}O_{11}ClNa^+$ 479.1296, Found 479.1315.

1-Azido-3,6-dioxa-8-octyl *O*-(2,3,4,6-tetra-*O*-benzyl-2-D-galactopyranolsyl)-(1 → 3)-2,4,6-tri-*O*-acetyl β-D-galactopyranoside (19). Under an atmosphere of nitrogen, to a flame-dried 500 mL 3-neck flask equipped with an addition funnel, was added **17** (10.0 g, 21.9 mmol), methyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside (**18**) (25.1 g, 44 mmol), 2,6-di-*t*-butyl 4-methyl pyridine (6.8 g, 33 mmol), 4 Å MS (10 g) and absolute ether (300 mL). After the mixture was stirred for 1 h, a solution of methyl triflate (7.2 mL) in ether (50 mL) was added dropwise during 36 h. After 48 h, the acceptor was completely consumed. The reaction mixture was filtered through a celite pad and washed with ether. The filtrate was concentrated under reduced pressure and chromatographed eluting with ethyl acetate and hexane (4/1 and 1/1) to give the disaccharide 1-chloro-3,6-dioxa-8-octyl *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranoside (18.1 g, 84%) as a coloress oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.83 (s, 3H, OAc), 1.97 (s, 3H, OAc), 2.07 (s, 3H, OAc), 3.53 (d, J = 6.5 Hz, 2H, PhCH_2 –), 3.62–3.76 (m, 12H), 3.84 (m, 2H), 3.88 (dd, J_1 = 3.5 Hz, J_2 = 10.5 Hz, 1H, H-2′), 3.95, (m, 2H, H-5, H-5′), 4.02 (dd, J_1 = 3.0 Hz, J_2 = 9.5, 1H, H-3), 4.10–4.93 (m, 9H, 2PhCH_2 –, 2H-6, 2H-6′, H-1), 5.10 (d, J = 3.5 Hz, 1H, H-1′), 5.21 (dd, J_1 = 8.0 Hz, J_2 = 10.0, 1H, H-2), 5.47 (d, J = 3.0 Hz, 1H, H-4), 7.26–7.39 (m, 20H, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.2, 171.1, 169.9, 139.4, 139.3 (m), 138.8, 129.1 (m), 128.9, 128.8 (m), 128.7, 128.4 (m),

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128.3, 128.2 (m), 128.1, 102.2, 95.3, 79.2, 76.5, 76.0, 75.5, 74.3, 73.9 (m), 73.2, 72.1, 71.8, 71.4 (m), 71.0, 70.8, 70.5, 69.6, 69.4, 65.8, 62.6, 43.5, 21.6, 21.5, 21.2. HR-FABMS Calcd for $C_{52}H_{63}O_{16}ClNa^+$ 1001.3702, Found 1001.3719. Sodium azide (0.68 g, 10.42 mmol) was added to a solution of 1-chloro-3,6-dioxa-8-octyl-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranoside) (5.10 g, 5.21 mmol) in *N*,*N*-dimethylformamide (50 mL). The mixture was stirred overnight at 80°C then cooled to room temperature. Chloroform (100 mL) was added and the mixture was washed with water (30 mL) and brine (30 mL), dried with Na_2SO_4, filtered and concentrated. The residue was chromatographed on silica gel (eluent: hexane/ethyl acetate: 2/1, 1/1, sequentially) to give **19** (4.90 g, 95 %) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.83 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.07 (s, 3H, OAc), 3.87 (t, J = 4.5 Hz, 2H, -CH_2N_3), 3.54 (d, J = 6.5 Hz, 2H,), 3.64–3.74 (m, 10H), 3.84 (m, 2H), 3.89 (dd, J_1 = 3.5 Hz, J_2 = 10.5, 1H, H-2′), 3.95 (m, 2H, H-5, H-5′), 4.02 (dd, J_1 = 3.0 Hz, J_2 = 9.5, 1H, H-3), 4.10–4.95 (m, 9H, 2PhCH_2 _, 2H-6, 2H-6′, H-1), 5.10 (d, J = 3.5 Hz, 1H, H-1′), 5.21 (dd, J_1 = 8.0 Hz, J_2 = 10.0, 1H, H-2), 5.47 (d, J = 3.0 Hz, 1H, H-4′), 7.25–7.39 (m, 20H, ArH).^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.2, 171.1, 169.9, 139.5, 139.4, 139.3, 138.8, 129.1 (m), 128.9, 128.8 (m), 128.7, 128.4 (m), 128.3, 128.2 (m), 128.1, 102.3, 95.3, 79.2, 78.1, 76.5, 76.0, 75.5, 74.2, 73.9 (m), 73.2, 71.8, 71.4 (m), 71.0, 70.8, 70.7, 70.5, 69.7, 69.4, 65.8, 62.6, 51.4, 21.6, 21.5, 21.2. HR-FABMS Calcd for $C_{52}H_{63}N_3O_{16}Na^+$ 1008.4106, Found 1008.4158.

