Bioconjugate Chemistry

Technical Note

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N-Acetylgalactosamino Dendrons as Clearing Agents to Enhance Liver Targeting of Model Antibody-Fusion Protein

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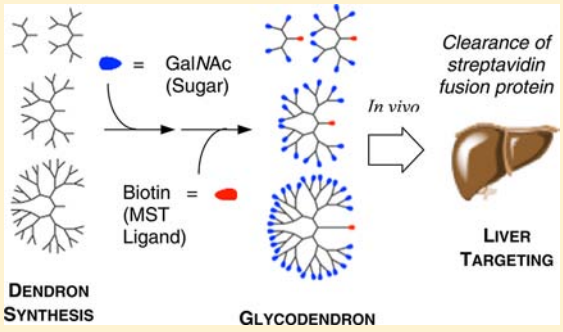
Supporting Information

ABSTRACT: Dendrimer *clearing agents* represent a unique class of compounds for use in multistep targeting (MST) in radioimmunotherapy and imaging. These compounds were developed to facilitate the removal of excess tumor-targeting monoclonal antibody (mAb) prior to administration of the radionuclide to minimize exposure of normal tissue to radiation. Clearing agents are designed to capture the circulating mAb, and target it to the liver for metabolism. Glycodendrons are ideally suited for MST applications as these highly branched compounds are chemically well-defined, thus advantageous over heterogeneous macromolecules. Previous studies have described glycodendron **3** as a clearing agent for use in three-step MST protocols, and early *in vivo* assessment of **3** showed promise. However, synthetic challenges have hampered its availability for further development. In this report we describe a new sequence of chemical steps which enables the straightforward synthesis and analytical characterization of this class of dendrons. With accessibility and analytical identification solved, we sought to evaluate both lower and higher generation dendrons for hepatocyte targeting as well as clearance of a model protein. We prepared a series of clearing agents where a single biotin is connected to glycodendrons displaying four, eight, sixteen or thirty-two α-thio-*N*-acetylgalactosamine (α-SGalNAc) units, resulting in compounds with molecular weights ranging from 2 to 17 kDa, respectively. These compounds were fully characterized by LCMS and NMR. We then evaluated the capacity of these agents to clear a model ^{131}I-labeled single chain variable fragment antibody-streptavidin (^{131}I-scFv-SAv) fusion protein from blood and tissue in mice, and compared their clearing efficiencies to that of a 500 kDa dextran-biotin conjugate. Glycodendrons and dextran−biotin exhibited enhanced blood clearance of the scFv-SAv construct. Biodistribution analysis showed liver targeting/uptake of the scFv-SAv construct to be 2-fold higher for compounds **1** to **4**, as well as for the 500 kDa dextran, over saline. Additionally, the data suggest the glycodendrons clear through the liver, whereas the dextran through reticuloendothelial system (RES) metabolism.



INTRODUCTION

Dendrimers have broad application in the fields of biology and medicine. For example, in cancer therapy, these highly branched polymers have been utilized as multivalent carriers for antimetastatic activity, the stimulation of immune responses, tumor targeting, as well as delivery vehicles for chemotherapeutics.[1,2] One specific application where carbohydrate containing dendrimers, or glycodendrimers, have shown promise is as *clearing agents* in multistep targeting (MST) strategies for radioimmunotherapy (RIT) and imaging. MST clearing agents are bifunctional compounds designed to facilitate the rapid clearance of an unbound tumor-targeting protein, such as a recombinant monoclonal antibody (mAb), from the vasculature and off-target tissue prior to administration of the imaging tracer or drug.[3] This step is often essential to achieving a high therapeutic index. The clearance mechanisms exploited by these agents are through recognition by the reticuloendothelial system (RES) and/or active targeting to the liver through hepatocyte lectins.[4]

Dendrimers provide an ideal platform to fulfill the molecular requirements of clearing agents for clinical MST applications. They can allow for precise construction of a carbohydrate array with optimum density and geometry for efficient lectin recognition, while incorporating an orthogonal functional group for binding of the tumor-targeting mAb. Additionally, the polarity and size of dendrimers can be tuned to restrict diffusion into extravascular compartments, such as tumors, thus restricting access to the prelocalized mAb bound to the tumor. While conjugates of macromolecules − such as glycoconjugates of albumin with biotin ligands,[5] and derivatized dextrans[6] − have been demonstrated to be effective clearing agents, they possess a high degree of compositional heterogeneity. This poses a barrier to reproducible synthesis and, eventually, dosing. From a biological standpoint, use of these molecules

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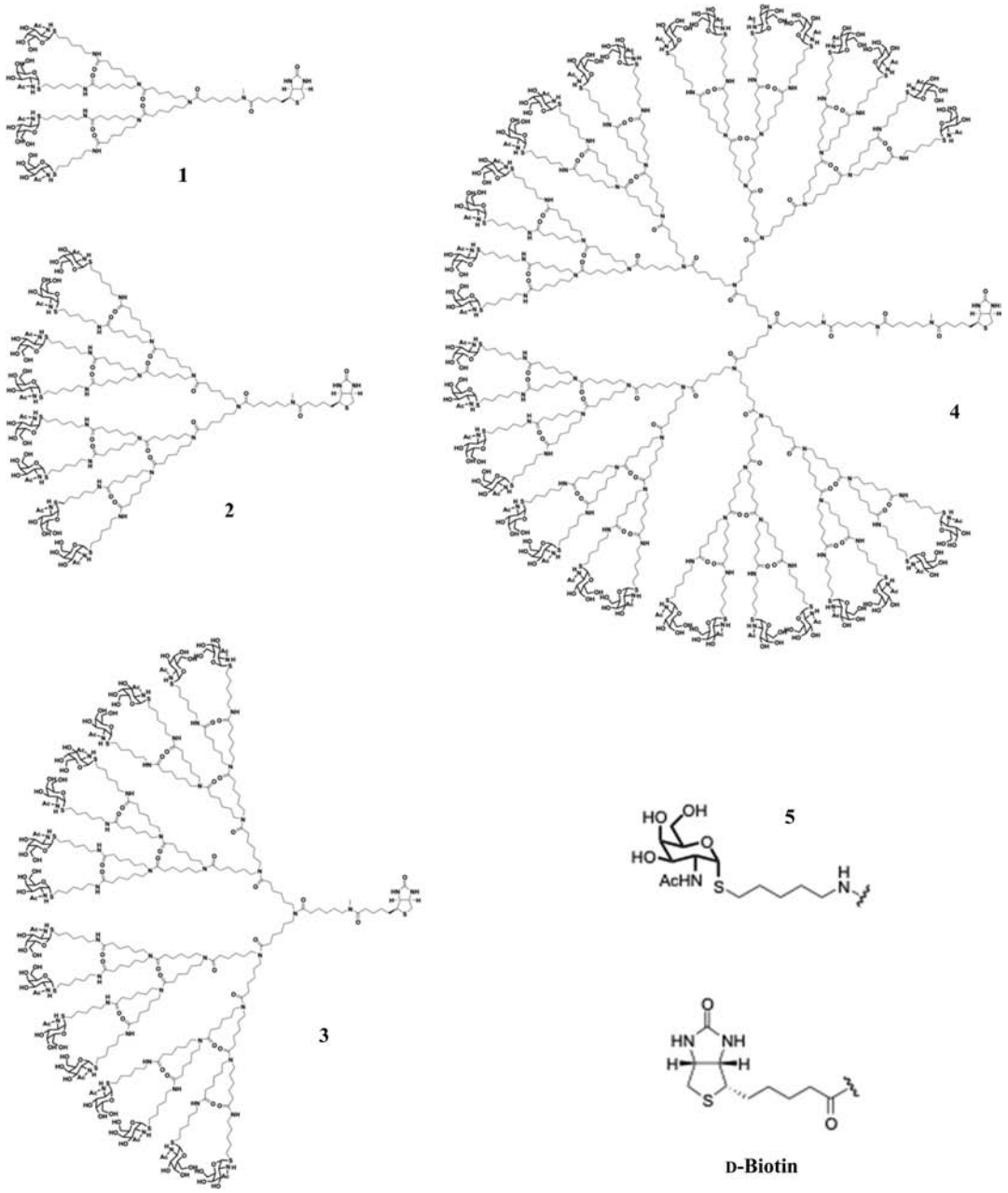

Figure 1. Glycodendron clearing agents (**1**–**4**), α-thio-GalNAc end group (**5**), and biotin core.

can lead to interference with subsequently administered imaging tracer or drug at target tissue, since processing of the macromolecules by the RES can lead to the introduction of metabolites back into circulation which can saturate binding sites (for the imaging tracer or drug) on the targeting mAb.[3,7]

The *N*-acetylgalactoseamine functionalized dendron **3** (Figure 1) has proven effective in MST, and has been utilized in preclinical and clinical studies using the biotin/streptavidin based three-step pretargeting approach.[8] This glycodendron contains a single biotin attached to a dendron scaffold composed of aminohexanoic aliphatic arms connected through tertiary amides, with sixteen α-configured thio-*N*-acetylgalactosamine end groups **5**. These monosaccharides are used for liver targeting, as they serve as ligands for the asialylglycoprotein (ASGP) receptors expressed on hepatocytes.[9,10] The ASGP receptor is a lectin that binds desialylated glycoproteins with particular preference for terminal galactose and *N*-acetyl galactosamine (GalNAc) units.[11] In addition, other studies have shown that significant binding enhancements to the ASGP receptor were obtained with branched polyvalent presentation of the carbohydrate groups,[11] further underscoring the advantage of a dendron scaffold. On the glycodendron, monosaccharides are *S*-linked, as opposed to *O*-linked, which imparts greater resistance to hydrolysis. The single biotin is attached to an *N*-methyl amide, which has been shown to provide stability against biotinases *in vivo*.[12] While compound **3** has been utilized in MST studies with promising results,[5,8,13−15] synthesis of this glycodendron has remained a challenge, and analytical characterization has not been previously described. In addition, we had particular interest in evaluating lower generation variants in order to further optimize structures that may be less complex and more readily obtained for clinical use.

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Absent clear answers as well as analytical data, and present the urge to gain insights into the clearing mechanism and properties of these glycodendrons, we sought to synthesize compounds **1** to **4** and subsequently test their clearing abilities. Most prior work was limited to patents. Be that as it may, the lack of a clear synthesis path, analytical data, and clearing profiles dictated that we revisit all the above problems. With clinical development and its need for large amounts of the best clearing agent in mind, we embarked on the search for a better clearing agent by solving all the above problems. From the synthesis point of view, one could clearly discern an advantage in more converging strategies that couple advanced intermediates of the dendron or branches thereof bearing the GalNac units in late stages of the synthesis to lead to the final compound. However, despite previously reported methods to obtain **3** we have found most convergent routes to be much more problematic at several levels. At the level of chemistry, there needed to be longer reaction times to ensure completion, which, due the large number of protecting groups, led to partial loss of some under different coupling conditions. At the purification level, numerous purification steps ensued which proved to be unpredictable, time-consuming, and material wasting.

In this report, we devised a new synthetic approach to readily obtain the aliphatic dendrons **11** to **14** using a semiconvergent methodology where the building block **7** is successively used to increase dendron generations, and later at dendron completion, appropriately protected aminoalkylthiosugar moieties were introduced prior to global deprotection. The incorporation of an orthogonal protecting groups strategy provided the versatility to site-specifically attach biotin as well as 4 to 32 α-thio-GalNAc units, and led to the desired family of glycodendrons (**1** to **4**) with molecular weights ranging from approximately 2 to 17 kDa, respectively (Figure 1). Additionally, we have explored solid phase synthesis as a faster means for development of such molecules. However, solid phase synthesis has proven incompatible with the growing dendrimer skeleton, and coupling became inefficient by the second generation. Overall the homogeneous synthesis approach is highly reproducible and efficient, requiring a significantly reduced number of purification steps. Compounds **1** to **4** and intermediates **11** to **14** were fully characterized by LCMS and NMR to ensure product integrity and purity. To verify biological efficacy and utility for MST *in vivo*, we examined the blood clearance kinetics of a model antibody−streptavidin fusion protein following administration of glycodendrons **1** to **4** in normal biotin-starved mice by serial-blood sampling. In addition, we conducted biodistribution studies to determine tracer uptake and retention in the liver, as well as other RES-associated organs. The model antibody−streptavidin protein used for this study is the ^{131}I-labeled scFv-CC49-SAv, a fusion protein containing the single chain variable fragment (scFv) of the murine mAb CC49 and streptavidin.[15−18] The CC49 fragment is specific for the tumor associated glycoprotein-72 (TAG72), which is known to be widely expressed on adenocarcinomas.[18]

EXPERIMENTAL PROCEDURES

General. Solvents and reagents purchased from commercial sources were used without further purification. Ethyl 6-bromohexanoate, *N*-methylcaprolactam, potassium iodide (KI), 4-methoxybenzylamine (PBM), di-*tert*-butyl carbonate, *p*-toluenesulfonyl chloride (TsCl), 4-(dimethylamino)pyridine (DMAP), Lawesson's reagent, sodium bromide (NaBr), tetrabutylammonium bromide (Bu_4NBr), acetonitrile, diethyl ether, dimethylformamide (DMF), ethyl acetate, hexanes, methylene chloride (DCM), diisopropylethylamine (DIEA), and triethylamine (TEA) were obtained from Sigma-Aldrich. Concentrated HCl was obtained from Fisher Scientific. D-Biotin was obtained from Fluka. 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was obtained from Genescript. 2-Acetomido-2-deoxy-3,4,6-tri-*O*-acetyl-1-thio-α-D-galactopyranose and *tert*-butyl (5-hydroxypentyl)carbamate were obtained from BOC Sciences. Deuterium oxide and $CDCl_3$ were obtained from Cambridge Isotopes. Solid-phase synthesis was conducted in polypropylene fritted syringes from Torviq. All resins were obtained from Novabiochem. ^{131}I was purchased in the form of carrier-free $Na^{131}I$ in NaOH containing 0.02 M Na_2SO_4 from Nordion (Canada) Inc. The CC49 single chain variable fragment scFv-streptavidin (scFv-CC49-SAv) fusion protein was provided by the NeoRx Corporation.[16] Pierce precoated iodination tubes (50 μg of Iodogen/tube) were purchased from Thermo Scientific. L-Tyrosine, glycerol, and xylene cyanol, were purchased from Sigma-Aldrich. Silica gel IB-F reverse-phase thin-layer chromatography (TLC) sheets were obtained from J.T. Baker. All buffers and solutions were prepared using ultrapure water (18 MΩ-cm resistivity). Outbred female athymic nude mice (4−6 weeks of age) were obtained from Harlan Sprague−Dawley Inc. Biotin-deficient diet (5836) was obtained from Purina Mills. The human cell carcinoma line LS174T was obtained from ATCC (CL-188).

