MULTIVALENTLY PRESENTED CARBOHYDRATES CAN BE USED AS DRUG DELIVERY VEHICLES AND TO STUDY PROTEIN CARBOHYDRATE INTERACTIONS

by

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This was for me, in honor of my parents and family.

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ABSTRACT

Dendrimers in general excel as drug delivery vehicles since there are many different ways they can be assembled and different ways to tailor them to the system being studied. Glycodendrimers are generally nontoxic and can be further developed to meet the needs of what is being studied. For instance, in the studies below, a quaternity ammonium compound (QAC) has been attached to a glycodendrimer to determine the antimicrobial activity of a multivalently presented QAC in studies of minimum inhibitory concentration (MIC), biofilm prevention, and bacterial resistance. Results include comparable MICs to those of established antibiotics, prevention of biofilm formation but not disruption of an established biofilm, and establishment of multivalency as a strategy to counteract bacterial resistance. Another heterogeneously functionalized dendrimer was synthesized to study drug release characteristics of a prodrug attached to a cleavable substrate. In these studies, the upregulation of several proteins during cancer progression was taken advantage of including; MMP-2, -7, -9, and galectin-3.

Glycodendrimers are tools used to study protein carbohydrate interactions. Study of galectins and their corresponding β-galactosides have illuminated their role in several essential biological processes. Multivalency plays a crucial role in many protein-carbohydrate interactions. Galectins are known to interact multivalently with various ligands. Although the role of galectins in this process is not yet fully understood, galectins have been proposed to serve as protective proteins during periods of high oxidative stress. We describe the synthesis of Galβ1-4Fuc functionalized poly(amidoamine) (PAMAM) dendrimers in order to test *C. elegans*' response to high oxidative stress. In order to test the function of Galβ1-4Fuc *in vivo*, *C. elegans* were treated with RNAi to knockdown *lec-1* or *lec-10,* and then treated with glycodendrimer and exposed to oxidative stress. *C. elegans* that were pre-treated with the glycodendrimers were less susceptible to oxidative stress than untreated controls. The glycodendrimers mainly appeared within the digestive tract of the worms, and uptake into the vulva and proximal gonads could also be observed in some instances. This study indicates that multivalently presented Galβ1-4Fuc can protect *C. elegans* from oxidative stress by binding to galectins.

Keywords: Dendrimer, Glycodendrimer, PAMAM, Mannose, Quaternary Ammonium Compound, Matrix metalloproteinase (MMP), Galectin-3, *C. elegans,* oxidative stress, multivalency, Galβ1-4Fuc, Lactose

CHAPTER ONE

INTRODUCTION TO CARBOHYDRATES, GALECTINS, AND HOW MULTIVALENCY CAN BE USED TO STUDY BIOLOGICAL SYSTEMS

Four macromolecule classes are the fundamental building blocks for living organisms; proteins, DNA, lipids, and carbohydrates. Each of the classes of macromolecules plays a different biological role. Proteins have a wide range of functions including functioning as enzymes regulating different cellular behavior and structural components of cells such as forming extracellular matrices.[1-2] DNA is genetic information that is stored molecularly and codes for all protein products and also plays a role in regulating fundamental cellular processes.[3-4] Lipids are a different class all together and can be described as a biological molecule that is not water soluble.[3, 5] They can be small molecules such as steroids or the more traditional membrane subunit structure such as a phospholipid. Some of the lipids such as the steroids have functions in cell signaling, energy storage, and lipids also form cell membranes.[3] Carbohydrates differ from the other classes of macromolecules. DNA and proteins are almost exclusively linear, and while carbohydrates can be linear, it is their ability to branch and the variety of different ways they can be connected that helps distinguishes them from the other classes.

Proteins and DNA are assembled with specific linkages: peptide bonds and phosphodiester bonds, respectively. Unlike proteins and DNA, carbohydrates possess the ability to link together two monomers with multiple connection choices. In fact, because of the diversity of connections between monosaccharides,

carbohydrates can be assembled into an astonishingly complex array that is characterized by successive branching. This complexity gives them unique properties and contributes to their specificity for their receptor. However, this complexity can also lead to difficulties in studying carbohydrates. It is well known that cells are covered in carbohydrates[6-7] typically expressed as oligosaccharides. They appear to serve as recognition markers[8] and are a form of posttranslational modification of proteins and lipids that modifies/modulates the protein's or lipid's activity.[9] Carbohydrates are the fundamental unit that, when crosslinked, can form structural components such as cellulose. The interaction between expressed carbohydrates and their protein binding partners is the focus of the remainder of this thesis.

Overview of Multivalently Presented Carbohydrates Using Dendritic Scaffold for the Study of Protein-Carbohydrate interactions

Eliciting a response from a biological system typically requires a ligand to bind with a receptor. This can either be done when a single ligand interacts with a receptor. However, more significant responses for weakly associating receptor/ligand pairs may require many ligands to bind to many different receptors. Multivalency occurs when several ligands on one entity bind to multiple receptor binding sites. Here, valency is defined as the number of interactions between a ligand and receptor. A multivalent interaction can overcome the relatively weak interaction that a monovalent interaction can experience due to an enhancement to the functional avidity. This results in a response that is more significant than a monovalent interaction.

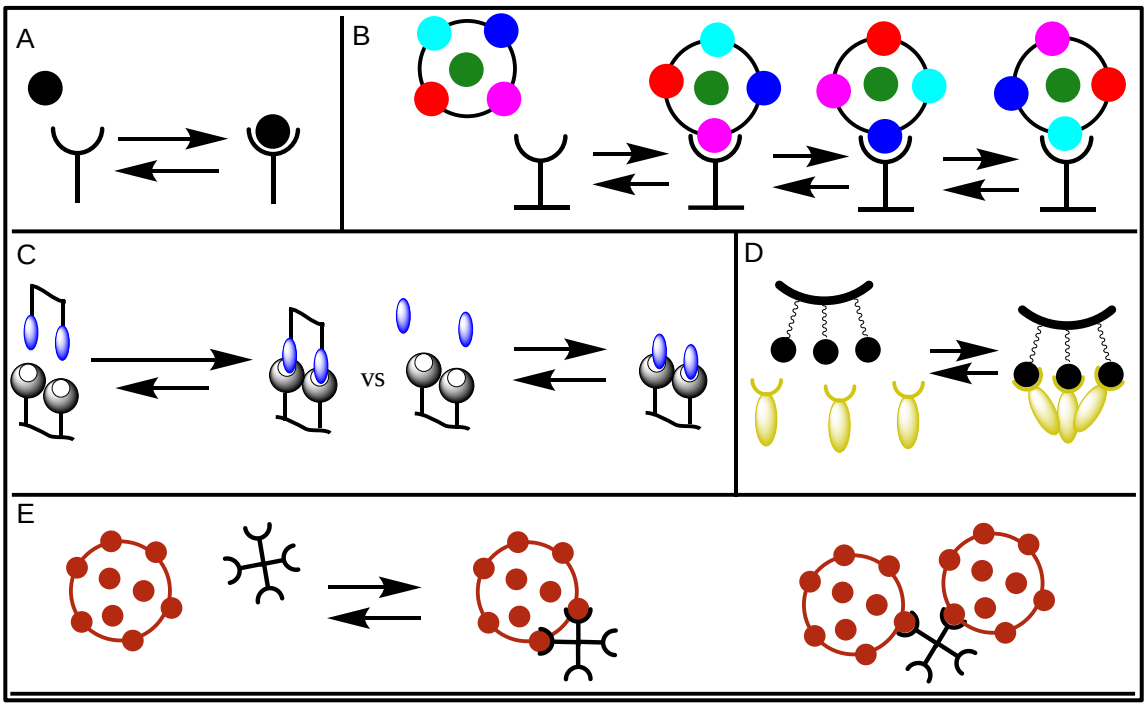

Figure 1. Examples of Multivalent binding.
A) Monovalent binding: The black circle represents the ligand while the "y" shaped structure is the receptor. In this example, it is important to note there is only a single ligand per receptor. B) Statistical effect: there are so many ligands around this receptor that as one ligand and receptor experience dissociation, another ligand is available nearby to bind to the receptor. C) Chelate effect: an entropic cost is avoided in binding when the two ligands are attached before binding to their receptor. D) Receptor clustering: multiple instances of ligand and receptor binding result in a clustering of the receptors. This can elicit a biological response. E) Bivalent interactions: Two different examples of bivalent interactions are shown. On the left, a ligand on a large framework is presented multivalently and thus can bind more than once to a multivalent receptor. On the right, the receptor is shown crosslinking molecules.

As seen in Figure 1A, a monovalent binding even occurs when a single ligand interacts with its receptor. When the same ligand is presented multivalently, there is a high localized concentration that results in the receptor being in the bound state more often.[10] This can be seen in Figure 1B. Further, by attaching the ligands to a scaffold the entropic cost is paid before the ligands interact with their

receptor.[10-12] An example of this can be seen in Figure 1C. In the bound state two individual entities interact instead of three had they not been connected. A result of tethering ligands together could be the resulting clustering of receptors (Figure 1D). Clustered receptors can sometimes be necessary for eliciting a biological response.[12-13] When receptors are also multivalently presented, two modes of interaction can occur. Consider a bivalent interaction which involves two ligands and two receptors. A bivalent interaction can occur between one entity of ligands and one of receptors. A bivalent interaction can also occur between one of the ligand and two entities with receptors as seen in Figure 1E. This leads to crosslinking between entities and can be used to describe aggregation.[14]

Protein-carbohydrate interactions are of particular interest since they have been implicated in a wide range of critical biological processes.[15-17] Typically, biologists characterize the proteins using site specific mutagenesis to determine how they interact with their ligands. This is often followed by studies of synthetically produced ligands and arrays in order to determine binding specificity and other important properties of protein-carbohydrates interactions. The research described in this thesis will employ organic synthesis to produce multivalent carbohydrate arrays for the study of protein-carbohydrate interactions. Specifically, in chapter 3 the galectins in *C. elegans* will be studied using galactose-fucose functionalized dendrimers.

When studying protein-carbohydrate interactions that can be weak (10^{-3} to 10^{-4} M),[18]; it is typically necessary to display multiple copies of the carbohydrate to

elicit a biological response. In order to benefit from the binding enhancement that results from presenting multiple glycosides at once, the spacing between them needs to be relatively similar to the spacing between receptors.[6] Lee et al, were able to obtain a 500-fold enhancement in binding for a rat hepatic lectin (RHL) when they presented multiple copies of GalNAc on BSA (bovine serum albumin) with an appropriate spacing between glycosides. In the paper, the authors attributed the observed lack of binding enhancement for their multivalently displayed mannosides as arising from unoptimized spacing of the mannose groups. Later, it was shown that multiple copies of mannose binding to Concanavalin A can benefit from clustered glycosides.[19] This boost in apparent affinity is referred to as the “cluster glycoside effect.”[20] This term was first used in the literature in 1999 when Dimick *et al*. described a single entity with several mannosides binding to Concanavalin A more strongly than monomer mannosides. The core concept for the boost in binding is multivalency. In this research, they were able to obtain a 30-fold enhancement on a per valency basis (per carbohydrate).[20]

Galectins

Lectin are a family of proteins that bind carbohydrates, are found in plants and animals indicating an importance to biological functions,[21] and can be further broken down into different classes. One class of lectins are the Ca^{2+}-dependent lectins, which are referred to as the C-type lectins and are found in serum, extracellular matrix, and membranes.[22] Another main class of lectins are the S-type lectins, which earned their name due to the reduced thiols that were necessary to maintain the protein's activity. The sulphur atoms on the cysteine residues were oxidized during failed isolation attempts.[15] The S-type lectins are found in a wide range of tissues and are not usually membrane bound. This class of lectins is also referred to as galectins due to their ability to engage with β-galactosides. Galectins can be classified based on their carbohydrate ligands, subcellular localization, associated biological processes, and need for a metal ion to properly function.[22-23] These proteins are in the size range of 14 KDa to 39 KDa.[24] While their overall structure can vary widely, galectins all contain a conserved carbohydrate recognition domain (CRD) that physically interacts with its carbohydrate ligand. There are eight amino acids that are conserved in the galectin CRD and are essential for natural ligand receptor duties.[25-27] These proteins have a wide range of biological responsibilities, making them an attractive area of study.

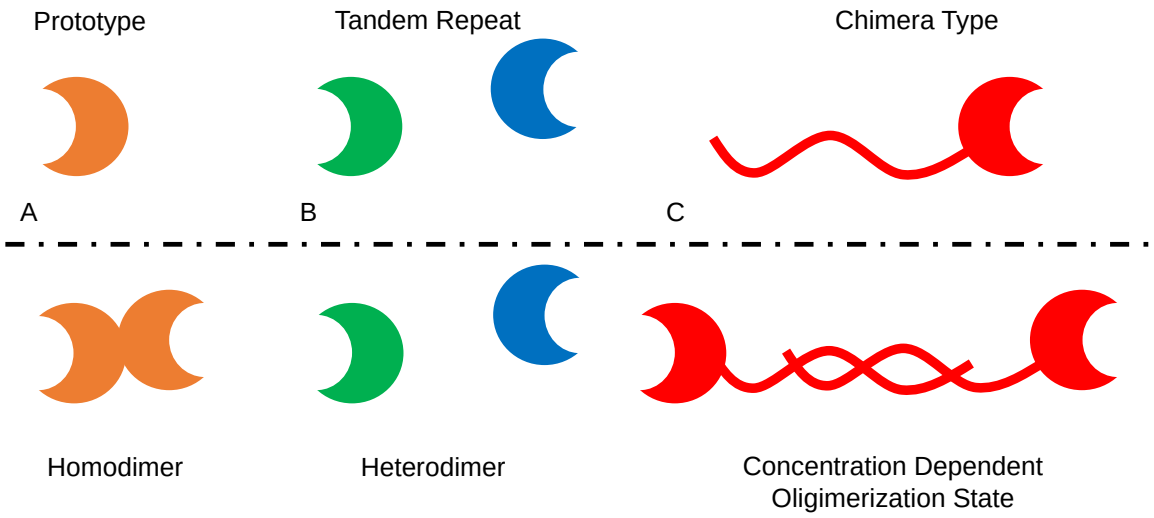

Figure 2. The subfamilies of galectins.
A) Prototype galectins can form homodimers. These galectins include galectin- 1, 2, 5, 7, 10, 11, 13, 14, and 15. B) Tandem repeats have two CRDs connected by a short peptide sequence. The tandem repeat subfamily consists of galectin- 4, 6, 8, 9, and 12. C) Chimera type galectin have a CRD with a collagen-like tail that interaction with other chimera type galectins in a concentration dependent manner to form increasing oligermization states. Galectin-3 is the only member of this subfamily.

Galectins are divided into three subfamilies. These can be seen above in Figure 2. Human galectins- 1, 2, 5, 7, 10, 11, 13, 14, and 15 all belong to the prototype subfamily and are capable of forming homodimers.[28-29] The next subfamily is characterized by having two carbohydrate recognition domains connected by a short peptide sequence. Their members include human galectin-4, 6, 8, 9, and 12.[28] Human galectin-3 is the only known member of the third family, denoted chimera type galectin.[28, 30] The most notable features of galectin-3 include a carbohydrate recognition domain with an extended N-terminal domain that is essential to galectin-3 concentration dependent oligomerization. This collagen-like tail can be cleaved by proteases such as MMPs.[31]

Galectin-3

Of all of the members of the human galectin family of proteins, galectin-3 is the most studied. Although galectins are necessary for basic biological functions, their abnormal expression has been used a potential biomarker in several types of cancer.[28] There appears to be a correlation between the concentration of circulating galectin-3 and cancer progression.[13, 32] Galectin-3 is upregulated in several forms of cancer including thyroid[33] and pituitary tumors[34]. In addition, MMPs are also often over expressed in several cancers.[35] MMP2, MMP9, and MMP7 can cleave the collagen-like tail of galectin-3.[31, 36] The Thomson-Friedenreich antigen is exposed in several forms of cancer, has the strongest dissociation constant with human galectin-3,[37] and can be used as a diagnostic indicator of the aggressiveness of cancer.[38-39] Although, the Thomson-Friedenreich antigen is not elaborated on further in this text, that fact that this disaccharide is the putative native ligand for galectin-3 deserves a mention. Several saccharides contain the β-galactose motif and interact with galectins. The β-galactosides examples included in this work are lactose (Galβ1-3-Glc, or lactose), and Galβ1-4Fuc.

Galectin Research in this Thesis

In chapter 3, this thesis will cover how galectins function with their ligands to play a protective role in oxidative stress. In chapter 4, research showing that endogenous galectin-3 can be exploited to design an anticancer therapy is presented.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are enzymes that are named because they require a metal in order to exhibit activity. There are at least 25 different members of the vertebrate MMP family and at least 24 human analogs.[40] MMPs degrade and process proteins extracellularly and are essential for cells to undergo development as well as advancing disease related processes. While each MMP has different functions, virtually all MMPs degrade extracellular matrix proteins. The potent ability of MMPs to degrade matrices distinguishes this family of enzymes from other metalloproteinases.[41] MMPs are produced as proenzymes or zymogens and need to be activated before they can assume normal function. A sulfhydryl group acts as a fourth ligand to the active site of the zinc ion. The sulfhydryl group on a cysteine residue near the C-terminal end of the MMP protein needs to be removed or disturbed out of its native position before hydrolysis of the target protein can occur.[42] The catalytic domain contains a stranded β-sheet and three α-helices.

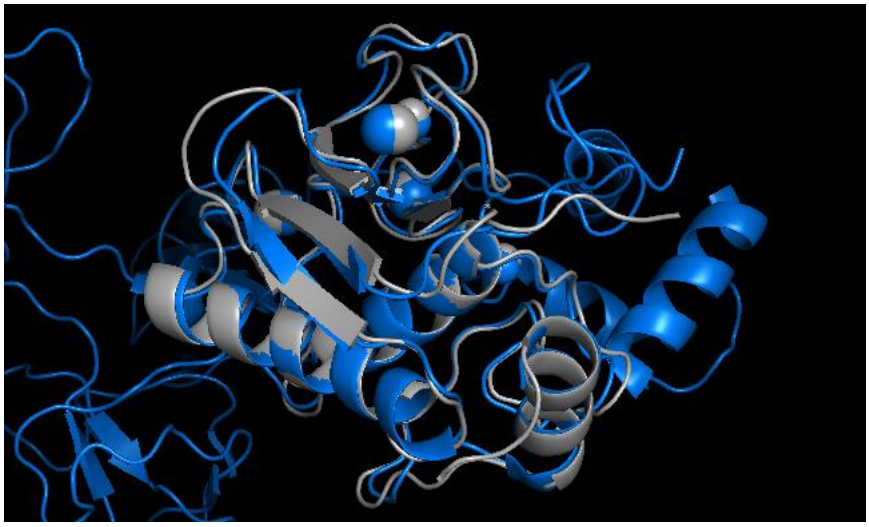

Figure 3. Crystal structure overlay of the catalytic domain of MMP2 and MMP9.

The overlay of the MMP2 and MMP9 show in Figure 3 exemplifies how similar their catalytic domains are, which is consistent with other members of the MMP family. MMP2 and MMP9 are also referred to Gelatinase A and Gelatinase B, respectively. A distinguishing feature of their catalytic domain is three head-to-tail cysteine rich repeat regions.[43-44] Like most MMPs, MMP9 is regulated at the transcription level. However, MMP2 is unique because it is regulated by enzymes.[41]

MMP2 and MMP9 play a role in several typical degradation and repair of tissue processes.[45-46] Their upregulation has also been associated with inflammation and several malignancies including pancreatic cancer.[47] MMP2 and MMP9 are upregulated in pancreatic cancer and have proved to be a diagnostic marker for the malignancy.[48] MMP2 and MP9 have been implicated in metastasis and tumor progression. Thus, their inhibition is an attractive treatment option, and

development of inhibitors made it to clinical trial.[45, 47, 49] However, the inhibitors that made it to the trial ultimately failed because designing selective inhibitors proved to be challenging.[49] Galectin-3 is upregulated in several cancers[50-51] along with MMPs.[35, 52] In breast cancer, MMP2 and MMP9 are upregulated and have been shown to cleave the collagen region of galectin-3.[31] The spatial and temporal importance of the regulation of MMPs are now the focus of studies.[53-54] Getting an inhibitor to the right place at the right time could be the key to clinical success.[41, 49]

Like MMP2 and MMP9, MMP7 has also been associated with upregulation in several forms of cancer such as pancreatic ductal adenocarcinoma, and lung adenocarcinomas and ovarian cancers.[55-57]

Lactose can be presented multivalently and can attenuate the aggregation characteristics of several cancer cell lines as shown by previous members of the Cloninger research group.[58] In these studies, various sizes of glycodendrimer were synthesized in a successful attempt to modulate the formation of aggregation of A549, HT1080, and DU145. They found that generation 2, G(2), glycodendrimers coated with lactose were most effective in creating aggregates from free cells. In chapter 4, these G(2) glycodendrimers will be used to study drug release to A549 cells.

Using Synthetically Conjugated Carbohydrates on a PAMAM Scaffold as Nanotherapies and to Study Protein Carbohydrate Interactions

In chemical terms, carbohydrates are defined as molecules that can fit into the following formula: $C_m(H_2O)_n$. They can exist as a straight chain or as a ring. All of the carbohydrates discussed here will be in the pyran ring form. Carbohydrates are named by describing the configuration of the hydroxyl functional group and the number of carbons. Because carbohydrates are hydrates on a carbon scaffold, each carbon has two different orientations of hydroxyl groups that make the carbons chiral and result in a single carbohydrate that has a considerable amount of information stored in it. For example, a pyranose ring can have five different chiral centers, and a furanose ring has four. The collection of chiral centers is what give the carbohydrates their configuration. Branching of carbohydrates in oligosaccharides, especially when the oligosaccharides have different configurations, greatly increases the complexity so that they can suit different biological needs.[8, 25]

A particular configuration of hydroxyl groups in a carbohydrate is usually necessary for proper binding to their receptor. During searches into the endogenous glyco-epitope for the galectins in *C. elegans*, other investigators determined that the two constituent monosaccharides were galactose and fucose.[59] The linkage was unknown, and therefore these investigators sought to determine which sugar interacted more strongly and therefore had a smaller K_d with galectins of interest (LEC-1 and LEC-6). Galβ1-4Fuc and Galβ1-3Fuc were

synthesized with a 2-aminopyridine tether for array construction. They included other oligosaccharides in their comparisons referred to as E3 and PA041. These oligosaccharides are endogenous ligands in *C. elegans* and do not contain a Galβ-Fuc moiety. Results showed that the 1-4 connection afforded higher affinity binding for LEC-1 and LEC-6 than 1-3 disaccharides. E3 was comparable to the 1-3 linkage.[60] This is an excellent example as to why configuration matters. This also means that the complexity can be enhanced because two different rings can be connected in many different ways leading to disaccharides and eventually oligosaccharides. Thus, complex carbohydrates have access to a high number of chiral centers and connectivities with different binding properties to various proteins. This is how carbohydrates accommodate the wide range of biological processes from apoptosis to protection from oxidative stress to signaling blood type.[8]

Furthermore, the other macromolecules can be chemically altered with the addition of a carbohydrate in a process called glycosylation. The process of glycosylation can take place enzymatically[61] or by synthetic manipulation of carbohydrates.[62] Ultimately, the glycosylation state can regulate the activity of macromolecules such as proteins and lipids. Carbohydrates can also be presented on a synthetic scaffold which can be further synthetically altered to accommodate the system being studied. Ligands that are linked together with an appropriate space between ligands take advantage of multivalency. A framework such as a polymer that allows multiple copies of a ligand or receptor to be bound is ideal.

Some examples of good frameworks for the display of carbohydrates include dendrimers, polyglycerols, and linear polymers.

Here, we describe the use of poly(amidoamine) (PAMAM) dendrimers for multivalent display of carbohydrates.[63] PAMAM dendrimers are advantageous for biological assays because they are biologically compatible can be synthesized or purchased in a variety of sizes.[63-64] PAMAM dendrimers are symmetric, highly-branched macromolecules for which the degree of branching can be noted by generation number. Larger generations have more branch points and can be functionalized with more endgroups. PAMAM dendrimers are attractive in terms of presenting carbohydrates multivalently in a biological system because they are protein-like and easy to functionalize. The number of carbohydrates as well as the diameter of the glycodendrimer can be systematically modified based on dendrimer generation to fit the needs of the biological system.

The size of the dendrimer is referred to as the generation, and with each increasing generation of a PAMAM dendrimer, there is a doubling of the number of endgroups.[63] Methods used to characterize dendrimers have been firmly established. Their synthesis starts with ethylene diamine reacting with methyl acrylate in a Michael addition followed by another treatment of ethylene diamine. This affords the G(0) or generation zero PAMAM that has a total of 4 primary amines.[63] G(1) would then have 8 amino endgroups, G(2) has 16 amino endgroups, etc. For this work, generations 2, 3, 4, and 6 are discussed. A generation 2, G(2), dendrimer can be seen below in Figure 4. The main reason

why a change in dendrimer generation would be desirable is because size and shape are likely to play a crucial role in the multivalent interactions of interest. This can be further explained by exploring how the properties of the dendrimer change as a function of size. The smaller generations are much more amorphous in shape than the larger generational counterparts, and G(2) PAMAM dendrimers are highly asymetrical.[65] As the generation number increases the dendrimers become more spherical in shape. G(4), marks the transition from asymmetrical to spherical and its shape can best be described as globular.[65] The larger generations including G(6) are spherical in shape.[65]

Despite their advantages, PAMAM dendrimers do have some drawbacks. They can be difficult to dissolve which can lead to difficulties in their characterization. Furthermore, the PAMAM dendrimers used as starting materials for functionalization in this research were coated in primary amines. These primary amines are convenient due to the multitude of reactions to attach groups on them. However, at physiological pH, these primary amines are protonated, and can best be described as a quaternary ammonium compound (QAC) that can be highly hemolytic or may have other toxic effects.[64, 66] When designing nanotherapeutics using PAMAM dendrimer, careful consideration such as blocking of the primary amine endgroups is necessary as to mediate any potential toxic effects.

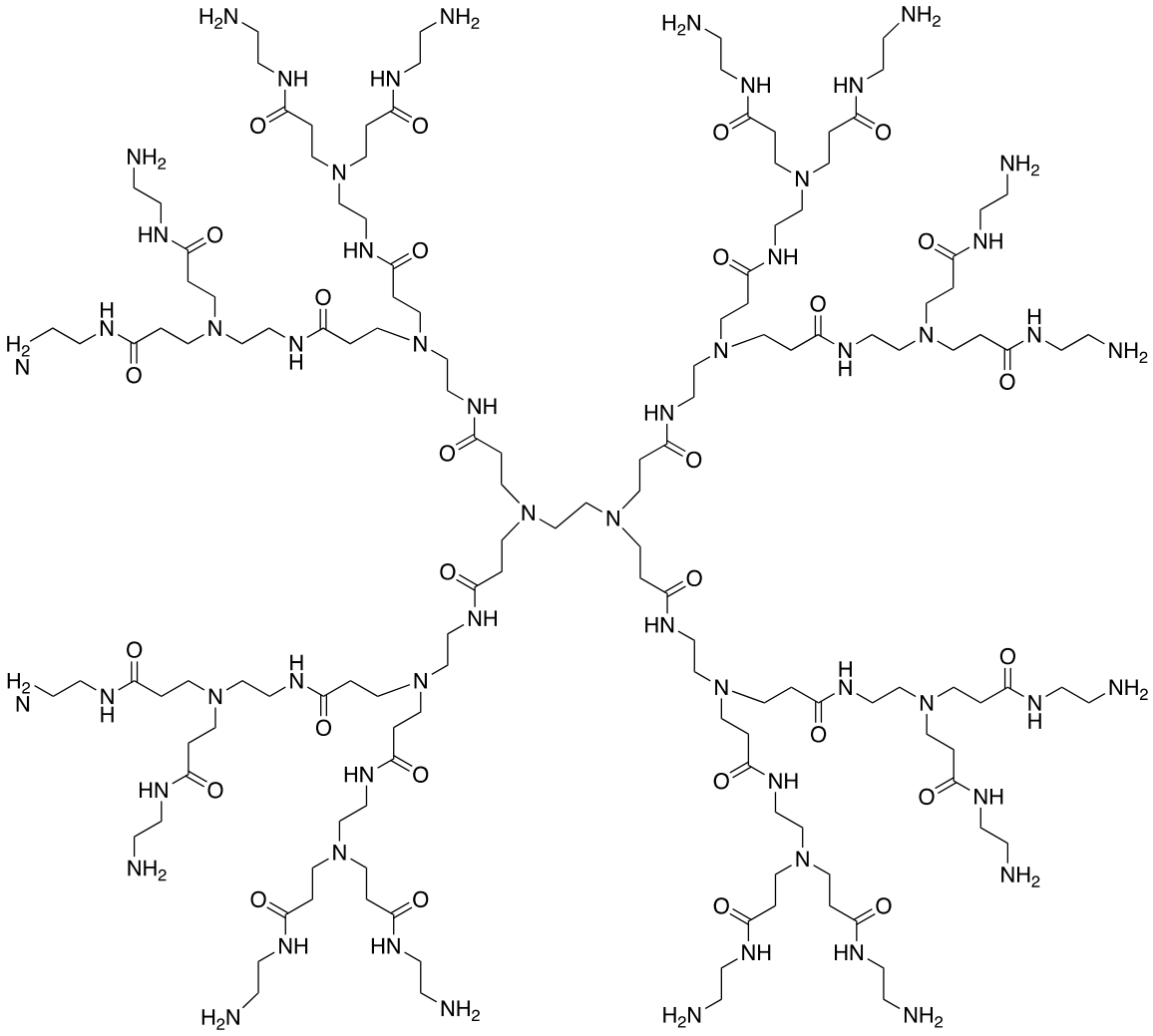

Figure 4. An amine terminated generation 2, G(2) PAMAM dendrimer.

RNAi induced gene suppression to study protein carbohydrate interactions

The 1980s were an exciting time to be part of genomic research. For the first time, RNA was found to exhibit enzymatic activity. From there on, it was a race to see how these RNA fragments could be used in a research setting.[67] While the understanding of the mechanism of action is still incomplete, RNAi regulation has been found in a large number of species including humans. RNAi can provide a selective and potent inhibitor of a protein and is considered a post translational form of regulation.[68] A plasmid contains the complementary information needed to interfere with mRNA of interest. This plasmid is double stranded and contains two T7 promoters that face towards each other. The gene that is to be interfered/suppressed is placed between these two promoters.[69] The system is placed under the regulation of a lac operon and is therefore inducible with IPTG (isopropylthiogalactopyranoside). As the dsRNA enters the cell, a protein complex called Dicer cuts the dsRNA into small pieces ~23 bp. This is followed by RNA-induced silencing complex (RISC), which unwinds the genetic material so that it can bind with its complementary mRNA.[70]

C. elegans had its complete genome sequenced in the late 1990s,[71] and over 40 percent of its predicted protein products match other organism making it an ideal candidate for studying the effects of silencing a gene using RNAi. There are two main methods to deliver the dsRNA into the organism: injection and feeding. For the most consistent suppression, inhibition feeding is the method of choice.[68-69] In addition, it is much easier to simply feed the organisms *E. coli* that

produce the dsRNA of interest than it is to inject the microscopic organism. It has been shown that this method of gene silencing is quite effective.[69] Only a couple of molecules of dsRNA per cell are required for gene silencing.[68]

RNAi can be used to suppress galectins in *C. elegans*. Paired with a glycodendrimer, *C. elegans* variants with suppressed galectin expression can be used to study the role galectins play in regulating natural processes. This is the subject of chapter 4 of this thesis.

Summary of Chapter One

Multivalently presented carbohydrates can be used to study a variety of systems used to study biological change. In the following chapters this concept will be employed to study: how multivalently presented ligands can be used to study if bacterial resistance can be reduced, to explore the galectin pathway in *C. elegans* when exposed to high levels of oxidative stress, and how over expression of MMPs in adenocarcinoma cells can be used to release a cleavable drug or prodrug. In all of the following work carbohydrate coated PAMAM dendrimers were used.

CHAPTER TWO

SYNTHESIS AND BIOLOGICAL ACTIVITY OF HIGHLY CATIONIC DENDRIMER ANTIBIOTICS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2
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Contributions: Main contributor of written text, synthesis, MIC testing, and resistant selection studies.

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CHAPTER 2

SYNTHESIS AND EVALUATION OF THE BIOLOGICAL ACTIVITY OF HIGHLY CATIONIC DENDRIMER ANTIBIOTIC

Introduction

Bacteria are dynamic and adaptive. They grow and proliferate in a wide range of pHs and temperatures and in many seemingly inhospitable environments including in vivo.[72,73] Over time, the constant exposure of bacteria to antibiotics results in resistance. Antimicrobial resistance is one of the greatest threats to human health worldwide.[74,75] Despite an increasing number of drug resistant bacteria being discovered, the pipeline for new antibiotics has slowed.[76,77] Essentially, we are losing the evolutionary arms race with pathogenic bacteria. Without appropriate drugs to treat infections, it becomes increasingly likely that an epidemic will occur.[78] With bacterial resistance on the rise, new effective antibiotics that can circumvent resistance are becoming highly desirable.[79]

Bacteria intrinsically have strategies for developing resistance.[80,81] One strategy by which bacteria can acquire resistance is by horizontal gene transfer. These genes are usually in the form of a plasmid that can be transferred between one bacterium and another even across genus.[81-82] Alternatively, bacteria can produce enzymes that break down certain types of antibiotics (such as β-lactamases breaking down β-lactam scaffold antibiotics).[81,83,84] Bacteria are also capable of altering the binding site of certain antibiotic targets, thus reducing the antibiotic's binding affinity. Methicillin resistant Staphylococcus aureus (MRSA) is

an example of bacteria altering a targeted binding site.[81] Moreover, bacteria can attenuate metabolic pathways in order to be less susceptible to an antibiotic. Gram negative bacteria, for example, are known to possess efflux pumps that can effectively reduce the accumulation of an antibiotic inside a bacterium.[81,83,85]

Armed with this knowledge, strategies to circumvent resistance are being developed.[80] An antibiotic that has several targets, for example, would decrease the likelihood of resistance since the bacteria would have to make several changes in order to reduce their susceptibility. Alternatively, a strategy that would target the bacterium's metabolic processes, cell membrane biosynthesis, or another essential target would greatly reduce the likelihood that resistance could be developed. Polymeric and macromolecular systems are currently actively being developed[86,87] because of their ability to multivalently[88,11] interact with bacterial targets. Dendrimers, macromolecules with branches emanating from the central core,[89] are often used as the synthetic multivalent framework for antimicrobials. Several recent reviews of antibacterial dendrimers are available.[90,91]

Various dendrimer-based antibacterials using this multivalent strategy have been described. Amino acid functionalized dendrimers have been synthesized on a poly(amidoamine) scaffold for drug delivery with low cytotoxicity.[92] Ortega *et al*. have reported the synthesis and activity of amine and ammonium functionalized chloromethylsilane-ended dendrimers effective against Escherichia coli and Staphylococcus aureus.[93] Grinstaff et al. prepared amphiphilic anionic dendrimers, with selective activity toward Bacillus subtilis over HUVECs.[94] Vancomycin

conjugated onto a fifth generation poly(amidoamine) showed several orders of magnitude improvement over free vancomycin.[95] Furthermore, this multivalent strategy has been employed to tackle difficult to treat viral infections such as anionic dendrimers with activity against HIV type-1 and herpes simplex virus type-2 (HSV-2), which are currently in phase 3 clinical trials.[96] Peptide dendrimers are another kind of macromolecules suited for drug delivery,[97] such as an antimicrobial for instance, and have been employed against multidrug-resistant *Acinetobacter baumanni* and *Pseudomonas aeruginosa*.[98]

Specifically, the dendrimers reported here are fourth generation poly(amidoamine) dendrimers (G(4)-PAMAMs)[63] functionalized with quaternary ammonium endgroups (Figure 5). Quaternary ammonium compounds (QACs) represent a well-established type of cationic surfactant known for having antibacterial activity.[73] The QAC chosen for the studies reported in this paper is 4-aza-1-hexadecylazoniabicylo-[2.2.2]octane, or C_{16}-DABCO.[99]

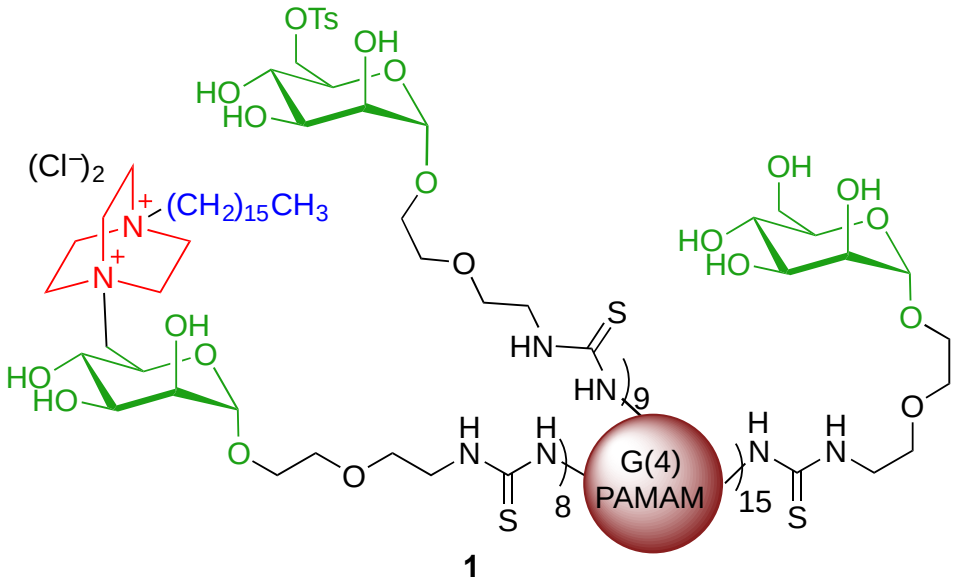


Figure 5**.** Highly cationic dendrimer antibiotic **1**.
The red sphere represents the G(4)-PAMAM. The black portion is the linker to the carbohydrate, the mannoside is shown in green, DABCO is shown in red, and the hexadecyl hydrocarbon chain is blue.

In addition to the C_{16}-DABCO endgroups, mannoside endgroups have also been incorporated into the design of the antimicrobial dendrimers since bacteria can be intercepted before they adhere to the host cell surface by multivalent carbohydrates. Small mannose-containing glycodendrimers, for example, inhibit the interaction between *E. coli* and mannan 10 to 100-fold more effectively than methyl mannoside.[100] The C-6 primary hydroxyl group on mannose has the added advantage of providing a convenient point of attachment for the C_{16}-DABCO endgroup. Thus, the C_{16}-DABCO and mannoside functionalized PAMAM dendrimers **1** are designed to deliver a catastrophic dose of positive charge to the bacteria in a highly localized (multivalent) fashion. In this case, this high localized concentration of active group delivered by the dendrimer results in a more effective

species than a less localized concentration of active group that is not presented multivalently.

In addition to reporting the synthesis and characterization of **1**, the minimum inhibitory concentration (MIC) values for **1** were obtained against a series of Gram positive and Gram negative bacteria, and these values are reported here. MIC values for dendrimers were compared to monomeric control compounds. Red blood cell hemolysis and mammalian cell toxicity assays were performed, and results were compared to the MIC values obtained against bacteria. Complete inhibition of the growth of *S. aureus* biofilm was observed for membranes pre-treated with **1**. Finally, in order to determine whether bacteria are more or less likely to develop resistance to multivalent antibacterial compounds such as **1** than to monovalent compounds, MIC values for *E. coli* and *B. cereus* that were grown in the maximum tolerable sub-inhibitory concentrations of **1** were determined for up to 50 growth cycles. The MIC values, biofilm inhibition studies, and resistance assays all indicate that **1** is an interesting new multivalent antibacterial compound.

