

THE SYNTHESIS AND CHARACTERIZATION OF FLUORESCENTLY LABELED, LACTOSE-
FUNCTIONALIZED POLY(AMIDOAMINE) (PAMAM) DENDRIMERS

by

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DEDICATION

To Everyone, for your support and second chance. To myself, for not giving up.

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NOMENCLATURE

PAMAM: Poly(amidoamine)

NMR: Nuclear Magnetic Resonance

UV: Ultraviolet

DOL: Degree of Labeling

G(2): Generation 2

G(3): Generation 3

G(4): Generation 4

G(6): Generation 6

α -Gal: galactosyl-(1–3) galactose

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

OTf: Triflate

DCM: Dichloromethane

PEG: Polyethylene glycol

siRNA: Silencing RNA

CRD: Carbohydrate Recognition Domain

MIC: Minimum Inhibitory Concentration

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

MWCO: Molecular Weight Cut-Off

DI: Deionized

SEC: Size Exclusion Column

NOMENCLATURE CONTINUED

PES: Polyethersulfone

COSY: Correlation Spectroscopy

HSQC: Heteronuclear Single Quantum Coherence

HMBC: Heteronuclear Multiple Bond Correlation

s: Singlet

bs: Broad Singlet

d: Doublet

ddd: Doublet of Doublet of Doublets

dd: Doublet of Doublets

bd: Broad Doublets

t: Triplets

bt: Broad Triplets

m: Multiplet

ABSTRACT

Cellular uptake of lactose-functionalized poly(amidoamine) dendrimers (PAMAM) has yet to be fully understood and deeply studied. Before sufficient cellular uptake studies can be made, optimization of the synthesis of the lactoside, and the coupling and purification of dye-tagged lactose-functionalized PAMAM had to be completed, as reported here. The synthesis of the requisite lactoside derivative for dendrimer functionalization was optimized. The coupling of the dye, Alexa Fluor 647, to the lactoside-functionalized PAMAM was performed in the presence of a sodium acetate buffer and utilized size separation methods to ensure purity. The structures of the lactoside derivatives and of lactose functionalized PAMAMs were confirmed via nuclear magnetic resonance (NMR) spectroscopy. The purity and degree of labeling (DOL) of the dye labeled, lactose-functionalized PAMAMs were determined with UV-vis. Results show high success of yield and purity resulting from the optimized procedure described in this study.

CHAPTER ONE

INTRODUCTION

Polymers and Macromolecules in Chemical Biology

Polymers and macromolecules have a wide range of uses in chemical applications such as improving solubility, biodegradability, soft/hardness of materials, and melting points. In addition, natural polymers can hold biological information that is important for cell signaling, genomic expression, and structural integrity.¹ Synthetic polymers and macromolecules have been developed to mimic biological polymers. Thus, polymers can be useful for the development of non-toxic drug delivery systems by improving their bioavailability (such as PEGylation for improving solubility) and can also serve an important role in fundamental studies of cellular processes. Polymers can be linear, can be comprised of repeats of the same monomer units, or can be synthetically made to include a diverse make-up of monomers (copolymers). Polymers can also be branched, having linear “hairs” or branches hanging off the backbone, can be crosslinked by covalent bonds, or can form a complex 3-D network with structural integrity.² Large macromolecule systems such as dendrimers and star polymers benefit from having a more defined quaternary structure that is approximately spherical. All of these systems have been used in biomedical applications due to the low cost of large-scale production and the ease of functional adjustment for a broad range of applications.

Synthetic polymers and macromolecules can be used for antimicrobial studies with great effect. Leslie A. Rank, *et. al.*, utilized linear nylon-3 copolymers as mimics of natural-defense peptides to inhibit antimicrobial growth without toxicity to eukaryotic cells.³ By adjusting the

copolymer structures, they were able to gain insight to the differences in membranes between eukaryotic and prokaryotic cells. They provide valuable work for future development of synthetic antimicrobial polymers that offer an alternate low-cost production of polypeptides for biomedical uses.³ Nicolas Perez-Soto, *et. al.*, synthesized cationic linear polymers that were able to enhance the quorum sensing of *Vibrio cholerae*, upon inducing biofilm aggregation, through rapid autoinduction.⁴ The enhanced quorum sensing induced by the polymer led to control the regulation of certain phenotypes that impact the level of luminescence (under certain conditions) and prevent dissipation specifically in *V. cholerae* which is useful for preventing the spread of water contamination. In a study done by the Cloninger group, they used mannose-functionalized dendrimers were used as a multivalent quaternary antimicrobial to study mutation responses in *Bacillus cereus*.⁵ These synthetic macromolecules helped gain insight into the mechanism of bacteria antimicrobial resistance showing that the compounds induce membrane fortification within the bacteria that led to increased mutations.

Another class of synthetic polymers are glycopolymers, or synthetic polymers with pendant carbohydrates. Glycopolymers have been gaining interest for improving cellular uptake of anti-cancer drugs and insulin and targeting viruses. They can serve as delivery agents for nucleic acid and gene therapy.⁶ The glycopolymers are especially useful for targeting specific cells that overexpress lectin receptors (**figure 1**). Lectins are a class of carbohydrate-binding proteins that are highly specific in their interactions and mediate many cell-signaling processes that can be associated with disease development. Mannose-functionalized polymers have been used as a drug delivery system for immunotherapy in cancer cell lines due to the high affinity specific macrophages have for mannose.⁶ Likewise, mannose-functionalized glycodendrimers have been used in studies of influenza uptake.⁷ The affinity for certain glycopolymers are dependent on the

type of lectins that are over-expressed on the cell surfaces and can be taken advantage of with glycopolymers to study these cells.

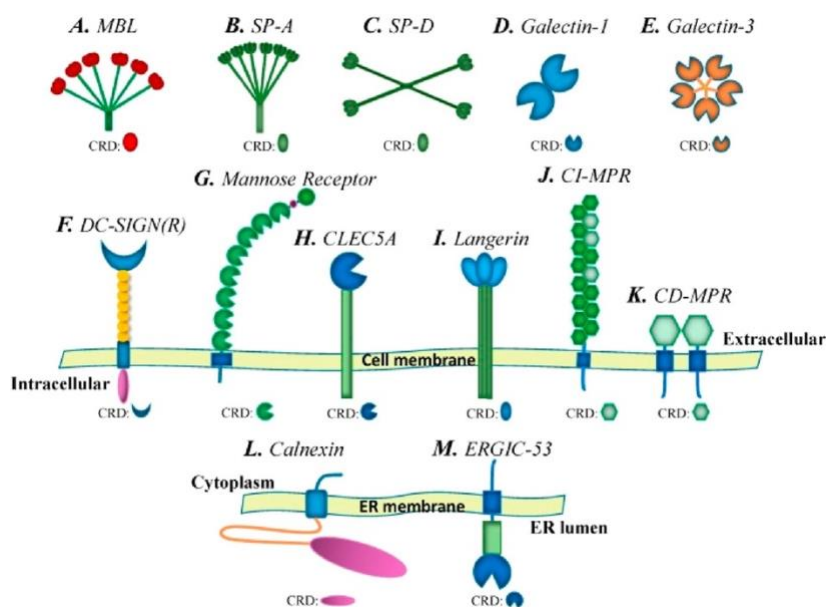


Figure 1. Examples of few noteworthy lectins that are free and/or surface-bound that can be potential targets of interest for glycoproteins in biomedical use for disease treatment.³

Other than linear polymer structures, branched polymers such as dendrimers have also proven useful for biological studies. Dendrimers are a globular, soft polymer nanoparticle that are made with branched layers (generations).⁸ Their appeal stems from its precise and controlled synthesis that results in highly monodispersed macromolecules. Poly(amidoamine) dendrimers (PAMAM) are a popular dendrimer to improve delivery of siRNA, antibodies, therapeutics, and many ligands that affect gene expression for cancer research (**figure 2**).^{8, 9} One example from Azadeh Reshadmanesh, *et. al.*, used an anti-HER2 nanobody-conjugated PAMAM nanoparticle that bound to HER2 cell surface markers in breast cancer. Their nanoparticle was shown to improve the quantity and specificity of cellular uptake, resulting in increased gene expression for apoptosis.¹⁰

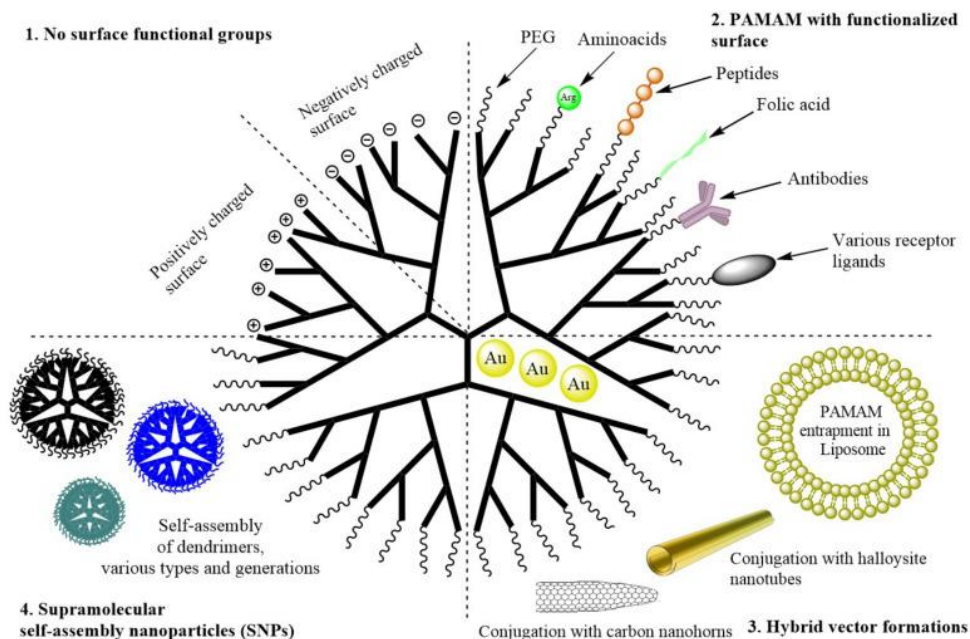


Figure 2. Depiction of the diverse methods to utilize PAMAMs surfaces for drug delivery in biological systems.⁵

Taking Advantage of Multivalent Interactions in Biological Systems

Biological interactions between a receptor and a monomer ligand are sometimes too weakly binding to be physiologically relevant. In these cases, multivalent interactions between ligands and receptors often occur synergistically to create a stronger, more selective interaction.¹¹ Multivalent interactions are utilized by biological systems to carry out many functions spanning from regulating cell signaling processes to viral infection of a host cell. For example, hemagglutinin, a homotrimeric receptor on avian influenza, relies on multivalent interactions with multiple sugar moieties on the host cell's surface to create a tight hold for inducing infection.¹² Another case of naturally occurring multivalent interactions involves the recognition of an antigen by an antibody. For example, immunoglobulin G (IgG) antibodies have an Fc (fragment crystallizable) region to bind to Fc receptor proteins that are expressed on a variety of cells to help

the immune system differentiate a healthy cell from an infected one. A monovalent interaction with the Fc region is insufficient to transmit an activation signal; a multivalent interaction to crosslink surface receptors is required to initiate an immune response (**figure 3**).^{13, 14, 15}

There are different mechanisms through which multivalent interactions can occur. There is a chelating effect in which oligomeric receptors can bind synergistically to multivalent ligands. Multivalent ligands can also create receptor clustering on a cell surface.¹⁵

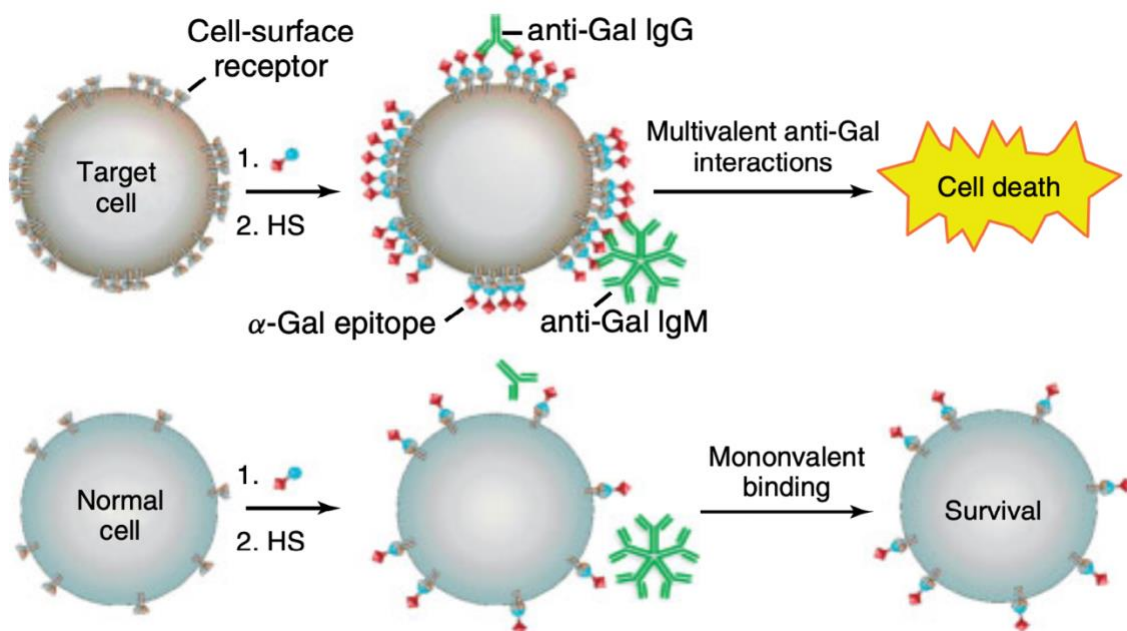


Figure 3. *Top schematic:* A multivalent interaction between the antibodies immunoglobulin M (IgM) and immunoglobulin G (IgG), that are naturally present in the human body, with the introduced galactosyl-(1–3) galactose (α -Gal) (red diamond connected to a blue circle) that decorate the target cell containing overexpressed receptors. The multivalent interactions between the antibodies and the disaccharide through receptor clustering, aided the antibodies in recognizing the target cell to trigger cell death. *Bottom schematic:* In a normal cell, there are less receptors present, therefore a multivalent interaction cannot be induced between the disaccharide and the antibodies, allowing the cell to live.¹³

Lectins use multivalency to increase affinity and specificity of protein-carbohydrate interactions by crosslinking or clustering receptors and/or glycans at the cell surface to mediate

significant biological events such as cellular recognition and differentiation, signaling, proliferation, and surface adhesion.^{16, 17} Many cell surface receptors and lectins in the immune system can recognize multivalent carbohydrate ligands which help regulate the body's immune response such as I-type lectins (a subset of the immunoglobulin superfamily) and fucoselectins.^{18, 19} Gaining a better understanding of these protein-carbohydrate interactions is essential due to their critical roles in disease development. Development of carbohydrate-based drugs for managing inflammatory responses and arresting tumor growth depend on understanding multivalent protein-carbohydrate interactions. The native interactions can also be altered, and thereby better understood, by introducing carbohydrate functionalized synthetic multivalent systems.

Synthetic multivalent systems have been developed using polymer scaffolds to inhibit viral infections, promote desired cell responses, and increase potency of drugs.^{20, 21} Whitesides developed polymers containing multiple sialosides that displayed polyvalency to inhibit influenza receptors with a greater binding affinity to its receptors than its natural ligand.²² Whitesides shows that using a polymer scaffold that displays multivalency can induce a greater effect over natural biological interactions, which can be taken advantage of to inhibit disease development.

PAMAM dendrimers offer an interesting multivalent scaffold for biological applications because the size and the number of end groups can be systematically varied. The number of end groups on PAMAM dendrimers doubles with each successive generation, and its defined shape makes analysis simpler. A G(0) PAMAM has 4 terminal primary amines, while a G(10) theoretically has 4,096 primary amines available for chemical modification, peptide coupling, fluorescent tagging, etc. Another advantage of dendritic polymeric frameworks is the potential for heterogeneous functionalization to fine tune the dendrimer's selectivity, specificity, drug delivery, solubility, and detection (**figure 5**). It is important to mention that functionalization determination

of the dendrimers is a statistical average. A smaller generation dendrimer may have easy access to all its attachment sites, but as the generation increases the macromolecule becomes more globular in shape making some terminal endgroups harder to access due to sterics. Despite this, a larger generation dendrimer has sufficient endgroups to enable synergistic binding even when less than full functionalization is achieved.^{21, 23} Even homogeneously functionalized small generation dendrimers will have lower valencies than large dendrimers.

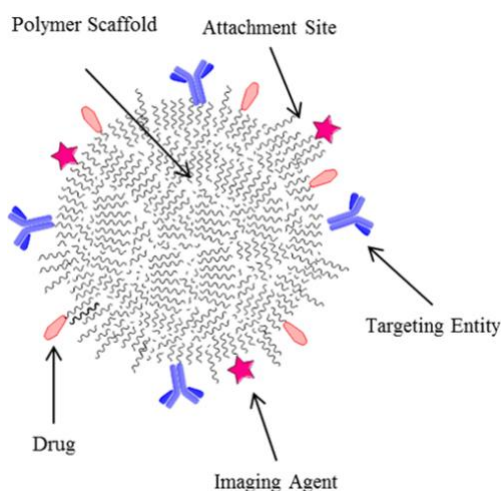


Figure 4. A generalized model of how a polymer, specifically a dendrimer, scaffold can hold diverse functional endgroups. Dendrimers enable the attachment of an imaging agent to visualize in cell studies as well as sites for attachment of targeting, prodrug, solubilizing, and/or immunomodulatory groups.¹⁹

Cellular Uptake Studies of Glycopolymers/Glycodendrimers

Understanding how well a synthetic molecule is taken up by cells is important for evaluating the bioactivity of a synthetic molecule before use in cell-based assays. Knowing whether a synthetic glycopolymer enters the cell or not is essential for understanding the role that the glycodendrimers play in mediating cellular processes. There are studies that demonstrate synthetic linear glycopolymers being used to study cellular uptake pathways, such as with the

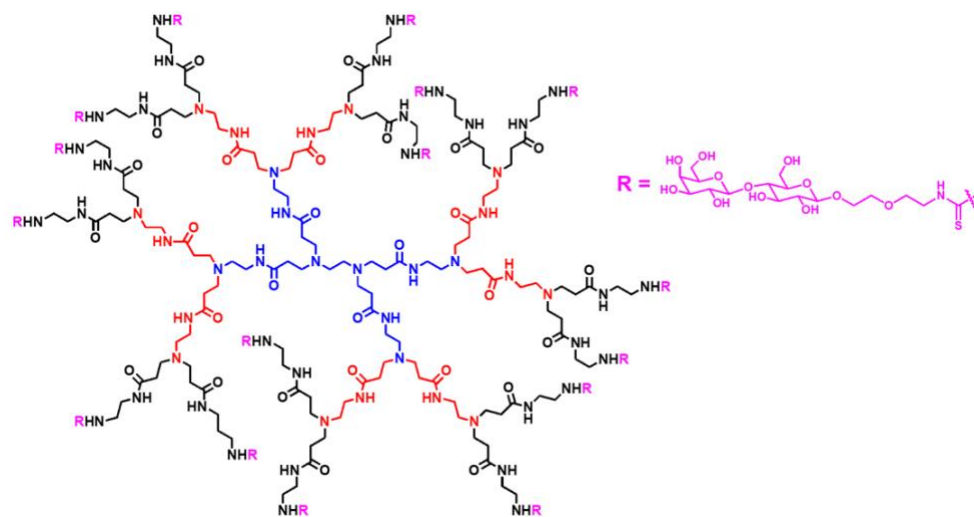
Kiessling group utilizing a mannose-functionalized linear polymers to study the uptake of glycosylated antigens into nanodomain pockets located on dendritic cell surface. In another key example, Bertozzi's group created a synthetic linear glycopolymer to serve as a mucin mimic, which embedded into the cell membrane. The Bertozzi polymers were shown to be taken up through an endocytic pathway into the membrane.^{24, 25} Imaging for studies, such as the Kiessling and Bertozzi research, was performed by attaching a fluorescent probe to the polymer without inhibiting the function of the polymer.

Despite the cytotoxicity of the unfunctionalized PAMAMs that bear amino termini which are protonated at physiological pH, PAMAMs are nontoxic to cells if the surface is functionalized with nontoxic endgroups. Due to the rising interest of using PAMAM dendrimers as a drug delivery scaffold for cancer therapies, there have been multiple cellular uptake studies done to evaluate solubility and specificity with promising results.¹⁰ However, different sized polymers can have different uptake pathways into the cell. Specifically, cellular uptake of a series of generations of lactose-functionalized (and other beta-galactoside functionalized) PAMAM dendrimers has yet to be understood.

Purpose

The purpose of this project is to functionalize (G(2), G(3), G(4), and G(6) PAMAM dendrimers with a lactose endgroup (**figure 5a**) followed by labelling approximately one lactoside per dendrimer with a fluorescent dye, Alexa Fluor 647 (**figure 5b**). These glycodendrimers will be further used for studying the cellular uptake of lactose-functionalized dendrimers via fluorescent imaging.

We hypothesize that the lactose-functionalized glycodendrimers will have important multivalent protein-carbohydrate interactions at the cell surface while also being readily taken up by cancer cells. Four generations of fluorescently-labeled, lactose-functionalized glycodendrimers reported herein will be imaged during cellular uptake assays to determine whether they penetrate the cell or remain exterior. Although the synthesis of lactose-functionalized glycodendrimers has been previously reported,²⁶ some steps with low and variable yields needed to be optimized. The fully optimized synthesis of the lactose derivative required for attachment to the PAMAM dendrimers will be described in chapter 2 of this thesis. The methods used for attaching the fluorescent dye onto the glycodendrimers and for purifying and characterizing the fluorescently labeled, lactose-functionalized glycodendrimers is reported in chapter 3. Conclusions from the synthesis component of the project and a brief description of planned cell studies are presented in chapter 4.