LCMS Analysis and Purification. All liquid chromatography mass spectrometry (LCMS) data was obtained with a Waters Autopure system comprising the following: 2767 Sample Manager, 2545 Binary Gradient Module, System Fluidics Organizer, 2424 Evaporative Light Scattering Detector, 2998 Photodiode Array Detector, 3100 Mass Detector. *Binary solvent system:* solvent *A*, 0.05% TFA in water; solvent *B*, 0.05% TFA in acetonitrile. *Analytical method (unless otherwise noted):* 5−95% solvent *B* in 10 min, 1.2 mL/min flow rate. *Analytical columns:* Waters XBridge, BEH300, C4, 3.5 μm, 4.6 × 50 mm and C18, 5 μm, 4.6 × 50 mm. *Preparative method:* 5−95% solvent *B* in 30 min, 20 mL/min flow rate. *Preparative Columns:* Waters XBridge Prep C18, 5 μm, OBD, 19 × 150 mm.

Silica Gel Purification. Silica gel purifications were performed on the Teledyne ISCO CombiFlash Companion Purification System. RediSep normal phase flash columns from Teledyne ISCO were used for purifications.

NMR Characterization. All NMR data were obtained with either a Bruker AV 500 or AV 600 instruments. The following abbreviations are used: singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), pentet (p), doublet of a doublet (dd), multiplet (m).

Diethyl *N*,*N*-6,6′-((4-Methoxybenzyl)amino)-dihexanoate (6). 4-Methoxybenzylamine (92 g, 87 mL, 0.673 mol) and KI (143 g, 0.861 mol, 1.3 equiv) were mixed in a 2 L flask. The reaction was heated in an oil bath to 75−80 °C for 5 min, and DIEA (382 g, 514 mL, 2.959 mol, 4.4 equiv) was added. The resulting suspension was allowed to heat for another 15 min, and then ethyl 6-bromohexanoate (300 g, 239 mL, 1.345 mol, 2.0 equiv) was introduced and reflux was continued overnight. The mixture was cooled to ambient temperature, filtered over a Büchner funnel, and the filtrate evaporated. The residue was resuspended in ethyl acetate and washed with water, 3×. The organic layer was dried with

sodium sulfate, evaporated, and purified on silica gel using a hexane/ethyl acetate gradient. The amine was isolated as a slightly tan oil in typically 70−80% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.21 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 8.5 Hz, 2H), 4.12 (q, J = 7 Hz, 4H), 3.80 (s, 3H), 3.46 (s, 2H), 2.36 (t, J = 7.3 Hz, 4H), 2.25 (t, J = 7.5 Hz, 4H), 1.59 (p, J = 7.5 Hz, 4H), 1.45 (p, J = 7.5 Hz, 4H), 1.27 (m, 10H). ^{13}C NMR ($CDCl_3$, 500 MHz) δ = 173.8, 158.4, 132.0, 129.9, 113.5, 60.2, 57.9, 55.2, 53.4, 34.4, 27.0, 26.7, 24.9, 14.3. ESI-MS (m/z) for $C_{24}H_{39}NO_5$ (*exact mass* 421.28): $[M+H]^+$ *calc.* 422.29; *obs.* 422.3.

Aminodiethylhexanoate (7). Compound **6** (50 g, 119 mmol) was placed under a blanket of argon in a 1 L stainless steel autoclave equipped with a magnetic stirrer. Ethanol (500 mL) was then introduced followed by ammonium formate (25 g, 392 mmol, 3.3 equiv). The vessel was then flooded with argon such that the addition of palladium 10% on carbon (13 g, 119 mmol, 1 equiv) does not spark. The autoclave was then sealed and the reaction was heated in a 110 °C oil bath for 5 h. After cooling, the reaction vessel was depressurized and flushed with argon before opening. The mixture was then filtered on a thick pad of Celite, and evaporated *in vacuo*. The remaining yellow oil was then purified on silica gel using ethyl acetate and ethanol to provide 21 g (60%) of **7** as a brown oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 4.12 (q, J = 7.1 Hz, 4H), 2.6 (t, J = 7.3 Hz, 4H), 2.3 (t, J = 7.5 Hz, 4H), 1.64 (p, J = 7.6 Hz, 4H), 1.50 (p, J = 7.5 Hz, 4H), 1.35 (m, 4H), 1.25 (t, J = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$, 500 MHz, 298K) δ = 173.7, 60.2, 49.6, 34.4, 34.3, 29.4, 26.9, 25.0, 24.8, 14.3. ESI-MS (m/z) for $C_{16}H_{31}NO_4$ (*exact mass* 301.23): $[M+H]^+$ *calc.* 302.23; *obs.* 302.3.

***N*-Methyl Caproic Acid·HCl (8).** *N*-Methylcaprolactam (10 g, 78.8 mmol) was added to a 500 mL three-neck flask equipped with a water condenser and pressure equalizing addition funnel and cooled in an ice bath. Concentrated HCl (250 mL) was then carefully added dropwise, and the reaction was allowed to reflux for two days. The reaction was evaporated to dryness, and the product was crystallized in ethanol/ether to give 14.1 g (99%) of a white solid (HCl salt). *Analytical data for free base:* ^{1}H NMR (D_2O, 600 MHz) δ = 2.96 (t, J = 7.7 Hz, 2H), 2.63 (s, 1H), 2.32 (t, J = 7.4 Hz, 2H), 1.62 (p, J = 7.7 Hz, 2H), 1.56 (p, J = 7.6 Hz, 2H), 1.33 (m, 2H). ^{13}C NMR (D_2O, 600 MHz, 298K) δ = 178.82, 48.86, 33.43, 32.60, 25.03, 23.66. ESI-MS (m/z) for $C_7H_{15}NO_2$ (*exact mass* 145.11): $[M+H]^+$ *calc.* 146.12; *obs.* 146.3.

***N*-Methyl-(*tert*-Butyloxycarbonyl)-Carproic Acid (9).** *N*-Methyl caproic acid·HCl salt **8** (3.5 g, 0.019 mol) was suspended in 100 mL DCM. *tert*-Butyloxy carbamate (4.5 g, 0.021 mol, 1.1 equiv) was then added, followed by TEA (5.6 mL, 0.04 mol, 2.1 equiv). The mixture was stirred for 60 min, and the DCM/TEA was removed under reduced pressure. The contents were resuspended in ethyl acetate, and washed with 0.2 N HCl. The organic layer was dried over $MgSO_4$, filtered, and the volatiles were removed under reduced pressure to afford **9** as a colorless oil (4.4 g, 95%). ^{1}H NMR ($CDCl_3$, 500 MHz, 298K) δ = 3.20 (bs, 2H), 2.83 (bs, 3H), 2.35 (t, J = 7.4 Hz, 2H), 1.66 (p, J = 7.6 Hz, 2H), 1.53 (p, J = 7.5 Hz, 2H), 1.45 (s, 9H), 1.33 (p, 2H). ^{13}C NMR ($CDCl_3$, 600 MHz, 298K) δ = 179.1, 155.9, 79.3, 48.6, 48.2, 34.1, 33.9, 28.5, 27.6, 27.3, 26.1, 24.4. ESI-MS (m/z) for $C_{12}H_{23}NO_4$ (*exact mass* 245.16): $[M+H]^+$ *calc.* 246.17; *obs.* 246.3.

Generation 0 (G^0) Dendron (10). Compound **9** (125 mg, 0.5 mmol) was suspended in 1 mL DMF. Compound **7** (228 mg, 0.6 mmol, 1.2 equiv) was then added, followed by DIEA (340 uL, 252 mg, 1.75 mmol, 3.5 equiv). HATU (374 mg, 0.902 mmol, 1.2 equiv) was dissolved in 1 mL DMF then added to **9**. After 20 min, solvent was partially removed under high vacuum at room temperature, and the residue was transferred to a separatory funnel containing diethyl ether. The organic layer was washed with 0.1 N HCl (3×) and then with water (1×). The ether and residual water was removed under reduced pressure, then high vacuum. 253 mg (95%) of **10** was obtained as a light brown oil and was used without further purification. ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 4.13 (m, J = 7.1, 2.9 Hz, 4H), 3.28 (t, J = 6.3 Hz, 2H), 3.20 (t, J = 6.5 Hz, 4H), 2.83 (s, 3H), 2.29 (m, 6H), 1.65 (m, 6H), 1.54 (m, 6H), 1.45 (s, 9H), 1.31 (m, 6H), 1.26 (q, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$, 600 MHz): 173.7, 173.4, 155.8, 79.1, 60.3, 60.2, 47.8, 45.7, 34.2, 34.1, 33.1, 28.9, 27.5, 26.7, 26.6, 26.4, 25.2, 24.7, 24.7. ESI-MS (m/z) for $C_{28}H_{52}N_2O_7$ (*exact mass* 528.38): $[M+H]^+$ *calc.* 529.38; *obs.* 529.4.

Generation 1 (G^1) Dendron (11). *Hydrolysis of Ethyl Esters of G^0 Dendron.* Compound **10** (253 mg, 0.5 mmol) was dissolved in 2 mL ethanol and 2 mL of 2 N NaOH. The hydrolysis was conducted in a 40 °C water bath for 1 h. The solution was transferred to a separatory funnel and then neutralized with 1 N HCl. Diethyl ether was added and the organic layer was washed with 0.1 N HCl (3×). The organic layer was collected and the solvent was removed under reduced pressure resulting in a tan oil. *Coupling of 7 to form G^1 dendron:* The oil was dissolved in 3 mL DMF. To this, compound **7** (440 mg, 1.3 mmol, 2.6 equiv) was added followed by DIEA (681 uL, 505 mg, 2 mmol, 4 equiv). HATU (456 mg, 1.2 mmol, 2.4 equiv) was dissolved in 2 mL DMF, then added. The reaction was allowed to proceed for 20 min, at which point the solvent was partially removed *in vacuo*, and transferred to a separatory funnel with diethyl ether. The organic layer was washed with water (1×), 0.1 N HCl (3×), and then water (1×). The organic layer was collected and subjected to reduced pressure to remove solvents. **11** was obtained as a light brown oil (470 mg, 90%) and used without further purification. ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 4.13 (m, J = 7.1, 3.6 Hz, 8H), 3.28 (q, J = 7.6, 6H), 3.2 (q, 8H), 2.83 (s, 3H), 2.29 (m, 14H), 1.65 (m, 14H), 1.54 (m, 14H), 1.45 (s, 9H), 1.31 (m, 14H), 1.26 (q, J = 7.1 Hz, 12H). ^{13}C NMR ($CDCl_3$, 600 MHz) δ = 173.68, 173.65, 173.44, 173.41, 172.39, 172.00, 155.83, 79.07, 60.35, 60.33, 60.22, 60.20, 47.80, 45.80, 45.72, 34.26, 34.24, 34.13, 34.10, 33.09, 32.89, 29.22, 28.93, 28.50, 27.78, 27.53, 27.51, 27.02, 26.87, 26.69, 26.59, 26.45, 25.28, 25.11, 24.73, 24.68, 24.65 14.26. ESI-MS (m/z) for $C_{56}H_{102}N_4O_{13}$ (*exact mass* 1038.74): $[M+H]^+$ *calc.* 1039.75, *obs.* 1040.06.

