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Piperidine-based glycodendrons as protein N-glycan prosthetics

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ABSTRACT

The generation of homogeneously glycosylated proteins is essential for defining glycoform-specific activity and improving protein-based therapeutics. We present a novel glycodendrimer prosthetic which can be site-selectively appended to recombinant proteins to create “N-glycosylated” glycoprotein mimics. Using computational modelling, we designed the dendrimer scaffold and protein attachment point to resemble the native N-glycan architecture. Three piperidine-melamine glycodendrimers were synthesized via a chemoenzymatic route and attached to human growth hormone and the Fc region of human IgG. These products represent a new class of engineered biosimilars bearing novel glycodendrimer structures.

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1. Introduction

Glycosylation modulates the biological function, solubility, and stability of many eukaryotic proteins and is an essential feature of numerous biotherapeutics.^{1–3} Despite the importance of glycoproteins as medicines, we remain constrained in our ability to generate glycoproteins as chemically-defined entities. Instead, they are expressed in cells as a heterogeneous mixture of glycoforms, where each glycosylation state may have drastically different properties and biological effects.^{4–6} This is especially relevant to biopharmaceutical production as glycosylated proteins make up a majority of top selling pharmaceuticals currently on the market.⁷ Simply ignoring protein glycosylation can have perilous consequences;^{8,9} exemplified by the severe anaphylactic reactions seen in patients who receive antibodies bearing contaminating galactose- α -1,3-galactose glycans.¹⁰

In similar predicaments where biological heterogeneity has been an obstacle to understanding function and increasing efficacy, chemical synthesis has served as a reliable solution.^{11,12} Indeed, modern innovations in chemical methods have made the total synthesis of functional glycoproteins a reality.¹³ However, the synthetic methodology leading to glycan-modified polypeptides remains a challenging hurdle. To circumvent this issue, a hybrid approach has emerged in which recombinant proteins are produced in cell hosts and later chemically glycosylated.^{14–16} To achieve site-specific glycan conjugation,

these efforts often employ the chemoselective reaction of defined glycan structures with non- natural amino acid side chains. To date, this synthetic glycoengineering approach has encountered one main limitation: the need to produce large quantities of a chemically-defined and functionalized glycan that matches the complexity and length seen in natural structures.^{17,18}

Fig. 1 Reaction of an fGly residue with an aminoxy glycodendrimer produces an oxime glycan that resembles the natural N-glycan structure. (a) Natural Complex Type N-glycan structure attached to Asn (b) Synthetic oxime glycodendrimer attached to fGly.

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Table 1. Molecular mechanics modeling measurements^a of known synthetic dendrimer structures compared to the natural N-glycan.

Dendron Type	Length 1	Length 2	Length 3	Angle 1	Angle 2	Volume
N-Glycan	12.99	6.11	6.29	86.60	52.40	859.45
Benzyl	8.95	7.98	8.17	71.80	45.10	562.19
Phenyl	11.49	9.46	9.46	112.70	112.60	555.68
Piperazine/Melamine	11.68	9.25	9.96	121.80	104.40	722.88
Piperidine/Melamine	11.97	9.82	10.33	138.80	86.80	681.25
PAMAM	7.38	5.71	7.19	97.40	80.40	598.94
Morpholino	14.66	10.44	11.16	127.70	101.10	942.98
Benzyl Ether	10.77	10.95	11.66	30.10	30.30	523.33

^aThe measurements are of distances between each monomer from the core branching unit (lengths 1,2,3), the branch angles (angles 1,2), and total model volume after energy minimization in an OPLS force field.

Glycodendrimers - branched polymers bearing glycan antennae - have previously been conjugated to proteins to modulate their serum half-life or lectin avidity.^{19–21} Yet these approaches have only encompassed simple, unstructured dendron scaffolds that bear little resemblance to native glycans. Seldom have dendron backbones been designed to match natural glycan structures²² and recapitulate the properties of the native glycoproteins. Herein, we report on the synthesis of a novel glycodendron structure that was selected through *in silico* modeling to best mimic the N-glycan architecture. Addition of an aminoxy functionality to the dendron terminus enabled its attachment to recombinant proteins via oxime formation with the formylglycine (fGly) residue of the aldehyde tag.^{23,24} Using this methodology, we were able to construct chemically-glycosylated therapeutic proteins bearing site-specific glycodendrons that greatly resemble native glycoprotein structures (Fig. 1).

2. Results and discussion

The attachment of branched oligosaccharides to Asn residues of nascent polypeptides, termed N-glycosylation, dominates in mammalian proteins. Several studies have demonstrated how N-glycosylation can enhance a protein's *in vivo* function and stability.^{1–3} Unfortunately, the size and complexity of N-glycans also require numerous manipulations to synthesize.^{17,18} To create a more tractable platform toward N-glycan production, we imagined that dendrimer chemistry could be implemented to replace the physical properties of the core N-glycan structure. This core dendrimer would then be appended with distinct terminal sugars such as sialic acid to preserve their specific biological functions (Fig. 1). The role of terminal sugars such as sialic acid in antibody efficacy is well documented,^{25–27} though whether these same properties can be recapitulated with a non-natural scaffold is the purpose for this work.

2.1. Modelling the N-glycan structure

Fig. 2 The structure of three N-glycan dendrimer mimics **1**, **2**, and **3**. Dendrons **1** and **2** contain sialic acid with different regiochemistry while **3** possesses a single Gal found in many asialo glycoproteins.

In choosing a dendrimer scaffold, we wished to maintain the natural N-glycan's ability to 1) prolong circulation half-life by improving protein stability, resistance to proteolytic degradation and pharmacokinetics^{28–30} and 2) modulate receptor binding by fixing protein conformation.^{31,32} We reasoned that the dendrimer monomers should be hydrophilic to match a glycan's ability to increase protein solubility. The assembled dendrimer should also mimic the space filling attributes of an N-glycan to recapitulate conformational space bias and degradation protection. Lastly, the linkage point should closely resemble the natural glycosylation site to ensure stabilizing contacts of the dendrimer monomers with hydrophobic amino acid side chains of the protein.

To select a dendrimer scaffold which could satisfy these desired properties, we used molecular mechanics to model dendrimer structures against a natural N-glycan. Using an OPLS force field, the lowest energy conformation of a N-glycan core heptasaccharide was compared to several branched dendrimer heptamers. Implicit solvation was performed using the generalized Born and surface area solvation method with a "water" dielectric constant set to 1. For simplicity, the dendrimer charge state in water was not considered. Selection criteria were based on the length and conformation angle of the arms about the first mannose (Man) branching point and total space filling of the conformer (Table 1, Fig. S1, ESI). A percent difference was calculated for each category against the modeled N-glycan values. The categories were then weighted for importance with Length 1 and Volume given the highest weight. Finally, weighted scores for all categories were summed for each dendrimer class.

The best match was a dendrimer bearing a melamine core with piperidine or piperazine arms. We ultimately chose the piperidine framework as the synthesis was simpler and has also been utilized in other therapeutically relevant materials.³³ In this scaffold, the melamine serves as a mimic for the core branching Man while the piperidines are hydrophilic heterocycles ($\log D = -1.77$) representing the N-acetylglucosamine ($\log D = -1.63$) and Man ($\log D = -2.79$) hexoses (Fig. 1). To preserve the close proximity between the glycan mimic and protein, we chose the fGly amino acid as the non-natural amino acid conjugation site. Oxime formation with the aldehyde side chain of fGly closely mimics that of the natural glycosylation linkage (Fig. 1).²³ Additionally, the fGly amino acid can be easily introduced into proteins by inclusion of the genetically encoded aldehyde tag motif, CxPxR, as we and others have previously demonstrated.^{24,34}

We set out to synthesize three dendrons with the same piperidine-melamine core but with different terminal sugars (Fig. 2). These targets were designed based on the complex type bi-antennary N-glycans which are frequently found on secreted

Scheme 1 Synthesis of galactosyl dipiperidine arm

Scheme 2 Synthesis of *N*-pentenoyl aminoxy dipiperidine arm

proteins and biotherapeutics (Fig. 1).⁵ We chose to append terminal galactose (Gal) as well as sialic acid with two distinct linkages: α -2,6 (**1**) and α -2,3 (**2**). Sialylation is known to affect protein serum half-life, and these constructs could help define how linkage regiochemistry affects physical and biological properties.³⁵ For assembly, we envisioned using a chemoenzymatic synthesis in which galactose dendron **3** could be generated by synthetic methods and subsequent enzymatic sialylation at the C-6 and C-3 positions of Gal would afford dendron **1** and **2**, respectively. Each dendrimer arm would be addressed separately employing reliable coupling chemistries, while the final glycodendrimer would then be assembled via convergent substitution with cyanuric chloride.