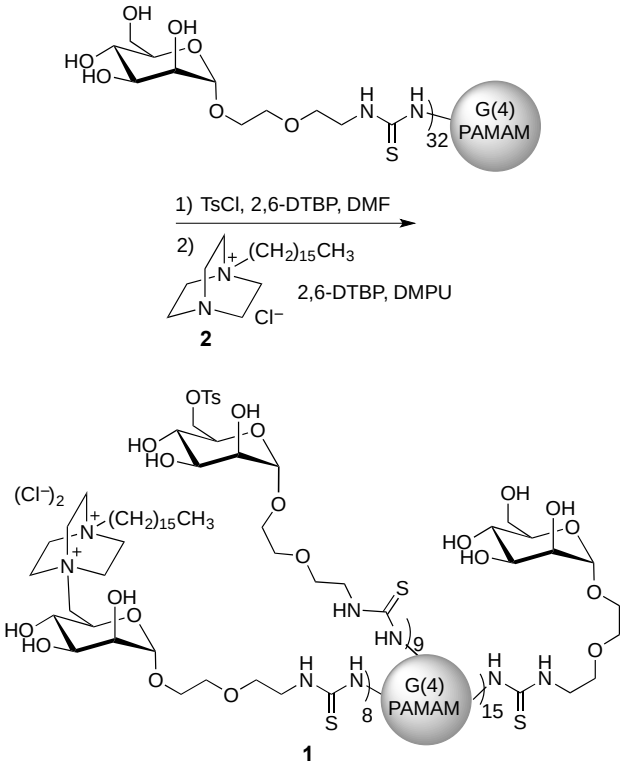
Results and Discussion

Synthesis of C_{16}-DABCO Dendrimers.

The synthesis of **1** is shown in Scheme 1. Mannose functionalized G(4)-PAMAM dendrimers were prepared using previously described methods Woller, et al. [19] The primary hydroxyl group of mannose was tosylated, and S_N2 displacement of the tosyl group using chloro 4-aza-1-hexadecyl-azoniabicylo[2.2.2]octane[99] (**2**) afforded product **1**. The degree of mannose functionalization of the substrate dendrimer was determined using MALDI-TOF MS with both the mannose functionalized dendrimers and their precursor peracetylated mannose functionalized dendrimers.[19] The average number of C_{16}-DABCO endgroups on **1** was determined using the weight average molecular weight (M_W) as determined by MALDI-TOF MS, ratio of C_{16}-DABCO and tosyl groups were found using the ratios of methyl group on both C_{16}-DABCO and tosyl from ^{1}H NMR, and chemical transformations were also confirmed by ^{1}H NMR. For dendrimer **1**, the M_W values indicate that an average of 32 terminal amines of the G(4)-PAMAM dendrimer were functionalized with mannose, and 8 of those 32 received a C_{16}-DABCO. Nine mannosides per dendrimer retained their tosyl group on average. The C_{16}-DABCO was not able to completely displace the tosyl group presumably due to the steric bulk of the dendrimer and the C_{16}-DABCO nucleophile. Zeta potential measurements on the mannose-functionalized dendrimer indicate that the unfunctionalized amines on the mannose-functionalized dendrimer substrate are not surface accessible (Figure 43). Mannose functionalized PAMAM dendrimers

typically have a neutral zeta potential whereas, the addition of C_{16}-DABCO to the dendrimers results in a positive zeta potential indicating these dendrimers are cationic. (Figure 43)

Scheme 1. Synthesis of C_{16}-DABCO and mannose functionalized dendrimer **1**.



Determination of the Minimum Inhibitory Concentrations.

The antimicrobial activities, in terms of MIC (minimum inhibitory concentration, or the lowest concentration that prevents bacteria from proliferating), of C_{16}-DABCO dendrimer **1** and of the monomeric control compound **2** have been determined and are shown in Table 1. Values in Table 1 are reported on a per active unit (per C_{16}-DABCO basis), and in addition, concentration per molecule/dendrimer are shown in parenthesis afterward. Control MICs can be found in Table 3 in the appendix. Both Gram positive (*S. oralis*, *S. aureus*, and *B. cereus*) and Gram negative (*P. aeruginosa* and *E. coli*) strains were studied. Of all the strains of bacteria that were tested, *Streptococcus oralis* had the highest MIC value by at least an order of magnitude compared to the MIC values obtained for the rest of the strains. The MIC for C_{16}-DABCO dendrimer **1** with *S. oralis* is denoted as greater than 170 μM (20 μM, per dendrimer) because this was the maximum amount of **1** that was readily dissolved, and the MIC value was above the solubility limit. The rest of the Gram positive strains had MIC values with **1** that were two orders of magnitude lower than the MIC value for *S. oralis*. *B. cereus* and *S. aureus* required the lowest concentration of **1** to achieve inhibition at 1.1 μM (0.13 μM, per dendrimer). The MIC values for the two Gram negative strains (*P. aeruginosa* at 16 μM (2.0 μM, per dendrimer) and *E. coli* at 1.1 μM (0.13 μM, per dendrimer)) were one order of magnitude less than the MIC value for *S. oralis*. C_{16}-DABCO dendrimer **1** appears to be more effective against *S. aureus* and *B. cereus* than *P. aeruginosa* and *E. coli* bacteria. *S. oralis* had the highest MIC tested. The

explanation for the high MIC could lie in the composition of the outer surface of the bacteria.

Table 1. Minimum Inhibitory Concentrations (MICs) for Dendrimer **1** and Monomer **2**.

Gram	Microorganism	MIC C_{16}-DABCO Dendrimer **1** per C_{16}-DABCO **(**per dendrimer)	MIC C_{16}-DABCO Monomer **2**
+	*Streptococcus oralis*	>170 (μM) (20 μM)	3000 (μM)
+	*Staphylococcus aureus*	1.1 (0.13)	11
+	*Bacillus cereus*	1.1 (0.13)	17
-	*Pseudomonas aeruginosa*	16 (2.0)	330
-	*Escherichia coli*	11 (1.1)	150

When dendrimer **1** and monomer **2** are compared, it becomes clear that multivalency does improve the MIC. In all cases, the MIC value is at least 10-fold higher for the C_{16}-DABCO monomer 2 than for C_{16}-DABCO dendrimer **1** on a per C_{16}-DABCO basis. Both the dendrimer and the monomer are below their critical micelle concentrations (CMC), indicating that they are not acting as aggregated species (Figure 44). One likely explanation for the multivalent enhancement in activity exhibited by the highly cationic dendrimer antibiotic relative to the monomer is that the polycationic dendrimer displaces native cations associated with surface associated adhesion molecules, lipopolysaccharides (LPS) for Gram negative bacteria or lipoteichoic acid (LTA) of Gram positive bacteria. This interaction of **1** with LPS or LTA likely alters the standard LPS or LTA cross-linking interactions, destabilizing the outer membrane.[101,102] Zeta potential measurements on **1** suggest that the dendrimer does not become polycationic until conjugation with C_{16}-DABCO (Figure 43). Since the polycationic form of an unmodified PAMAM dendrimer generally has more antimicrobial activity over neutral or anionic unmodified PAMAM dendrimers and the data suggests the primary amines are not available for membrane interaction, a positive control for the unmodified PAMAM dendrimer was not performed. However, other groups have performed experiments to determine the antimicrobial activity of an unmodified PAMAM dendrimer.[103,104]

Hemolysis and Mammalian Cell Toxicity Assays.

Hemolysis of red blood cells (RBCs) serves as a good indication of how compounds interact with cellular membranes, particularly mammalian membranes. RBC hemolysis assays were performed with **1**, **2**, mannose functionalized G(4)-PAMAM dendrimer, and unfunctionalized G(4)-PAMAM dendrimer (Table 2). Results with dendrimers and **2** were compared to control trials in phosphate buffered saline (PBS) alone (unlysed RBC control) and in PBS with 1% Triton-X100 (near total lysis control). G(4)-PAMAM up to a concentration of 34 μM showed negligible lysis, comparable to the PBS control. Significant lysis occurred only when the concentration of G(4)-PAMAM reached 68 μM. (Figure 48 shows a graph of the hemolysis data.)

Table 2. Minimum hemolysis concentrations.

COMPOUND	MINIMUM CONCENTRATION WITH OBSERVED HEMOLYSIS
DENDRIMER **1**	2.5 (0.6, per dendrimer) μM
MONOMER **2**	1.2 μM
MANNOSE-FUNCTIONALIZED G(4)-PAMAM	Not hemolytic at concentrations studied
UNFUNCTIONALIZED G(4)-PAMAM	68 μM
PBS	Not detected
1% TRITON-X100 IN PBS	Hemolytic control (near total hemolysis)

Mannose functionalized G(4)-PAMAM dendrimer displayed significantly lower hemolytic activity when compared to unfunctionalized G(4)-PAMAM dendrimer at similar concentrations most likely due to the burying of the primary amines after the addition of mannose (mannose functionalized dendrimers did not induce hemolysis at the concentrations tested). Hemolysis of RBCs was observed at and above 2.5 μM (0.6 μM, per dendrimer) for C_{16}-DABCO functionalized dendrimer **1** (concentrations reported per C_{16}-DABCO). The most potently hemolytic compound tested was C_{16}-DABCO monomer control compound **2**, and significant hemolysis was observed at 1.2 μM. The concentration required for C_{16}-DABCO dendrimers to lyse RBCs is within the same order of magnitude as the MIC for Gram positive and Gram negative organisms tested, indicating broad rather than selective activity for **1**. Since hemolysis was not observed for mannose functionalized dendrimers prior to the addition of C_{16}-DABCO, the activity of the dendrimer was attributed to the C_{16}-DABCO endgroups.

Additional studies to determine toxicity of C_{16}-DABCO dendrimers and C_{16}-DABCO monomers were performed using MTS and A549 human lung carcinoma cells (ATCC# CCL-185). Observable toxicity of C_{16}-DABCO dendrimer **1** toward A549 cancer cells was seen at 8.8 μM (1.1 μM, per dendrimer). This value is similar to the MIC values obtained against the Gram negative bacteria tested and is almost an order of magnitude larger than the MIC values for **1** with *E. coli* and *P. aeruginosa* (Table 1) suggesting there is little to no selectivity for **1** acting against A549 over the bacterial strains tested. The C_{16}-DABCO dendrimer **1** was more

than an order of magnitude more potent than monomer **2** (8.8 μM (per C_{16}-DABCO) and 190 μM for **1** and **2**, respectively).

Taken together, the results from the hemolysis and the toxicity assays indicate that **1** is active against mammalian cells, presumably their membranes. In terms of toxicity, addition of C_{16}-DABCO to the dendrimer scaffold generally increases the toxicity with the exception being red blood cells. The multivalent presentation of C_{16}-DABCO appears to increase membrane activity. Changing alkyl chain length on the DABCO subunit or altering the ratios of unfunctionalized mannose and C_{16}-DABCO residues could increase the selectivity for bacteria over human cells. Alternatively, compounds such as **2** may be more ideally suited for broader decontamination protocols in which activity against human cells and microorganisms is desired.

Biofilm Disruption and Formation Studies.

Experiments involving biofilm disruption and inhibition of formation were carried out to determine if the C_{16}-DABCO dendrimers had activity against biofilms. To determine whether 1 disrupts biofilm stability after it has been established, the established biofilms were treated during day 1 and day 3 with C_{16}-DABCO dendrimer **1**, C_{16}-DABCO monomer **2**, or mannose functionalized G(4)-PAMAM dendrimer. No inhibition or knock-back of biofilms in several species (Gram positive and negative) was observed, as determined by log-kill platings (data not shown). The most likely explanation for the dendrimers' lack of activity in biofilm disruption assays is that the dendrimers are too large to penetrate into the biofilm and are restricted to the biofilm surface. This result is as expected, as relatively few compounds are lethal to established biofilms.[105,106-107,35,41]

Experiments aimed at inhibiting biofilm growth at the membrane surface where biofilms are inoculated and attach exhibited much more dramatic results. Membranes pre-treated with C_{16}-DABCO dendrimer **1** at 274 μM (33.3 μM, per dendrimer) C_{16}-DABCO, mannose functionalized G(4)-PAMAM dendrimer, saline control (negative) or 10% bleach control (positive) were inoculated with *S. aureus* and allowed to grow for three growth cycles, after which membranes were disrupted and diluted in DE neutralizing broth or PBS for log plating. Figure 6 shows a representative trial for triplicate experiments revealing the results of dilution log platings of day three *S. aureus* biofilms from membranes pre-treated with 10% bleach (left column), 274 μM (33.3 μM, per dendrimer) **1** (middle column)

and saline (right column). No visible biofilms were present on membranes pre-treated with either 10% bleach or C_{16}-DABCO dendrimer **1** at day three, and no colonies grew from dilution log platings representing those membranes (Figure 6 left and middle columns). In contrast, robust biofilms were observed on membranes pre-treated with saline and colony growth was evident from dilution log platings of those membranes, as expected (Figure 6 right column). Pre-treatment of membranes with a lower concentration of C_{16}-DABCO dendrimer **1** (137 μM (16.6 μM, per dendrimer)) or with mannose functionalized G(4)-PAMAM dendrimers lacking the C_{16}-DABCO endgroups at 1mg/mL or 68 μM did not inhibit biofilm formation, nor did pre-treatment with a 1 mg/mL or 2962 μM solution of **2**. This suggests a high concentration of **1** is necessary to inhibit *S. aureus* biofilms.

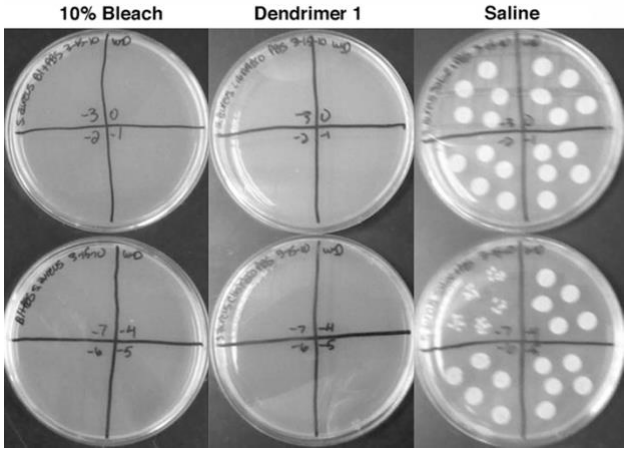


Figure 6. Biofilm disruption studies.
Dilution plating of *S. aureus* day three biofilms. Membranes were pre-treated with 10% bleach (column 1), 1mg/mL (274 μM (33 μM, per dendrimer)) C_{16}-DABCO dendrimer **1** (column 2) or saline (column 3) and allowed to dry before inoculation with *S. aureus*.

As shown in Figure 6, the pre-treatment of the biofilm inoculation surface with an appropriate dose of **1** causes complete inhibition of the growth of *S. aureus* biofilms, and these results may have important implications for development of new materials that inhibit biofilm growth for medical and other commercial applications.[106,108]

Multistep Resistance Selections Studies.

Because development of resistance is such a widespread problem for antimicrobial agents,[74, 79, 82] studies were performed to determine the degree to which bacteria are able to develop resistance to dendrimer **1**. Following the protocol put forward by the International Organization for Standardization,[109] bacteria were grown in the highest tolerated sub-inhibitory concentration of **1** or **2**, and MIC values were determined after each growth cycle. The results for the multistep resistance selection studies are shown in Figure 7, where MIC values are shown as a function of growth cycle. Figure 7a shows the effect that repeatedly growing the *E. coli* in the highest tolerated sub-inhibitory concentration of **2** has on the MIC values against the *E. coli* for **2**, for cephalexin, and for ampicillin. Figure 7a reveals that the MIC value of the monomeric C_{16}-DABCO control compound **2** increased significantly more over the seventeen growth cycles than the MIC values increase for cephalexin and ampicillin for *E. coli* grown in the presence of 2.

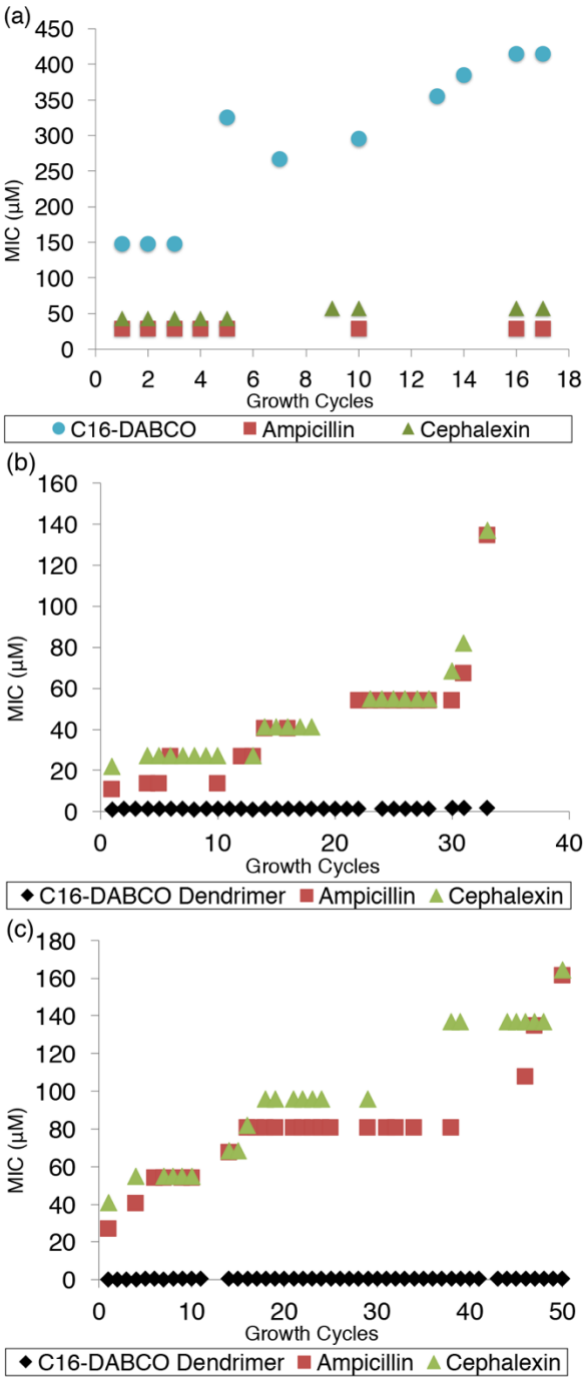


Figure 7. Resistance selection studies.
All graphs represent bacteria grown in sub inhibitory concentration of antibiotics and show the MIC changes over time. (A) *E. coli* grown in sub inhibitory concentration of C16-DABCO monomer **2**. (B) *E. coli* grown in sub inhibitory concentration of C16-DABCO dendrimer **1**. (C) *B. cereus* grown in sub inhibitory concentrations of C16-DABCO dendrimers **1**.

In Figure 7b and 7c, respectively, results are shown for *E. coli* and *B. cereus* that were repeatedly grown in the presence of the highest tolerated sub-inhibitory concentration of **1**. As shown in Figure 7b, the MIC value for **1** only increased from 1.1 μM (0.13 μM, per dendrimer) to 1.6 μM (0.20 μM, per dendrimer) over a 33 day period, while the MIC values for cephalexin and ampicillin increased much more: 22 μM to 137 μM for cephalexin and 11 μM to 134 μM for ampicillin. As shown in Figure 7c, the MIC value for **1** increased from 0.11 μM (0.013 μM, per dendrimer) to 0.60 μM (0.073 μM, per dendrimer) over a 50 day period, while the MIC values for cephalexin and ampicillin increased much more: 41 μM to 164 μM for cephalexin and 27 μM to 162 μM for ampicillin.

The graphs in Figure 7b and 7c reveal a pattern in which the MIC values do not change for several growth cycles, and then an increase in MIC value is observed. These increases in the MIC values most likely occur because of alterations undergone by the bacteria in order to decrease their susceptibility to the multivalent dendrimer antibiotic. When compared to the ampicillin and cephalexin controls, a much smaller increase in MIC value was obtained for 1 with both *E. coli* and *B. cereus*. The MIC values for **1** remained significantly lower for the duration of the assay than the values for cephalexin and ampicillin.

The difference between the results with multivalent **1** when compared to monomeric **2** and to the cephalexin and ampicillin controls suggests that multivalency is a viable concept for the development of antibacterial compounds to which bacteria are less likely to develop resistance.

Conclusion

The synthesis and characterization of a mannose functionalized G(4)-PAMAM dendrimer bearing quaternary ammonium endgroups comprised of DABCO with a hexadecyl hydrocarbon chain are reported. This dendrimer (compound **1**) presumably utilizes both the mannoside and the quaternary ammonium endgroups to interact detrimentally with the bacterial cell wall. The antibacterial properties of the dendrimer were assessed using both Gram positive and Gram negative strains of bacteria. Minimum inhibitory concentration values for **1** against *S. oralis*, *S. aureus*, *B. cereus*, *P. aeruginosa*, and *E. coli* were obtained and were compared to the MIC values of monomeric C_{16}-DABCO control compound **2**. Dendrimer **1** is in all cases at least 10 times more potent as an antibiotic than the monomeric control compound **2**. Of the bacterial strains tested, dendrimer 1 is most active against Gram positive bacteria *S. aureus* and *B. cereus*, which both have MIC values in the low micromolar range. Toxicity studies with A549 lung cancer cells and hemolysis assays with red blood cells indicate compound **1** is active against mammalian cells as well. When presented multivalently, C_{16}-DABCO is more toxic to bacteria and mammalian cells. Although biofilm disruption was not observed using **1**, complete inhibition of the growth of *S. aureus* biofilm was achieved for membranes that were pre-treated with **1**. Finally, using a series of resistance assays, bacteria were shown to be less likely to successfully develop resistance to multivalent antibacterial compound **1** than to the monovalent comparison compound **2** or to other small molecule antibiotics.

MIC values for *E. coli* and *B. cereus* that were grown in the maximum tolerable sub-inhibitory concentrations of **1** were determined for up to 50 growth cycles. Overall, the results reported here for C_{16}-DABCO functionalized dendrimer **1** indicate that multivalent displays of antimicrobial agents afford macromolecules with increased activity against bacteria both in solution and in biofilm relative to the monomeric endgroups (active group) alone and that multivalency can be used to thwart the development of bacterial resistance.

Materials and Methods

Materials. All standard chemicals and reagents were purchased from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Alfa Aesar (Johnson Matthey Company, Ward Hill, MA, USA), or Fisher (Fisher Scientific, Hampton, New Hampshire, USA) and were used without further purification. PAMAM dendrimers were purchased from Dendritech (Dendritech, Midland, MI, USA). C_{16}-DABCO monomer was provided by Dr. Robert Engel (R. Engel Laboratory, Queens, NY, USA). Cephalexin was obtained from MP Biomedicals (Santa Ana, California). Both ampicillin and streptomycin were purchased from Fisher (Fisher Scientific, Hampton, New Hampshire, USA). Bacterial cultures and adenocarcinoma cell line (A549) were obtained from the American Type Culture Collection and are as follows: *Escherichia coli* (ATCC #25922), *Bacillus cereus* (ATCC #11778), *Pseudomonas aeruginosa* (ATCC #27853), *Staphylococcus aureus* (ATCC #29213), *Streptococcus oralis* (ATCC #35037), and A549 cell line (ATCC #CCL-185). Rabbit blood was purchased from QuadFive (Ryegate, Montana) with EDTA as the anticoagulant.

Methods. Reactions were monitored via TLC. TLC was performed on silica gel glass plates containing 60 G F -254 and visualization was achieved with a UV light or a cerium ammonium molybdate stain. Column chromatography was performed on Silicycle 230-400 mesh silica gel. ^{1}H and ^{13}C spectra were performed on Bruker DRX500 (500 MHz) or Bruker DRX600 (600 MHz). Chemical shifts (δ) were reported in ppm downfield from internal TMS standard. Absorbances were

determined on a Molecular Devices Spectramax Plus 384. MALDI spectra were recorded on a Bruker III Biflex with a 337 nm nitrogen laser using freshly recrystallized trans-3-indolacrylic acid as a matrix. Electrophoretic mobility measurements were determined on a Wyatt Technologies Mobiuζ DLS instrument. Critical micelle concentration measurements were taken on a 90 plus Particle Size Analyzer made by Brookhaven Instruments Corporation.

Synthesis of generation 4 PAMAM-based thiourea 1-O-(5-thiourea-3-oxapentyl)-6-(1-hexadecyl-1-azonia-4-azabicyclo-[2.2.2]octane)-α-D-mannopyranoside dendrimer **1**. Mannose functionalized dendrimer (300 mg, 0.01 mmol) was dissolved in DMF (1.5 mL) and cooled to 0 °C. TsCl (115 mg, 0.6 mmol) was added with 2,6-di-*tert*-butylpyridine (115 mg, 0.134 mL, 0.6 mmol). The reaction was allowed to warm to RT and was stirred for 2.5 h. DMF was removed at reduced pressure leaving a pale yellow fluffy solid. This solid was dissolved in DMPU to make a 75 mmol solution with respect to the dendrimer, 2,6-DTBP (112.8 mg, 0.59 mmol) was added, and C_{16}-DABCO (165.0 mg, 0.488 mmol) was also added. The reaction was allowed to stir for two days. The reaction mixture was dialyzed in water (1000 M_w cutoff), and lyophilized to a white fluffy powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.49 (d, J = 7.7 Hz, 3H), 7.12 (d, J = 7.9 Hz, 3H), 4.88 – 4.46 (m, 3H), 3.80 – 3.29 (m, 2H), 3.28 – 3.07 (m, 15H), 3.07 – 2.94 (m, 9H), 2.75 (s, 1H), 2.29 (s, 3H), 1.75 – 1.55 (m, 2H), 1.24 (s, 24H), 0.85 (t, J = 6.7 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 146.14, 146.13, 138.06, 138.06, 128.49, 125.93, 63.81, 51.18, 47.82, 46.97, 44.17, 40.89, 40.57, 40.48, 40.41, 40.32, 40.15, 39.98,

39.82, 39.65, 39.48, 31.72, 29.49, 29.45, 29.37, 29.20, 29.13, 28.92, 26.15, 22.53, 21.59, 21.22, 14.40. MALDI-TOF MS 30,074 g/mol. (NMR data can be found in Figure 45 and Figure 46. MALDI data can be found in Figure 47)

General Procedures for MIC Determinations. The broth microdilution method described in ISO/FDIS 20776-1:2006(E)[109] was used to determine the minimum inhibitory concentration or MIC. The assay was performed in Nunc brand 96-well plates with Nunclon Delta Surface with an accompanying lid. These plates were chosen because they had the least amount of desiccation after an incubation period (18±2 hours). In addition, the wells closest to the edge were filled with deionized water to prevent the desiccation of the inner wells. Dilutions of each compound and controls were plated in triplicate. Cephalexin was obtained from MP Biomedicals (Santa Ana, California). Both ampicillin and streptomycin were purchased from Fisher Scientific (Hampton, New Hampshire).

General Procedure for Hemolysis Assay. Rabbit blood was purchased from QuadFive (Ryegate, Montana) in EDTA as the anticoagulant. Red blood cells (RBCs) were separated by centrifugation of 5 ml of received blood for 10 minutes at 900 rpm. Serum fraction was removed and discarded, and RBCs were washed with freshly prepared cold 0.9% saline three times. After the final wash, supernatant was removed and the volume adjusted to 5 ml with cold PBS and furthered diluted 1:10 in PBS as described previously.[92] 200 µl of RBCs were added to 800 µl of PBS with containing various concentrations of C16-DABCO dendrimer, C16-DABCO monomer, G(4)-PAMAM dendrimer, and mannose

functionalized G(4)-PAMAM dendrimer, 1% Triton X-100 as positive control, and PBS alone as negative control. Samples were incubated for 3 hours at 37 ºC and mixed by inversion every 30 minutes. After incubation samples were centrifuged for five minutes at 1300 rpm. Absorbance was determined at 540 nm and hemolysis was calculated using equation 1.

$$\%\ Hemolysis = \frac{[(OD\ of\ sample) - (OD\ of\ blank)]}{OD\ of\ positive\ control} \times 100 \qquad (1)$$

General Procedure for Toxicity Assay. Once a plate of A549 cells became fifty-percent confluent, growth medium was drained from plate and replaced with an EDTA solution. After a ten-minute incubation, cells were removed from the plate and centrifuged until a pellet was formed. EDTA solution was replaced with 2 mL of growth medium. Cells were diluted and stained in order to enumerate cells. 10,000 cells per 100 µL were aliquoted into a 96-well plate in each well. Analytes were added, Promega's CellTiter 96 AQ_{eous} One Solution Cell Proliferation Assay was performed. Using this method, the number of viable cells after exposure to an analyte was determined at A_{400}.

Resistance Selection Studies. Multistep resistance selection was performed using the broth microdilution method described in ISO/FDIS 20776-1:2006(E).[109] The procedure was slightly adapted from Kosowska-Shick et al.[110] The multistep resistance selection study is a MIC study with serial daily passages. The bacteria for the next day of the assay came from the MIC plate and the wells chosen were one dilution less than the MIC value. These wells had an optical density that was similar to the growth control wells. Since all of the dilutions were performed in

triplicate, all three wells of bacteria (~300 µL) were taken for the next day because the C_{16}-DABCO dendrimer caused noticeable lethargy in growth. The process was repeated daily for a maximum of 50 days or until the MIC had changed appreciably.

General Procedure for MALDI Sample Preparation. Compound **1** was dissolved in DMF at a concentration of about 6 mg/mL or about 12 pmol/µL. *Trans*-3-indoleacrylic acid (IAA) was dissolved in DMF at a concentration of 2.25 mg/mL or 12000 pmol/µL. IAA was recrystallized in warm ethanol. Compound **1** was mixed with IAA in concentrations ranging from 1:500 to 1:30000 where 1 µL compound **1** was mixed with 10 µL IAA to obtain a 1:1000. External calibration was performed with proteins such as myoglobin, bovine serum albumen, and trypsinogen in IAA as a matrix to obtain the reported *m/z*. Standards were dissolved in 80% acetonitrile : 20% water. Spectra were obtained on a Bruker Biflex III with a 337 nm nitrogen laser. *Trans*-3-indoleacrylic acid, myoglobin, bovine serum albumen, and trypsinogen were all obtained from Sigma-Aldrich.

CHAPTER THREE

PROBING THE LEC-1 AND LEC-10 OXIDATIVE STRESS PATHWAY IN *CAENORHABDITIS ELEGANS* USING GALβ1-4FUC DENDRIMERS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3
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Contributions: [Performed synthesis and oxidative stress assays]
Co-Author: [Rebecca Moore]
Contributions: [Aided in *C. elegans* studies including preparation of animals and collection of fluorescent images.]
Co-Author: [Dr. Mary Cloninger]
Contributions: [Principle investigator and aided in oxidative stress assays]
Co-Author: [Dr. Colleen Murphy]
Contributions: [Provided oversight of *C. elegans* and edited manuscript]

Manuscript Information

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CHAPTER THREE

PROBING THE LEC-1 AND LEC-10 OXIDATIVE STRESS PATHWAY IN *CAENORHABDITIS ELEGANS* USING GALβ1-4FUC DENDRIMERS

Introduction

Reactive oxygen species (ROS) have long been thought to play a critical role in the process of aging. It has been reported that lifespan is anti-correlated with metabolism (i.e., organisms with longer lifespans have lower rates of metabolism. Harman *et al.* proposed that ROS byproducts from essential metabolism can cause cellular senescence,[111] which contributes to and promotes faster aging. In order to study such a complicated process, a model organism with well-understood biochemical machinery is desirable to study the role of oxidative stress in aging. *C. elegans* is an excellent model organism to study aging due to its short life cycle, relatively short lifespan, amenable genetic and biochemical assays, and fully sequenced genome which has revealed many analogs between *C. elegans'* and humans' biochemical machinery.

In the *C. elegans* genome, many genes have been identified that have a role in both lifespan and resistance to oxidative stress. Mutations in the insulin/insulin-like growth factor signaling pathway (IIS), for example, lead to a lifespan extension. Mutations in *daf-2*, the sole *C. elegans* homolog of the mammalian insulin-like receptor, or in *age-1*,[112-113] a PI(3)K homolog that functions downstream of *daf-2*,[114] result in an extension in lifespan through the regulation of

DAF-16/FOXO.[115-116] Additionally, mutations in *old-1*,[117] a putative receptor of tyrosine kinase,[118] also result in an extension of lifespan. Mutations in all of these genes also led to a greater resistance to oxidative stress[2-7], due to the expression of a set of genes downstream of DAF-16/FOXO that includes several oxidative stress resistance genes[119], each of whom contribute to *daf-2*'s longevity. Therefore, understanding the role that oxidative stress plays in typical cellular activity is a challenging.

Galectins are known for their β-galactoside binding properties. These glycan binding proteins are conserved across nature, can be traced back to all metazoans[120], and are found in both humans and *C. elegans*. In humans, galectins have been implicated in many biological phenomena such as cellular crosstalk[16], embryonic development[121], tumor metastassis[122-123], proliferation[124-125], immunity[126], oxidative stress[127-128], and infection[129]. Roles of galectins in oxidative stress have also been proposed in *C. elegans*.[130-131] All galectins have a conserved eight-amino acid sequence.[23]

In order to explore the behavior of galectins in *C. elegans*, the major glyco-epitope in *C. elegans* needed to be identified. A glyco-epitope refers to a carbohydrate with a specific configuration. The major human epitopes such as Gal-GlcNAc have not been isolated in *C. elegans*,[59] so it was desirable to find an epitope that was naturally occurring in *C. elegans*. Takeuchi, *et. al.* demonstrated that a Gal-Fuc residue was recognized with good affinity by LEC-6, which shares binding partners with LEC-1 and LEC-10 and is endogenous to *C. elegans*.[59]

Possible Gal-Fuc ligands with different linkages were labeled with a fluorescent tag for binding studies with galectins in *C. elegans*, and Galβ1-4Fuc was shown to bind well to LEC-1 and LEC-6.[60] Therefore, Galβ1-4Fuc, which has been identified in several invertebrates including C. *elegans,*[132] was proposed to be the endogenous glyco-epitope in *C. elegans*.[60, 133] Human galectins have also been shown to bind this disaccharide.[134]

To date, eleven members of the *C. elegans* galectin protein family have been isolated and purified.[23] LEC-1 (a tandem repeat galectin)[135] and LEC-10 (a chimeric galectin)[135] have been studied in oxidative stress experiments.[130-131] *Lec-1* knockout mutants have increased susceptibility to both hydrogen peroxide and paraquat, two oxidizing agents, and *lec-10* knockout mutants are more susceptible to oxidative stress (hydrogen peroxide).[130] These results indicate that *lec-1* and *lec-10* play a role in protecting *C. elegans* from the effects of oxidative stress.

Multivalency is important for many biological processes including galectin/galactoside binding in mammalian systems.[18] Binding events between monovalent carbohydrate ligands and protein binding partners are typically weak and in the range of 10^{-3} to 10^{-4}M.[18] A significant enhancement in apparent affinity can be seen when the carbohydrate ligands are presented multivalently in what is referred to as the “cluster glycoside effect.”[6] We hypothesized that a multivalent presentation of the Galβ1-4Fuc ligand would take advantage of the cluster glycoside effect when binding to LEC-1 and LEC-10 in *C. elegans*. We further

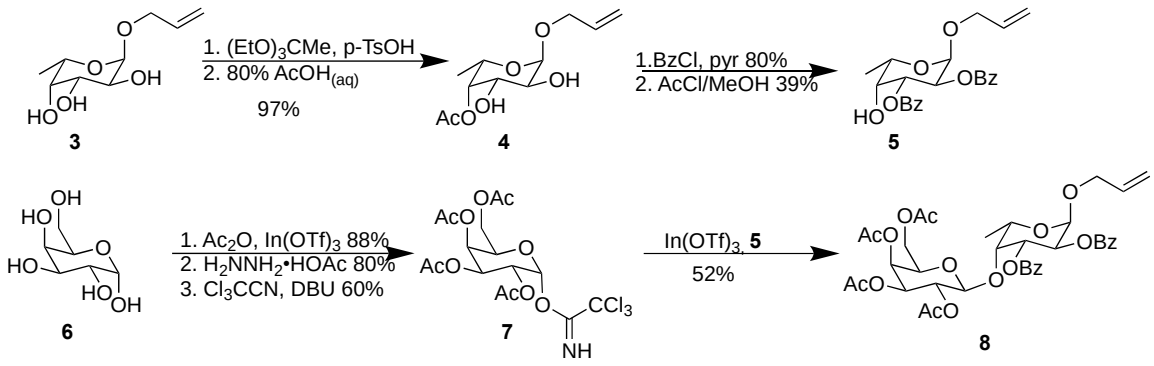
hypothesized that this multivalent association could function to protect the organism during periods of high oxidative stress.

In this study, Galβ1-4Fuc functionalized dendrimers were synthesized and used in oxidative stress assays with *C. elegans*. Different generations of dendrimers were synthesized to determine whether the size of the framework or the number of carbohydrate ligands impacts the multivalent protein-carbohydrate interaction in *C. elegans*. In addition, a fluorescent label was appended to Galβ1-4Fuc functionalized dendrimers, and these glycodendrimers were used to determine the localization of the glycodendrimers in *C. elegans*.

Results

Synthesis of Galβ1-4Fuc.

Scheme 2. Synthesis of protected Galβ1-4Fuc.

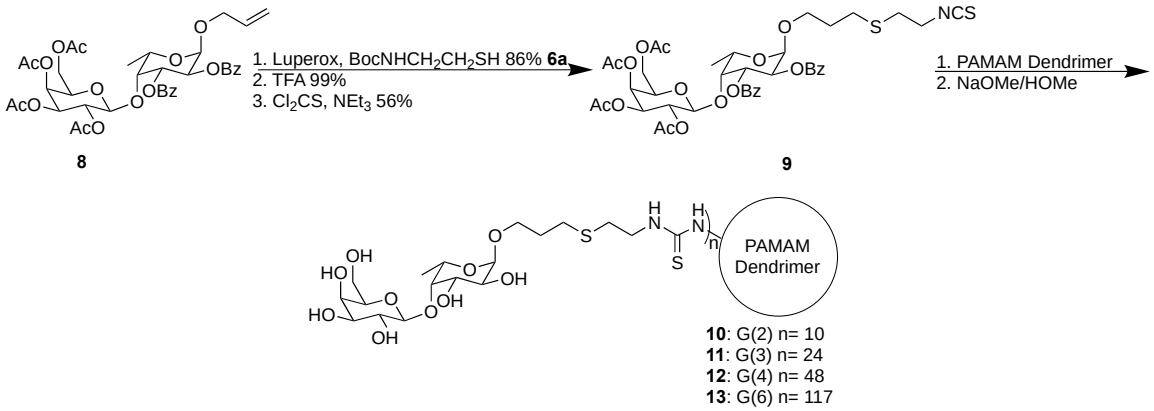


The synthesis of the fully protected disaccharide is shown in Scheme 2. α-L-Allylfucoside[136] **3** was treated with trimethylorthoacetate to generate an orthoester intermediate, which was treated with 80% aqueous acetic acid to afford 4-acetylallylfucoside (**4**) using a previously established procedure.[137] The remaining hydroxyl groups were protected using benzoyl chloride and standard conditions. In order to form the 1-4 linkage, the acetyl group needed to be selectively cleaved. This was done using dilute acidic conditions following an established procedure to afford product **5**.[133]

Synthesis of trichloroacetimidate **7** began with the peracetylation of galactose using $In(OTf)_3$ as the Lewis-acid mediator[138] followed by selective removal of the acetyl group at the anomeric position with hydrazine acetate. Using DBU as a base, treatment with trichloroacetonitrile formed the trichloroacetimidate

7. The 1-4 linkage of disaccharide **8** was formed by reacting both requisite halves **5** and **7** with indium triflate.[62]

Scheme 3. Synthesis of Galβ1-4Fuc functionalized dendrimers.



With disaccharide **8** in hand, the allyl group was elaborated using Boc protected aminoethanethiol as shown in Scheme 3 to form **8a** (not shown). Once liberated, the free amine was converted to isothiocyanate **9** with thiophosgene. The formation of a thiourea linked the disaccharide to the dendrimer. Protecting groups were removed using methanolic sodium methoxide (Zemplén conditions). Galβ1-4Fuc functionalized generation 2, 3, 4, and 6 dendrimers **10-13** were synthesized in this manner. The degree of Galβ1-4Fuc functionalization of the substrate dendrimer was determined using MALDI-TOF MS and ^{1}H NMR with both the Galβ1-4Fuc functionalized dendrimers and their precursor, ester protected Galβ1-4Fuc functionalized dendrimers. The average number of Galβ1-4Fuc was determined using the weight-average molecular weight (M_W) as determined by MALDI-TOF MS. Chemical transformations were confirmed with ^{1}H and ^{13}C NMR, and mass spectrometry.