Generation 2 (G^2) Dendron (12). *Hydrolysis of Ethyl Esters of G^1 Dendron.* Compound **11** (97 mg, 0.1 mmol) was dissolved in 2 mL ethanol, and 2 mL of 2.0 N NaOH were added. The hydrolysis was conducted in a 40 °C water bath for 1 h. The solution was transferred to a separatory funnel and then neutralized with 1.0 N HCl. A solution of diethyl ether and DCM (10:1, v:v) was added and the organic layer was washed with 0.1 N HCl (3×). The organic layer was collected and subjected to reduced pressure to remove solvents and obtain a light brown oil. *Coupling of 7 to form G^2 dendron:* The oil was dissolved in 4 mL DMF. To this was added compound **7** (236 mg, 0.7 mmol, 7 equiv), followed by DIEA (280 uL, 207 mg, 1.6 mmol, 4 equiv) and HATU (228 mg, 0.6 mmol, 6 equiv) as dissolved solution in 1 mL DMF. The reaction was allowed to proceed for 20 min, at which point the solvent was partially removed under reduced pressure, and the mixture was

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transferred to a separatory funnel containing diethyl ether. The organic layer was washed with 0.2 N HCl (3×), and then with water (2×). The organic layer was collected and subjected to reduced pressure to remove solvent. 176 mg (92%) of **12** was obtained as a tan oil, and used without further purification. ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 4.12 (m, J = 7.1, 2.8 Hz, 16H), 3.28 (q, J = 7.6 Hz, 14H), 3.21 (q, 16H), 2.83 (s, 3H), 2.29 (m, 30H), 1.65 (m, 30H), 1.54 (m, 30H), 1.45 (s, 9H), 1.31 (m, 30H), 1.26 (q, J = 6.9 Hz, 24H). ^{13}C NMR ($CDCl_3$, 600 MHz) δ = 173.68, 173.42, 172.52, 172.09, 79.07, 60.34, 60.21, 47.84, 45.97, 45.85, 45.78, 34.25, 34.12, 34.10, 33.07, 32.87, 29.22, 28.92, 28.50, 27.79, 27.74, 27.50, 27.04, 26.87, 26.59, 26.44, 25.34, 25.28, 25.16, 25.10, 24.73, 24.67, 14.27, 14.26. ESI-MS (m/z) for $C_{12}H_{202}N_8O_{25}$ (*exact mass* 2059.48): $[M+H]^+$ *calc.* 2060.49; $[M+2H]^{2+}$ *calc.* 1030.75, *obs.* 1031.59.

Generation 3 (G^3) Dendron (13). Compound **12** (90 mg, 0.043 mmol) was dissolved in 5 mL ethanol, and 4 mL of 2.0 N NaOH was added. The hydrolysis was conducted in a 40 °C water bath for 1 h. The solution was transferred to a separatory funnel and then neutralized with 1.0 N HCl. A solution of diethyl ether and DCM (10:1; v:v) was added and the organic layer was washed with 0.1 N HCl (3×). The organic layer was collected and subjected to reduced pressure to remove solvent. The resulting oil was diluted in DMF (4 mL). To this, **7** (245 mg, 0.09 mmol, 16 equiv) was added followed by DIEA (280 uL, 207 mg, 1.6 mmol, 4 equiv). HATU (233 mg, 0.6 mmol, 6 equiv) was first dissolved in DMF (1 mL) then added. The reaction was allowed to proceed for 20 min, at which point the reaction was partially concentrated *in vacuo*, and the residue was transferred to a separatory funnel preloaded with a diethyl ether and DCM. The organic layer was washed with 0.1 N HCl (3×) and with water (2×). Evaporation under reduced pressure provided 168 mg (95% crude yield) of **13** was obtained as a light brown oil, and was used without further purification. ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 4.12 (m, J = 7.1, 2.9 Hz, 32H), 3.28 (q, J = 7.5 Hz, 30H), 3.20 (q, 32H), 2.83 (s, 3H), 2.29 (m, 62H), 1.65 (m, 62H), 1.54 (m, 62H), 1.44 (s, 9H), 1.31 (m, 62H), 1.26 (q, J = 6.9 Hz, 48H). ^{13}C NMR ($CDCl_3$, 600 MHz) δ = 173.67, 173.43, 172.42, 172.08, 60.32, 60.21, 47.86, 47.82, 45.82, 45.76, 34.25, 34.13, 34.10, 33.09, 32.90, 29.21, 28.92, 27.84, 27.51, 27.07, 26.88, 26.59, 26.44, 25.27, 25.15, 24.73, 24.68, 24.65, 14.28, 14.27. ESI-MS (m/z) for $C_{224}H_{402}N_{16}O_{49}$ (*exact mass* 4100.95): $[M+H]^+$ *calc.* 4101.95; $[M+3H]^{3+}$ *calc.* 1367.99, *obs.* 1368.40; $[M+4H]^{4+}$ *calc.* 1026.24, *obs.* 1026.89.

Generation 4 (G^4) Dendron (14). *Extension of Linker.* Compound **13** (101 mg, 0.025 mmol) was treated with neat TFA (1 mL) for 10 min at room temperature to remove the BOC protecting group. TFA was then removed by vacuum, and the residual dendron was lyophilized overnight. The dendron was resuspended in 1 mL anhydrous DMF to which DIEA (66 uL, 49 mg, 0.38 mmol, 15 equiv) was added. Compound **9** (12 mg, 0.050 mmol, 2 equiv) was premixed with HATU (16 mg, 0.043 mmol, 1.7 equiv) in 500 uL of DMF and 44 uL DIEA (32 mg, 0.25 mmol, 10 equiv) was then added to the dendron and stirred at room temperature for 20 min. DCM (10 mL) was then added, and the reaction solution was washed with water (1×), 0.1 N HCl (3×), and water (1×). The DCM layer was evaporated, then left to dry overnight under high vacuum, resulting in an oil. To further extend the linker one more round, the procedure above was reiterated in its entirety where the dendron was treated with neat TFA (~1 mL) for 10 min at room temperature. TFA was removed *in vacuo*, and the dendron was dissolved in water:acetonitrile (1:1), frozen, and lyophilized overnight. The dendron was dissolved in 1 mL DMF, and DIEA (44 uL, 32 mg, 0.043 mmol, 10 equiv) was added. Compound **9** (12 mg, 0.050 mmol, 2 equiv) was premixed with HATU (16 mg, 0.043 mmol, 1.7 equiv) in 200 uL of DMF; to which DIEA (44 uL, 32 mg, 0.25 mmol, 10 equiv) was then added. This solution was then added to the dendron and stirred at room temperature for 20 min. For workup, DCM (10 mL) was added, and the organic solution was washed with water (1×), 0.1 N HCl (3×), and then water (1×). After evaporation, the residue was left to dry overnight under high vacuum, to give an oil.

Increasing Dendron Generation. The sixteen ethyl esters of the dendron were hydrolyzed in 1 mL of 1.0 N NaOH and 1 mL ethanol, then left to stir overnight at ambient temperature. The volatiles were then removed under reduced pressure, and residue was later transferred to a separatory funnel and then neutralized with 1.0 N HCl. A solution of diethyl ether, tBuOH, and DCM (6:3:1; v:v:v) was added and the organic layer was washed with 0.1 N HCl (3×), then collected and evaporated under vacuum. The residual brown oil was diluted in anhydrous DMF (2 mL), and **7** (116 mg, 0.384 mmol, 16 equiv) was added followed by DIEA (134 uL, 99 mg, 0.768 mmol, 32 equiv), then HATU (124 mg, 0.326 mmol, 0.041 equiv). The reaction was allowed to proceed for 20 min under stirring, at which point the mixture was transferred to a separatory funnel with diethyl ether and DCM. The organic layer was washed with 0.1 N HCl (3×), then water (2×), before it was collected and evaporated under reduced pressure. 161 mg (79%) of **14** was obtained as a brown oil, which was used without further purification. ^{1}H NMR ($CDCl_3$, 600 MHz) δ = 4.12 (m, J = 7.1, 2.8 H, 64H), 3.28−3.21 (m, 140H), 2.97 (m, 3H), 2.90 (m, 3H), 2.83 (s, 3H), 2.29 (m, 130H), 1.65 (m, 130H), 1.55 (m, 130H), 1.45 (s, 9H), 1.32 (m, 130H), 1.25 (q, J = 6.8 Hz, 96H). ^{13}C NMR ($CDCl_3$, 600 MHz) δ = 173.66, 173.39, 172.41, 172.03, 60.31, 60.19, 47.88 47.80, 45.98, 45.81, 45.74, 34.25, 34.12, 34.10, 33.14, 33.09, 32.90, 29.23, 28.92, 28.50, 27.79, 27.51, 27.19, 27.07, 26.94, 26.87, 26.59, 26.43, 25.26, 25.14, 24.73, 24.67, 24.65, 14.29. ESI-MS (m/z) for $C_{462}H_{828}N_{34}O_{99}$ (*exact mass:* 8438.08): $[M+5H]^{5+}$ *calc.* 1688.62, *obs.* 1689.85; $[M+6H]^{6+}$ *calc.* 1407.35, *obs.* 1408.37.

5-((*tert*-Butoxycarbonyl)amino)pentyl 4-Methylbenzenesulfulfonate (15). *tert*-Butyl (5-hydroxypentyl)-carbamate (28.6 g, 0.14 mmol) was dissolved in DCM (200 mL), and TEA (29 mL, 0.21 mol) at 0 °C, then TsCl (32.2 g, 0.169 mol) and a crystal DMAP were added to the solution. After overnight stirring at RT, the reaction mixture was washed by brine, briefly dried over Na_2SO_4, and concentrated under reduced pressure. Silica gel chromatography using hexane/ethyl acetate gradient from 9/1 to 3/1 provided the desired tosylate 40 g (80%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.78 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 8.2 Hz, 2H), 4.56 (bs, 1H), 4.03 (t, J = 6.4 Hz, 2H), 3.05 (m, 2H), 2.45 (s, 3H), 1.65 (m, 2H), 1.40 (s, 9H), 1.36 (m, 2H), 1.32 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 155.96, 144.76, 133.07, 129.86, 127.87, 79.10, 70.37, 40.20, 29.41, 28.41, 22.65, 21.64. ESI-MS (m/z) for $C_{17}H_{27}NO_5S$ (*exact mass:* 357.46): $[M+H]^+$ *calc.* 358, *obs.* 358.

***tert*-Butyl (5-Bromoxypentyl)carbamate (16).** The tosylate **15** (39 g, 0.109 mmol) was dissolved in acetonitrile (100 mL), then NaBr (45 g, 0.437 mmol) and Bu_4NBr (1.4 g, 0.0043 mmol) were added. The resulting mixture was heated to reflux for an overnight period. Solvents were then removed under vacuum, and the residue was dissolved in DCM (400 mL) and washed by brine. Drying over Na_2SO_4, filtration,

concentration, and separation by silica gel chromatography with hexane/ethyl acetate gradient from 10/1 to 6/1 provided the desired primary bromide 24 g (83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.53 (bs, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.13 (m, 2H), 1.88 (m, 2H),1.59−1.47 (m, 4H), 1.44 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 155.97, 78.92, 40.24, 33.60, 32.29, 29.20, 28.38, 25.29. ESI-MS (m/z) for $C_{10}H_{20}BrNO_2$ (*exact mass:* 265.07): $[M+H]^+$ *calc.* 265, *obs.* 265.

5-((*tert*-Butoxycarbonyl)amino)pentyl-2-acetamido-2-deoxy-1-thio-3,4,6-tri-*O*-acetyl-α-D-galactopyranoside (18). To a suspension of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (50 g, 0.128 mol) in toluene (500 mL) was added Lawesson's reagent (44.2 g, 0.109 mol, 0.85 equiv), and the resulting mixture was heated up to reflux for 2.5 h. LCMS indicated the reaction to be complete. After cooling down to ambient temperature the crude mixture was directly loaded onto a silica gel (500 g) column and eluted with ethyl acetate. Collection and concentration of appropriate fractions gave a brownish foam (**17**) which was dissolved in methanol (400 mL), and then 36 mL of a mixture of TFA and water (1:1) was added, and stirring was continued for 1.5 h at room temperature. Solvents were then removed and 400 mL of DMF was added followed by DIEA (35 mL) and **16** (40 g, 0.15 mol). The reaction was stirred at room temperature overnight. The volatiles were removed (DIEA and DMF) under low, then high vacuum, and the residue was dissolved in ethyl acetate (700 mL), washed with brine (3×), and dried briefly over Na_2SO_4. After filtration over Celite, the filtrate was concentrated and purified by column chromatography using a hexane/ethyl acetate gradient − from 4/1 to 1/1 as the eluant to provide 53.9 g (89%) of the desired BOC-protected amine. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 5.67 (d, J = 8.8 Hz, 1H), 5.49 (d, J = 5.0 Hz, 1H), 5.38 (d, J = 2.5 Hz, 1H), 5.05 (dd, J = 2.5 Hz, J = 11.7 Hz, 1H), 4.77 (m, 1H), 4.61 (bs, 1H), 4.55 (t, J = 6.5 Hz, 1H), 4.13 (dd, J = 6.5 Hz, J = 11.4 Hz, 1H), 4.10 (dd, J = 6.5 Hz, J = 11.4 Hz, 1H), 3.11 (m, 2H), 2.60 (m, 2H), 2.16, 2.05, 2.01, 1.98 (4 s, 12H), 1.64 (m, 2H), 1.49 (m, 2H), 1.44 (s, 9H), 1.38 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.01, 170.41, 170.29, 170.16, 155.98, 84.92, 79.11, 68.53, 67.34, 67.23, 61.83, 48.32, 40.38, 30.97, 29.62, 29.28, 28.43, 25.94, 23.35, 20.76, 20.72. ESI-MS (m/z) for $C_{24}H_{40}N_2O_{10}S$ (*exact mass:* 548.24): $[M+H]^+$ *calc.* 549, *obs.* 549.