2.2. Synthesis of glycodendrimer arms

To generate the first arm (Scheme 1), we began with the galactosyl piperidine **4** prepared by a Schmidt glycosylation of a galactose imidate with Boc-protected piperidinemethanol. The amine of **4** was deprotected by addition of TFA followed by a reductive amination with piperidine carboxaldehyde **6** to afford the fully protected galactosyl dipiperidine arm **7**. The secondary amine was obtained by another Boc deprotection in 10% TFA to give arm **8**, which was then ready for attachment to the core triazine.

Construction of the second arm bearing the aminoxy functionality (Scheme 2) began with the generation of the *N*-methoxypiperidinyl phthalimide **9** through a Mitsunobu reaction with Boc-protected piperidinemethanol and *N*-hydroxyphthalimide. At this point, the phthalimide functionality was exchanged for a pentenoyl protecting group as the *N*-hydroxypentenamide is more stable to a variety of reaction conditions and its removal with aqueous iodine is compatible with unprotected glycans.²³ Treatment of **9** with hydrazine to remove the phthalimide and subsequent acylation with pentenoic anhydride (pent anhyd) afforded the *N*-pentenylmethoxy piperidine **10**. The second piperidine was again conjugated by reductive amination to give the fully protected aminoxy dipiperidine arm **12**. Boc deprotection in 10% TFA afforded the

free amine **13**, providing the final arm for coupling to the core triazine.

2.3. Dendrimer assembly and sialylation

With the two arms in hand, we began assembly of the final dendron structure (Scheme 3). First, two equivalents of Gal dipiperidine **8** was added to the core triazine by DIPEA-promoted nucleophilic substitution with cyanuric chloride. As previously found with melamine-based dendrimers, addition of the first arm proceeded quickly at 0 °C while addition of the second arm was completed at rt after several hours.³⁶ The final addition of the aminoxy arm **13** onto the triazine chloride **14** required heating at 60 °C for days to reach completion. Benzoyl esters on the resulting dendron **15** were removed by sodium hydroxide saponification in a wet MeCN/CH₂Cl₂ mixture, necessary for solubility. Finally, the free galactosyl dendron **16** was reacted with I₂ in acidic MeCN/MeOH to remove the pentenoyl and afford the aminoxy galactosyl dendron **3**.

To complete the synthesis of dendrons **1** and **2**, sialic acid was installed onto pentenoyl protected **16** using a one-pot, three-enzyme system introduced by Chen and coworkers (Scheme 4).^{37,38} In this method, cytidine monophosphate (CMP)-sialic acid is generated from *N*-acetylmannosamine (ManNAc), pyruvate and cytidine triphosphate (CTP) by the combined use of an *E. coli* aldolase and the CMP-sialic acid synthase, NmCSS. Transfer of the sialic acid to either the 6- or the 3-hydroxyl of galactosyl dendron **16** was performed by a bacterial α -2,6-sialyltransferase (Pd2,6ST) or α -2,3-sialyltransferase (PmST1) to yield the sialylated dendrons **17** and **18** respectively. It should be noted that the pentenoyl protecting group was necessary at this stage as a free aminoxy group would undergo side reactions with the ketones and aldehydes of the starting enzymatic substrates. The final aminoxy sialyl galactose dendrons **1** and **2** were obtained by treatment of **19** and **20** with I₂.

2.4. Assembly of chemically-glycosylated proteins

With the synthesis of the three dendron structures completed, we commenced preparation of the defined N-glycan mimic-protein conjugates. To examine oxime formation between dendrons **1-3** and an fGly bearing protein, we initially chose a human growth hormone (hGH) construct bearing a C-terminal aldehyde tag. We have previously demonstrated that the aldehyde tag in this construct bears high fGly content when coexpressed with the fGly-generating enzyme (FGE) and is amenable to efficient chemical glycosylation with aminoxy glycans.^{23,24} Reaction of aminoxy dendrons **1**, **2**, and **3** with hGH-fGly was performed at 37 °C in a phosphate-citrate buffer, pH 4.2 (Fig. 3a). The conjugation efficiency was assessed by lectin blot analysis and electrospray ionization mass spectrometry (ESI-MS; Figs. 3b, 4).

The glycodendrons conjugated well to the aldehyde-tagged hGH-fGly, as assessed by the gel shift observed by Ponceau staining and binding of FITC-labelled lectins (Fig. 3). The conjugation of dendron **1** was assessed by *Sambucus nigra* lectin

Scheme 3 Assembly and deprotection of Gal N-glycan dendron **1**

(SNA) which binds α -2,6-sialic acid, dendron **2** conjugation by *Maackia amurensis* lectin (MAA-II) which binds α -2,3-sialic acid/galactose and dendron **3** conjugation with soybean agglutinin (SBA) which binds only antennary galactose. Lanes 1 show minimal background lectin staining of the fGly-hGH starting material without dendron addition while lanes 2-4 demonstrate conjugation with each subsequent dendron. Lanes 5 contain reactions of the stated dendron with a CtoA mutant, which lacks the critical Cys residue in the aldehyde tag. Since the fGly residue is not present in these constructs they also show minimal background labelling in the lectin blots. The high background seen in lanes 2 and 4 of the MAA-II blot are due to the promiscuity of the lectin though the highest signal is seen in lane 3 with conjugation of the α 2,3-sialic acid dendron **2**. We attribute the presence of an additional higher MW band to partially folded unreduced protein as it can be found in all lanes. The high reactivity of this material to MAA-II upon conjugation is an interesting but unremarkable feature of the lectin as the staining is not fully recapitulated in lane 1.

Further, we were able to resolve each glycodendron-hGH species using intact protein ESI-MS and found that the conjugation efficiency was high for each construct (Fig. 4). In Fig. 4a, we found the expected mass of the unconjugated hGH-fGly plus the addition of water as the aldehyde is a hydrated geminal diol at steady state. Conjugation of dendrimers **1** and **3** proceeded with near quantitative yield without any evidence of hGH-fGly starting material. Despite use of the same hGH protein preparation, addition of dendrimer **2** proved more difficult without full consumption of free hGH-fGly and may highlight steric constraints in tertiary structure of dendrimer **2** conjugates.

The ultimate utility of our glycoengineering approach lies in the ability to conjugate the dendrimer scaffold at natural N-glycosylation sites. We felt that using our prosthetics to replace the N-glycan of IgG antibody would be exceptionally relevant. The N-glycan structure on the F_c region of IgG is known to control its pharmacokinetics and is essential for F_c receptor binding and effector function.^{25,26,39,40} Furthermore, we have previously generated a human IgG F_c fragment bearing a CxPxR motif at the endogenous N-glycan site which could be conjugated with small aminooxy glycan moieties.⁴¹ Reaction of aminooxy dendrimer **3** with the aldehyde-tagged F_c fragment was performed at 60 °C for 16 h in acetate buffer, pH 4. Analysis of the product by lectin blot demonstrated attachment of the Gal dendrimer to the human protein (Fig. S2, ESI).

3. Conclusion

In conclusion, we present a new platform for the facile generation of glycoprotein mimics. Our method encompasses a novel glycodendron that highly mimics the natural N-glycan structure. This architecture in conjunction with the aldehyde tag technology provides a system that streamlines the construction of recombinant proteins bearing site-specific glycosylation. The conjugation of polymeric material, such as polyethyleneglycol (PEG), to proteins has been used for decades to engineer therapeutics with increased potency. The N-glycan dendrons augment this approach with an improved, rationally designed synthetic scaffold that mimics biological structures to create a new class of engineered biosimilars.