B)

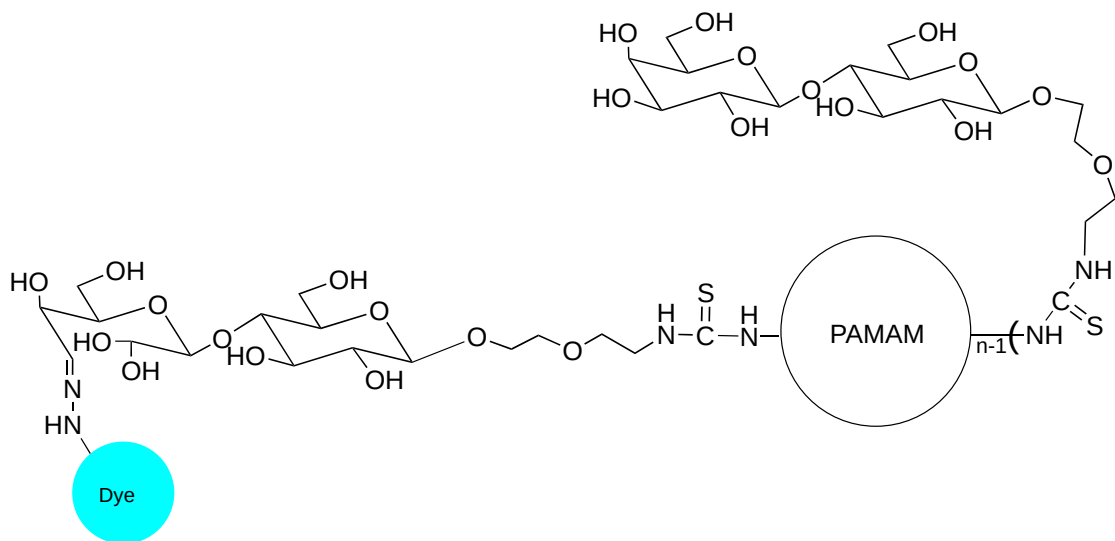


Figure 5. A) Target moiety that was synthesized for the first part of this project. Depicted is a G(2) PAMAM dendrimer with each generation before it highlighted a different color. This G(2) is functionalized with glycosylated lactose (pink R groups). B) A depiction of a functionalized dendrimer with a label present.

CHAPTER TWO

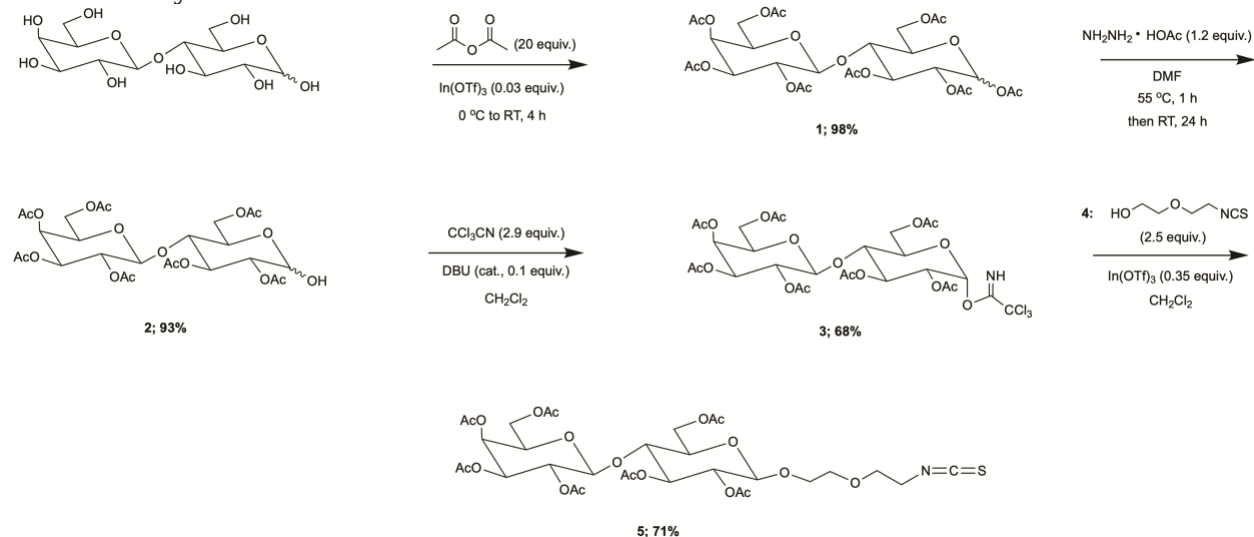
SYNTHETIC APPROACH TO LACTOSIDE FUNCTIONALIZED DENDRIMERS

Introduction

This section provides an analysis of the synthesis of lactose derived small molecules for use in the functionalization of PAMAM dendrimers. All procedures had been previously developed,²⁶ although additional optimization of several steps was required and is reported herein. Notably, optimization of the glycosylation reaction to convert trichloroacetimidate **3** to compound **5**, affording an increased yield of isothiocyanate **5** for use in dendrimer functionalization, is described (**scheme 1**).

Synthesis of Glycosylated Lactose

Scheme 1. Synthetic Route for Lactose Derived Small Molecules



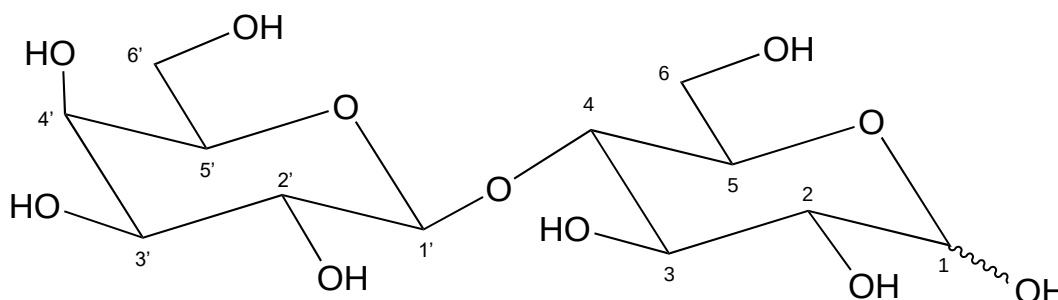
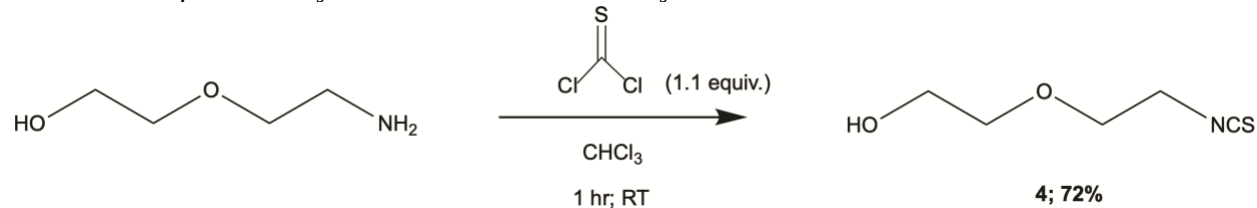


Figure 6. This figure is shown to be used as a guide for when carbon positions of the lactose structure are referred to in the text.

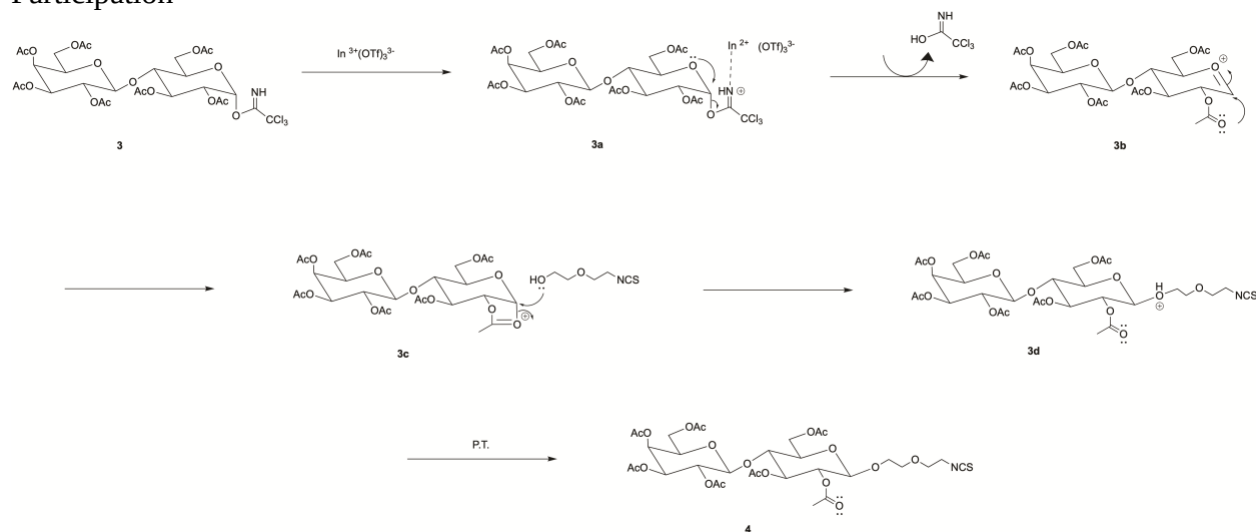
Once the C1 (**figure 6**) anomeric hydroxyl group is unmasked, **2** is then reacted with 2.9 equivalents of trichloroacetonitrile using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a soft base catalyst to afford trichloroacetimidate **3**. This reaction was thermodynamically controlled to afford the trichloroacetimidate group in the alpha position at the anomeric site. Stereoselective addition of 2-(2-isothiocyanatoethoxy) ethanol **4** affords isothiocyanate **5**. Rigorous purification of **3** prior to performing the glycosylation step was essential to avoid formation of byproducts and increase the yield of desired product **5**. Careful maintenance of anhydrous conditions for the glycosylation reaction was also necessary.

Formation of isothiocyanate **4** required additional optimization as well. Initially, the salt of the alcohol was isolated from ethanol when thiophosgene (Cl_2CS) was used without aqueous workup. The procedure was changed to using thiophosgene in chloroform, followed by aqueous workup and purification by silica gel column chromatography (**scheme 2**). This reaction was performed on a large scale (7 grams of aminoethoxyethanol) in good yield (72%).

Scheme 2. Optimized Synthetic Route for Isothiocyanate **4**

The glycosylation step is quite sensitive to water. The reaction was performed in the presence of 4 Å molecular sieves under argon for 24 hours to minimize exposure to moisture. Extra steps were taken to keep indium triflate dry by weighing it in a dried vial purged with argon. Dichloromethane (CH₂Cl₂) was dried over basic alumina, and the solution of **3** in CH₂Cl₂ was stirred with 4 Å molecular sieves for 24 hours prior to the addition of **4** and the catalyst, indium triflate. This results in formation of the target isothiocyanatoethoxyethanol-functionalized heptaacetylated lactoside **5**, with the linker in the beta (equatorial) position, in 71% yield. Beta selectivity was achieved in the glycosylation reaction by neighboring group participation in which the acetyl group at the C2 position blocks nucleophilic approach to the axial position at the anomeric (C1) carbon (**scheme 3**).

Scheme 3. Stereoselective Addition of Isothiocyanate **5** by Use of Neighboring-Group Participation



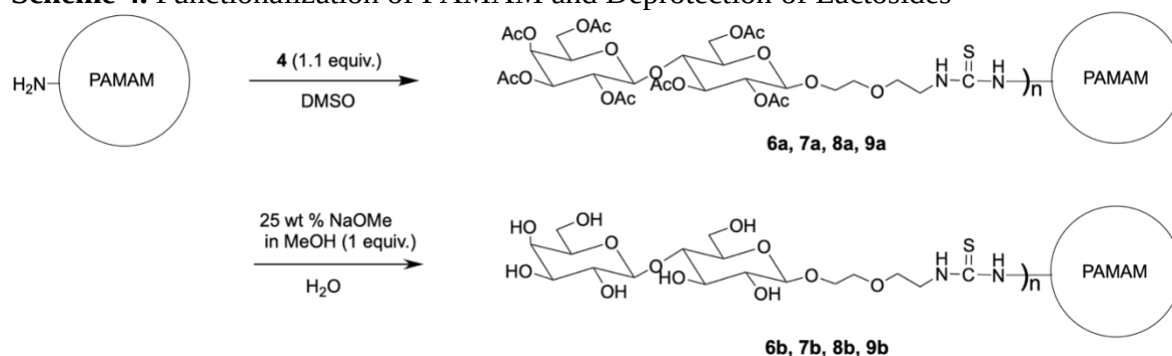
If the reaction is performed under less rigorously dried conditions, a sugar byproduct becomes the predominant product of the reaction. This byproduct was isolated via silica gel column chromatography and was obtained as a white, crystalline solid. Previously, this reaction would have a 40% yield after the purification process, and two chromatography columns were required. The second column was essential prior to PAMAM functionalization because the impurity in **5** either reacted with or was tightly bound by the PAMAM dendrimer and could not be separated from the final product. Decomposition and byproducts were greatly reduced by ensuring all reagents were dry. This resulted in the requirement for only one chromatography purification of **5** prior to dendrimer functionalization. In addition, the yield of **5** from **3** was reproducibly improved from 40% to 70%.

Functionalization of PAMAM Dendrimers with Lactosides

PAMAM Dendrimer G(2), G(3), G(4), and G(6) were all purchased from Dendritech as an aqueous solution.

Vials of each generation were stirred with **5** in DMSO without any promoter for 2 days (**scheme 4**). Initially, dialysis was performed after this step on **6a-9a**. However, we determined that omitting the first dialysis and only performing dialysis on the final glycodendrimers afforded a higher yield of pure materials for the final deprotected glycodendrimers **6b-9b**.

Scheme 4. Functionalization of PAMAM and Deprotection of Lactosides



The average number of lactosides that were added to each generation was determined via ^1H NMR by integrating the thiourea peak at 7.5 ppm and comparing the area of this peak to that of the amide peaks from the PAMAM frameworks as shown for peracetylated lactose-functionalized G(6)-PAMAM **9a** in **figure 7**.

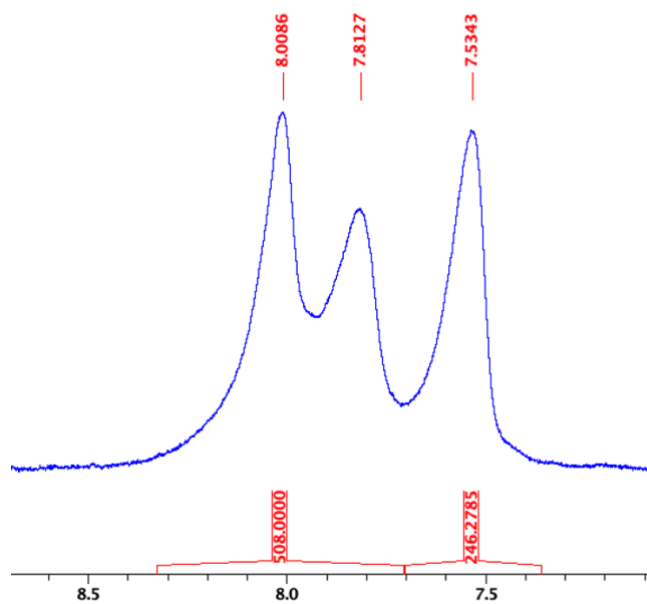


Figure 7. Integration of **9a** comparing the amide signals (8.00 and 7.81 ppm) to the thiourea signal (7.53 ppm). The amide signals were calibrated to be the number of the total amides in the framework. G(6) had 805 total amide proton signals to give the total number of protons (246) from the thiourea linkage. The thiourea protons appear as one signal, but there are two protons per linkage. 246 divided by 2 gives the total number of lactosides present on the dendrimer (123).

Table 1. Results of Functionalization for Each Generation of PAMAM

Generation	% Functionalization	Average Functionalized End Groups	Number of Surface Binding Sites	Average Molecular Weight
2, 6a	94 %	15	16	15,508
3, 7a	78 %	25	32	31,412
4, 8a	75 %	48	64	63,222
6, 9a	48%	123	256	254,075

The degree of functionalization with disaccharide endgroups decreases as the size of the dendrimer increases due to the increased steric hindrance at the amino groups on the larger

generation PAMAMs relative to the smaller generations.²³ ^1H NMR typically gives a reliable value for the number of endgroups **5** that are present on **6a-9a**.

The deprotection of all PAMAM generations occurred under Zemplen conditions (**scheme 4**). The methoxy solution was carefully added in 0.25 equivalent amounts each hour and monitored by a change in pH to avoid amide bond cleavage. The deprotection was complete once the pH was maintained at 11 for an hour. Dendrimers **6b-9b** were then purified by dialysis. Completion of the deprotection was confirmed by ^1H NMR (**figure 8**) indicated by the absence of resonances for the acetyl groups in the 2 ppm region.

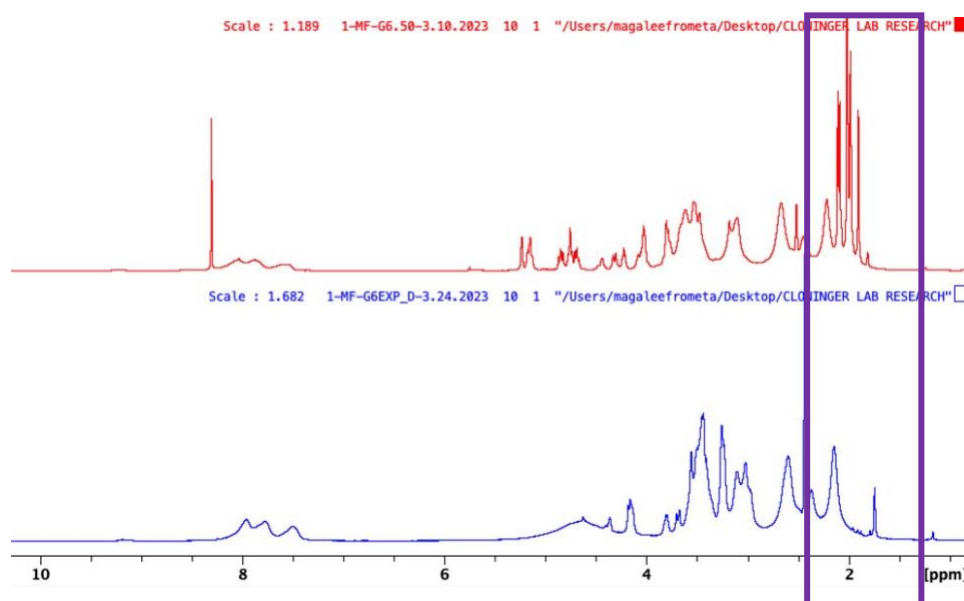


Figure 8. ^1H NMR comparison of protected and deprotected lactose-functionalized PAMAMs. Red spectrum (top) shows the presence of acetyl-protected lactosides in **9a** indicated by the signals at 2 ppm. Blue spectrum (bottom) shows the absence of the acetyl signals at 2 ppm in lactose-functionalized PAMAM **9b**.

The average molecular weights of all dendrimer compounds are described in **table 2**. These weights were determined by finding the average number of lactosides loaded on each dendrimer via ^1H NMR.

Table 2. Molecular Weights of Dendrimers After Deprotection of Lactosides for Compounds **6b-9b**

Compounds	Molecular Weight Unfunctionalized (g/mol)	Average Number of Lactose Groups Added	Molecular Weight with Lactose-Functionalization (g/mol)
6b	3,256	15	10,321
7b	6,909	25	13,974
8b	14,215	48	36,823
9b	58,048	123	170,617

Experimental

General reagents were purchased from Fischer Scientific, Sigma-Aldrich, and Honeywell Chemical Companies. PAMAM dendrimers of generations 2, 3, 4, and 6 were purchased from Dendritech. Published procedures for synthesis of lactose functionalized PAMAM dendrimers²³ were optimized as described below. All ¹³C NMR and ¹H NMR data were obtained on a Bruker Ascend™ 400. The silica gel used for flash chromatography was SiliCycle® SiliaFlash®, 40-63 μm, 60 Å purchased from SiliCycle® Inc. Thin layer chromatography used in purifications were HPTLC Silica Gel 60 GF254 purchased from ChemScene LLC. Dry solvents were obtained from a JC Meyer solvent system. Sodium alumino-silicate molecular sieves with a pore diameter of 4 Å were used and purchased from Sigma-Aldrich. Molecular sieves were activated at 350-400 °C for 4 hours using a Glas-Col heating mantel and stored in an oven at 150 °C.

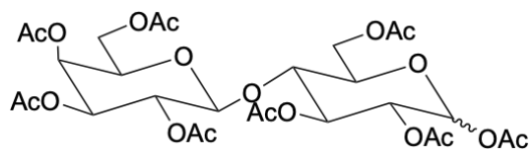


Figure 9. 2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-[1 $\rightarrow$ 4]-1,2,3,6-tetra-O-acetyl- β -D-glucopyranoside **1**

α -D-Lactose (3.03 g, MW: 342.3 g/mol, 8.42 mmol, 1 equiv.) and acetic anhydride (18 mL, MW: 102.0 g/mol, 191.0 mmol, 22.5 equiv.) were added to a round bottomed flask and brought to 0 °C for the addition of the catalyst, In(OTf)₃ (0.152 g, MW: 562.0 g/mol, 0.270 mmol, 0.032 equiv.). The reaction stirred at 0 °C for 35 min, then stirred at room temperature for approximately 5 h. The mixture was neutralized with a 1:1 volume of aqueous sodium carbonate solution with the mixture, giving a cloudy appearance. The product was then separated from the aqueous phase by washing with ethyl acetate twice into a separatory funnel. The organic layer was collected and dried with sodium sulfate. The solution was filtered and dried *in vacuo* to give the final product. The final product was used without further purification. ¹H NMR (400 MHz, CDCl₃) δ 6.26 (d, 1H, **H1**, J = 3.9 Hz), 5.46 (t, 1H, **H3**, J = 10.0, 10.0 Hz), 5.36 (d, 1H, **H4'**, J = 3.4 Hz), 5.01 (dd, 1H, **H2'**, J = 3.7, 10.4 Hz), 4.96 (dd, 1H, **H3'**, J = 3.4, 10.4 Hz), 4.48 (d, 1H, **H2**, J = 8.1 Hz), 4.45 (dd, 1H, **H1'**, J = 12.3, 2.0 Hz), 4.19-4.05 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 4.00 (ddd, 1H, **H5**, J = 10.2, 3.9, 2.0 Hz), 3.88 (t, 1H, **H5'**, J = 6.8, 6.8), 3.81 (t, 1H, **H4**, J = 9.5, 9.5 Hz), 1.96 (s, 3H, OAc: **H₃CC=OO-**), 1.93 (s, 3H, OAc: **H₃CC=OO-**), 1.89 (s, 3H, OAc: **H₃CC=OO-**), 1.84 (overlapping s, 9H, OAc: **H₃CC=OO-**), 1.79 (s, 3H, OAc: **H₃CC=OO-**), 1.73 (s, 3H, OAc: **H₃CC=OO-**). ¹³C NMR (125 MHz, CDCl₃) δ 170.57, 169.97, 169.93, 169.82, 169.64, 169.62, 169.27, 168.80, 168.63 (OAc: **H₃C $\underline{C}$ =OO-**); 100.77 (**C1**); 88.66 (**C1'**); 75.49 (**C4'**); 70.74, 70.55, 70.42, 69.36,

69.24, 68.98 (C2, C3, C5, C2', C3', C5'); 66.67 (C4); 61.47, 60.82 (C6, C6'); 21.77, 20.61, 20.47, 20.40, 20.26, 20.12, 20.1 (OAc: H₃CC=OO-).