5-Amino-pentyl-2-Acetamido-2-deoxy-1-thio-3,4,6-tri-*O*-acetyl-α-D-galactopyranoside Trifluoroacetic Acid Salt (19). A solution of the BOC-protected compound above (700 mg, 1.28 mmol) in TFA/DCM (7 mL, 2:5 (v:v)) was stirred at room temperature for 1 h. The solvents were removed, and the residue was dried over vacuum for 2 h, and directly used for next step without purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.52 (bs, 2H), 6.26 (d, J = 8.0 Hz, 1H), 5.59 (d, J = 5.3 Hz, 1H), 5.38 (d, J = 2.2 Hz, 1H), 5.04 (dd, J = 2.2 Hz, J = 11.6 Hz, 1H), 4.67 (m, 1H), 4.53 (t, J = 6.5 Hz, 1H), 4.19 (dd, J = 6.5 Hz, J = 11.6 Hz, 1H), 4.05 (dd, J = 6.5 Hz, J = 11.6 Hz, 1H), 3.10 (m, 2H), 2.69, 2.61 (2 m, 2H), 2.18, 2.06, 2.05, 2.03 (4 s, 12H), 1.72 (m, 2H), 1.68 (m, 2H), 1.51 (m, 2H). ESI-MS (m/z) for $C_{19}H_{32}N_2O_8S$ (*exact mass:* 448.19): $[M+H]^+$ *calc.* 449, *obs.* 449.

Clearing Agent 1. *Hydrolysis of Ethyl Esters of G^1 Dendron.* Compound **11** (50 mg, 0.05 mmol, 0.2 mmol of ethyl ester) was dissolved in 2 mL ethanol and 2 mL of 2.0 N NaOH was added. The hydrolysis was conducted in a 40 °C water bath for 2 h. The solution was transferred to a separatory funnel and then neutralized with 1.0 N HCl. Ether was added and the organic layer was washed w/0.1 N HCl (3×). The organic layer was collected and subjected to low then high vacuum to remove volatiles and obtain a tan oil.

*Coupling of αSGalNAc **19** to G^1 Dendron.* The oil was diluted in anhydrous DMF (1 mL) and treated with DIEA (210 uL, 155 mg, 1.2 mmol, 24 equiv). A solution of compound **10** (180 mg, 0.4 mmol, 8 equiv) in DMF (1 mL) was added, followed by a solution of HATU (114 mg, 0.3 mmol, 6 equiv) in DMF (1 mL). The resulting mixture was stirred for 30 min, after which volatiles were partially removed under reduced pressure, and the residue transferred to a separatory funnel. DCM was added, and the DCM layer was washed with water (1×) followed by 0.1 N HCl (3×), then water (1×). The DCM layer was then collected, evaporated under reduced pressure resulting in a brown oil. *Coupling of D-biotin:* The oil was treated with neat TFA and stirred for 10 min, and the TFA was removed *in vacuo*. The oil was dissolved in 5 mL of water:acetonitrile (1:1), frozen, and lyophilized overnight. The oil was suspended in DMF and a solution of preactivated D-biotin (25 mg, 0.1 mmol, 2 equiv) was added and stirred for 30 min (see below). The solution was transferred to a separatory funnel, and DCM was added, and washed with water (4×). The DCM layer was evaporated resulting in a tan solid (132 mg). 40 mg of the crude tan solid was dissolved in a 1:1 solution of methanol:0.2 N NaOH for 30 min, then neutralized with 1.0 N HCl. The solution was evacuated to remove residual methanol, and the remaining solution was purified by reversed-phase HPLC. 25 mg of pure compound **1** was recovered as a white powder. ^{1}H NMR (D_2O, 600 MHz) δ = 5.42 (d, J = 5.52, 4H), 4.54 (m, 1H), 4.35 (m, 1H), 4.26 (dd, J = 5.9, 5.5 Hz, 4H), 4.18 (t, J = 6.1 Hz, 4H), 3.91 (d, J = 3.1 Hz, 4H), 3.75 (dd, J = 8.2, 3.2 Hz, 4H), 3.69 (d, J = 6.1 Hz, 8H), 3.25 (m, 15H), 3.09 (t, 8H), 2.98−2.83 (m, 4H), 2.70 (d, J = 13.0 Hz, 1H), 2.58 (m, 4H), 2.50 (m, 4H), 2.32 (m, 8H), 2.16 (q, J = 7.3 Hz, 8H), 1.96 (s, 12H), 1.69−1.22 (m, 72H). ^{13}C NMR (D_2O, 600 MHz, 298K) δ = 176.38, 176.19, 174.38, 71.59, 68.44, 67.70, 62.14, 61.08, 60.26, 55.51, 50.21, 48.34, 45.84, 39.81, 39.11, 35.70, 35.66, 32.52, 30.19, 28.58, 28.03, 28.00, 26.68, 25.97, 25.80, 25.65, 25.52, 25.34, 25.25, 25.22, 25.17, 21.97. ESI-MS (m/z) for $C_{105}H_{188}N_{14}O_{29}S_5$ (*exact mass* 2269.23): $[M+2H]^{2+}$ *calc.* 1135.62, *obs.* 1136.36; $[M+3H]^{3+}$ *calc.* 757.42, *obs.* 758.07.

Preactivated D-Biotin. For a typical reaction, D-biotin (2 equiv relative to amine) was dissolved in DMF (5 mg of D-biotin per 1 mL DMF) with DIEA (52 uL, 39 mg, 0.3 mmol, 6 equiv), and HATU (38 mg, 0.1 mmol, 1.8 equiv) in DMF was added and allowed to mix for 10 min.

Clearing Agent 2. *Hydrolysis of Ethyl Esters of G^2 Dendron.* Compound **12** (44 mg, 0.02 mmol, 0.16 mmol of ethyl ester) was dissolved in 2 mL ethanol and 2 mL of 2 N NaOH. The hydrolysis was conducted in a 40 °C water bath for 2 h. The solution was transferred to a separatory funnel and then neutralized with 1.0 N HCl. Ether was added and the organic layer was washed with 0.1 N HCl (3×). The organic layer was collected and subjected to reduced pressure to remove solvent resulting in a tan oil.

*Coupling of αSGalNAc **19** to G^2 Dendron.* The tan oil (assumed to be 0.02 mmol dendron, and 0.16 mmol of carboxylic acid as from the previous step) was suspended in DMF (1 mL) and DIEA (167 uL, 124 mg, 0.96 mmol, 48 equiv). A solution of compound **19** (175 mg, 0.32 mmol, 16 equiv) in DMF (1 mL) was added, followed by a solution of HATU (92 mg, 0.24 mmol, 12 equiv) in DMF (1 mL). The

reaction was allowed to proceeded for 30 min, then subject to high vacuum for 30 min to partially remove solvent. The remaining volume was transferred to a separatory funnel, DCM was added, and the DCM layer was washed with water (1×), then with 0.1 N HCl (3×), and finally with water (1×). The DCM layer was collected and evaporated resulting in a brown oil.

Coupling of D-Biotin. The brown oil above was treated with neat TFA and stirred for 10 min, and TFA was removed *in vacuo* for 60 min. The oil was resuspended in DMF and a solution of preactivated D-biotin (10 mg, 0.04 mmol, 2 equiv) was added and stirred for 30 min. The solution was transferred to a separatory funnel, DCM was added, and washed with water (4×). The DCM layer was evaporated resulting in a tan solid (102 mg). 20 mg of the crude tan solid was dissolved in a 1:1 solution of methanol:0.2 N NaOH for 30 min, then the mixtrure was neutralized with 1.0 N HCl. The solution was evacuated to remove residual methanol, and the remaining solution was purified by reversed-phase HPLC. 12 mg of pure compound **2** was obtained as a white powder (73% recovery from HPLC purification). ^{1}H NMR (D_2O, 600 MHz) δ = 5.42 (pd, J = 5.4 Hz, 8H), 4.53 (m, 1H), 4.34 (m, 1H), 4.26 (dd, J = 11.3, 5.5 Hz, 8H), 4.16 (t, J = 5.9 Hz, 8H), 3.91 (d, J = 3.2 Hz, 8H), 3.75 (dd, J = 11.3, 3.2 Hz, 16H), 3.68 (d, J = 6.1 Hz, 16H), 3.24 (m, 31H), 3.08 (t, J = 6.8 Hz, 16H), 2.97−2.82 (m, 4H), 2.69 (d, J = 12.8, 1H), 2.57−2.50 (m, m, 16H), 2.31 (m, 16H), 2.15 (q, J = 7.4 Hz, 16H), 1.96 (s, 24H), 1.53−1.22 (m, 144H). ^{13}C NMR (D_2O, 600 MHz) δ = 174.33, 83.77, 71.59, 68.44, 67.72, 61.07, 50.24, 45.83, 39.15, 35.69, 30.21, 28.66, 28.13, 26.79, 25.66, 25.41, 25.28, 22.01. ESI-MS (m/z) for $C_{205}H_{368}N_{26}O_{57}S_9$ (*exact mass* 4394.42): $[M+3H]^{3+}$ *calc.* 1465.8, *obs.* 1466.9; $[M+4H]^{4+}$ *calc.* 1099.6, *obs.* 1100.5; $[M+5H]^{5+}$ *calc.* 879.9, *obs.* 880.8.

Clearing Agent 3. *Hydrolysis of Ethyl Esters of G^3 Dendron.* Compound **13** (48 mg, 0.012 mmol) was dissolved in 1 mL ethanol, and 1 mL of 1.0 N NaOH was added. The hydrolysis was conducted overnight, at room temperature. The solution was transferred to a separatory funnel and then neutralized with 1.0 N HCl. A solution of diethyl ether, tBuOH, and DCM (6:3:1; v:v:v) was added and the organic layer was washed with 0.1 N HCl (3×), and water (3×). The organic layer was transferred into a round bottomed flask and evaporated under reduced pressure, then high vacuum, to produce a brown oil.

Coupling of GalNAc Groups. The brown oil above was suspended in 2 mL anhydrous DMF. DIEA (210 uL, 155 mg, 1.2 mmol, 96 equiv) was added followed by a solution of **19** (215 mg, 0.384 mmol, 32 equiv) as a solution in DMF. HATU (114 mg, 0.3 mmol, 24 equiv) dissolved in DMF was then added. The reaction was allowed to proceed for 30 min, at which point the solvent was partially removed under reduced pressure, and the mixture was transferred to a separatory funnel. A solution of diethyl ether, tBuOH, and DCM (3:3:1; v:v:v) was added and the organic layer was washed with 0.1 N HCl (3×), and with water (2×). The organic layer was collected and subjected to reduced pressure to remove volatiles. 150 mg of a tan oil was obtained.