Reduction of Galectin Expression Increases Susceptibility to Oxidative Stress

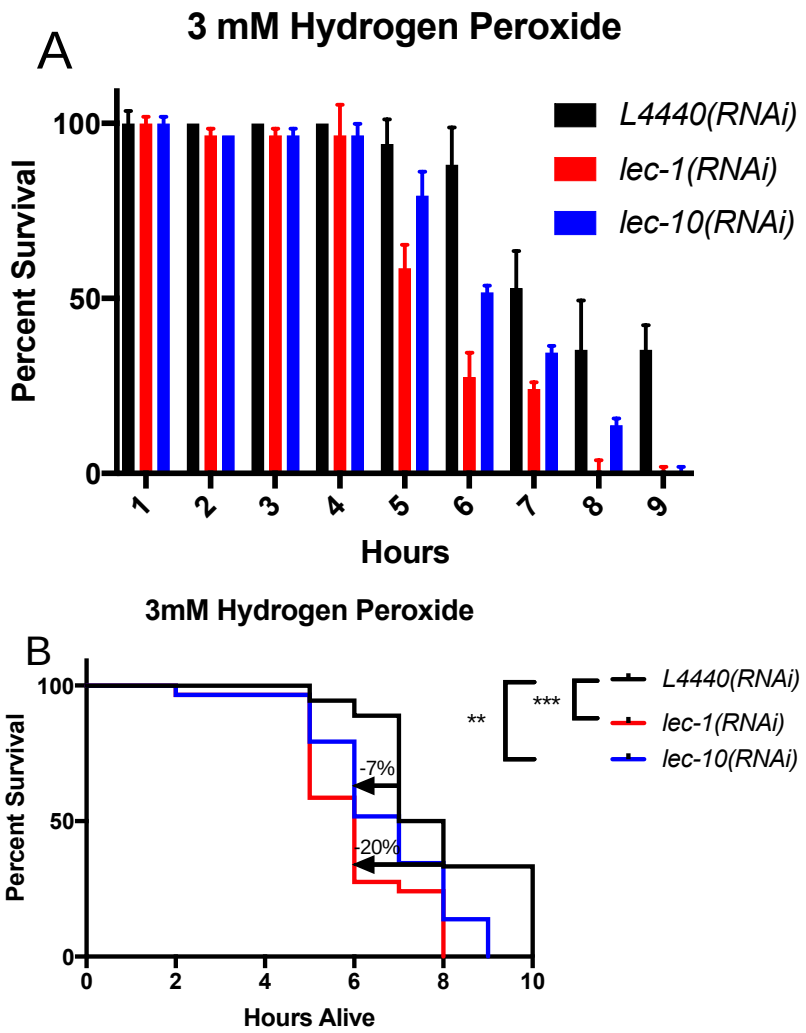


Figure 8. Oxidative stress assay with galectin knockouts.
C. elegans are sensitive to oxidative stress, such as hydrogen peroxide. Knockdown of *lec-1* or *lec-10* with RNAi result in increased susceptibility to hydrogen peroxide compared to the organisms given the empty vector (*L4440(RNAi)*) treated control. A) Average survival at each point (n = 30) at 3 mM hydrogen peroxide. B) Kaplan-Meir survival analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. *Lec-1(RNAi)* median survival was 7% lower than *L4440(RNAi)* and *lec-10(RNAi)* had a median survival 20% less than *L4440(RNAi).*

In order to study galectin/glycan interactions in *C. elegans*, survival assays in the presence of 3 mM hydrogen peroxide were performed on animals treated with RNAi to knockdown *lec-1* (referred to *as lec-1(RNAi)*) and *lec-10* (referred to *as lec-10*i). As shown in Figure 8, *lec-1(RNAi)* and *lec-10(RNAi)* worms are more susceptible to oxidative stress than the empty vector treated controls (referred to as *L4440(RNAi)*, which is consistent with prior results using *lec-1 or lec-10* knockout mutants.[130-131] A 7 percent reduction in median survival for *lec-1(RNAi)* compared *L4440(RNAi)* and a 20 percent reduction for *lec-10(RNAi)* can be seen. A survival assay was also performed in the presence of 6 mM hydrogen peroxide, which was also consistent with prior results (6 mM oxidative stress experiments not shown).

Considering multivalency is important for galectin/galactoside binding in mammalian systems, we hypothesized that *C. elegans* incubated with Galβ1-4Fuc functionalized dendrimers prior to oxidative stress (i.e., hydrogen peroxide) could display increased survival rates when challenged with oxidative stress agents, such as hydrogen peroxide. *C. elegans* were subjected to survival assays as previously described, except worms were incubated with Galβ1-4Fuc functionalized dendrimers **10**, **11**, **12**, or **13** for one hour prior to hydrogen peroxide addition.

Figure 9A and Figure 10A-C show the percent survival of *C. elegans* in the presence of Galβ1-4Fuc functionalized generation 2 (G2) dendrimer **10** and hydrogen peroxide. *lec-1(RNAi)* worms had greater survival rates when pre-

incubated with **10** than worms that had not received pretreatment with dendrimer **10**. For the first two hours after peroxide addition, all three RNAi treated worms had indistinguishable survival rates. A significant difference in survival was noted 3h post challenge with hydrogen peroxide. Both *L4440(RNAi) and lec-10(RNAi)* treated worms had better survival in the presence of G2 dendrimer **10** than those that did not receive glycodendrimer, indicating that dendrimer **10** does protect *C. elegans* from oxidative stress. The survival plots displayed in Figure 10A and 10C show that both *L4440(RNAi)* and *lec-10(RNAi)* worms had a 33 percent increase in their median survival. It appears that *lec-1(RNAi)* pre-treated worms did not benefit from the glycodendrimer but did seem to benefit from the 1 h pre-incubation period in M9 or in dendrimer (compare Figure 8B with Figure 10B).

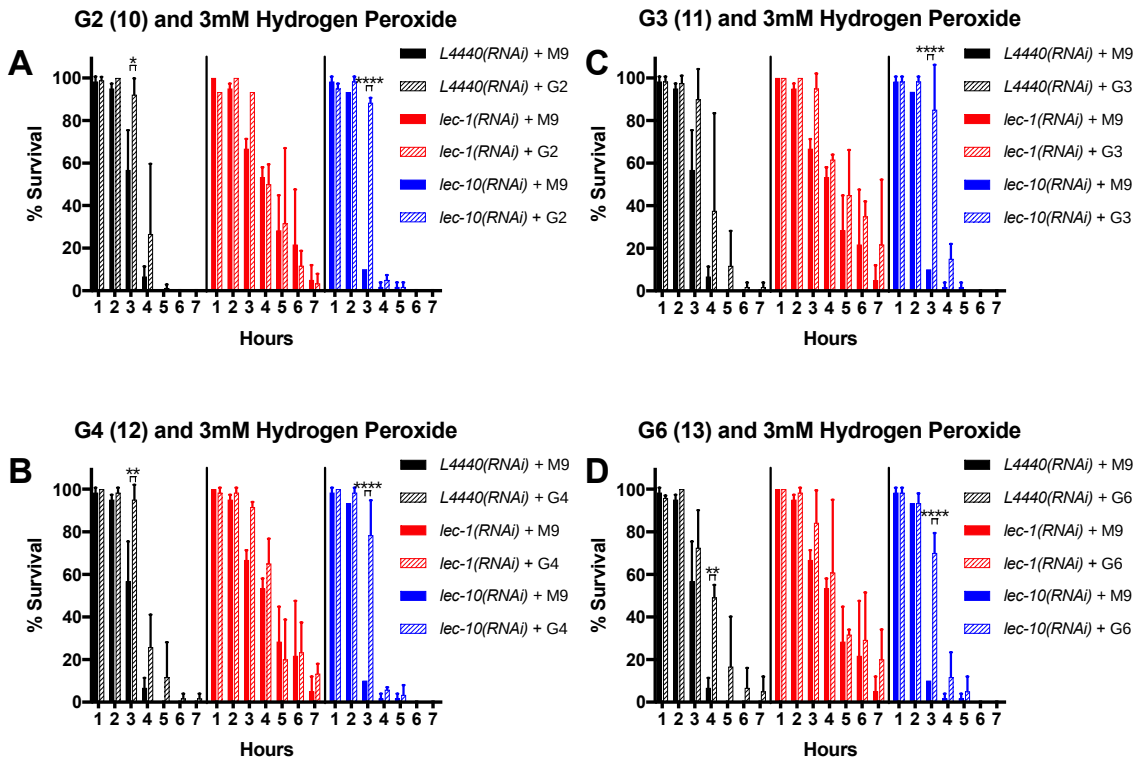


Figure 9. Oxidative stress assays with dendrimers.
Galβ1-4Fuc functionalized multivalent glycodendrimers improve survival of *C. elegans* challenged with hydrogen peroxide. All panels present the comparison of survival rates for *lec-1(RNAi)*, *lec-10(RNAi)*, and L4440i worms in the presence or absence of glycodendrimers (4 mg/mL). These worms were incubated for an hour in glycodendrimer solution or M9 buffer before the hydrogen peroxide (3 mM) was added. A) Galβ1-4Fuc functionalized generation 2 PAMAM dendrimer **10** (0.56 mM). B) Galβ1-4Fuc functionalized generation 4 PAMAM dendrimer **12** (0.11 mM) C) Galβ1-4Fuc functionalized generation 3 PAMAM dendrimer **11** (0.24 mM) D) Galβ1-4Fuc functionalized generation 6 PAMAM dendrimer **13** (0.04 mM). *p<0.05, **p<0.01, ****p<0.0001.

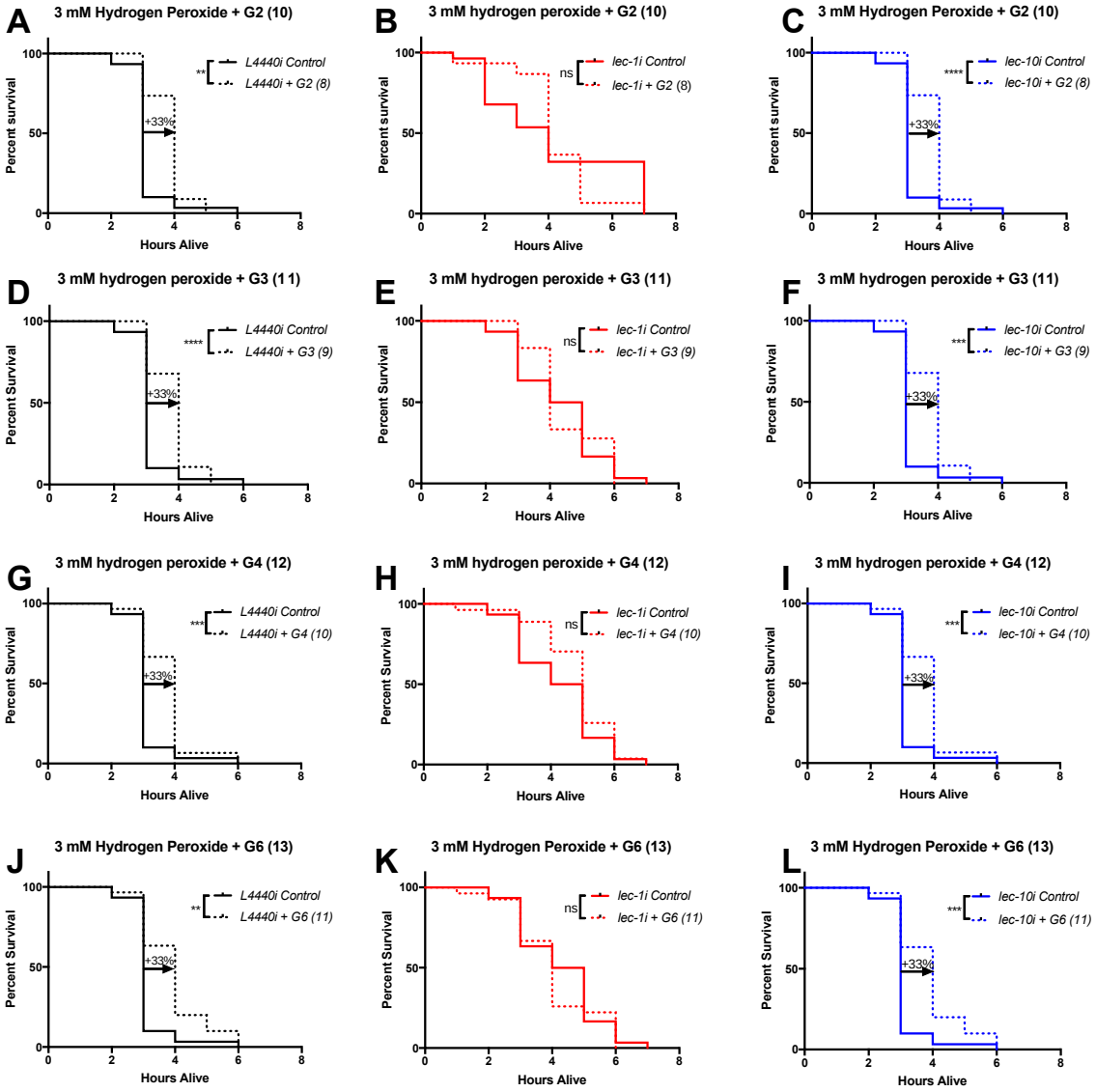


Figure 10. Survival plots of *C. elegans* knockouts.
Galβ1-4Fuc functionalized multivalent glycodendrimers improve survival of *C. elegans* challenged with hydrogen peroxide. All panels present the comparison of survival rates for *lec-1(RNAi)*, *lec*-10i, and *L4440*i worms in the presence or absence of glycodendrimers (4 mg/mL). These worms were incubated for an hour in glycodendrimer solution or M9 buffer before the hydrogen peroxide (3 mM) was added. A-C) Galβ1-4Fuc functionalized generation 2 PAMAM dendrimer **10** (0.56 mM). D-F) Galβ1-4Fuc functionalized generation 3 PAMAM dendrimer **11** (0.24 mM) G-I) Galβ1-4Fuc functionalized generation 4 PAMAM dendrimer **12** (0.11 mM) J-L) Galβ1-4Fuc functionalized generation 6 PAMAM dendrimer **13** (0.04 mM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Survival assays using Galβ1-4Fuc functionalized G3 dendrimers **11** are shown in Figure 9C and Figure 10D-F. Control worms pre-treated with G3 dendrimer **11** have survival rates similar to those pre-treated with G2 dendrimer **10** (compare Figure 9A and 9C). The survival *lec-1(RNAi)* knockdowns pre-treated with G3 dendrimer **11** prior to exposure to hydrogen peroxide was indistinguishable from untreated *lec-1(RNAi)* worms without G3 dendrimer **11** pretreatment (Figure 9C, Figure 10E). However, it again appears that *lec-1(RNAi)* worms benefitted from the 1h pretreatment period in either M9 or dendrimer. *Lec-10(RNAi)* worms treated with G3 dendrimer **11** had significantly better survival rates compared to untreated worms (Figure 9B). Figure 10F shows a 25 percent increase in survival rate between 3h and 4h. The glycodendrimer **11** also benefitted *L4440(RNAi)* worm; pre-treatment with G3 dendrimer **11** (Figure 9B) afforded a 33 percent increase in median survival rate from (Figure 10D).

L4440i (vector control) animals treated with G4 glycodendrimer **12** had significantly greater median survival rates (33 percent) with the most significant difference at 3h relative to untreated animals (Figure 9B and Figure 10G) than controls. As with **10** and **11**, *lec-1(RNAi)* worms pre-treated with G4 dendrimer **12** displayed insignificant difference to control worms without dendrimer (Figure 9B, Figure 10H). *lec-10(RNAi)* worms pre-treated with G4 dendrimer **12** prior to exposure to hydrogen peroxide survived longer than those pre-treated with M9, with a significant difference observed at 3h seen in Figure 9B (33 percent increase of median survival, Figure 10I). Control worms that were treated with G6 dendrimer

13 lived longer compared to worms that were treated with dendrimers **10**, **11, or 12** (Figure 9D). Both *L4440(RNAi)* control worms and *lec-10(RNAi)* worms displayed in increase in their median survival (33 percent for both, Figure 10J and 10L) when challenged with hydrogen peroxide.

The survival rate of *lec-1(RNAi)* worms treated with all dendrimers tested showed no survival advantage compared to the worms pre-treated with M9 followed by hydrogen peroxide. It is noteworthy that after an hour of incubation with hydrogen peroxide only, *lec-1(RNAi)* worms had a greater median survival than *L4440(RNAi)* or *lec-10(RNAi)* worms. This data suggests LEC-1 may have a more complicated role than directly protecting the organism from oxidative stress or LEC-10 could function redundantly for LEC-1. *lec-10(RNAi)* worms pretreated with all generations of dendrimers consistently had 33 percent greater median survival rate than those pre-treated with M9 followed by hydrogen peroxide challenge (Figure 9A-D and Figure 10C, 10F, 10I, 10L). All generations of dendrimers provided protection to control, *L4440(RNAi),* and *lec-10(RNAi)* knockdowns from oxidative stress. Therefore, carbohydrate/galectin binding is protective during oxidative stress and Galβ1-4Fuc functionalized dendrimers can provide protection *in vivo* when *C. elegans* are challenged with oxidative stress such as hydrogen peroxide.

Fluorescent microscopy studies show localization of dendrimers

In order to better ascertain the functional *in vivo* location of the dendrimers on or within the worms being tested, a fluorescent tag (AlexaFlour 488) was added to all generations of dendrimers. Approximately one tag was added per dendrimer, as described in the materials and methods section. Worms were incubated in the fluorescently-tagged glycodendrimer for 1h before being anesthetized (Levamisole, 7 μL of a 1 mM solution in M9 buffer) and imaged. As shown in Figure 11, the fluorescent signal was observed in the pharynx, gut, vulva, and sometimes in the neurons (fluorescein derivatives are known to collect in amphid and phasmid neurons).[139] However, G6 dendrimer **13** was not observed in the neurons. This could be due to the overwhelmingly strong signal in the gut masking any smaller signal in a neuron, or because G6 glycodendrimer **13** may be too large to pass readily out of the gut, or into a neuron. A likely explanation of the observed biodistribution is that the glycodendrimers are binding to lectins in the *C. elegans*.

LEC-1 is primarily expressed in the cuticle and pharynx, and LEC-10 is mainly expressed in the cytoplasm of intestinal cells.[140, 141] Differences in the overall localization of dendrimer in the three different RNAi knockdowns used were small, but control *L4440(RNAi)* worms had a larger physical area of fluorescence compared to the knockdowns. This is presumably because galectins are not suppressed in *L4440(RNAi)* treated animals. In other words, the control worms are expressing all of their galectins, enabling more binding of the fluorescently tagged glycodendrimers. Embryos, oocytes, and somatic gonads of more mature worms

(i.e., Day 1 adults with fertilized embryos) were illuminated by fluorescently tagged dendrimer, as shown in Figure 12. Less fluorescence was observed in the neurons of *lec-1(RNAi)* worms. In addition, *lec-10i* worms treated with dendrimers **12** or **13** had decreased dye update in the vulva relative to *lec-1(RNAi)* and *L4440(RNAi)* worms. An overall decrease of signal in the vulva was also observed using dendrimers **10** and **11** in *lec-1(RNAi)* and *lec-10(RNAi)* knockdowns compared *L4440(RNAi)* based on exposure time. A fluorescent signal was also observed in the proximal gonads. More studies are needed in order to fully rationalize the differences in observed distribution patterns for the different generations of fluorescently labeled dendrimers. *C. elegans* are able to uptake the glycodendrimers, and the glycodendrimers are distributed to different tissues throughout the organism.

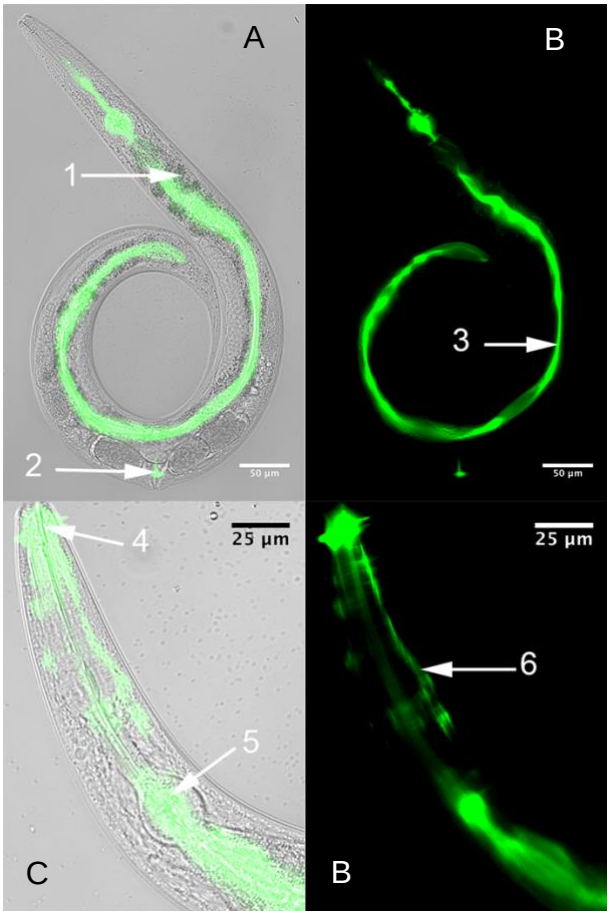


Figure 11. Fluorescent microscopy images of *L4440(RNAi)* worms with fluorescent Galβ1-4Fuc functionalized G2 dendrimer **10**.
A and C) DITC/FITC channel overlay. B and D) FITC channel. These are two different animals. As can be seen in A and B, the fluorescent signal is seen throughout the pharynx, intestine, and can also be seen in the vulva. C and D show a significant signal in the head of the animal, possibly the amphid neurons. B exposure time 327 ms. D exposure time 1 s. ((1) Proximal gonads 2) vulva 3) intestine/ gut 4) buccal cavity 5) pharynx 6) amphid neural channels)

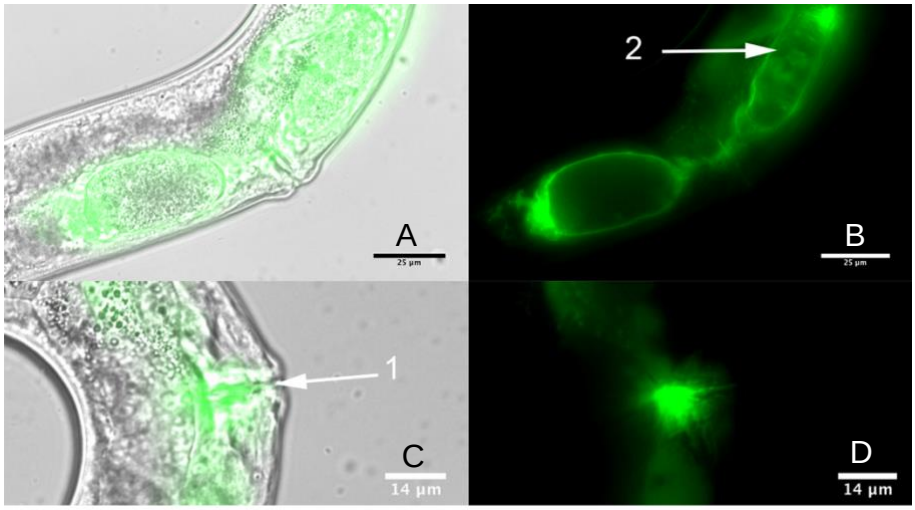


Figure 12. Fluorescent microscopy images of wildtype *C. elegans* treated with fluorescent Galβ1-4Fuc G2 dendrimers.
A and B) Wildtype worms treated with fluorescent G2 dendrimer **10**. C and D) *lec-10(RNAi)* worms treated with fluorescent dendrimer **8**. A and C) DITC/FITC channel overlay. B and D) FITC channel. In images A and B, a strong fluorescence can be seen surrounding the eggs or oocytes (2), but not in the vulva. In images C and D, fluorescence in the vulva (1) can be seen. B exposure time 1 s. D exposure time was 865 ms.

RNAi knockdown of *lec-1* or *lec-10* did not cause any obvious phenotypical abnormalities, which is consistent with previously reported observations for the mutants.[130-131] Although LEC-1 is expressed mainly in the cuticle and pharynx[140] and LEC-10 is primarily expressed in the intestine,[141] only small differences were observed between the fluorescence images of *lec-1(RNAi)* and *lec-10(RNAi)* worms. This may be due to compensatory or redundant lectins binding to the glycodendrimers: for example previous studies have reported that LEC-10 and LEC-6 share similar carbohydrate binding partners,[141] and LEC-1 and LEC-6 can be co-isolated.[142] More studies are needed to fully elucidate the binding behavior of the glycodendrimers to the galectins in *C. elegans*. The studies reported here

suggest that additional evaluation, although beyond the scope of this publication, is merited.

Future Directions

For the oxidative stress assays Figure 8 is different from Figure 9 and Figure 10. In Figure 8 these worms were transferred from an agar plate into a well containing hydrogen peroxide. For the worms in Figure 9 and Figure 10, they received an hour incubation in an 8 mg/mL solution of dendrimer before the hydrogen peroxide was added to the well plate. The worms in Figure 9 looked noticeably more stressed than those seen in Figure 8. A noticeable discrepancy can be seen in the time the worms survived from these two graphs. The best explanation for the differences in the time it took worms to completely die off can be explained by the initial incubation with the glycodendrimers.

The intent for the hour incubation for the worms with the dendrimer was to provide an opportunity for the dendrimer to absorb into the worm and interact with galectins. During the time while the worms are in a solution of dendrimers, the dendrimers are likely adsorbing onto the surface of the intestine essentially providing the worm with an internal coat of glycodendrimers such that when they are exposed to oxidative stress it is the dendrimer that gets exposed to the oxidative stress before the worms themselves. A better understanding of what the dendrimer is binding to would be beneficial. The addition of more control would help elucidate the interaction that are occurring. For example, a dendrimer with a different sugar or a hydroxyl terminated dendrimer lacking the galβ1-4fuc

disaccharide would allow assessment of whether or not the disaccharide is interacting specifically with some protein, most likely galectins, or the glycodendrimer is nonspecifically providing an additional barrier to the oxidative stress. Further, it would be interesting to challenge the worms with hydrogen peroxide and then treat them with fluorescent dendrimers to see if localization changes.

Conclusion

Taken together, the results from oxidative stress assays and fluorescent images showing the *in vivo* localization of glycodendrimers suggest that the Galβ1-4Fuc functionalized dendrimers **10**-**13** bind to *C. elegans* galectins and are able to protect the worms from oxidative stress. The concentrations of glycodendrimers that were used in these studies display no generational dependence in oxidative stress assays, with all four generations affording similar results. *C. elegans L4440(RNAi)* and *lec-10(RNAi)* that were pre-incubated with Galβ1-4Fuc functionalized dendrimers before being exposed to oxidative stress were afforded a significant survival advantage. These data suggest that multivalently presented Galβ1-4Fuc can protect *C. elegans* from oxidative stress by binding to galectins including LEC-10. More studies need to be done to determine if the protection is afforded to the animal due to interactions with Galβ1-4Fuc and not because the dendrimers are interacting nonspecifically to offer a coating of protection.

Materials and Methods

Worm strains: All strains used were N2.

RNAi strains: *L4440(RNAi)*, *lec-1(RNAi)*, and *lec-10(RNAi)* are all from the Ahringer collection[143] and were all sequence verified.

Bacteria and nematode growth. RNAi bacteria were grown overnight in Luria-Broth with 12.5 mg/mL of tetracycline and 100 mg/mL of carbenicillin at 37 °C from glycerol stocks. The next morning, 1 mL of the bacteria culture was plated onto NGM plates that had 1M IPTG and 100 mg/mL of carbenicillin. Plates were left at room temperature to dry overnight. Prior to bleaching adult *C. elegans,*[144] plates were treated with 200 μL of 0.1M IPTG. Worms were bleached and eggs were grown on their respective RNAi until adulthood whole life.

Fluorescent Microscopy Procedure. Day 1 adult *C. elegans* were added to 20 μL of an 8 mg/mL solution of Alexafluor-488 labeled **10**, **11, 12**, or **13** in M9 in a 96-well plate for 1h. The worms were transferred to a small pad of agar before being anesthetized (Levamisole, 7 μL of a 1 mM solution in M9) and imaged. Images were taken with a Nikon Ti-E, and the FITC/DITC overlay images were prepared using FIJI.[145]

Oxidative Stress Assay. Adult hermaphrodite worms (20 worms/group) were transferred to a 96-well plate containing 40 μL of a 3 mM or a 6 mM hydrogen peroxide solution prepared in M9 buffer. Worms were incubated at 20 ˚C, and survivability was assessed every hour. Worms that did not respond to repeated touching with a platinum wire were considered dead.

Oxidative Stress Assay with Glycodendrimers. Adult hermaphrodite worms (30 worms/group) were transferred to a 96-well plate containing 20 μL of an 8 mg/mL solution of glycodendrimer that had been prepared in M9 buffer. The worms were covered and incubated at 20˚C for 1h before solution of hydrogen peroxide was added such that the final concentration of peroxide was 3 mM and the final total volume in the well was 40 μL. Every hour, the number of animals that had survived was assessed. Worms that did not respond to repeated touching with a platinum wire were considered dead. Three independent experiments were performed.

Statistics. Statistical analyses were performed using a two-way ANOVA comparing the *C. elegans* variant with and without the dendrimer compared across each time point. Error bars correspond to + standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p < 0.0001$.

Synthetic materials. All standard chemicals and reagents were purchased from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Alfa Aesar (Johnson Matthey Company, Ward Hill, MA, USA), or Fisher (Fisher Scientific, Hampton, New Hampshire, USA), or Chem-Impex (Chem-Impex International, Wood Dale, IL, USA) and were used without further purification. PAMAM dendrimers were purchased from Dendritech (Dendritech, Midland, MI, USA). Compounds **2** through **6** were synthesized using previously established procedures.[133]

Methods. Reactions were monitored via thin-layer chromatography (TLC). TLC was performed on silica gel glass plates containing 60 G F −254, and visualization was achieved with a UV light, a cerium ammonium molybdate, or ninhydrin stain. Column chromatography was performed on Silicycle 230−400 mesh silica gel. ^{1}H spectra and ^{13}C spectra were performed on Bruker DRX500 (500 MHz) or Bruker DRX600 (600 MHz). Chemical shifts (δ) were reported in ppm downfield from an internal TMS standard. MALDI spectra were recorded on a Bruker III Biflex with a 337 nm nitrogen laser using freshly recrystallized trans-3-indolacrylic acid (IAA) or 2,5-dihydroxybenzoic acid (DHB) as a matrix.

1-O-(6-t-Butylcarbamate-4-thiohexyl)-2,3,4,6-Tetra-O-acetyl-b-D-galactopyranosyl-(1→4)-2,3-di-O-benzoyl-α-L-fucopyranoside (**8a**). Product **8** (243 mg, 0.330 mmol) and Boc-protected aminoethanethiol (292 mg, 1.65 mmol) were transferred to a 25 mL flask, and 3x2.5 mL aliquots of toluene were used to azeotrope off excess water. Once disaccharide **8** was dry, **8** was dissolved in 5 mL of toluene, and the solution was degassed with argon for 30 min. Toluene was brought to reflux for 10 min before Luperox (1,1-bis(*tert*-butylperoxy)cyclohexane) (64.4 mg, 0.25 mmol) was added. Reaction was allowed to reflux for 5 h or until TLC indication reaction had completed. The crude reaction mixture was concentrated *in vacuo* and was subjected to column chromatography (1:1 hexanes to ethyl acetate). Product **8a** was obtained in 86% yield (261.2 mg). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 1.30 (d, *J* = 6.6 Hz, 3H), 1.44 (s, 9H), 1.82 (p, *J* = 6.9 Hz, 2H), 1.93 (s, 3H), 1.96 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.48 (t, *J* = 6.5 Hz, 2H),

2.55 (ddd, *J* = 19.0, 12.4, 7.2 Hz, 1H), 3.09 – 3.31 (m, 4H), 3.48 (dt, *J* = 10.0, 6.1 Hz, 1H), 3.63 (td, *J* = 6.9, 1.2 Hz, 1H), 3.80 (dt, *J* = 10.1, 5.8 Hz, 1H), 4.16 (dd, *J* = 13.9, 7.2 Hz, 1H), 4.25 (d, *J* = 1.4 Hz, 1H), 4.43 (d, *J* = 7.9 Hz, 1H), 4.83 (s, 1H), 4.94 (dd, *J* = 10.5, 3.5 Hz, 1H), 5.17 (dd, *J* = 3.5, 1.2 Hz, 1H), 5.20 (d, *J* = 2.7 Hz, 1H), 5.27 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.50 – 5.55 (m, 2H), 7.33 – 7.41 (m, 4H), 7.46 – 7.55 (m, 2H), 7.97 (dd, *J* = 8.4, 1.4 Hz, 2H), 8.03 – 8.07 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 16.00, 20.56, 20.58, 20.60, 20.67, 20.82, 28.35, 28.41, 29.34, 32.28, 39.60, 60.40, 65.30, 66.43, 66.82, 68.79, 69.35, 69.88, 70.52, 70.85, 76.50, 76.78, 77.03, 77.23, 77.28, 79.40, 96.42, 101.76, 128.14, 128.41, 128.59, 129.71, 129.75, 130.03, 130.08, 133.09, 133.17, 155.74, 165.80, 166.18, 169.18, 170.06, 170.21. HR-MS (ESI) *m/z*: 920.3373 $[M+H]^+$, Calcd for $C_{44}H_{57}NO_{18}S$ 920.3369 $[M+H]^+$.

1-O-(6-isothiocyanato-4-thiohexyl) 2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3-di-Obenzoyl-a-L-fucopyranoside (**9**). Carbamate **8a** (156.4. mg, 0.17 mmol) was transferred to 3 mL of methylene chloride and was chilled to 0 ˚C before (164 µL, 174 mg) trifluoroacetic acid (TFA) was added, and the solution was allowed to stir overnight. Once TLC indicated the reaction had completed, product was concentrated *in vacuo*. Removal of the carbamate was nearly quantitative. Free amine (134 mg, 0.17 mmol) was dissolved in 2.0 mL of dry methylene chloride along with 1.40 mL (0.37 mmol) of triethylamine. This solution was cooled to 0 ˚C and placed under argon. Separately, thiophosgene (18 µL, 0.2 mmol) was dissolved in dry methylene chloride (5.6 mL). The thiophosgene

solution was added dropwise over the course of 2h. The solution was allowed to stir for an additional hour while it warmed to RT. After TLC indication the reaction had completed, 1 mL of ultra-pure water was added to quench any remaining thiophosgene. After 30 minutes, mixture was poured into DI water and extracted (3x10 mL) with methylene chloride, and brine (10 mL), dried over $MgSO_4$, and concentrated *in vacuo*. Column chromatography was performed using 1:1 hexanes and ethylacetate to afford 82.8 mg (0.10 mmol) of pure product (56%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 1.31 (d, *J* = 6.7 Hz, 3H), 1.77 – 1.90 (m, 2H), 1.93 (s, 3H), 1.97 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 2.50 – 2.65 (m, 3H), 3.18 (dd, *J* = 11.3, 6.6 Hz, 1H), 3.23 (dd, *J* = 11.3, 7.2 Hz, 1H), 3.49 (t, *J* = 6.9 Hz, 2H), 3.63 (t, *J* = 6.8 Hz, 1H), 3.77 – 3.85 (m, 1H), 4.16 (q, *J* = 6.7 Hz, 1H), 4.26 (d, *J* = 1.2 Hz, 1H), 4.44 (d, *J* = 7.9 Hz, 1H), 4.94 (dd, *J* = 10.5, 3.4 Hz, 1H), 5.17 (dd, *J* = 3.5, 1.1 Hz, 1H), 5.23 (d, *J* = 2.6 Hz, 1H), 5.28 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.48 – 5.57 (m, 2H), 7.35 – 7.42 (m, 4H), 7.49 – 7.56 (m, 2H), 7.96 – 7.99 (m, 2H), 8.04 – 8.09 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 14.12, 16.02, 20.56, 20.60, 20.82, 22.70, 29.06, 29.28, 29.37, 29.71, 31.94, 32.25, 45.01, 60.42, 65.33, 66.18, 66.82, 68.89, 69.34, 69.64, 69.82, 70.53, 70.56, 70.84, 76.47, 76.76, 77.02, 77.22, 77.27, 96.36, 101.77, 128.15, 128.18, 128.47, 128.62, 129.68, 129.74, 130.02, 130.09, 133.11, 133.20, 133.27, 165.76, 166.18, 169.18, 170.03, 170.05, 170.20. HR-MS (ESI) *m/z*: 884.2136 (M+Na), Calcd for 884.2234 (M+Na). IR (CH_2Cl_2) ν_{max}: 2112 (NCS) cm^{-1}.

Galβ1-4Fuc functionalized dendrimers **10-13**. Approximately 40 mg of PAMAM dendrimer was taken from stock and lyophilized to remove any water. Once the dendrimers were dried to a constant mass, a 100 mg/mL solution was prepared using DMSO as the solvent, and 20.7 mg of dendrimer in DMSO was transferred to a conical vial charged with isothiocyanate **9**. The reaction volume was adjusted to 2 mL, and the reaction was let stand for 3 days. The crude reaction mixture was transferred to dialysis tubing (1 kDa MWCO) and dialyzed against DMSO (3x 2h solvent change and 1x overnight). Lyophilization afforded a yellow oil. This oil was suspended in 1:1 MeOH : H_2O, and 0.8 M NaOMe prepared in methanol was added dropwise with the pH taken at 1 h intervals. When the pH remained basic, the solution was neutralized with 0.5 M AcOH. MeOH and AcOH were removed *in vacuo*, and the remaining aqueous solution was dialyzed against Millipore water (3x 2h solvent change and 1x overnight) and lyophilized to yield product as a tan fluffy material.

G2 (**10**). ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.19 (d, J = 6.4 Hz, 3H), 1.70 – 1.84 (m, 2H), 2.23 (s, 6H), 2.46 (s, 2H), 2.65 (d, J = 43.2 Hz, 10H), 2.87 (s, 1H), 3.03 – 3.20 (m, 7H), 3.25 – 3.78 (m, 25H), 3.87 (q, J = 6.7 Hz, 1H), 4.13 (d, J = 7.7 Hz, 1H), 4.20 (s, 1H), 4.54 (s, 1H), 4.60 (d, J = 3.6 Hz, 3H), 5.07 (s, 1H), 7.71 (s, 2H), 7.83 – 8.19 (m, 4H), 8.26 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 14.43, 16.64, 17.16, 18.88, 18.94, 23.13, 28.13, 29.77, 30.54, 33.53, 36.90, 37.25, 38.64, 38.82, 38.88, 39.19, 39.55, 39.69, 39.83, 39.97, 40.11, 40.24, 40.38, 40.50, 40.88, 43.70, 49.83, 49.98, 52.56, 60.46, 66.11, 66.59, 68.34, 69.00, 69.56, 71.06, 73.86, 75.83,

82.71, 99.53, 105.14, 170.01, 171.44, 171.79, 172.06, 172.22, 172.72, 183.20. MALDI-TOF MS M_W=7,200 m/z.

G3 (**11**). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15 (d, J = 6.3 Hz, 3H), 1.62 – 1.84 (m, 2H), 2.16 (s, 4H), 2.25 – 2.48 (m, 4H), 2.52 – 2.83 (m, 8H), 3.04 (s, 3H), 3.13 (s, 3H), 3.27 (d, J = 8.7 Hz, 2H), 3.30 – 3.44 (m, 6H), 3.44 – 3.55 (m, 4H), 3.57 (dd, J = 10.3, 4.9 Hz, 1H), 3.59 – 3.71 (m, 3H), 3.83 (q, J = 6.6 Hz, 1H), 4.09 (d, J = 7.5 Hz, 1H), 4.56 (d, J = 3.6 Hz, 2H), 7.46 – 7.94 (m, 3H), 8.00 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 16.64, 28.16, 29.79, 30.56, 33.70, 37.37, 38.66, 39.45, 39.62, 39.78, 39.95, 40.04, 40.12, 40.21, 40.28, 40.37, 40.45, 40.86, 43.82, 50.04, 52.61, 60.55, 66.15, 66.60, 68.42, 69.03, 69.59, 71.05, 73.85, 75.84, 82.67, 99.53, 105.13, 171.93, 172.40. MALDI-TOF MS M_W=16,900 m/z.