4. Experimental section

4.1. Chemical Synthesis

4.1.1. General materials

All chemical reagents were purchased from Sigma-Aldrich, Acros, and TCI and used without purification unless noted otherwise. Anhydrous DMF, MeOH, and pyridine were purchased from Acros in sealed bottles; all other anhydrous solvents were obtained from an alumina column solvent purification system. Molecular sieves were ground to powder, flame-dried under hi-vacuum and used immediately after cooling. All reactions were carried out in flame-dried glassware under N₂ unless otherwise noted. In all cases, solvent was removed by reduced pressure with a Buchi Rotovapor R-114 equipped with a Welch self-cleaning dry vacuum. Products were further dried by reduced pressure with an Edwards RV3 high vacuum. Lyophilization was performed on a LABCONCO FreeZone® instrument equipped with an Edwards RV2 pump. Thin layer chromatography was performed with Silicycle 60 Å silica gel plates and detected by UV lamp or charring with p-anisaldehyde in acidic EtOH. Flash chromatography was performed using Silicycle® 60 Å 230-400 mesh silica. HPLC was performed on a Varian system attached to a absorption detector using a C18 reverse phase column (5 µm, 250 x 4.6 mm, Agilent(Varian); Carlsbad, CA) for analytical or a C18 reverse phase column (8 µm, 250 x 21.4 mm, Agilent(Varian); Carlsbad, CA) for preparative purifications. HPLC solvents were A: ddH₂O with 0.1% formic acid and B: MeCN with 0.1% formic acid. All ¹H and ¹³C NMR spectra are reported in ppm and referenced to solvent peaks. Spectra were obtained on Bruker AVQ-400, AVB-400, DRX-500, AV-500, or AV-600 instruments. High resolution electrospray ionization (ESI) mass spectra were obtained from the UC Berkeley Mass Spectrometry Facility. Protein structures were generated using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

4.1.2. Synthetic procedures and characterization

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxyamine]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-(5-acetamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-2-nonulopyranosyl-onic acid)-(2 $\rightarrow$ 6)- β -*D*-galactopyranosyl]-melamine (1).** A solution of *N*-pentenoyl aminoxy sialoside dendrimer **17** (1.1 mg, 0.64 µmol) in MeCN/MeOH/FA (3:1:0.001, v:v:v; 0.6 mL) was stirred at rt with dropwise addition of I₂ (10 µmol, 0.5 M solution in THF). The mixture was heated to 35 °C and stirred for 3 h followed by 16 h at rt after which the reaction was quenched with the addition of aqueous Na₂S₂O₃ until the disappearance of color. The solvent was removed and the remaining residue was dissolved in ddH₂O followed by purification via HPLC. The product eluted at 13 min in a gradient of 5% to 35% MeCN in ddH₂O (0.05% formic acid) over 20 min. The product peaks were collected and lyophilized to obtain aminoxy 2,6-SiaGal dendrimer **1** as a white powder (0.6 mg, 57%). ¹H NMR (600 MHz, D₂O) δ 4.62 (d, *J* = 11.9 Hz, 6H), 4.35 (d, *J* = 8.3 Hz, 2H), 3.94 – 3.88 (m, 3H), 3.88 – 3.82 (m, 3H), 3.82 – 3.76 (m, 3H), 3.76 – 3.70 (m, 2H), 3.70 – 3.64 (m, 3H), 3.64 – 3.50 (m, 10H), 3.50 – 3.43 (m, 3H), 3.35 – 3.29 (m, 2H), 3.07 – 2.90 (m, 9H), 2.86 (t, *J* = 12.2 Hz, 5H), 2.71 (dd, *J* = 12.4, 5.1 Hz, 2H), 2.23 – 2.10 (m, 4H), 2.07 – 1.91 (m, 10H), 1.91 – 1.85 (m, 2H), 1.84 – 1.74 (m, 5H), 1.65 (t, *J* = 12.4 Hz, 2H), 1.59 – 1.42 (m, 6H), 1.27 – 1.11 (m, 6H); ¹³C NMR (151 MHz, D₂O) δ 175.07, 173.38, 164.36, 158.18, 115.96, 103.12, 100.46, 79.54, 79.26, 73.48, 72.62, 71.76, 70.68, 68.59, 68.16, 63.46, 62.66, 59.31, 53.04, 51.87, 45.50, 42.59, 40.15, 33.37, 31.67, 30.86, 29.07, 28.95, 25.78, 22.00; HRMS (ESI): calcd for C₇₃H₁₂₄N₁₂O₂₉ [M+2Na]²⁺ *m/z* = 839.4191, found: 839.4186

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxyamine]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-(5-acetamido-3,5-dideoxy-*D*-glycero- α -*D*-galacto-2-nonulopyranosyl-onic acid)-(2 $\rightarrow$ 3)-**

β -*D*-galactopyranosyl]-melamine (2). To a solution of *N*-pentenoyl aminoxy sialoside dendrimer **18** (5.4 mg, 3.1 µmol) in MeCN/MeOH/FA (3:1:0.002, v:v; 0.8 mL) was added I₂ (19 µmol, 0.5 M solution in THF) while stirring at rt. The mixture was heated to 35 °C and stirred for 6 h upon which the reaction was quenched with the addition of aqueous Na₂S₂O₃ until the disappearance of color. The solvent was removed and the remaining residue was dissolved in ddH₂O followed by purification via HPLC. The product eluted at 10 min in a gradient of 10% to 50% MeCN in ddH₂O (0.05% formic acid) over 25 min. The product peaks were collected and lyophilized to obtain aminoxy 2,3SiaGal dendrimer **2** as a white powder (5.1 mg, 99%). ¹H NMR (500 MHz, D₂O) δ 4.60 (d, *J* = 13.56 Hz, 6 H), 4.43 (d, *J* = 7.96 Hz, 2 H), 4.05 (dd, *J* = 9.81, 3.19 Hz, 2 H), 3.90 (m, *J* = 2.80 Hz, 2 H), 3.75 – 3.87 (m, 8 H), 3.68 – 3.72 (m, 4 H), 3.59 – 3.67 (m, 16 H), 3.51 – 3.58 (m, 6 H), 2.93 – 3.04 (m, 10 H), 2.86 (t, *J* = 12.26 Hz, 6 H), 2.72 (dd, *J* = 12.37, 4.49 Hz, 2 H), 1.92 – 2.08 (m, 16 H), 1.78 (m, 9 H), 1.46 – 1.56 (m, 4 H), 1.16 – 1.27 (m, 6 H); ¹³C NMR (126 MHz, D₂O) δ 175.0, 173.7, 164.6, 162.5, 102.6, 75.7, 74.9, 73.2, 72.8, 71.7, 70.7, 69.1, 68.3, 68.0, 67.4, 62.5, 61.9, 60.9, 52.9, 52.8, 51.6, 43.0, 39.6, 33.2, 32.0, 30.7, 29.0, 25.7, 25.5, 22.0; HRMS (ESI): calcd for C₇₃H₁₂₄N₁₂O₂₉ [M+2H]²⁺ *m/z* = 817.4371, found: 817.4367