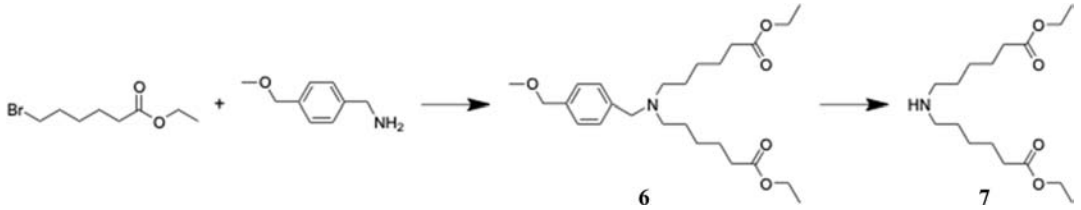
Coupling with D-Biotin. The oil was treated with neat TFA for 10 min at room temperature, to remove the BOC protecting group. TFA was then removed by under high vacuum. The resulting oil was diluted with DMF and a solution of preactivated D-biotin (6 mg, 0.024 mmol, 2 equiv) was added and stirred for 30 min (see above). The solution was transferred to a separatory funnel, DCM was added, and washed with water, 4×. The DCM layer was evaporated resulting in a tan solid (150 mg). *Final deprotection and purification:* 20 mg of the crude tan solid was dissolved in 2 mL of a 1:1 solution of methanol:0.2 N NaOH for 30 min, then neutralized with 1.0 N HCl. The solution was evacuated to remove residual methanol, and the remaining solution was purified by reversed-phase HPLC. 10 mg of pure compound **3** was isolated as a white powder. ^{1}H NMR (D_2O, 600 MHz) δ = 5.43 (d, J = 5.4 Hz, 16H), 4.54 (m, 1H), 4.35 (m, 1H), 4.26 (dd, J = 11.3, 5.5 Hz, 16H), 4.16 (t, J = 6.0 Hz, 16H), 3.92 (d, J = 3.2 Hz, 16H), 3.75 (dd, J = 3.1, 11.3 Hz, 16H), 3.68 (d, J = 6.1 Hz, 32H), 3.24 (m, 63H), 3.08 (t, J = 6.7 Hz, 32H), 2.97−2.83 (m, 4H), 2.69 (d, 1H), 2.53 (m, 32H), 2.31 (m, 32H), 1.96 (s, 48H), 1.70−1.23 (m, 288H). ^{13}C NMR (D_2O, 600 MHz, 298 K) δ = 174.3, 83.8, 71.6, 68.4, 67.7, 61.1, 50.2, 39.2, 35.7, 30.2, 28.7, 28.2, 25.5, 25.3, 22.0. ESI-MS (m/z) for $C_{405}H_{728}N_{50}O_{113}S_{17}$: $[M+H]^{+}$ *calc.* 8565.11; $[M+5H]^{5+}$ *calc.* 1713.83, *obs.* ; $[M+6H]^{6+}$ *calc.* 1428.36, *obs.*; $[M+7H]^{7+}$ *calc.* 1224.45, *obs.*

Clearing Agent 4. *Hydrolysis of Ethyl Esters of G^4 Dendron.* Compound **14** (19 mg, 2.25 umol,) was dissolved in 0.5 mL ethanol and 0.5 mL of 1.0 N NaOH and left overnight at room temperature. The solution was transferred to a separatory funnel with 10 mL of water, then neutralized with 1.0 N HCl. A volumetric mixture composed of diethyl ether, tBuOH, and DCM (6:3:1) was added and the organic layer was washed with 0.1 N HCl (3×), then water (3×). The organic layer was recovered and solvents were removed under vacuum, resulting in a brown oil. For an alternative workup after overnight hydrolysis, ethanol was partially removed from the reaction under reduced pressure. The dendron solution was neutralized with 1.0 N HCl, then acidified. This resulted in the precipitation of the dendron. After centrifugation (3000 rpm), a brown viscous pellet formed and was washed with water (3×). Residual water was removed under vacuum resulting a brown oil.

Coupling of GalNAc Groups. The dendron was dissolved in 500 uL of DMF and DIEA (75 uL, 56 mg, 0.432 mmol, 192 equiv) was added followed by **19** (65 mg, 0.144 mmol, 64 equiv) in 500 uL DMF, and HATU (114 mg, 0.3 mmol, equiv) also in 500 uL of DMF. The reaction was allowed to proceed for 1 h, at which point solvent was partially removed under reduced pressure for 30 min. DCM and ether (2:1) were added and the solution was transferred to a separatory funnel and decanted. The organic layer was then washed with 0.1 N HCl (3×), then with water (3×), before evaporation under reduced pressure.

Coupling with D-Biotin. The collected oil above was treated with neat TFA (1 mL) for 10 min at room temperature to remove the BOC protecting group. TFA was removed by evacuation and the dendron was diluted in a solution of water:acetonitrile (1:1), frozen, and lyophilized overnight. The resulting oil was dissolved in DMF and a solution of preactivated D-biotin (5 mg, 0.02 mmol) was added and mixed for 1 h (see above). The solution was transferred to a separatory funnel, DCM was added, and the organic layer washed with water (4×). The DCM layer was evaporated, resulting in a off-white solid. The material was dissolved in a solution of methanol:0.2 N NaOH (1:1) for 30 min, monitored by LCMS, then neutralized with 1 N HCl. The solution was evacuated to remove residual methanol, and the remaining solution was purified by reversed-phase HPLC. 13 mg of pure

Scheme 1. Synthesis of Amino-Diethyl-Hexanoate[a]



[a](a) Δ, KI, DIEA, overnight (70−80%); (b) PdC, 110 °C, 5 h (60%).

compound **4** was recovered as a white powder (33% yield starting from **14**). ^{1}H NMR (D_2O, 600 MHz) δ = 5.44 (pd, J = 5.16 Hz, 32H), 4.55 (m, 1H), 4.36 (m, 1H), 4.28 (dd, J = 5.9, 5.4 Hz, 32H), 4.17 (t, 32H), 3.93 (d, J = 2.9 Hz, 32H), 3.56 (dd, J = 11.3, 2.7 Hz, 32H), 3.69 (d, J = 5.8 Hz, 64H), 3.25 (m, 135H), 3.09 (t, 64H), 2.99−2.84 (m, 10H), 2.58 (m, 32H), 2.51 (m, 32H), 2.31 (m, 68H), 2.17 (m, 64H), 1.97 (s, 96H), 1.55−1.24 (m, 584H). ^{13}C NMR (D_2O, 600 MHz) δ = 174.2, 130.8, 117.4, 83.8, 71.6, 68.4, 67.7, 61.0, 50.3, 39.2, 35.7, 30.2, 28.8, 28.3 25.5 25.4, 22.1. ESI-MS (m/z) for $C_{819}H_{1474}N_{100}O_{227}S_{33}$ (*exact mass* 17399.77): $[M+9H]^{9+}$ *calc.* 1934.3, *obs.* 1934.3; $[M+10H]^{10+}$ *calc.* 1741.0, *obs.* 1741.1.

General Procedure for Solid-Phase Synthesis of Dendron Core. For a typical synthesis of **20** on solid support, 100 mg of Wang resin (0.5 mmol/g) was allowed to swell in DCM (2 mL) for 10 min. The DCM was removed and carbon tetrabromide (83 mg, 0.25 mmol, 5 equiv) and triphenylphosphine (66 mg, 0.25, 5 equiv) in DCM (2 mL) were added to the resin, and allowed to shake for 3 h at room temperature. The resin was washed 3× with DCM, and 3× with DMF. Compound **8** (73 mg, 0.5 mmol, 10 equiv) in DMF (4 mL) with DIEA (350 uL, 260 mg, 2 mmol, 40 equiv) was prepared and added to the resin, and the resulting mixture shaken overnight. The liquids were removed by suction filtration and the resin was washed 6× with DMF. *Coupling step:* Compound **7** (45 mg, 0.15 mmol, 3 equiv) in DMF (2 mL) was mixed with DIEA (52 uL, 39 mg, 0.3 mmol, 6 equiv) and added to the resin. A solution of HATU (57 mg, 0.15 mmol, 3 equiv) in DMF (1 mL) was then added and the reaction shaken for 60 min. The liquids were removed, the resin was washed 6× with DMF.

Deprotection Step. Sodium silanolate (10 equiv) was prepared in DMF/DCM (1:1, 5 mL). Due to incomplete solubility, the mixture was either filtered or centrifuged, and filtrate or supernatant was added to the resin, then allowed to shake for 2 h. The resin was washed 3× with DCM, 3× with DMF, 3× with 5% acetic acid in DMF (5 min/wash), and finally 6× with DMF. *Coupling step:* Similar to above, **8** (90 mg, 0.3 mmol, 6 equiv) in DMF (2 mL) with DIEA (105 uL, 78 mg, 0.6 mmol, 12 equiv) and added to the resin. HATU (114 mg, 0.3 mmol, 6 equiv) in DMF (1 mL) was added last, and the mixture was shaken for 60 min. After suction filtration, the resin was washed 6× with DMF. *Cleavage from resin:* Resin was treated with a 10% solution of chloroethylchloroformate in DCM for 60 min. The cleaved resin was then washed 3× with DCM and filtered. Methanol is added to the filtrate, and then subject to vacuum and residual products were analyzed by LCMS.

Radioiodination of scFv-CC49-SAv. The scFv-CC49-SAv protein was labeled with ^{131}I using the Iodogen method and previously described methods to a specific activity of 3.0 ± 2.1 μCi/μg (n = 3).[16,19] The radiochemical purity was determined to be ≥98.5% using instant thin-layer chromatography. The functional integrity of the tracer-antibody was evaluated using a binding assay with a TAG-72(+) cell-line (LS174T) according to the method by Lindmo et al. (59.3 ± 12.9%; n = 3).[20] No efforts were made to determine biotin-binding capacity.

Blood Clearance of ^{131}I-scFv-CC49-SAv. All animal experiments were approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center, and institutional guidelines for the proper and humane use of animals in research were followed. All mice were fed a biotin-free diet for one week prior to study. The antibody was prepared for injection by mixing the radioactive antibody with additional nonlabeled antibody to achieve 100 μg per dose. All blood sampling and injections were performed via the tail vein. Mice (n ≥ 3 per group) were intravenously administered 15−30 uCi/100 μg of ^{131}I-scFv-CC49-SAv (0.57 nmol). After 24 h, a blood sample was collected to determine activity concentration of the tracer ("baseline", t = 0)) and within 1−2 h the mice were injected with 50 ug of **1** (22 nmol) or 100 μg of **2**, **3**, and **4** (5.7−23 nmol) formulated in 100 μL of saline, or vehicle. Serial blood samples at various times 5−120 min postinjection (p.i.) of clearing agent (t = 5−120 min) were collected in preweighed microcapillary tubes and counted in the gamma counter for quantification of radioactivity. Differences between cohorts were statistically analyzed with Student's t test for paired data. Two-sided significance levels were calculated and P < 0.05 was considered statistically significant.

Biodistribution Analysis of ^{131}I-scFv-CC49-SAv with Clearing Agent Step. For all groups, animals were sacrificed via CO_2 asphyxiation 22−24 h post injection of the clearing agent (t = 22−24 h). Activities in blood, heart, liver, spleen, and kidney were determined. Tissues and organs were excised, rinsed with water and allowed to air-dry, weighed, and then counted in an automatic well counter (Perkin-Elmer Wallac Wizard 3″ Automatic Gamma Counter). The radioactive uptake was determined by decay-correcting the net count rate to the time of injection and expressing as the percentage of the injected dose per gram of the tissue (%ID/g).

RESULTS

Glycodendrons **1**−**4** were constructed in a stepwise manner where dendrons **11**−**14** were synthesized first, followed by the addition of the biotin to the dendron core, and then attachment of the prefunctionalized α-thio-*N*-acetylgalactosamine units **19**. Orthogonal protecting groups (ethyl esters and BOC) were utilized to site-specifically incorporate the desired functional groups, i.e., biotin and carbohydrates, with a final single deprotection step leading to the desired compounds **1**−**4**.

Synthesis of AB_2 Monomer 7 and Dendron Core. For the preparation of AB_2 type monomer **7**, a number of synthetic routes were initially considered. However, the route depicted in

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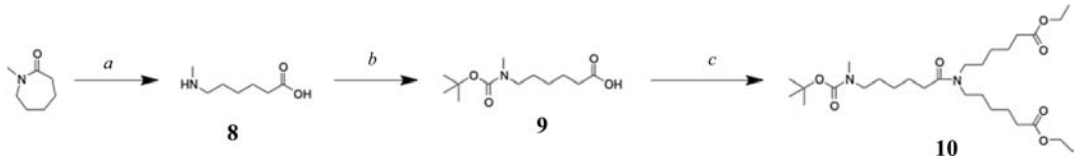
Scheme 2. Synthesis of Generation 0 (G^0) Dendron[a]



[a](a) Conc. HCl, reflux, 48 h (99%); (b) CH_2Cl_2, di-*tert*-butylcarbonate, 60 min (95%); (c) DMF, **7**, HATU, TEA, 20 min (Crude 95%).

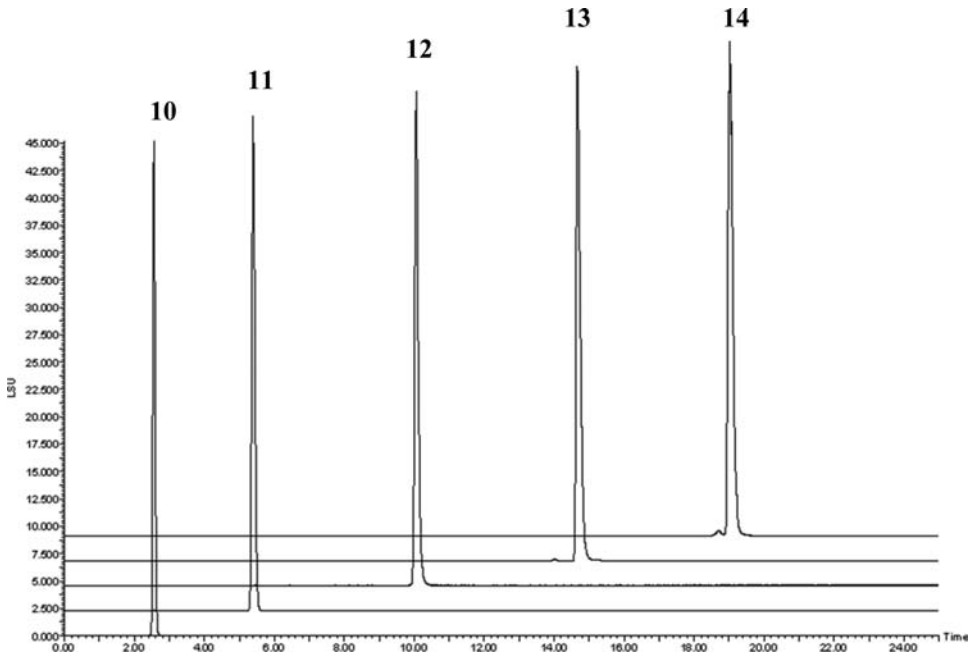


Figure 2. HPLC Analysis of Dendrons **10** (G^0), **11** (G^1), **12** (G^2), **13** (G^3), and **14** (G^4). Analysis conditions used: gradient of 45−95% acetonitrile (in water, 0.05% TFA) in 25 min; reversed phase C4 column (300 Å, 4.6 × 50 mm).