G4 (**12**). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15 (d, J = 6.3 Hz, 3H), 1.65 – 1.83 (m, 2H), 2.17 (s, 4H), 2.30 – 2.44 (m, 3H), 2.43 – 2.48 (m, 3H), 2.53 – 2.72 (m, 7H), 3.05 (s, 3H), 3.14 (s, 3H), 3.19 – 3.44 (m, 28H), 3.45 – 3.56 (m, 4H), 3.56 – 3.64 (m, 2H), 3.67 (s, 1H), 3.84 (d, J = 6.7 Hz, 1H), 4.09 (d, J = 7.4 Hz, 1H), 4.16 (s, 1H), 4.46 (s, 1H), 4.57 (d, J = 3.6 Hz, 2H), 4.71 (s, 1H), 4.81 (s, 0.29H), 5.02 (s, 1H), 7.59 (s, 2H), 7.76 (s, 1H), 7.97 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 16.65, 28.16, 29.79, 30.60, 33.70, 37.41, 38.67, 39.50, 39.66, 39.83, 40.00, 40.09, 40.16, 40.26, 40.33, 40.42, 40.50, 40.91, 43.66, 50.03, 52.67, 60.56, 66.17, 66.61, 68.44, 69.05, 69.60, 71.06, 73.86, 75.85, 82.73, 99.55, 105.17, 141.23. MALDI-TOF MS M_W=37,900 m/z.

G6 (**13**). ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.15 (d, J = 5.7 Hz, 3H), 1.67 – 1.84 (m, 2H), 2.44 – 2.48 (m, 5H), 2.52 – 2.87 (m, 11H), 2.94 – 3.21 (m, 7H), 3.33 – 3.74 (m, 19H), 3.84 (d, J = 6.6 Hz, 1H), 4.10 (s, 1H), 4.18 (s, 1H), 4.50 (s, 2H), 4.57 (s, 2H), 4.75 (s, 1H), 5.05 (s, 1H), 7.41 – 7.89 (m, 4H), 7.99 (s, 2H). ^{13}C NMR (151 MHz, DMSO) δ 16.19, 27.68, 29.31, 30.12, 33.10, 36.72, 39.10, 39.24, 39.38, 39.52, 39.66, 39.80, 39.94, 40.06, 40.43, 43.29, 44.30, 49.48, 60.09, 65.67, 66.14, 67.97, 68.57, 69.13, 70.57, 73.38, 75.37, 82.30, 99.05, 104.69, 171.30. MALDI-TOF MS M_W=98,900 m/z.

Alexa Fluor 488 Labeled Glycodendrimer. The following procedure is adapted from Cousin.[146] Alexa-Fluor 488 hydrazide powder (1 mg) was dissolved in 500 µL millipore H_2O to form a 2 mg/mL stock solution. Lyophilized Galβ1-4Fuc functionalized glycodendrimers were dissolved in millipore H_2O to make 2 mg/mL stock solutions. To the glycodendrimer solution, $NaIO_4$ was added (2 equiv. per dendrimer), and the reaction mixture was stirred at RT for 2 h. After 2 h, Alexa-Fluor 488 hydrazide (1 equiv. per dendrimer) was added and allowed to react for 0.5 h at RT. After 0.5 h, the reaction mixture was purified by dialysis against 1 kDa MWCO (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) in millipore H_2O. The purified reaction mixture was frozen and lyophilized to dryness.

Characterization of dendrimer labeling was determined using a UV-Vis Spectrometer (Molecular Devices, SpectraMax Plus, Softmax Pro 5). Absorbance was measured at 494 nm using an extinction coefficient of 71,000 $M^{-1}cm^{-1}$, indicating that a labeling ratio of 1 to 1 Alexa Flour 488 to dendrimer was obtained.

MALDI procedure. Sample preparation is critical in order to obtain successful MALDI data. Samples were dissolved in 5 mL of ultrapure water, frozen, and lyophilized. Material went from a yellowish oil to an off white fluffy solid which is ideal for MALDI sample preparation. A 1 mg/mL aqueous solution of dendrimer was prepared. This is the dendrimer stock solution. Either a 2.25 mg/mL solution (4:1, acetonitrile:water) of trans-3-indoleacrylic acid (IAA) or a 20 mg/mL aqueous solution of 2,5-dihydroxybenzoic acid (DHB) was used to prepare the samples on the plate. For IAA, dendrimer was mixed with matrix and then plated. For DHB samples, the dry drop method from Bruker was used.

CHAPTER FOUR

USING GLYCODENDRIMER WITH CLEAVABLE MMP SUBSTRATE TO STUDY DRUG RELEASE

Introduction

Matrix metalloproteinases or MMPs belong to the family of metalloproteinases (MPs). The unique feature of the MMPs is their collective ability to degrade all extracellular matrix components.[40] Abnormal expression of MMPs are associate with several types of diseases. They can be upregulated in cancer, vascular disease, and different types of inflammatory diseases.[40] The MMP family includes 25 members of vertebrate origin 24 of which have human paralogs. Some notable nonpathogenic behavior includes MMPs acting as the agent responsible for tail resorption during the metamorphosis of frogs.[49] MMPs are also involved in normal tissue turnover and repair such as wound healing. Further, their activity is required during embryogenesis and angiogenesis.[40] However, MMPs are more than extracellular matrix degrading proteins, their name describes only part of the role they play. Instead, ECM degradation could be a result of a more finely regulated system where growth factors are not necessarily released from the degradation of the ECM, but by the cleavage and release of cytokine masking carrier proteins that block growth factor function and activity.[147] In this way MMPs are less destructive enzymes and more cell signaling regulators.[148]

MMPs need precise spatial and temporal regulation in order to maintain homeostasis of the extracellular and pericellular environment.[40-41] MMPs are

produced as zymogens or proenzymes that require activation before they can carry out their function. Their regulation can be at the transcription level, and they can be controlled at the protein level with their endogenous activators and inhibitors in addition to other factors that can impact their localization.[41] MMP2 is distinguished by its rapid ability to degrade gelatin.[149] Rheumatoid arthritis and malignant diseases are associated with upregulated MMPs.[41] The more MMP upregulation, the worse the prognosis. A major hallmark of these kinds of diseases is the ability of cells to cross normal tissue boundaries. A good practical example of this is the metastasis of cancer that results in the spread of cancer to different parts of the body.

The specificity binding pocket, $S_{1`}$, is the main MMP subsite for substrate recognition found on MMPs.[49] Several first and second generations inhibitors have targeted this pocket for therapeutic use. However, although as these drugs made it to clinical trials, they ultimately failed due to severe side effect presumably due to the nature of MMPs. Some promote disease while other have a more protective effect.[49] In addition, the specificity binding pocket can be similar between two different MMPs therefore making selectivity a challenge.

Since MMPs are found in specific tissues and are upregulated in several different malignancies other therapeutics approaches have sought to use the MMPs ability to cleave specific peptide sequences. Albright *et. al.* synthesized a peptide fragment with five cleavable doxorubicin, an anthracycline natural product that is used to treat various forms of cancer.[150] They also demonstrated that

doxorubicin could be cleaved in the presence of MMPs effectively using mice with HT1080 xenographs. HT1080 is a fibrosarcoma cell line. What is so attractive about this research is the reduction in toxicity that was observed at similar doses when comparing the multivalent presentation of doxorubicin and the monomer, in addition to demonstrating an ability to cleave a prodrug off an MMP substrate.

Proteomics allows for the determination of specific markers for cancer progression and early drug targets[147] and has identified proteases upregulation as a marker for colorectal cancer (ex: MMP2, MMP9, MMP7).[151] The adenocarcinoma cell line A549 has increased expression of MMP2, MMP7, and MMP9. Based on of previous reports, MMP7 may be more prevalent than MMP2 and MMP9.[55] An alternative approach to traditional inhibitors would be to use the knowledge of upregulated proteinases that cleave a specific peptide sequence to deliver a drug payload to cells that have undesirable MMP upregulation such as the pentavalent doxorubicin peptide reported by Albright *el. al.*. Difficulties with this concept can arise when metastasis is either occurring or has occurred, due to the decrease in colocalization of the target cells. In other words, it is easier to treat a single large tumor instead of several small ones with unknown distribution. Here, the application of multivalency in a different way is proposed by capitalizing on the upregulation of another protein such as galectin-3 and its ability to interact with lactose. The lactose functionalized glycodendrimers serve to attenuate the aggregation characteristics of cancer cells while also affording a nontoxic delivery vehicle for chemotherapy.[152] Thus, heterogeneously functionalizing the dendrimer

with lactose (for modulation of toxicity and for cancer cellular aggregation) and an MMP substrate (for cleavage of a prodrug at the tumor) was hypothesized to afford a more effective treatment than a monovalent drug. The ability to tailor the release of a drug is necessary to improve drug delivery and efficacy while limiting exposure to healthy tissue.[153]

Kridel *et. al.* demonstrated that MMP9 can cleave a range of substrates but was more specific for some sequences than other MMPs.[154] The most prevalent sequence motif that is cleaved for MMP9 is Pro-X-X-Hy-(Ser/Thr). However, a search for putative physiologic substrates produced a limited list suggesting other residues enhance selectivity.[154] Other groups have studied MMP2 activity using denatured type I collagen, and found selectivity enhancements for glycine and hydrophobic residues with some additional exceptions. Further, a hydroxyproline was always found five residues away from the cleavage site and frequently the cleavage site was bracketed by hydroxyproline.[155] MMP7 cleaves between Leu and Ile in exposed regions between structural domains.[36] The sequence Pro-X-X-Hy, where Hy is a hydrophobic residue, appears to be a universal sequence cleave by most MMPs.[36, 149, 154, 156-157]

Dendrimers as Drug Delivery Vehicles

PAMAM dendrimers excel as drug delivery vehicles and have been used for a variety of purposes. For example a G0 PAMAM dendrimer has been use in conjunction with Naproxen in order to enhance the drug's permeation.[153] These dendrimers have also been used as solubility enhancers, as drug delivery vehicles, and as therapeutic agents. Vivagel® is a therapeutic agent that is PAMAM dendrimer based and is used as an anti-HIV agent and has successfully completed several clinical trials including phase III trials.[158] A. J. Khopade *et. al.* have shown dendrimers can be used to trap drugs such as MTX (methotrexate) and successfully release them over an eight hour period.[159] Xiaojie Li *et. al.* reported a pegylated PAMAM dendrimer that has been conjugated to a gold nanorods and doxorubicin displays release of the drug at slightly acidic pH and negligible release under normal physiological pH.[160]

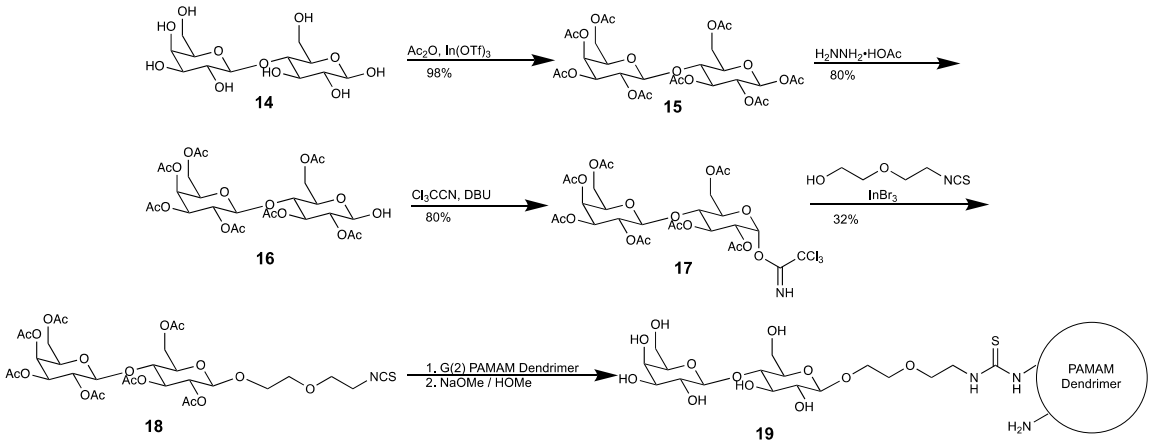
Several different forms of cancer have shown an upregulation of various MMPs including MMP-2, -7, -9 in A549, a lung carcinoma cell line.[55] This makes the A549 cell line a prime candidate to take advantage of this upregulation and have these MMPs cleave a substrate with a fluorogenic dye or prodrug. MMP substrates are commercially available in a FRET-active form and can be incorporated into the synthesis of a dendrimer.

In this work an MMP substrate has been conjugated to a G2 PAMAM dendrimer that is also functionalized with lactose. The MMP substrate has a fluorogenic dye attached to it that fluoresces upon cleavage. In this manner,

fluorescence monitoring can be used determine the rate of substrate cleavage. Two different macromolecules using lactose functionalized glycodendrimers as a precursor have been synthesized, one with a substrate specific for MMP2/9 and another that is specific for MMP7. Fluorescent lifetime measurements have been used to compare cleavage rates for MMP-functionalized dendrimers with monovalent MMP substrates in order to evaluate potential drug release characteristics.

Results and Discussion

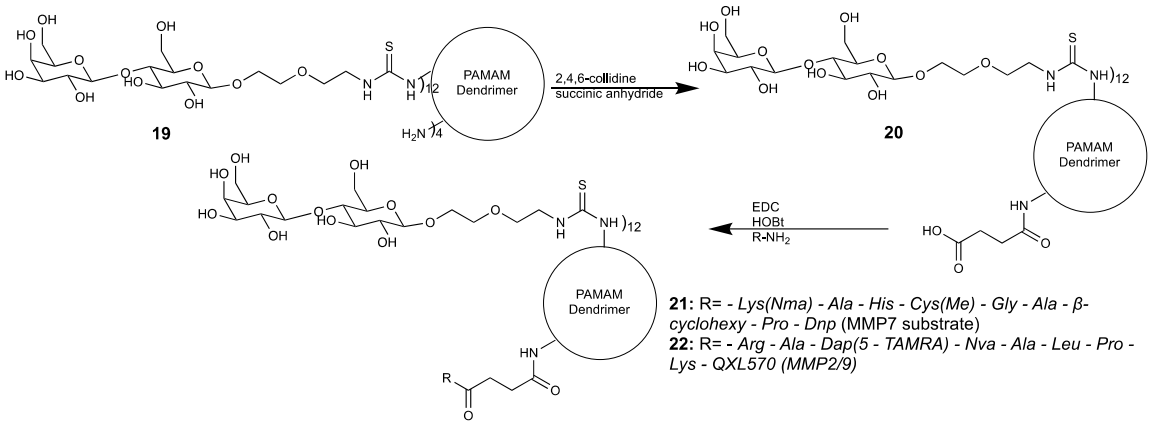
Scheme 4. Synthesis of Lactose functionalized dendrimers.



In Scheme 4, lactose (**14**) was peracetylated in acetic anhydride with indium triflate to yield **15**. Selective deprotection at the anomeric position with hydrazine acetate afforded **16**. Standard conditions of trichloroacetonitrile and DBU were used to create an alpha trichloroacetimidate (**17**). This group is used as a way of attaching the sugar to different nucleophiles. In this case an ethoxy ethanol liker with an isothiocyanate that is used to attach the lactose onto the dendrimers.

Indium(III) bromide was used as a Lewis acid mediator to attach the linker giving product **18**. Dendrimers were produced by reacting the a G(2) PAMAM dendrimer with **18** to give acetylated lactose functionalize G(2) PAMAM dendrimer that were deprotected with methanolic sodium methoxide creating a water-soluble dendrimer **19**. Succinic anhydride was coupled to any remaining primary amines with an EDC coupling on the dendrimer in order to create a point of attachment for the MMP substrate seen in **20**. A final EDC coupling forms an amide bond with **20** and an fluorogenic MMP substrate will afford **21** and **22**. This can be seen in Scheme 5.

Scheme 5. Synthesis of MMP functionalized dendrimers



Fluorescence Assay to Monitor Substrate Cleavage

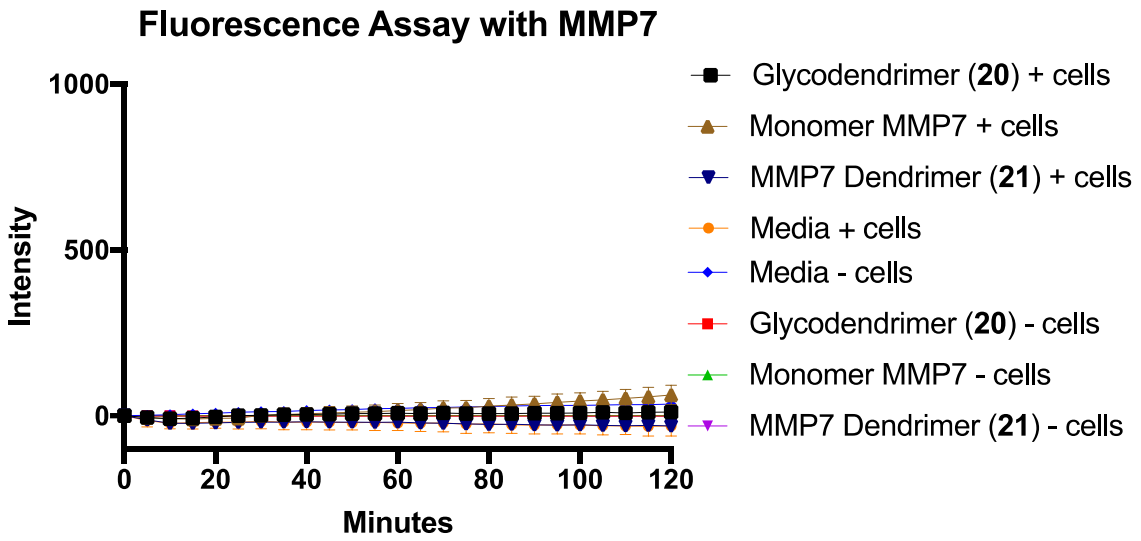


Figure 13. Fluorescence intensity of MMP7 substrates incubated with A549 cells. Error bars represent standard deviation. Dendrimer concentration is approximately a quarter of the free MMP7 substrate concentration. The y-axis is an order of magnitude smaller than the previous graph.

A similar assay was performed with free MMP7 substrate and MMP7 dendrimers and can be seen in Figure 13. Only the slightest signal increase was observed over the course of the assay with free MMP7 substrate. MMP7 dendrimer (**21**) did not display any increase in fluorescent intensity. Nor, did experiments with serum free media or lactose functionalized glycodendrimer (**20**), indicating either the dye was not cleaved or the dye has been quenched. The substrate's expected cleavage site was the Gly - Ala bond. Further experiments are required to rule out either of those possible outcomes.

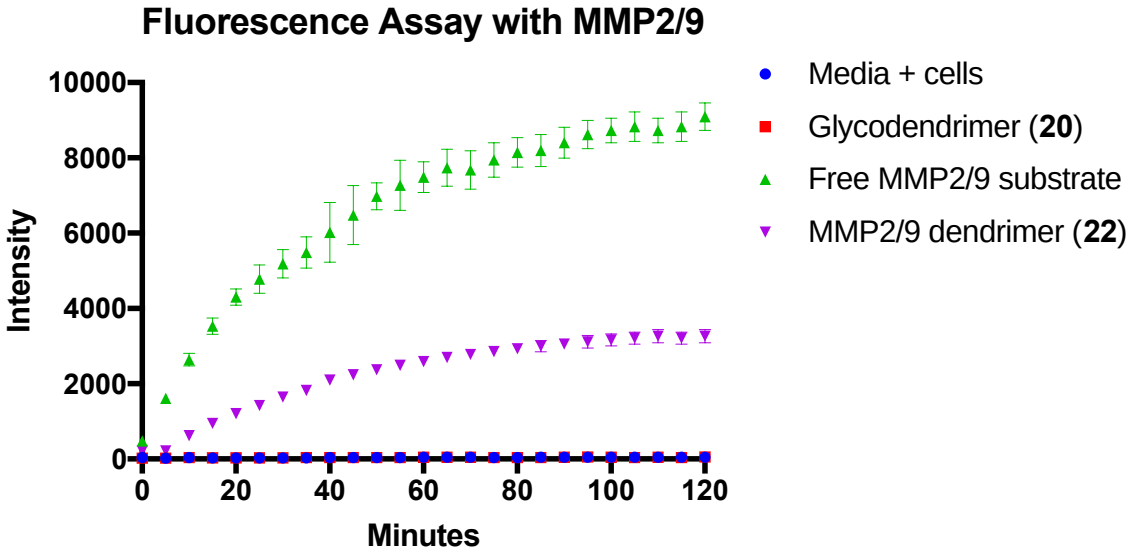


Figure 14.Fluorescence intensity of MMP2/9 substrates incubated with A549 cells. Results represent background-subtracted intensities based off of conditions without cells. Error bars represent standard deviation. Concentration of MMP substrate was kept the same for the free MMP substrate and the dendrimer attached MMP substrate.

Over the course of the assay the fluorescent signal for experiments containing MMP2/9 monomer (green triangle) and MMP2/9 dendrimer (**22,** purple triangle) increased, indicating cleavage of the substrate by cellular MMP2 and MMP9. As expected, there was negligible increase in signal from the lactose functionalized glycodendrimer (**20**, red square), and conditions with only cells (blue circle). The monomer (green triangle) gave significantly more signal than the dendrimer indicating possible loss of functional dye on dendrimer. Spectral analysis revealed an average of 0.33 MMP2/9 substrate for every dendrimer and based off of this concentration there were twice as many MMP2/9 substrates on dendrimer for every free MMP2/9 substrate tested. These results indicated a loss of function of the MMP substrates that can be indicative of sterics preventing the

cleavage of the substrate. The sterics issues arise from attaching the MMP substrate to a dendrimer, thus limiting the sides MMP2 and MMP9 can approach and cleave the substrate. The substrate's expected cleavage site was the Ala – Leu bond. Overall, results from the MMP2/9 fluorescence assay suggest a prodrug could be cleaved in the presence of cells over expressing MMP2 and MMP9.

Conclusion

Lactose functionalized PAMAM dendrimers have been synthesize with two MMP substrates to monitor the rate of the cleavage of the substrate and compare it to the free MMP substrate. The two substrates tested were MMP2/9 and MMP7. These substrates were chosen due to a putative up regulation of these substrates corresponding proteinases in A549 adenocarcinoma cells. Of the substrates tested only MMP2/9 displayed significant increase in fluorescent intensity. MMP7 dendrimers and free MMP7 substrate showed little to no increase in fluorescent signal. The free MMP2/9 substrate outperformed the dendrimer in this case. Despite a more efficient cleavage of the free MMP2/9 substrate, the fact that cleavage can be obtained is encouraging. Since dendrimers, are known drug delivery vehicles these results should encourage future development of MMP substrate dendrimers. For example, prodrugs can be attached, endgroups to encourage the aggregation of cells, and possibly several different MMP substrates enhancing selectivity for cancers that over express certain MMPs.

Materials and Methods

General Methods

All standard chemicals and reagents were purchased from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Alfa Aesar (Johnson Matthey Company, Ward Hill, MA, USA), or Fisher (Fisher Scientific, Hampton, New Hampshire, USA), or Chem-Impex (Chem-Impex International, Wood Dale, IL, USA) and were used without further purification. PAMAM dendrimers were purchased from Dendritech (Dendritech, Midland, MI, USA). Reactions were monitored via thin-layer chromatography (TLC). TLC was performed on silica gel glass plates containing 60 G F −254, and visualization was achieved with a UV light, a cerium ammonium molybdate, or ninhydrin stain. Column chromatography was performed on Silicycle 230−400 mesh silica gel. ^{1}H spectra and ^{13}C spectra were performed on Bruker DRX500 (500 MHz) or Bruker DRX600 (600 MHz). Chemical shifts (δ) were reported in ppm downfield from an internal TMS standard. MALDI spectra were recorded on a Bruker III Biflex with a 337 nm nitrogen laser using freshly recrystallized trans-3-indolacrylic acid (IAA) or 2,5-dihydroxybenzoic acid (DHB) as a matrix.

Synthesis of Carboxylic Acid-Terminated Lactose Functionalized Dendrimers. In a 3 mL conical vial a Teflon-coated stir bar was added to a 25 mg/mL solution of G2 PAMAM dendrimer in DMSO. The solution was made basic with the addition of 2,4,6-collidine (100 μL). An excess of succinic anhydride (25

eq.) was added to the reaction mixture and was allowed to stir at RT for 3 days. The reaction was purified via dialysis against 1000 MWCO dialysis tubing in 250 mL of DMSO overnight, followed by a change every 2h (3X) and one solvent change overnight. The contents of the dialysis tubing were frozen and lyophilized to yield the purified as an amorphous white solid that was analyzed by NMR and MALDI-TOF that matched previously synthesized material.

Addition of MMP Substrate on Carboxylic Acid-Terminated Lactose Functionalized Dendrimers. A 3 mL conical vial was charged with (4.1 mg), and a Teflon coated stir bar and dissolved in water. A solution of the MMP substrate (580 MMP FRET Substrate I) in Millipore water. A solution of 10 mg/mL of HOBt was prepared in Millipore water that had been made basic with 1 equivalent of sodium carbonate. A small aliquot was added to the dendrimer solution. A 20 mg/mL solution of 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was prepared in Millipore water, and from which a small aliquot was added to the reaction mixture. The MMP substrate was added last. The final concentration of the reaction was 400 nM with respect to the dendrimer. The reaction mixture was shielded from light and allowed to stir at RT for 3 days before being transferred to 3500 MWCO dialysis tubing in 250 mL of Millipore water. The water was changed ever 1.5h 3X and then one final exchange was done and allowed to dialyze overnight. In the following morning the reaction mixture was removed from the tubing, frozen, and lyophilized into a fluffy purple solid. Into a fluffy slight yellow solid.

MMP2/9 dendrimer. ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.97 (t, J = 7.2 Hz, 1H), 1.04 (d, J = 6.1 Hz, 4H), 1.23 (s, 2H), 1.48 (p, J = 7.0 Hz, 1H), 2.15 (s, 4H), 2.21 (s, 26H), 2.35 (s, 14H), 2.39 (dd, J = 3.6, 1.8 Hz, 4H), 2.44 (s, 37H), 2.51 – 2.53 (m, 7H), 2.54 (s, 8H), 2.61 (dd, J = 3.7, 1.8 Hz, 2H), 2.66 (s, 18H), 2.93 (s, 1H), 2.98 (dt, J = 12.7, 7.0 Hz, 4H), 3.09 (s, 14H), 3.17 (s, 23H), 3.22 – 3.37 (m, 53H), 3.37 – 3.45 (m, 31H), 3.45 – 3.63 (m, 96H), 3.66 (s, 23H), 3.77 (dt, J = 12.2, 6.1 Hz, 9H), 3.80 – 3.92 (m, 11H), 4.00 (s, 5H), 4.02 – 4.14 (m, 9H), 4.14 – 4.32 (m, 10H), 4.31 – 4.50 (m, 14H), 4.52 (s, 6H), 4.68 (s, 3H), 4.73 – 4.92 (m, 5H), 4.99 – 5.25 (m, 5H), 5.70 – 5.84 (m, 1H), 6.85 (s, 3H), 7.82 (s, 14H), 8.06 (s, 8H), 9.15 (s, 1H), 9.39 (d, J = 135.8 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 20.29, 23.80, 25.50, 29.34, 33.17, 36.85, 37.53, 38.27, 43.22, 44.45, 44.97, 49.52, 52.18, 56.62, 60.20, 60.39, 62.04, 66.60, 67.62, 68.22, 68.78, 69.25, 70.56, 120.92, 156.79, 171.35, 171.80, 172.21, 174.20, 189.70. MALDI-TOF MS M_w=12,800 m/z.

MMP7 dendrimer. ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.74 – 0.91 (m, 13H), 0.97 (t, J = 7.2 Hz, 8H), 1.04 (d, J = 6.1 Hz, 2H), 1.13 – 1.18 (m, 3H), 1.23 (s, 8H), 1.49 (p, J = 7.3 Hz, 12H), 1.90 (s, 2H), 2.15 (s, 27H), 2.21 (s, 142H), 2.35 (s, 88H), 2.44 (s, 226H), 2.54 (s, 45H), 2.59 – 2.62 (m, 13H), 2.66 (s, 92H), 2.98 (dt, J = 13.7, 6.9 Hz, 27H), 3.09 (s, 91H), 3.17 (s, 135H), 3.21 – 3.46 (m, 713H), 3.46 – 3.62 (m, 496H), 3.66 (s, 175H), 3.79 – 3.92 (m, 56H), 3.92 – 4.31 (m, 123H), 4.32 – 4.49 (m, 69H), 4.49 – 4.65 (m, 30H), 4.68 (s, 16H), 4.73 – 4.92 (m, 27H), 4.96 – 5.12 (m, 22H), 5.12 – 5.25 (m, 11H), 5.78 (d, J = 6.6 Hz, 8H), 6.85 (s, 21H), 6.90 – 7.56 (m, 9H), 7.73 (s, 33H), 7.81 (s, 44H), 8.05 (s, 40H), 8.89 (d, J = 2.8 Hz, 1H),

9.01 – 9.65 (m, 16H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 15.10, 19.66, 23.17, 27.15, 28.66, 32.53, 33.45, 36.24, 36.87, 37.61, 41.60, 42.71, 43.83, 44.29, 48.90, 51.57, 55.96, 59.57, 65.97, 66.99, 67.60, 68.14, 68.63, 68.84, 69.93, 72.24, 74.19, 79.91, 94.57, 94.71, 97.91, 102.05, 102.86, 103.24, 120.28, 146.39, 156.16, 157.46, 170.69, 171.16, 171.52, 173.40, 187.54, 189.06. MALDI-TOF MS M_w=12,200 m/z.

Fluorescence assay with free MMP substrate and MMP substrate glycodendrimers and A549 cells. Before performing the assay, the total amount of dye loaded on to the dendrimer photometrically determined and compared against a standard curve. The degree of loading for MMP7 dendrimers was determined at A_{351} and found 0.8 MMP7 substrates for every dendrimer. For MMP2/9 dendrimers at A_{548} an average of 0.3 MMP2/9 substrates per dendrimer was determined. Lung carcinoma A549 cells were purchased from ATCC and cultured as recommended by ATCC. Prior to use, cells were dissociated from petri dishes using a 0.02% EDTA / 0.25% trypsin (FAFC biosciences) and diluted to 500,000 cell/mL using serum free F12K media (SFM, Gibco). The assay was performed in a flat bottomed 96-well quartz plate (Hellma). The assay was performed with four different experimental conditions for each MMP substrate functionalized dendrimers: SFM, dendrimer, free MMP substrate, and the MMP substrate on the dendrimer. Samples were monitored every 5 min for 130 min for fluorescent intensity and changes in lifetime using a NovaFlour Fluorescence Lifetime Microplate Reader at 532/556 for MMP2/9 substrate and 280/360 for MMP7 substrate excitation/emission with 1s signal averaging 3 measurements per well per time

point. For MMP7 substrate a Thermo Scientific Varioskan Lux multimode microplate reader was used.

APPENDICES

APPENDIX A

Mode of Action and Routes to Optimize C_{16}-DABCO Dendrimers

C_{16}-DABCO can be considered a surfactant in that it has a polar head and a nonpolar tail; its overall structure resembles a lipid with a cationic head group. C_{16}-DABCO is a QAC. QACs are known to be membrane active and are used frequently in a laboratory or clinical setting. Salton proposed a sequence of events that occurs for the QAC to have a lytic effect on the bacteria. 1) QAC gets adsorbed and penetrates into the cell wall. 2) QAC interacts with lipids or proteins in the cell membrane that leads to membrane disorganization. 3) Small molecules with low molecular weight begin to leak out of the cell. 4) Proteins and nucleic acids degrade. 5) Bacterium undergoes lysis as a result of cell wall degrading autolytic enzymes.[161] The previous sequence of events suggest QAC mode of action is non-specific or acts in a general way. This makes it effective at limiting the resistance bacteria can develop, but also could result in poor selectivity. There are significant differences between bacteria and mammalian cells constitution of the cell membrane that could be taken advantage of to create a more selective antibiotic. DABCO dendrimers could be altered in such a way they selectively interact with bacterial membranes instead of mammalians cell membranes. Altering the chain length on the DABCO subunit, reaching the final product without tosyl groups would be ideal, and changing the generation size of the dendrimers could also aid in obtaining better selectivity. Some combination of these parameters could result in a more selective DABCO dendrimer. Further, using a more specific antibiotic piece instead of a QAC could certainly result in a more selective antibiotic.