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxyamine]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)- β -*D*-galactopyranosyl]-melamine (3).** A solution of *N*-pentenoyl aminoxy galactose dendrimer **16** (5.0 mg, 0.0044 mmol) in MeCN/MeOH/FA (4:1:0.001, v:v:v; 2 mL) was stirred at rt with dropwise addition of I₂ (0.035 mmol, 0.5 M solution in THF). The mixture was heated to 35 °C and stirred for 4 h after which the reaction was quenched with the addition of aqueous Na₂S₂O₃ until the disappearance of color. The solvent was removed and the remaining residue was dissolved in ddH₂O followed by purification via HPLC. The product eluted at 8 min in a gradient of 10% to 50% MeCN in ddH₂O (0.05% formic acid) over 25 min. The product peaks were collected and lyophilized to obtain aminoxy galactose dendrimer **3** as a white powder (2.3 mg, 50%). ¹H NMR (600 MHz, D₂O) δ 4.56 (d, *J* = 12.5 Hz, 6H), 4.37 (d, *J* = 7.7 Hz, 2H), 3.93 – 3.87 (m, 4H), 3.84 – 3.77 (m, 2H), 3.77 – 3.72 (m, 4H), 3.70 – 3.60 (m, 9H), 3.60 – 3.53 (m, 2H), 3.53 – 3.46 (m, 2H), 3.36 – 3.27 (m, 2H), 3.18 – 3.11 (m, 1H), 3.10 – 3.02 (m, 5H), 3.02 – 2.90 (m, 10H), 2.30 – 2.19 (m, 3H), 2.13 – 1.93 (m, 9H), 1.83 (d, *J* = 11.4 Hz, 6H), 1.64 – 1.48 (m, 6H), 1.38 – 1.24 (m, 6H); ¹³C NMR (151 MHz, D₂O) δ 160.11, 122.44, 121.02, 103.01, 75.13, 73.42, 72.74, 70.77, 68.61, 61.87, 60.95, 53.05, 43.50, 33.28, 30.64, 28.95, 25.77, 25.41; HRMS (ESI): calcd for C₅₁H₉₀N₁₀O₁₃ [M+H]⁺ *m/z* = 1051.6762, found: 1051.6764

***N*-Boc-6-(piperidin-4-ylmethoxy) *O*-2,3,4,6-tetra-*O*-benzoyl- β -*D*-galactopyranoside (4).** Known galactosyl trichloroacetimidate⁴² (738 mg, 0.996 mmol) and *N*-Boc-4-piperidinemethanol (750 mg, 1.1 mmol) were dried by coevaporation with anhydrous toluene and left under high vacuum. To the dried mixture was added 4 Å MS and stirred in CH₂Cl₂ (4 mL) for 1 h at rt. The solution was cooled to -20 °C upon which TMSOTf (9 µL, 0.05 mmol) was added dropwise and allowed to warm to rt over 2 h. Upon completion, the reaction was quenched with TEA and filtered to remove sieves. The concentrated residue was purified by silica flash chromatography (Hex/EtOAc gradient) to obtain piperidine galactoside **4** as a white solid (182 mg, 73%). *R*_f = 0.36 (2:1, Hex/EtOAc); ¹H NMR (600 MHz, CDCl₃) δ 8.13 – 8.06 (m, 2H), 8.01 (dd, *J* = 12.3, 5.0 Hz, 2H), 7.95 (d, *J* = 7.8 Hz, 2H), 7.82 – 7.74 (m, 2H), 7.59 (t, *J* = 7.5 Hz, 1H), 7.55 – 7.43 (m, 4H), 7.43

– 7.33 (m, 5H), 7.22 (t, $J = 7.8$ Hz, 2H), 6.02 (d, $J = 3.3$ Hz, 1H), 5.80 (dd, $J = 10.3, 8.0$ Hz, 1H), 5.65 (dd, $J = 10.4, 3.4$ Hz, 1H), 4.82 (d, $J = 7.9$ Hz, 1H), 4.71 (dd, $J = 11.2, 6.4$ Hz, 1H), 4.43 (dd, $J = 11.2, 6.8$ Hz, 1H), 4.36 (t, $J = 6.6$ Hz, 1H), 4.04 – 3.86 (m, 2H), 3.85 (dd, $J = 9.6, 6.2$ Hz, 1H), 3.40 – 3.33 (m, 1H), 2.61 – 2.41 (m, 2H), 1.78 – 1.66 (m, 1H), 1.58 (dd, $J = 29.7, 12.8$ Hz, 2H), 1.09 – 0.92 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ 166.02, 165.58, 165.26, 154.72, 133.60, 133.31, 129.99, 129.73, 129.59, 129.39, 129.32, 129.00, 128.74, 128.62, 128.46, 128.42, 128.28, 101.94, 79.22, 74.93, 71.66, 71.28, 69.87, 68.15, 61.97, 36.19, 28.69, 28.41; HRMS (ESI): calcd for $\text{C}_{45}\text{H}_{47}\text{NO}_{12}$ $[\text{M}+\text{H}]^+$ $m/z = 794.3171$, found: 794.3210.

***N*-Boc-piperidine-4-carboxaldehyde (6).** *N*-Boc-4-piperidinemethanol (1.0 g, 4.6 mmol) was dissolved in dry MeCN (25 mL) and stirred with subsequent addition of $[\text{Cu}(\text{MeCN})_4]\text{PF}_6$ (86 mg, 0.23 mmol), 2,2'-bipyridine (36 mg, 0.23 mmol), *N*-methylimidazole (37 μL , 0.46 mmol), and 4-Acetamido-TEMPO (49 mg, 0.23 mmol). The reaction was stirred at rt for 22 h followed by solvent removal under reduced pressure and purification by silica flash chromatography (Hex/EtOAc gradient) to obtain piperidine aldehyde **6** as a white solid (772 mg, 78%). $R_f = 0.61$ (1:2, Hex/EtOAc); ^1H NMR (400 MHz, CDCl_3) δ 9.65 (s, 1H), 3.97 (d, $J = 9.9$ Hz, 2H), 2.98 – 2.83 (m, 2H), 2.47 – 2.32 (m, 1H), 1.88 (d, $J = 10.8$ Hz, 2H), 1.62 – 1.47 (m, 2H), 1.45 (s, 9H).

***N*-Boc-6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-O-2,3,4,6-tetra-O-benzoyl- β -D-galactopyranoside (7).** Boc protected piperidine **4** (789 mg, 0.995 mmol) was dissolved in 10% TFA/ CH_2Cl_2 (12 mL) and stirred at rt for 2 h. The reaction was concentrated under reduced pressure and excess TFA removed by coevaporation with toluene to yield deprotected piperidine **5**. $R_f = 0.26$ (1:9, MeOH/ CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ 8.09 (d, $J = 7.8$ Hz, 2H), 8.02 (d, $J = 7.9$ Hz, 2H), 7.96 (d, $J = 7.9$ Hz, 2H), 7.79 (d, $J = 8.0$ Hz, 2H), 7.62 (t, $J = 7.4$ Hz, 1H), 7.59 – 7.46 (m, 4H), 7.41 (dt, $J = 26.1, 7.7$ Hz, 6H), 7.26 – 7.22 (m, 3H), 6.02 (d, $J = 2.9$ Hz, 1H), 5.79 (dd, $J = 10.3, 8.0$ Hz, 1H), 5.65 (dd, $J = 10.4, 3.4$ Hz, 1H), 4.82 (d, $J = 7.9$ Hz, 1H), 4.71 (dd, $J = 11.3, 6.5$ Hz, 1H), 4.43 (dd, $J = 11.3, 6.6$ Hz, 1H), 4.37 (t, $J = 6.5$ Hz, 1H), 3.88 (dd, $J = 9.5, 5.9$ Hz, 1H), 3.40 (t, $J = 8.5$ Hz, 1H), 3.27 (dd, $J = 22.1, 12.5$ Hz, 2H), 2.78 – 2.62 (m, 2H), 1.83 (dd, $J = 29.9, 13.0$ Hz, 3H), 1.51 – 1.31 (m, 2H); HRMS (ESI): calcd for $\text{C}_{40}\text{H}_{39}\text{NO}_{10}$ $[\text{M}+\text{H}]^+$ $m/z = 694.2647$, found: 694.2642. To the resulting residue was added piperidine aldehyde **6** (255 mg, 1.19 mmol) and $\text{NaBH}(\text{OAc})_3$ (316 mg, 1.49 mmol) in 1,2-dichloroethane (12 mL) and stirred for 18 h at rt. The reaction was quenched with aqueous NaHCO_3 and extracted into CH_2Cl_2 followed by washing with a saturated NaCl solution and drying over Na_2SO_4 . The crude mixture was concentrated under vacuum and purified by silica column chromatography (MeOH/ CH_2Cl_2) to afford galactose dipiperidine **7** as a white solid (845 mg, 95%). $R_f = 0.35$ (1:9, MeOH/ CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3) δ 8.08 (d, $J = 7.4$ Hz, 2H), 8.01 (d, $J = 7.4$ Hz, 2H), 7.94 (d, $J = 7.4$ Hz, 2H), 7.81 – 7.74 (m, 2H), 7.60 (t, $J = 7.4$ Hz, 1H), 7.57 – 7.50 (m, 1H), 7.50 – 7.44 (m, 3H), 7.39 (dt, $J = 22.7, 7.7$ Hz, 5H), 7.23 (t, $J = 7.7$ Hz, 2H), 5.99 (d, $J = 3.4$ Hz, 1H), 5.78 (dd, $J = 10.3, 8.0$ Hz, 1H), 5.60 (dd, $J = 10.4, 3.4$ Hz, 1H), 4.79 (d, $J = 7.9$ Hz, 1H), 4.68 (dd, $J = 11.2, 6.4$ Hz, 1H), 4.40 (dd, $J = 11.2, 6.8$ Hz, 1H), 4.31 (dd, $J = 15.3, 8.6$ Hz, 1H), 4.04 (s, 2H), 3.84 (dd, $J = 9.6, 5.7$ Hz, 1H), 3.45 (t, $J = 8.6$ Hz, 1H), 3.37 (dd, $J = 9.5, 7.1$ Hz, 1H), 2.76 – 2.60 (m, 5H), 2.03 (d, $J = 6.7$ Hz, 2H), 1.79 – 1.48 (m, 10H), 1.43 (s, 9H), 1.16 – 1.07 (m, 2H), 1.05 – 0.93 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 166.48, 166.01, 165.68, 155.35, 134.02, 133.73, 130.47, 130.20, 130.09, 129.84, 129.46, 129.20, 129.04, 128.90, 128.79, 128.71, 102.48, 79.61, 72.13, 71.67, 70.30, 68.55, 62.41, 28.90;