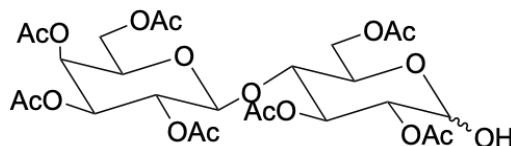


Figure 10. 2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-[1 $\rightarrow$ 4]-2,3,6-tri-O-acetyl- β -D-glucopyranoside **2**

Hydrazine acetate (0.554 g, 6.01 mmol, 1.2 equiv.) was added directly to the flask of dried compound **1** (3.09 g, 4.56 mmol, 1 equiv.) with 15 mL of DMF. The flask was then placed in an oil bath and kept at 55 °C for 1 h, then removed to stir at room temperature overnight resulting in a transparent solution. The solution was transferred to a separatory funnel with DCM. The organic layer was washed with saturated aqueous sodium bicarbonate (NaHCO₃) (x2), DI water (x2), brine (x2), and then dried with sodium sulfate. The solution was filtered and dried via rotary evaporator, with heat not exceeding 70 °C, followed by lyophilization. This resulted in a 93% yield (4.92 g) of **2** and used without further purification. ¹H NMR (400 MHz, CDCl₃) δ 5.53 (t, 1H, **H3**, J = 9.7, 9.7 Hz), 5.36 (overlapping d, 2H, **H1**, **H1'** J = 3.8 and J = 3.1 Hz), 5.15-5.07 (m, 1H, **H2'**), 4.96 (dd, 1H, **H3'**, J = 10.4, 3.3 Hz), 4.84 (dd, 1H, **H2**, J = 3.74, 10.11 Hz), 4.53-4.47 (overlapping d, 2H, **H4**, **H4'**), 4.21-4.03 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 3.88 (t, 1H, **H5'**, J = 6.57, 6.57 Hz), 3.77 (t, 1H, **H5**, J = 9.5, 9.5), 2.16 (s, 3H, OAc: **H3**CC=OO-), 2.13 (s, 3H, OAc: **H3**CC=OO-), 2.08 (s, 3H, OAc: **H3**CC=OO-), 2.07-2.02 (m, 9H, OAc: **H3**CC=OO-), 1.97 (s, 3H, OAc: **H3**CC=OO-). ¹³C NMR (125 MHz, CDCl₃) δ 170.57, 169.97, 169.93, 169.82, 169.64, 169.62, 169.27, 168.80, 168.63 (OAc: H₃CC=OO-); 100.77 (C1); 88.66 (C1'); 75.49 (C4'); 70.74, 70.55, 70.42, 69.36,

69.24, 68.98 (C2, C3, C5, C2', C3', C5'); 66.67 (C4); 61.47, 60.82 (C-COCOCH₃); 21.77, 20.61, 20.47, 20.40, 20.26, 20.12, 20.1 (OAc: H₃CC=OO-).

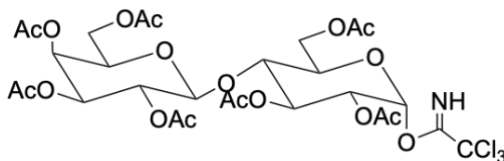


Figure 11. Trichloroacetimido-2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-[1 $\rightarrow$ 4]-2,3,6-tri-O-acetyl- β -D-glucopyranoside **3**

Trichloroacetonitrile (1.63 mL, MW: 144.38 g/mol, 1.44 g/mL, 16.3 mmol, 2.9 equiv.) and DCM as solvent were added directly to **2**. The flask was placed in an ice bath and left to stir. DBU (83.8 μ L, MW: 144.38 g/mol, 1.02 g/mL, 0.56 mmol, 0.1 equiv.) was diluted in 420 μ L of DCM and added dropwise to the stirring solution over the course of 30 min to prevent rapid decomposition. The reaction was left to stir overnight, allowing it to warm to room temperature. DCM was added and the diluted crude reaction mixture was washed twice with saturated aqueous sodium bicarbonate solution. The organic layer was then washed with DI water twice, causing an emulsion. To ensure all product was collected, more DCM was added to separate the layers more during this wash. The organic layer was then washed twice with brine, and dried *in vacuo*. Purification was done with a wet-loaded silica column with a mobile phase of 1:4 ethyl acetate to DCM to afford 68% yield (2.99 g) of **3**. ¹H NMR (400 MHz, CDCl₃) δ 8.66 (s, 1H, =NH), 6.44 (d, 1H, **H1**, *J*= 3.8 Hz), 5.51 (t, 1H, **H3**, *J*= 9.76, 9.76 Hz), 5.31 (d, 1H, **H4'**, *J*= 3.25 Hz), 5.08 (dd, 1H, **H2'**, *J*= 7.94, 10.38 Hz), 5.01 (dd, 1H, **H3'**, *J*= 3.72, 10.23 Hz), 4.92 (dd, 1H, **H1'**, *J*= 3.3, 10.4 Hz), 4.49 (d, 1H, **H4**, *J*= 4.48 Hz), 4.47-4.37 (m, 1H, **H2**), 4.14-4.02 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 3.89-3.83 (m, 2H, **H5**, **H5'**), 2.11 (s, 3H, OAc: H₃CC=OO-), 2.06 (s, 3H, OAc: H₃CC=OO-), 2.03-2.02

(overlapping s, 6H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.00 (s, 3H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.96 (s, 3H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.92 (s, 3H, OAc: $\text{H}_3\text{CC}=\text{OO}-$). ^{13}C NMR (125 MHz, CDCl_3) δ 170.23, 170.14, 170.05, 169.97, 169.28, 168.97 (OAc: $\text{H}_3\text{CC}=\text{OO}-$); 160.85 ($-\text{C}=\text{NH}$); 101.13 ($\text{C1}'$); 92.84 ppm (C1); 90.66 ppm ($-\text{C}\text{Cl}_3$); 75.82 ppm ($\text{C4}'$); 71.07, 70.91, 70.67, 69.92, 69.52, 69.11 ppm ($\text{C2}'$, $\text{C3}'$, $\text{C5}'$, C2 , C3 , C5); 66.61 ppm (C4); 61.49, 60.80 ppm ($\text{C}-\text{CH}_2\text{OCOCH}_3$); 20.80, 20.73, 20.58, 20.43, 20.40 ppm (OAc: $\text{H}_3\text{CC}=\text{OO}-$).

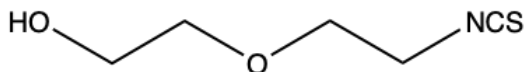


Figure 12. 2-(2-isothiocyanatoethoxy) ethanol **4**

Thiophosgene (5.40 mL, MW: 114.98 g/mol, 70 mmol, 1 equiv.) was added to 400 mL of chloroform while stirring. In a separate beaker, 2-(2-aminoethoxy)-ethanol (7.00 mL, MW: 105.14 g/mol, 70 mmol, 1 equiv.) was diluted with 100 mL of chloroform. The ethanol solution was added dropwise to the thiophosgene mixture over a course of an hour to prevent rapid decomposition resulting in a deep orange color. Immediately after all the ethanol mixture was added, the reaction flask was removed from stirring and dried *in vacuo* to remove solvent. After all the chloroform had been removed, the product residue was dissolved in 100 mL of DI water and washed twice with DCM, then DI water (x2), and dried over magnesium sulfate. The product was dried *in vacuo* and purified through a wet-loaded silica column with a mobile phase of 1:4 ethyl acetate to hexane to afford a 72% yield (7.47 g) of **4**. ^1H NMR (400 MHz, CDCl_3) δ 3.74-3.53 (AA'BB', 8H), 2.13 (m, 1H, -OH). ^{13}C NMR (125 MHz, CDCl_3) δ 133 (**C5**), 72.53 (**C2**), 69.27 (**C3**), 61.81 (**C1**), 45.38 (**C4**).

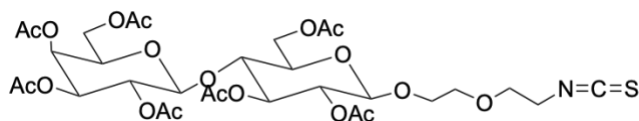
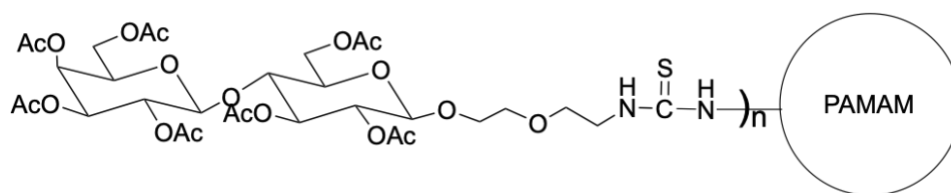


Figure 13. 5-Isothiocyanato-3-oxapentyl 2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-[1 $\rightarrow$ 4]-2,3,6-tri-O-acetyl- β -D-glucopyranoside **5**

A 250 mL round bottomed flask equipped with a stir bar was flame dried, filled with argon, and immediately capped with a rubber septum. Activated 4 Å molecular sieves were added to the argon-purged reaction flask. Once the flask had cooled, approximately 45 mL of dry DCM was added directly to the reaction flask straight from the solvent system to minimize any introduction of water. The capped flask was equipped with an argon balloon and left stirring for an hour to dry the DCM further with sieves. After 1 h, compound **3** (1.55 g, MW: 780.0 g/mol, 1.98 mmol, 1 equiv.) was added to the argon flask and left stirring overnight. Compound **4** (0.73 mL, 147.12 g/mol, 4.96 mmol, 2.5 equiv.) was added via syringe to the reaction flask and left to stir for 30 min. The flask was then placed into an ice bath, continuing to stir, as the catalyst $\text{In}(\text{OTf})_3$ (0.390 g, MW: 562.0 g/mol, 0.694 mmol, 0.35 equiv.) was added. As a note, it is important that even the catalyst is kept dry while weighing. Therefore, a flame dried vial filled with argon was prepared for the weighing of the catalyst. The reaction was left stirring for 24 h, allowing the ice to melt. Sodium bicarbonate was added in a 1:1 ratio with the amount of catalyst used to neutralize and minimize workup effort. The flask was left to stir for approximately 20 min to ensure neutralization of the solution. The neutralized crude reaction mixture was filtered twice to remove the base. The crude product, which will still come through cloudy because this is a highly concentrated solution, was collected in a pre-weighed round bottom flask and dried in vacuo resulting in 1.52 grams of “sticky” golden crystal/oil **5**. Purification was done with a wet-loaded silica column with a mobile phase of 2% MeOH in DCM to afford 72% (1.09 g) of pure **5**. ^1H NMR (400 MHz, CDCl_3) δ 5.34

(d, 1H, **H4'**, $J = 3.31$ Hz), 5.20 (t, 1H, **H3**, $J = 9.38, 9.38$ Hz), 5.07 (dd, 1H, **H2'**, $J = 7.92, 10.37$ Hz), 5.00 (dd, 1H, **H3'**, $J = 3.25, 3.25$ Hz), 4.88 (t, 1H, **H1'**, $J = 8.30, 8.30$ Hz), 4.62-4.53 (overlapping d, 3H, **H1**, **H2**, **H4**, $J = 7.94, 7.94$ Hz), 4.50 (d, 1H, **Ha**, $J = 11.94$ Hz), 4.15-4.05 (m, 3H, **Hb**, **Hc**, **Hd**), 3.98-3.89 (m, 2H, **H5**, **H5'**), 3.77-3.63 (m, 8H, **He**, **Hf**, **Hg**, **Hi**, **Hj**, **Hk**, **Hl**, **Hm**,), 2.15 (s, 3H, OAc: **H₃CC=OO-**), 2.13 (3H, OAc: **H₃CC=OO-**), 2.08-2.02 (overlapping s, 12H, OAc: **H₃CC=OO-**), 1.96 (s, 3H, OAc: **H₃CC=OO-**). ^{13}C NMR (125 MHz, CDCl_3) δ 170.24, 170.16, 169.97, 169.82, 169.59, 169.45, 168.94 (OAc: **H₃CC=OO-**); 132.35 (-**NCS**, not resolved from baseline); 100.74 (**C1'**); 100.40 (**C1**); 76.06 (**C4'**); 72.62 (**Cb**), 72.52 (**Ca**), 71.54 (**Cc**); 70.82, 70.46, 70.19, 69.15, 69.02, 68.99 (**C2'**, **C3'**, **C5'**, **C2**, **C3**, **C5**); 66.65 (**C4**); 61.49, 60.80 (**C6** and **C6'**); 45.20 (**Ca**); 20.77, 20.62, 20.58-20.47, 20.38 (OAc: **H₃CC=OO-**).



n = 15 G (2); **6a**

15 G (3); **7a**

32 G (4); **8a**

125 G (6); **9a**

Figure 14. Peracetylated-Lactoside-Functionalized-PAMAMs (**6a – 9a**)

G (n)-PAMAM (62.0 mg, 1 equiv.) was first dissolved in DMSO (0.774 mL) and stirred in a small vial. Isothiocyanate **5** was dissolved in DMSO (1.2 equiv.) then added to the vials of each generation of PAMAM and left to stir for 2 days. The product was then dried in vacuo and characterized using ^1H NMR. Amounts that were added for each generation are shown in **table 3**.

6a: ^1H NMR (400 MHz, DMSO- d_6) δ 8.01-7.74 (*m*, 28H, **amide protons**), 7.75-7.48 (*bs*, 10.6H, **2 thiourea protons**), 5.23 (*app d*, 4.5H, $J = 2.84$ Hz), 5.20-5.90 (*m*, 10H), 4.85 (*dd*, 5.8H, $J = 8.05, 8.05$ Hz), 4.75 (*t*, 8.5H, $J = 8.73, 8.73$), 4.72-4.64 (*m*, 6.2H), 4.51-4.38 (*m*, 7.4H), 4.31 (*d*, 5.8H, $J = 11.25$), 4.23 (*t*, 5.2H, $J = 6.99, 6.99$ Hz), 4.14-3.96 (*m*, 19.2H), 3.86-3.72 (*m*, 18.6H), 3.72-3.28 (*m*, 105.7H), 3.23-3.01 (*m*, 86.9H), 2.74-2.56 (*m*, 103.6H), 2.44 (*s*, 34.5H), 2.21 (*s*, 74H), 2.11 (*s*, 13.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.09 (*s*, 14.4H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.01 (*s*, 33.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.98 (*overlapping s*, 27.7H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.90 (*s*, 13.3H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.81 (*s*, 5.4H).

7a: ^1H NMR (400 MHz, DMSO- d_6) δ 8.00-7.73 (*m*, 60H, **amide protons**), 7.73-7.47 (*bs*, 25.1H, **2 thiourea protons**), 5.23 (*app s*, 11.9H), 5.20-5.08 (*m*, 24H), 4.85 (*t*, 14.2H, $J = 8.98, 8.98$ Hz), 4.76 (*t*, 22.4H, $J = 8.62, 8.62$ Hz), 4.68 (*app t*, 14.1H, $J = 8.7, 8.7$ Hz), 4.50-4.37 (*m*, 17.3H), 4.31

(*d*, 14.5H, *J*= 11.48), 4.26-4.16 (*bt*, 14.5H, *J*= 6.67, 6.67 Hz), 4.14-3.95 (*m*, 48H), 3.86-3.72 (*m*, 46.1H), 3.72-3.34 (*m*, 243H), 3.24-3.02 (*m*, 194H), 2.66 (*bs*, 234.6H), 2.44 (*bs*, 78.5H), 2.21 (*s*, 38.5H), 2.08 (*s*, 34.5H, OAc: H₃CC=OO-), 2.01 (*s*, 72.9H, OAc: H₃CC=OO-), 1.98 (*overlapping s*, 65.6H, OAc: H₃CC=OO-), 1.90 (*s*, 33.6H, OAc: H₃CC=OO-), 1.81 (*s*, 10H).

8a: ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.17-7.74 (*m*, 124H, **amide protons**), 7.58 (*bs*, 35.4H, **2 thiourea protons**), 5.24 (*app s*, 15.6H), 5.20-5.10 (*m*, 32.6H), 4.85 (*t*, 14.2H, *J*= 9.05, 9.05 Hz), 4.76 (*t*, 29.2H, *J*= 8.50, 8.50 Hz), 4.68 (*app t*, 19.1H, *J*= 8.7, 8.7 Hz), 4.51-4.38 (*m*, 22.8H), 4.32 (*d*, 18.6H, *J*= 11.34), 4.26-4.16 (*bt*, 18.1H, *J*= 6.59, 6.59 Hz), 4.14-3.97 (*m*, 62.3H), 3.86-3.72 (*m*, 58.1H), 3.72-3.36 (*m*, 285.6H), 3.24-3.02 (*m*, 259.1H), 2.66 (*bs*, 289.4H), 2.44 (*bs*, 97.4H), 2.21 (*s*, 212.5H), 2.01 (*s*, 49.2H, OAc: H₃CC=OO-), 2.10 (*s*, 45.0H, OAc: H₃CC=OO-), 2.01 (*s*, 98.9H, OAc: H₃CC=OO-), 1.98 (*overlapping s*, 88.5H, OAc: H₃CC=OO-), 1.90 (*s*, 40.2H, OAc: H₃CC=OO-), 1.81 (*s*, 9.9H).

9a: ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.21-7.71 (*m*, 508H, **amide protons**), 7.56 (*bs*, 193.4, **2 thiourea protons**), 5.24 (*app s*, 95.4H), 5.20-5.10 (*m*, 156.2H), 4.88-4.80 (*m*, 103.4H), 4.79-4.72 (*m*, 134.4H), 4.72-4.65 (*m*, 92.3H), 4.51-4.39 (*m*, 335.4H), 4.35-4.26 (*m*, 115.1H), 4.26-4.19 (*m*, 123.4H), 4.14-3.95 (*m*, 446.8H), 3.85-3.74 (*m*, 304.3H), 3.72-3.63 (*s*, 403.3H), 3.63-3.40 (*m*, 1,564.8H), 3.34-3.01 (*m*, 1,099.8H), 2.66 (*bs*, 895.4H), 2.44 (*bs*, 331.7H), 2.21 (*s*, 768.8H), 2.14-2.06 (*m*, 689.9H, OAc: H₃CC=OO-), 2.01 (*s*, 631H, OAc: H₃CC=OO-), 1.98 (*overlapping s*, 408.8H, OAc: H₃CC=OO-), 1.90 (*s*, 256H, OAc: H₃CC=OO-), 1.81 (*s*, 92.1H).

Table 3. Example of Calculated Amounts of Lactose Derivative to Add for Each Generation

PAMAM Generation	Amount of PAMAM (mg)	Mmoles of PAMAM Endgroups	1.2 Equiv of Lactose (mg)	Mmoles of Lactose
G(2)	62	0.295	270	0.353
G(3)	61	0.278	234	0.306
G(4)	62	0.270	228	0.298
G(6)	63	0.265	223	0.291

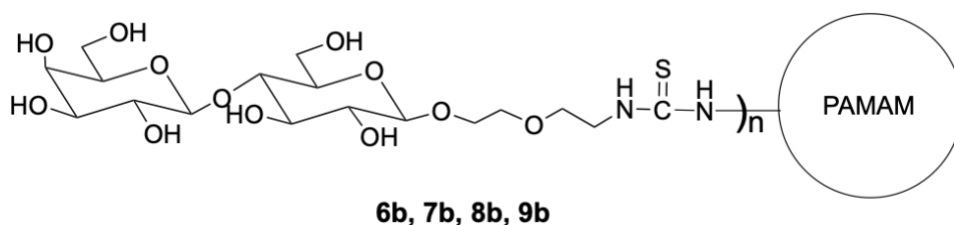


Figure 15. Deprotection of Peracetylated-Lactoside-Functionalized-PAMAMs (**6b-9b**)

Each functionalized dendrimer was dried *in vacuo*. Exactly 1 mL of a 1:1 MeOH and distilled water solution was added to each vial, resulting in a cloudy white solution. An equivalence of 0.2 of 25% wt NaOMe in MeOH was added to each dendrimer every hour until the pH remained at 11 for an hour after addition. This will likely require a total of 1 equivalent of the NaOMe/MeOH solution. Amounts are shown in **table 4**. Once reaction was completed, the dendrimers were dialyzed in water, changing the solution five times in 12 h intervals, then dried *in vacuo*. Characterization was done with ^1H NMR.