APPENDIX B

Spectra

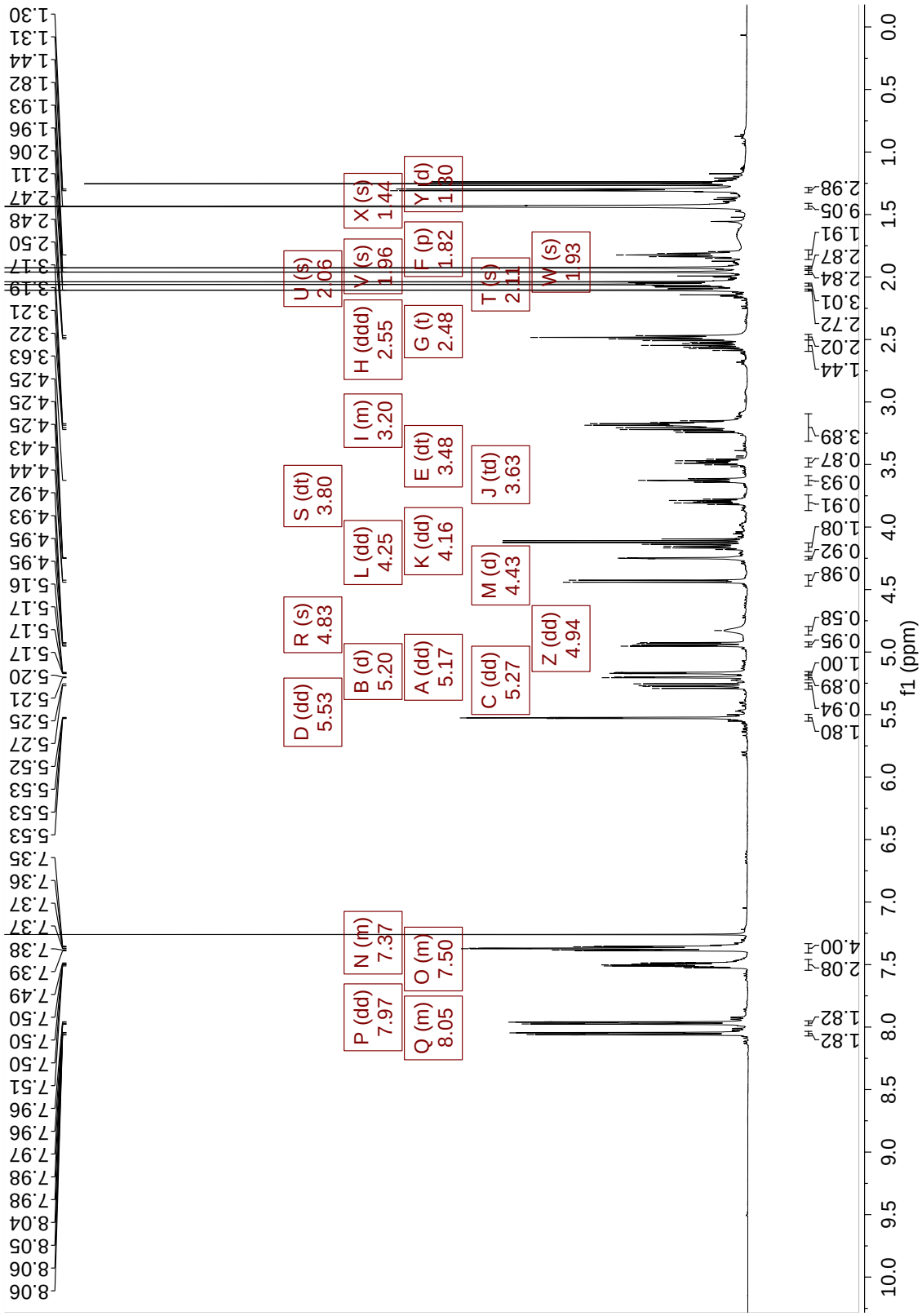


Figure 15. ^{1}H NMR of compound **8a.**

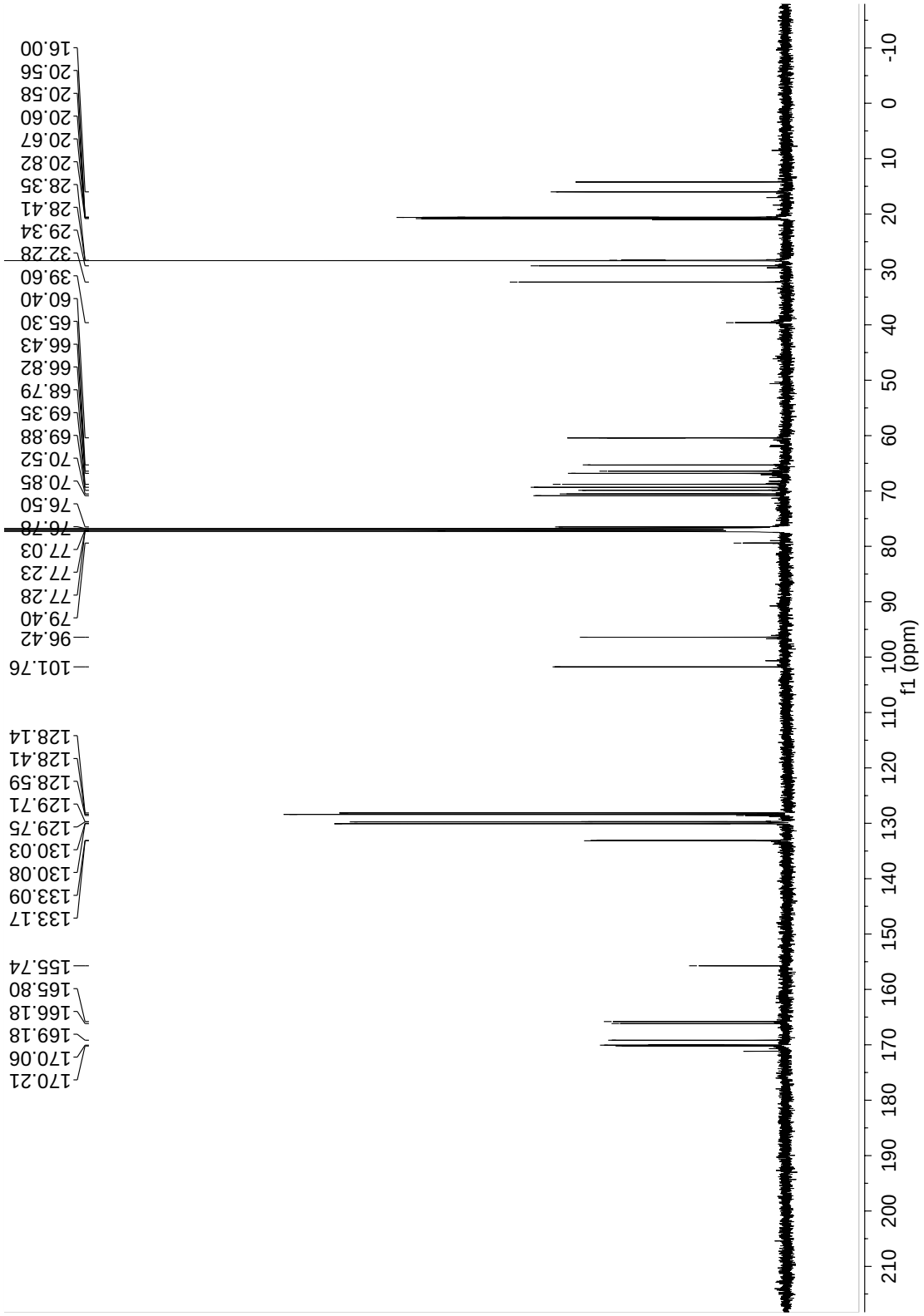


Figure 16. ^{13}C NMR of **8a.**

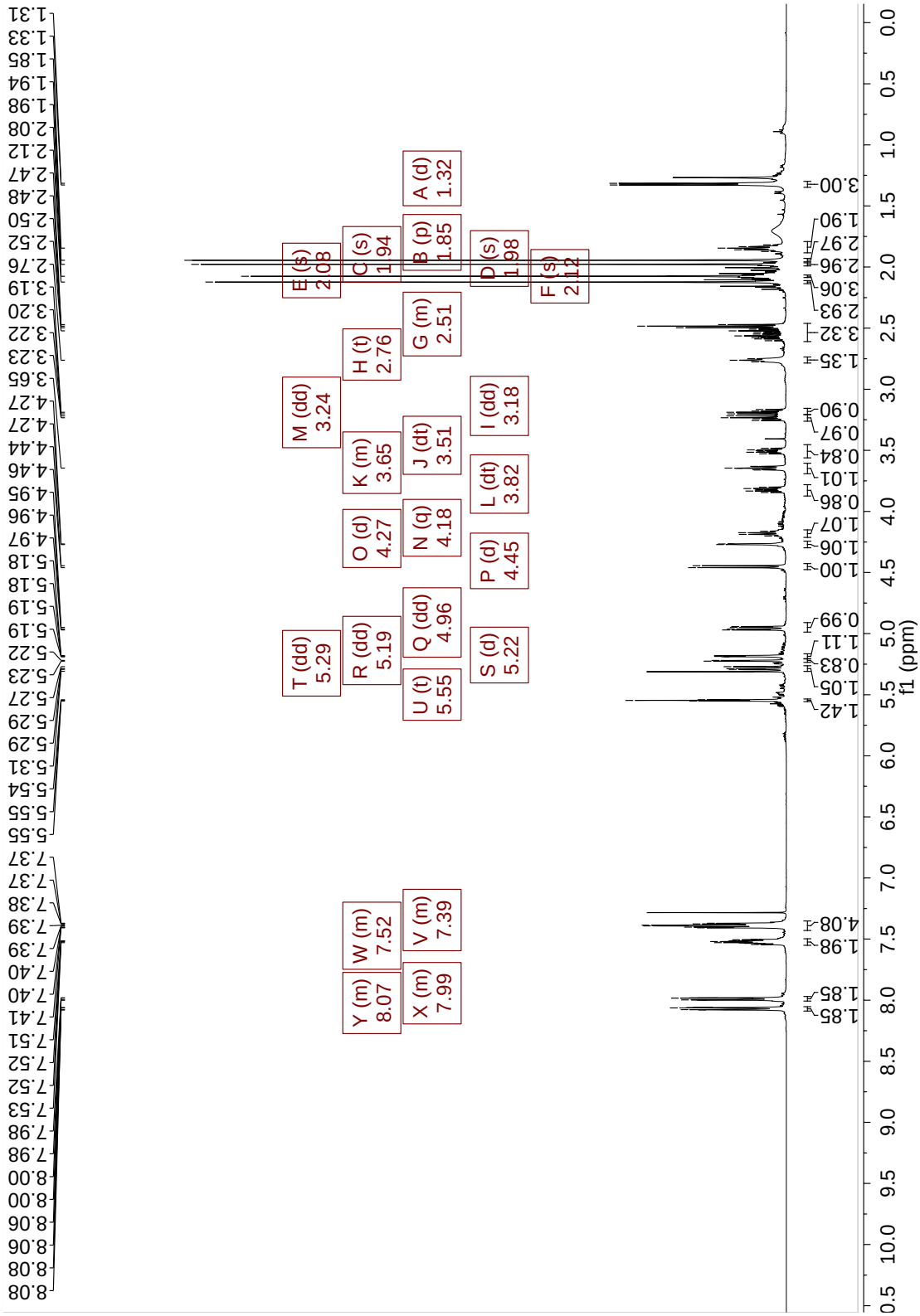


Figure 17. ^{1}H NMR of product **8b.**

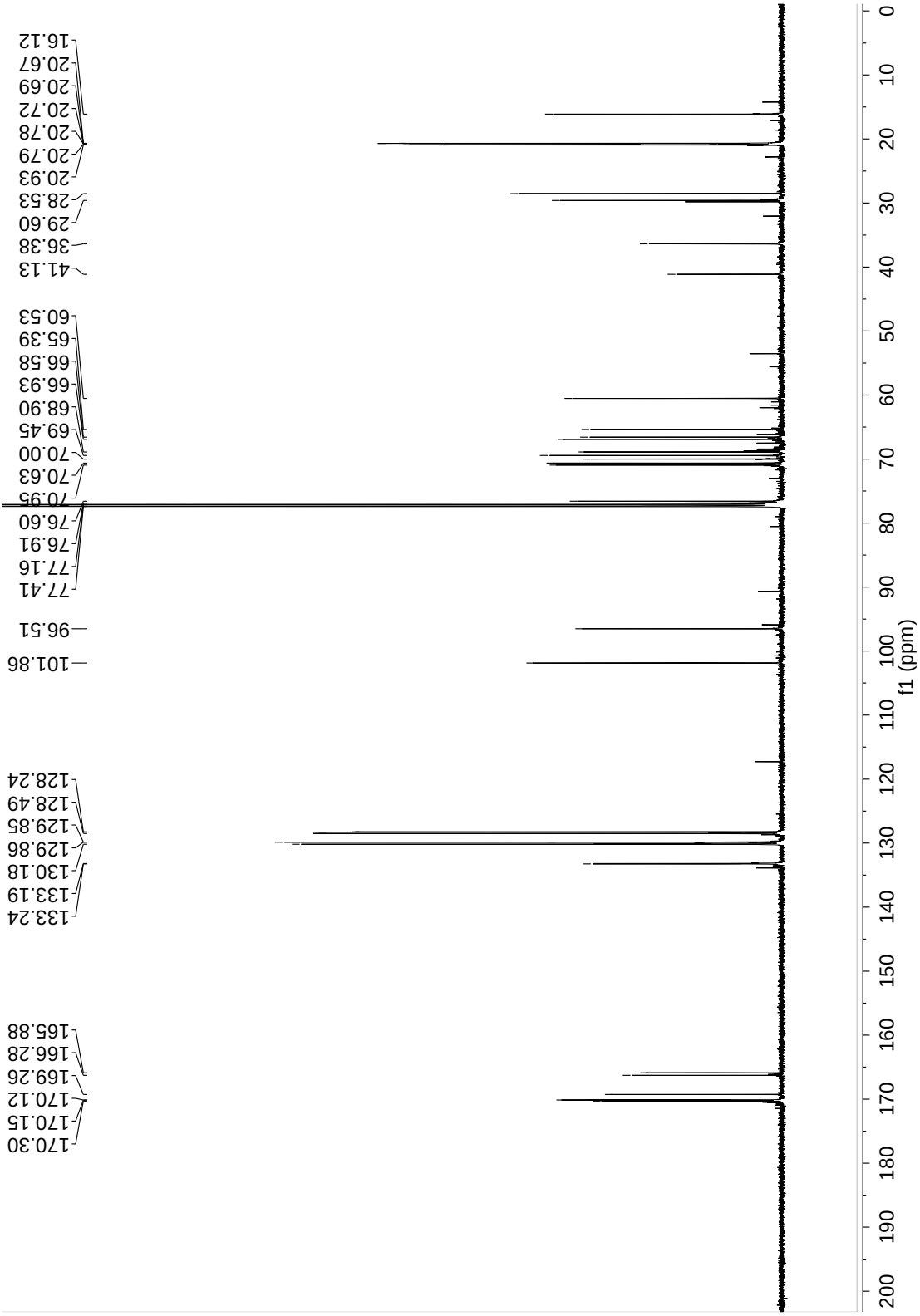


Figure 18. ^{13}C NMR of product **8b**.

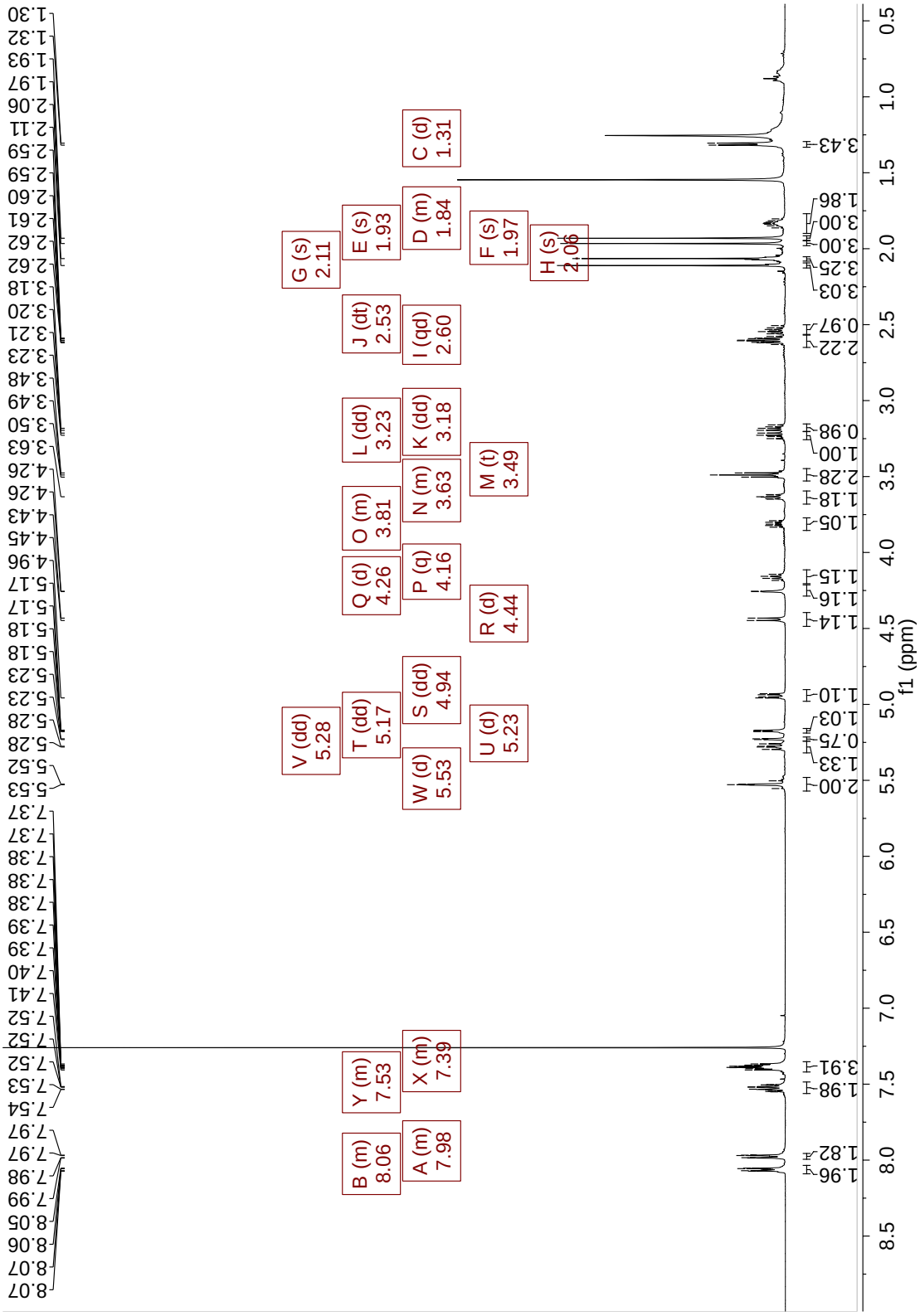


Figure 19. ^{1}H NMR of product **9**.

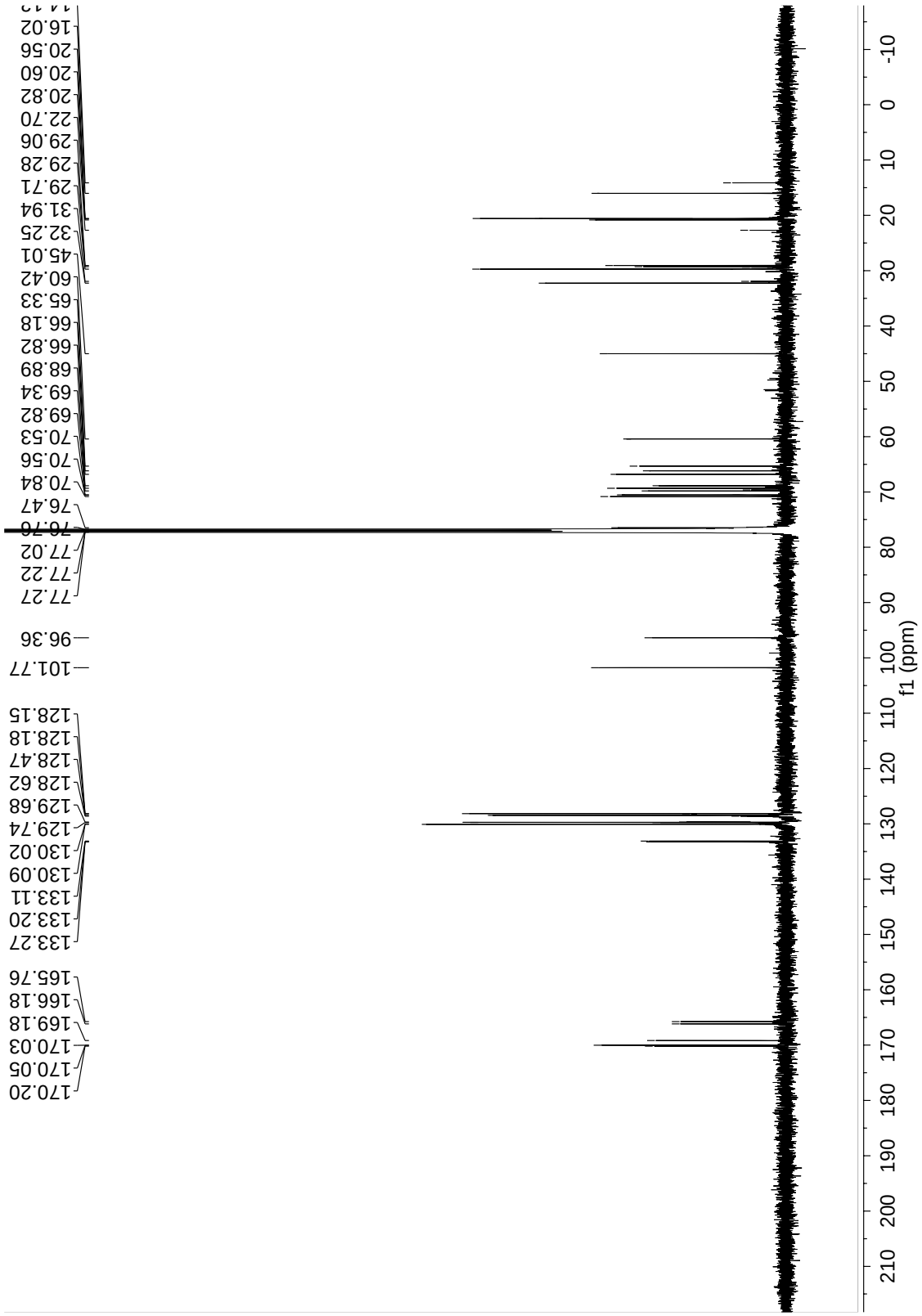


Figure 20. ^{13}C NMR of product **9**.

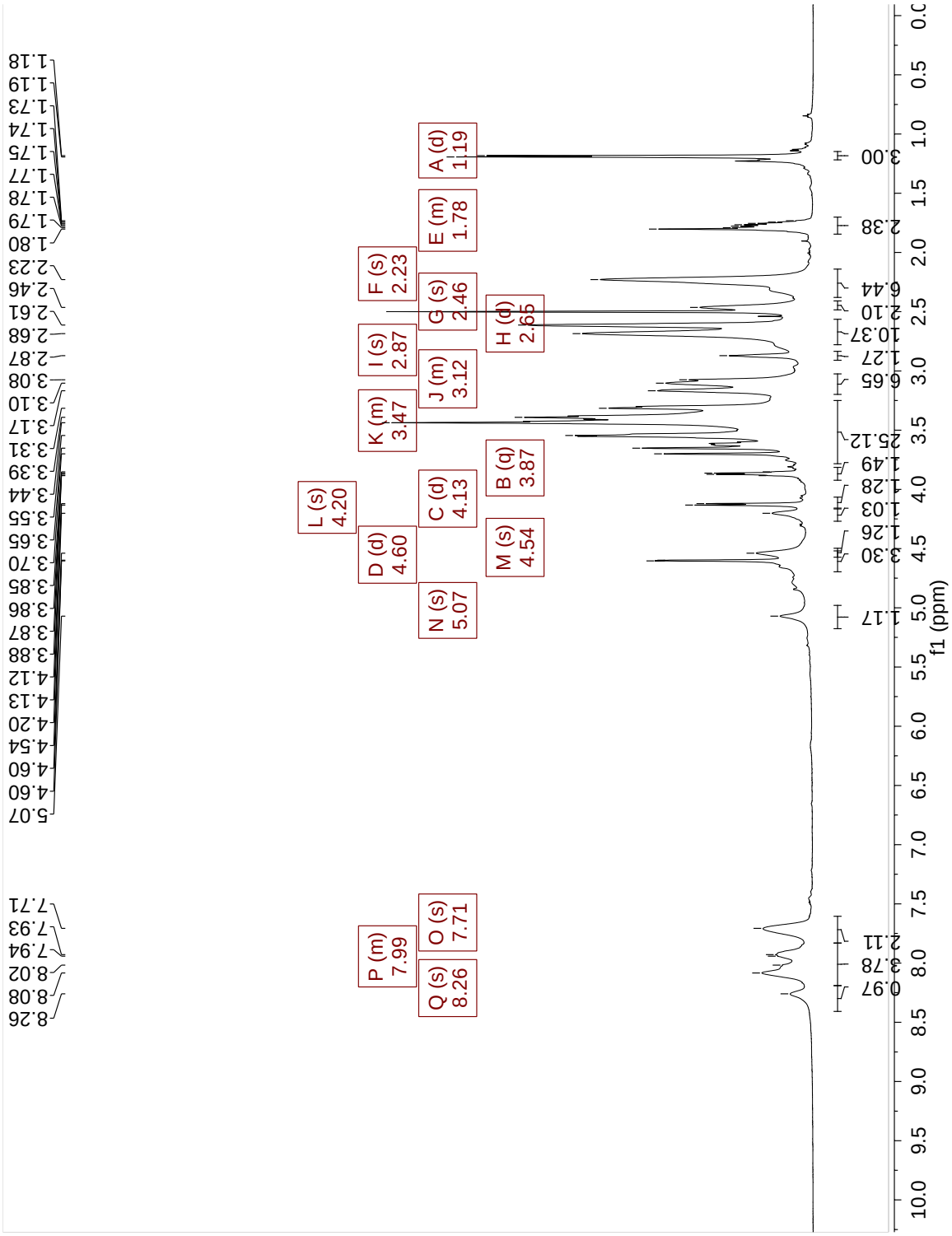


Figure 21. ^{1}H NMR of product **10.**

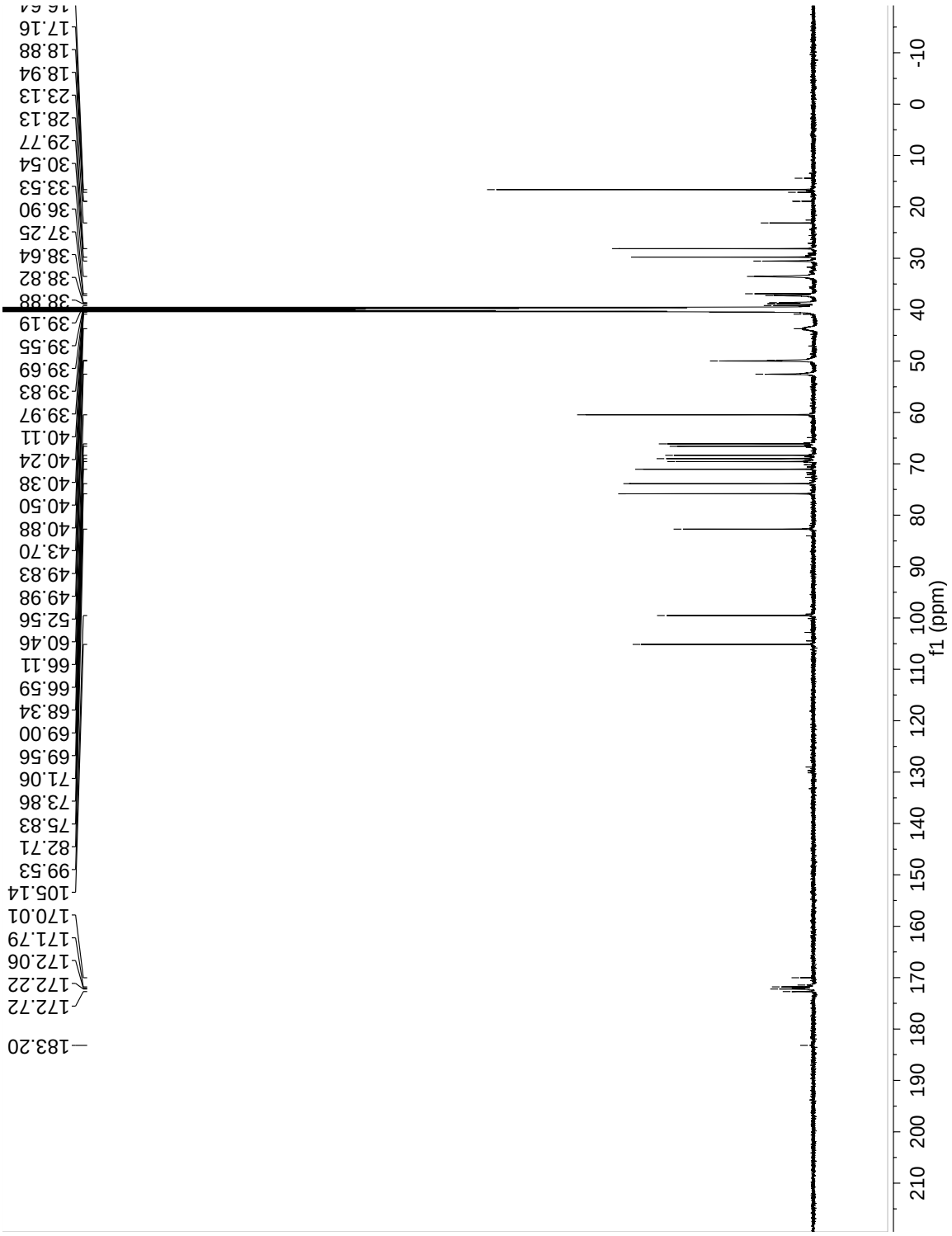


Figure 22. ^{13}C NMR of product **10**.

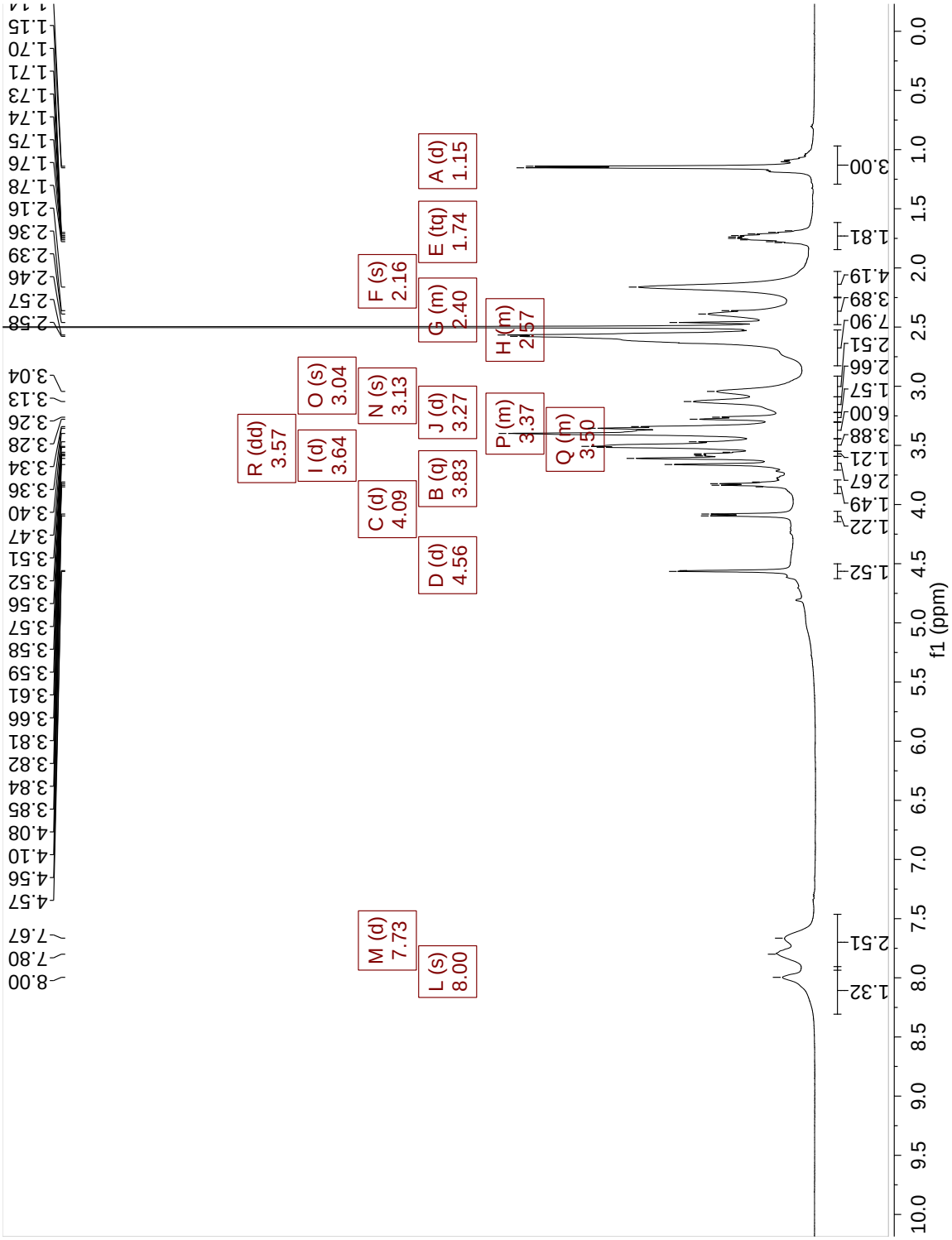


Figure 23. ^{1}H NMR of product **11.**

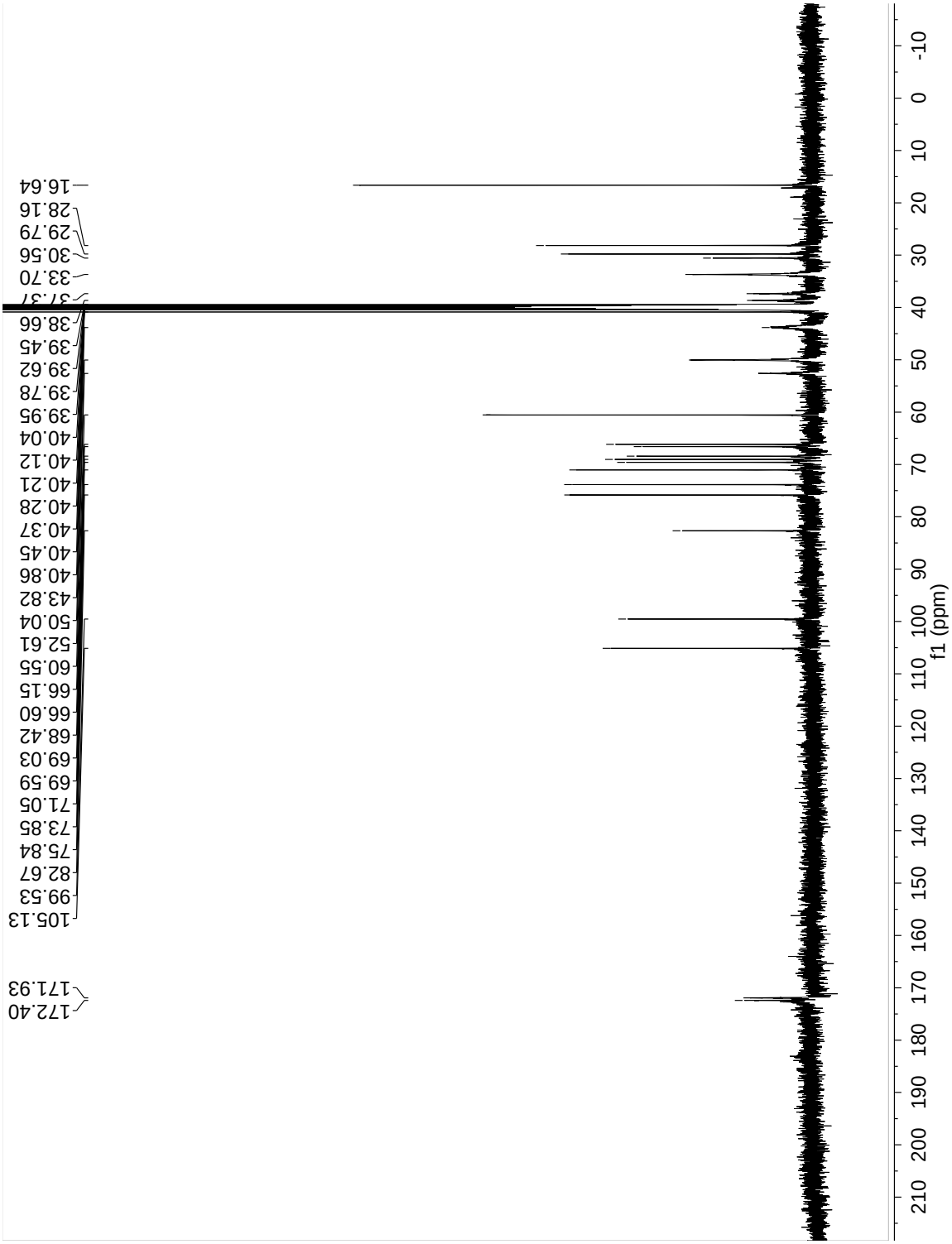


Figure 24. ^{13}C NMR of product **11**.

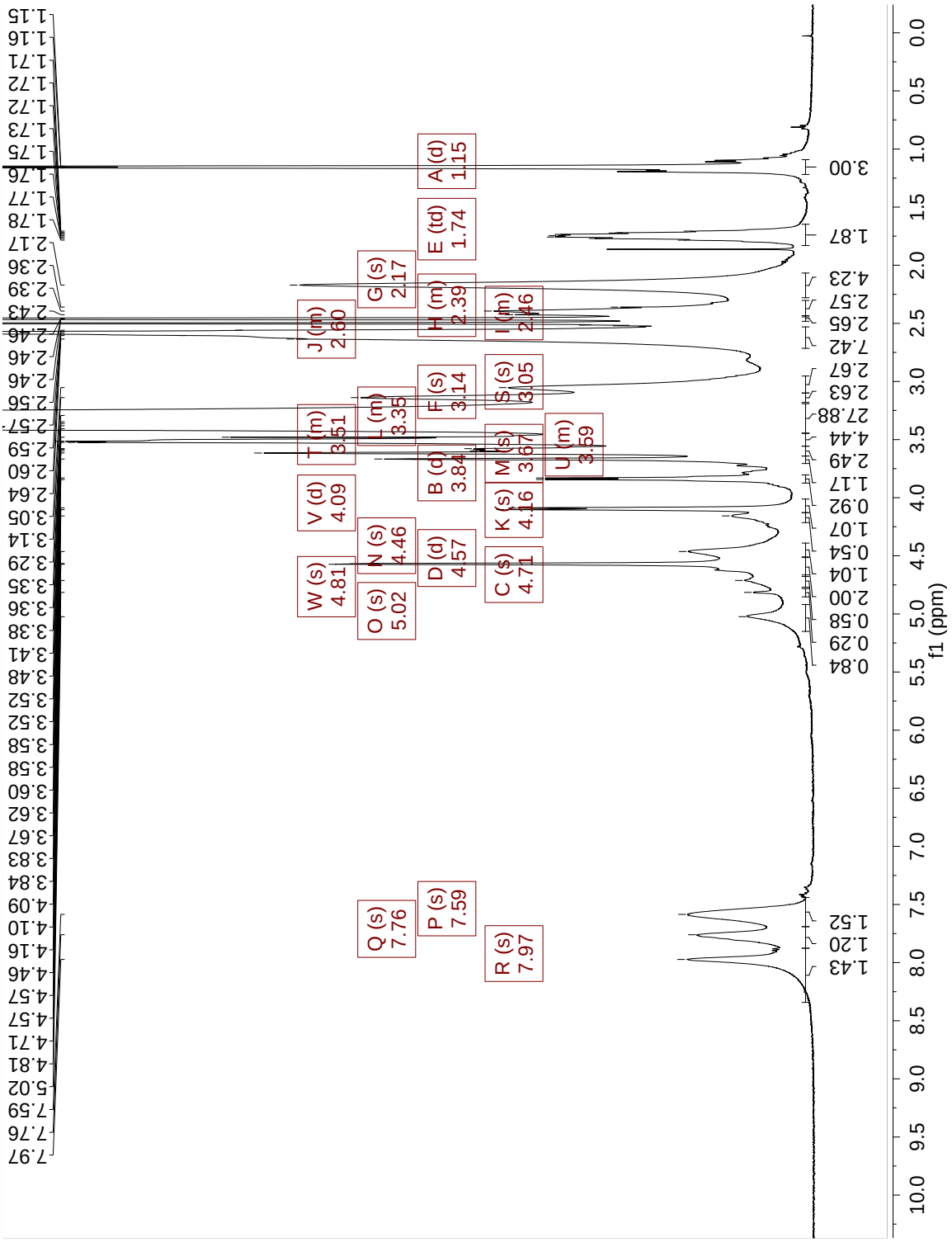


Figure 25. ^{1}H NMR of product **12.**

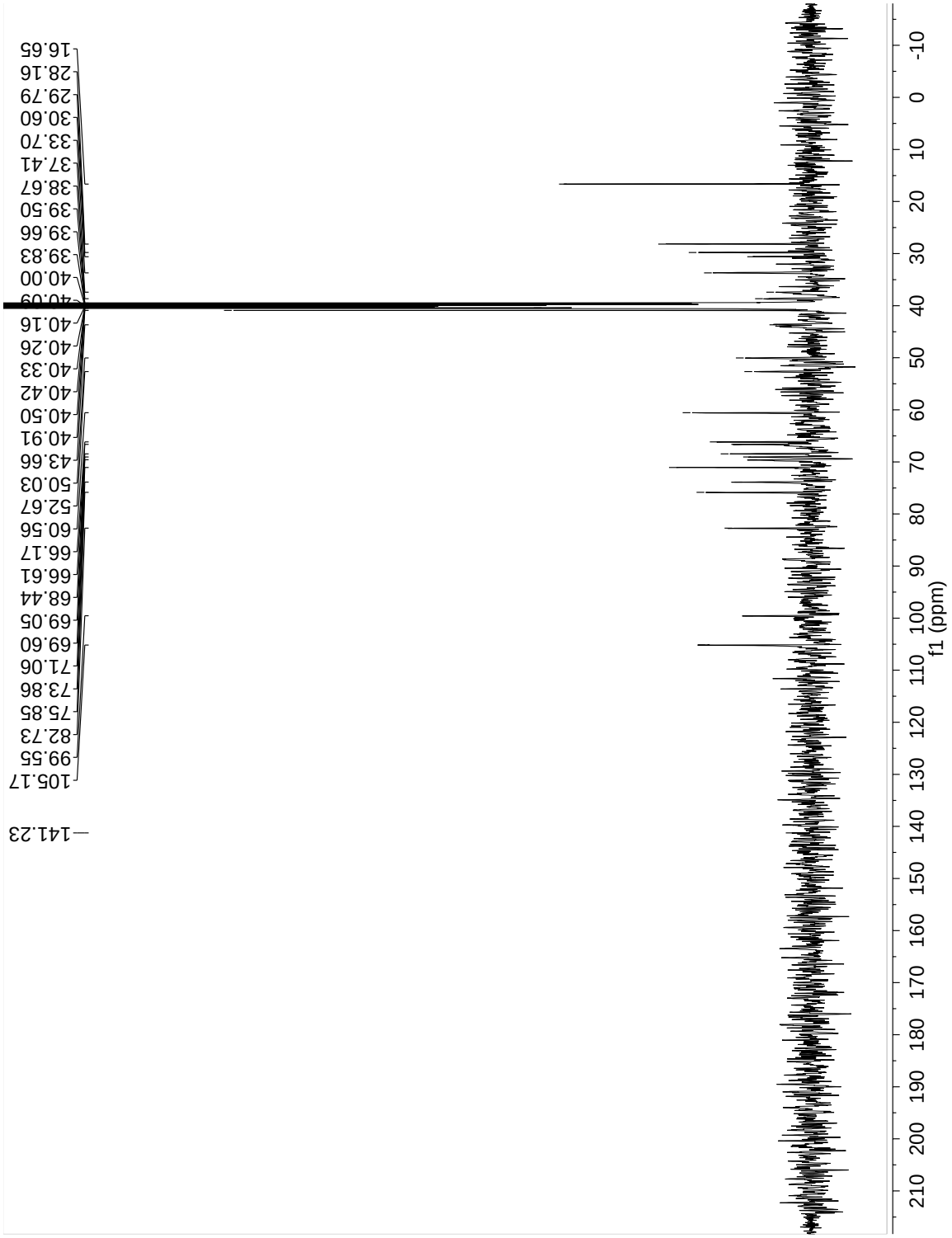


Figure 26. ^{13}C NMR of product **12**.

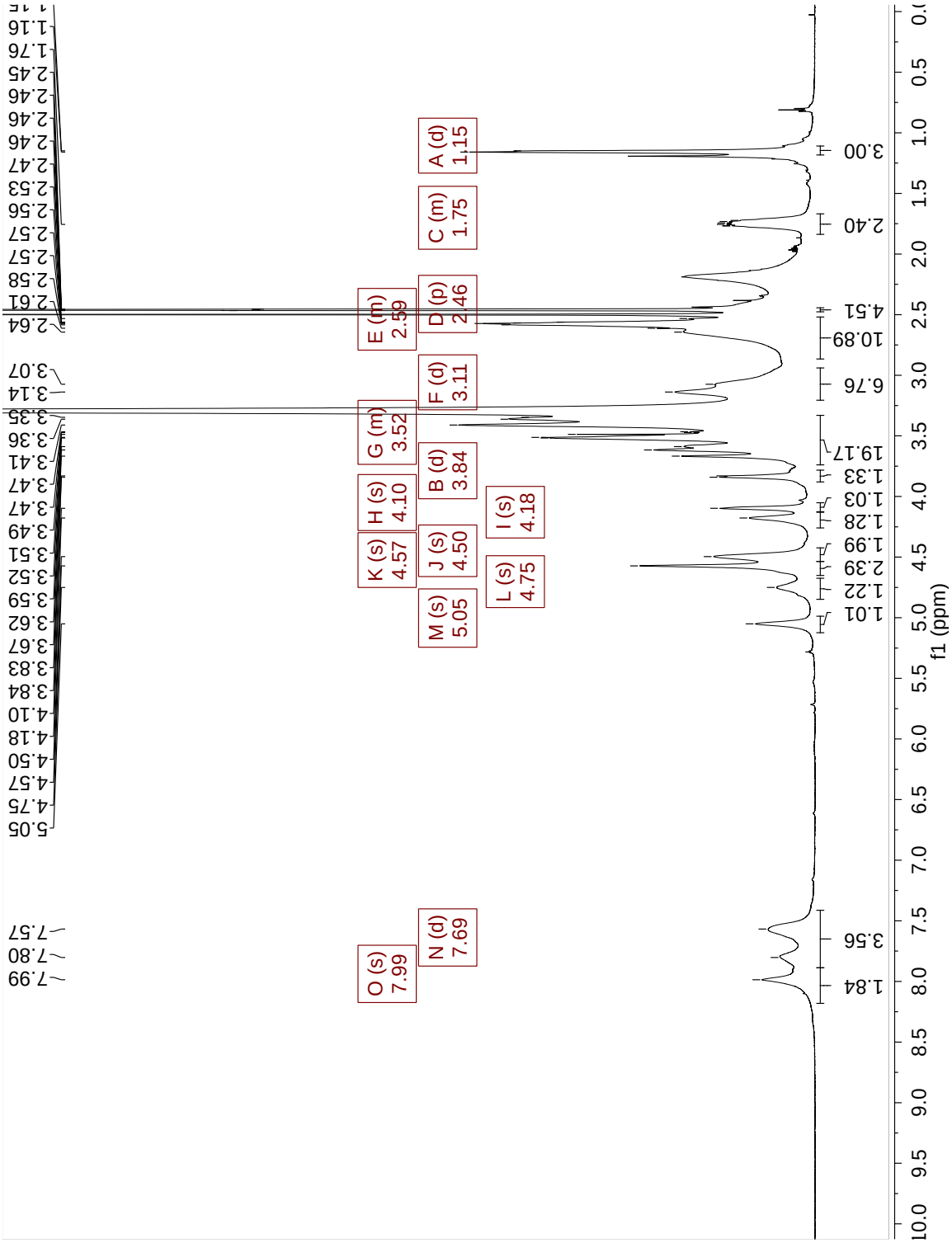


Figure 27. ^{1}H NMR of product **13**.