HRMS (ESI): calcd for $\text{C}_{51}\text{H}_{58}\text{N}_2\text{O}_{12}$ $[\text{M}+\text{H}]^+$ $m/z = 891.4063$, found: 891.4085.

***N*-(*N*-Boc-piperidin-4-yl)methoxyphthalimide (9).** To *N*-Boc-4-piperidinemethanol (1.0 g, 4.6 mmol) was added *N*-hydroxyphthalimide (1.14 g, 6.97 mmol) and triphenylphosphine (1.6 g, 6.1 mmol) and cooled to 0 °C while stirring in THF (30 mL). Diethyl azodicarboxylate (877 μL , 5.57 mmol) was added dropwise and allowed to warm to rt over 4 h. The reaction was diluted in CH_2Cl_2 and quenched by washing with sat. NH_4Cl and a saturated NaCl solution followed by drying over Na_2SO_4 . The crude mixture was concentrated by evaporation under reduced pressure and purified by silica flash chromatography (Hex/EtOAc gradient) to afford the phthalimide piperidine **9** as a white solid (1.57 g, 94%). $R_f = 0.59$ (1:1, Hex/EtOAc); ^1H NMR (400 MHz, CDCl_3) δ 7.74 – 7.57 (m, 4H), 4.09 – 3.97 (m, 2H), 3.93 (d, $J = 6.5$ Hz, 2H), 2.64 (s, 2H), 1.94 – 1.81 (m, 1H), 1.78 (d, $J = 12.5$ Hz, 2H), 1.34 (s, 9H), 1.25 – 1.04 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 163.44, 154.69, 134.49, 128.79, 123.43, 82.45, 79.30, 76.97, 35.21, 28.39, 21.93; HRMS (ESI): calcd for $\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$ $m/z = 361.1758$, found: 361.1767.

***N*-(*N*-Boc-piperidin-4-yl)methoxypent-4-enamide (10).** *N*-methoxypiperidinyl phthalimide **9** (615 mg, 1.71 mmol) was dissolved in MeOH (12 mL) followed by dropwise addition of hydrazine hydrate (91 μL , 1.88 mmol) and stirred at rt for 1 h. The crude reaction was concentrated under reduced pressure, redissolved in CH_2Cl_2 , filtered, and the concentrated residue was purified by silica column chromatography (Hex/EtOAc) to afford the free aminoxy piperidine (306 mg, 78%). To this was added triethylamine (280 μL , 2.0 mmol) in MeOH (8 mL) and cooled to 0 °C. Pentenoic anhydride (486 μL , 2.66 mmol) was added dropwise and stirred for 1.5 h followed by removal of solvent under vacuum. The crude mixture was purified by silica flash chromatography to yield the *N*-methoxypiperidinyl pentenamide **10** (374 mg, 90%). $R_f = 0.72$ (1:2, Hex/EtOAc); ^1H NMR (400 MHz, CDCl_3) δ 9.77 (s, 1H), 5.86 – 5.59 (m, 1H), 4.96 (dd, $J = 24.8, 13.6$ Hz, 2H), 4.12 – 3.88 (m, 2H), 3.66 (d, $J = 5.2$ Hz, 2H), 2.77 – 2.50 (m, 2H), 2.39 – 2.23 (m, 2H), 2.14 (d, $J = 6.9$ Hz, 2H), 1.89 – 1.59 (m, 3H), 1.38 (s, 9H), 1.21 – 0.97 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 170.31, 154.84, 136.60, 134.28, 130.19, 123.39, 115.63, 115.33, 80.60, 79.54, 76.93, 34.93, 33.32, 32.26, 29.32, 28.76, 28.66, 28.40. HRMS (ESI): calcd for $\text{C}_{16}\text{H}_{27}\text{N}_2\text{O}_4$ $[\text{M}+\text{Na}]^+$ $m/z = 335.1941$, found: 335.1934.

***N*-(1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxypent-4-enamide (12).** Boc protected piperidine **10** (290 mg, 0.928 mmol) was dissolved in 10% TFA/ CH_2Cl_2 (5 mL) and stirred at rt for 2 h. The reaction was concentrated under reduced pressure, excess TFA removed by coevaporation with toluene, and run through a silica plug (MeOH/ CH_2Cl_2) to yield deprotected piperidine **11**. $R_f = 0.17$ (1:9, MeOH/ CH_2Cl_2); ^1H NMR (400 MHz, MeOD) δ 5.69 (ddt, $J = 16.9, 10.2, 6.6$ Hz, 1H), 5.03 – 4.82 (m, 2H), 3.61 (d, $J = 6.1$ Hz, 2H), 3.29 (d, $J = 12.7$ Hz, 2H), 2.88 (dt, $J = 12.9, 6.6$ Hz, 2H), 2.23 (q, $J = 7.1$ Hz, 2H), 2.06 (t, $J = 7.3$ Hz, 2H), 1.97 – 1.81 (m, 3H), 1.48 – 1.27 (m, 2H). To the resulting residue (145 mg, 0.464 mmol) was added piperidine aldehyde **6** (119 mg, 0.557) and $\text{NaBH}(\text{OAc})_3$ (148 mg, 0.696 mmol) in 1,2-dichloroethane with cat. AcOH and stirred for 18 h at rt. The reaction was quenched with aqueous NaHCO_3 and extracted into CH_2Cl_2 followed by washing with a saturated NaCl solution and drying over Na_2SO_4 . The crude mixture was concentrated under vacuum and purified by silica column chromatography (MeOH/ CH_2Cl_2) to afford *N*-hydroxypentenyl dipiperidine **12** as a clear residue (92 mg, 48%). $R_f = 0.30$ (1:9, MeOH/ CH_2Cl_2); ^1H NMR (400 MHz, CDCl_3) δ 5.99 – 5.68 (m, 1H), 5.06 (dd, $J = 24.4, 13.6$ Hz, 2H),

4.25 – 3.91 (m, 2H), 3.84 – 3.60 (m, 2H), 2.88 (d, $J = 11.2$ Hz, 2H), 2.70 (t, $J = 11.5$ Hz, 2H), 2.42 (dd, $J = 14.5, 6.7$ Hz, 2H), 2.18 (d, $J = 6.8$ Hz, 3H), 1.94 (t, $J = 11.2$ Hz, 2H), 1.84 – 1.55 (m, 7H), 1.47 (s, 9H), 1.37 – 1.26 (m, 2H), 1.08 (qd, $J = 12.7, 4.2$ Hz, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 154.95, 136.68, 115.71, 81.16, 79.33, 64.85, 53.86, 34.75, 33.65, 32.39, 30.80, 29.36, 28.85, 28.46; HRMS (ESI): calcd for $\text{C}_{22}\text{H}_{39}\text{N}_3\text{O}_4$ [$\text{M}+\text{Na}$] $^+$ $m/z = 410.3013$, found: 410.3005.