6b: ^1H NMR (400 MHz, DMSO- d_6) δ 7.90-7.66 (*bs*, 60H, **amide protons**), 7.65-7.38 (*bs*, 34.3H, **2 thiourea protons**), 4.60-4.07 (*m*, 186.8H), 3.85-3.75 (*m*, 22H), 3.69 (*d*, 29H, $J=10.23\text{Hz}$), 3.63-3.30 (*m*, 319H), 3.29-3.17 (*m*, 99.4H), 3.17-2.84 (*m*, 225H), 2.37 (*s*, 92H), 2.14 (*s*, 1861H), 1.73 (*s*, 8H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 190.18, 188.62, 172.24, 171.76, 104.40, 103.10, 81.14, 76.03, 75.43, 75.30, 73.73, 73.59, 72.59, 71.02, 69.96, 69.19, 68.65, 68.05, 60.87, 60.64, 52.63, 50.01, 45.14, 44.86, 38.79, 37.37, 33.67, 23.11.

7b: ^1H NMR (400 MHz, DMSO- d_6) δ 7.90-7.66 (*bs*, 60H, **amide protons**), 7.65-7.38 (*bs*, 34.3H, **2 thiourea protons**), 4.60-4.07 (*m*, 186.8H), 3.85-3.75 (*m*, 22H), 3.69 (*d*, 29H, $J=10.23\text{Hz}$), 3.63-3.30 (*m*, 319H), 3.29-3.17 (*m*, 99.4H), 3.17-2.84 (*m*, 225H), 2.37 (*s*, 92H), 2.14 (*s*, 1861H), 1.73 (*s*, 8H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 190.18, 188.62, 172.22, 171.76, 169.99, 162.78,

104.27, 103.12, 81.17, 75.98, 75.42, 75.29, 73.70, 73.57, 72.58, 72.58, 71.02, 69.95, 69.18, 68.64, 68.43, 68.03, 60.87, 60.63, 52.61, 50.01, 45.14, 44.85, 38.80, 37.37, 36.25, 33.67, 31.25, 23.10.

8b: ^1H NMR (400 MHz, DMSO- d_6) δ 7.86-7.65 (*bs*, 124H, **amide protons**), 7.65-7.39 (*bs*, 84.6H, **2 thiourea protons**), 4.34-3.91 (*m*, 388.8H), 3.84-3.76 (*m*, 52.2H), 3.69 (*d*, 62.8H, $J=10.28\text{Hz}$), 3.63-3.30 (*m*, 730.7H), 3.30-3.18 (*m*, 225.9H), 3.17-2.87 (*m*, 558.1H), 2.36 (*s*, 230.0H), 2.14 (*s*, 492.9H), 1.73 (*s*, 22.7H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 190.15, 190.09, 188.61, 172.26, 171.80, 170.02, 104.31, 103.11, 81.15, 75.98, 75.42, 75.29, 73.70, 73.57, 72.58, 71.02, 69.95, 69.18, 68.64, 68.43, 68.03, 60.88, 60.64, 52.61, 50.00, 45.14, 44.85, 38.90, 38.68, 37.37, 33.67, 23.11.

9b: ^1H NMR (400 MHz, DMSO- d_6) δ 8.13-7.63 (*bd*, 508H, **amide protons**), 7.62-7.36 (*bs*, 228.1H, **2 thiourea protons**), 5.00-4.26 (*bs*, 688.4H), 4.23-4.07 (*m*, 236.7H), 3.80 (*bs*, 126.9H), 3.67 (*d*, 29H, $J=11.12\text{Hz}$), 3.63-3.30 (*m*, 1,744.7H), 3.30-3.17 (*m*, 575H), 3.17-2.86 (*m*, 1,096.5H), 2.59 (*bs*, 847.0H), 2.37 (*s*, 373H), 2.14 (*s*, 947.5H), 1.74 (*s*, 101.0H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 190.18, 188.62, 172.24, 171.76, 104.40, 103.10, 81.14, 76.03, 75.43, 75.30, 73.73, 73.59, 72.59, 71.02, 69.96, 69.19, 68.65, 68.05, 60.87, 60.64, 52.63, 50.01, 45.14, 44.86, 38.79, 37.37, 33.67, 23.11.

Table 4. Calculated Amounts of NaOMe/MeOH Solution to Add for Each Generation

	Moles PAMAM^a	# of Endgroups^b	Mmol NaOMe 1 Equiv^c	Mmol NaOMe 0.2 Equiv^d	NaOMe/ MeOH (μL)	0.2 Equiv Aliquot (μL)
6a	1.66x10 ⁻⁵	15	1.74	0.348	376	75.3
7a	9.55x10 ⁻⁶	25	1.67	0.334	361	72.3
8a	4.57x10 ⁻⁶	51	1.63	0.326	353	70.6
9a	1.07x10 ⁻⁶	123	0.920	0.184	199	39.7

^a Amount was calculated using the molecular weights after functionalization found in **table 2**.

^b Value is a determined average found with ¹H NMR integrations.

^c Amount was found using a 1:1 equivalence to the number of moles of acetyl groups for each endgroup on the dendrimer (7 acetyl groups for each lactoside).

^d The number of mmol of NaOMe for 1 equiv was divided by the number of aliquots (five) for 0.2 equiv.

CHAPTER THREE

FLOURESCENT LABELING OF FUNCTIONALIZED PAMAM DENDRIMERS

Introduction

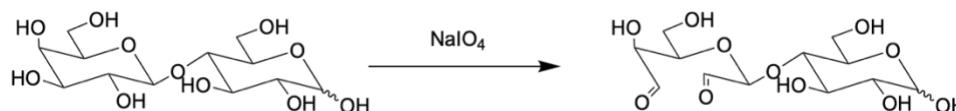
This section of the work is in collaboration with the Haag lab at Freie University in Berlin, Germany. This work is a revision for an unpublished manuscript for Biomolecules, previously prepared by past members of the Cloninger lab. The fluorescently-labeled lactose-functionalized dendrimers that were synthesized, purified, and characterized as described below will be sent to our collaborators, where they will conduct confocal cellular imaging studies. These studies will provide greater insight into uptake pathways of our compounds both in the presence and absence of exogenous galectin-3 in lung cancer cells. Here, the optimization of the synthesis, purification, and characterization of the compounds is described. Cellular imaging and uptake studies will be reported separately.

Fluorescent Labeling of the Functionalized PAMAM Dendrimers

The use of sodium periodate (NaIO_4) in water, a known method of sugar (diol) oxidation, was used to prepare the lactoside species for coupling to the Alexa Fluor 647 hydrazide dye. This oxidation method targets vicinal diols and converts them to aldehydes (**scheme 5**).²⁸ Using 2 equivalence of NaIO_4 to 1 dendrimer molecule, the goal was to oxidize at least 1 lactose group on every dendrimer molecule. To ensure at least every dendrimer is targeted, the amount of NaIO_4 needed for oxidation of one lactose endgroup was doubled. From this step, the oxidized dendrimers were taken through a buffer exchange process using a centrifugal concentrator. The water solvent was replaced with a sodium acetate buffer at a pH of 5.5 for the removal of NaIO_4 and for the

conjugation of the dye to the aldehyde. This procedure was adapted from Min Yen Lee, *et al.* for carbohydrate staining.²⁹ Because the sodium acetate buffer at pH 5.5 worked well, other buffers and pH ranges were not explored for our compounds and were not of interest for the scope of this paper.

Scheme 5. Schematic of NaIO_4 Oxidation of a Vicinal Diol on Lactose (regioselectivity is likely to vary).



The conjugation of the hydrazide on Alexa Fluor 647 with an aldehyde to form a hydrazone (**scheme 6**) works well in a buffer with a low pH. The coupling relies on the release of water during formation of the hydrazone. The release of water from the structure is a slow process and therefore the rate limiting step (**figure 9**). If the reaction occurs at neutral pH in water, this release is extremely slow and not favored, resulting in a very low amount of dye coupled to each dendrimer.³⁰ Therefore, the buffer conditions help drive the process forward to result in a higher recovery of tagged dendrimers. The second aldehyde most likely hydrolyzes to form a geminal diol.

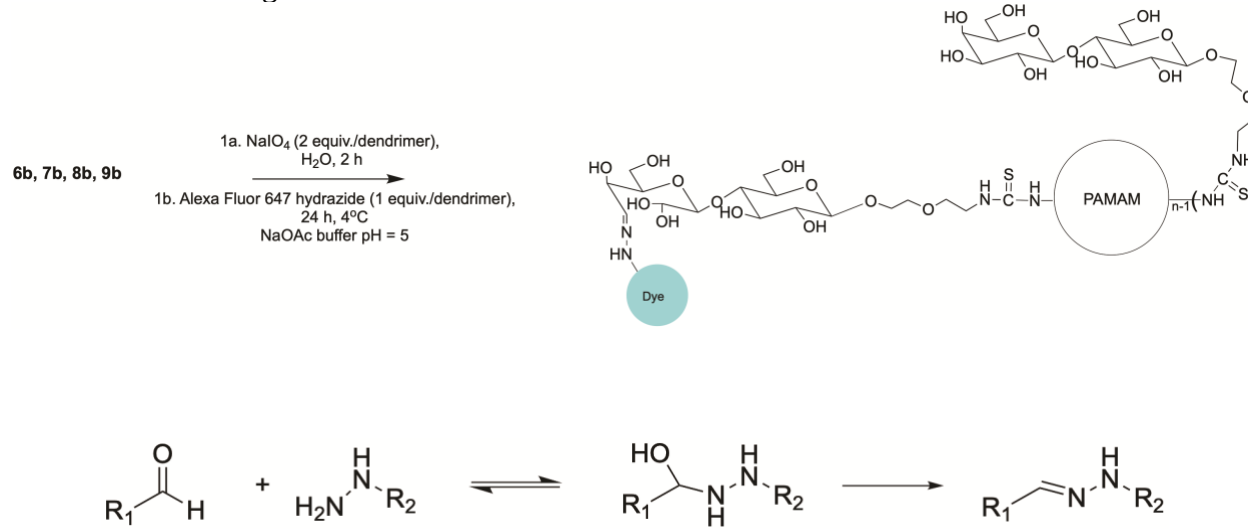
Scheme 6. Labelling of the Lactoside-Functionalized PAMAM Dendrimers

Figure 16. Schematic of hydrazine adding to an aldehyde and protonating the oxygen in equilibrium. The release of the hydroxyl as water has been shown to be the rate limiting step.

Alexa Fluor 647 hydrazide was used to label the lactoside. The nitrogen of the hydrazide functional group will perform a nucleophilic attack on the carbon of one of the aldehydes. The oxygen will then become protonated to a hydroxyl. This step occurs in equilibrium, having the second protonation of the hydroxyl group and leaving as water, be the rate determining step.

The Alexa Fluor 647 hydrazide salt was dissolved in 500 µL (2 mg/mL) of the sodium acetate buffer and then reacted with the dendrimers for 24 h in the dark at 4°C. The solution was purified using a SEC spin column and Millipore water (Vivaspin, **figure 10** (right)) to remove any unbound dye and salt from the buffer. G(2) and G(3) used a column with a MWCO of 7 kDa, while G4 and G6 used a column with a MWCO of 40 kDa. Due to the sensitivity of the dye when exposed to light and moisture, confirmation of purity was done qualitatively by observing if excess dye was retained in the column's resin (**figure 10**). Considering the significant size differences between functionalized dendrimers and dye monomer, it was reasonable to assume that smaller molecules would not flow through the SEC column after one spin.

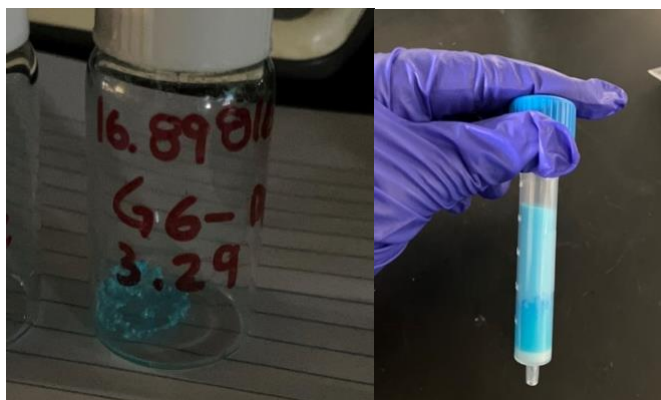


Figure 17. (Left) Dried lactose functionalized G(6) tagged with Alexa Flour 647 recovered after SEC spin column. (Right) The result after running G(3) through the spin column. Shown in the resin is retained excess dye.

After purification, UV-Vis was utilized to measure the absorbance of the dye present in the samples. The diluted concentration of the dye present was determined using the absorbance from UV-Vis in Beer's Law (**eq 1a**). A sample calculation of G(4) is shown below using $270,000 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient³¹ (**eq 1a-1c**). The initial concentration of the dye was then found using the dilution equation (**eq 2a-2c**). From the molarity, the moles of the dye were calculated (**eq 3a-3b**).

$$A_{647} = l \epsilon c \quad (1a)$$

$$0.792 = (1 \text{ cm})(270,000 \text{ M}^{-1} \text{ cm}^{-1})c \quad (1b)$$

$$c = 2.93 \times 10^{-6} \text{ M of dye} \quad (1c)$$

$$M_1 V_1 = M_2 V_2 \quad (2a)$$

$$M_1 (5.0 \times 10^{-4} \text{ L}) = (2.93 \times 10^{-6} \text{ M})(0.0025 \text{ L}) \quad (2b)$$

$$M_1 = 1.47 \times 10^{-5} \text{ M of dye} \quad (2c)$$

$$\text{Moles} = (1.47 \times 10^{-5} \text{ M})(0.00355 \text{ L}) \quad (3a)$$

$$\text{Moles of dye} = 5.20 \times 10^{-8} \text{ moles} \quad (3b)$$

The ratio of dendrimer to dye was determined using the moles found from the initial mass of the glycodendrimer and the moles of the dye found from absorbance. A sample calculation of the ratio of dendrimer to dye is shown with G(4) (**eq 4**) where 9.78×10^{-8} mol (3.6 mg) of lactose-functionalized G(4)-PAMAM are present. The results for all generations can be found in **table 5**.

$$\text{Dendrimer/Dye} = \frac{9.78 \times 10^{-8} \text{ mol G(4)PAMAM}}{5.20 \times 10^{-8} \text{ mol Dye}} = 1.88 \quad (4)$$

Table 5. Dendrimer/Dye Ratio Results for Each PAMAM Generation

PAMAM Generation	Dendrimer/Dye ^a
2 (6c)	1.8
3 (7c)	1.4
4 (8c)	1.9
6 (9c)	1.0

^aValues were calculated from experimentally determined values of one set of dendrimers using Eq 4.

The results from **table 5** suggest approximately half the dendrimers have been functionalized with the dye. Since the molecular weights of the labeled glycodendrimer products and the unlabeled glycodendrimer starting materials are not significantly different, the theoretical masses of the products of the dye-labeling reactions are almost the same as the masses of the starting material. However, because rigorously dried glycodendrimers are difficult to redissolve in water, the products were not fully dried, and the actual masses of products obtained includes some water (having water present will not impact the cell studies). Due to this, the mass of starting material glycodendrimers, rather than the mass of labeled product glycodendrimers plus water, was used to calculate the degree of fluorescence labeling reported in **table 5** since it was concluded that there should not be a mass of glycodendrimers higher than what was started with. It is recognized that not knowing the actual amount of glycodendrimers in the end could lead to an issue of overestimating the amount of glycodendrimers used in determining the ratio between dye. A solution for this that is currently in the works, is to also use absorbance the same way it was used to determine the dye amount. For this, we would need to experimentally determine the extinction coefficient of each generation of glycodendrimers.

Experimental

General Methods

Lactose functionalized PAMAM dendrimers were synthesized as described in chapter 2. Zeba™ Spin Desalting Columns (5 mL, 40 kDa and 7 kDa) and Vivaspin® Turbo 4 and 15 with a 3,000 – 50,000 MWCO PES were used. A benchtop Sorvall ST 8R centrifuge and a Sorvall Lynx 4000 centrifuge were used for centrifugal purification procedures. UV-Vis measurements were taken on a Genesys 10S UV-Vis Spectrophotometer.

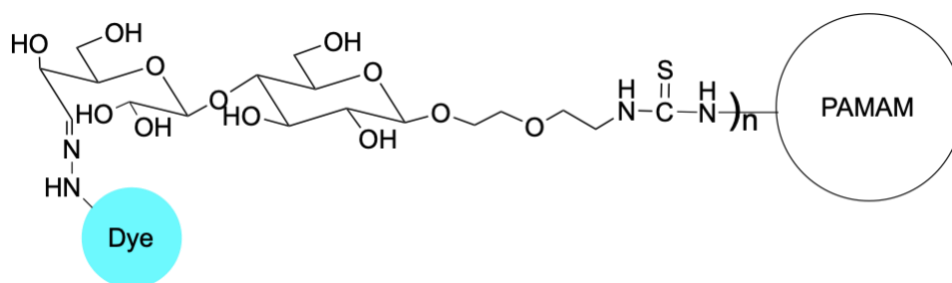


Figure 18. Fluorescent Tagging of Lactoside-functionalized G(2), G(3), G(4), and G(6) Dendrimers (**6c-9c**)

Approximately 3 mg of lactose functionalized dendrimer compounds **6b-9b** were dissolved, in their respective vials, in 1.5 mL of Millipore water for a concentration of 2 mg/mL. An aqueous solution of 2 mg/mL NaIO₄ (2 equiv. / dendrimer; 62 μ L for G(2), 46 μ L for G(3), 17 μ L for G(4), and 4 μ L for G6) was then added to each dendrimer and allowed to stir for 2 h in the dark. NaIO₄ was then washed from the dendrimer solution using a centrifugal concentrator (MWCO of 3 kDa for G(2) and G(3), and 10 kDa for G(4) and G(6)). The water solvent was displaced by adding 2 mL of a sodium acetate buffer (pH of 5.5). G(2) and G(3) were spun 4 times at 4,000 xg for 60 min each spin. G(4) and G(6) was also spun 4 times but at 2,000 xg for 60 min each spin. Once the dendrimer intermediate bearing aldehydes was in sodium acetate buffer, Alexa Fluor 647 dye (1 mg) was dissolved in 500 μ L of the sodium acetate buffer and was added to each reaction vial (1 equiv./dendrimer; 233 μ L for G(2), 128 μ L for G(3), 56 μ L for G(4), and 20 μ L for G6). The vials were then incubated in the dark for 24 h at 4 °C, then purified via SEC spin column (G(2) and G(3) used a spin column with a MWCO of 7 kDa, and G(4) and G(6) used a MWCO of 40 kDa). All generations were spun at 1,000 xg for 4 min once after thorough washing of resin was done in accordance with the manual.³² Characterization was done using UV-Vis with an extinction coefficient of 270,000 M⁻¹ cm⁻¹ at a wavelength of 647 nm. MilliQ water was used as the blank for measurements.

Sodium Acetate Buffer²⁶

The amounts were increased for a larger volume. Sodium acetate (2.398 g) was dissolved in 195 mL of Millipore water. 1 M of HCl was added to the buffer dropwise until a pH of 5.5 was reached.

CHAPTER FOUR

CONCLUSION

The methods described in this thesis provide optimized protocols for the synthesis, purification, and characterization of fluorescently labeled lactose functionalized glycodendrimers for future use in cell-based assays. Establishing these methods is an important step toward formulating an answer to a critical open question: do lactose-functionalized glycodendrimers only function extracellularly with cancer cells, or are they also taken up by the cell? If they are taken up by the cell, does extracellular binding of galectins alter cellular uptake? Finally, if the glycodendrimers are taken up by the cell, what is their fate? What are their intracellular binding partners, and where are they localized?

The advancement of this work seeks to understand the effects our glycodendrimers have in cell assays with greater confidence. Using confocal microscopy, the labelled dendrimers can be imaged to determine whether the dendrimers can be taken into the cell through normal endocytosis or non-traditionally. These studies can then be further used to better understand protein-carbohydrate interactions and multivalency in biological systems. Specifically, while looking at galectin-3 and its unique involvement in cellular processes.

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APPENDIX

NMR SPECTRA

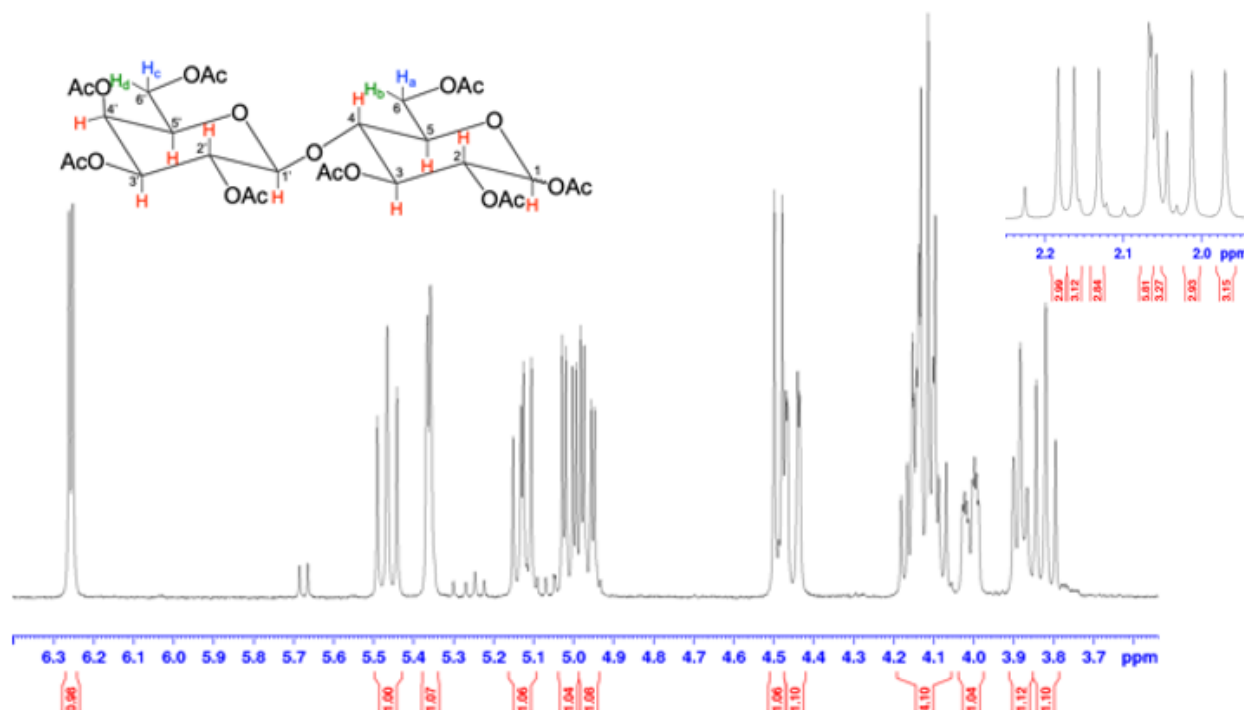


Figure S1. ^1H NMR of **1**. This reaction is not stereoselective at the anomeric position. Signals reported are of the major conformation. There is presence of ethyl acetate. ^1H NMR (400 MHz, CDCl_3) δ 6.26 (d, 1H, **H1**, J = 3.9 Hz), 5.46 (t, 1H, **H3**, J = 10.0, 10.0 Hz), 5.36 (d, 1H, **H4'**, J = 3.4 Hz), 5.01 (dd, 1H, **H2'**, J = 3.7, 10.4 Hz), 4.96 (dd, 1H, **H3'**, J = 3.4, 10.4 Hz), 4.48 (d, 1H, **H2**, J = 8.1 Hz), 4.45 (dd, 1H, **H1'**, J = 12.3, 2.0 Hz), 4.19-4.05 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 4.00 (ddd, 1H, **H5**, J = 10.2, 3.9, 2.0 Hz), 3.88 (t, 1H, **H5'**, J = 6.8, 6.8), 3.81 (t, 1H, **H4**, J = 9.5, 9.5 Hz), 1.96 (s, 3H, OAc: **H₃CC=OO-**), 1.93 (s, 3H, OAc: **H₃CC=OO-**), 1.89 (s, 3H, OAc: **H₃CC=OO-**), 1.84 (overlapping s, 9H, OAc: **H₃CC=OO-**), 1.79 (s, 3H, OAc: **H₃CC=OO-**), 1.73 (s, 3H, OAc: **H₃CC=OO-**).