Bis{*N*,*N*′-[*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-(2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 8)-3,6-dioxaoctyl]}terephthalamide (21). A solution of **19** (0.30g, 0.31 mmol) in methanol (10 ml) was hydrogenated (4 atm) at room temperature with platinum (IV) oxide hydrate (40 mg) as the catalyst. After 1 h, the TLC showed that all starting material had been consumed. The reaction mixture was diluted with methanol (30 mL), filtered and concentrated under reduced pressure to give a white solid. The solid was dissolved in anhydrous dichloromethane (10 mL) and triethylamine (87 μL, 0.62 mmol) was added. The reaction mixture was then cooled to − 30°C. Terephthaloyl chloride (**20**, 24 mg, 0.12 mmol) was added dropwise to the stirred mixture at − 30°C. Stirring was continued for 1 h at the same temperature and the mixture was kept stirring for 12 h. Chloroform (30 mL) was added, and the mixture was washed successively with 1M aqueous sodium bicarbonate (20 mL) , water (20 mL) and brine (20 mL) at room temperature. The organic layer was dried with sodium sulfate and the solvents were evaporated in vacuo. The residue was chromatographed on silica gel (eluent: chloroform/methanol: 40/1) to afford **21** (0.21g, 66% from **19**, 88% based on terephthaloyl chloride **20**) as a white foamy solid.^{1}H NMR (300 MHz, $CDCl_3$) δ 1.79 (s, 6H, OAc), 1.96 (s, 6H, OAc), 2.05 (s, 6H, OAc), 3.51–3.73 (m, 28H), 3.79–4.09 (m, 16H), 4.34–4.89 (m, 18H, 4PhCH_2 _, 4H-6, 4H-6′, 2H-1), 5.07 (d, J = 3.3, 2H, 2H-1′), 5.19 (dd, J_1 = 8.4 Hz, J_2 = 10.2 Hz, 2H, 2H-2), 5.45 (d, J = 2.4 Hz, 2H, 2H-4), 7.20–7.40 (m, 40H, ArH), 7.88 (s, 4H, ArH). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.4, 170.3, 169.3, 166.5, 138.7, 138.6, 138.1, 137.0, 128.3, 128.2, 128.1, 127.9. 127.6, 127.5, 127.4, 127.3, 101.5, 94.7, 78.5, 75.7, 75.7, 75.3, 74.7, 73.5, 73.2, 72.5, 71.1, 70.5, 70.2, 70.1, 70.0, 69.8, 69.6, 69.0, 68.7, 65.1, 61.8, 39.8, 20.8, 20.7, 20.4. HR-FABMS Calcd for $C_{112}H_{132}N_2O_{34}Na^+$ 2071.8559, Found 2071.8535.