Scheme 1 was selected due to its high reproducibility, good product yields, and low cost of reagents. Monomer **7** was obtained by reacting 4-methoxybenzylamine (PMBA) with an excess of ethyl 6-bromo-hexanoate in the presence of base (DIEA) and NaI, while stirred overnight at room temperature (Scheme 1). After an aqueous workup and silica gel purification, the tertiary amine **6** was isolated in high yield. Removal of the PMB group was achieved through catalytic hydrogenolysis resulting in the desired product **7**. Purity and identity of the monomer were assessed by LCMS and ^{1}H NMR.

It should be emphasized that the AB_2 type monomer **7** is the most important component in the synthesis of these dendrons. Extreme care was taken to ensure the complete absence of impurities in **7**, especially amine byproducts which have proven to adversely complicate purification and isolation of homogeneous dendron products later in the synthesis. LCMS analysis was essential for identifying impurities not readily observed by ^{1}H NMR. For example, in the early synthesis of **7**, low levels, typically less than 1% of ethyl-6-aminohexanoate were observed by LCMS but difficult to detect by ^{1}H NMR, reacted much faster than higher order dendrons, and gave inseparable contaminations in later steps (data not shown).

The dendron core **9**, was obtained in two steps (Scheme 2) where the *N*-methylcaprolactam was hydrolyzed in refluxing concentrated HCl to quantitatively provide caproate **8**. The resulting amine was then protected as the respective *tert*-butoxycarbamate **9**. The acid-labile BOC group was utilized to provide an orthogonal protection strategy against the base sensitive ethyl esters in monomer **7**. The product **9** was obtained in excellent yield and purity, which was confirmed by LCMS and ^{1}H NMR.

Synthesis of Dendrons 11−14. Dendrons **11−14** were constructed where the AB_2 type monomer **7** was reiteratively introduced through a series of coupling-deprotection steps. This route provided the flexibility to obtain dendrons of successive generations, in contrast to more convergent methods that have been previously described.[21] In order to evaluate the molecular identity, purity, and structural integrity of the dendron products, while monitoring for impurities such as truncated molecules, LCMS and ^{1}H NMR were extensively used during the course of the syntheses. High performance liquid chromatography (HPLC) with three modes of detection − mass, evaporative light scattering (ELS), and standard UV absorption − was utilized for determining product identity and purity. Dendron products **11−14** were also analyzed by ^{1}H NMR, which was used to assess product purity, as well provide supporting structural information. For example, the integration values for two specific resonance − a singlet ($\delta \approx 2.83$) from the *N*-methyl group(s) of the dendron core and a multiplet ($\delta \approx 4.13$) from the ethyl ester ($CH_3C\underline{H}_2O-$) end groups − were used to determine the relative number of *N*-methyl to ethyl ester protons (Supporting Information). This served as an additional means to verify the dendron products.

The generation zero (G^0) dendron **10** was obtained by coupling compounds **9** and **7** with HATU in the presence of

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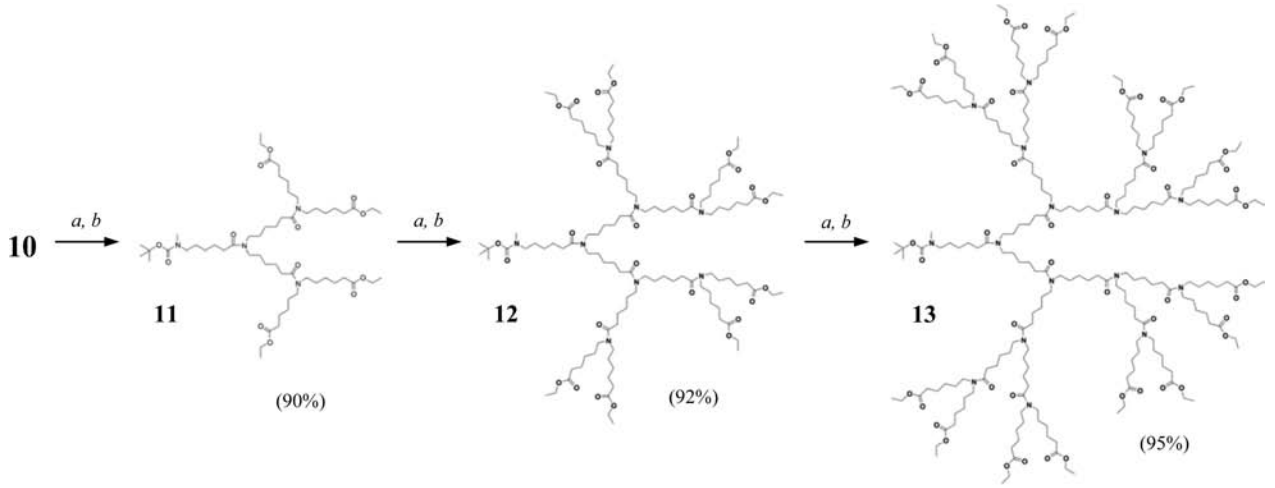
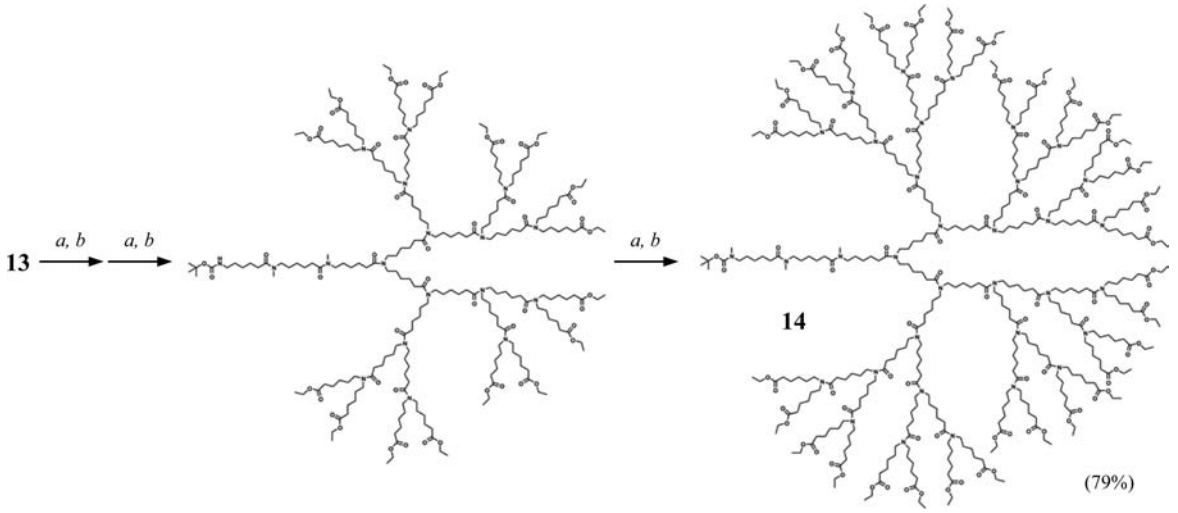
Scheme 3. Synthesis of Dendron Scaffold 11−13[a]



[a](a) *Ethyl Ester Deprotection*: 1 N NaOH, 40 °C, 1−2 h; (b) *Coupling*: **7**, HATU, DIEA in DMF, 20−30 min (Crude yields are shown).

Scheme 4. Synthesis of Dendron Scaffold 14[a]



[a](a) *BOC Deprotection*: TFA, 10 min; (b) *Coupling*: **9**, HATU, TEA in DMF, 20 min; (c) *Ethyl Ester Deprotection*: 1 N NaOH, overnight; (d) *Coupling*: **7**, HATU, TEA in DMF, 20 min.

DIEA (Scheme 2). This reaction was complete within 20 min, and was followed by an acidic workup to remove unreacted **7** as well as HATU byproducts. Analysis of the crude product by LCMS revealed a single peak (Figure 2), with a molecular ion at 529.36 m/z (*calc.* $C_{28}H_{53}N_2O_7$: 528.3775), indicative of a product of high purity. The ^{1}H NMR spectrum of the product was consistent with the desired compound **10**. The resonances for the *N*-methyl singlet and the $CH_3C\underline{H}_2O-$ multiplet for the methylene in the ethyl esters were determined to be 3 and 4 respectively, as expected (Supporting Information, Figure S2).

Continuing with the deprotection-coupling cycle, dendrons **11−13** were synthesized as shown in Scheme 3. Starting with compound **10**, dendron **11** was obtained in two steps. First, ester **10** was saponified with 1.0 N NaOH, followed by an acidic workup, yielding two free carboxylic acids. Two equivalents of monomer **7** were then coupled to the two exposed carboxylic acids using HATU/DIEA in anhydrous DMF. The reaction was complete within 30 min as determined by LCMS. An acidic workup was used to remove any unreacted monomer **7** as well as HATU related contaminants from **11**. LCMS analysis of **11** showed a single peak (Figure 2) with molecular ions of $[M+H]^+$ m/z = 1039.59 (*calc.* $C_{56}H_{103}N_4O_{13}$: 1039.7443) corresponding to the G^1 tetraethyl ester product. The ratio of *N*-methyl to ethyl ester ($CH_3C\underline{H}_2O-$) protons was 3:8 as determined by ^{1}H NMR (Figure S5).

To obtain the G^2 dendron **12**, the deprotection−coupling procedure above was repeated with **11** (Scheme 3). Ester hydrolysis of **11** yielded four free carboxylic acids, which were coupled with the monomer **7** resulting in the G^2 octaethyl ester dendron **12**. LCMS analysis for the final product **12** showed a single peak (Figure 2) with multiply charged ion peaks $[M+2H]^{2+}$ m/z = 1031.04 and $[M+3H]^{3+}$ m/z = 687.77 (*calc.* $C_{112}H_{202}N_8O_{25}$: 2059.4781). ^{1}H NMR integration data further confirmed the presence of the desired product ratio of 3:15.4 (Figure S8).

Similarly, the G^3 dendron was obtained by hydrolysis of the ethyl esters of **12** resulting in eight carboxylic acids; and HATU/DIEA mediated monomer addition yielded the hexadeca-ethyl ester **13** (Scheme 3). After workup the final product exhibited a single peak by HPLC (Figure 2), with the

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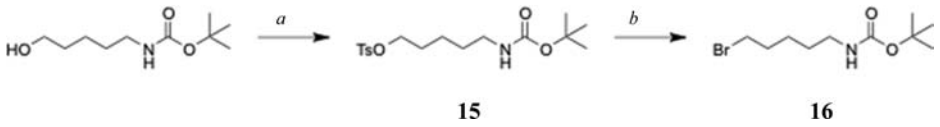
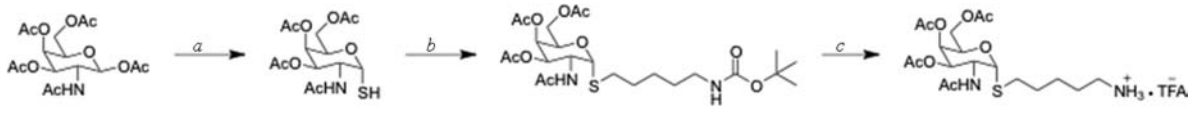
Scheme 5. Synthesis of *tert*-Butyl-(5-Bromoxypentyl)carbamate 16[a]



15 16

[a](a) TEA, TsCl, DMAP (cat.), 0 °C; overnight at RT (80%); (b) ACN, NaBr, Bu_4NBr, reflux overnight (83%).

Scheme 6. Synthesis of *N*-*tert*-Butyloxycarbonyl-*S*-(2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-5-Thiopentylamine Trifluoroacetic Acid Salt 19[a]



17 18 19

[a](a) Toluene, Lawesson's reagent, reflux 2.5 h; (b) DMF, DIEA, **16** (two steps 89%); (c) TFA/DCM (2:5, v/v), 2 h.

expected masses (*calc.* $C_{224}H_{402}N_{16}O_{49}$: 4100.9457, *obs.* $[M+3H]^{3+}$ m/z = 1368.57, $[M+4H]^{4+}$ m/z = 1026.77). ^1H NMR spectrum was also consistent with the desired dendron−proton ratios of 3 to 32.3 from the *N*-methyl and ethyl ester groups, respectively, were observed (Figure S11).