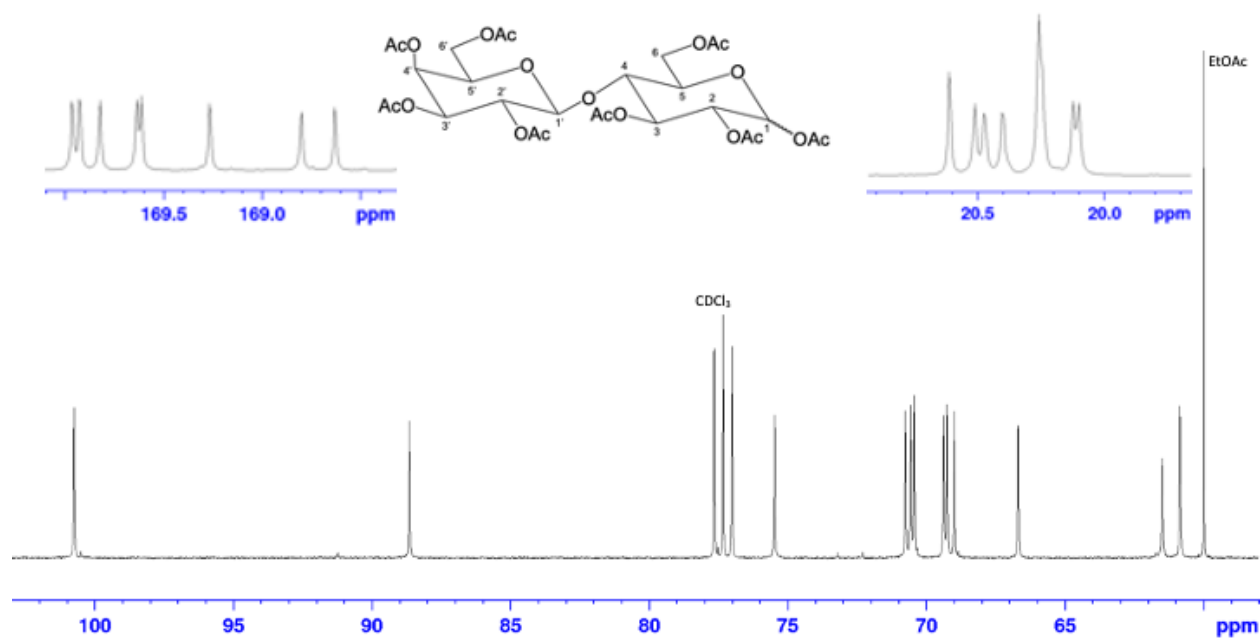


Figure S2. ^{13}C NMR of **1**. ^{13}C NMR (125 MHz, CDCl_3) δ 170.57, 169.97, 169.93, 169.82, 169.64, 169.62, 169.27, 168.80, 168.63 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$); 100.77 (**C1**); 88.66 (**C1'**); 75.49 (**C4'**); 70.74, 70.55, 70.42, 69.36, 69.24, 68.98 (**C2**, **C3**, **C5**, **C2'**, **C3'**, **C5'**); 66.67 (**C4**); 61.47, 60.82 ($\text{C}-\text{COCCH}_3$); 21.77, 20.61, 20.47, 20.40, 20.26, 20.12, 20.1 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$).

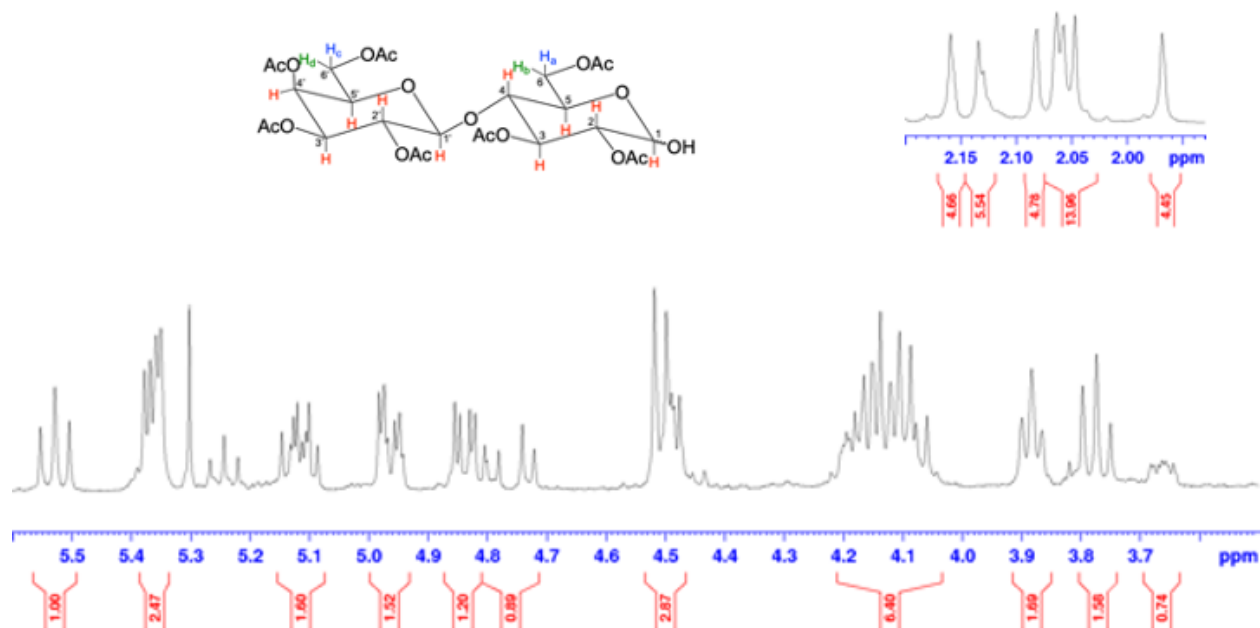


Figure S3. ^1H NMR of **2**. Note that the proton NMR will contain both rotamers of the lactose and signals will overlap. Reported signals are of the major configuration. ^1H NMR (400 MHz, CDCl_3) δ 5.53 (t, 1H, **H3**, J = 9.7, 9.7 Hz), 5.36 (overlapping d, 2H, **H1**, **H1'** J = 3.8 and J = 3.1 Hz), 5.15-5.07 (m, 1H, **H2'**), 4.96 (dd, 1H, **H3'**, J = 10.4, 3.3 Hz), 4.84 (dd, 1H, **H2**, J = 3.74, 10.11 Hz), 4.53-4.47 (overlapping d, 2H, **H4**, **H4'**), 4.21-4.03 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 3.88 (t, 1H, **H5'**, J = 6.57, 6.57 Hz), 3.77 (t, 1H, **H5**, J = 9.5, 9.5), 2.16 (s, 3H, OAc: **H₃**CC=OO-), 2.13 (s, 3H, OAc: **H₃**CC=OO-), 2.08 (s, 3H, OAc: **H₃**CC=OO-), 2.07-2.02 (m, 9H, OAc: **H₃**CC=OO-), 1.97 (s, 3H, OAc: **H₃**CC=OO-).

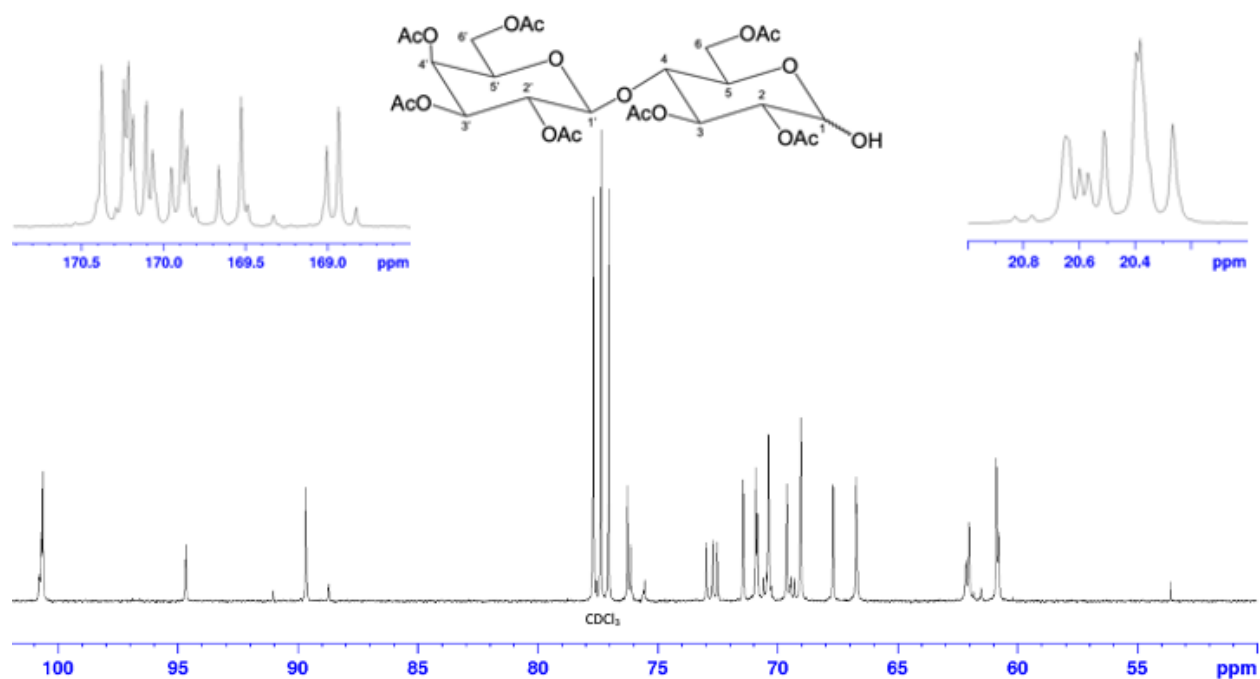


Figure S4. ^{13}C NMR of **2**. ^{13}C NMR (125 MHz, CDCl_3) δ 170.38-168.93 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$); 162.77, 100.69 (**C1**); 89.64 (**C1'**); 76.19 (**C4'**); 71.41, 70.86, 70.37, 69.61, 69.02, 67.66 (**C2**, **C3**, **C5**, **C2'**, **C3'**, **C5'**); 66.68 (**C4**); 61.47, 60.82 ($\text{C}-\text{C}(\text{O})\text{COCH}_3$); 21.77, 20.61, 20.47, 20.40, 20.26, 20.12, 20.1 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$).

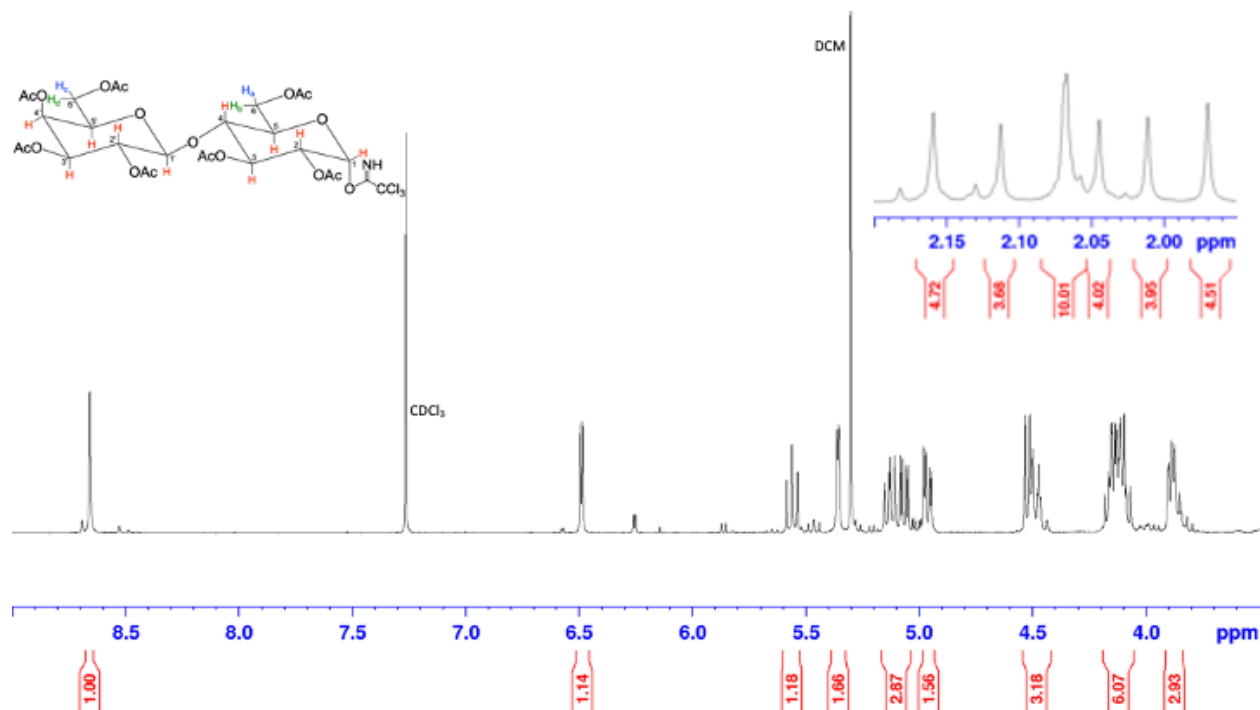


Figure S5. ^1H NMR of **3**. ^1H NMR (400 MHz, CDCl_3) δ 8.66 (s, 1H, =NH), 6.44 (d, 1H, **H1**, J = 3.8 Hz), 5.51 (t, 1H, **H3**, J = 9.76, 9.76 Hz), 5.31 (d, 1H, **H4'**, J = 3.25 Hz), 5.08 (dd, 1H, **H2'**, J = 7.94, 10.38 Hz), 5.01 (dd, 1H, **H3'**, J = 3.72, 10.23 Hz), 4.92 (dd, 1H, **H1'**, J = 3.3, 10.4 Hz), 4.49 (d, 1H, **H4**, J = 4.48 Hz), 4.47-4.37 (m, 1H, **H2**), 4.14-4.02 (m, 4H, **Ha**, **Hb**, **Hc**, **Hd**), 3.89-3.83 (m, 2H, **H5**, **H5'**), 2.11 (s, 3H, OAc: **H3**CC=OO-), 2.06 (s, 3H, OAc: **H3**CC=OO-), 2.03-2.02 (overlapping s, 6H, OAc: **H3**CC=OO-), 2.00 (s, 3H, OAc: **H3**CC=OO-), 1.96 (s, 3H, OAc: **H3**CC=OO-), 1.92 (s, 3H, OAc: **H3**CC=OO-).

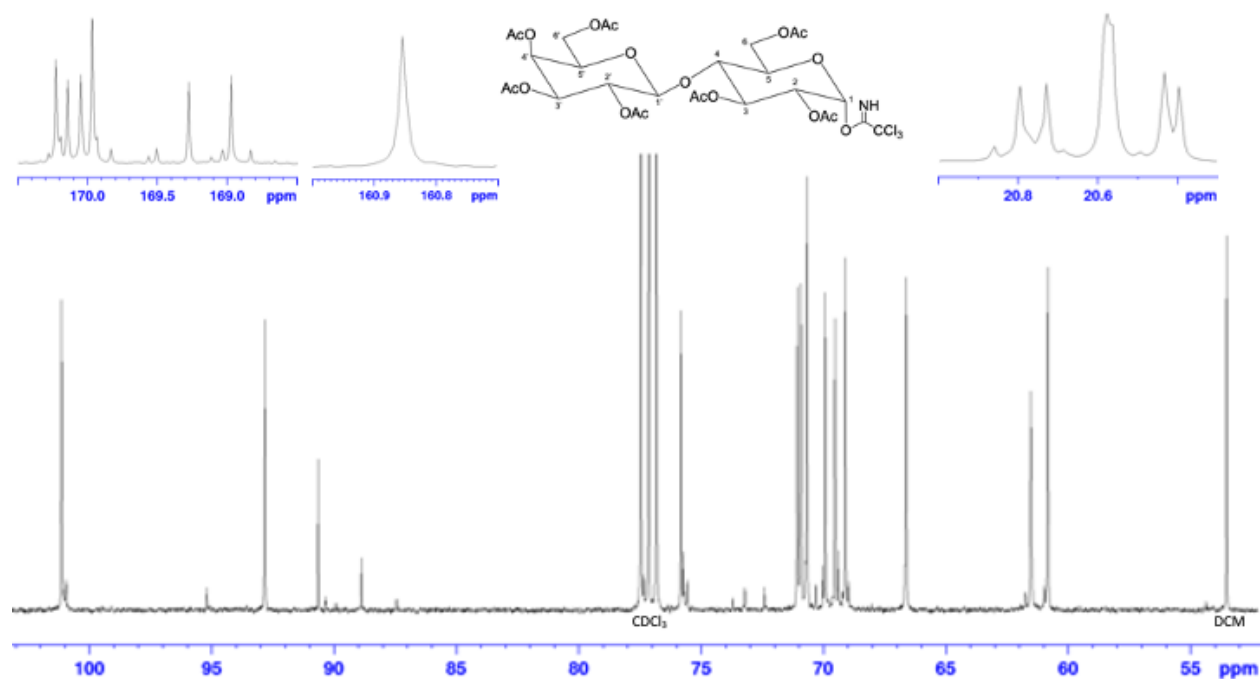


Figure S6. ^{13}C NMR of **3**. ^{13}C NMR (125 MHz, CDCl_3) δ 170.23, 170.14, 170.05, 169.97, 169.28, 168.97 (OAc: $\text{H}_3\text{C}-\text{C}(=\text{O})-\text{O}-$); 160.85 ($-\text{C}=\text{NH}-$); 101.13 (**C1'**); 92.84 (**C1**); 90.66 ($-\text{C}-\text{Cl}_3$); 75.82 (**C4'**); 71.07, 70.91, 70.67, 69.92, 69.52, 69.11 (**C2'**, **C3'**, **C5'**, **C2**, **C3**, **C5**); 66.61 (**C4**); 61.49, 60.80 ($\text{C}-\text{CH}_2\text{OCOCH}_3$); 20.80, 20.73, 20.58, 20.43, 20.40 (OAc: $\text{H}_3\text{C}-\text{C}(=\text{O})-\text{O}-$).

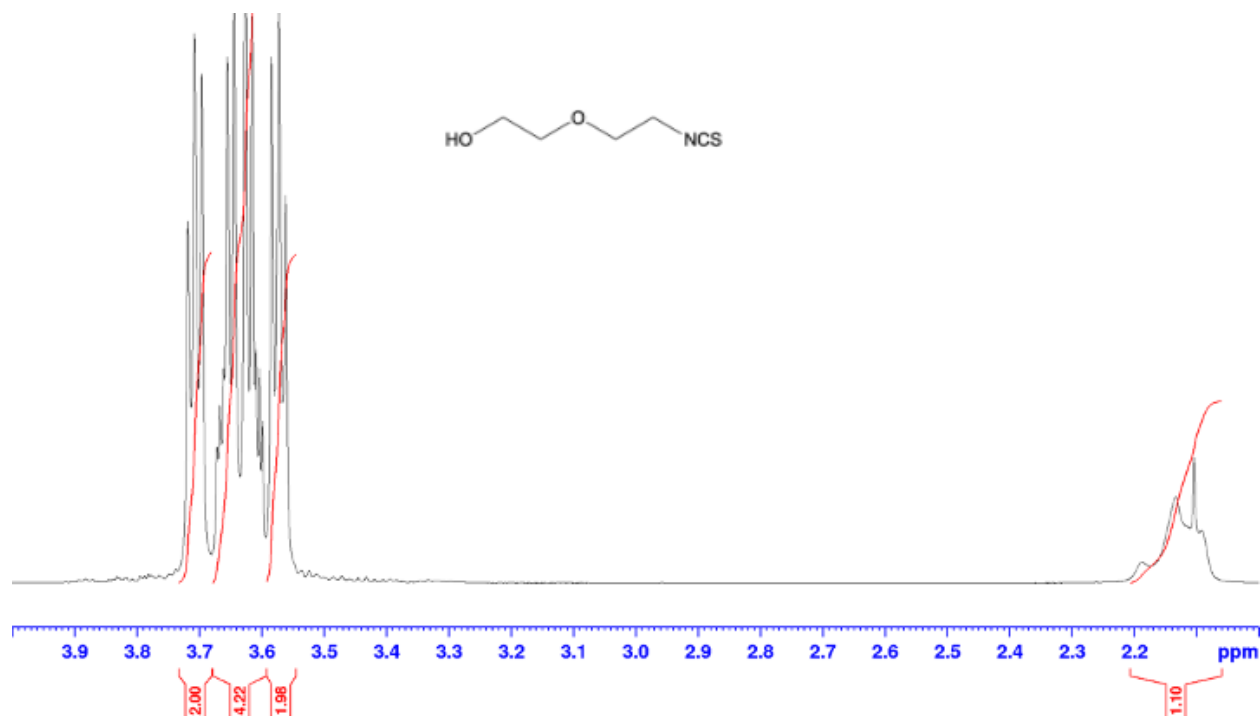


Figure S7. ¹H NMR of 4. ¹H NMR (400 MHz, CDCl₃) δ 3.74-3.53 (AA'BB', 8H), 2.13 (m, 1H, -OH).