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1,4-Bis{*N*-[*O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl]-(1 → 8)-3,6-dioxa-octyl]carbamido}benzene (22). A solution of **21** (0.15g, 0.073 mmol) in methanol (15 mL) and acetic acid (1 mL) was hydrogenated (4 atm) at room temperature with palladium hydroxide on carbon (20%) (20 mg) as catalyst for 24 h. The reaction mixture was diluted with methanol (60 mL), filtered through a pad of Celite and concentrated to give a debenzylated residue. The residue was dissolved in absolute methanol (100 mL), sodium methoxide was added and the pH of the solution was adjusted to ca. 9. The reaction mixture was left stirring at room temperature overnight and precipitated material was dissolved in the minimum amount of water, neutralized with Dowex 50WX2-100 (H^+) resin, filtered and washed with methanol. The solvents were removed in vacuo. Purification by Bio-Gel (P2) column afforded the product **22** (0.65g, 83%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 3.42–3.54 (m, 34H), 3.62 (m, 2H, 2H-5′), 3.68 (dd, J_1 = 3.6 Hz, J_2 = 10.4 Hz, 2H, 2H-2′), 3.75 (dd, J_1 = 3.1 Hz, J_2 = 10.4 Hz, 2H, 2H-3′), 3.81 (d, J = 3.1 Hz, 2H, 2H-4′), 3.85 (m, 2H, 2H-5), 3.99 (m, 4H, 2H-4, 2H-6a), 4.25 (d, J = 8.0 Hz, 2H, 2H-1), 4.96 (d, J = 3.6 Hz, 2H, 2H-1′), 7.68 (s, 4H, ArH). ^{13}C NMR (125.7 MHz, D_2O) δ 170.0, 136.6, 127.4, 102.5, 95.2, 77.1, 74.8, 70.7, 69.6, 69.5, 69.3, 69.2, 69.1, 69.0, 68.7, 68.5, 68.1, 64.7, 60.8, 39.5. HR-FABMS Calcd for $C_{44}H_{72}N_2O_{28}Na^+$ 1099.4169, Found 1069.4179.

1,3,5-Tris{*N*-[*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-(1 → 3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 → 8)-3,6-dioxaoctyl] carbanmido}benzene (23). A solution of **19** (0.38g, 0.39 mmol) in methanol (20 mL) was hydrogenated (4 atm) at room with platinum (IV) oxide hydrate (50 mg) as the catalyst. After 1 h, TLC showed the starting material had been consumed. The reaction mixture was diluted with methanol (40 mL), filtered and concentated under reduced pressure to give a white solid. The solid was dissolved in anhydrous dichloromethane (10 mL) and triethylamine (0.16 ml, 1.17 mmol) was added. The reaction mixture was then cooled down to − 30°C. 1,3,5-benzentricarbonyl trichloride (25 mg, 0.096 mmol) was added dropwise to the stirred mixture at − 30°C. Stirring was continued for 1 h at the same temperature and left stirring for 12 h at room temperature. Chloroform (30 ml) was added and the mixture washed successively with 0.5 N HCl and 1M aqueous sodium bicarbonate (20 mL), water (20 mL) and brine (20 mL). The solution was dried with sodium sulfate and the solvents were evaporated in vacuo. The residue was chromatographed on silica gel (eluent: chloroform/methanol: 40/1) to afford **23** (0.32g, 62% from **19**, 83% based on 1,3,5-benzentricarbonyl trichloride **20**) as a white foamy solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.81 (s, 9H, OAc), 1.95 (s, 9H, OAc), 2.04 (s, 9H, OAc), 3.51 (d, J = 6.6 Hz, 6H), 3.60–3.71 (m, 36H), 3.79–4.05 (m, 24H), 4.36–4.85 (m, 27H, 6PhCH_2–, 6H-6, 6H-6′, 3H-1), 5.07 (d, J = 3.6 Hz, 3H, H-1′), 5.16 (dd, J_1 = 7.8Hz, J_2 = 9.9 Hz, 3H, H-2), 5.44 (d, J = 3.0 Hz, 3H, H-4), 7.24–7.38 (m, 60H, ArH), 8.39 (s, 3H, ArH). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.4, 170.3, 169.1, 165.7, 138.7, 138.6, 138.1, 135.2, 128.3–127.4, 101.5, 94.7, 78.4, 75.7, 75.3, 74.7, 73.5, 73.2, 72.6, 71.0, 70.6, 70.3, 70.1, 69.7, 69.6, 68.9, 68.7, 65.1, 61.8, 40.1, 20.8, 20.7, 20.4.