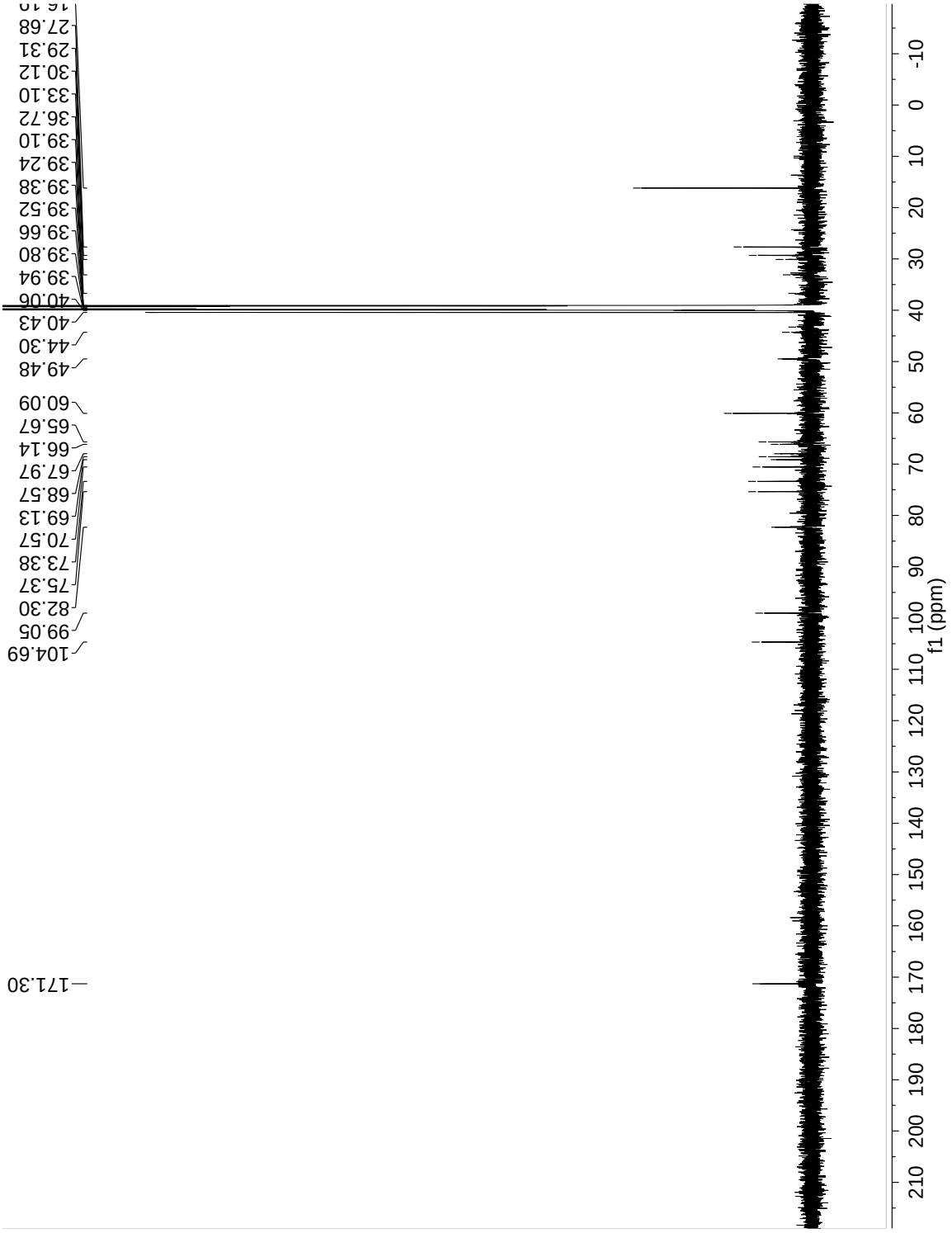


Figure 28. ^{13}C NMR of product **13**.

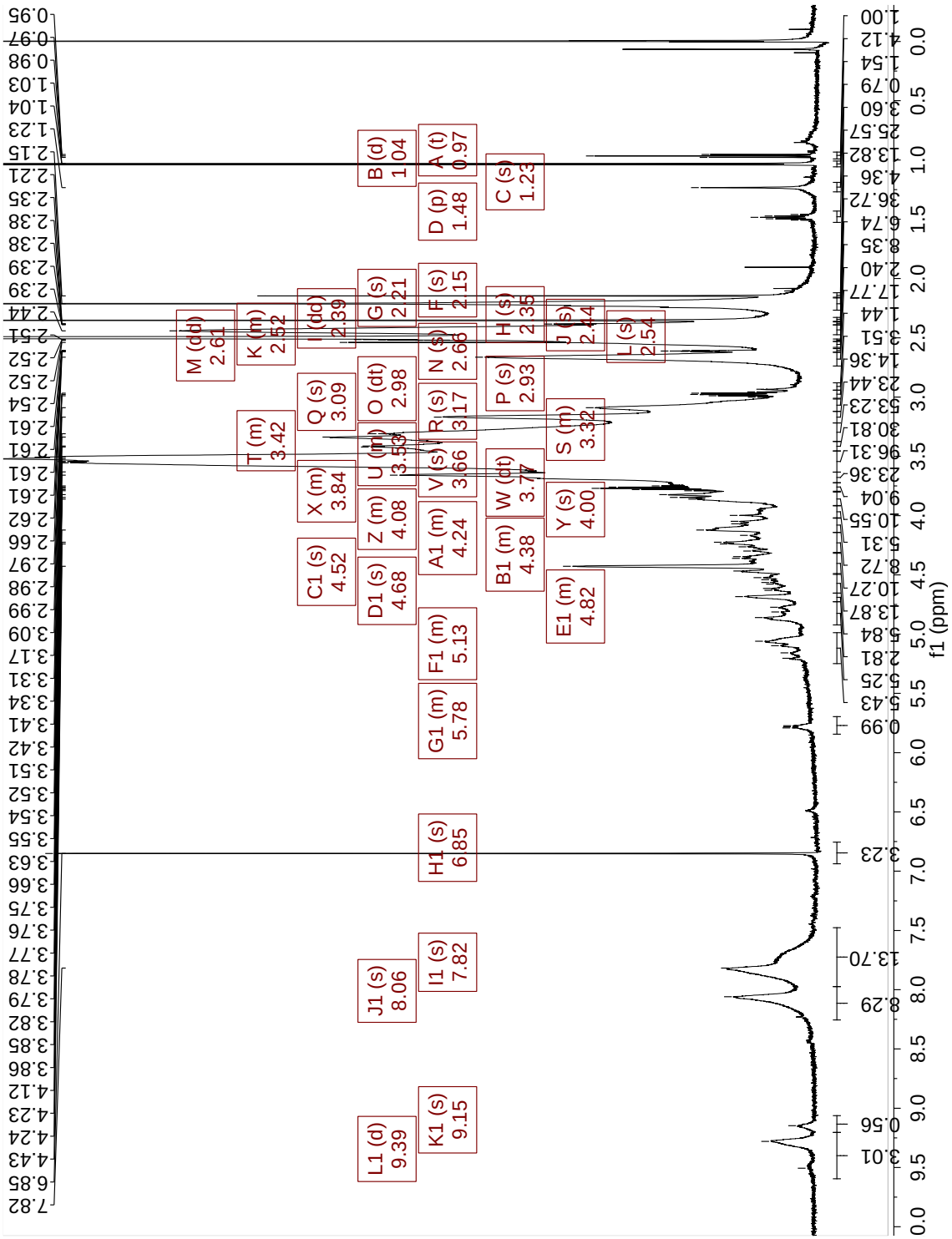


Figure 29. ^{1}H NMR of product **21**.

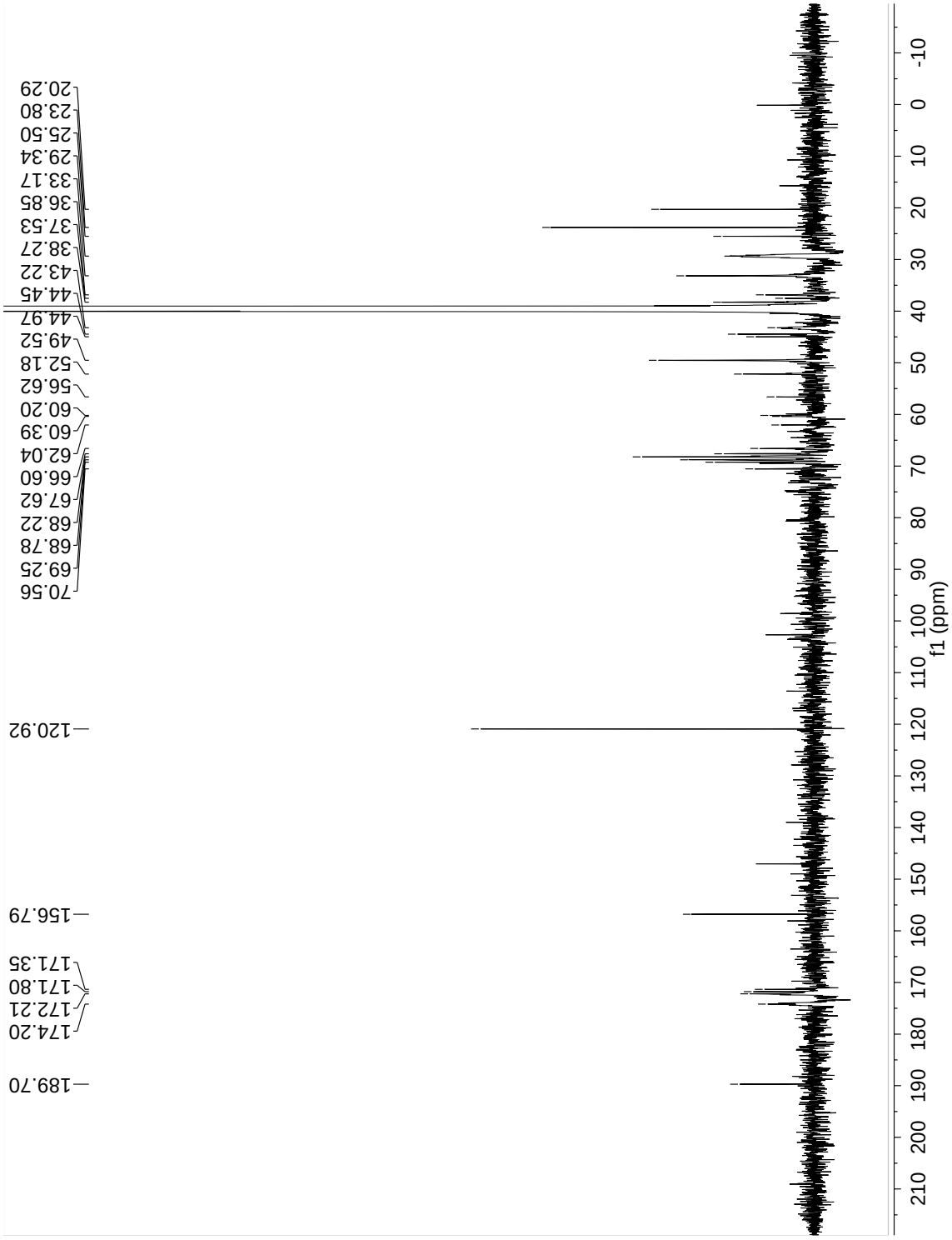


Figure 30. ^{13}C NMR of product **21**.

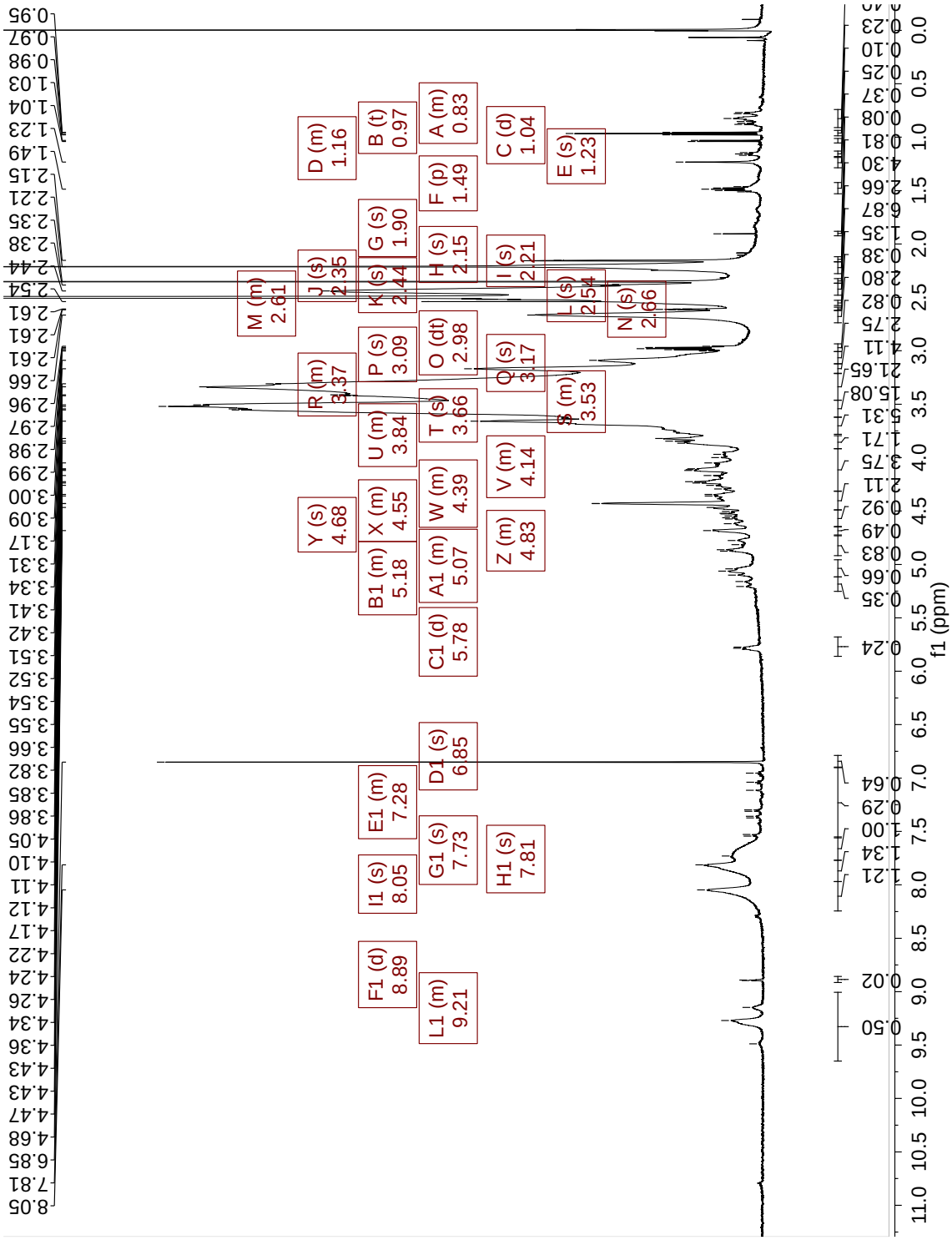


Figure 31. ^{1}H NMR of product **22**.

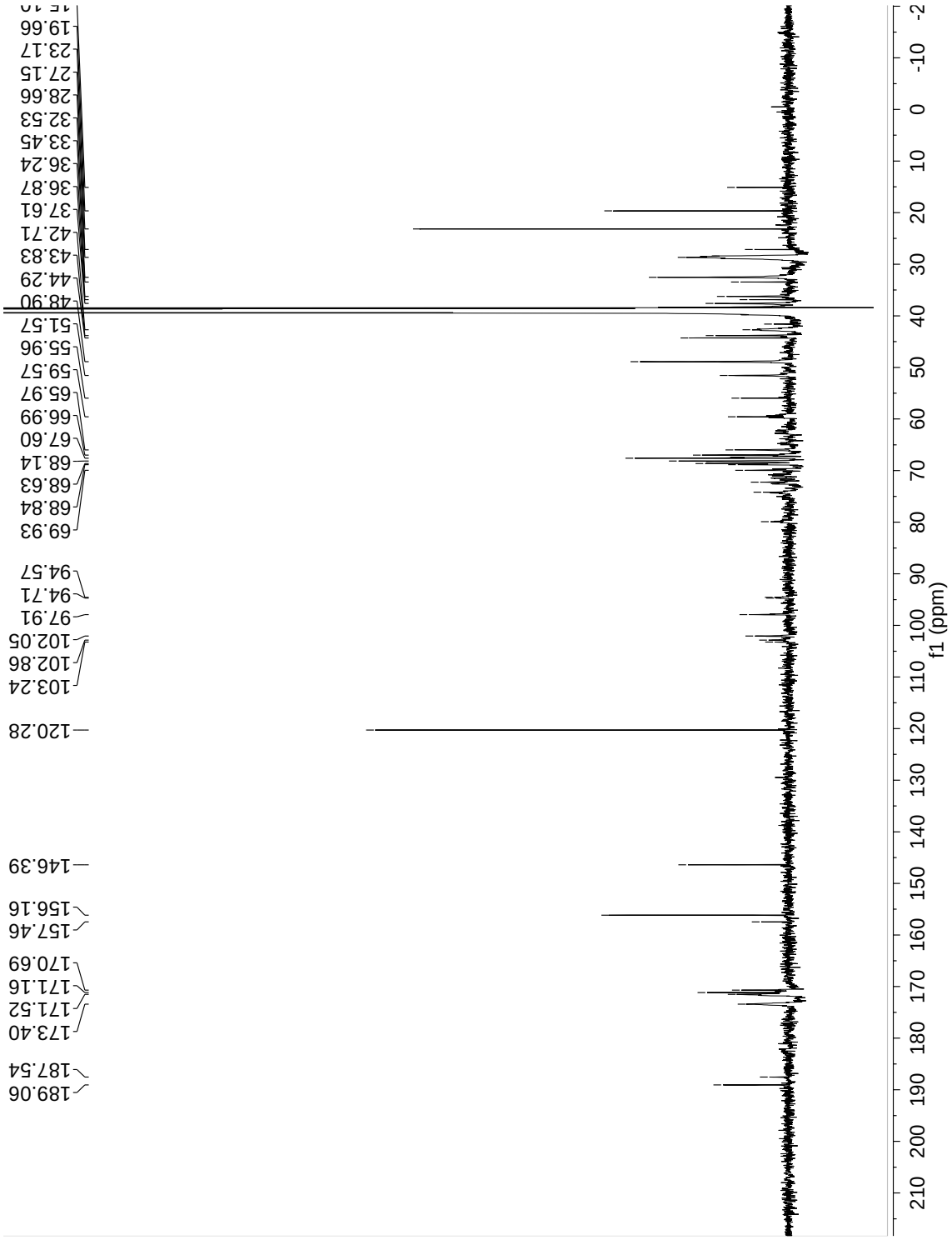


Figure 32. ^{13}C NMR of product **22**.

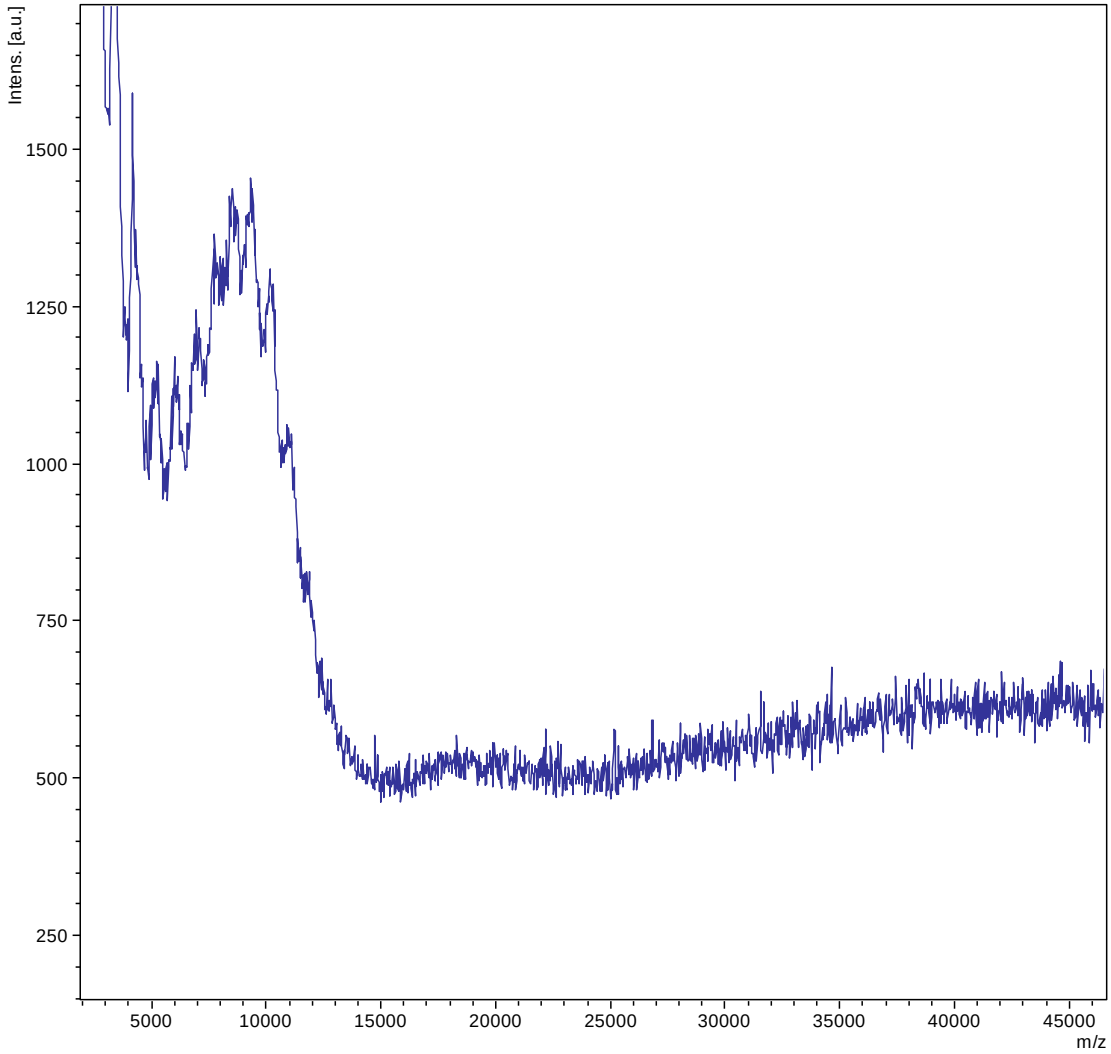


Figure 33. MALDI spectrum of the protected precursor to **10**.

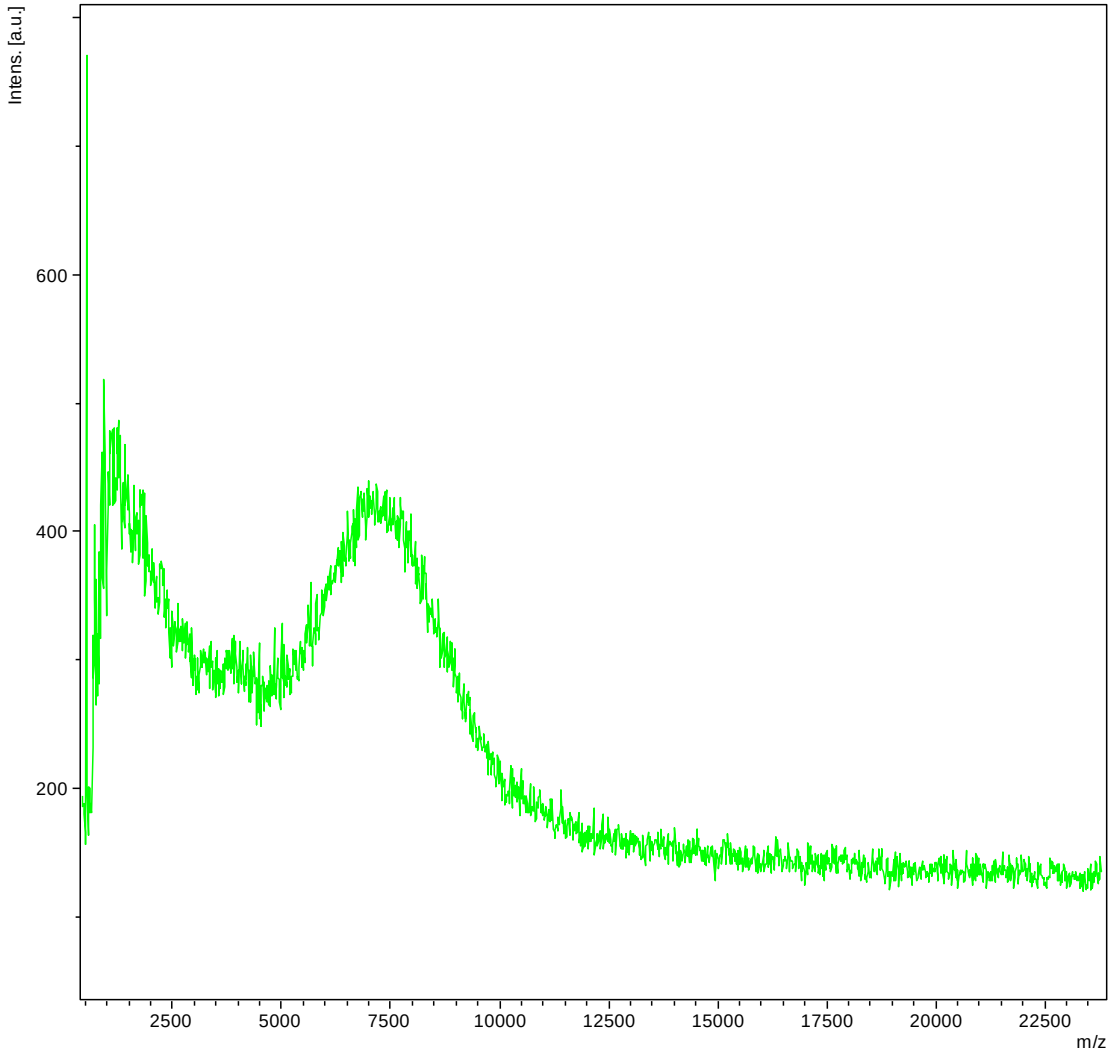


Figure 34. MALDI spectra of **10**.

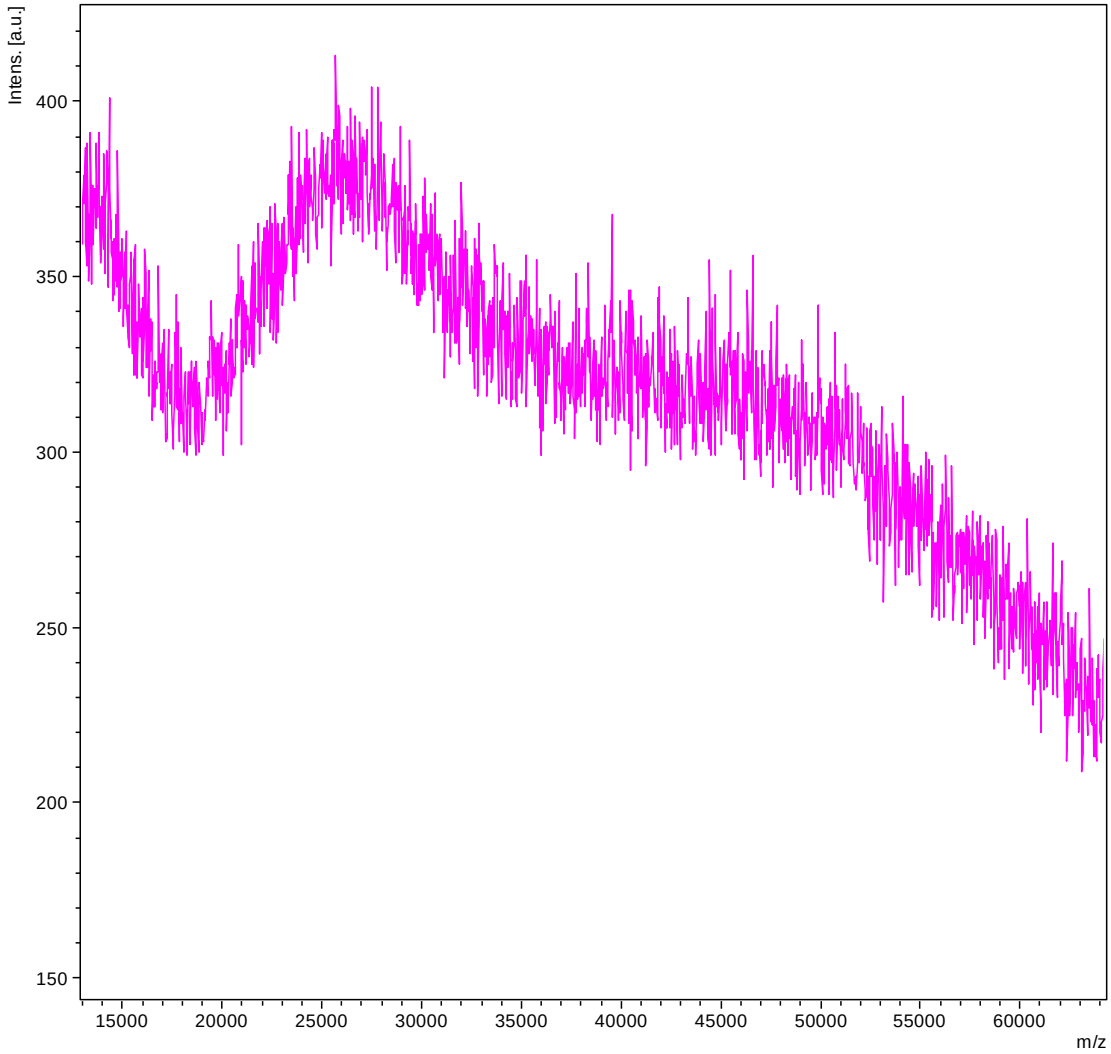


Figure 35. MALDI spectrum of the protected precursor to **11**.

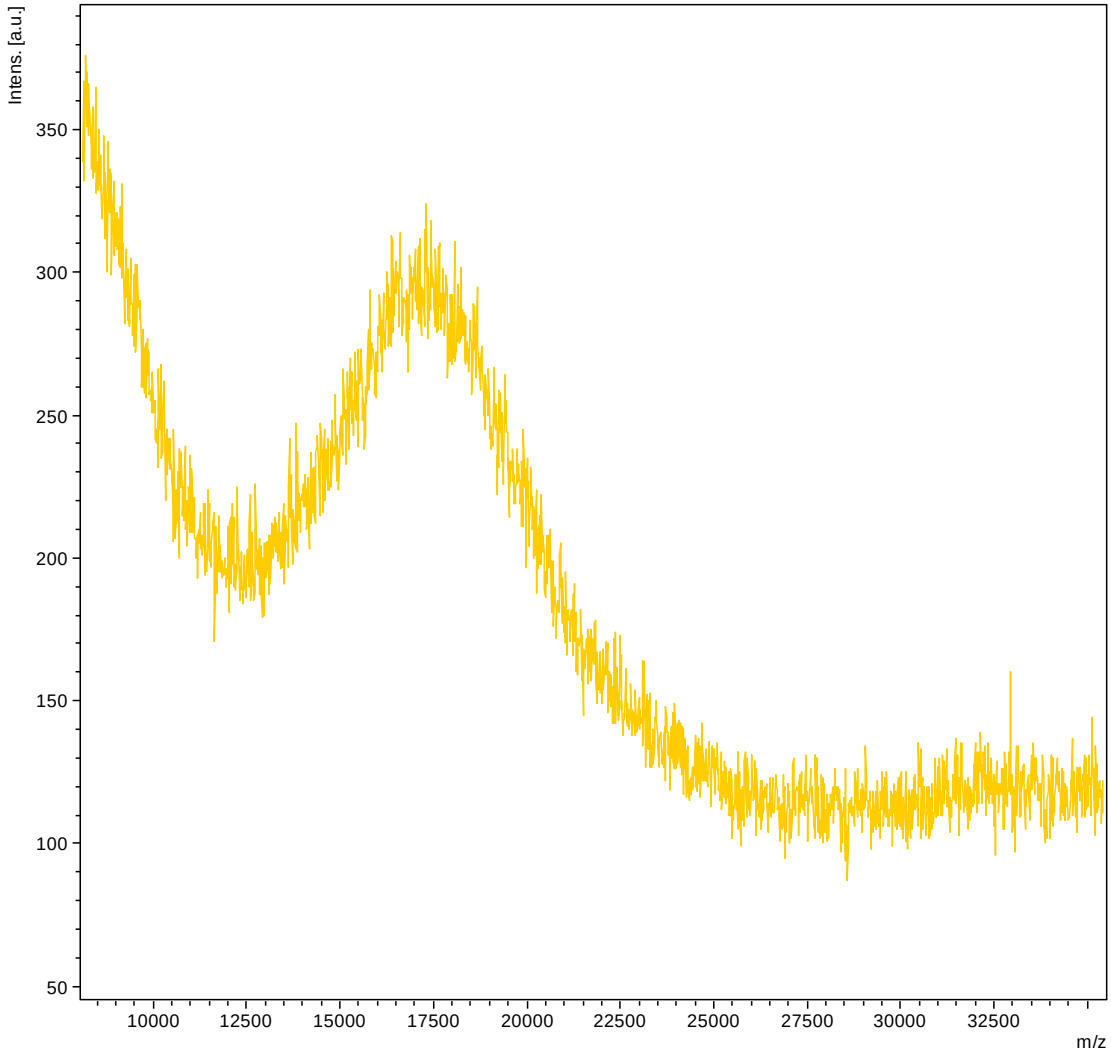


Figure 36. MALDI spectrum of **11**.

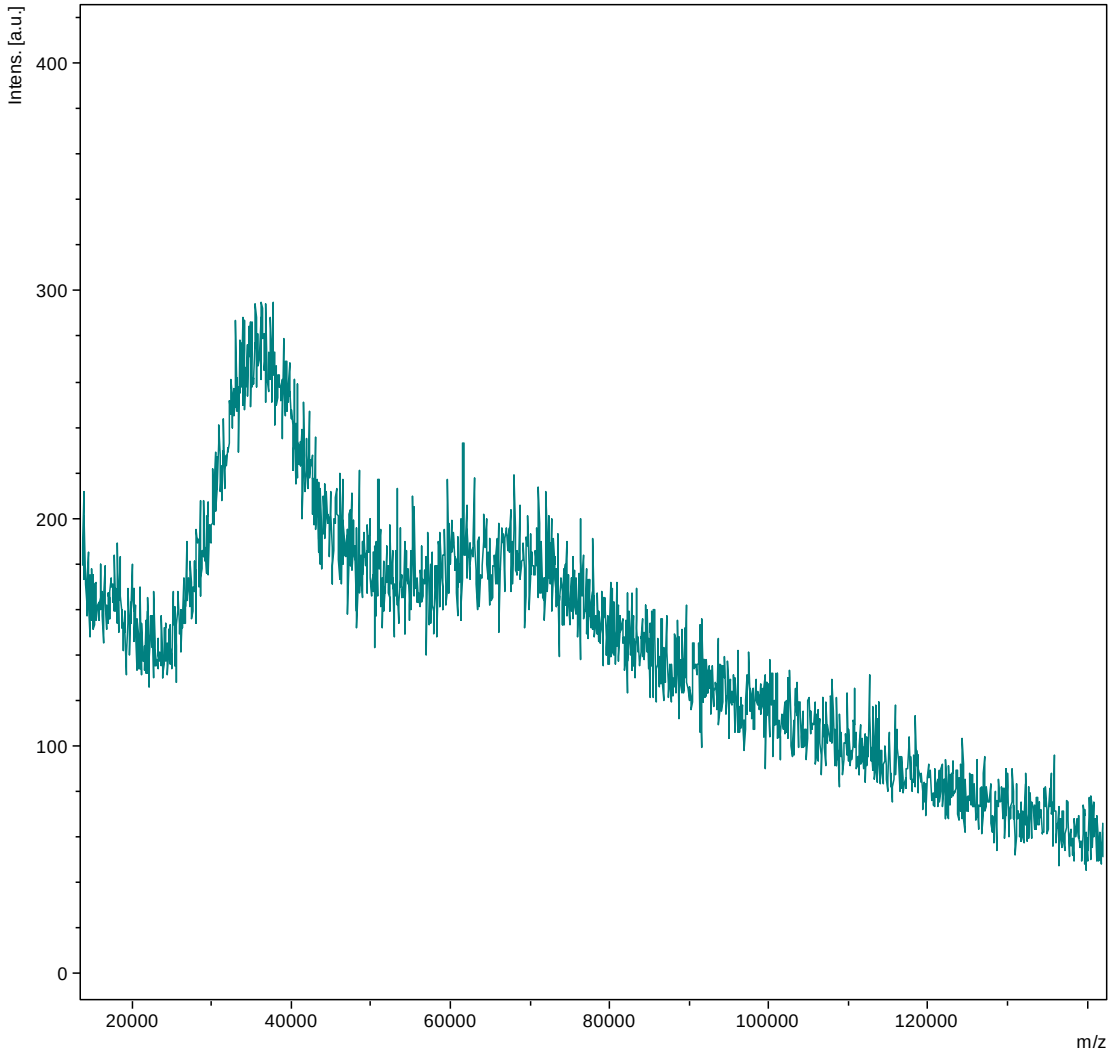


Figure 37. MALDI spectrum of the protected precursor **12**.