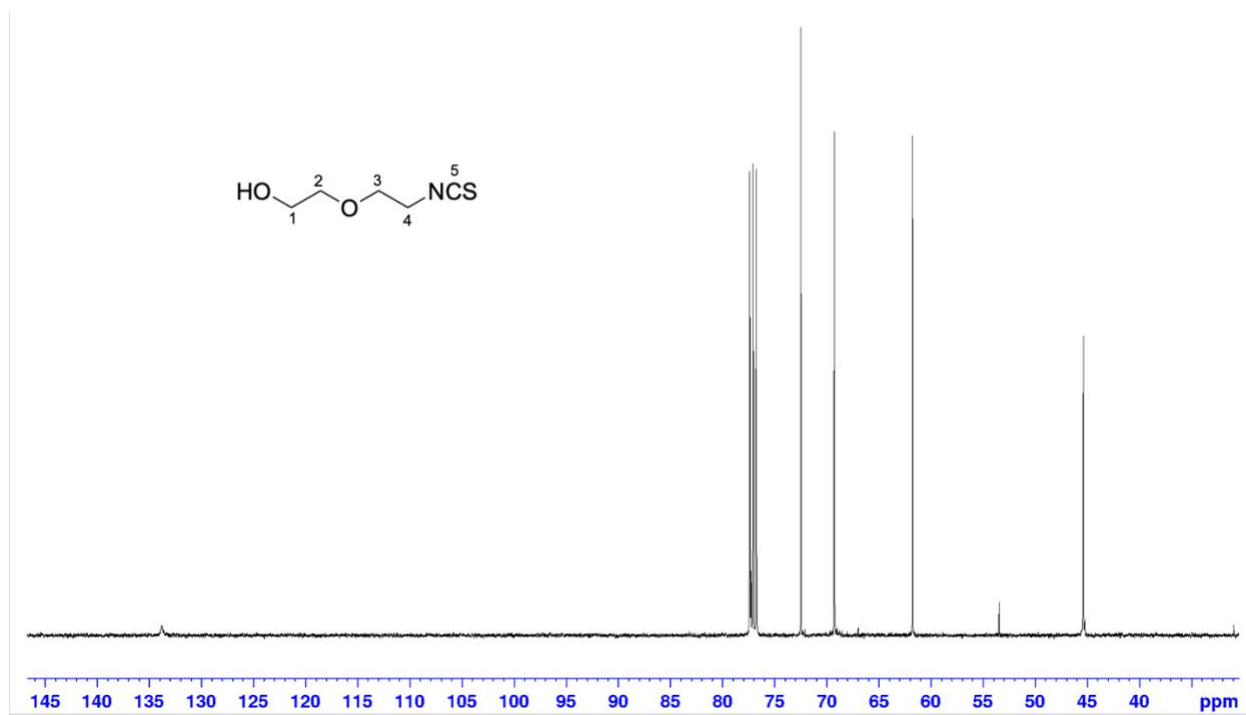


Figure S8. ¹³C NMR of 4. ¹³C NMR (125 MHz, CDCl₃) δ 133 (C5), 72.53 (C2), 69.27 (C3), 61.81 (C1), 45.38(C4).

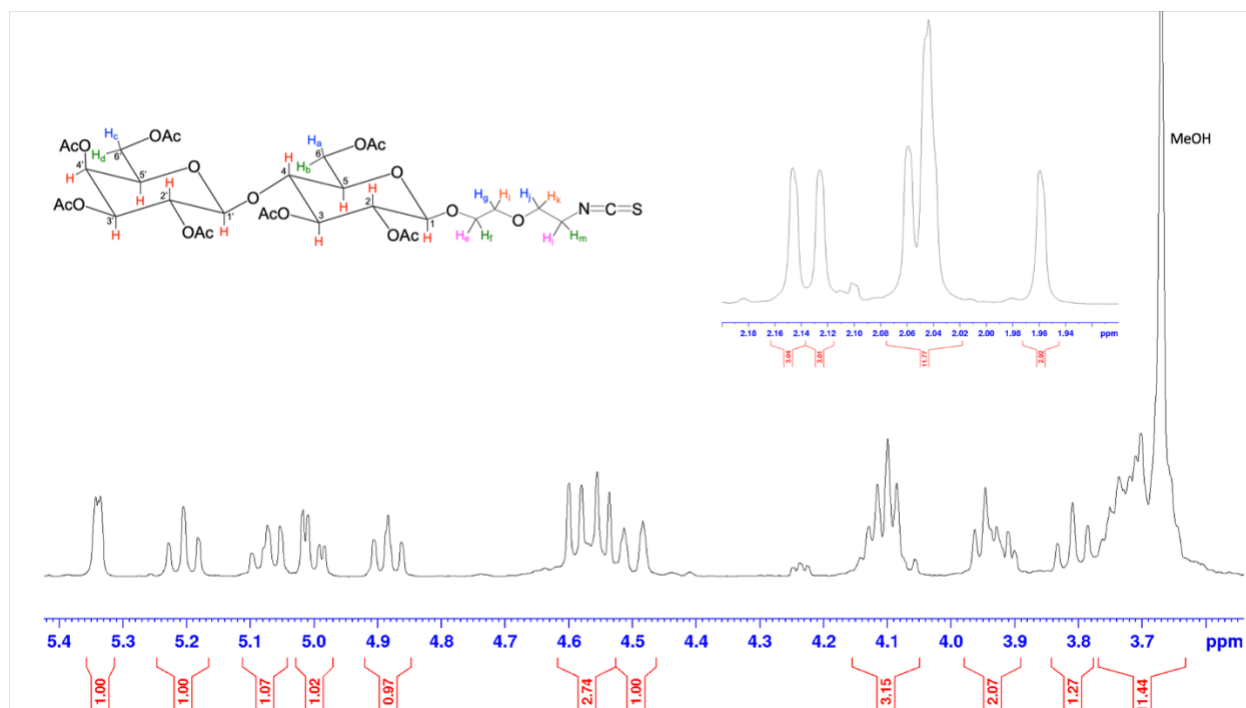


Figure S9. ^1H NMR of **5**. ^1H NMR (400 MHz, CDCl_3) δ 5.34 (d, 1H, **H4'**, J = 3.31 Hz), 5.20 (t, 1H, **H3**, J = 9.38, 9.38 Hz), 5.07 (dd, 1H, **H2'**, J = 7.92, 10.37 Hz), 5.00 (dd, 1H, **H3'**, J = 3.25, 3.25 Hz), 4.88 (t, 1H, **H1'**, J = 8.30, 8.30 Hz), 4.62-4.53 (overlapping d, 3H, **H1**, **H2**, **H4**, J = 7.94, 7.94 Hz), 4.50 (d, 1H, **Ha**, J = 11.94 Hz), 4.15-4.05 (m, 3H, **Hb**, **Hc**, **Hd**), 3.98-3.89 (m, 2H, **H5**, **H5'**), 3.77-3.63 (m, 8H, **He**, **Hf**, **Hg**, **Hi**, **Hj**, **Hk**, **Hl**, **Hm**,), 2.15 (s, 3H, OAc: **H3**CC=OO-), 2.13 (3H, OAc: **H3**CC=OO-), 2.08-2.02 (overlapping s, 12H, OAc: **H3**CC=OO-), 1.96 (s, 3H, OAc: **H3**CC=OO-).

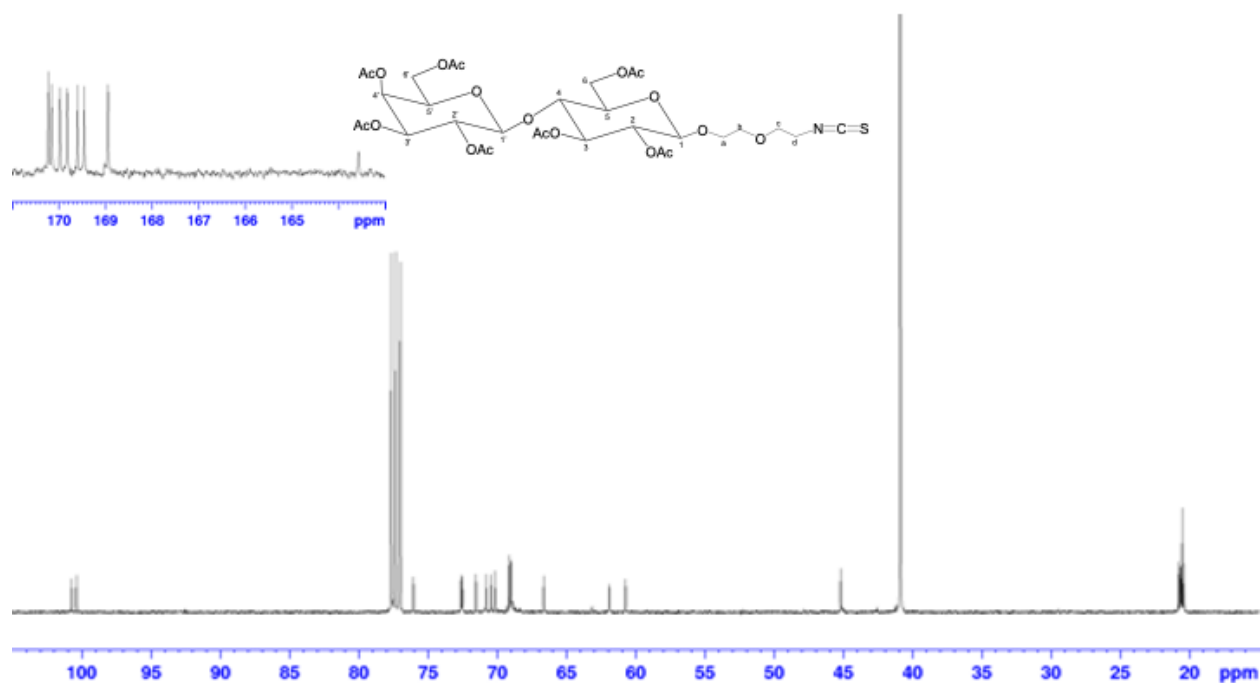


Figure S10. ^{13}C NMR of **5**. ^{13}C NMR (125 MHz, CDCl_3) δ 170.24, 170.16, 169.97, 169.82, 169.59, 169.45, 168.94 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$); 132.35 ($-\text{N}=\text{C}=\text{S}$, not resolved from baseline); 100.74 (**C1'**); 100.40 (**C1**); 76.06 (**C4'**); 72.62 (**C_b**), 72.52 (**C_a**), 71.54 (**C_c**); 70.82, 70.46, 70.19, 69.15, 69.02, 68.99 (**C2'**, **C3'**, **C5'**, **C2**, **C3**, **C5**); 66.65 (**C4**); 61.49, 60.80 (**C6** and **C6'**); 45.20 (**C_d**); 20.77, 20.62, 20.58-20.47, 20.38 (OAc: $\text{H}_3\text{C}\text{C}(=\text{O})\text{O}-$).

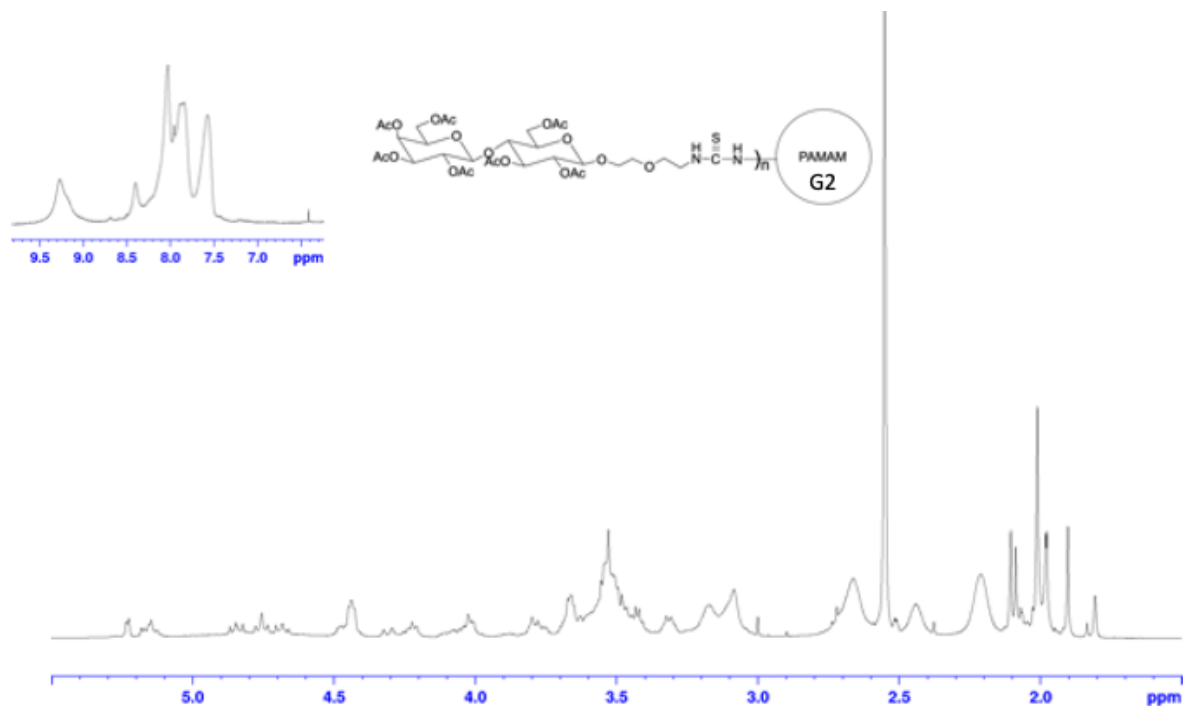


Figure S11. ^1H NMR of **6a**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be in the broad peak from 2.68-2.46 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO- d_6) δ 8.01-7.74 (m, 28H, **amide protons**), 7.75-7.48 (bs, 10.6H, **2 thiourea protons**), 5.23 (app d, 4.5H, $J = 2.84$ Hz), 5.20-5.90 (m, 10H), 4.85 (dd, 5.8H, $J = 8.05, 8.05$ Hz), 4.75 (t, 8.5H, $J = 8.73, 8.73$), 4.72-4.64 (m, 6.2H), 4.51-4.38 (m, 7.4H), 4.31 (d, 5.8H, $J = 11.25$), 4.23 (t, 5.2H, $J = 6.99, 6.99$ Hz), 4.14-3.96 (m, 19.2H), 3.86-3.72 (m, 18.6H), 3.72-3.28 (m, 105.7H), 3.23-3.01 (m, 86.9H), 2.74-2.56 (m, 103.6H), 2.44 (s, 34.5H), 2.21 (s, 74H), 2.11 (s, 13.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.09 (s, 14.4H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.01 (s, 33.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.98 (overlapping s, 27.7H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.90 (s, 13.3H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.81 (s, 5.4H).

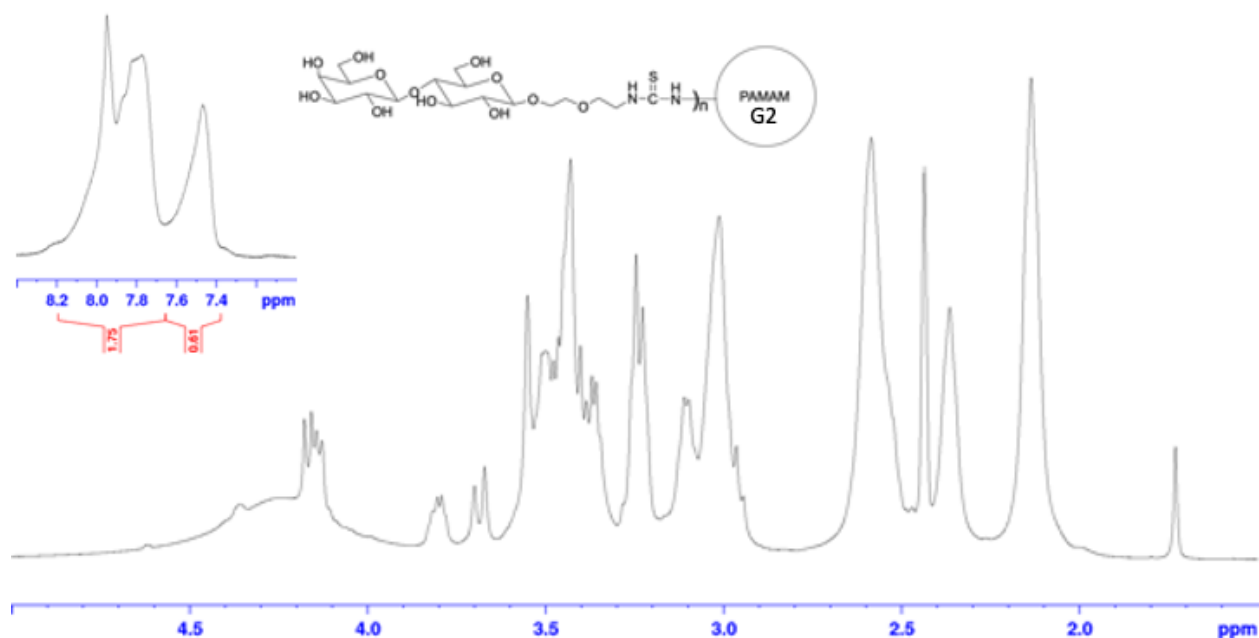


Figure S12. ^1H NMR of **6b**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be the broad peak from 2.68-2.46 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO-d_6) δ 7.90-7.66 (bs, 28H, **amide protons**), 7.65-7.38 (bs, 16.6H, **2 thiourea protons**), 4.59-3.90 (m, 86H), 3.85-3.75 (m, 10H), 3.69 (d, 11H, $J=12.63\text{Hz}$), 3.63-3.30 (m, 146H), 3.29-3.19 (m, 41.5H), 3.15-2.92 (m, 98H), 2.37 (s, 39H), 2.14 (s, 81H), 1.73 (s, 4.6H).

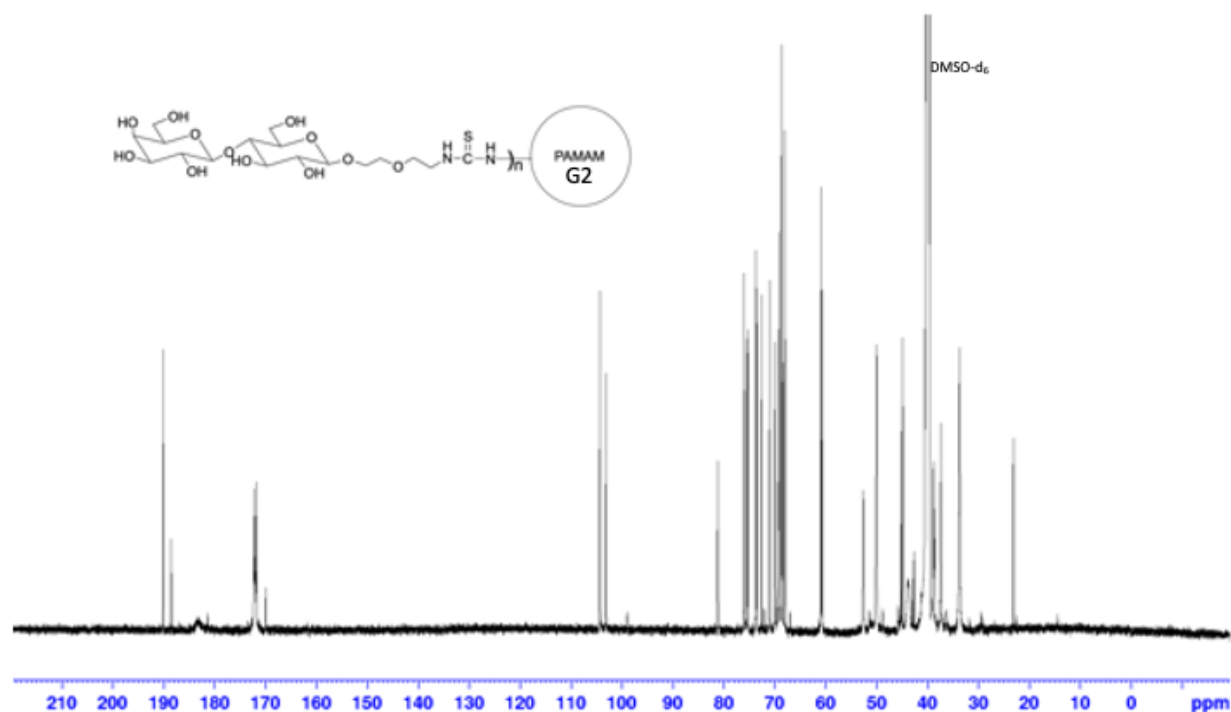


Figure S13. ^{13}C NMR of **6b**. Some peaks may not have been resolved from the baseline or were too broad. ^{13}C NMR (151 MHz, DMSO-d_6) δ 190.18, 188.62, 172.24, 171.76, 104.40, 103.10, 81.14, 76.03, 75.43, 75.30, 73.73, 73.59, 72.59, 71.02, 69.96, 69.19, 68.65, 68.05, 60.87, 60.64, 52.63, 50.01, 45.14, 44.86, 38.79, 37.37, 33.67, 23.11.