1,3,5-Tris{*N*-[*O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 8)-3,6-dioxa-octyl]carbamido}benzene (24). A solution of **23** (0.24g, 0.079 mmol) in methanol (20 mL) and acetic acid (1 mL) was hydrogenated (4 atm) at room with

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palladium hydroxide on carbon (5%) (80 mg) for 24 h. The reaction mixture was diluted with methanol (60 mL), filtered through a pad of Celite, and concentrated to give a debenzylated residue. The residue was dissolved in absolute methanol (100 mL). Sodium methoxide was added and the pH of the solution was adjusted to ca. 9. The reaction mixture was left stirring at room temperature overnight and precipitated material was dissolved in the minimum amount of water. The reaction was then neutralized with Dowex 50WX2-100 (H^+) resin, filtered, and washed with methanol. The solvents were removed in vacuo. Purification by Bio-Gel (P2) column afforded the product **24** (0.10g, 80%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 3.44–3.54 (m, 51H), 3.65 (m, 3H, 3H-5′), 3.70 (dd, J_1 = 3.6 Hz, J_2 = 10.2 Hz, 3H, 3H-2′), 3.76 (dd, J_1 = 3.2 Hz, J_2 = 10.2 Hz, 3H, 3H-3′), 3.82 (d, J = 3.2 Hz, 3H, 3H-4′), 3.85 (m, 3H, 3H-5), 3.99 (m, 6H, 3H-4, 3H-6a), 4.26 (d, J = 8.0 Hz, 3H, 3H-1), 4.96 (d, J = 3.6 Hz, 3H, 3H-1′), 8.14 (s, 3H, ArH). ^{13}C NMR (125.7 MHz, D_2O) δ 168.9, 134.8, 128.9, 102.5, 95.2, 77.1, 74.6, 70.7, 69.9, 69.5, 69.3, 69.2, 69.1, 69.0, 68.7, 68.5, 68.1, 64.7, 60.8, 39.6. HR-FABMS Calcd for $C_{63}H_{105}N_3O_{42}Na^+$ 1598.6070, Found 1598.5996.