6-chloro-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-*O*-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranosyl]-1,3,5-triazine-2,4-diamine (14). Boc protected dipiperidine **7** (1.09 g, 1.22 mmol) was dissolved in 10% TFA/ CH_2Cl_2 (16 mL) and stirred at rt for 1.5 h. The reaction was concentrated under reduced pressure, excess TFA removed by coevaporation with toluene, and run through a silica column ($\text{MeOH}/\text{CH}_2\text{Cl}_2$) to yield deprotected galactose dipiperidine **8** in quantitative yield. $R_f = 0.14$ (1:9, $\text{MeOH}/\text{CH}_2\text{Cl}_2$); ^1H NMR (500 MHz, CDCl_3) δ 8.07 (d, $J = 7.4$ Hz, 2H), 7.99 (d, $J = 7.4$ Hz, 2H), 7.92 (t, $J = 7.4$ Hz, 2H), 7.76 (d, $J = 7.4$ Hz, 2H), 7.59 (q, $J = 7.3$ Hz, 1H), 7.54 (dd, $J = 13.9, 6.5$ Hz, 1H), 7.51 – 7.44 (m, 3H), 7.39 (dt, $J = 21.6, 7.7$ Hz, 5H), 7.23 (t, $J = 7.8$ Hz, 2H), 5.99 (d, $J = 3.2$ Hz, 1H), 5.75 (dd, $J = 10.2, 8.1$ Hz, 1H), 5.61 (dd, $J = 10.4, 3.3$ Hz, 1H), 4.80 (d, $J = 7.9$ Hz, 1H), 4.68 (dd, $J = 11.0, 6.3$ Hz, 1H), 4.43 – 4.36 (m, 1H), 4.34 (t, $J = 6.3$ Hz, 1H), 3.84 (s, 1H), 3.59 – 3.47 (m, 1H), 3.47 – 3.39 (m, 1H), 3.39 – 3.22 (m, 3H), 2.96 – 2.73 (m, 5H), 2.57 – 2.37 (m, 2H), 2.18 – 2.06 (m, 1H), 2.00 – 1.73 (m, 6H), 1.67 – 1.45 (m, 4H); HRMS (ESI): calcd for $\text{C}_{46}\text{H}_{50}\text{N}_{10}\text{O}_{10}$ [$\text{M}+\text{H}$] $^+$ $m/z = 791.3538$, found: 791.3546. The resulting residue was dissolved in MeCN (18 mL) and *N,N*-diisopropylethylamine (320 μL , 1.8 mmol) was added dropwise followed by cooling to 0 °C. Cyanuric chloride (113 mg, 0.611 mmol) was added and the mixture was allowed to warm to rt and stirred for 2 h followed by concentration under reduced pressure. The crude product was purified by a silica flash column ($\text{MeOH}/\text{CH}_2\text{Cl}_2$) to afford the two-arm galactose melamine dendron **4.14** (936 mg, 90%). $R_f = 0.69$ (1:9, $\text{MeOH}/\text{CH}_2\text{Cl}_2$); ^1H NMR (600 MHz, CDCl_3) δ 8.07 (d, $J = 7.6$ Hz, 4H), 8.00 (d, $J = 7.6$ Hz, 4H), 7.93 (d, $J = 7.6$ Hz, 4H), 7.77 (d, $J = 7.7$ Hz, 4H), 7.61 (d, $J = 7.0$ Hz, 2H), 7.53 (dt, $J = 24.4, 7.3$ Hz, 4H), 7.48 (t, $J = 7.4$ Hz, 4H), 7.40 (dt, $J = 19.8, 7.7$ Hz, 10H), 7.23 (t, $J = 7.7$ Hz, 4H), 5.99 (d, $J = 2.4$ Hz, 2H), 5.76 (dd, $J = 9.9, 8.3$ Hz, 2H), 5.61 (d, $J = 9.2$ Hz, 2H), 4.80 (d, $J = 7.9$ Hz, 2H), 4.68 (d, $J = 6.2$ Hz, 6H), 4.41 (dd, $J = 11.2, 6.4$ Hz, 2H), 4.34 (t, $J = 6.3$ Hz, 2H), 3.99 – 3.79 (m, 2H), 3.65 – 3.25 (m, 6H), 2.93 – 2.58 (m, 9H), 2.50 – 2.27 (m, 3H), 2.05 – 1.48 (m, 17H), 1.30 – 1.07 (m, 5H); ^{13}C NMR (151 MHz, CDCl_3) δ 165.89, 165.41, 165.37, 164.07, 133.53, 133.28, 129.85, 129.60, 129.49, 129.23, 128.88, 128.52, 128.40, 128.18, 101.72, 71.36, 69.71, 68.00, 61.93, 42.83; HRMS (ESI): calcd for $\text{C}_{95}\text{H}_{98}\text{N}_7\text{O}_{20}\text{Cl}$ [$\text{M}+\text{H}$] $^+$ $m/z = 1692.6628$, found: 1692.6578.

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxypent-4-enamide]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-*O*-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranosyl]-melamine (15).** Boc protected dipiperidine **12** (95 mg, 0.232 mmol) was dissolved in 10% TFA/ CH_2Cl_2 (2.5 mL) and stirred at rt for 1 h. The reaction was concentrated under reduced pressure and excess TFA removed by coevaporation with toluene to yield deprotected *N*-methoxypentenamide dipiperidine **13**. The resulting residue was dissolved in THF/MeCN (1:1, v:v; 4 mL) and the two-arm galactose melamine dendron **14** (140 mg, 0.083 mmol) was added followed by dropwise addition of *N,N*-diisopropylethylamine (87 μL , 0.50 mmol). The reaction was heated at 60 °C for 7 days upon which the solvent was removed under reduced pressure and purified by silica column

chromatography ($\text{MeOH}/\text{CH}_2\text{Cl}_2$) to afford the fully protected *N*-pentenoyl aminooxy digalactose melamine dendron **15** as a clear residue (123 mg, 75%). $R_f = 0.52$ (3:17, $\text{MeOH}/\text{CH}_2\text{Cl}_2$); ^1H NMR (600 MHz, CDCl_3) δ 8.07 (d, $J = 7.6$ Hz, 4H), 7.99 (t, $J = 14.7$ Hz, 4H), 7.93 (d, $J = 7.7$ Hz, 4H), 7.77 (d, $J = 7.7$ Hz, 4H), 7.61 (t, $J = 7.2$ Hz, 2H), 7.53 (dt, $J = 20.2, 7.2$ Hz, 4H), 7.46 (dd, $J = 16.2, 8.7$ Hz, 4H), 7.40 (dt, $J = 21.7, 7.6$ Hz, 10H), 7.23 (t, $J = 7.5$ Hz, 4H), 5.99 (d, $J = 2.1$ Hz, 2H), 5.83 – 5.71 (m, 3H), 5.61 (dd, $J = 10.3, 2.6$ Hz, 2H), 5.00 (dd, $J = 40.2, 13.5$ Hz, 2H), 4.80 (d, $J = 7.8$ Hz, 2H), 4.73 – 4.61 (m, 8H), 4.40 (dd, $J = 11.1, 6.5$ Hz, 2H), 4.33 (t, $J = 6.2$ Hz, 2H), 3.95 – 3.81 (m, 2H), 3.81 – 3.69 (m, 2H), 3.50 – 3.17 (m, 6H), 2.77 – 2.62 (m, 9H), 2.62 – 2.41 (m, 5H), 2.41 – 2.29 (m, 4H), 2.18 – 2.11 (m, 2H), 1.97 – 1.87 (m, 4H), 1.84 – 1.64 (m, 15H), 1.62 – 1.39 (m, 4H), 1.30 – 1.02 (m, 9H); ^{13}C NMR (151 MHz, CDCl_3) δ 166.00, 165.53, 165.50, 165.28, 162.20, 161.97, 133.62, 133.44, 133.36, 133.31, 129.97, 129.71, 129.60, 129.35, 129.16, 128.99, 128.69, 128.62, 128.49, 128.28, 128.19, 117.73, 115.79, 115.71, 101.85, 71.54, 71.40, 69.83, 68.11, 62.01, 42.90, 30.05, 29.65; HRMS (ESI): calcd for $\text{C}_{112}\text{H}_{128}\text{N}_{10}\text{O}_{22}$ [$\text{M}+\text{H}$] $^+$ $m/z = 1965.9277$, found: 1965.9256.