In preparation of the G^4 dotriaconta-ethyl ester dendron, the core of **13** was further extended by two *N*-methyl-6-hexanoic acid units (Scheme 4). Since three-dimensional structures of these dendrons are not available, there were general concerns that, for the G^4 glycodendron, the central amine and the biotin may be cloaked by 32 densely associated carbohydrate groups thereby precluding binding to circulating/off-target antibody−streptavidin fusion protein. To circumvent this possibility, we extended the linker attachment to the biotin by 14 atoms. Hence, **13** was elongated with two units of *N*-methyl-6-hexanoic acid via the corresponding BOC analog **9**. *N*-Methyl-6-hexanoic acid groups were sequentially added, i.e., after removal of the central BOC group with TFA, **9** was coupled onto the exposed *N*-methyl amino terminus; and these steps were repeated for the addition of another molecule of **9**. Following final BOC deprotection and the incorporation of D-biotin, ethyl esters hydrolyses resulted in sixteen carboxylic acid groups which were then extended by the addition of **7** to afford the G^4 dendron **14**. As shown in Figure 2, the final product exhibited a single peak by LCMS (*calc.* $C_{462}H_{828}N_{34}O_{99}$: 8438.0802, *obs.* $[M+5H]^{5+}$ m/z = 1689.53, $[M+6H]^{6+}$ m/z = 1407.99, $[M+7H]^{7+}$ m/z = 1207.06). The ^1H NMR spectrum revealed three well resolved *N*-methyl resonances (3H, δ = 2.83; 3H, δ = 2.90; 3H, δ = 2.97) along with the $CH_3C\underline{H_2}O-$ multiplet (64H, δ = 4.12) with integrations of 3.0, 3.0, 3.2, and 63.9, respectively (Figure S14).

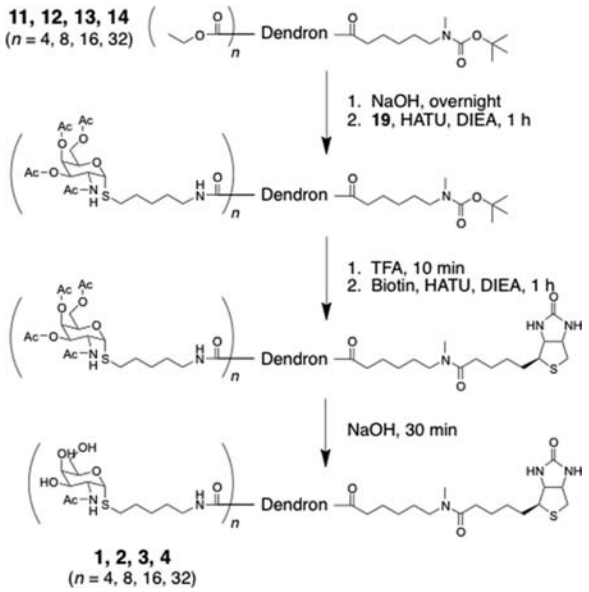
In contrast to prior work which used BOP or other carboxylic acid activators,[7,21] we utilized 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) for these coupling reactions. HATU has been shown to be highly effective in the condensation of carboxylic acids and secondary amines.[22,23] Indeed, for the reactions described in this work, HATU proved to be efficient in forming amide bonds between the AB_2 monomer **7** and the free carboxylic acid end groups. All reactions typically required only a slight excess of monomer (1.2−3.0 equiv per free carboxylic acid), and were complete within 30 min. Additionally, the HATU byproducts could be removed with a standard acidic workup, further helping to minimize impurities in the final dendron products.

Synthesis of Amino-alkyl Peracetylated α-Thio-GalNAc 19. The prefunctionalized α-thio-*N*-acetylgalactosamine **19** was synthesized as shown in Schemes 5 and 6. *N*-BOC-aminopentanol was tosylated (**15**), then converted into **16** (Scheme 5). To obtain the desired α-GalNAc mercaptan **17** (2-acetomido-2-deoxy-3,4,6-tri-*O*-acetyl-1-thio-α-D-galactopyranose), commercially available peracetylated β-galactosamine was treated with Lawesson's reagent, 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide, resulting in the α-thioglycoside (Scheme 6).[24] **16** and **17** were then combined through a displacement reaction in the presence of base to yield the BOC protected peracetylated α-sugar **18**. Finally acid was used to remove the BOC group, and the aminopentyl conjugate **19** was obtained as a TFA salt. Purity and identity of the intermediates and final thioglycoside were assessed by LCMS and ^1H NMR.

Synthesis of Clearing Agents 1−4. To obtain clearing agents **1**−**4**, the end groups for dendrons **11**−**14** were first modified with the carbohydrate moieties, followed by the addition of D-biotin (Scheme 7). Starting with dendrons **11**−**14** the ethyl esters were hydrolyzed, exposing 4, 8, 16, or 32 carboxylic acid groups, respectively. This was followed by HATU/DIEA mediated coupling of tetraacetyl-α-galactosamino-5-thiopentylamine groups **19** to the free acids (Scheme 7). The lone BOC group on the glycodendrons was then removed by brief treatment with TFA, and the resulting amine was coupled with D-biotin. Finally, global deprotection of all the *O*-acetyl groups with 0.2 N NaOH afforded the desired clearing agents **1**, **2**, **3**, and **4** as determined by LCMS (**1**, *calc.* $C_{105}H_{184}N_{10}O_{33}S_5$: 2273.1631, *obs.* $[M+2H]^{2+}$ m/z = 1135.96, $[M+3H]^{3+}$ m/z = 757.69; **2**, *calc.* $C_{205}H_{368}N_{26}O_{57}S_9$: 4394.4183, *obs.* $[M+3H]^{3+}$ m/z = 1466.42, $[M+4H]^{4+}$ m/z = 1100.08 $[M+5H]^{5+}$ m/z = 880.07; **3**, *calc.* $C_{405}H_{728}N_{50}O_{113}S_{17}$: 8644.8009, *obs.* $[M+5H]^{5+}$ m/z = 1731.08, $[M+6H]^{6+}$ m/z = 1442.69, $[M+7H]^{7+}$ m/z = 1236.61; **4**, *calc.* $C_{819}H_{1474}N_{100}O_{227}S_{33}$: 17399.7654, *obs.* $[M+9H]^{9+}$ m/z = 1935.47, $[M+10H]^{10+}$ m/z = 1741.91). Crude products **1**, **2**, **3**, and **4** were typically obtained in >90% purity and required a single purification step using reversed-phase HPLC of the final product (Figure 3).

Solid-Phase Synthesis of Dendrons. Given the sequential and repetitive nature of the dendron synthesis, we also investigated the use of solid-phase methods as a possible

Scheme 7. Synthesis of Clearing Agents 1−4



expedient route to access limited quantities of the clearing agents in order to accelerate development (Supporting Information, Figure S28). Hydroxymethylphenyl resins of various loading capacities (0.2−1.0 mmol/g) were converted into the corresponding bromomethyl form upon treatment with excess of carbon tetrabromide. The amine of the *N*-methyl-6-aminohexanoic acid (Figure S28) was then reacted with the bromo-functionalized resin in the presence of DIEA, and dendron was grown from resulting carboxylic acid using a repetitive coupling/deprotection cycles which were compatible with solid phase synthesis. Hence, on-resin hydrolysis of the ethyl esters was achieved by the use of sodium trimethylsilanolate in DCM/DMF,[25,26] and 1-chloroethyl chloroformate was used to cleave dendron products from the resin[27] for the purposes of efficacy analyses. Following this approach, compounds **20**, **21**, and **22** were characterized by LCMS. However, attempts to grow the dendron beyond generation 3 were not successful, and we could not isolate product **23** by this method. Interestingly, upon coupling of **7** to the second generation dendron-resin, the resin became visually distorted and lost the capacity to swell in solvent. It is worth noting, however, that some preliminary work suggests that the synthesis of higher generation dendrons may be possible on larger PEG-based resins and/or rigid macroporous nonswelling beads (Synbeads).[28]

Evaluation of Blood Clearance of ^{131}I-scFv-CC49-SAv. Experiments were conducted *in vivo* to evaluate the kinetics of blood clearance of the scFv-CC49-SAv model protein following a separate injection (24 h later) of an excess dose of clearing agent to antibody (10:1 to ∼40:1 molar ratio). Additionally, to provide further insights into the biology of these compounds and for comparison, we also evaluated a high-molecular-weight (500 kDa) biotinylated-dextran. The scFv-CC49-SAv was radioiodinated with ^{131}I using standard Iodogen procedures to directly measure levels of the fusion protein.[20] Time activity data for the ^{131}I-tracer in blood were calculated as %ID/g relative to baseline collected prior to injection of compounds (t = 0 min). As shown in Figure 5, clearing agents **1** to **4** produced an almost immediate effect on blood activity, with radioactivity levels dropping as much as 40% from baseline within 30 min p.i. (post injection). During the same time interval, the clearance of activity in animals given vehicle or biotinylated dextran was ≥15% to a concentration of ≥85% of baseline. At 120 min all cohorts given either glycodendron or biotinylated-dextran had blood activities significantly less than vehicle (P < 0.05 for biotinylated-dextran and **1**, and P < 0.005 for **2**, **3**, and **4**).

Biodistribution Analysis. At 24 h p.i. of compounds or controls, biodistribution analysis was conducted to determine

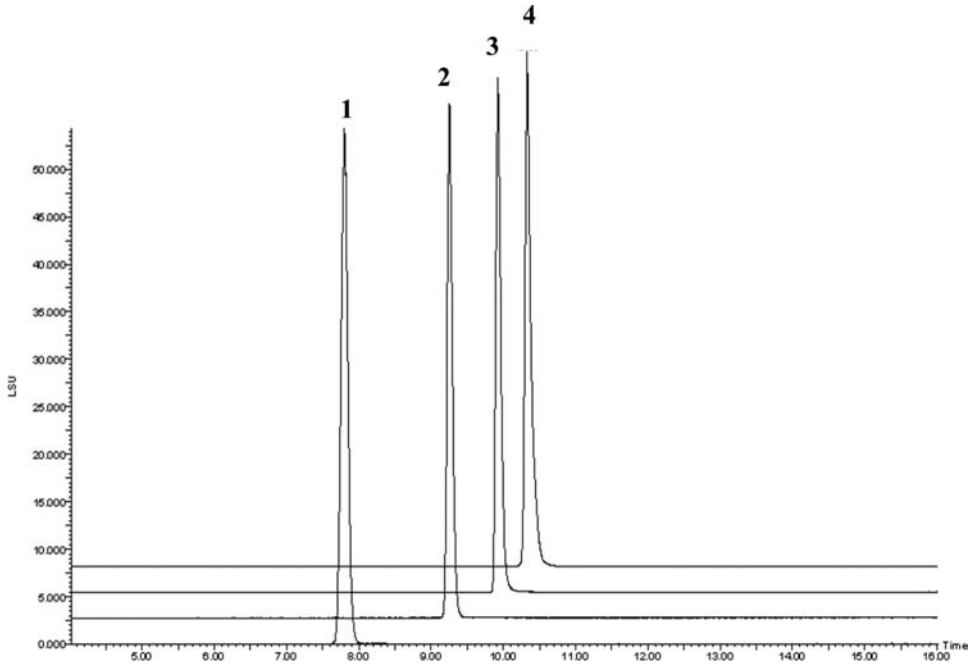


Figure 3. HPLC analysis of clearing agents **1**−**4**. Analysis conditions used: gradient of 20−40% acetonitrile (in water, 0.05% TFA) in 20 min; reversed phase C4 column (300 Å, 4.6 × 50 mm).

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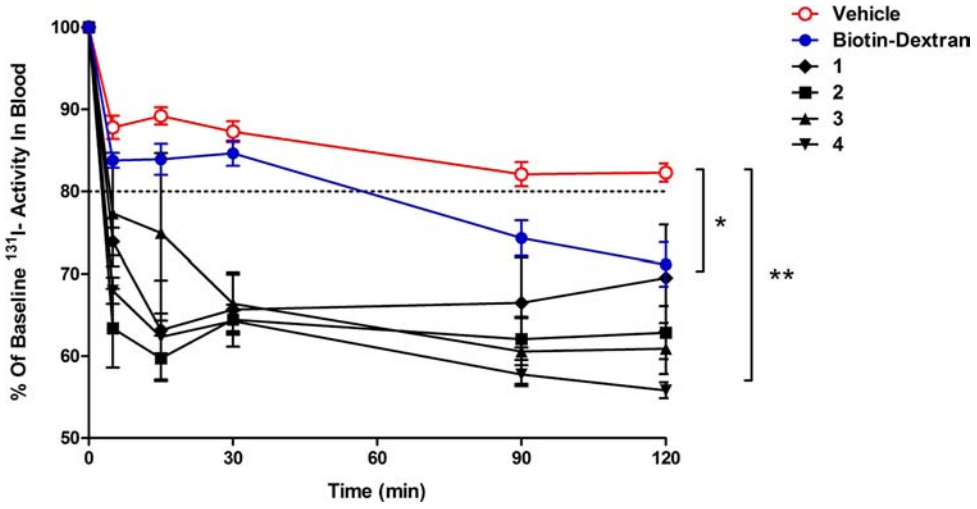

Figure 4. Blood clearance of ^{131}I-scFv-CC49-SAv. Time activity curve for ^{131}I-activity in blood determined by serial blood collection up to 120 min following injection of ^{131}I-scFv-CC49-SAv (t = −22 h to −24 h) and compounds **1**−**4** (t = 0) in biotin-starved mice. Cohorts given vehicle or 100 μg of biotinylated-dextran were included as controls. Data is shown as mean ± SEM, *P < 0.05, **P < 0.005. Note: scale on y-axis is 50−100%.