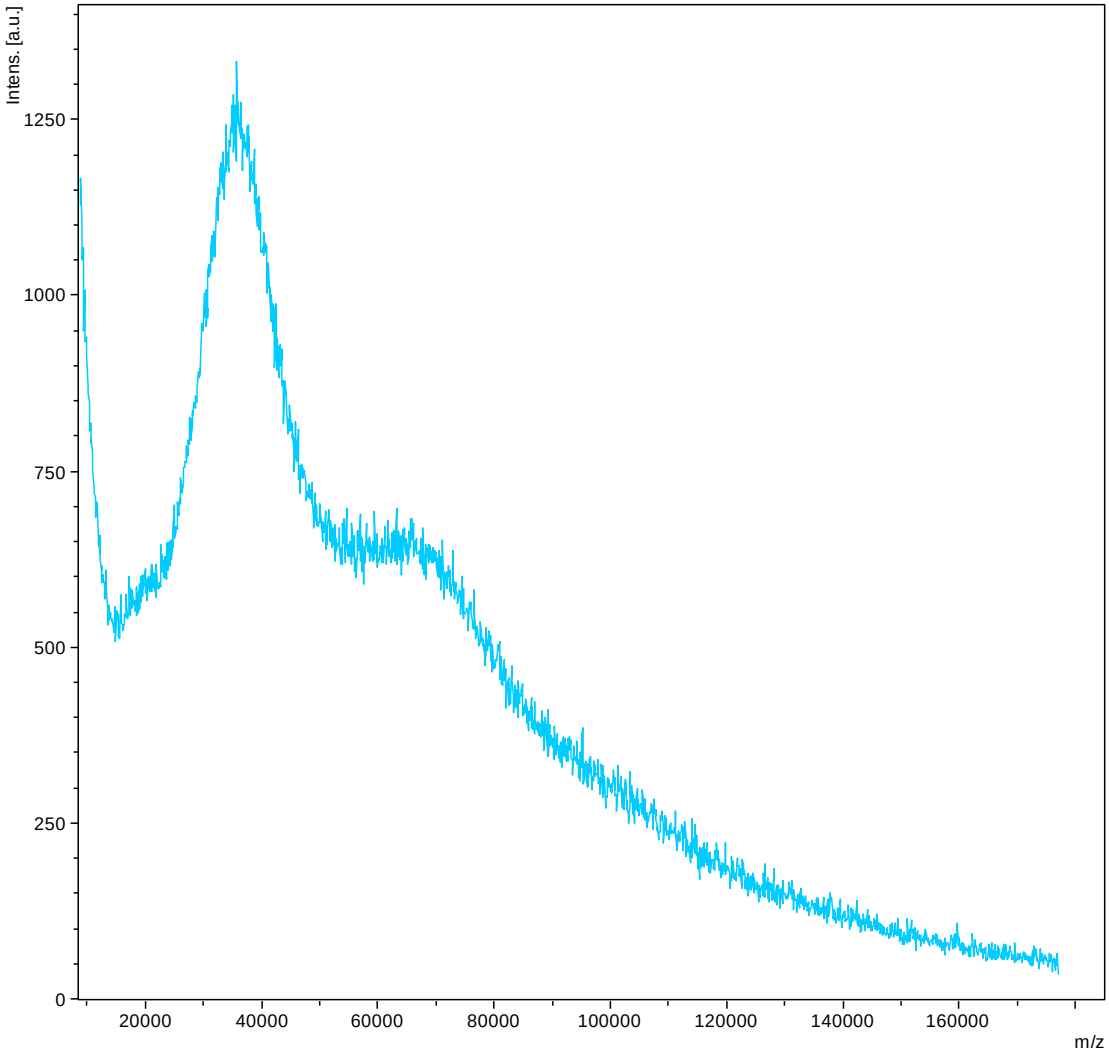


Figure 38. MALDI spectrum of product **12**.

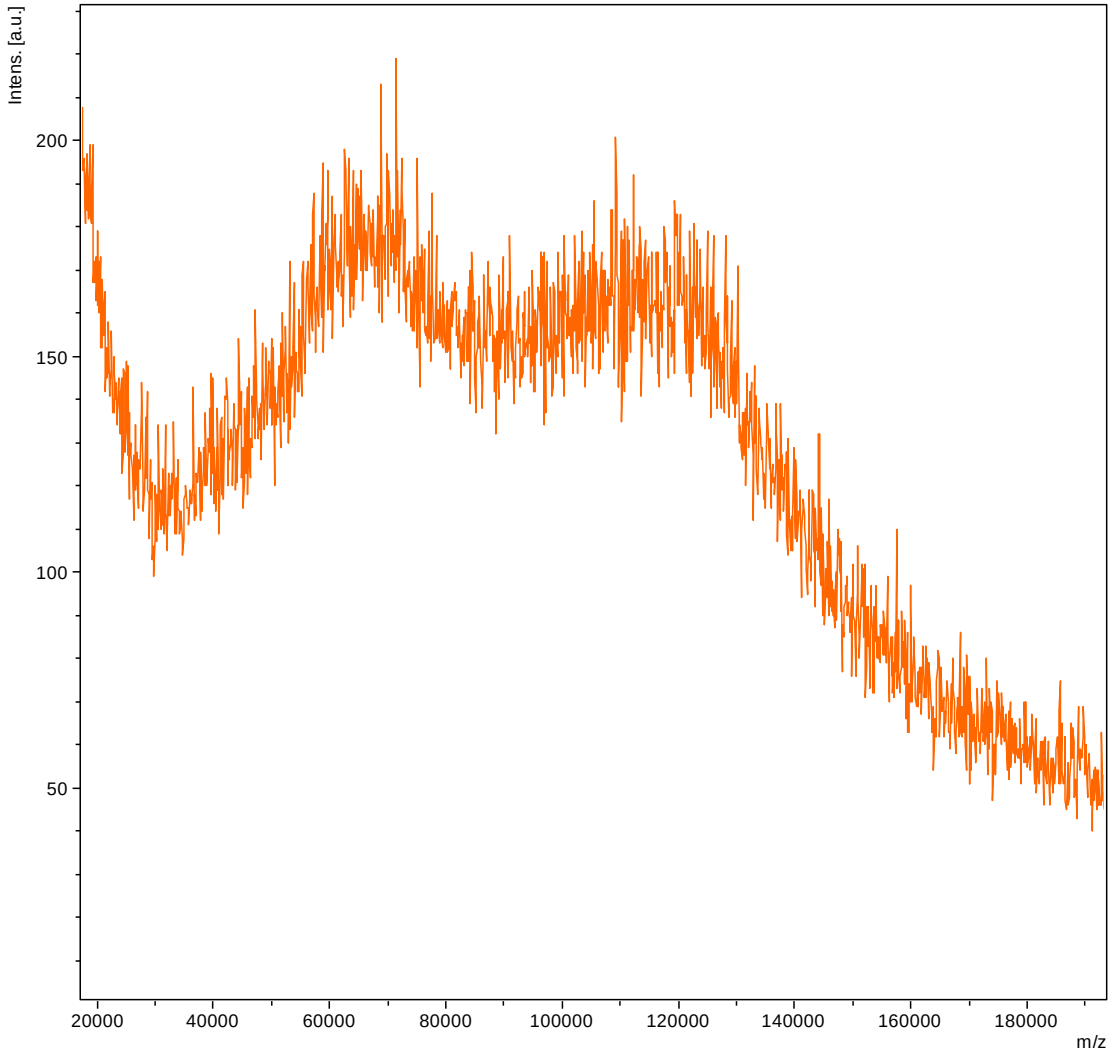


Figure 39. MALDI spectra of the precursor to **13**.

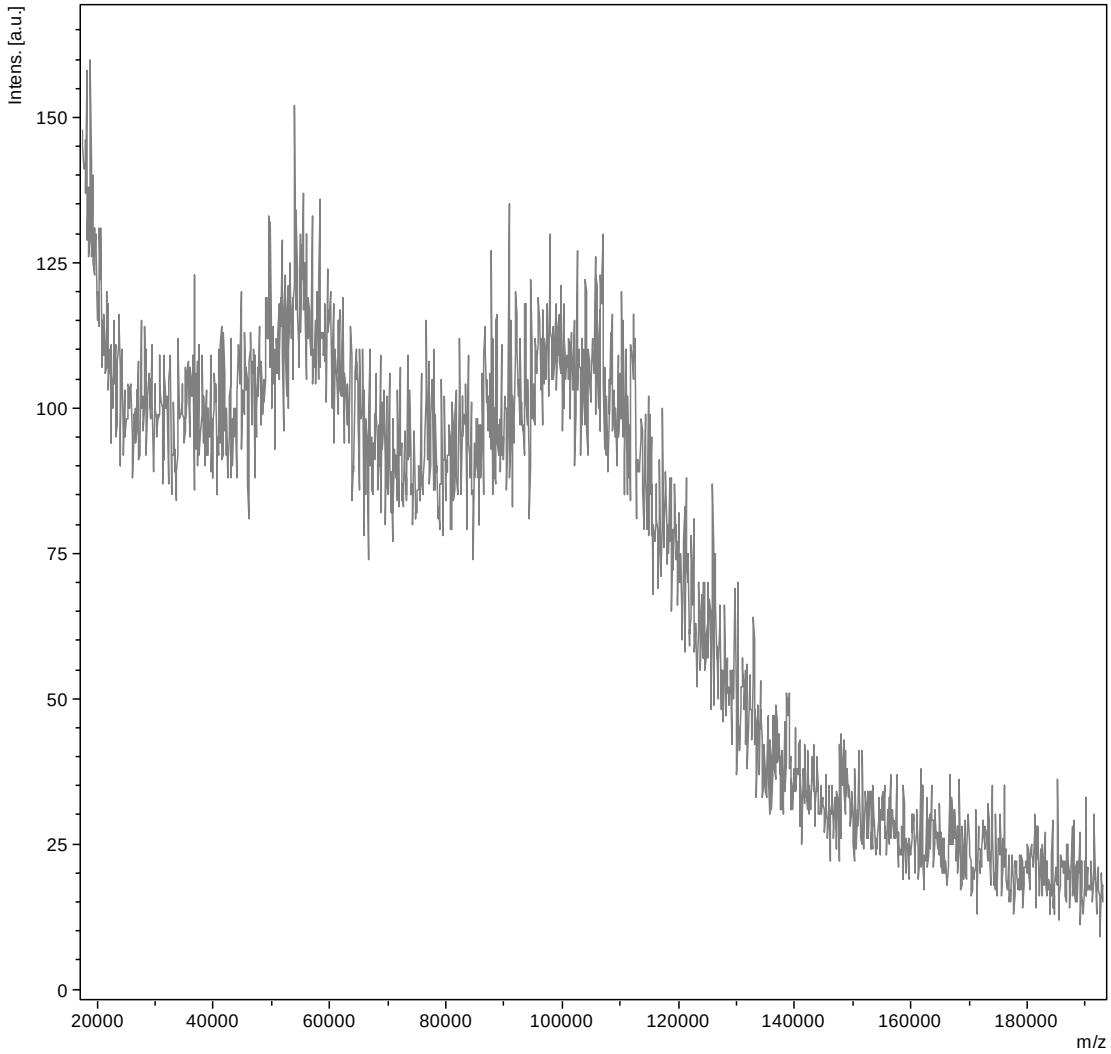


Figure 40. MALDI spectrum of **13**.

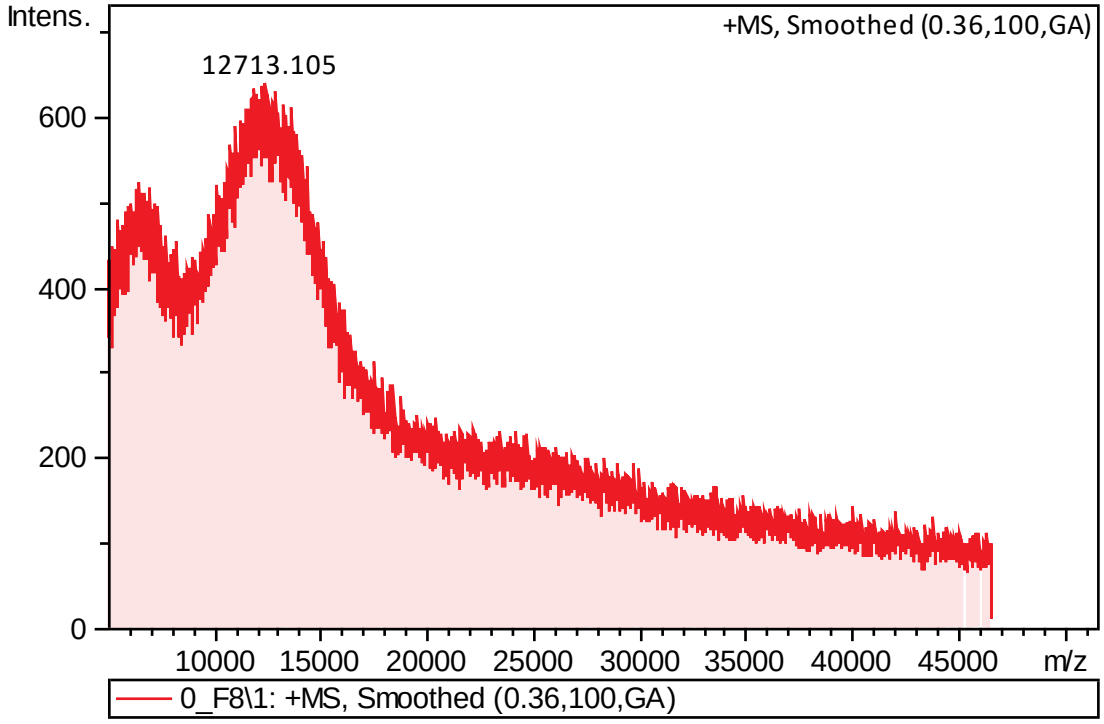


Figure 41. MALDI spectrum of **21**.

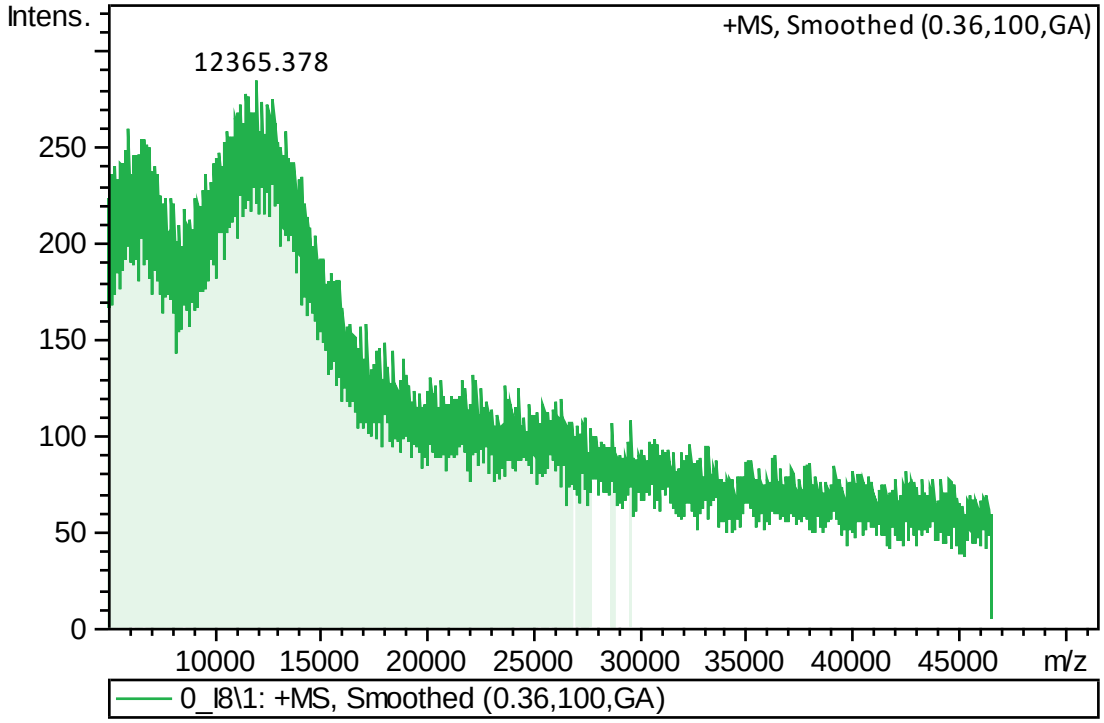


Figure 42. MALDI spectrum of **21**.

General procedure for determining charge of C_{16}-DABCO dendrimers. A 0.01 mM solution of C_{16}-DABCO dendrimers was dissolved in filtered (0.022 μm filter) Millipore water. 100 μL of the solution was placed into Wyatt Technologies' Mobius Dip Cell to perform the electrophoretic mobility experiment. The triangle extending above the y-axis indicates C_{16}-DABCO dendrimers are positively charged. It is noteworthy, that the positive charge appears after the addition of C16-DABCO. Generally speaking, unfunctionalized glycodendrimers are neutral in charge.

General Procedure for Determining Critical Micelle Concentration. Experiments were performed to ensure that C_{16}-DABCO was below the critical micelle concentration (CMC). Several concentrations of C_{16}-DABCO were dissolved in water (Millipore) and filtered through a 0.22 μm syringe filter to ensure no dust or other contaminants were present in the sample. Samples were analyzed on a 90 plus Particle Size Analyzer made by Brookhaven Instruments Corporation. The graph in Figure S4 represents several overlapping data acquisitions. The two regression lines represent the intensity changes as a function of concentration of C_{16}-DABCO. One line shows data prior to micelle formation, and the other line is after formation of micellular aggregates. The intersection point shows the concentration at which micelles begin to form. From this experimental data, the critical micelle concentration was determined to be 0.685 mM. This concentration

is well above the concentrations reported throughout this publication for C16-DABCO use in assays.

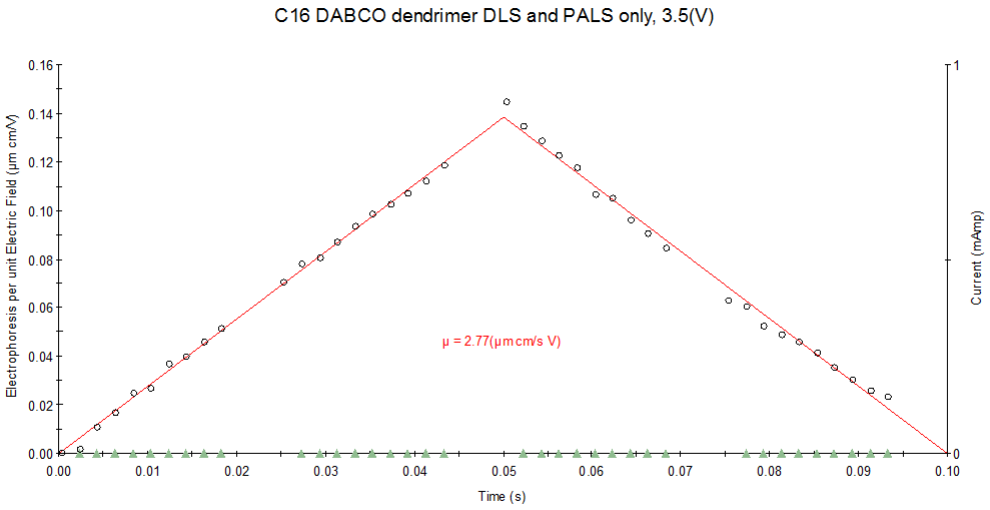


Figure 43. Electrophoretic mobility plot for C_{16}-DABCO dendrimers.

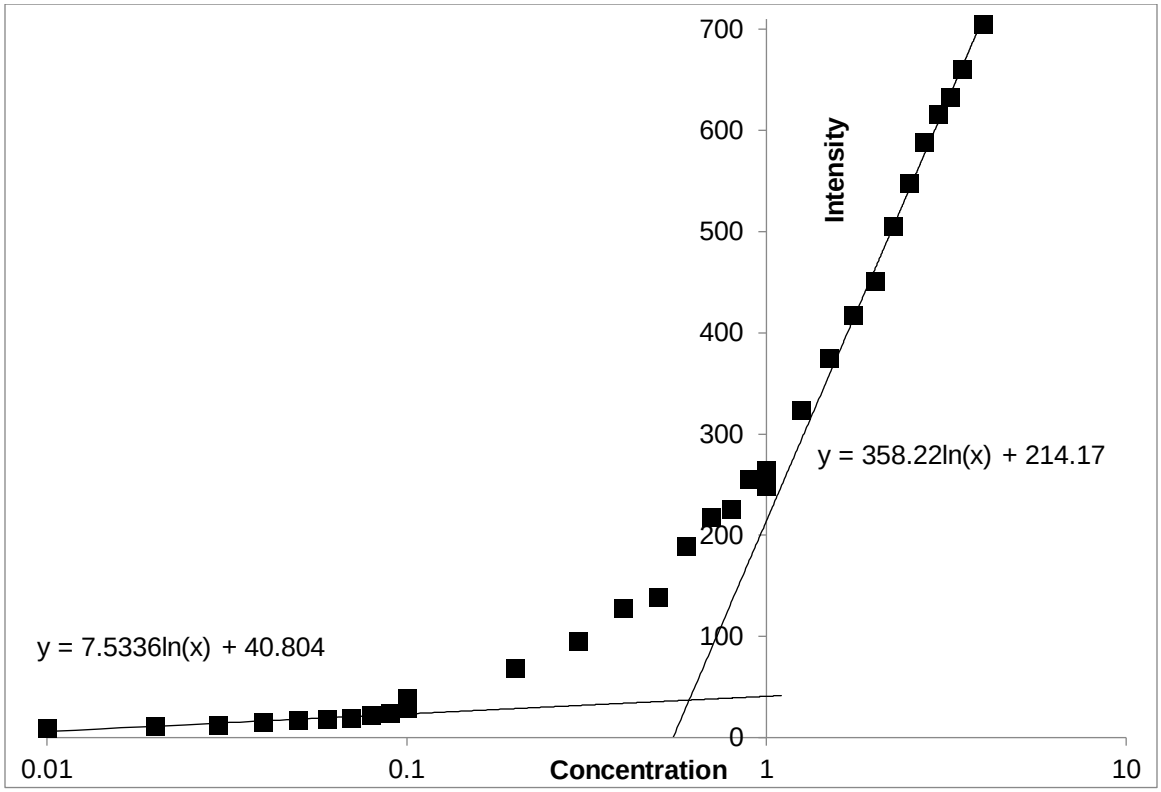


Figure 44. CMC determination for **1**.

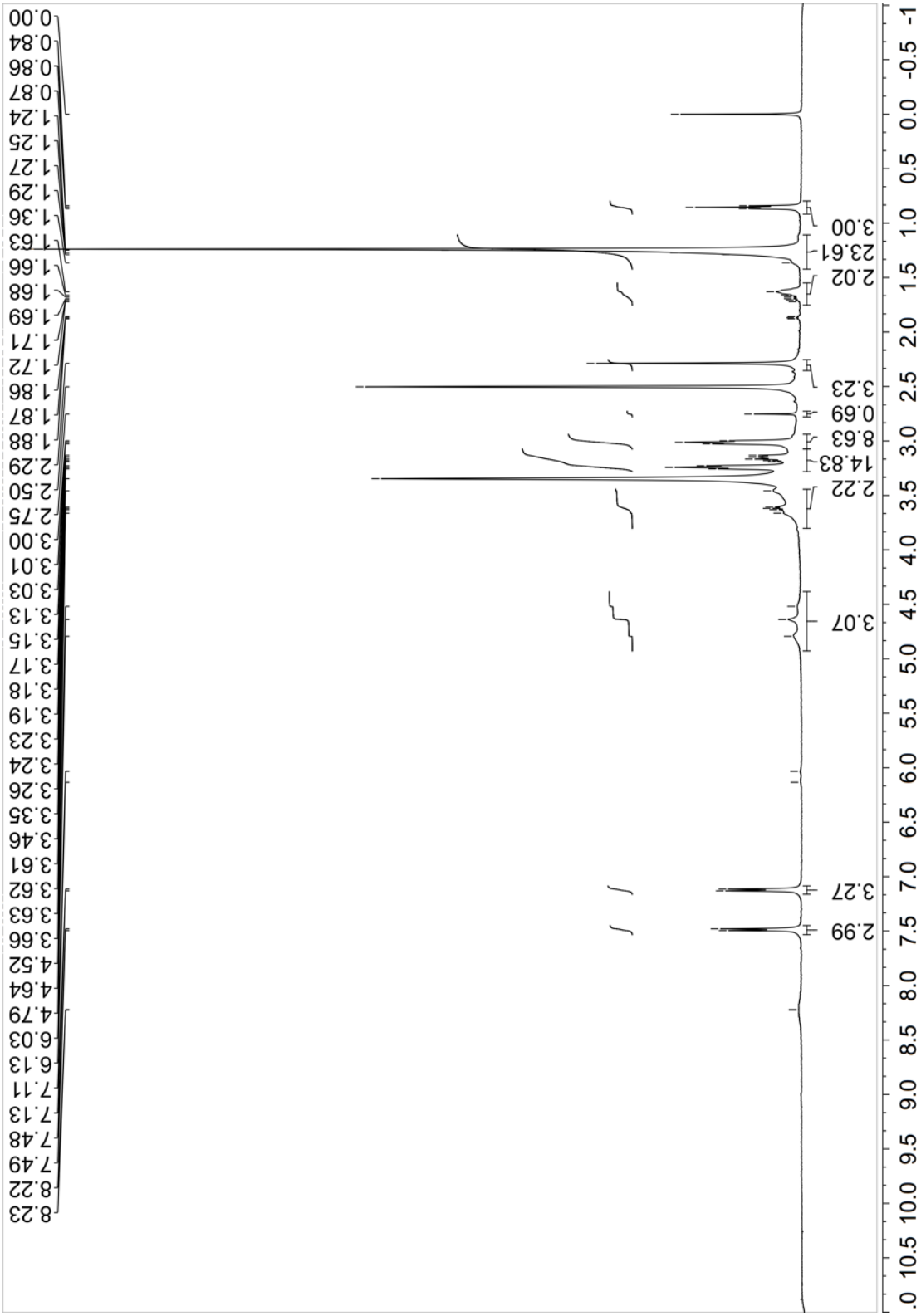


Figure 45. ^{1}H NMR of product **1**.

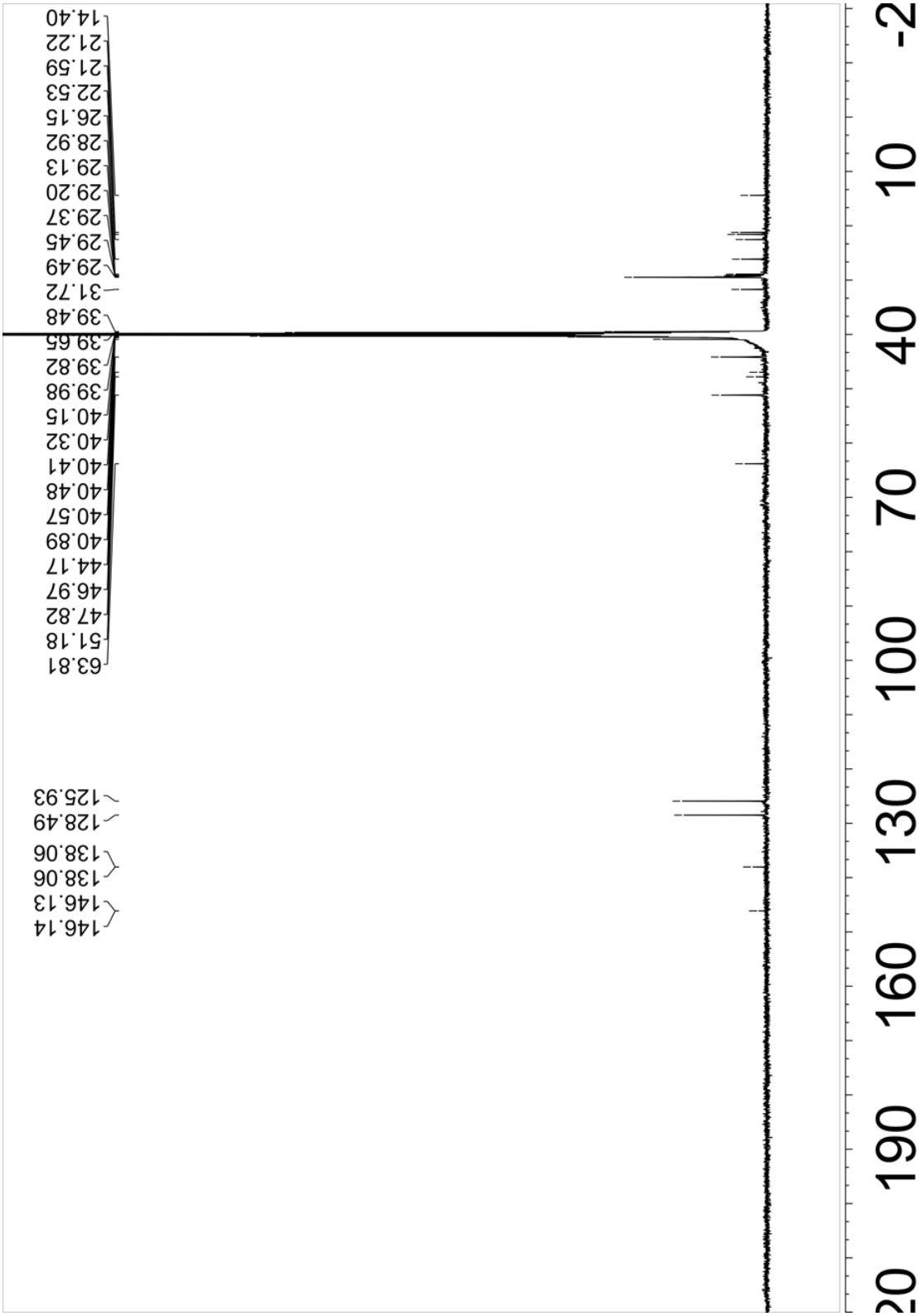


Figure 46. ^{13}C NMR spectrum of **1**.

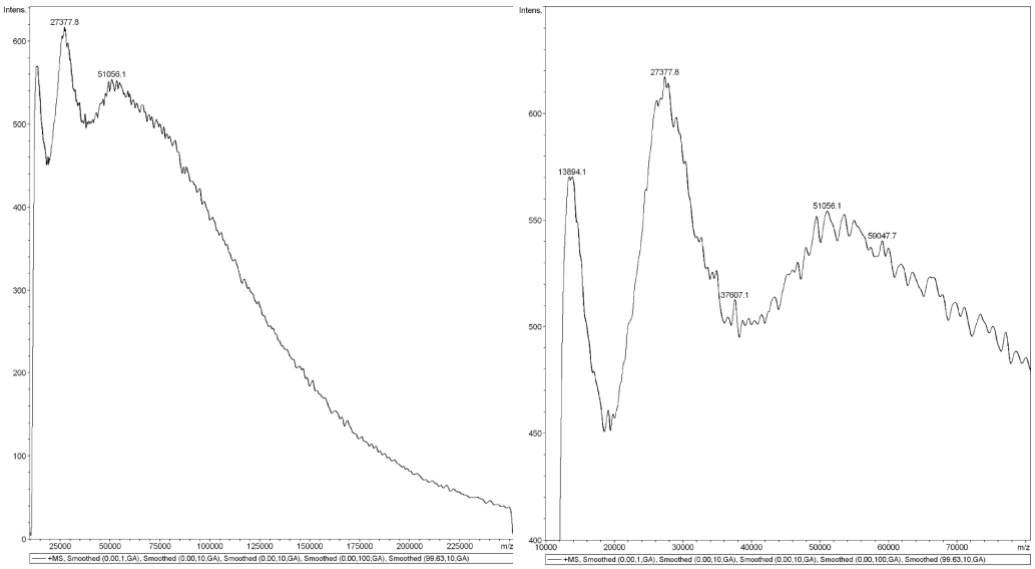


Figure 47. MALDI-TOF MS of **1**. Full spectrum on left and zoom view on right.

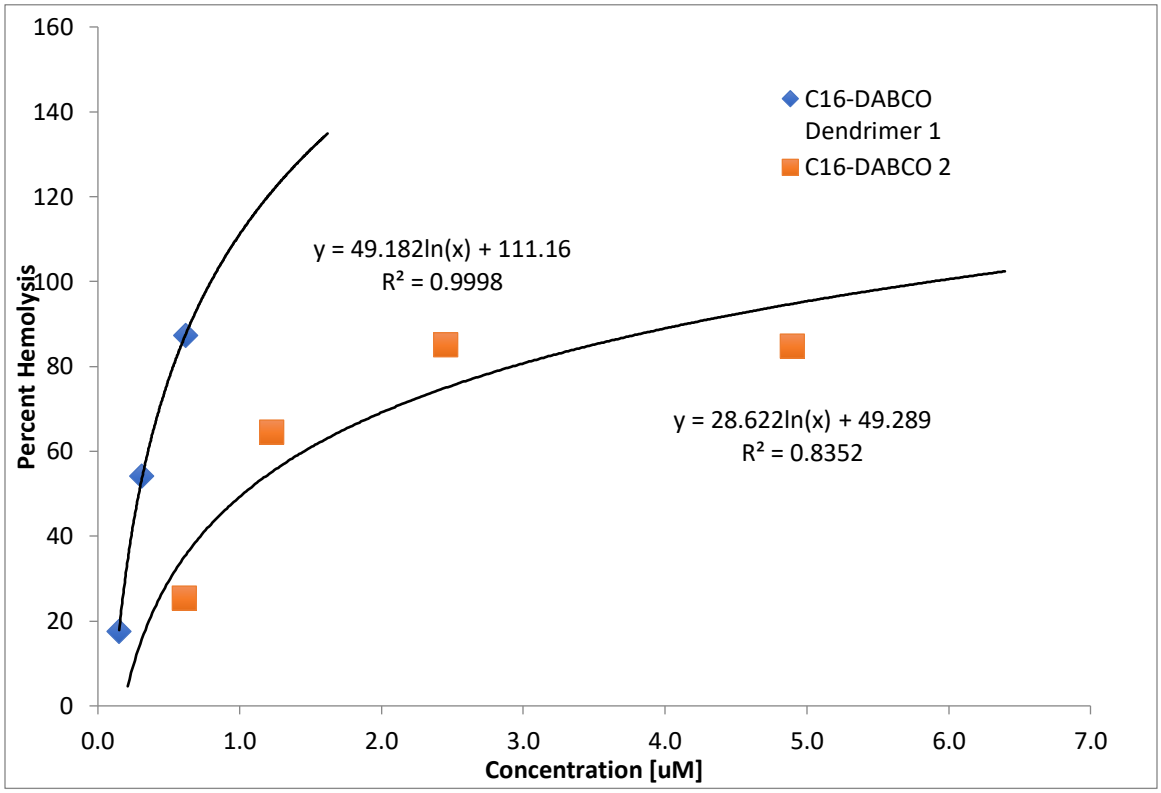


Figure 48. Red blood cell hemolysis for C_{16}-DABCO dendrimer **1** and C_{16}-DABCO monomer **2**.

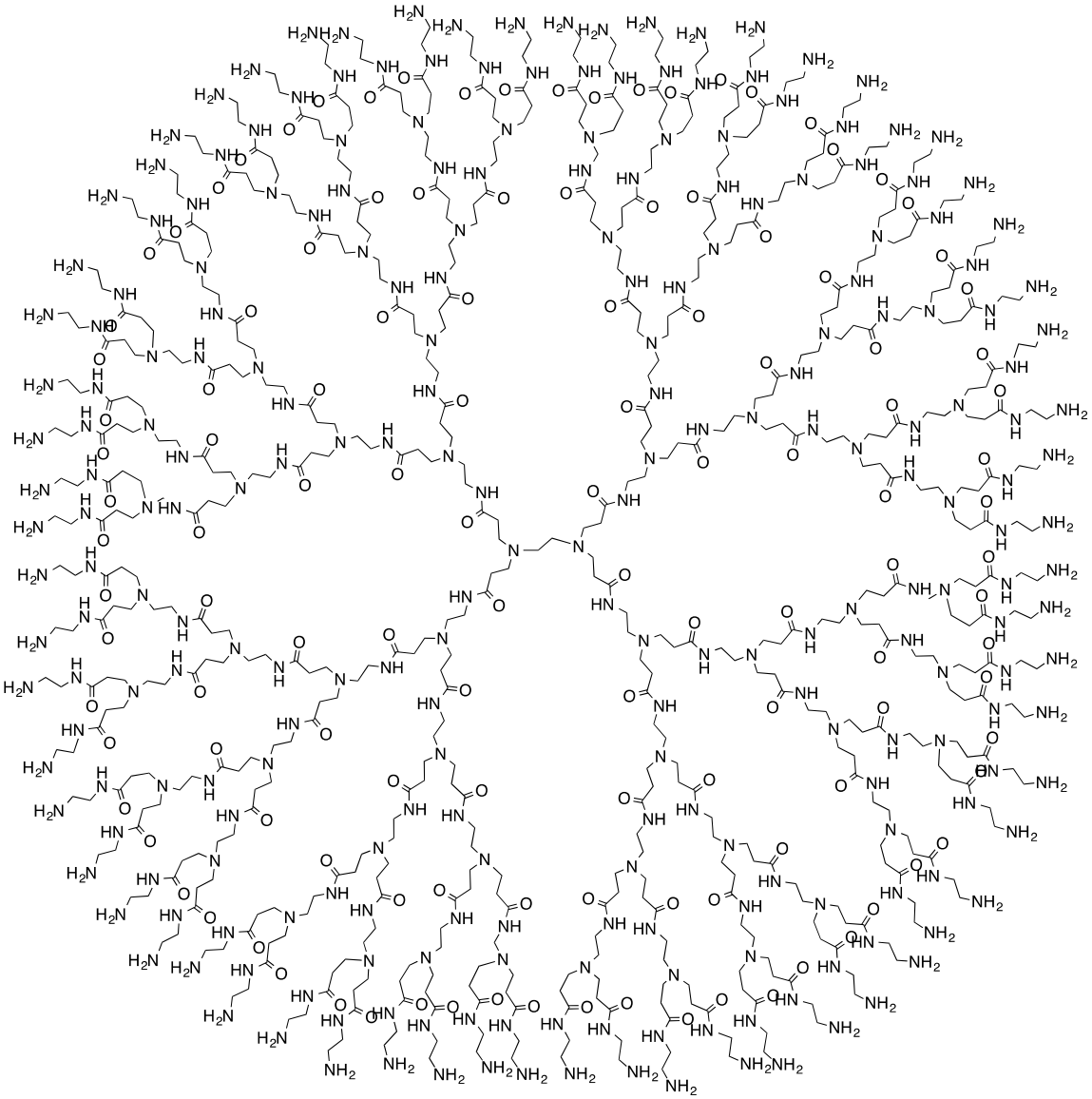


Figure 49. Fourth generation poly(amidoamine) dendrimer, i.e. G(4)-PAMAM.

Table 3 Minimum Inhibitory Concentrations (MICs) for Dendrimer **1** and Monomer **2**.

Gram	Microorganism	C_{16}-DABCO Dendrimer **1** (per C_{16}-DABCO group)	C_{16}-DABCO Dendrimer **1** (per dendrimer)	C_{16}-DABCO Monomer **2**	C_{16}-DABCO Dendrimer **1** (per dendrimer)	C_{16}-DABCO Monomer **2**	Ampicillin (uM)	Cephalexin (uM)	Streptomycin (uM)
+	*Streptococcus oralis*	>165 uM	>20.0 uM	3033 uM	>600 (ug/mL)	1024 (ug/mL)	N/A	N/A	<34
+	*Staphylococcus aureus*	1.1	0.133	11.8	4	4	<67	<68	<17
+	*Bacillus cereus*	1.1	0.133	17.7	4	6	27	41	N/A
–	*Pseudomonas aeruginosa*	16	2.00	331.7	60	112	N/A	N/A	<17
–	*Escherichia coli*	11	1.09	148.1	40	50	11	22	N/A

Sample Calculation for Determining Concentration of Active Group.

From mass concentration of dendrimer to molar

$$x\frac{mg}{mL} * \frac{1\ mol\ dendrimer}{30{,}074\ g\ dendrimer} * \frac{1\ g\ dendrimer}{10^3\ mg\ dendrimer} * \frac{1\ mL}{10^{-3}L} * \frac{1\ \mu mol}{10^{-6}\ mol} = y\ \frac{\mu mol}{L} = y\ \mu M$$

Equation 1. Solving for concentration of active group (per dendrimer basis)

$$(mass\ of\ a\ tosyl\ group) * (number\ of\ tosyl\ groups)$$
$$+(mass\ of\ a\ C16DABCO\ group) * (number\ of\ tosyl\ groups)$$
$$= (mass\ after\ tosyl\ and\ C16DABCO\ addition - mass\ before\ tosyl\ and\ C16DABCO\ addition)$$

Equation 2. Determining ratio active group to non-active group vis NMR analysis

$$ratio\ of\ tosyl\ methyl\ to\ C16DABCO\ methyl\ (NMR) = \frac{number\ of\ tosyl\ groups}{number\ of\ C16DABCO\ groups}$$

Two equations with two unknowns.

Solve for one variable in **equation 2** in terms of the other variable, put answer in **equation 1**, solve for the only variable and put answer back into **equation 1** to determine second variable. Variable are number of tosyl and C16DABCO groups.