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxypent-4-enamide]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)- β -D-galactopyranosyl]-melamine (16).** Fully protected dendron **15** (100 mg, 0.05 mmol) was dissolved in MeCN/ $\text{H}_2\text{O}/\text{CH}_2\text{Cl}_2$ (4:1:1, v:v; 3 mL) and NaOH (1 M in NaOH, 150 μL) was added dropwise while stirring at rt. The mixture was chilled to 0 °C and reacted for 48 h after which AcOH was added dropwise until the pH reached 7. Excess solvent was removed under reduced pressure and the resulting residue was dissolved in dd H_2O /MeCN (9:1, v:v; 1.5 mL) followed by HPLC purification. The product eluted at 14 min in a gradient of 10% to 50% MeCN in dd H_2O (0.1% TFA) over 25 min. The product peaks were collected and lyophilized to obtain *N*-pentenoyl aminooxy galactose dendrimer **16** as a white powder (55 mg, 95%). ^1H NMR (500 MHz, D_2O) δ 5.87 – 5.66 (m, 1H), 5.03 (dd, $J = 17.8, 14.1$ Hz, 2H), 4.45 (d, $J = 12.7$ Hz, 6H), 4.34 (d, $J = 7.8$ Hz, 2H), 3.88 (d, $J = 2.8$ Hz, 3H), 3.82 – 3.67 (m, 9H), 3.66 – 3.56 (m, 11H), 3.54 (dd, $J = 10.1, 6.3$ Hz, 2H), 3.47 (dd, $J = 9.8, 8.0$ Hz, 2H), 3.36 – 3.23 (m, 2H), 3.08 – 3.00 (m, 12H), 2.96 (t, $J = 11.9$ Hz, 6H), 2.36 – 2.27 (m, 2H), 2.27 – 2.19 (m, 5H), 2.09 – 1.91 (m, 10H), 1.86 – 1.80 (m, 6H), 1.59 – 1.44 (m, 6H), 1.32 – 1.23 (m, 6H); ^{13}C NMR (126 MHz, CDCl_3) δ 172.29, 163.18, 162.90, 162.61, 162.33, 156.58, 136.35, 119.74, 117.42, 115.93, 115.10, 112.78, 102.96, 79.57, 75.09, 73.35, 72.67, 70.70, 68.54, 61.69, 60.89, 52.96, 52.83, 44.14, 33.22, 31.83, 31.62, 30.34, 28.94, 28.81, 25.70, 25.50; HRMS (ESI): calcd for $\text{C}_{56}\text{H}_{96}\text{N}_{10}\text{O}_{14}$ [$\text{M}+\text{H}$] $^+$ $m/z = 1133.7180$, found: 1133.7173.

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxypent-4-enamide]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosyl-onic acid)-(2 $\rightarrow$ 6)- β -D-galactopyranosyl]-melamine (17).** To *N*-pentenoyl galactose dendrimer **16** (4.8 mg, 0.0042 mmol) was added *N*-acetylmannosamine (2.8 mg, 0.013 mmol), sodium pyruvate (4.7 mg, 0.042 mmol), and CTP•Na (7.2 mg, 0.013 mmol) and dissolved in dd H_2O (1 mL). A concentrated stock of Tris-HCl buffer pH 8.5 with MgCl_2 was added to a final concentration of 100 mM Tris, 20 mM MgCl_2 . Recombinant *E. coli* K12 sialic acid aldolase (1 U), *N. meningitidis* CMP-sialic acid synthetase (0.5 U), and *P. damsela* α -2,6-sialyltransferase (0.5 U) were added and the reaction mixture was incubated at 37 °C for 4 h followed by shaking at rt for 16 h. The reaction was monitored by LCMS (5% to 50% MeCN in dd H_2O over 15 min) and after

24 h, the reaction mixture was quenched with cold MeOH (1 mL) and incubated on ice 20 min. The mixture was centrifuged, precipitates removed, and concentrated under vacuum. The crude mixture was redissolved in ddH₂O (1 mL) followed by the readdition of 0.5 U of each enzyme and incubation at rt for 48 h. The reaction mixture was quenched with cold MeOH (1 mL) and precipitates removed followed by concentration under vacuum. The product was purified by HPLC with elution at 11 min in a gradient of 10% to 50% MeCN in ddH₂O (0.05% formic acid) over 25 min. The product peaks were collected and lyophilized to obtain *N*-pentenoyl 2,6-sialylgalactose dendrimer **17** as a white powder (3.8 mg, 52%). ¹H NMR (600 MHz, D₂O) δ 5.88 – 5.73 (m, 1H), 5.05 (dd, *J* = 22.1, 13.9 Hz, 2H), 4.62 (d, *J* = 12.0 Hz, 6H), 4.39 – 4.32 (m, 2H), 3.91 (t, *J* = 8.8 Hz, 5H), 3.88 – 3.83 (m, 4H), 3.82 – 3.73 (m, 8H), 3.72 – 3.70 (m, 2H), 3.70 – 3.59 (m, 15H), 3.58 – 3.53 (m, 2H), 3.53 – 3.45 (m, 2H), 3.39 – 3.22 (m, 2H), 3.16 – 3.02 (m, 7H), 2.98 (t, *J* = 12.0 Hz, 5H), 2.87 (t, *J* = 11.8 Hz, 6H), 2.71 (dd, *J* = 12.4, 4.6 Hz, 2H), 2.39 – 2.29 (m, 2H), 2.29 – 2.15 (m, 5H), 2.14 – 2.03 (m, 8H), 2.01 (s, 6H), 1.86 – 1.76 (m, 6H), 1.66 (t, *J* = 12.2 Hz, 2H), 1.64 – 1.45 (m, 6H), 1.33 – 1.13 (m, 6H); ¹³C NMR (126 MHz, D₂O) δ 175.0, 173.4, 172.3, 170.0, 163.7, 136.4, 116.0, 103.1, 100.4, 79.6, 73.8, 73.4, 72.6, 72.5, 71.7, 70.6, 68.5, 68.1, 63.4, 62.6, 62.0, 53.0, 52.8, 51.8, 42.7, 40.1, 33.3, 31.9, 31.6, 30.8, 29.0, 29.0, 25.7, 25.5, 22.0; HRMS (ESI): calcd for C₇₈H₁₃₁N₁₂O₃₀ [M+H]⁺ *m/z* = 858.4581, found: 858.4587