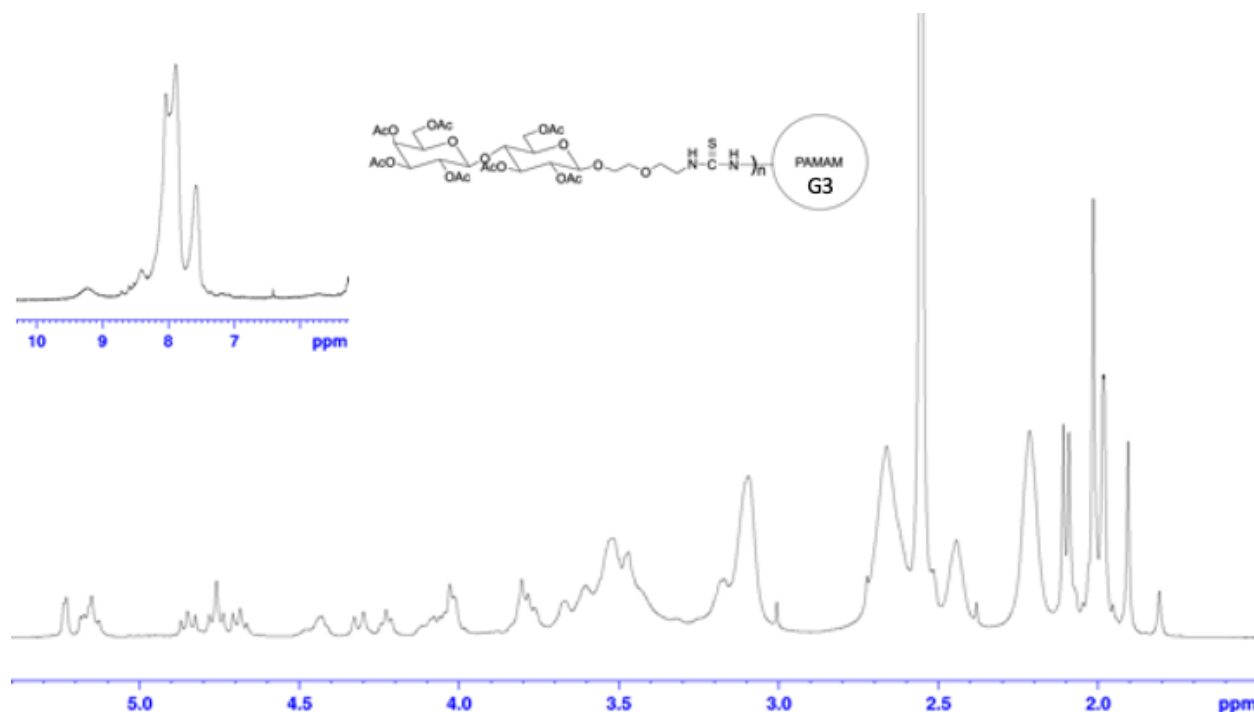


Figure S14. ^1H NMR of **7a**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be in the broad peak from 2.68-2.46 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO- d_6) δ 8.00-7.73 (*m*, 60H, **amide protons**), 7.73-7.47 (*bs*, 25.1H, **2 thiourea protons**), 5.23 (*app s*, 11.9H), 5.20-5.08 (*m*, 24H), 4.85 (*t*, 14.2H, $J = 8.98, 8.98$ Hz), 4.76 (*t*, 22.4H, $J = 8.62, 8.62$ Hz), 4.68 (*app t*, 14.1H, $J = 8.7, 8.7$ Hz), 4.50-4.37 (*m*, 17.3H), 4.31 (*d*, 14.5H, $J = 11.48$), 4.26-4.16 (*bt*, 14.5H, $J = 6.67, 6.67$ Hz), 4.14-3.95 (*m*, 48H), 3.86-3.72 (*m*, 46.1H), 3.72-3.34 (*m*, 243H), 3.24-3.02 (*m*, 194H), 2.66 (*bs*, 234.6H), 2.44 (*bs*, 78.5H), 2.21 (*s*, 38.5H), 2.08 (*s*, 34.5H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.01 (*s*, 72.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.98 (*overlapping s*, 65.6H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.90 (*s*, 33.6H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.81 (*s*, 10H).

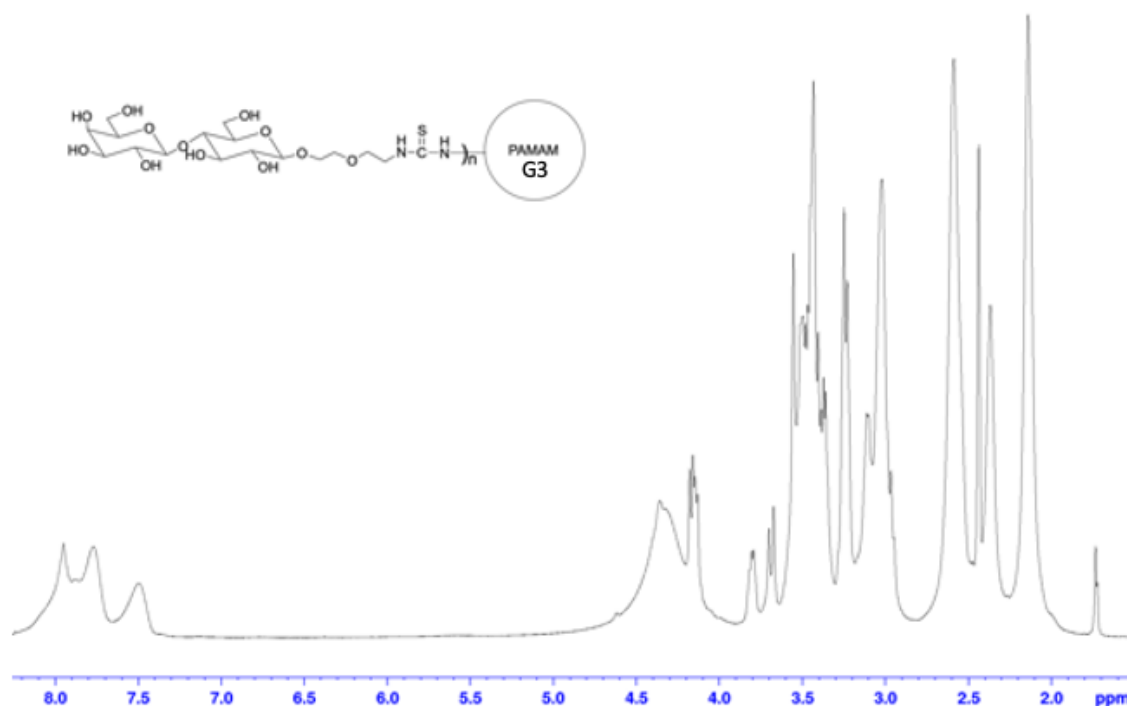


Figure S15. ^1H NMR of **7b**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be the broad peak from 2.68-2.50 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO- d_6) δ 7.90-7.66 (bs, 60H, **amide protons**), 7.65-7.38 (bs, 34.3H, **2 thiourea protons**), 4.60-4.07 (m, 186.8H), 3.85-3.75 (m, 22H), 3.69 (d, 29H, $J=10.23\text{Hz}$), 3.63-3.30 (m, 319H), 3.29-3.17 (m, 99.4H), 3.17-2.84 (m, 225H), 2.37 (s, 92H), 2.14 (s, 1861H), 1.73 (s, 8H).

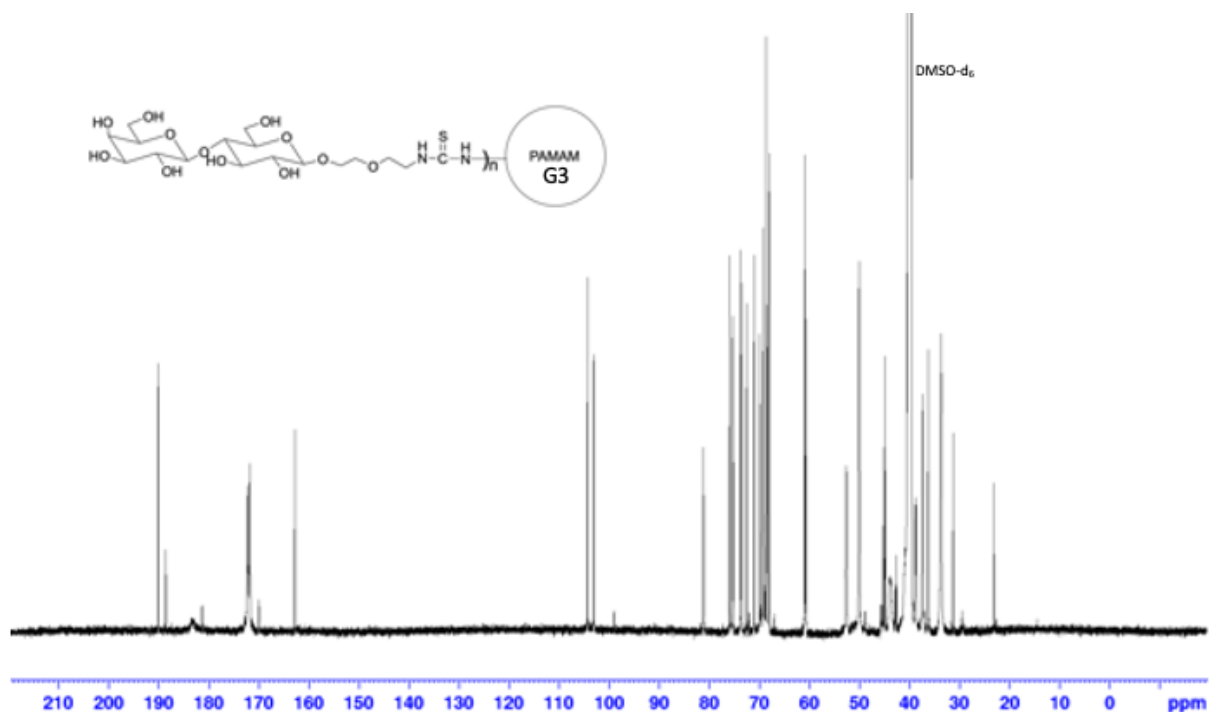


Figure S16. ^{13}C NMR of **7b**. Some peaks may not have been resolved from the baseline or were too broad. ^{13}C NMR (151 MHz, DMSO-d_6) δ 190.18, 188.62, 172.22, 171.76, 169.99, 162.78, 104.27, 103.12, 81.17, 75.98, 75.42, 75.29, 73.70, 73.57, 72.58, 72.58, 71.02, 69.95, 69.18, 68.64, 68.43, 68.03, 60.87, 60.63, 52.61, 50.01, 45.14, 44.85, 38.80, 37.37, 36.25, 33.67, 31.25, 23.10.

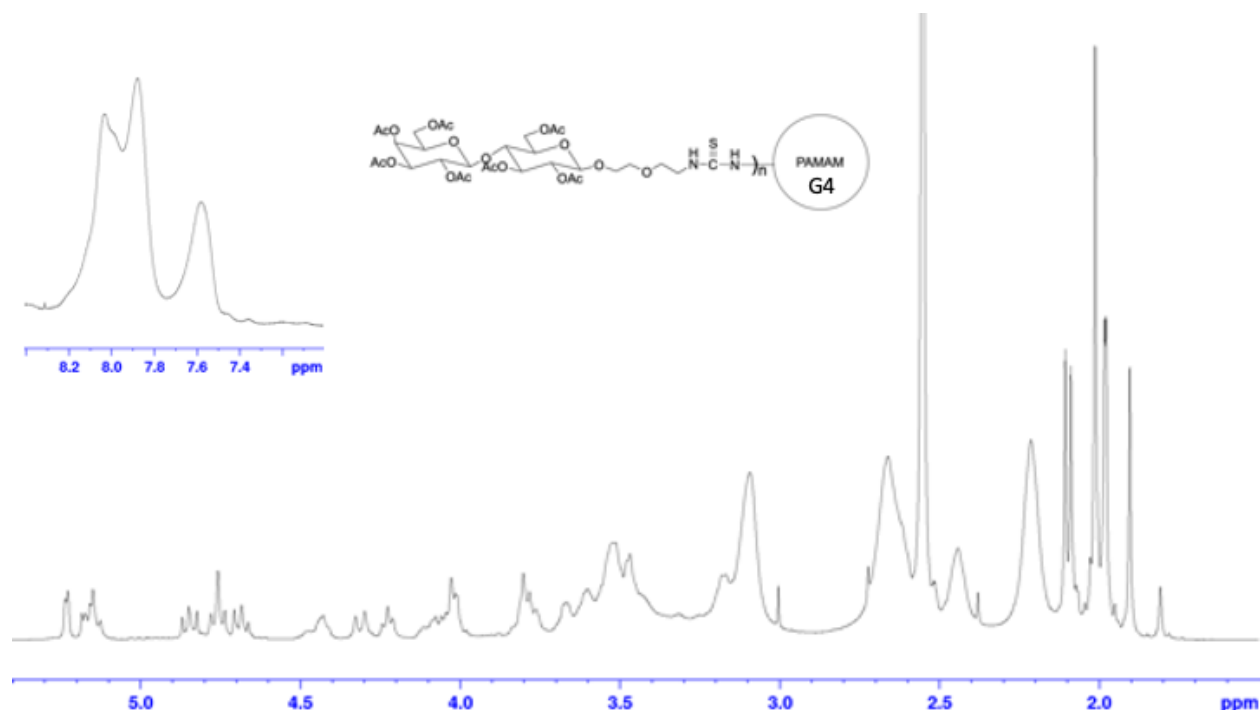


Figure S17. ¹H NMR of **8a**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO-d₆ residual may be in the broad peak from 2.68-2.46 ppm and DMSO the sharp singlet at 2.44 ppm. ¹H NMR (400 MHz, DMSO-d₆) δ 8.17-7.74 (*m*, 124H, **amide protons**), 7.58 (*bs*, 35.4H, **2 thiourea protons**), 5.24 (*app s*, 15.6H), 5.20-5.10 (*m*, 32.6H), 4.85 (*t*, 14.2H, *J*= 9.05, 9.05 Hz), 4.76 (*t*, 29.2H, *J*= 8.50, 8.50 Hz), 4.68 (*app t*, 19.1H, *J*= 8.7, 8.7 Hz), 4.51-4.38 (*m*, 22.8H), 4.32 (*d*, 18.6H, *J*= 11.34), 4.26-4.16 (*bt*, 18.1H, *J*= 6.59, 6.59 Hz), 4.14-3.97 (*m*, 62.3H), 3.86-3.72 (*m*, 58.1H), 3.72-3.36 (*m*, 285.6H), 3.24-3.02 (*m*, 259.1H), 2.66 (*bs*, 289.4H), 2.44 (*bs*, 97.4H), 2.21 (*s*, 212.5H), 2.01 (*s*, 49.2H, OAc: **H₃CC=OO-**), 2.10 (*s*, 45.0H, OAc: **H₃CC=OO-**), 2.01 (*s*, 98.9H, OAc: **H₃CC=OO-**), 1.98 (*overlapping s*, 88.5H, OAc: **H₃CC=OO-**), 1.90 (*s*, 40.2H, OAc: **H₃CC=OO-**), 1.81 (*s*, 9.9H).

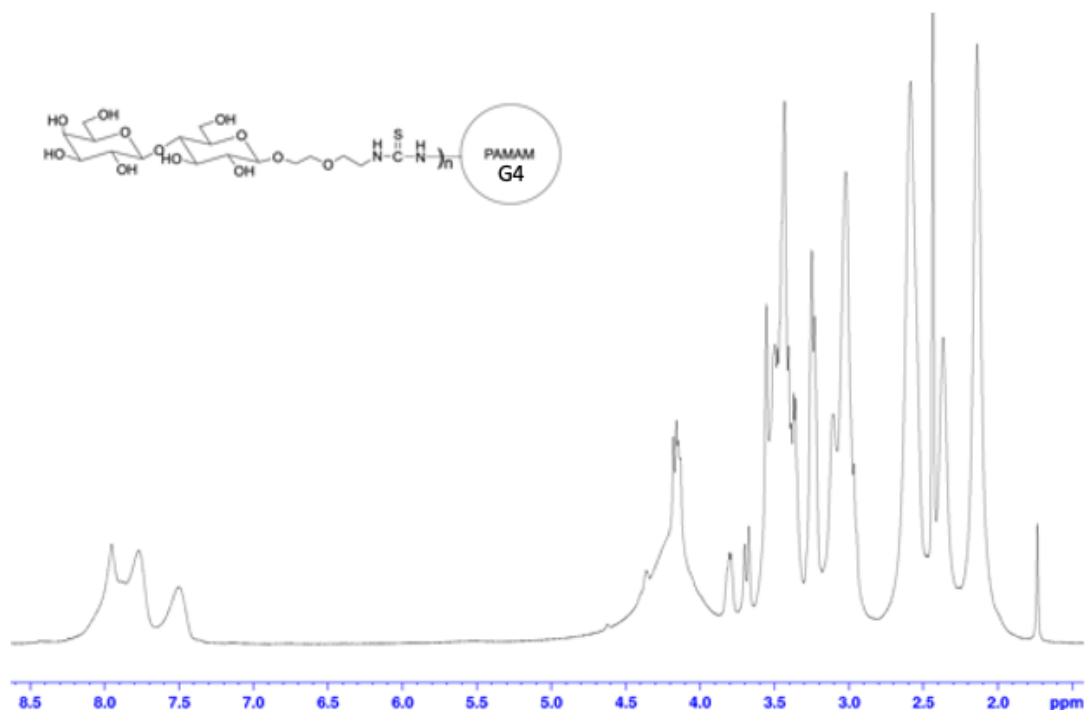


Figure S18. ^1H NMR of **8b**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be the broad peak from 2.68-2.50 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO- d_6) δ 7.86-7.65 (*bs*, 124H, **amide protons**), 7.65-7.39 (*bs*, 84.6H, **2 thiourea protons**), 4.34-3.91 (*m*, 388.8H), 3.84-3.76 (*m*, 52.2H), 3.69 (*d*, 62.8H, $J=10.28\text{Hz}$), 3.63-3.30 (*m*, 730.7H), 3.30-3.18 (*m*, 225.9H), 3.17-2.87 (*m*, 558.1H), 2.36 (*s*, 230.0H), 2.14 (*s*, 492.9H), 1.73 (*s*, 22.7H).

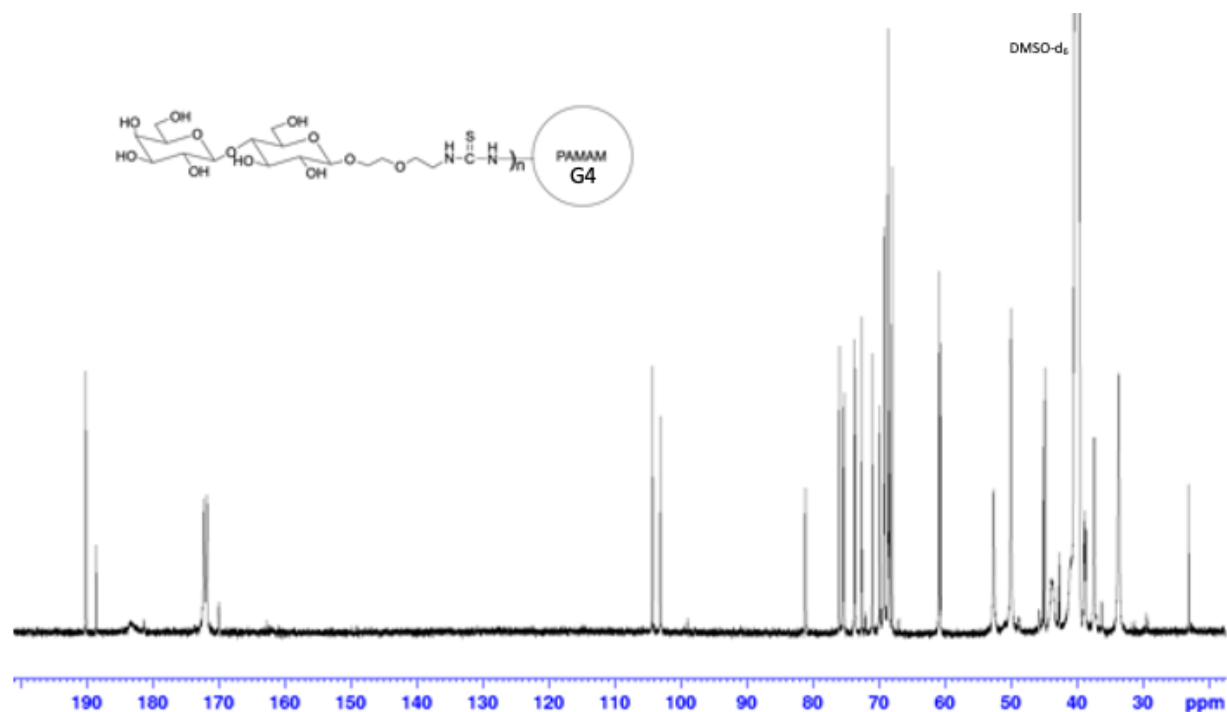


Figure S19. ^{13}C NMR of **8b**. Some peaks may not have been resolved from the baseline or were too broad. ^{13}C NMR (151 MHz, DMSO-d_6) δ 190.15, 190.09, 188.61, 172.26, 171.80, 170.02, 104.31, 103.11, 81.15, 75.98, 75.42, 75.29, 73.70, 73.57, 72.58, 71.02, 69.95, 69.18, 68.64, 68.43, 68.03, 60.88, 60.64, 52.61, 50.00, 45.14, 44.85, 38.90, 38.68, 37.37, 33.67, 23.11.