Enzymatic synthesis of the α-Gal trivalent compound 26. Trilactose **25** (4.3 mg, 3.8 μmol), UDP-Gal (7.7 mg, 12.3 μmol) and 2 μL of alkaline phosphatase (1 U/μL) were added to a 1.5-mL solution of α1,3-Gal T (from calf thymus, 40 mU/mL in 30 mM sodium cacodylate, 20 mM $MnCl_2$, 0.1 % Triton X-100, pH 6.5). The mixture was incubated with gentle rotation at room temperature for 4 days with addition of UDP-Gal (2 mg) and α1,3-Gal T (600 μL) every two days. The reaction mixture was clarified by centrifugation (Microsepconcentrate, NMWL cut of 30 kDa) and the sample was purified on a Bio-Gel (P2) column. Further purification was carried out by loading the crude product onto two sequential C18 Sep-Pak cartridges. To remove Triton-X-100, the cartridges were washed with water (80 mL) and 50% aqueous MeOH (30 mL). The eluate was concentrated, redissolved in water, filtered through a Miller-GV 0.22 μM filter and lyophilized to yield **26** (3.9 mg, 64%) as a white solid: ^{1}H NMR (600 MHz, D_2O) δ 3.33 (dd, J_1 = 7.8 Hz, J_2 = 9.3 Hz, 3H-2 of β-Glc), 3.60–3.82 (m, 36H), 3.86 (dd, J_1 = 3.8 Hz, J_2 = 10.4 Hz, 3H-2 of α-Gal), 3.95 (dd, J_1 = 3.6 Hz, J_2 = 10.4 Hz, 3H-3 of α-Gal), 3.99 (dd, J_1 = 2.0 Hz, J_2 = 12.6 Hz, 3H-3 of β-Gal), 4.02 (d, J = 3.3 Hz, 3H-4 of β-Glc), 4.18 (d, J = 3.3 Hz, 3H-4 of β-Gal), 4.20 (d, J = 4.0 Hz, 3H-4 of α-Gal), 4.21 (d, J = 11.2 Hz, 3Ha of NO_2-Tris), 4.46 (d, J = 11.2 Hz, $3H_b$ of NO_2-Tris), 4.51 (d, J = 7.8 Hz, 3H-1 of β-Glc),), 4.52 (d, J = 7.8 Hz, 3H-1 of β-Gal),), 5.15 (d, J = 3.8 Hz, 3H-1 of α-Gal). HR-ESMS: Calcd for $C_{58}H_{99}NO_{50}Na^+$ 1632.5132, Found 1632.5128.

Enzymatic synthesis of α-Gal terminated *N-linked* oligosaccharide 28. Tyrosinamide *N*-linked oligosaccharide **27** (7 mg, 3.0 μmol), UDP-Gal (0.109 g, 60 μmol) was added to a 3-mL solution of bovine α1,3-galactosyltransferase[42] (2.8 U/3mL in 100 mM Tris buffer, pH 7.0, 500 mM NaCl, 30 mM $MnCl_2$, 1% BSA, 50 U of alkaline phosphatase). The mixture was incubated with gentle shaking at room temperature for 7 days. The reaction mixture was filtered through glass wool and then ultrafiltered (NMWL 10,000) to remove the protein. The filtrate was lyophilized and purified on a Bio-Gel (P4) column with water as eluent. Further purification was carried out using semi-preparative HPLC. The sample was dissolved in water and applied to a 1 × 25 cm semi-preparative C18 reverse phase (RP) HPLC column and eluted

isocratically with 12% acetonitrile at 1 mL/min. The eluting peaks detected by UV absorbance at 280 nm (0.3 aufs) were pooled separately and lyophilized (Figure 4). The major peak (1) corresponded to the desired α-Gal terminated *N*-linked oligosaccharide **28** (5.2 mg, 61%) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 1.34 (s, br, 9H, Boc), 1.98 (s, 3H, 1-NAc), 2.05 (s, br, 6H, 5,5′-NAc), 2.08 (s, 3H, 7-NAc), 2.09 (s, 3H, 2-NAc), 2.82 (m, 1H, PhCH_2 _), 3.00 (m, 1H, PhCH_2 _), 3.49 (t, J = 9.5 Hz, 1H, $PhCH_2CH$-), 3.56–4.06 (m), 4.12 (d, J = 3.1 Hz, 1H), 4.18–4.25 (m, 8H), 4.53–4.56 (m, 4H, H-1 of 6, 8, 6′, 7), 4.57 (d, J = 7.1 Hz, 1H, H-1 of 5′), 4.58 (d, J = 7.8 Hz, 1H, H-1 of 5), 4.62 (d, J = 8.0 Hz, 1H, H-1 of 2), 4.74 (overlap with H_2O, 1H, H-1 of β-Man), 4.93 (s, 1H, H-1 of 4′), 5.02 (d, J = 9.7 Hz, 1H, H-1 of 1), 5.12 (s, 1H, H-1 of 4), 5.15 (d, J = 3.6 Hz, 3H, H-1 of G, G′, G″). ESMS: m/z 2777 (MNa^+), 1400 ($M + 2Na^+$).

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