***N*-[1-((*N*-Boc-piperidin-4-yl)methyl)piperidin-4-yl)methoxypent-4-enamide]-*N,N*-[6-((1-(piperidin-4-ylmethyl)piperidin-4-yl)methoxy)-(5-acetamido-3,5-dideoxy-*D*-glycero-*α*-*D*-galacto-2-nonulopyranosyl-onic acid)-(2→3)-*β*-*D*-galactopyranosyl]-melamine (**18**).** To *N*-pentenoyl galactose dendrimer **16** (4.5 mg, 3.9 μmol) was added *N*-acetylmannosamine (2.6 mg, 0.012 mmol), sodium pyruvate (4.3 mg, 0.039 mmol), and CTP•Na (6.8 mg, 0.012 mmol) and dissolved in ddH₂O (1 mL). A concentrated stock of Tris-HCl buffer pH 8.5 with MgCl₂ was added to a final concentration of 100 mM Tris, 20 mM MgCl₂. Recombinant *E. coli* K12 sialic acid aldolase (1 U), *N. meningitidis* CMP-sialic acid synthetase (1.5 U), and *P. multocida* *α*-2,3-sialyltransferase (0.4 U) were added and the reaction mixture was incubated at 37 °C for 4 h followed by shaking at rt for 16 h. The reaction was monitored by LCMS (5% to 50% MeCN in ddH₂O over 15 min) and after 24 h, the reaction mixture was quenched with cold MeOH (1 mL) and incubated on ice 20 min. The mixture was centrifuged, precipitates removed, and concentrated under vacuum. The product was purified by HPLC with elution at 11 min in a gradient of 10% to 50% MeCN in ddH₂O (0.05% formic acid) over 25 min. The product peaks were collected and lyophilized to obtain *N*-pentenoyl 2,3-sialylgalactose dendrimer **18** as a white powder (3.7 mg, 55%). ¹H NMR (600 MHz, D₂O) δ 5.86 – 5.75 (m, 1H), 5.06 (dd, *J* = 22.7, 13.8 Hz, 2H), 4.63 (d, *J* = 12.1 Hz, 6H), 4.45 (d, *J* = 7.9 Hz, 2H), 4.07 (d, *J* = 9.8 Hz, 2H), 3.96 – 3.89 (m, 2H), 3.89 – 3.76 (m, 9H), 3.76 – 3.70 (m, 4H), 3.70 – 3.56 (m, 15H), 3.57 – 3.49 (m, 3H), 3.11 – 2.91 (m, 10H), 2.87 (t, *J* = 12.1 Hz, 6H), 2.74 (dd, *J* = 11.2, 2.7 Hz, 2H), 2.38 – 2.29 (m, 2H), 2.24 (t, *J* = 7.0 Hz, 2H), 2.22 – 2.10 (m, 3H), 2.10 – 1.93 (m, 13H), 1.89 – 1.71 (m, 8H), 1.66 – 1.46 (m, 6H), 1.29 – 1.14 (m, 6H); ¹³C NMR (126 MHz, D₂O) δ 175.0, 173.8, 172.3, 170.9, 164.5, 136.4, 115.9, 102.7, 99.8, 75.8, 74.9, 72.8, 71.7, 69.1, 68.3, 68.0, 67.4, 62.5, 60.9, 51.6, 42.5, 39.6, 31.7, 30.9, 29.0, 29.0, 22.0; HRMS (ESI): calcd for C₇₈H₁₃₁N₁₂O₃₀ [M+H]⁺ *m/z* = 858.4581, found: 858.4585

4.2. Expression and purification of aldehyde-tagged proteins

Recombinant hGH was expressed in *E. coli* as previously described.²⁴ In short, BL21(DE3) *E. coli* harboring both a pET plasmid containing the aldehyde tagged-protein and a pBAD plasmid containing the *M. tuberculosis* FGE were grown in LB media supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 37° C. When OD₆₀₀ reached 0.5, arabinose was added at 0.25% and the culture was shaken at 37° C for an additional 1 h. The temperature was then reduced to 18° C for 1 h and IPTG (0.1 mM) was added with further shaking for 14-18 h. Cells were lysed by homogenization, and the His₆-tagged protein was purified using Ni-NTA-agarose beads (Qiagen) under standard purification procedures. hGH was eluted in 50 mM Tris, 500 mM NaCl, 300 mM imidazole, 10% glycerol, pH 7.5 with 1 mM DTT, TCEP, and methionine to protect against oxidation and frozen directly for storage at -20 °C. The Fc construct was expressed and purified according to Smith *et al.*⁴¹ Briefly, CHO cells were transiently transfected with the Fc-containing plasmid, and the media was collected two days after transfection. After concentration, the aldehyde-tagged Fc was purified using a Protein A/G agarose resin (Pierce). Protein concentrations were determined using the calculated absorbances at 280 nm.

When required, proteins were treated with additional FGE *in vitro* to fully convert Cys-to-fGly if intracellular conversion was sub-optimal. Briefly, aldehyde-tagged proteins were incubated in 50 mM Tris, 100 mM NaCl, 0.1-1 mM β-Me, pH 9 with either 0.05 equiv. (for hGH) or 1 equiv. (for Fc) of *M. tuberculosis* FGE⁴³ for 20 h at rt. Proteins were then purified by size exclusion and buffer exchanged by spin concentration and stored in PBS with 10% glycerol.

4.3. Protein conjugation and analysis

Proteins were concentrated and buffer exchanged by dilution in Amicon Ultra-0.5 centrifugal filter devices (MWCO 10K or 30K, Millipore). Proteins were resolved by SDS-PAGE on Bis-Tris Criterion Gels (10% or 4-12%; Bio-Rad). Western blot analysis was performed by wet transfer (Tris-glycine, 20% MeOH) onto nitrocellulose at 100V for 60 min. Protein loading was confirmed by Ponceau stain (0.2% ponceau in 3% AcOH). Blocking and antibody incubation conditions were conducted in 1x Dulbecco's phosphate buffered saline with 0.1% Tween-20 (PBST) with component concentrations as described. For intact protein mass spectrometry analysis, samples were diluted to a final concentration of 0.3-1 μM in H₂O/MeCN/FA (95:5:0.1). LC-ESI-MS (liquid chromatography electrospray ionization mass spectrometry) was performed using an EASY-nLC 1000 containing a C8 capillary column (75 μm ID x 10 cm, packed in-house) connected in-line to an LTQ XL linear ion trap mass spectrometer (Thermo Scientific). Mass spectra were deconvoluted using ProMass for Xcalibur software (version 2.8 rev. 5, Novatia).

General procedure for dendrimer conjugation: Aldehyde-tagged hGH at 30 μM was reacted with 2 mM aminoxy compound in 5 μL NaCit/NaH₂PO₄ buffer pH 4.5 for 16 h at 37 °C. Aldehyde-tagged Fc (5 μM) was reacted with 500 μM of Gal dendrimer **3** in NaCit/NaH₂PO₄ buffer, pH 4.5 for 16 h at 60 °C. The reactions were quenched with 1 μL of 1 M Tris, pH 7.5, 4x SDS loading dye, samples boiled, and were run on a 10% SDS-PAGE gel. The protein was transferred to nitrocellulose and blocked in PBST (0.5% Tween-20, 2 mM CaCl₂, MgCl₂). The presence of sugars was probed with SBA-FITC, SNA-FITC, or MAA-FITC (EY Labs, 1:1,000 dilution) in PBST with 2 mM CaCl₂ and MgCl₂. Membranes were developed by chemiluminescence using the SuperSignal West Pico kit (ThermoFisher) or scanned for fluorescence on a Typhoon 9410 Imaging System (Amersham). For mass spectrometry analysis,

aliquots were taken before SDS-PAGE, and prepared as above. Percent conjugation was determined by comparing peak areas of unreacted aldehyde-tagged protein to dendrimer conjugated peaks.

4.4. Molecular modeling

The N-glycan core structure (Fig S1) along with seven candidate synthetic glycodendrons were energetically minimized using Schrödinger molecular modeling software and OPLS 2005 force field and conformational search algorithm.⁴⁴ The base length, arm length, branch angle, and electrostatic volume for each minimized glycodendron were measured and compared to the native N-glycan core (Table 1). An example weighted calculation for the piperidine dendrimer follows: percent difference for Piperidine, $\text{Length1} = \frac{\text{absolute value}(\text{Length1, Piperidine} - \text{Length1, N-Glycan})}{(\text{Length1, N-Glycan})} = 0.0785$. This percent difference was then converted into a score for each category. Score for Piperidine, $\text{Length1} = 1 - \text{Percent difference for Piperidine, Length1}$. The score then was 0.9215. The scores for each category were 0.9215 (Length1), 0.3928 (Length2), 0.3577 (Length3), 0.3972 (Angle1), 0.3435 (Angle2), 0.7927 (Volume). The scores were then weighted by the factors - 0.35, 0.1, 0.1, 0.05, 0.05, 0.35. The final score for Piperidine was 0.712/10. This was completed for all seven candidate structures.

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Supplementary Material

Supplementary material associated with this article including additional figures and NMR data can be found, in the online version, at

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