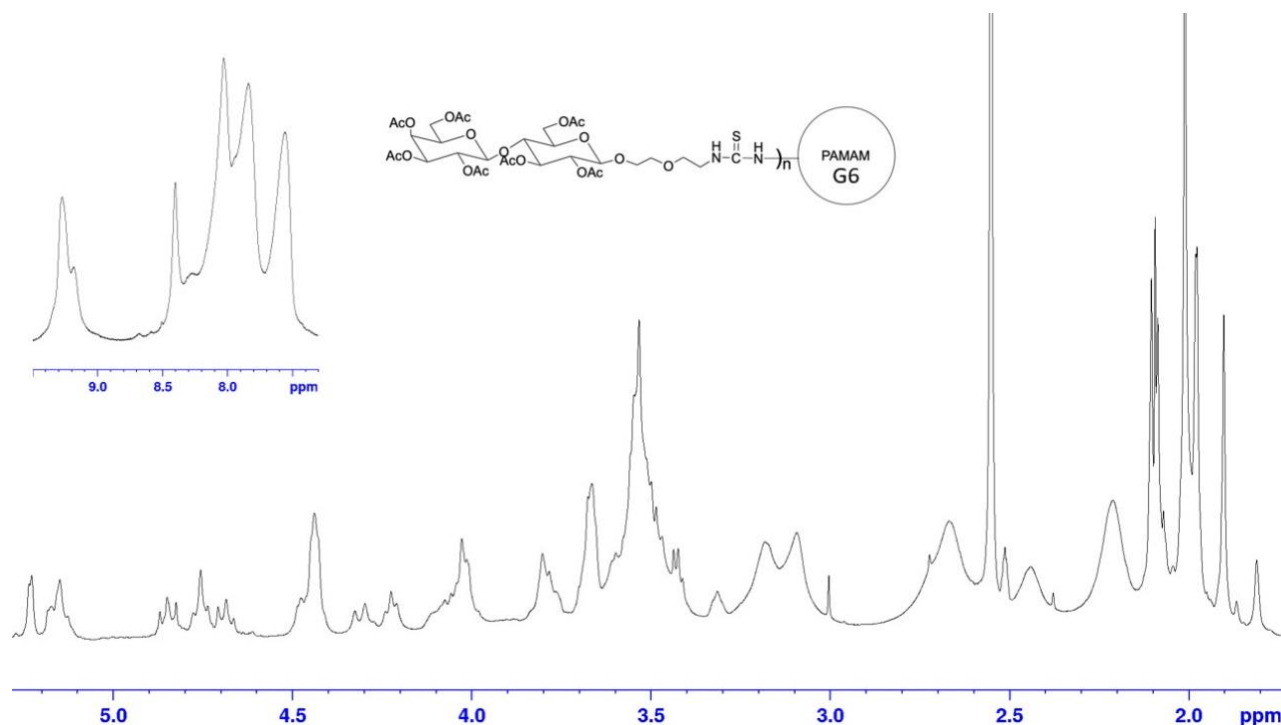


Figure S20. ^1H NMR of **9a**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO- d_6 residual may be in the broad peak from 2.68-2.46 ppm and DMSO the sharp singlet at 2.44 ppm. ^1H NMR (400 MHz, DMSO- d_6) δ 8.21-7.71 (m, 508H, **amide protons**), 7.56 (bs, 193.4, **2 thiourea protons**), 5.24 (app s, 95.4H), 5.20-5.10 (m, 156.2H), 4.88-4.80 (m, 103.4H), 4.79-4.72 (m, 134.4H), 4.72-4.65 (m, 92.3H), 4.51-4.39 (m, 335.4H), 4.35-4.26 (m, 115.1H), 4.26-4.19 (m, 123.4H), 4.14-3.95 (m, 446.8H), 3.85-3.74 (m, 304.3H), 3.72-3.63 (s, 403.3H), 3.63-3.40 (m, 1,564.8H), 3.34-3.01 (m, 1,099.8H), 2.66 (bs, 895.4H), 2.44 (bs, 331.7H), 2.21 (s, 768.8H), 2.14-2.06 (m, 689.9H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 2.01 (s, 631H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.98 (overlapping s, 408.8H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.90 (s, 256H, OAc: $\text{H}_3\text{CC}=\text{OO}-$), 1.81 (s, 92.1H).

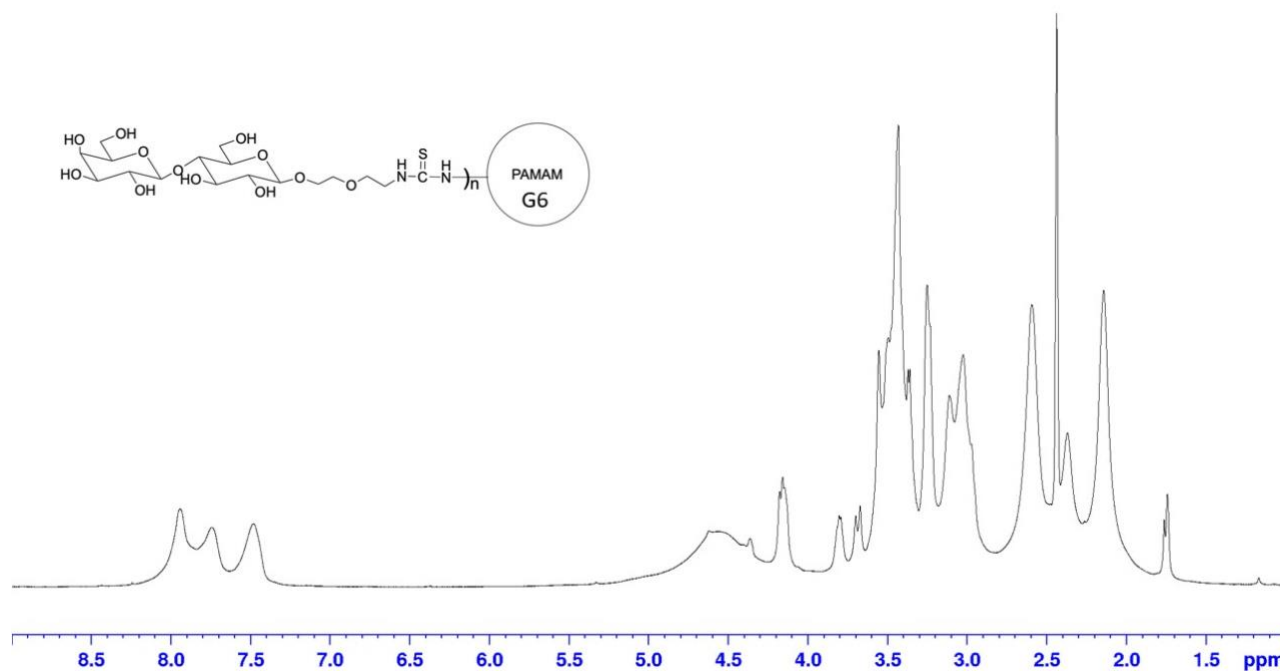


Figure S21. ¹H NMR of **9b**. Note that only our current diagnostic signals can be defined and identified. Some signals may not have been identified due to broadening, therefore there may be more or less protons accounted for than expected. DMSO-d₆ residual may be in the broad peak from 2.68-2.50 ppm and DMSO the sharp singlet at 2.44 ppm. ¹H NMR (400 MHz, DMSO-d₆) δ 8.13-7.63 (*bd*, 508H, **amide protons**), 7.62-7.36 (*bs*, 228.1H, **2 thiourea protons**), 5.00-4.26 (*bs*, 688.4H), 4.23-4.07 (*m*, 236.7H), 3.80 (*bs*, 126.9H), 3.67 (*d*, 29H, $J=11.12\text{Hz}$), 3.63-3.30 (*m*, 1,744.7H), 3.30-3.17 (*m*, 575H), 3.17-2.86 (*m*, 1,096.5H), 2.59 (*bs*, 847.0H), 2.37 (*s*, 373H), 2.14 (*s*, 947.5H), 1.74 (*s*, 101.0H).

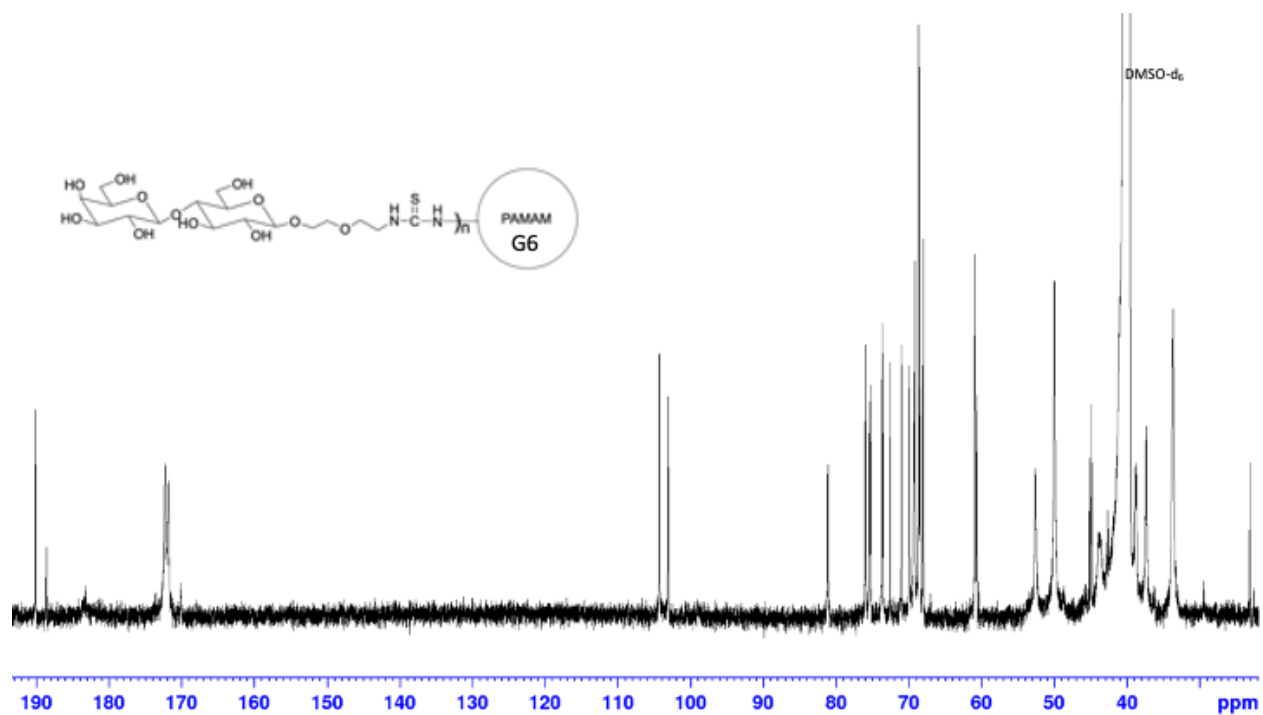


Figure S22. ^{13}C NMR of **9b**. Some peaks may not have been resolved from the baseline or were too broad. ^{13}C NMR (151 MHz, DMSO-d_6) δ 190.15, 188.62, 172.33, 171.87, 104.31, 103.11, 81.14, 75.98, 75.41, 75.29, 73.70, 73.57, 72.58, 71.02, 69.95, 69.19, 68.66, 68.44, 68.02, 60.89, 60.66, 52.62, 50.00, 45.14, 44.85, 38.92, 38.83, 38.68, 37.36, 33.66, 23.11.

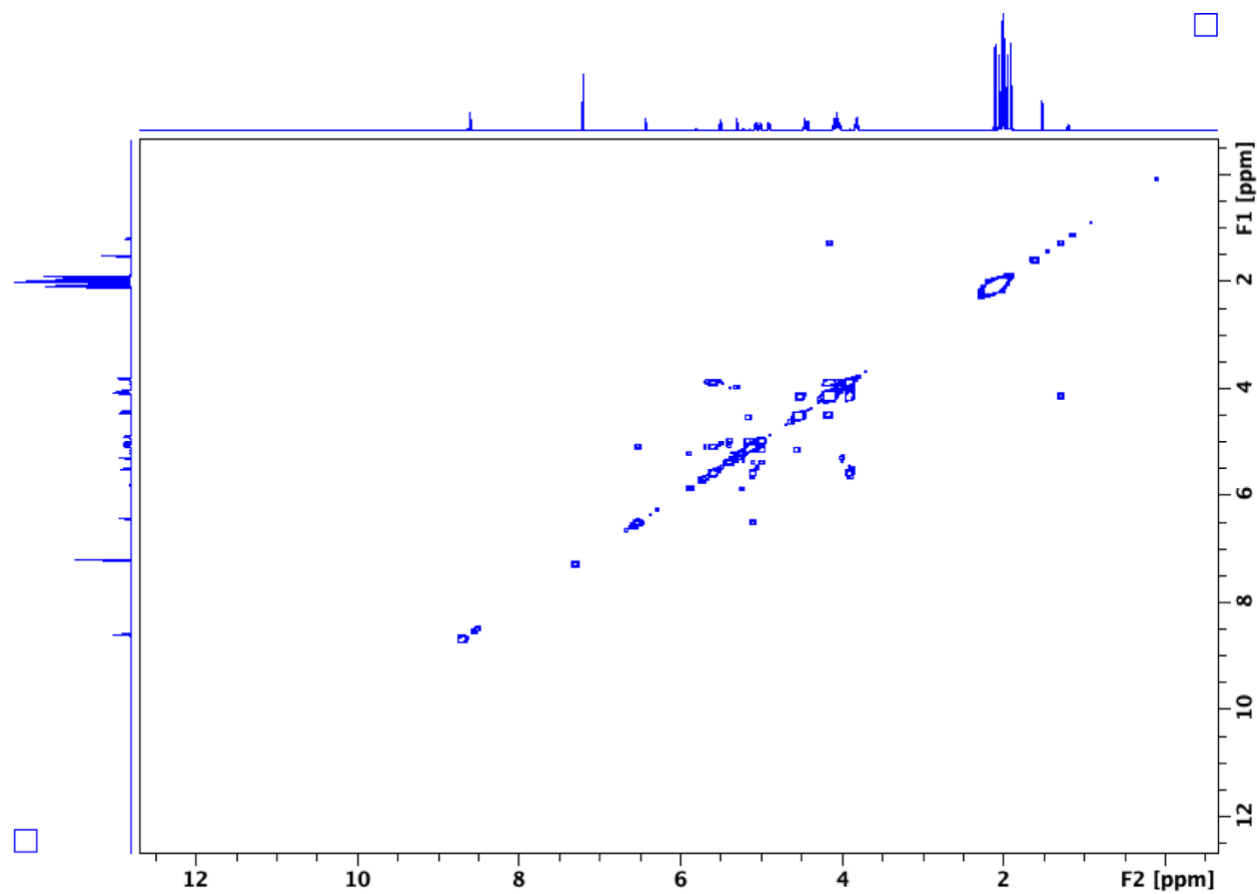


Figure S23. COSY of **3** (600 MHz, CDCl₃). Spectra was provided by Ramat Tahir.

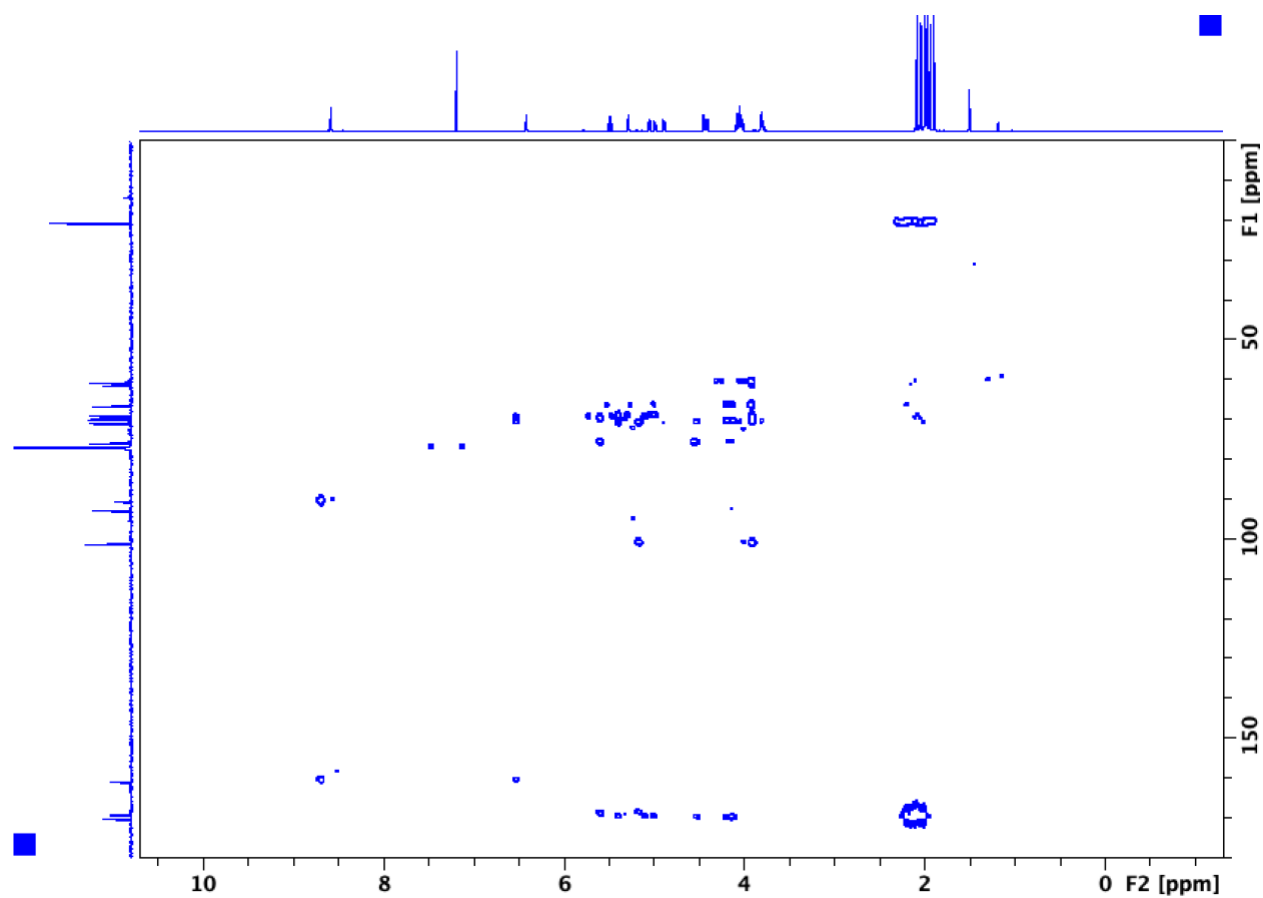


Figure S24. HMBC of **3** (F1: 150 MHz, F2: 600 MHz, CDCl₃). Spectra was provided by Ramat Tahir.

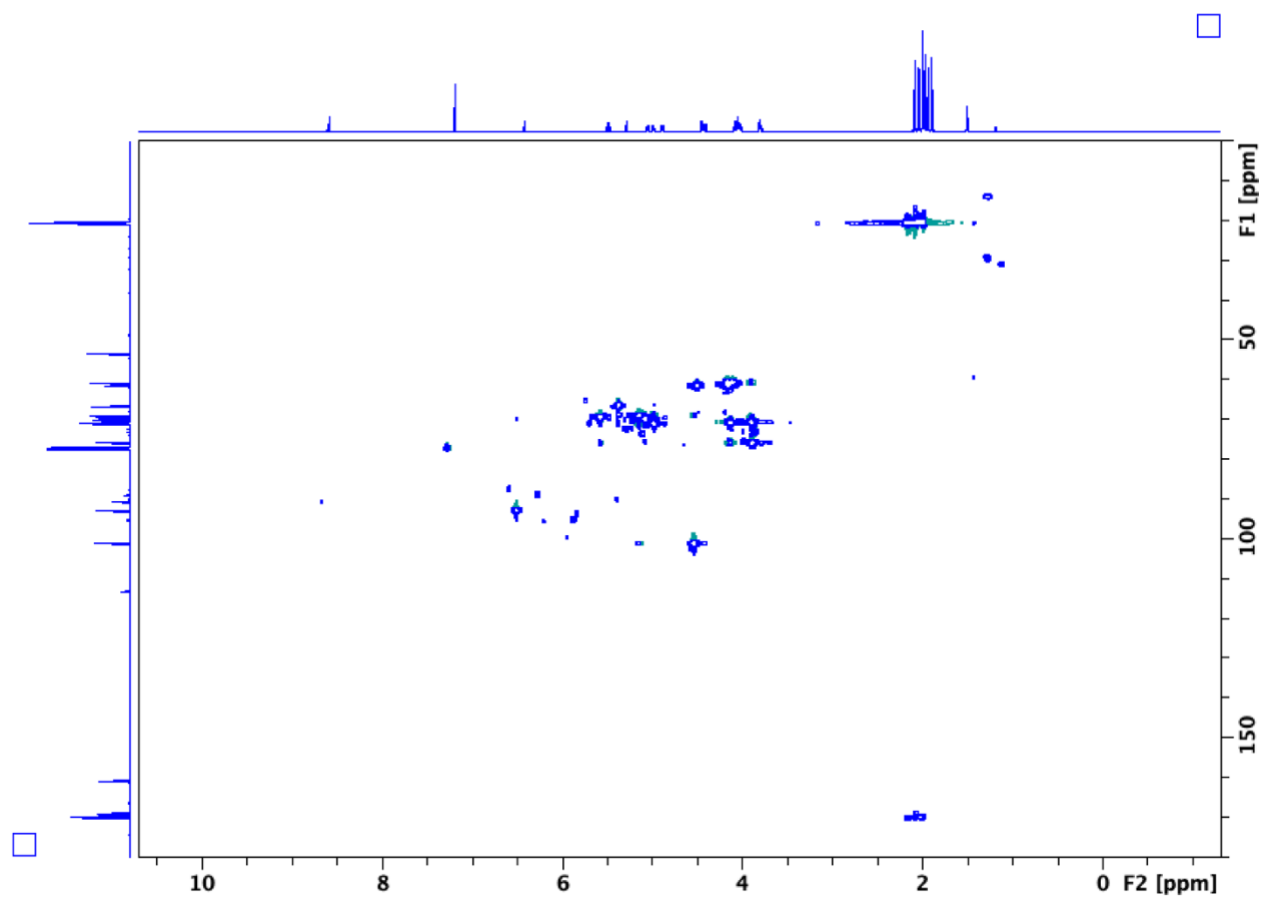


Figure S25. HSQC of **3** (F1: 150 MHz, F2: 600 MHz, CDCl₃). Spectra was provided by Ramat Tahir.