Finally,

$$number\ of\ C16DABCO\ per\ dendrimer * molarity\ of\ dendrimer\ (y) = molarity\ of\ C16DABCO\ \text{per dendrimer}$$

REFERENCES CITED

1. Alberts B, J. A., Lewis J, et al., The Shape and Structure of Proteins. In *Molecular Biology of the Cell*, Garland Science: New York, 2002.

2. Alberts B, J. A., Lewis J, et al., Protein Function. In *Molecular Biology of the Cell*, Garland Science: New York, 2002.

3. Cooper, G.; Hausman, R., The Composition of Cells. In *The Cell a Molecular Approach*, The American Society for Microbiology: Washington, DC, 2007.

4. Alberts B, J. A., Lewis J, et al., Structure and FunctIon of DNA. In *Molecular Biology of the Cell*, 4 ed.; Garland Science: New York, 2002.

5. IUPAC, Lipids. In *Compendium of Chemical Terminology*, Blackwell Scientific Publications: Oxford, 1997.

6. Lee, Y. C.; Lee, R. T., CARBOHYDRATE-PROTEIN INTERACTIONS - BASIS OF GLYCOBIOLOGY. *Accounts of Chemical Research* **1995,** *28* (8), 321-327.

7. Dwek, R. A., Glycobiology: Toward understanding the function of sugars. *Chem. Rev.* **1996,** *96* (2), 683-720.

8. Weir, D. M., Carbohydrates as recognition molecules in infection and immunity. *FEMS microbiology immunology* **1989,** *1* (6-7), 331-40.

9. Turco, S. J., MODIFICATION OF OLIGOSACCHARIDE-LIPID SYNTHESIS AND PROTEIN GLYCOSYLATION IN GLUCOSE-DEPRIVED CELLS. *Archives of Biochemistry and Biophysics* **1980,** *205* (2), 330-339.

10. Rao, J. H.; Lahiri, J.; Isaacs, L.; Weis, R. M.; Whitesides, G. M., A trivalent system from vancomycin center dot D-Ala-D-Ala with higher affinity than avidin center dot biotin. *Science* **1998,** *280* (5364), 708-711.

11. Page, M. I.; Jencks, W. P., ENTROPIC CONTRIBUTIONS TO RATE ACCELERATIONS IN ENZYMIC AND INTRAMOLECULAR REACTIONS AND CHELATE EFFECT. *Proceedings of the National Academy of Sciences of the United States of America* **1971,** *68* (8), 1678-&.

12. Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E., Synthetic multivalent ligands in the exploration of cell-surface interactions. *Current Opinion in Chemical Biology* **2000,** *4* (6), 696-703.

13. Zhao, Q.; Guo, X.; Nash, G. B.; Stone, P. C.; Hilkens, J.; Rhodes, J. M.; Yu, L.-G., Circulating Galectin-3 Promotes Metastasis by Modifying MUC1 Localization on Cancer Cell Surface. *Cancer Research* **2009,** *69* (17), 6799-6806.

14. Sharon, N.; Lis, H., LECTINS - CELL-AGGLUTINATING AND SUGAR-SPECIFIC PROTEINS. *Science* **1972,** *177* (4053), 949-&.

15. Kamili, N. A.; Arthur, C. M.; Gerner-Smidt, C.; Tafesse, E.; Blenda, A.; Dias-Baruffi, M.; Stowell, S. R., Key regulators of galectin-glycan interactions. *Proteomics* **2016,** *16* (24), 3111-3125.

16. Perillo, N. L.; Marcus, M. E.; Baum, L. G., Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *Journal of Molecular Medicine-Jmm* **1998,** *76* (6), 402-412.

17. Liu, F. T.; Yang, R. Y.; Hsu, D. K., Galectins in acute and chronic inflammation. *Ann N Y Acad Sci* **2012,** *1253*, 80-91.

18. Cloninger, M. J.; Bilgicer, B.; Li, L.; Mangold, S. L.; Phillips, S. T.; Wolfenden, M. L., Multivalency. In *Supramolecular Chemistry: From Molecules to Nanomaterials*, John Wiley & Sons, Ltd.: 2012.

19. Woller, E. K.; Walter, E. D.; Morgan, J. R.; Singel, D. J.; Cloninger, M. J., Altering the strength of lectin binding interactions and controlling the amount of lectin clustering using mannose/hydroxyl-functionalized dendrimers. *Journal of the American Chemical Society* **2003,** *125* (29), 8820-8826.

20. Dimick, S. M.; Powell, S. C.; McMahon, S. A.; Moothoo, D. N.; Naismith, J. H.; Toone, E. J., On the meaning of affinity: Cluster glycoside effects and concanavalin A. *Journal of the American Chemical Society* **1999,** *121* (44), 10286-10296.

21. Hirabayashi, J.; Kasai, K., THE FAMILY OF METAZOAN METAL-INDEPENDENT BETA-GALACTOSIDE-BINDING LECTINS - STRUCTURE, FUNCTION AND MOLECULAR EVOLUTION. *Glycobiology* **1993,** *3* (4), 297-304.

22. Drickamer, K.; Taylor, M. E., BIOLOGY OF ANIMAL LECTINS. *Annual Review of Cell Biology* **1993,** *9*, 237-264.

23. Arthur, C. M.; Baruffi, M. D.; Cummings, R. D.; Stowell, S. R., Evolving Mechanistic Insights into Galectin Functions. *Galectins: Methods and Protocols* **2015,** *1207*, 1-35.

24. Leffler, H.; Carlsson, S.; Hedlund, M.; Qian, Y. N.; Poirier, F., Introduction to galectins. *Glycoconjugate Journal* **2002,** *19* (7-9), 433-440.

25. Hirabayashi, J.; Hashidate, T.; Arata, Y.; Nishi, N.; Nakamura, T.; Hirashima, M.; Urashima, T.; Oka, T.; Futai, M.; Muller, W. E. G.; Yagi, F.; Kasai, K., Oligosaccharide specificity of galectins: a search by frontal affinity

chromatography. *Biochimica Et Biophysica Acta-General Subjects* **2002,** *1572* (2-3), 232-254.

26. Hirabayashi, J.; Kasai, K., EFFECT OF AMINO-ACID SUBSTITUTION BY SITE-DIRECTED MUTAGENESIS ON THE CARBOHYDRATE RECOGNITION AND STABILITY OF HUMAN 14-KDA BETA-GALACTOSIDE-BINDING LECTIN. *Journal of Biological Chemistry* **1991,** *266* (35), 23648-23653.

27. Lobsanov, Y. D.; Gitt, M. A.; Leffler, H.; Barondes, S. H.; Rini, J. M., X-RAY CRYSTAL-STRUCTURE OF THE HUMAN DIMERIC S-LAC LECTIN, L-14-II, IN COMPLEX WITH LACTOSE AT 2.9-ANGSTROM RESOLUTION. *Journal of Biological Chemistry* **1993,** *268* (36), 27034-27038.

28. Balan, V.; Nangia-Makker, P.; Raz, A., Galectins as cancer biomarkers. *Cancers* **2010,** *2* (2), 592-610.

29. Rabinovich, G. A., Galectin-1 as a potential cancer target. *Br. J. Cancer* **2005,** *92* (7), 1188-1192.

30. Norling, L. V.; Perretti, M.; Cooper, D., Endogenous galectins and the control of the host inflammatory response. *Journal of Endocrinology* **2009,** *201* (2), 169-184.

31. Nangia-Makker, P.; Wang, Y.; Raz, T.; Tait, L.; Balan, V.; Hogan, V.; Raz, A., Cleavage of galectin-3 by matrix metalloproteases induces angiogenesis in breast cancer. *International Journal of Cancer* **2010,** *127* (11), 2530-2541.

32. Zhao, Q. C.; Barclay, M.; Hilkens, J.; Guo, X. L.; Barrow, H.; Rhodes, J. M.; Yu, L. G., Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis. *Molecular Cancer* **2010,** *9*.

33. Takenaka, Y.; Inohara, H.; Yoshii, T.; Oshima, K.; Nakahara, S.; Akahani, S.; Honjo, Y.; Yamamoto, Y.; Raz, A.; Kubo, T., Malignant transformation of thyroid follicular cells by galectin-3. *Cancer Letters* **2003,** *195* (1), 111-119.

34. Diao, B.; Liu, Y.; Xu, G. Z.; Zhang, Y.; Xie, J.; Gong, J., The role of galectin-3 in the tumorigenesis and progression of pituitary tumors. *Oncol Lett* **2018,** *15* (4), 4919-4925.

35. Nangia-Makker, P.; Balan, V.; Raz, A., Regulation of Tumor Progression by Extracellular Galectin-3. *Cancer Microenvironment* **2008,** *1* (1), 43-51.

36. Matrisian, L. M., Matrix Metallopeptidase-7/Matrilysin. *Handbook of Proteolytic Enzymes, Vols 1 and 2, 3rd Edition* **2013**, 786-795.

37. Galectins and Their Functions in Plain Language. In *Galectins*.

38. Dippold, W.; Steinborn, A.; Zumbuschenfelde, K. H. M., THE ROLE OF THE THOMSEN-FRIEDENREICH ANTIGEN AS A TUMOR-ASSOCIATED MOLECULE. *Environmental Health Perspectives* **1990,** *88*, 255-257.

39. Glinsky, V. V.; Glinsky, G. V.; Rittenhouse-Olson, K.; Huflejt, M. E.; Glinskii, O. V.; Deutscher, S. L.; Quinn, T. P., The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. *Cancer Research* **2001,** *61* (12), 4851-4857.

40. Fanjul-Fernandez, M.; Folgueras, A. R.; Cabrera, S.; Lopez-Otin, C., Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models. *Biochimica Et Biophysica Acta-Molecular Cell Research* **2010,** *1803* (1), 3-19.

41. Sternlicht, M. D.; Werb, Z., How matrix metalloproteinases regulate cell behavior. *Annual Review of Cell and Developmental Biology* **2001,** *17*, 463-516.

42. Vanwart, H. E.; Birkedalhansen, H., THE CYSTEINE SWITCH - A PRINCIPLE OF REGULATION OF METALLOPROTEINASE ACTIVITY WITH POTENTIAL APPLICABILITY TO THE ENTIRE MATRIX METALLOPROTEINASE GENE FAMILY. *Proceedings of the National Academy of Sciences of the United States of America* **1990,** *87* (14), 5578-5582.

43. Murphy, G.; Nguyen, Q.; Cockett, M. I.; Atkinson, S. J.; Allan, J. A.; Knight, C. G.; Willenbrock, F.; Docherty, A. J. P., ASSESSMENT OF THE ROLE OF THE FIBRONECTIN-LIKE DOMAIN OF GELATINASE-A BY ANALYSIS OF A DELETION MUTANT. *Journal of Biological Chemistry* **1994,** *269* (9), 6632-6636.

44. Shipley, J. M.; Doyle, G. A. R.; Fliszar, C. J.; Ye, Q. Z.; Johnson, L. L.; Shapiro, S. D.; Welgus, H. G.; Senior, R. M., The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases. *Journal of Biological Chemistry* **1996,** *271* (8), 4335-4341.

45. Nelson, A. R.; Fingleton, B.; Rothenberg, M. L.; Matrisian, L. M., Matrix metalloproteinases: Biologic activity and clinical implications. *Journal of Clinical Oncology* **2000,** *18* (5), 1135-1149.

46. Sternlicht, M. D.; Coussens, L. M.; Vu, T. H.; Werb, Z., Biology and regulation of the matrix metalloproteinases. In *Cancer Drug Discovery and Development. Matrix metalloproteinase inhibitors in cancer therapy*, Clendeninn, N. J.; Appelt, K., Eds. Humana Press Inc. {a} , 999 Riverview Drive, Suite 208, Totowa, NJ, 07512, USA: 2001; pp 1-37.

47. Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H., Design and therapeutic application of matrix metalloproteinase inhibitors. *Chem. Rev.* **1999,** *99* (9), 2735-2776.

48. Bloomston, M.; Zervos, E. E.; Rosemurgy, A. S., Matrix metalloproteinases and their role in pancreatic cancer: A review of preclinical studies and clinical trials. *Annals of Surgical Oncology* **2002,** *9* (7), 668-674.

49. Tallant, C.; Marrero, A.; Xavier Gomis-Ruth, F., Matrix metalloproteinases: Fold and function of their catalytic domains. *Biochimica Et Biophysica Acta-Molecular Cell Research* **2010,** *1803* (1), 20-28.

50. Califice, S.; Castronovo, V.; Van Den Brule, F., Galectin-3 and cancer. *International Journal of Oncology* **2004,** *25* (4), 983-992.

51. Newlaczyl, A. U.; Yu, L.-G., Galectin-3-A jack-of-all-trades in cancer. *Cancer Letters* **2011,** *313* (2), 123-128.

52. Kim, S.-J.; Shin, J.-Y.; Lee, K.-D.; Bae, Y.-K.; Choi, I.-J.; Park, S. H.; Chun, K.-H., Galectin-3 Facilitates Cell Motility in Gastric Cancer by Up-Regulating Protease-Activated Receptor-1(PAR-1) and Matrix Metalloproteinase-1(MMP-1). *Plos One* **2011,** *6* (9).

53. Overall, C. M.; Kleifeld, O., Towards third generation matrix metalloproteinase inhibitors for cancer therapy. *Br. J. Cancer* **2006,** *94* (7), 941-946.

54. Overall, C. M.; Lopez-Otin, C., Strategies for MMP inhibition in cancer: Innovations for the post-trial era. *Nature Reviews Cancer* **2002,** *2* (9), 657-672.

55. Lee, S. H.; Jaganath, I. B.; Manikam, R.; Sekaran, S. D., Inhibition of Raf-MEK-ERK and Hypoxia pathways by Phyllanthus prevents metastasis in human lung (A549) cancer cell line. *Bmc Complementary and Alternative Medicine* **2013,** *13*.

56. Fukuda, A.; Wang, S. C.; Morris, J. P.; Folias, A. E.; Liou, A.; Kim, G. E.; Akira, S.; Boucher, K. M.; Firpo, M. A.; Mulvihill, S. J.; Hebrok, M., Stat3 and MMP7 Contribute to Pancreatic Ductal Adenocarcinoma Initiation and Progression. *Cancer Cell* **2011,** *19* (4), 441-455.

57. Koebel, M.; Kalloger, S. E.; Boyd, N.; McKinney, S.; Mehl, E.; Palmer, C.; Leung, S.; Bowen, N. J.; Ionescu, D. N.; Rajput, A.; Prentice, L. M.; Miller, D.; Santos, J.; Swenerton, K.; Gilks, C. B.; Huntsman, D., Ovarian Carcinoma Subtypes Are Different Diseases: Implications for Biomarker Studies. *Plos Medicine* **2008,** *5* (12), 1749-1760.

58. Michel, A. K.; Nangia-Makker, P.; Raz, A.; Cloninger, M. J., Lactose-Functionalized Dendrimers Arbitrate the Interaction of Galectin-3/MUC1 Mediated Cancer Cellular Aggregation. *Chembiochem* **2014,** *15* (14), 2106-2112.

59. Takeuchi, T.; Hayama, K.; Hirabayashi, J.; Kasai, K.-i., Caenorhabditis elegans N-glycans containing a Gal-Fuc disaccharide unit linked to the innermost GlcNAc residue are recognized by C. elegans galectin LEC-6. *Glycobiology* **2008,** *18* (11), 882-890.

60. Takeuchi, T.; Nishiyama, K.; Sugiura, K.; Takahashi, M.; Yamada, A.; Kobayashi, S.; Takahashi, H.; Natsugari, H.; Kasai, K., Caenorhabditis elegans galectins LEC-6 and LEC-1 recognize a chemically synthesized Galbeta1-4Fuc disaccharide unit which is present in Protostomia glycoconjugates. *Glycobiology* **2009,** *19* (12), 1503-10.

61. Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A., Glycosylation and the immune system. *Science* **2001,** *291* (5512), 2370-2376.

62. Mattson, A. L.; Michel, A. K.; Cloninger, M. J., Using In(III) as a promoter for glycosylation. *Carbohydrate Research* **2012,** *347* (1), 142-146.

63. Esfand, R.; Tomalia, D. A., Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug Discovery Today* **2001,** *6* (8), 427-436.

64. Duncan, R.; Izzo, L., Dendrimer biocompatibility and toxicity. *Adv. Drug Deliv. Rev.* **2005,** *57* (15), 2215-2237.

65. Maiti, P. K.; Cagin, T.; Wang, G. F.; Goddard, W. A., Structure of PAMAM dendrimers: Generations 1 through 11. *Macromolecules* **2004,** *37* (16), 6236-6254.

66. Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N. K., Dendrimer toxicity: Let's meet the challenge. *International Journal of Pharmaceutics* **2010,** *394* (1-2), 122-142.

67. Hannon, G. J.; Rossi, J. J., Unlocking the potential of the human genome with RNA interference. *Nature* **2004,** *431* (7006), 371-378.

68. Fire, A.; Xu, S. Q.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C., Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **1998,** *391* (6669), 806-811.

69. Timmons, L.; Fire, A., Specific interference by ingested dsRNA. *Nature* **1998,** *395* (6705), 854-854.

70. Andersen, J.; Krichevsky, A.; Leheste, J. R.; Moloney, D. J., Caenorhabditis elegans as an undergraduate educational tool for teaching RNAi. *Biochem Mol Biol Educ* **2008,** *36* (6), 417-27.

71. Consortium, C. e. S., Genome sequence of the nematode C-elegans: A platform for investigating biology. *Science* **1998,** *282* (5396), 2012-2018.

72. Health, N. I. o. NIH Human Microbiome Project defines normal bacterial makeup of the body. (accessed September 22).

73. McDonnell, G.; Russell, A. D., Antiseptics and disinfectants: Activity, action, and resistance. *Clinical Microbiology Reviews* **1999,** *12* (1), 147-+.

74. Organization, W. H. *Antimicrobial resistance: global report on surveillance*; World Health Organization: France, 2014.

75. Magiorakos, A. P.; Srinivasan, A.; Carey, R. B.; Carmeli, Y.; Falagas, M. E.; Giske, C. G.; Harbarth, S.; Hindler, J. F.; Kahlmeter, G.; Olsson-Liljequist, B.; Paterson, D. L.; Rice, L. B.; Stelling, J.; Struelens, M. J.; Vatopoulos, A.; Weber, J. T.; Monnet, D. L., Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **2012,** *18* (3), 268-281.

76. Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E., Jr.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J., Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases* **2009,** *48* (1), 1-12.

77. Gilbert, D. N.; Guidos, R. J.; Boucher, H. W.; Talbot, G. H.; Spellberg, B.; Edwards, J. E., Jr.; Scheld, W. M.; Bradley, J. S.; Bartlett, J. G.; Infect Dis Soc, A., The 10 x '20 Initiative: Pursuing a Global Commitment to Develop 10 New Antibacterial Drugs by 2020. *Clinical Infectious Diseases* **2010,** *50* (8), 1081-1083.

78. Appelbaum, P. C., 2012 and beyond: potential for the start of a second pre-antibiotic era? *Journal of Antimicrobial Chemotherapy* **2012,** *67* (9), 2062-2068.

79. MacDougall, C.; Polk, R. E., Antimicrobial stewardship programs in health care systems. *Clinical Microbiology Reviews* **2005,** *18* (4), 638-+.

80. Engler, A. C.; Wiradharma, N.; Ong, Z. Y.; Coady, D. J.; Hedrick, J. L.; Yang, Y.-Y., Emerging trends in macromolecular antimicrobials to fight multi-drug-resistant infections. *Nano Today* **2012,** *7* (3), 201-222.

81. Neu, H. C., THE CRISIS IN ANTIBIOTIC-RESISTANCE. *Science* **1992,** *257* (5073), 1064-1073.

82. Davies, J.; Davies, D., Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews* **2010,** *74* (3), 417-+.

83. Walsh, C., Molecular mechanisms that confer antibacterial drug resistance. *Nature* **2000,** *406* (6797), 775-781.

84. Drawz, S. M.; Bonomo, R. A., Three Decades of beta-Lactamase Inhibitors. *Clinical Microbiology Reviews* **2010,** *23* (1), 160-+.

85. Poole, K., Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **2005,** *56* (1), 20-51.

86. Ganewatta, M. S.; Tang, C., Controlling macromolecular structures towards effective antimicrobial polymers. *Polymer* **2015,** *63*, A1-A29.

87. Munoz-Bonilla, A.; Fernandez-Garcia, M., The roadmap of antimicrobial polymeric materials in macromolecular nanotechnology. *European Polymer Journal* **2015,** *65*, 46-62.

88. Mammen, M.; Choi, S. K.; Whitesides, G. M., Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angewandte Chemie-International Edition* **1998,** *37* (20), 2755-2794.

89. Newkome, G.; Moorefield, C.; Vogtle, F., *Dendrimers and Dendrons: Concepts, Syntheses, Applications*. Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2001; p 623.

90. Mintzer, M. A.; Dane, E. L.; O'Toole, G. A.; Grinstaff, M. W., Exploiting Dendrimer Multivalency To Combat Emerging and Re-Emerging Infectious Diseases. *Molecular Pharmaceutics* **2012,** *9* (3), 342-354.

91. Kannan, R. M.; Nance, E.; Kannan, S.; Tomalia, D. A., Emerging concepts in dendrimer-based nanomedicine: from design principles to clinical applications. *Journal of Internal Medicine* **2014,** *276* (6), 579-617.

92. Navath, R. S.; Menjoge, A. R.; Wang, B.; Romero, R.; Kannan, S.; Kannan, R. M., Amino Acid-Functionalized Dendrimers with Heterobifunctional Chemoselective Peripheral Groups for Drug Delivery Applications. *Biomacromolecules* **2010,** *11* (6), 1544-1563.

93. Ortega, P.; Copa-Patino, J. L.; Munoz-Fernandez, M. A.; Soliveri, J.; Gomez, R.; de la Mata, F. J., Amine and ammonium functionalization of chloromethylsilane-ended dendrimers. Antimicrobial activity studies. *Organic & Biomolecular Chemistry* **2008,** *6* (18), 3264-3269.

94. Meyers, S. R.; Juhn, F. S.; Griset, A. P.; Luman, N. R.; Grinstaff, M. W., Anionic Amphiphilic Dendrimers as Antibacterial Agents. *Journal of the American Chemical Society* **2008,** *130* (44), 14444-+.

95. Choi, S. K.; Myc, A.; Silpe, J. E.; Sumit, M.; Wong, P. T.; McCarthy, K.; Desai, A. M.; Thomas, T. P.; Kotlyar, A.; Holl, M. M. B.; Orr, B. G.; Baker, J. R., Jr., Dendrimer-Based Multivalent Vancomycin Nanoplatform for Targeting the Drug-Resistant Bacterial Surface. *Acs Nano* **2013,** *7* (1), 214-228.

96. Telwatte, S.; Moore, K.; Johnson, A.; Tyssen, D.; Sterjovski, J.; Aldunate, M.; Gorry, P. R.; Ramsland, P. A.; Lewis, G. R.; Paull, J. R. A.; Sonza, S.; Tachedjian, G., Virucidal activity of the dendrimer microbicide SPL7013 against HIV-1. *Antiviral Research* **2011,** *90* (3), 195-199.

97. Sadler, K.; Tam, J. P., Peptide dendrimers: applications and synthesis. *Journal of biotechnology* **2002,** *90* (3-4), 195-229.

98. Pires, J.; Siriwardena, T. N.; Stach, M.; Tinguely, R.; Kasraian, S.; Luzzaro, F.; Leib, S. L.; Darbre, T.; Reymond, J. L.; Endimiani, A., In Vitro Activity of the Novel Antimicrobial Peptide Dendrimer G3KL against Multidrug-Resistant Acinetobacter baumannii and Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy* **2015,** *59* (12), 7915-7918.

99. Abel, T.; Cohen, J. I.; Engel, R.; Filshtinskaya, M.; Melkonian, A.; Melkonian, K., Preparation and investigation of antibacterial carbohydrate-based surfaces. *Carbohydrate Research* **2002,** *337* (24), 2495-2499.

100. Heidecke, C. D.; Lindhorst, T. K., Iterative synthesis of spacered glycodendrons as oligomannoside mimetics and evaluation of their antiadhesive properties. *Chemistry-a European Journal* **2007,** *13* (32), 9056-9067.

101. Hancock, R. E. W., ALTERATIONS IN OUTER-MEMBRANE PERMEABILITY. *Annual Review of Microbiology* **1984,** *38*, 237-264.

102. Percy, M. G.; Grundling, A., Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annual Review of Microbiology, Vol 68* **2014,** *68*, 81-100.

103. Wang, B.; Navath, R. S.; Menjoge, A. R.; Balakrishnan, B.; Bellair, R.; Dai, H.; Romero, R.; Kannan, S.; Kannan, R. M., Inhibition of bacterial growth and intramniotic infection in a guinea pig model of chorioamnionitis using PAMAM dendrimers. *International Journal of Pharmaceutics* **2010,** *395* (1-2), 298-308.

104. Zarena, A. S.; Gopal, S., Dendrimer a New Dimension in Targeting Biofilms. *Mini-Reviews in Medicinal Chemistry* **2013,** *13* (10), 1448-1461.

105. Mah, T. F. C.; O'Toole, G. A., Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **2001,** *9* (1), 34-39.

106. Stewart, P. S.; Franklin, M. J., Physiological heterogeneity in biofilms. *Nature Reviews Microbiology* **2008,** *6* (3), 199-210.

107. Stewart, P. S.; Costerton, J. W., Antibiotic resistance of bacteria in biofilms. *Lancet* **2001,** *358* (9276), 135-138.

108. Mah, T. F.; Pitts, B.; Pellock, B.; Walker, G. C.; Stewart, P. S.; O'Toole, G. A., A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. *Nature* **2003,** *426* (6964), 306-310.

109. ISO/FDIS, Clinical Laboratory Testing and in Vitro Diagnostic Test Systems. In *Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices -- Part 1: Reference Method for Testing the in Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases.* , International Organization of Standardization: Geneva, Switzerland, 2006; Vol. Iso 20776-1:2006.

110. Kosowska-Shick, K.; Clark, C.; Pankuch, G. A.; McGhee, P.; Dewasse, B.; Beachel, L.; Appelbaum, P. C., Activity of Telavancin against Staphylococci and Enterococci Determined by MIC and Resistance Selection Studies. *Antimicrobial Agents and Chemotherapy* **2009,** *53* (10), 4217-4224.

111. Harman, D., AGING - A THEORY BASED ON FREE-RADICAL AND RADIATION-CHEMISTRY. *Journals of Gerontology* **1956,** *11* (3), 298-300.

112. Vanfleteren, J. R., OXIDATIVE STRESS AND AGING IN CAENORHABDITIS-ELEGANS. *Biochemical Journal* **1993,** *292*, 605-608.

113. Larsen, P. L., AGING AND RESISTANCE TO OXIDATIVE DAMAGE IN CAENORHABDITIS-ELEGANS. *Proceedings of the National Academy of Sciences of the United States of America* **1993,** *90* (19), 8905-8909.

114. Kenyon, C.; Chang, J.; Gensch, E.; Rudner, A.; Tabtiang, R., A C-ELEGANS MUTANT THAT LIVES TWICE AS LONG AS WILD-TYPE. *Nature* **1993,** *366* (6454), 461-464.

115. Cahill, C. M.; Tzivion, G.; Nasrin, N.; Ogg, S.; Dore, J.; Ruvkun, G.; Alexander-Bridges, M., Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. *Journal of Biological Chemistry* **2001,** *276* (16), 13402-13410.

116. Lin, K.; Hsin, H.; Libina, N.; Kenyon, C., Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nature Genetics* **2001,** *28* (2), 139-145.

117. Murakami, S.; Johnson, T. E., Life extension and stress resistance in Caenorhabditis elegans modulated by the tkr-1 gene. *Current Biology* **1998,** *8* (19), 1091-1094.

118. Finkel, T.; Holbrook, N. J., Oxidants, oxidative stress and the biology of ageing. *Nature* **2000,** *408* (6809), 239-247.

119. Murphy, C. T.; McCarroll, S. A.; Bargmann, C. I.; Fraser, A.; Kamath, R. S.; Ahringer, J.; Li, H.; Kenyon, C., Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature* **2003,** *424* (6946), 277-284.

120. Cooper, D. N. W.; Barondes, S. H., God must love galectins; He made so many of them. *Glycobiology* **1999,** *9* (10), 979-984.

121. Metzler, M.; Gertz, A.; Sarkar, M.; Schachter, H.; Schrader, J. W.; Marth, J. D., COMPLEX ASPARAGINE-LINKED OLIGOSACCHARIDES ARE REQUIRED FOR MORPHOGENIC EVENTS DURING POSTIMPLANTATION DEVELOPMENT. *Embo Journal* **1994,** *13* (9), 2056-2065.

122. Lotz, M. M.; Andrews, C. W.; Korzelius, C. A.; Lee, E. C.; Steele, G. D.; Clarke, A.; Mercurio, A. M., DECREASED EXPRESSION OF MAC-2 (CARBOHYDRATE BINDING PROTEIN-35) AND LOSS OF ITS NUCLEAR-LOCALIZATION ARE ASSOCIATED WITH THE NEOPLASTIC PROGRESSION OF COLON-CARCINOMA. *Proceedings of the National Academy of Sciences of the United States of America* **1993,** *90* (8), 3466-3470.

123. Schoeppner, H. L.; Raz, A.; Ho, S. B.; Bresalier, R. S., EXPRESSION OF AN ENDOGENOUS GALACTOSE-BINDING LECTIN CORRELATES WITH NEOPLASTIC PROGRESSION IN THE COLON. *Cancer* **1995,** *75* (12), 2818-2826.

124. Sanford, G. L.; Harrishooker, S., STIMULATION OF VASCULAR CELL-PROLIFERATION BY BETA-GALACTOSIDE SPECIFIC LECTINS. *Faseb Journal* **1990,** *4* (11), 2912-2918.

125. Raimond, J.; Rouleux, F.; Monsigny, M.; Legrand, A., THE 2ND INTRON OF THE HUMAN GALECTIN-3 GENE HAS A STRONG PROMOTER ACTIVITY DOWN-REGULATED BY P53. *Febs Letters* **1995,** *363* (1-2), 165-169.

126. Sato, S.; Nieminen, J., Seeing strangers or announcing "danger": Galectin-3 in two models of innate immunity. *Glycoconjugate Journal* **2004,** *19* (7-9), 583-591.

127. Takeuchi, T.; Nemoto-Sasaki, Y.; Sugiura, K.; Arata, Y.; Kasai, K., Galectin LEC-1 plays a defensive role against damage due to oxidative stress in Caenorhabditis elegans. *J Biochem* **2013,** *154* (5), 455-64.

128. Suzuki, Y.; Inoue, T.; Yoshimaru, T.; Ra, C., Galectin-3 but not galectin-1 induces mast cell death by oxidative stress and mitochondrial permeability transition. *Biochim Biophys Acta* **2008,** *1783* (5), 924-34.

129. Vasta, G. R., Roles of galectins in infection. *Nature Reviews Microbiology* **2009,** *7* (6), 424-438.

130. Nemoto-Sasaki, Y.; Kasai, K., Deletion of lec-10, a Galectin-Encoding Gene, Increases Susceptibility to Oxidative Stress in Caenorhabditis elegans. *Biological & Pharmaceutical Bulletin* **2009,** *32* (12), 1973-1977.

131. Takeuchi, T.; Nemoto-Sasaki, Y.; Sugiura, K.; Arata, Y.; Kasai, K., Galectin LEC-1 plays a defensive role against damage due to oxidative stress in Caenorhabditis elegans. *Journal of Biochemistry* **2013,** *154* (5), 455-464.

132. Takeuchi, T.; Arata, Y.; Kasai, K. I., Galactosebeta1-4fucose: A unique disaccharide unit found in N-glycans of invertebrates including nematodes. *Proteomics* **2016,** *16* (24), 3137-3147.

133. Nishiyama, K.; Yamada, A.; Takahashi, M.; Takeuchi, T.; Kasai, K.-i.; Kobayashi, S.; Natsugari, H.; Takahashi, H., Synthesis of Fluorescence-Labeled Gal beta 1-3Fuc and Gal beta 1-4Fuc as Probes for the Endogenous Glyco-Epitope Recognized by Galectins in Caenorhabditis elegans. *Chemical & Pharmaceutical Bulletin* **2010,** *58* (4), 495-500.

134. Takeuchi, T.; Tamura, M.; Nishiyama, K.; Iwaki, J.; Hirabayashi, J.; Takahashi, H.; Natsugari, H.; Arata, Y.; Kasai, K., Mammalian galectins bind galactosebeta1-4fucose disaccharide, a unique structural component of protostomial N-type glycoproteins. *Biochem Biophys Res Commun* **2013,** *436* (3), 509-13.

135. Hirabayashi, J.; Arata, Y.; Hayama, K.; Kasai, K., Galectins from the nematode Caenorhabditis elegans and the glycome project. *Trends in Glycoscience and Glycotechnology* **2001,** *13* (73), 533-549.

136. Garegg, R. J.; Norberg, T., Synthesis of O-beta-D-glucopyranosyl-(1 linked to 3)-O-alpha-L-fucopyranosyl-L-threonine. *Carbohydrate research* **1976,** *52*, 235-40.

137. Khatuntseva, E. A.; Ustuzhanina, N. E.; Zatonskii, G. V.; Shashkov, A. S.; Usov, A. I.; Nifant'ev, N. E., Synthesis, NMR and Conformational Studies of Fucoidan Fragments 1:1Desulfated 2,3- and 3,4-Branched Trisaccharide

Fragments and Constituting Disaccharides. *Journal of Carbohydrate Chemistry* **2000,** *19* (9), 1151-1173.

138. Bizier, N. P.; Atkins, S. R.; Helland, L. C.; Colvin, S. F.; Twitchell, J. R.; Cloninger, M. J., Indium triflate catalyzed peracetylation of carbohydrates. *Carbohydrate Research* **2008,** *343* (10-11), 1814-1818.

139. Perkins, L. A.; Hedgecock, E. M.; Thomson, J. N.; Culotti, J. G., MUTANT SENSORY CILIA IN THE NEMATODE CAENORHABDITIS-ELEGANS. *Developmental Biology* **1986,** *117* (2), 456-487.

140. Arata, Y.; Akimoto, Y.; Hirabayashi, J.; Kasai, K.; Hirano, H., Immunohistochemical study of the 32-kDa galectin (beta-galactoside-binding lectin) in the nematode Caenorhabditis elegans. *Histochemical Journal* **1996,** *28* (3), 201-207.

141. Maduzia, L. L.; Yu, E.; Zhang, Y., Caenorhabditis elegans Galectins LEC-6 and LEC-10 Interact with Similar Glycoconjugates in the Intestine. *Journal of Biological Chemistry* **2011,** *286* (6), 4371-4381.

142. Takeuchi, T.; Nemoto-Sasaki, Y.; Arata, Y.; Kasai, K., Galectin LEC-6 Interacts with Glycoprotein F57F4.4 to Cooperatively Regulate the Growth of Caenorhabditis elegans. *Biological & Pharmaceutical Bulletin* **2011,** *34* (7), 1139-1142.

143. Fraser, A. G.; Kamath, R. S.; Zipperlen, P.; Martinez-Campos, M.; Sohrmann, M.; Ahringer, J., Functional genomic analysis of C-elegans chromosome I by systematic RNA interference. *Nature* **2000,** *408* (6810), 325-330.

144. Brenner, S., GENETICS OF CAENORHABDITIS-ELEGANS. *Genetics* **1974,** *77* (1), 71-94.

145. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J.-Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A., Fiji: an open-source platform for biological-image analysis. *Nature Methods* **2012,** *9* (7), 676-682.

146. Cousin, J. GLYCODENDRIMERS: TOOLS TO STUDY MULTIVALENT GALECTIN-1 INTERACTIONS. Montana State University, 2015.

147. Rodriguez, D.; Morrison, C. J.; Overall, C. M., Matrix metalloproteinases: What do they not do? New substrates and biological roles identified by murine models and proteomics. *Biochimica Et Biophysica Acta-Molecular Cell Research* **2010,** *1803* (1), 39-54.

148. Overall, C. M., Dilating the degradome: matrix metalloproteinase 2 (MMP-2) cuts to the heart of the matter. *The Biochemical journal* **2004,** *383* (Pt. 3), e5-7.

149. Murphy, G., Matrix Metallopeptidase-2/Gelatinase A. *Handbook of Proteolytic Enzymes, Vols 1 and 2, 3rd Edition* **2013**, 747-753.

150. Albright, C. F.; Graciani, N.; Han, W.; Yue, E.; Stein, R.; Lai, Z. H.; Diamond, M.; Dowling, R.; Grimminger, L.; Zhang, S. Y.; Behrens, D.; Musselman, A.; Bruckner, R.; Zhang, M. Z.; Jiang, X.; Hu, D.; Higley, A.; DiMeo, S.; Rafalski, M.; Mandlekar, S.; Car, B.; Yeleswaram, S.; Stern, A.; Copeland, R. A.; Combs, A.; Seitz, S. P.; Trainor, G. L.; Taub, R.; Huang, P.; Oliff, A., Matrix metalloproteinase-activated doxorubicin prodrugs inhibit HT1080 xenograft growth doxorubicin with less toxicity. *Molecular Cancer Therapeutics* **2005,** *4* (5), 751-760.

151. McKerrow, J. H.; Bhargava, V.; Hansell, E.; Huling, S.; Kuwahara, T.; Matley, M.; Coussens, L.; Warren, R., A functional proteomics screen of proteases in colorectal carcinoma. *Molecular Medicine* **2000,** *6* (5), 450-460.

152. Goodman, C. K.; Wolfenden, M. L.; Nangia-Makker, P.; Michel, A. K.; Raz, A.; Cloninger, M. J., Multivalent scaffolds induce galectin-3 aggregation into nanoparticles. *Beilstein Journal of Organic Chemistry* **2014,** *10*, 1570-1577.

153. Menjoge, A. R.; Kannan, R. M.; Tomalia, D. A., Dendrimer-based drug and imaging conjugates: design considerations for nanomedical applications. *Drug Discovery Today* **2010,** *15* (5-6), 171-185.

154. Kridel, S. J.; Chen, E.; Kotra, L. P.; Howard, E. W.; Mobashery, S.; Smith, J. M., Substrate hydrolysis by matrix metalloproteinase-9. *Journal of Biological Chemistry* **2001,** *276* (23), 20572-20578.

155. Seltzer, J. L.; Akers, K. T.; Weingarten, H.; Grant, G. A.; McCourt, D. W.; Eisen, A. Z., CLEAVAGE SPECIFICITY OF HUMAN SKIN TYPE-IV COLLAGENASE (GELATINASE) - IDENTIFICATION OF CLEAVAGE SITES IN TYPE-I GELATIN, WITH CONFIRMATION USING SYNTHETIC PEPTIDES. *Journal of Biological Chemistry* **1990,** *265* (33), 20409-20413.

156. Chen, E. I.; Kridel, S. J.; Howard, E. W.; Li, W. Z.; Godzik, A.; Smith, J. W., A unique substrate recognition profile for matrix metalloproteinase-2. *Journal of Biological Chemistry* **2002,** *277* (6), 4485-4491.

157. Eisen, A. Z.; Sarkar, S. K.; Neuman, K. C.; Goldberg, G. I., Matrix Metallopeptidase 9/Gelatinase B. *Handbook of Proteolytic Enzymes, Vols 1 and 2, 3rd Edition* **2013**, 754-763.

158. Starpharma http://www.starpharma.com/vivagel/vivagel_clinical_trials.

159. Khopade, A. J.; Caruso, F.; Tripathi, P.; Nagaich, S.; Jain, N. K., Effect of dendrimer on entrapment and release of bioactive from liposomes. *International Journal of Pharmaceutics* **2002,** *232* (1-2), 157-162.

160. Li, X.; Takashima, M.; Yuba, E.; Harada, A.; Kono, K., PEGylated PAMAM dendrimer-doxorubicin conjugate-hybridized gold nanorod for combined photothermal-chemotherapy. *Biomaterials* **2014,** *35* (24), 6576-6584.

161. Salton, M. R., Lytic agents, cell permeability, and monolayer penetrability. *The Journal of general physiology* **1968,** *52* (1), 227-52.