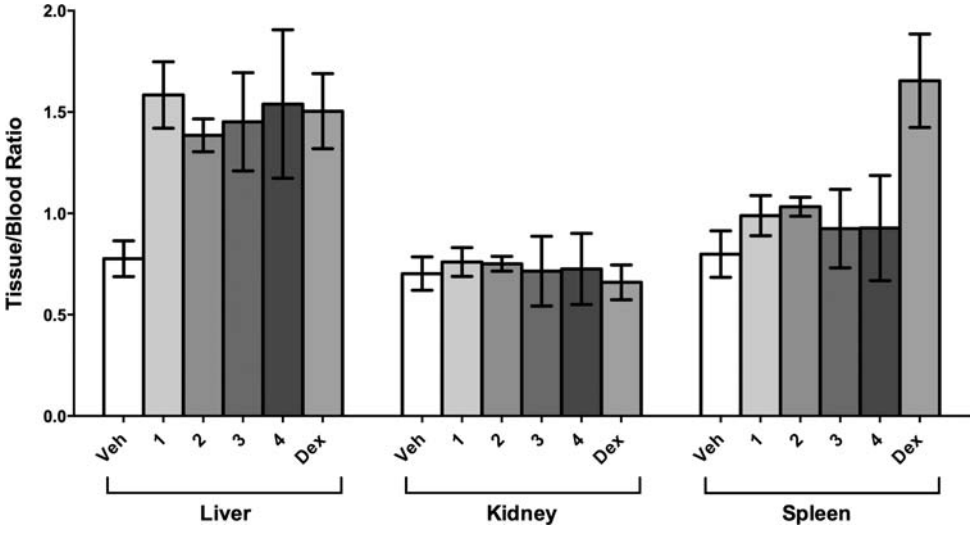

Figure 5. Glycodendrons enhance liver targeting/uptake of ^{131}I-scFv-CC49-SAv. Tissue-to-blood ratios determined from biodistribution obtained 24 h p.i. of vehicle (Veh), glycodendrons (**1**, **2**, **3**, **4**), or biotin-dextran (Dex) (Figure S29). Data is shown as mean ± SEM.

activities of ^{131}I-tracer in the blood, kidneys, liver, and spleen (Figure S29). Animals treated with clearing agents **1** and **4** consistently showed lower levels of ^{131}I in the blood, spleen, and kidneys, but notably higher levels (3.14 ± 0.24 and 3.22 ± 0.68%ID/g) in the liver, relative to saline (2.2 ± 0.31%ID/g). Compounds **2** and **3** showed radioactivity levels similar or moderately higher than saline for all except the liver where nearly twice as much ^{131}I accumulation was observed (4.30 ± 0.41 and 4.25 ± 0.07%ID/g). The 500 kDa dextran−biotin conjugate followed a similar trend to glycodendrons **1** and **4**, except for the spleen where notably higher radioactivity levels were observed (3.17 ± 0.67% ID/g).

The tissue-to-blood ratios were calculated to evaluate tracer retention (Figure 5). Mice treated with glycodendrons or the 500 kDa dextran−biotin compounds showed liver/blood ratios >1:1 compared with vehicle, suggesting retention of the tracer. For spleen/blood, the biotinylated-dextran cohort was 1.5:1, whereas for glycodendrons and control, the ratio was ≤1:1. In the kidney, no differences were observed between groups, and the kidney/blood ratio was ∼0.75:1.

Overall, the *in vivo* data suggests enhanced blood clearance of the ^{131}I-scFv-CC49-SAv when a glycodendron compound or the 500 kDa biotin−dextran conjugate is administered. Additionally, the biodistribution results reveal increased liver retention of the ^{131}I-tracer, suggesting that all clearing agents facilitate ^{131}I-scFv-CC49-SAv targeting to the liver.

DISCUSSION

The goal of this work is to evaluate and advance pretargeted RIT approaches for cancer imaging and therapy. Our laboratory has been interested in the synthesis of effective clearing agents to be used in MST applications. The basic design strategy used thus far has been to construct bimodal molecules that have the capacity to target both the desired antibody and hepatocytes. Early clearing agents utilized human serum albumin (HSA) as a scaffold to which biotin and galactose were conjugated.[29] Macromolecular dextrans with biotin, as shown here, have also been used as clearing agents. The primary drawback with these agents is the heterogeneity of the final product as well as the challenges of synthetic reproducibility. While adequate for small

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scale preclinical work, such issues become significant barriers to clinical studies in humans. This lead to the design of glycodendron **3**, which has been used in several preclinical and clinical PRIT related studies.[5,8,13−15] The use of a dendron polymer offers the advantages of incorporating multiple carbohydrate units and biotin in a predetermined and controlled manner, resulting in a product that is chemically well-defined and monodisperse. However, due to past difficulties to synthesize compound **3**, efforts to explore lower molecular weight compounds using rational design approaches with multiple biotin ligands have been reported.[30]

In this study we describe an alternative solution to efficiently obtain compound **3** as well as its lower and higher generation homologues. Previously reported synthetic protocols for **3** followed a more convergent approach where more advanced glycodendron intermediates were linked together to form the final product.[7] However, efforts to repeat these steps resulted in polydisperse products which were difficult to purify. To avoid potential steric issues associated with joining high molecular weight fragments and partial loss of protecting groups, we focused on a more linear route where high purity low molecular weight building blocks are used to first construct the dendron scaffold, followed by carbohydrate and biotin conjugations. Our approach relies upon a new route to synthesize high purity AB_2 monomer **7**, which was used as the basic dendron building block. Dendrons **11** to **14** were then readily obtained by implementing iterative deprotection/coupling cycles reminiscent of solid phase synthesis. To minimize purification steps, coupling reactions were optimized to ensure that all reactions were complete before proceeding. We were then able to take advantage of the aliphatic nature of the dendron products and use simple aqueous workup procedures to remove impurities. This enabled us to forego several column chromatography purification steps while affording the desired dendrons with high purities and good yields as determined by LCMS and NMR (Figure 2 and Supporting Information). Once dendrons **11** to **14** were obtained, biotin and sugar **19** could be readily conjugated to obtain glycodendrons **1** to **4**. A single HPLC purification step was performed resulting in materials of excellent quality (Figure 3 and Supporting Information). An analogous methodology which used a different protecting group schema has been described for similar aliphatic dendrons. In this example a reduced number of synthetic steps were used, but potentially difficult purification steps were necessary to obtain dendrons of good purity.[21]

We conducted biological testing of the glycodendrons to verify hepatocyte targeting *in vivo*. For utility in MST, the glycodendron must demonstrate the capacity to redirect the tumor targeting mAb from circulation to the liver. We administered ^{131}I- scFv-CC49-SAv, a familiar MST-antibody tracer, to biotin-starved mice followed by an excess dose of clearing agent relative to antibody dose. We conducted serial-blood sampling for kinetic analysis of radioactivity, as well as biodistribution studies to determine if animals given glycodendrons showed increased liver/blood ratios, indicating increased uptake and retention (i.e., liver targeting). The inclusion of biotinylated-dextran was twofold: a dextran-based clearing agent was reported recently to be an effective clearing agent for 3-step MST, and supporting biodistribution experiments described here with tracer and dextran-clearing agent showed significant uptake in the spleen and liver 4 h p.i. (30%ID/g and ∼10%ID/g, respectively), suggesting that RES metabolism is the predominant clearing mechanism for dextran and its complexes.[6]

Kinetic analysis of blood clearance of ^{131}I-scFv-CC49-SAv following administration of compounds **1** to **4** indicate that all can bind the protein *in vivo* and efficiently remove it from circulation within 15 min following administration of an intravenous bolus dose ≤100 μg with no adverse effects. For this experiment the primary goal was to determine basic blood clearing activity for compounds **1** to **4**. Starting with 0.57 nmol of the labeled fusion protein (i.e., ^{131}I-scFv-CC49-SAv), a convenient dose of each CA was chosen to achieve a >10-fold molar excess, relative to protein. Consequently, 50−100 μg of CA were used (50 μg of **1**, 100 μg of **2**−**4**). This is in contrast to previously reported work where 1.4 to 3.5 nmol protein and 50−100 μg of **3** were typically employed.[31−33] These amounts were optimized for pretargeting protocols where the higher fusion protein doses were necessary to allow for saturation of the tumor target (by the fusion protein). Consequently, direct comparisons between Figure 4 and analogous pharmacokinetic profiles from previous pretargeting studies may not be possible. Additionally, the presence of low levels of endogenous biotin in mice may significantly impact the CA mediated clearance of ^{131}I-scFv-CC49-SAv at the lower dose used here. This may explain why greater relative percentage drops in circulating fusion protein (Figure 4) were not achieved in our experiments.

The pharmacokinetic profiles in Figure 4 provide little insight as to which glycodendron is best suited for MST applications. More rigorous studies are certainly needed, especially in the context of a pretargeting protocol. The advantages of lower molecular weight CAs, such as compounds **1** and **2**, are clear: they require fewer synthetic steps, and are more readily obtained at lower cost. However, their small size can potentially lead to unwanted complications, such as extravasation and accumulation at the tumor site, leading to binding of the tumor prelocalized mAb-SAv. Conversely, the larger CAs, such as compounds **3** and **4**, are synthetically more demanding, thus more expensive. Yet their size may afford reduced tissue penetration and increased residence time in the blood thereby facilitating clearance of excess mAb-SAv.

Apart from the use of compounds **1** to **4** as clearing agents in the context of MST, we also sought to evaluate these glycondendrons as general liver targeting agents. To measure CA dependent liver targeting/uptake of the fusion protein, biodistribution analysis for ^{131}I-scFv-CC49-SAv 24 h p.i. of glycodendrons in select organs was performed (Figure S29). When ^{131}I in the tissue are normalized to blood levels, we observe an enhancement in liver targeting over the spleen and kidneys when **1** to **4** are used (Figure 5). Additionally, the data suggest liver uptake and retention to be the predominant mechanism for the glycodendron dependent clearance of the fusion protein. This study does not reveal a correlation between sugar valency (or molecular weight) and liver targeting efficiency, so further studies would be needed to address this point.

The glycodendrons described here provide a highly versatile molecular scaffold upon which to design further optimized clearing agents as well as incorporate other ligand/receptor pairs for MST applications. The dendron scaffold is ideal for undertaking further structure−activity relationship (SAR) studies to identify modifications that could possibly enhance its function. For example, the chain lengths between the carbohydrates and the dendron and/or the biotin and dendron can be further extended to ensure optimal binding to the

respective receptor or binding partner. It would be of particular benefit to evaluate such glycodendrons in protein, liver membrane, or cell based binding assays and correlate the results with *in vivo* clearance data.[34,35]

In addition, the central amine functionality on the dendron linker can be used to orthogonally introduce different targeting moieties. Biotin can be replaced with other molecules to utilize different ligand/receptor pairs. While biotin/streptavidin has been shown to be effective, the presence of biotin in humans as well the immunogenicity of streptavidin may limit its use.[18] Consequently, different ligand/receptor pairs are being investigated.[18,36−40] In particular, bispecific mAbs, which recognize a tumor antigen and a small molecule such as metal bound DOTA, have made promising advances.[3,6] We are interested in utilizing these glycodendrons in the context of such bispecific mAbs MST protocols, and our results will be reported in due course.

In conclusion, we describe the synthesis and characterization of a class of glycodendron clearing agents. The synthesis comprises high yielding steps, potentially amenable to scalability, where crude products are obtained with good purity. We then evaluated the capacity for these glycodendrons compared to biotin−dextran conjugates to clear a circulating radiolabeled mAb streptavidin fusion protein, ^{131}I-scFv-CC49-SAv, in mice. We find that all the glycodendrons and biotin−dextran conjugate enhance blood clearance of the fusion protein, and observe higher accumulation of the radiolabel in the liver, suggesting appropriate targeting of the ASGP receptor. Experiments seeking to evaluate these compounds in tumor pretargeting experiments are underway and will be reported in due time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

LCMS, ^{1}H NMR, ^{13}C NMR for dendrons and biodistribution data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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